MEASUREMENT OF BIOPROCESS CONTAINMENT BY QUANTITATIVE POLYMERASE CHAIN REACTION

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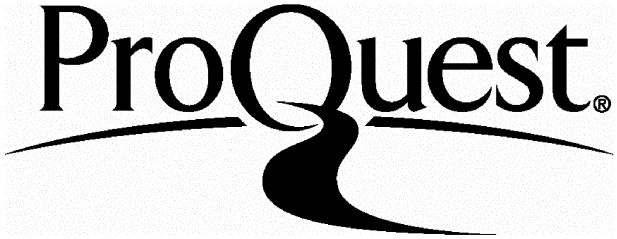

ABSTRACT

This thesis describes the development and application of a method for the measurement of the release of genetically modified micro-organisms from large scale bioprocesses. Polymerase chain reaction (PCR) assays for two *E. coli* K-12 strains have been shown to be specific for the target strain and have sufficiently low limits of detection (less than 50 cells per PCR) for monitoring of bioprocess release. A quantitative PCR assay, using a competitive internal standard, for one *E. coli* strain allows measurement of the concentration of the bacteria over a range of up to 6 orders of magnitude with a measurement error of $\pm$ 0.11 logs. This method has been applied to samples taken from an Aerojet General Cyclone air sampling device allowing the determination of the number of whole cells of the target organism in a sampled aerosol. Using this method, good correlation has been observed between the number of cells released by atomisation into a fixed, contained volume and the number of cells captured and enumerated. Aspects of large scale fermentation, homogenisation and centrifugation unit operations have been studied to determine the effectiveness of their containment. Airborne release of process micro-organisms has been detected in some instances, but the scale of the release was generally found to be small considering the total biomass involved in the bioprocess. Implications of the methodology and the findings from model and case studies on current engineering practice and bioprocess risk assessment are discussed. Areas for further improvement of the method and applications outside of bioprocess containment validation are identified.

for Marianne

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LIST OF ABBREVIATIONS

$A_{260/280}$	absorbance at 260/280 nm
ACBE	Advanced Centre for Biochemical Engineering
ACDP	Advisory Committee on Dangerous Pathogens
ACGM	Advisory Committee on Genetic Manipulation
AMS	Anderson microbial sampler
ATP	adenosine triphosphate
bp	base pair(s)
BST	bovine somatotrophin
CAS-QPCR	cyclone air sampling - QPCR
CD	cell disruption
CEN	European Committee on Standardisation
CER	carbon evolution rate
CFD	computational fluid dynamics
CFU	colony forming units
CGE	capillary gel electrophoresis
CIP	clean in place
COSHH	control of substances hazardous to health
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DOT	dissolved oxygen tension
DVC	direct viable count
FA	fluorescent antibody
FCM	flow cytometry
GILSP	good industrial large scale practice
GMMO	genetically modified micro-organism
GMO	genetically modified organism
GWMRC	Glaxo Wellcome Medicines Research Centre
HASAW	Health and Safety at Work Act
HEPA	high efficiency particulate air
HPLC	high performance liquid chromatography
HSC	Health and Safety Commission
HSE	Health and Safety Executive
IS, IS(T), IS(A)	internal standard, for transketlase construct (pQR701), for amylase construct (pQR126)
Kb	1000 bp

LIF	laser induced fluorescence
LOD	limit of detection
LSCC	large scale containment category
MPN	most probable number
mRNA	messenger RNA
OAF	open air factor
OD_{600}	optical density at 600 nm
OECD	Organisation for Economic Co-operation and Development
OUR	oxygen uptake rate
PCR	polymerase chain reaction
PPG	polypropylene glycol
QPCR	quantitative PCR
QRA	quantitative risk assessment
r	Pearson product-moment correlation coefficient
rDNA	recombinant DNA
RH	relative humidity
RNA	Ribonucleic acid
RO	reverse osmosis
RODAC	replicate organism direct contact plate
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase - PCR
SAS	Surface Air System
SDS	sodium dodecyl sulphate
SROW	sterile RO water
TESEO	empiric technique to estimate operator's error
TRS	thiosulphate ringers solution

1. INTRODUCTION

1.1 Biotechnology and biosafety.

Although the term 'biotechnology' was coined as long ago as 1917 (Bud, 1993), there are still many different definitions. The best known is probably that of the Organisation for Economic Co-operation and Development (OECD) - "biotechnology is the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services". This is an all-encompassing definition and could be used to describe, say, conventional agriculture. At the other extreme, biotechnology is equated solely with recombinant DNA techniques, genetic modification. The use of micro-organisms in industrial processes (bioprocesses) is undoubtedly a cornerstone of biotechnology.

Ancient processes using micro-organisms can be traced back many thousands of years. Before 6000 BC yeasts were first employed in brewing processes and somewhere around 4000 BC yeasts were used in the production of leavened bread. In more recent times, the ability of bacteria to form a wide range of chemical syntheses, such as the production of acetone, butanol and glycerol, has been recognised. In 1929, JBS Haldane commented "why trouble to make compounds yourself when a bug will do it for you?" The benefits that can be envisaged from the use of biotechnology are enormous and have application in medicine, agriculture and food production, energy production and in industry (see Table 1.1).

The publication of surveys of laboratory associated infections (Collins, 1992) has demonstrated that there is an element of risk involved when working with micro-organisms, due to the potential for infection. However, because the organisms used in 'traditional' biotechnological processes that have been practised over the centuries are almost universally non-pathogenic, there is no history of illness associated with such methods. Traditional biotechnology, even in the pharmaceutical industries, where pathogens are used in the manufacture of vaccines, has been generally regarded as safe, although there have been some incidents where operations in bioprocesses have given rise to illness (see Section 1.4.2).

In the late 1960s and early 1970s there were some considerable breakthroughs in the field of molecular genetics. Chief amongst these was Kornberg's finding that it was possible to biochemically synthesise a viral gene (Kornberg, 1989). Following these achievements, came the marriage between genetic technology and biotechnology which led, eventually, to the large scale fermentations using genetically modified micro-

TABLE 1.1 Milestones in Biotechnology (adapted from Prentis (1984))

Activity	Date
Yeasts employed to make wine and beer	before 6000BC
Leavened bread produced with the aid of yeasts	approx. 4000BC
Copper mined with aid of microbes, Rio Tinto, Spain	before 1670
Leeuwenhoek first sees microbes with his newly designed microscope	1680
Pasteur identifies extraneous microbes as a cause of failed beer fermentations	1876
Buchner discovers that enzymes extracted from yeast can convert sugar to alcohol	1897
Large-scale sewage purification systems employing microbes are established	approx. 1910
Three important industrial chemicals (acetone, butanol and glycerol) obtained from bacteria	1912-1914
Large-scale production of penicillin begins	1944
Double helix structure of DNA revealed	1953
Mining of uranium with aid of microbes begins in Canada	1962
First successful genetic modification experiments	1973
Monoclonal antibodies developed	1975
US National Institutes of Health introduce guidelines for genetic modification work	1976
US Court decides that genetically modified micro-organisms can be patented	1980
Biotechnology firm Cetus sets Wall Street record for first public offering of stock (£80m)	1981
Genetically engineered insulin approved for use in diabetics in US and UK	1982
Polymerase chain reaction patented by Cetus	1985
First deliberate release of GMMO into the environment	1987
Gene for cystic fibrosis mapped	1989
Human genome project formally launched	1990
PCR patent rights sold for £200m	1991
EU identifies biotechnology as a key sector in its white paper on growth, competitiveness and employment	1994
Market size for biotechnology products estimated at £3.8bn	1994
Offerings and commercial partnerships involving biotechnology sector raise £1.5bn	1995
First complete bacterial genome sequenced (*Haemophilus influenzae*)	1995
First complete eukaryotic genome sequenced (*Saccharomyces cerevisiae*)	1996
Market size for biotechnology products estimated at £60bn	*2000*
Estimated completion of human genome sequence	*2003*

organisms (GMMOs) that are common today.

However, throughout the history of genetic modification of organisms, there has been a great deal of public concern, not only about the safety of the techniques and the processes involved, but also about possible ethical implications (Bud, 1993). When the implications of the new technology available were realised a moratorium was imposed on certain experiments and large scale processes (see Section 1.2.1). However, since many of these safety related fears were found to be largely conjectural (Berg and Singer, 1995), industry has proposed not only to grow GMMOs at a large scale in contained facilities but also to deliberately release such organisms into the environment where they may demonstrate a valuable role in, for instance, pollution degradation or crop protection.

In order to carry out large scale bioprocess operations using GMMOs, industry has been subject to a series of regulations, culminating, in the EU, in the 1992 European Directive on Genetically Modified Organisms (Contained Use) and the subsequent amendments which came into force in 1995 (Council of European Communities, 1990; Commission of the European Communities, 1994). In principle, these regulations are like many before them in that in order to carry out an operation, there is first a requirement to assess the risks that may be posed by the operation involving the micro-organism, to assign the process to a certain level of containment that can be provided by the operator and to inform the regulatory authorities of the outcome of this risk assessment procedure. If the regulatory authorities, in this country the Health and Safety Executive (HSE), are satisfied that the risk assessment has been correctly carried out and the containment provided is appropriate, then the planned work can take place. However, at the centre of this seemingly simple and effective piece of legislation is the lack of an adequate definition of containment due to the scarcity of practical data available. Since the various levels of containment are not defined in practical terms, say, the number of GMMOs that may be released per unit time, then it is not possible to confidently design bioprocesses in a way such that the number of organisms released is (inversely) related to the hazard posed by the organism. This is due, in part, to the lack of suitable methods for the measurement of release of micro-organisms from bioprocesses. What can be achieved is the use of more elaborate and expensive engineering design with increasing containment levels. Whether this implies more effective containment is another matter, as some argue that more elaborate design might increase the chances of accidental failure or human error giving rise to release.

So, the lack of adequate methods for the quantitative estimation of bioprocess release

has been identified as a problem in the interpretation of existing regulations governing contained use of GMMOs. This problem can be addressed by the development of new methods that do allow bioprocess release to be measured, and hence practical data to be generated. Once such a method is in place, it will then be possible to apply more rationale to plant design and to determine the scale and relative importance of incidental (or operational) and accidental modes of release.

1.2 Regulations

1.2.1 Development of regulations

The Health and Safety at Work etc. Act 1974 (HASAW, 1974) makes general provisions for the health and safety of individuals which apply to all workplaces, including those where micro-organisms are handled. The key clause of HASAW is the legal responsibility of employers under Section 2(1) to ensure, as far as is reasonably practicable, the health, safety and welfare at work of all their employees. The enforcing authority for HASAW is the HSE, which is the executive arm of the policy making body the Health and Safety Commission (HSC). Before HASAW, there were no safety regulations which governed all places of work and the administration of regulations for different industries was governed by different bodies. HASAW can therefore be seen as an overall framework for safety in the workplace; other, more specific, regulations are seen within this context. Interestingly, HASAW adopted the policy of prospective rather than retrospective legislation. It had already been noted that changes in preventative legislation had often been introduced in the wake of previous unforeseen events. The Robens Report of 1972 (Robens, 1972) stated that "in an age of rapid change in industrial structures and technologies as well as in social attitudes and expectations this traditional empirical approach can not keep pace".

The principle of forward planning as a means of preventing accidents in the workplace was further embodied by another piece of umbrella legislation, the Control of Substances Hazardous to Health Regulations (Health and Safety Commission, 1988). A key element of COSHH, which covers all hazardous substances including micro-organisms, is that risk assessment of operations should be carried out before the work is to take place. The risk assessment should highlight the use of any substance which may give rise to a hazard to the operator or environment and should detail any accident control procedures. Regulations specifically regarding the use of pathogens or GMMOs in laboratory or industrial environments should be seen in the light of the general requirements put forward by these two pieces of legislation.

The first attempt to regulate the use of recombinant DNA technology was made at the Gordon Research Conference on Nucleic Acids held in the USA in 1973 (Singer and Soll, 1973). It was generally recognised at this conference that while the recent advances in the production of hybrid DNA molecules heralded the beginnings of a new understanding of fundamental biological processes and human health problems, there was the potential of such molecules to prove hazardous to laboratory workers and the public. Although no actual hazard had been identified, it was considered prudent to treat these potential hazards very seriously. This conference led to the formation of a Committee on Recombinant DNA Molecules, set up by the US National Academy of Sciences, which called for a voluntary embargo on certain types of DNA manipulations (Berg *et al*, 1974). Among the types of experiment forbidden was the introduction of plasmids carrying antibiotic resistance or bacterial toxin synthesis into strains not known to carry these traits and the introduction of segments of oncogenes or animal viruses into bacterial plasmids or other viral DNA. Other types of experiments were allowed, although caution was urged in some cases. This therefore represented the first classification of rDNA manipulation experiments into various classes according to the perceived hazard of the activity (Turner, 1989).

The Asilomar Conference on Recombinant DNA Molecules (Berg *et al*, 1975) concluded that, with regard to DNA manipulation experiments: (i) containment be made an essential consideration in the experimental design; and (ii) the effectiveness of containment should match as closely as possible the estimated risk. The conference defined four containment categories based on the risk involved and went on to match the type of experiments planned with the containment category required. A set of experiments were described which were generally agreed should not be carried out using the containment facilities then available. It was acknowledged at this conference that with the information available, estimating the risks was difficult and intuitive, but would improve as additional knowledge was acquired. The conference was also notable in that it stated that the most significant contribution to limiting the spread of recombinant DNA is the use of biological barriers. These barriers are of two types: fastidious bacterial hosts unable to survive in natural environments; and non-transmissible and equally fastidious vectors (plasmids, bacteriophages or other viruses) able to grow only in specified hosts. Physical containment provides an additional factor of safety. To this end, the conference advocated research into the development of safer vectors and hosts and the survival of recombinant organisms in nature.

With regard to large-scale operations, it was noted that such experiments would seem to be riskier than equivalent experiments done on a small scale and would therefore require

more stringent containment procedures. Consequently, experiments of a scale of over 10 L of culture were included in the list marked 'to be deferred'.

As a consequence of two outbreaks of smallpox, derived from clinical laboratories, in the UK in the 1970s (Schofield, 1992) the Advisory Committee on Dangerous Pathogens (ACDP) was set up. The ACDP defined a system of classification of naturally occurring organisms into four different hazard groups according to a set of criteria that include the pathogenicity of the organism and the availability of prophylaxis. Corresponding levels of containment commensurate with the risk posed by each group of organisms were also described.

In the UK, regulations on the use of GMOs were first made in 1978 - the Health and Safety (Genetic Manipulation) Regulations - and were based on a voluntary code of conduct and backed up by a series of guidance notes issued by the HSE. The Advisory Committee on Genetic Manipulation (ACGM) was set up in 1984 according to the same structure as the ACDP. The ACGM (HSE, 1988) has developed a protocol for the assessment of hazards involved with the use of GMOs. This assessment is based on: (i) access - host vector system, its survival and mobilisation; (ii) expression - a measure of the anticipated or known level of expression of the inserted DNA; and (iii) damage - a measure of the likelihood of harm being done to a person by exposure to a GMO. Each of these factors is given a numerical value ranging from 10^{-12} to 1, according to the assessment carried out, this is known as the Brenner scale, and the product of the three is used to define the category of containment required.

In the years following the Asilomar Conference, as research experience accumulated, so the initial concerns abated, but as the manufacture of proteins with recombinant organisms grew in scale it became necessary to control the larger-scale processes. Much of the guidance on large-scale use is based on the recommendations and conclusions of a major international study by the OECD (1986). The containment measures which the OECD suggested range from Good Industrial Large Scale Practice (GILSP) through a series of three increasingly stringent levels of containment (see Table 1.2). GILSP is an approach to the handling of micro-organisms on a large scale that was based on good industrial practices and was formalised in this report. Although not a containment category *per se*, GILSP could be applied to the handling of certain GMMOs on the basis that the vast majority of organisms used in traditional industries can be regarded as safe, due to an accumulated knowledge of their use, and that by inserting segments of DNA that are well characterised and free from known harmful sequences into such organisms to improve their performance, then the consequent GMMO can be considered

TABLE 1.2 OECD Containment Levels 1 to 3 (OECD, 1986)

Specifications	Containment Category		
	1	2	3
1. Viable organisms should be handled in a system which physically separates the process from the environment (closed system)	Yes	Yes	Yes
2. Exhaust gases from the closed system should be treated so as to:	Minimize release	Prevent release	Prevent release
3. Sample collection, addition of materials to a closed system and transfer of viable organisms to another closed system, should be performed so as to:	Minimize release	Prevent release	Prevent release
4. Bulk culture fluids should not be removed from the closed system unless the viable organisms have been:	Inactivated by validated means	Inactivated by validated chemical or physical means	Inactivated by validated chemical or physical means
5. Seals should be designed so as to:	Minimize release	Prevent release	Prevent release
6. Closed systems should be located within a controlled area	Optional	Optional	Yes, and purpose-built
(a) Biohazard signs should be posted	Optional	Yes	Yes
(b) Access should be restricted to nominated personnel only	Optional	Yes	Yes, via an airlock
(c) Personnel should wear protective clothing	Yes, work clothing	Yes	Yes, a complete change
(d) Decontamination and washing facilities should be provided for personnel	Yes	Yes	Yes
(e) Personnel should shower before leaving the controlled area	No	Optional	Yes
(f) Effluent from sinks and showers should be collected and inactivated before release	No	Optional	Yes
(g) The controlled area should be adequately ventilated to minimize air contamination	Optional	Optional	Yes
(h) The controlled area should be maintained at an air pressure negative to atmosphere	No	Optional	Yes
(i) Input air and extract air to the controlled area should be HEPA filtered	No	Optional	Yes
(j) The controlled area should be designed to contain spillage of the entire contents of the closed system	No	Optional	Yes
(k) The controlled area should be sealable to permit fumigation	No	Optional	Yes
7. Effluent treatment before final discharge	Inactivated by validated means	Inactivated by validated chemical or physical means	Inactivated by validated chemical or physical means

to be unlikely to pose any risk. The OECD report suggested criteria for defining these organisms. How, then, can GILSP and OECD containment category 1, which recommends that release be minimised, not prevented, be considered as containment categories. The answer is that in both these situations the GMMO used must have built-in environmental limitations, i.e. biological containment is operating (see Section 1.3.3).

The sets of large-scale guidelines from the US National Institutes of Health (NIH, 1986) and the ACGM for the UK (ACGM, 1987) are similar in outline to those from the OECD (1986). All are structured into a set of three increasingly stringent levels of containment above a lower level comparable to GILSP. For a comparison of the three sets of guidelines refer to Turner (1989).

The Royal Commission on Environmental Pollution Report (1989) stated that organisms with simple engineered gene deletions cannot be considered safe when released into the environment. However, the implications of GILSP and OECD containment category 1 in large scale manufacture using GMMOs is that some release is inevitable but is not likely to pose any hazard due to the inherent biological containment of the recombinant organism used. This apparent contradiction between different sets of legislation is due to the fact that the RCEP report considers deliberate release into the environment, where the GMMOs released will have at least a limited degree of competence. This should not be the case with GMMOs released into the environment via escape from industrial processes. Hence the difference between the two situations is in the degree of biological containment available.

1.2.2 Current regulations governing large scale work

In 1992 a European Directive, Genetically Modified Organisms (Contained Use) Regulations (90/219/EEC) (Council of European Communities, 1990), was introduced which covers both human health and safety and environmental protection (Health and Safety Executive, 1993). In these regulations recombinant organisms were classified into either of two groups: Group I or Group II. The criteria for classification of organisms into Group I include: the nature of the recipient or parental organisms (i.e. it must be non-pathogenic and have an extended history of safe use); the nature of the vector/insert (i.e. it must be well characterized and poorly mobilizable); and the nature of the GMMO (i.e. it must be non-pathogenic). Organisms that do not satisfy these criteria fall into Group II. Recently an amendment to these regulations has been introduced (Commission of the European Communities, 1994). The main purpose of this amendment is to simplify the procedure for classifying organisms into Group I.

Using these criteria for classification, it is noticeable that an organism can be a Group II GMMO on environmental grounds alone, irrespective of any hazards that it may pose to humans. In order to aid the classification of GMMOs into either Group I or Group II, the ACGM/HSE/DOE Note 7 (HSE, 1993) has provided some guidance utilizing the pre-existing ACGM risk assessment method. For instance, when considering the vector, the access factor, used in the ACGM assessment method, the Brenner scale, will provide some information on whether the vector is poorly mobilizable and well characterized.

The types of operation that can be carried out are similarly divided into two; Type A and Type B. Type A operations are defined as those used for teaching, research, development, or non-industrial or non-commercial purposes and are usually of a small scale (e.g. 10 L culture volume or less). Type B operations are any that can not be classified as Type A.

The level of containment must also be determined for the planned operations. These containment levels are designated as B1 (equivalent to GILSP), B2, B3 and B4 (equivalent to OECD large scale categories 1, 2 and 3, see Table 1.2). For Type B operations using micro-organisms from Group II, the containment levels are chosen from levels B2-B4 as appropriate to the micro-organism and the operation in question in order to ensure the protection of health of the general population and the environment. It is noted that Type B operations should be considered in terms of their unit operations and that each operation should be considered separately in order to ensure adequate and safe containment. It is also interesting to note that the risk and effect of equipment failure are seen as important factors that should be considered in the engineering design.

1.2.3 Implications of regulations on bioprocess design

Current UK and EC legislation is drafted in very general terms. The qualitative terms such as "minimize release" and "prevent release" that are used can not be translated directly into terms used in mechanical engineering design. This is because neither "minimize" nor "prevent" are adequately defined, precluding quantitative specification.

The consequence of this is that when bioprocess systems are designed there is no appropriate engineering standards to adhere to. The interpretation of the design of seals at different containment levels has led to some disagreement (Leaver and Hambleton, 1992; Titchener-Hooker *et al*, 1993) which illustrates the problems that may arise in these circumstances. Chapman (1989) proposed the following relationship between seal specification and containment category:

- single static seal for B2 (equivalent to large scale containment category 1, LSCC1)
- double static seal for B3 (LSCC2)
- double static seal with barrier fluid/steam trace for B4 (LSCC3).

However, this interpretation of the legislation has been criticised as a simplistic model lacking any real validation. Titchener-Hooker *et al* (1993) have pointed out that the assumption that two static seals are better than one is not necessarily valid. Double static seals can be criticised on several counts: they are more difficult to assemble correctly and so risk of failure may be increased; it is difficult to detect failure of either seal in operation; both seals will have similar histories therefore the possibility of simultaneous failure might be higher than anticipated; and the dead space between seals may create sterility problems. Titchener-Hooker *et al* also point out that US practice is to use single static seals at BL2-LS (roughly equivalent to B3). The interpretation of the regulations by Chapman (1989) may therefore lead to the adoption of inappropriate, and possibly costly, engineering features.

The source of this conflict of opinions has been identified as the lack of quantitative data on seal failure rates and failure-modes and on the extent of release that a failure causes. In Germany the approach to design of bioprocess systems required for certain levels of containment is to provide guidelines which describe in detail the engineering features necessary (Titchener-Hooker *et al*, 1993). However, this approach does not allow design flexibility or improvement as the optimum design today may be superseded by a more efficient solution in the future. The European approach to biotechnology standards for equipment, whose introduction is the responsibility of the Comité Européen de Normalisation (CEN), the European Committee for Standardisation, is therefore centred on performance rather than design criteria. One of the objectives of CEN is to implement a series of performance criteria relating to specific types of equipment such as homogenizers and shaft seals. The standards that are set should therefore reflect the degree of containment intended, i.e. that no detectable release will occur when high risk organisms are used and that some release is tolerable with low risk processes. Kirsop (1993) has stated that assigning numerical values to situations where some release is acceptable is extremely difficult, especially because there are few data available for existing processes.

It is apparent, therefore, that there is a real need for a programme designed to quantitatively assess the release of micro-organisms from bioprocess equipment under a variety of conditions. When there is data available on the scale of accidental and incidental (inherent or routine) release of micro-organisms from such equipment, then

performance criteria can be more confidently written. As a consequence, contained processes may be designed with reference to a measurable set of performance parameters.

1.3 Hazards associated with the release of GMMOs

This section will deal with the potential harm that may be caused by the inadvertent release of GMMOs from bioprocesses. The prospect of up to 10^{17} recombinant organisms being inadvertently released into the environment during some type of catastrophic industrial accident has ignited considerable interest in the determination of the likely consequences of such an event. The potential to cause harm to the environment and to human health by release of GMMOs will be discussed. In addition, the ability of GMMOs released into the environment, to transfer some part of their recombinant sequences to indigenous species will be assessed.

However, it is important to consider such release in the light of the regulations governing contained use. For instance, those GMMOs that might be used in situations where some release is likely (i.e. conditions of GILSP), must have been assessed and regarded as safe. Also, it is necessary to consider biological containment when discussing release. The potential for harm that might occur on release of a GMMO is clearly dependant on its ability to survive outside of, for example, the fermenter as well as its pathogenicity and environmental impact.

One final aspect to be covered is the attitude of the public to inadvertent release of GMMOs. There is a degree of public concern over the use of genetic modification techniques (see, for example, the Eurobarometer survey carried out by the Commission of the European Communities (1993)), and this is likely to be exacerbated by any well publicised accidents involving the technology.

1.3.1 Environment

There is a great deal of literature concerning the environmental implications of deliberate release of GMMOs for purposes such as improvement of crop growth, protection of crops against pests and frost damage, and biodegradation of toxic compounds in the environment (Trevors *et al*, 1987; Mullis, 1990; Bej *et al*, 1991d; Pickup *et al*, 1991; Sayre and Miller, 1991; Israeli *et al*, 1993; Iwasaki *et al*, 1993; Lenski, 1993). Although deliberate release of GMMOs to carry out a predetermined function in the environment has different implications to inadvertent release from bioprocesses, there are a number of features that are common to the two situations. The main difference between the two

scenarios is in the type of GMMO released, in bioprocess release from a GILSP process the GMMOs will be generally regarded as safe, and unlikely to survive in the environment, i.e. they will be biologically contained. Deliberate release organisms must play some role in the environment into which they are released, otherwise there would be no point in the operation. Biological containment of process organisms will be discussed in Section 1.3.3. For now, the general considerations applicable to any GMMO released into the environment will be outlined.

In general, there are two types of potential hazard involved in the introduction of GMMOs into the environment: (i) host cells containing the rDNA could demonstrate previously unknown pathogenicity, presenting the risk of infection to plants and animals; and (ii) GMMOs or subsequent hosts for the rDNA may have detrimental effects on natural ecosystems, giving one organism a selective advantage that would alter the microbial consortium and potentially the nutrient cycle, energy flow and ecosystem properties. Of particular concern for risk assessment is the fact that micro-organisms are self replicating entities, so that it may be impossible to control an adverse effect simply by discontinuing further releases of the recombinant organism. Moreover, it has been noted that deliberate release or accidental spillage will ultimately lead to some GMMOs becoming dispersed through run-off water systems into lakes and streams (Barnthouse and Palumbo, 1986).

The possibility of unforeseen pathogenicity arising from the introduction of rDNA into an organism is a remote one. Knowledge of the molecular basis of pathogenicity indicates that several specific genes working interactively are required if a bacterium is to cause an infectious disease (Smith, 1989). Concern has therefore focused on the effects that GMMOs might have on the environment, and the possibility of transfer of rDNA by conjugation, transduction or transformation.

Predicting the effect of introduction of GMMOs on the environment is no easy matter. Pickup *et al* (1991) have pointed out that field trials of released GMMOs have produced few surprises in terms of the behaviour of the release strain and the environmental effects caused by such a release. Such studies have provided some comfort for those who attempt to predict the behaviour of GMMOs in the environment by extrapolation from microcosm studies. Other reports however, do demonstrate that the unexpected may occur. Short *et al* (1991) examined the response of natural soil communities to the introduction of a plasmid-bearing or plasmid-less strain of *Pseudomonas putida* into soil contaminated with a substrate for plasmid encoded functions. The unanticipated appearance of a toxic intermediate (2,4-dichlorophenol) in the degradation of 2,4-

dichlorophenoxyacetate by the plasmid bearing organism corresponded with a significant change in the community structure, most notably a greater than 400-fold decline in fungal propagules. Altered function was also indicated by a marked reduction in the rate of CO_2 production. These effects were not observed after introduction of the plasmid-less strain and were not seen in a different soil type. Therefore it is important to consider, and difficult to predict, the interactions between introduced organism and the specific environmental and community response.

With regard to the release of GMMOs from bioprocess systems, it has been calculated that, due to the significant atmospheric dilution effect, a release will result in the arrival at niches on ground level of single organisms (Winkler and Parke, 1992). However, this calculation is somewhat simplistic as it does not take into account the likelihood that larger aerosol particles will each contain a high number of micro-organisms and that such particles will sediment very quickly. The probability of finding a suitable niche depends upon the host organism used; *Bacillus* species are far more likely to find such a niche than, say, *E. coli* K-12. In addition, the chances of survival in aerosols should be considered (see Section 1.4.3.2); bacterial spores and Gram-positive bacteria with a high internal osmotic pressure survive better in aerosols than Gram-negative bacteria (Winkler and Parke, 1992).

When the potential hazards of releasing GMMOs into the environment were identified (earlier in this section), it was noted that GMMOs or subsequent hosts for the rDNA may have detrimental effects on natural ecosystems. The phrase "subsequent hosts" implies that there is a concern that rDNA will be transferred in the environment from the GMMO to indigenous micro-organisms. Such "horizontal gene transfer" has recently been implicated in the emergence of a novel epidemic *Vibrio cholerae* strain (Mooi and Bik, 1995). Horizontal gene transfer may feasibly occur by conjugation, transduction or transformation.

Conjugation is the transfer of plasmid DNA through cell-to-cell contact between both donor and recipient cells. Since conjugation involves no extracellular nucleic acid, the DNA is protected from degradation by nucleases or heavy metals. Plasmid transfer by conjugation has been demonstrated, under certain conditions in a variety of environmental situations (Pickup *et al*, 1991) although the presence of indigenous flora generally reduces the frequency of transfer (Trevors *et al*, 1987; Neilson *et al*, 1994). Conjugation has assumed significance in studies of gene transfer in nature as a result of recent findings indicating a wider host range for this process than previously thought. In a review of the subject of gene transfer between distantly related bacteria, Mazodier and

Davies (1991) have surmised that "conjugation is likely to be the most effective means of transfer between a wide range of bacterial species, and even eukaryotic microbes".

Transduction is the transfer of bacterial genes by bacteriophages, which act as naturally occuring 'syringes' in picking-up, carrying and injecting DNA into a new host. Transfer would have to occur between host populations subjected to infection by the same phage strain; the narrow host range of most phages reduces the likelihood of gene transfer within a mixed bacterial population. Plasmid DNA has been shown to be transduced although, again, the presence of natural microflora reduced the transfer frequency (Saye *et al*, 1987).

Active DNA transfer, that is by conjugation or transduction, is thought to be unlikely to occur from organisms at GILSP level because of the requirement that these GMMOs do not carry self-transmitting plasmids or transducing phages (Winkler and Parke, 1992). However, because bacteriophages are known to be present in the environment (Bergh and co-workers (1989) have shown that in aquatic environments there may be as many as 10^8 particles mL^{-1}), it is possible that transduction could occur following the uptake of a phage into a GMMO.

The process in which free DNA is taken up from the environment into a cell is known as transformation. The presence of extracellular DNA in water and soil environments has been shown (Pickup *et al*, 1991), although the stability of the DNA may be low. In addition, strong non-specific adsorption of DNA to clay or humus particles is likely to delay the process of transformation (Winkler and Parke, 1992), although it can protect the DNA from degradative enzymes and thereby enhance the stability (Paget *et al*, 1992).

An important point to note is that even if a gene is moving at random through an interconnected, mixed, microbial population it is unlikely that the transconjugant will benefit unless there is an appropriate selection pressure. The detection of plasmids encoding antibiotic resistance has been directly correlated with the developing use of antibiotics and their release in the environment (Anderson, 1975). Davies and co-workers (Webb and Davies, 1993, 1994) have recently proposed that the spread of antibiotic resistance that has occurred since the onset of widespread use of penicillin in the 1940's may have come about through transformation. By using the polymerase chain reaction (PCR, see Section 1.5.2.2) it has been shown that a number of antibiotic preparations are contaminated with DNA from the organism used in the fermentative production of the antibiotic. Since the DNA of the production organism will inevitably include antibiotic resistance genes, then it becomes apparent that these resistance

determinants are co-administered with the antibiotic. Davies and his group propose that under the simultaneous selection pressure of the antibiotic, uptake of one or more resistance genes by members of the microbial population of the host would lead to antibiotic resistance organisms being constructed. Subsequent inter- and intraspecific transfers would permit other microbes to become resistant to the antibiotic. Notably, Frischer *et al* (1994) have demostrated the transfer of plasmid DNA to members of indigenous marine bacterial populations by natural transformation, the first report of this process for any microbial community. The findings that non-culturable cells may be implicated in gene transfer (Higgins, 1992) and that antibiotics may act as agents actively promoting bacterial gene transfer (Mazodier and Davies, 1991) lend weight to the theory.

It is worth recalling at this point that, currently, the majority of GMMOs used in bioprocesses contain antibiotic resistance genes encoded on plasmids and that plasmid stability is maintained in the fermenter by the application of a selective pressure: the use of antibiotics. If a release of GMMOs were to occur then it is possible that it would be accompanied by resistance encoding genes and the antibiotic itself. Although it has been shown that *E. coli* K-12 does not transfer plasmid encoded antibiotic resistance, even in the presence of a selection pressure ((Levine *et al*, 1983) and Section 1.3.2), if the plasmid were to exist as free, extracellular DNA, then this situation would resemble that which Davies and colleagues postulate gives rise to the spread of antibiotic resistance (Webb and Davies, 1993, 1994).

It can be concluded therefore, that there are methods whereby rDNA from a GMMO could feasibly be transferred to indigenous micro-organisms. However, the likelihood of this type of event, considering the chances of survival of the GMMO in the environment and the absence of self-transmitting plasmids or transducing phages is thought to be low. A proposed mechanism for spread of antibiotic resistance does illustrate that rDNA may be exchanged within a host organism and subsequently disseminated throughout the environment. However, a recent study on the field release of a genetically modified *Pseudomonas fluorescens* strain has shown that transfer of marker genes to indigenous micro-organisms was not recorded (Thompson, *et al*, 1995).

1.3.2 Health

Many of the issues that concern the possibility of deleterious effects on human health from the release of GMMOs are common to those in the environment. For instance, the question "can a harmless microbe accidentally become pathogenic by the introduction of rDNA" is equally valid, and the answer is again that pathogenic determinants are generally controlled by several genes acting in concert and so introduction of a well characterised piece of rDNA is very unlikely to give rise to an unexpected pathogenicity. This premise has been validated in a series of experiments (Winkler and Parke, 1992).

However, the work of Avery, Macleod and McCarty (1944) in demonstrating that DNA is the genetic material is interesting to note at this stage. The landmark discovery was made by showing that when a mixture of a heat killed pathogenic strain of *Pneumococcus* and a live, attenuated, non-pathogenic strain was injected into mice, the mixture was lethal to the mice and live pathogenic cells were recovered from the blood. Live pathogenic organisms were therefore generated *in vivo* by transfer of genetic material, in this case a single gene which encoded an enzyme for the synthesis of capsular polysaccharide. Although this pathogen arose after transfer of DNA from known pathogenic sequences, a situation that would not occur with GMMOs, the principle that pathogens can arise in this way should be noted.

Although it can be anticipated that organisms, such as *Bacillus subtilis* and *Saccharomyces cerevisiae*, that have no known history of pathogenicity are unlikely to cause any harm to human health, it would seem paradoxical that one of the most commonly used host strains for genetic modification work, *E. coli* K-12, was isolated from the faeces of a diphtheria patient. However, this particular strain was isolated in 1922 and years of laboratory cultivation have led to the loss of the K and O antigens, weakening, or attenuating, the strain so that it has lost the ability to grow in its natural habitat (Bogosian and Kane, 1991; Muth *et al*, 1993). Because so much is known about the physiology, biochemistry and genetics of this strain, it has become the workhorse of genetic modification technology. Since *E.coli* K-12 is so widely used, an extensive amount of work has occurred in an attempt to determine its ability to survive outside of bioprocess streams, i.e. its biological containment. This will be considered in Section 1.3.3, for now the general principles of potential dangers to human health will be discussed.

Apart from the possibility of unforeseen generation of pathogenic organisms, the other potential dangers to health can be identified as: (i) the ability to transfer DNA to animal cells; (ii) the ability to transfer DNA to the commensal flora; and (iii) the risk from an

rDNA gene product (Winkler and Parke, 1992). Clearly, there is a strong relationship between all of these three criteria and the ability of the GMMO to survive and multiply within the human body. If an organism cannot establish itself in humans then it is less likely that any of the other criteria will give cause for concern, however the potential for extracellular, naked DNA to spread to other organisms has been highlighted by the proposed mechanism of spread of antibiotic resistance (see Section 1.3.1).

The possibility of DNA transfer to animal cells has been studied by Israel *et al* (1979). This work showed that when mice were injected with *E.coli* K-12 with the complete genome of polyoma virus inserted, there was no infection with the equivalent of 10^{12} virus particles. However, the average infective dose for native polyoma virus is 200 particles and for naked polyoma virus DNA is around the equivalent of 10^{7} virus particles. DNA packed into *E.coli* K-12 is therefore at least 10^{9} times less dangerous to mice than the virus particles. Using a wild type *E. coli* with polyoma virus inserted, it was shown that although the organisms survived in the gut of mice for 6 weeks, there was no illness or antibody production against the virus. The probability of transfer was estimated at less than 2 in 10^{13} per bacterium per day. In *E. coli* K-12, which does not colonise the gut, the probability of transfer would be even lower. With regard to DNA transfer to the commensal flora the concern is mainly limited to transfer from *E. coli* K-12 to wild type *E. coli* or Enterobacteriaceae.

In an extensive review on the subject, Bogosian and Kane (1991) conclude that strains of *E. coli* K-12 do not persist in the mammalian intestinal tract, although they could, in some circumstances, colonise germ-free or antibiotic treated rats and mice. This is presumably due to the inability of *E. coli* K-12 to complete with the indigenous microflora, however it infers that *E. coli* K-12 strains are not completely defective in colonisation ability. The studies reviewed suggest that *E. coli* K-12 strains do not survive in the mammalian intestinal tract long enough to participate in active conjugational transfer. Levine *et al* (1983) showed that wild type *E. coli*, harbouring a self-transmitting plasmid as well as the non-mobilizable plasmid pBR325, did not transfer the tetracycline resistance carried by pBR325, unless selection was applied by giving tetracycline. *E. coli* K-12, carrying both plasmids, did not transfer the resistance even with the selection pressure. This is seen as a decisive result as the conjugation for introducing the self-transmitting plasmid had already taken place in the laboratory. The risk of DNA transfer, from appropriately constructed GMMOs, to commensals *in vivo* appears negligible. Again, with reference to the proposed mechanism of spread of antibiotic resistance (Section 1.3.1), the potential for uptake of free DNA, derived from GMMOs, should not be overlooked.

The possibility of rDNA products being expressed inside the body and causing harm also seems a remote one. It has been calculated (Gilbert, 1981) that if the gut were completely colonised with *E. coli* K-12 producing insulin, growth hormone or interferon, then the quantities would be much too low to have any effect, even if the proteins were not degraded in the gut. In addition, the question of whether *E. coli* K-12 producing an animal protein could induce the production of antibodies and lead to autoimmune disease has also been addressed. In this case it was concluded that the quantities of antigen entering the host would be much too small (Paterson, 1981).

1.3.3 Biological containment

So far, the risk of deleterious effects arising from the release of GMMOs from bioprocesses has been considered and found to be very low. In addition to the likelihood for harm to occur once an organism is released from the fermenter into the environment, the chances of survival of that organism should also be assessed. The use of recombinant organisms that have a low chance of survival outside of the designed bioprocess streams has long been advocated (Berg *et al*, 1975). The inability of recombinant organisms to survive outside of the bioprocess, termed biological containment or biological disablement, provides an additional layer of safety and will now be considered.

Organisms that display built-in environmental limitations, such as sensitivity to UV light, inability to colonise certain niches and inability to survive in aerosol droplets, are said to be 'biologically contained'. Because there is no physical barrier that actually prevents the release of such organisms, and their inherent safety is due to their, at best, limited capacity to survive outside the bioprocess stream, then the term biological disablement is preferred by some authors (personal communication; Prof. M. K. Turner, Dept. of Chemical and Biochemical Engineering, UCL).

As mentioned earlier, strains of *E. coli* K-12 are the most commonly used host organism for genetic manipulation work. It has been established that *E. coli* K-12 strains lack the ability to colonise the conventional mammalian gastro-intestinal tract and are very unlikely to take part in rDNA transfer events with either mammalian cells or the commensal flora. Recently, a whole series of papers have been published which demonstrate that fermentations with commercially relevant *E. coli* K-12 strains do not present any environmental hazards (Bogosian *et al*, 1993; Heitkamp *et al*, 1993; Kane, 1993; Muth *et al*, 1993; Yancey *et al*, 1993).

Using PCR, Bogosian *et al* (1993) showed that an *E. coli* K-12 strain, containing the pBR322 based plasmid pBGH1, which is used by Monsanto Company for large scale

production of bovine somatotrophin (BST), had not transferred pBGH1 or the portion of pBGH1 including the BST structural gene to indigenous microbial inhabitants of the Missouri River in a microcosm study. This may not be surprising given the requirements needed to transfer a pBR322 based plasmid from an F^- strain.

Heitkamp *et al* (1993) described how a recombinant *E. coli* K-12 strain, could not establish itself in the microbial sewage community despite attempts to favour such an event. Muth *et al* (1993) demonstrated that an *E. coli* K-12 tetracycline-resistant host vector used by Eli Lilly for the production of BST was unable to survive in rats even when the antibiotic was included in the feed. Similarly, Yancey *et al* (1993) found that an *E. coli* K-12 host vector of the Upjohn Company was unable to colonise the intestinal tracts in conventional antibiotic-treated mice. Using hybridization studies it was also shown that there was no evidence for gene transfer from plasmid or chromosomal DNA of the recombinant organism to indigenous *E. coli* strains. All of these studies indicate that there is no intrinsic difference, with respect to biological containment, between commercial and laboratory *E. coli* K-12 strains. Furthermore, growing these strains to high cell densities does not confer upon them any advantage to survive outside the laboratory.

An alternative approach to biological containment is to incorporate into the GMMO an active mechanism for killing the cell outside of the intended contained environment. Many of these suicide mechanisms utilise a family of homogenous proteins: *hok* (Molin *et al*, 1987); *gef* (Jensen *et al*, 1993); and *relF* (Knudsen and Karlstrom, 1991), all of around 50 amino acids, whose function is unknown but are lethal when expressed in a variety of bacterial species, although other mechanisms have been used (Ahrenholtz *et al*, 1994; Munthali *et al*, 1996). The condition triggering suicide may be transfer of plasmids to wild-type strains, reduced growth rate or temperature or absence of a chemical compound outside controlled areas. This approach has the advantage that the suicide mechanism may be incorporated into different 'healthy' laboratory strains or production strains; debilitated organisms need not be used. An obvious benefit of this can be seen in deliberate release procedures, but it may also have application in contained processes whereby organisms other than, say, *E.coli* K-12 may be preferred for optimisation of production, although suicide systems have been developed for *E. coli* K-12 strains to further enhance their biological containment (Schweder *et al*, 1995). Many of the suicide mediated biological containment systems have suffered from the disadvantage that a considerable fraction of cells survives the induction of the suicide function. Knudsen and Karlstrom (1991) demonstrated that the factors limiting killing were the mutation rate of the suicide function and the reduced growth rate caused by a

basal level of expression of the suicide gene during normal growth, which can give a selective growth advantage to cells with mutated suicide functions. By tightening of the repression system it was possible to demonstrate an improved killing efficiency. Similarly, Jensen *et al* (1993) demonstrated that the rate of appearance of mutants resistant to killing decreased from 10^{-6} per cell per generation to 10^{-8} per cell per generation on introduction of two copies of the killing cassette per cell.

Biological containment may therefore be achieved by either of two general methods: use of micro-organisms that are inherently unfit outside of containment; or addition of an active suicide mechanism. The former method is currently predominant as *E. coli* K-12 is the most used host organism for genetic manipulation work.

1.3.4 Public perception

Although the benefits arising from the use of genetic techniques have been widely proclaimed, often comparing the applications in scope and impact to those provided by information and computer technologies, there is a certain amount of scepticism and hostility on the part of the public at large (Commission of the European Communities, 1993). This is despite the fact that biotechnology regulators are attempting to avoid the problems which have arisen in, for example, the chemical and nuclear industries by anticipating new hazards likely to arise from emissions and waste products. It is also notable that industry has been watchful of public attitudes over the use of genetic modification technology since, ultimately, consumption will depend upon acceptance. An illustration of the promoters of biotechnology's awareness of public perception is the renaming of the genetic alteration itself. Until recently, official documents and regulatory bodies have tended to adopt the terms genetic 'manipulation' or genetic 'engineering'. Because of the sinister nuance of these terms they have been replaced by 'modification', which presents GMMOs as merely modified, as a modest evolutionary step. Levidow and Tait (1992) have noted that there are three metaphors for GMMOs - as a reprogrammed genome, as a modest evolutionary extension, and as an enhanced natural efficiency. By portraying biotechnological creations as rooted in natural or familiar processes, such as plant breeding and beer brewing, these metaphors support industry's claim to be developing environmentally-friendly products.

However, public fear of genetic modification work in general is raised by incidents where researchers have given insufficient thought to safety considerations. For instance, the prohibition of a research programme at the University of Birmingham in February 1994 by the HSE received broad press coverage. In this work (Bown, 1994) the researchers had inserted oncogenic sequences into viruses, including an adenovirus similar to those

which cause the common cold, in an attempt to transform cultured human cells. Although the virus was disabled by deletion of genes necessary for replication, the HSE feared that the disabled oncogene carrying virus might be capable of repair if it entered a human cell already containing wild-type adenovirus. Judging that the containment provided was insufficient for the risks posed by the work, the HSE issued a prohibition order. Unsurprisingly, the media picked up on the story of the highly contagious common cold-like virus containing oncogenic sequences, and the apparent lack of containment.

There are many instances of planned deliberate release that have similarly aroused public anxiety (Hodgson, 1990; Coghlan, 1994). The attitudes of green pressure groups ranges from one of total opposition to willingness to aid in the framing of regulations and the monitoring of their operation (Hill, 1992). Public anxiety, most clearly articulated by ecologically concerned groups, relates to the poorly understood complex interactions of organisms and physical conditions such as climate and soils, so that it is very difficult to predict entirely how an organism with altered genes will fare. In addition, there are many examples of plants and animals which, when transported to different ecosystems, have had a competitive advantage over the native flora and fauna, and have turned into major pests. In this country such examples include Dutch elm disease (a fungus), rhododendrons, Japanese knotweed (Hill, 1992), the myxoma virus and the American crayfish.

Allied to these ecologically based fears is a scepticism, on the part of some groups, of the motives of multinational biotechnology companies. It has been argued (Levidow and Tait, 1992) that the use of biotechnology will make farmers more dependent upon expensive inputs and dispossess many of them, as they lose the ability to use traditional methods to control their crops. There are related concerns that industry models of agricultural efficiency may result in pests and diseases adapting to biotechnology's single-gene solutions: the familiar 'chemical treadmill' would be replaced or supplemented by a 'genetic treadmill'.

Most of the concerns for the release of GMMOs into the environment have been related to deliberate release or release of organisms that might, in the public's eyes, be perceived as highly pathogenic. How does accidental or incidental release of process GMMOs fit into the public awareness? Because the regulations for large scale use anticipate some release of organisms that are considered safe, then there appears to be a tacit admission that the limited release of these organisms into the environment is not considered a matter of public concern. With higher risk organisms, the containment provided should

avoid any release into the environment, even in the event of a catastrophic accident. Whether provision can be made for incidents such as terrorism or aeroplane crashes is another matter. The point is that if an inadvertent release of organisms that are allowed to be used only under a high degree of containment into the environment were to occur, the implications to public perceptions of genetic modification could be severe.

1.4 Release of GMMOs from bioprocesses

1.4.1 Types of bioprocess

Industrial bioprocesses, such as the recovery of intracellular microbial enzymes, are made up of a sequence of unit operations which transform the input materials into the recovered product. The steps involved in the purification of a microbial intracellular protein are shown in Figure 1.1, where they are contrasted with the recovery of a protein excreted by micro-organisms. In terms of biosafety, each unit operation may pose a different level of hazard, but in general the greatest risks are thought to occur from the time the broth leaves the fermenter to the post precipitation stages (Dunnill, 1982).

The bioprocess as a whole can be represented in a simplified block diagram with a number of unit operations connected to the external environment through its inputs and outputs (Turner, 1989). Each of the unit operations has inlets, which receive the input materials, and outlets, which pass material to the next stage. If the outlet discharges directly to the environment, then the operation is said to be open. If, on the other hand, there is no outlet to the external environment then the operation is said to be closed.

Closed systems can be achieved by either of two approaches; primary and secondary containment. In the former, containment is provided by appropriate choice of equipment, that is, equipment that by its very design will not allow escape of products to the environment. Secondary containment involves the use of a physical barrier, such as the design of the facility, that surrounds the process and isolates it from the external environment. This method can therefore be used to contain unit operations that may be incapable of affording primary containment. Inevitably with secondary containment, the outer barrier will be breached at some point by personnel. An important aspect of operating under any system of containment is therefore that standard operating procedures are correctly drawn up and followed, so that containment is as effective as possible. The fact that containment is not absolute and can never be entirely achieved should be acknowledged, the term closed containment is realistically an engineering design concept (Turner, 1989).

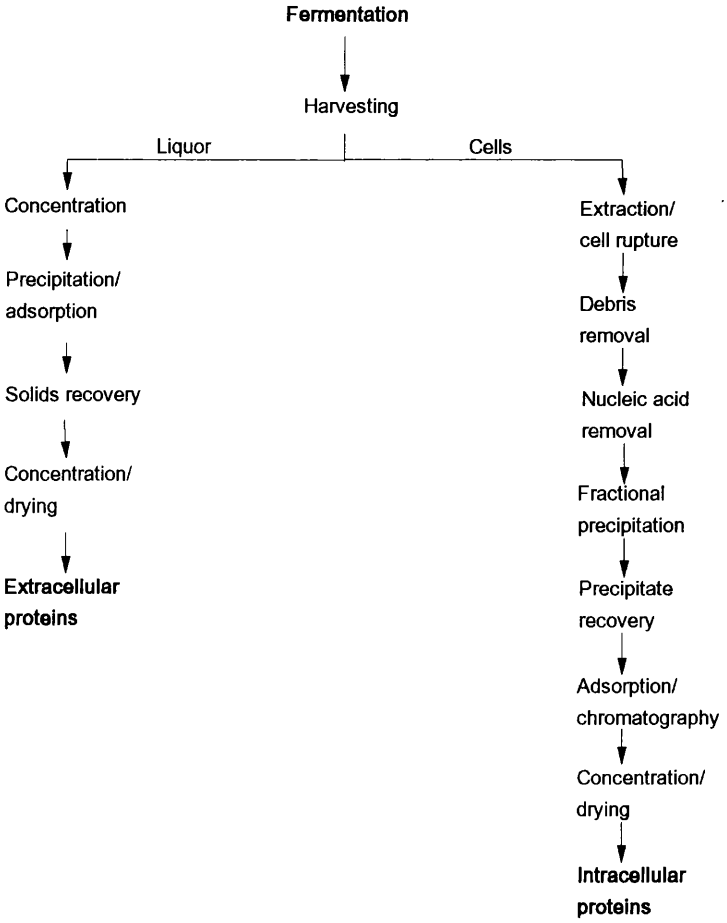


FIGURE 1.1 Comparison of the isolation of extracellular and intracellular microbial proteins (from Dunnill (1982))

1.4.2 Operations causing release

Many of the various unit processes of manufacture have the potential to generate biohazards. There have been several reported incidents where the use of centrifuges has caused health problems to the workers. In 1938 the improper use of an enclosed tubular bowl centrifuge led to the generation of an aerosol of *Brucella abortus* which was disseminated throughout a building, infecting 45 people, one fatally (Hambleton *et al*, 1992). In a French pharmaceutical factory, severe allergic effects in four workers were caused by the generation of a tuberculin aerosol by a centrifuge (Hambleton *et al*, 1992). Here, at University College London, Dunnill (1982) has reported that in the 1960's five workers were affected by *Pseudomonas aeruginosa* cell debris during a tubular bowl centrifugation operation. In this last example, modification of the type of centrifuge used has been successful in preventing any recurrence of this type of incident. While causes of individual incidents of release can be rectified, and safety design considerations of plants have been well documented (Vranch, 1992), there still exists a certain capacity of unit operations in bioprocesses to release organisms or potentially allergenic cellular debris. Other operations that may give rise to release include homogenization, fermenter

sampling and even the turning of a valve on a pipeline.

From the examples quoted above, it is clear that the release of cellular debris as well as viable micro-organisms may pose a threat to human health. However, because the prime concern here is to generate data for assisting in interpretation of the guidelines covering large scale use, and these guidelines specifically refer to the release of viable micro-organisms, then this is the only aspect that will be considered.

1.4.3 Forms of release

The manner in which a release of micro-organisms might occur is a very important factor as it will determine the potential of the release to cause harm and will influence the choice of detection method. If a bioprocess is running, there are two general ways in which an organism may be released: (i) incidental, that is the inherent release characteristic of the equipment due to the lack of 100 % efficient containment; and (ii) accidental where a single (or series of) unforeseen event(s), often caused by human error, come into play. The general interpretation given to the regulations (see Section 1.2) is that incidental release of organisms should be very strictly controlled, and so the measurement of this type of release will form a major goal of this project. However, whether continuous low level (incidental) release or occasional high level (accidental) release poses the most significant hazard to health and the environment is an important question. Only when sufficient data that enables accurate quantification of the incidental release from bioprocesses is available, can the relative contributions to overall release from incidental and accidental sources be assessed. At that stage it will be possible to determine the most important potential mode of release and to design bioprocess equipment accordingly.

Before moving on to the forms of incidental release, some of the possible accidents that may occur in bioprocesses resulting in release of GMMOs will be discussed. Ashcroft and Pomeroy (1983) considered four different accidents that might occur during the operation of a fermenter: failure of the exit gas filter; failure of the antifoam supply; failure of the pipework connecting the fermenter to the downstream operations; and breakage of the culture vessel. By simulating these events, using a 1 L fermenter with 500 mL of culture, and detecting release of aerosol and spillage by methods which require culture (and are therefore likely to underestimate the number of viable organisms released, see Section 1.5.1.1), they found that the number of cells released in liquid was between 2×10^{10} and 4×10^{12} and in aerosol was nil (in the case of filter failure) to 5×10^{7}. In terms of aerosol release, the percentage was greatest in the cases of anti-foam failure and, when the fermenter was of metal construct, vessel rupture. There are

numerous other scenarios that could be imagined for accidental release of micro-organisms ranging from the far fetched, such as aeroplane crashes and terrorist activity, to the more mundane, such as safety valve rupture and transmission via workers' clothing. Although spillages and leaks can cause gross local contamination of equipment surfaces and buildings, uncontrolled release beyond the production area can be limited by appropriate facility design and the use of effective decontamination procedures. Personnel exposed to such emissions are generally at risk of being contaminated by ingestion or skin contact. These are both relatively easily avoided by the provision and use of suitable protective clothing and the application of appropriate instructions for work (standard operating procedures). The possibility of discharge of incompletely inactive effluent, containing GMMOs, should also be considered as a potential source of release (Hambleton *et al*, 1992). However, it should always be the case that any inactivation processes are fully validated.

A lot of attention has focused on incidental release of micro-organisms in aerosols, since, in such a state the released organisms may pose a threat to health and the environment and can not be easily contained (Hambleton *et al*, 1992). In addition, bioaerosols are implicated in the transmission of disease in a variety of different situations ranging from sewage worker exposure to 'sick building syndrome' (Griffiths and DeCosemo, 1994). Studies have shown that airborne micro-organisms can be detected at a distance of 1.2 Km from sewage treatment plants (Adams and Spendlove, 1970). It is now widely acknowledged that aerosols may form as a result of large scale laboratory based microbiological procedures (Brunius, 1992). Any process such as stirring or bubbling results in the formation of a thread of liquid which subsequently breaks down into small droplets which then evaporate to form an aerosol. The effectiveness of any particular aerosol to cause infection or escape containment will depend upon a range of characteristics of that aerosol. Sampling methods should therefore take such parameters into account.

1.4.3.1 Properties of aerosols

The properties of aerosols that are important in terms of biosafety are the concentration of hazardous material and the particle size distribution. In terms of micro-organisms, their concentration in the process liquid will be greatest towards the end of the fermentation and in the early stages of downstream processing, this will be reflected in the composition of any aerosols produced.

For the majority of biological aerosols, aerodynamic diameters are generally greater than 2 μm (Upton *et al*, 1994). The particle size distribution in an aerosol is dependant on the

manner in which the aerosol was produced and the nature of the liquid from which it is derived. The size of particles in an aerosol is critical in determining the length of time a particle will remain airborne. The larger aerosol droplets (>5 μm) sediment rapidly and the organisms they contain may contaminate the bench and the operators hands. Smaller droplets dry rapidly in the air and leave the microbial particles suspended. If particles of less than 5 μm diameter are inhaled, they may reach the alveoli and cause infection, larger droplets are filtered and removed in the upper respiratory tract (Collins, 1992).

Dispersal of micro-organisms in an aerosol depends on the properties of the aerosol, as discussed, and also on the factors acting on the aerosol. These factors are principally the wind, or air movement, temperature and humidity in the environment into which the aerosol has been released. The interplay between these will determine the likelihood of release of GMMOs into the environment or respiratory tract, once aerosolized.

1.4.3.2 Survival of GMMOs in aerosols

Transmission of viable GMMOs to the environment from bioprocesses via aerosols can only occur if the GMMO has the capacity to survive in the aerosolized state. Because of the recognition of the fact that disease can be spread by airborne micro-organisms, there has been a considerable amount of work directed at determining the ability of naturally occurring organisms to survive in aerosols, and the factors that influence their survival. Cox (1989) reviews much of this work and identifies many of the stress factors that organisms are likely to encounter in the aerosolized state. He points out that because changes in water content occur for all aerosolized micro-organisms, then this is likely to represent the most fundamental potential stress. The combined effect of stress caused by the relative humidity (RH) and temperature of the environment into which the aerosol is released probably affect the outer phospholipid membrane of gram negative organisms.

Apart from RH and temperature, the other stress sources that are likely to affect the survival of the micro-organisms are oxygen, ozone, radiation (UV, x-rays, γ-rays) and 'open air factor' (OAF). OAF, caused by olefin-ozone reaction products (Cox, 1989), was identified as the factor in fresh air that caused reduced survival of micro-organisms trapped in particles on microthreads when compared to 'aged' or contained air.
The ability of an organism to survive (whether culturable or not) is affected by a combination of all the stresses acting at any one time. Different organisms will react differently to each particular set of conditions. The response of a single particular strain of *E. coli* to a single variable, RH, is surprisingly complex (Cox, 1989). The pattern that emerges is one of critical narrow RH bands where loss of viability of *E. coli* is very much greater than that for adjacent regions, but generally viability is greatest at lower RH

values. *Franciscella tularensis* shows different RH versus survival patterns depending on whether the aerosol was disseminated from the wet state (where survival is greatest at high RH values) or the dry state (where lower RH is preferred). This infers a further point; that aerosol survival may be a function of suspending (or spray fluid) composition.

Bennett and Norris (1989) have noted that many of the findings reviewed by Cox are based on studies where the micro-organisms have been exhaustively washed of media prior to aerosolization. Because such cells are generally not representative of those used in bioprocess operations, care should be taken in extrapolating the findings to the incidental release scenario. Marthi *et al* (1990) showed that when cells were aerosolized in large droplets, unwashed cells, which had previously been grown in Luria-Bertani broth, survived significantly better than cells which had been washed three times with 10 mM phosphate. Although these findings were not in agreement with those for smaller particle aerosols, they indicate that industrial release from bioprocesses may result in aerosols containing, in addition to micro-organisms, growth medium which offers some degree of protection. An interesting, methodological aspect of the work of Marthi *et al* (1990), which has often been employed (Cox, 1968), is the use of spores of *B. subtilis* as tracer particles to counter for any dilution effects that may act on the aerosol. These spores are chosen because their survival is not affected by the most adverse conditions.

Of some relevance to aerosolization of organisms used in contained bioprocesses is the work of Cox (1968) on *E. coli* K-12 survival in aerosols. Although this work used distilled water, or solutions of glycerol or raffinose as the spray liquid, some points are of particular interest. These are: (i) that *E. coli* K-12 follows the general pattern of survival versus RH of other *E. coli* strains, but is the most unstable of four strains tested; (ii) that the mechanism of rehydration of the micro-organism on collection is important in terms of survival; (iii) that glycerol and raffinose as additives in the spray liquid had some protective effect; and (iv) that although the *E. coli* K-12 strain used carried a temperate phage, this was not operative in the death mechanism.

Bennett and Norris (1989) have rightly cautioned that although there are numerous factors that may cause airborne bacteria to die rapidly, it should not be presumed that bacterial aerosols present no hazard. It is, however, important to recall these potential adverse conditions when considering air sampling methods for the detection and measurement of aerosols containing bacteria.

1.4.4 Aerosol sampling methods

Because of the implications of bacteria-bearing aerosols on human health, many aerosol sampling devices that have been used have been designed to simulate the human respiratory tract. Such instruments may be optimized to sample only the inhalable fraction of aerosols. Other samplers are designed to maximize the collection efficiency and give particle size information, while some simply collect as much airborne material as possible, giving no indication of particle size.

As inferred from the above, all samplers have advantages and disadvantages in certain applications. However, in general, the quantity of viable micro-organisms recovered from aerosols may be lower than theoretically possible due to inefficiency arising from several sources. Inefficient sampling may be caused by physical effects such as failure to trap sample organisms in the collection medium (termed slippage) and biological effects such as inactivation of viable cells by the capture process. This latter effect is not of such importance where the enumeration method used can detect non-culturable as well as culturable cells. In addition to these considerations, it should be remembered that since these aerosol sampling devices merely collect airborne particles for subsequent analysis for target organisms, then the methods of collection and analysis must be compatible. Capture of aerosolized organisms onto agar surfaces is appropriate when culture based techniques are to be used for identification, but will be of no use if, say, PCR is to be used. In the latter case capture into liquid would be more suitable.

Aerosol sampling devices can be classified into several categories, depending on the mode of operation and the media type into which the organisms are collected.

1.4.4.1 Passive sampling techniques

Passive techniques rely on the settling out of particles from the air onto a sampling platform, such as an open petri dish containing agar or sterile collecting liquid. Tuijnenburg Muijs *et al* (1987) have pointed out that settle plates, using agar, have several disadvantages: they detect only sedimenting aerosol particles therefore smaller droplets which remain airborne will not be collected; and larger droplets may contain numerous viable cells which will grow into a single colony thereby underestimating the number of sedimenting cells. Additionally, culture of collected organisms is required for enumeration (see Section 1.5.1.1). These problems are largely overcome by using liquid collection media, where the collected cells can be separated out from clumps by vortexing, and direct counting techniques can be applied. However, passive sampling techniques do not permit quantitation of particles per unit volume of air (Burge and Solomon, 1987). Despite the obvious limitations of settle plates as a means of sampling

aerosols, they can be of use in tracking the proportion of cells that are released but do not remain airborne for long.

1.4.4.2 Active sampling methods

To enhance the detection of micro-organisms in air, active samplers with a mechanically induced air flow have been designed to force air through or over sampling surfaces. Active sampling methods can be further divided into those which collect particles onto solid or semi-solid surfaces (impactors) and those which collect particles into liquid (impingers). Centrifugal samplers, where organisms are collected as a result of either tangential impaction or impingement are sometimes classified in a class of their own (Stetzenbach *et al*, 1992; Hambleton *et al*, 1992), but they will be included in the classes of impactors or impingers, depending on the method of cell capture.

1.4.4.2.1 Impactors

Impactor samplers operate by causing airborne micro-organisms to deposit on solid or semi-solid surfaces. The surfaces may be an adhesive substance such as gelatin or nutrient agar. If agar is used, then collected organisms can be enumerated by colony counting after incubation. As mentioned previously, there are drawbacks to this approach, but it is a simple method of collecting and enumerating the number of droplets captured that contain culturable organisms. Unless additional steps are incorporated, impactor sampling methods are not really suitable for subsequent assays employing liquid samples, that is any direct counting techniques. There are a number of different impactor designs that have been used and these fall into two types; those that give particle size information and those that do not.

Impactors that give particle size information include the Andersen Microbial Sampler (AMS) (Andersen, 1958), the Marple Personal Cascade Impactor (Macher and First, 1984) and the May Ultimate Impactor (May, 1975). The AMS is a cascade sampler designed to direct airflow through a series of up to 8 stacked stages (Curtis *et al*, 1978) depositing organisms on an agar plate at a stage corresponding to their particle size. The principle is based on the differential deposition rates of the various particle sizes at different flow rates. The sampler draws in air at approximately 28 L min^{-1}, is portable and is relatively efficient (Barrett *et al*, 1984). Sawyer *et al* (1993) have used the AMS to monitor bacterial aerosol emission rates from municipal wastewater aeration tanks and Kastelein and Logtenberg (1989) have used this type of sampler to monitor releases from a centrifuge. The Marple Personal Cascade Impactor (Macher and First, 1984) works on the same principle as the AMS, but is a personal sampler that is worn on the body. It has a lower flow rate (2 L min^{-1}) than the AMS and can accommodate up to 8 stages. The

May Ultimate Impactor (May, 1975) separates airborne particles into several size ranges from 0.5 μm to 32 μm.

Impactors that do not give any particle size distribution data include the Casella slit sampler, the Biotest RCS and the Surface Air System (SAS) sampler. The Casella slit sampler (Lundholm, 1982), used by Tinnes and Hoare (1992) to monitor release from a disc-stack centrifuge, draws air through a narrow slit (at between 30 - 700 L min^{-1}) onto a rotating agar plate. The SAS (Lach, 1985) sampler is a hand held device which collects particles onto a RODAC plate (replicate organism direct contact plate, which has a convex surface rising above the rim of the petri dish). The efficiency of this sampler for particles of less than 4 μm diameter has been shown to be relatively poor (Lach, 1985). The same observation has been made of the Biotest RCS sampler (Hambleton *et al*, 1992), although Houwink (1988) found it to be the most practical for daily air monitoring in biotech. plants and labs. This is a centrifugal sampler where airborne particles impact onto the agar strip at high velocity. The air sampling rate of the Biotest RCS sampler is 40 L min^{-1}.

1.4.4.2.2 Impingers

Many impingers were originally designed to simulate air flow through nasal passages and operate by drawing air into a liquid medium (Cox, 1987). The liquid collection fluid can be used as an inoculum for culture media or examined by direct count techniques, this is what makes impingement sampling better suited for subsequent techniques such as PCR. An advantage of impingers is that because particles are collected into liquid, aggregates of bacteria will tend to break up, allowing more accurate quantitation than with impactors.

The May three-stage glass impinger (May, 1966) is designed to separate airborne particles into three size ranges which correspond to the principal deposition sites of the human respiratory system; the upper respiratory tract (>6 μm), the bronchioles (3 - 6 μm) and the alveoli (<3 μm). This impinger has a relatively gentle flow and has been suggested as the optimum choice for fragile bacteria (Cox, 1987).

Impingers that do not give particle size information include the Porton AGI-30 and the Aerojet General Cyclone. In the Porton AGI-30 (Cox, 1987; Zimmerman *et al*, 1987), the particles are drawn in through an inlet tube (to simulate the nasal passage) and then through a jet. This impinger samples air at 12.5 L min^{-1} and is efficient for microbial particles in the respirable size range (0.8 - 15 μm) and has been used as a reference method for this purpose since 1963 (Cox, 1987). Cyclones are centrifugal samplers, the

air flow into the device (up to 750 L min^{-1}) is directed in such a way that it moves in a circular path and organisms are collected as a result of tangential impingement into a stream of liquid circulating around the inner walls of the sampler. In the Aerojet General Cyclone the liquid stream is collected and then recycled back to the collection chamber so that the concentration of collected organisms in the liquid increases with time (Decker *et al*, 1969; Griffiths and DeCosemo, 1994). Cyclone samplers are of particular value where large volume air sampling is required. In addition, the Aerojet General Cyclone is a high efficiency air sampling device for particles above 2 μm in diameter (Upton *et al*, 1994). Although, Fannin (1980) has reported that recovery of culturable bacteria has been variable using these samplers, the use of an enumeration method that does not rely on culture overcomes this problem (Alvarez *et al*, 1995). In this study, an Aerojet General Cyclone will be used in conjunction with PCR as the detection method.

1.4.4.2.3 Other air sampling methods

Besides impingement and impacting methods of air sampling, filter samplers and electrostatic precipitation samplers may be used (Stetzenbach *et al*, 1992). Collection of micro-organisms by filtration, common in water microbiology (Cartwright *et al*, 1993), is not widely used for airborne cells due to the loss of viability through desiccation. Fannin (1980) has reported that recovery of airborne *Serratia marcescens* using filtration was only between 1-2 %. Another difficulty associated with the use of filters is the recovery of organisms that have been trapped. Rotter *et al* (1973) has used gelatin filters which can be dissolved into liquid to overcome this problem. Electrostatic precipitation samplers are primarily used in collecting particles under 1 μm in diameter. These systems operate by creating an electrical charge on particles passing through a collection tube and due to their high sampling rate are very efficient in sampling situations where low concentrations of bacteria or viruses are expected (Stetzenbach *et al*, 1992).

1.4.5 Studies on airborne release of micro-organisms from bioprocesses

Although there have been many studies on the release of GMMOs to the external environment by deliberate release, there is relatively little data on incidental or accidental release of GMMOs from contained facilities.

Dunnill (1982) has reported high airborne microbial counts (sampled using a slit-sampler) after whole cell solids were discharged from a disc centrifuge. Tinnes and Hoare (1992) also used a slit sampler to monitor a deliberate low pressure release of supernatant from a high speed disc bowl centrifuge. Additionally, settle plates were used close to and far from the leak. The presence of organisms in the settle plates far from the leak demonstrated that the leak was manifested, at least in part, as an aerosol.

Using pre-existing data on the number of organisms released by applied genetics activities (data was accrued by measurement and estimation following various assumptions), Lincoln *et al* (1985) developed a computer simulation model to determine the likely magnitude and source of release of GMMOs from applied genetics protocols. Using this model, a number of interesting points were made about which operations are likely to give rise to the most significant release and how the containment facilities affected the exposure of the worker and the release to the external environment. The results also showed that technicians could receive daily doses of 10^5 viable organisms through inhalation and 10^6 organisms through contamination of hands and clothes. Notably, no simple 'reduction factors' for releases as a function of containment level were found, although this had previously been claimed (Genetic Manipulation Advisory Group, 1978). Lincoln *et al* (1985) noted that "what can be done in support of modelling is to define some typical facilities, to focus on central issues and dispersal sources, to provide some benchmark data as a database for the model and to conduct some degree of model testing".

Attempts at generating such benchmark data have been made by Ferris (1995) who has shown that aerosols can be quantitatively sampled in a repeatable manner by the use of a cyclone and by Cameron *et al* (1987) who detected spores released from a fermenter on valve opening using a slit sampler. Additionally, Winkler (1987) and Kastelein and co-workers (1992) have looked at release from fermentation and homogenisation unit operations (these reports will be discussed further in sections 5.3.3 and 5.4 respectively). The significance of the work of Ferris is twofold: (i) that captured organisms were enumerated by a direct counting procedure so that no underestimate of numbers occurred due to the formation of viable but non-culturable organisms (Section 1.5.1.1); and (ii) that numbers of organisms captured can be directly related to the number airborne over the sampling period. The implication of the second point is that, when correctly calibrated, the number of organisms released from a certain bioprocessing unit operation can be measured, whereas previously monitoring devices were used to obtain an, at best, representative estimate of the number of organisms released.

The work of Palchak *et al* (1990) is interesting in so far as it measures inadvertent release of endotoxin from a bioprocess, and suggests a maximum threshold exposure level. Because previous studies had identified the average exposure level that causes clinically significant changes, it was possible to set a recommended level (the action level) above which the airborne level should not rise. Here, contrary to the situation with micro-organisms, the issues of release are relatively clear cut: the level at which harm is caused is known and the measurement of released endotoxin is relatively simple,

quantitative and uncluttered by issues such as viability or culturability.

1.5 Methods for the selective measurement of recombinant micro-organisms

This section will deal with the methods that are available for analysis of micro-organisms that have been captured by air sampling. Specifically, a survey of literature has been carried out to identify methods that may be developed for measuring the release of specific, viable, GMMOs from bioprocesses.

The range of techniques that have been used to specifically identify target micro-organisms for a variety of applications is extensive. Because of the implications of the two different approaches for the monitoring of GMMOs, it is convenient to classify these techniques into phenotypic and genotypic methods. The former rely on the expression of some factor for the identification to proceed, whereas the latter attempt to identify the micro-organism at the level of the genome.

1.5.1 Phenotypic methods

1.5.1.1 Selective culture

There are numerous methods for the identification of micro-organisms, often to species level, that are based on the growth of the organism under selective conditions that suppress the growth of other types of organism. Frequently such techniques rely on the ability of the selected organism to utilise a particular growth substrate and grow at a temperature or pH that is inhibitory to other organisms. For instance, the presumptive presence of the marker organism *E. coli* in drinking water supplies is routinely tested for by a method that involves detection of acid and gas production after overnight incubation of the sample in minerals modified glutamate medium broth at 37°C (Department of the Environment *et al*, 1983).

In order to achieve more definitive identification of an isolate, a combination of selective enrichment and the presence of a particular activity are often used. For instance, many selective agars that have been developed for the identification of food borne pathogenic organisms do not suppress the growth of all other organisms, but identification occurs as the target organism causes a colour change as it metabolizes an indicator that is a constituent of the medium. Baird-Parker agar (Baird-Parker, 1962) which was developed for the isolation and enumeration of coagulase-positive staphylococci depends on the inhibitory action of tellurite and sodium pyruvate and uses egg yolk emulsion as the indicator; clearing zones are produced around colonies after an appropriate incubation. *Staphylococcus aureus* produces black, shiny, convex colonies with a zone

of clearing within 24-36 hours at 37°C, whilst other organisms (e.g. *Bacillus* spp.) produce different colour colonies, do not produce a clearing zone or do not grow. Therefore, there may be a degree of interpretation, and skill, required to correctly read such an agar plate, especially in conditions where the target organism may be sublethally damaged (Van Netten *et al*, 1990).

An interesting development from Biolog Inc. is a novel method of identifying bacterial isolates that is dependant on the variety of substrates that the pure culture will grow on. In this method each well of a 96 well microtitre plate contains minimal medium plus a defined carbon source as well as a chromogenic growth indicator (a dye that is reduced by respiratory chain activity). A pure culture of the isolate is used to inoculate each well of the microplate which is then incubated. The pattern of growth (visualized by a colour change) over the microplate reflects the fastidiousness of the isolate and can therefore be used as an identifier. Biolog have built up a database of patterns and any unknown isolate can be matched against this to reveal its identity. At present there are approximately 800 strains of bacteria on the database.

For the identification of GMMOs by selective culture it is necessary for the recombinant organism to express traits that allow only its growth on a certain medium. This can be achieved by incorporating multiple antibiotic resistance genes into the GMMO, such that this organism and no other (at least a very low likelihood) will grow in the presence of a combination of antibiotics (Mallory *et al*, 1982). Similarly the mercury resistance gene could be used (Neilson *et al*, 1994). However, there are several problems associated with the use of antibiotic resistance genes as markers in GMMOs (Ford and Olson, 1988): antibiotic resistance genes are frequently transposable or contain insertion sequences or other components of transposition systems, possibly resulting in an increase in transfer of recombinant DNA to the environment; if the antibiotic resistance gene is found commonly in the environment then false negatives may arise; conversely, if the gene is rare, there are dangers in introducing it into the environment as this may promote resistance to antibiotics that may be of clinical importance.

The major drawbacks of selective culture methods for monitoring the release of GMMOs from bioprocesses are the time required for any results to be seen and the fact that many organisms may exist in a viable but non-culturable state (Colwell *et al*, 1985; Islam *et al*, 1993). Incubation times for selective culture methods vary, but are never less than 'overnight', rendering this type of technique of limited value in monitoring (rather than verifying) the release of recombinant process organisms during any bioprocess. The implications of the viable but non-culturable state of bacteria exposed

to adverse or low nutrient conditions on the monitoring of pathogens and GMMOs released into the environment has been considered by Colwell and colleagues (1985). This group showed that *E. coli*, *Vibrio cholerae*, *Salmonella* and *Shigella* spp. can all adopt a dormant state and that enumeration by direct methods, such as acridine orange direct count and fluorescence antibody direct viable counts (see Section 1.5.1.3) exceeds values obtained from cultural methods (plate counts) by up to 4 orders of magnitude. Moreover, when non-culturable *V. cholerae* cells were injected into the intestines of rabbits, positive virulence was observed that was comparable with the response to control *V. cholerae* cells. In addition, it has been found that human volunteers developed clinical symptoms after ingestion of non-culturable cells of *V. cholerae* which were subsequently isolated in culturable form from the stools of the hapless volunteers (Colwell *et al*, 1990).

Colwell noted the public health significance of these findings and also pointed out that methods for monitoring GMMOs in the environment should rely on direct detection rather than culture. Islam and co-workers (1993) echoed these recommendations by showing that *Shigella dysenteriae* type 1, the agent responsible for shigellosis outbreaks in Bangladesh, enters a viable but nonculturable state 2-3 weeks after inoculation into laboratory microcosms consisting of sterilised samples of pond, lake, river and drain water. After this time cultural methods failed to show the presence of the organism, which was detected by both fluorescent antibody and PCR techniques. By extrapolating from these findings to the natural environment, it was concluded that the current methods of detecting viability by conventional cultural techniques is inadequate. This hypothesis is corroborated by outbreaks of shigellosis in which no organism can be isolated from the suspected transmission vehicles by conventional cultural techniques (Rahaman *et al*, 1975).

Similarly, other workers (Gibbs and Hayes, 1988) have shown that enumeration of heterotrophic bacteria in drinking water by a pour plate method using yeast extract agar and 3 day incubation underestimates the number of bacteria by a factor of over 500 compared to a 7 day spread plate method on R2A agar (R2A agar is composed of a wide variety of nutrients at low concentrations). This type of work has shown that conventional, culture based methods have severe limitations for the detection and enumeration of well characterised micro-organisms and are therefore unlikely to be very useful in the monitoring of GMMOs.

1.5.1.2 Biochemical markers

Whilst many methods for the identification of micro-organisms rely on ability to grow under certain conditions combined with the presence of a particular activity, and may therefore be classified as 'selective culture' methods, there are also methods that rely to a greater extent on the presence of biochemical marker activities and that may feasibly be assayed without culture. Although for non-recombinant organisms, culture is frequently employed, the rationale behind classifying these methods as 'biochemical markers' is that the gene(s) responsible for the activity may be transferred to a GMMO, which can then be assayed directly.

There are several naturally occurring micro-organisms that can be characterised by a specific biochemical reaction that they carry out. The Colilert test, which has been proposed as an alternative to conventional procedures for water quality monitoring, is based on the detection of β-galactosidase activity, using a colorimetric reaction and the substrate *o*-nitrophenyl-β-galactopyranoside for total coliforms, and β-D-glucuronidase activity, using fluorogenic 4-methylumbelliferyl-β glucuronidide to indicate the presence of the faecal bacterium *E. coli* (Edberg *et al*, 1989).

For the monitoring of GMMOs in the environment several workers have inserted functional marker genes into the DNA of the organisms to be tracked (Drahos *et al*, 1986; Shaw and Kado, 1986; Morgan *et al*, 1989; Winstanley *et al*, 1989; Stewart, 1990; Neilson *et al*, 1994). This approach assumes that the gene products are not to be found naturally occurring in the environment in which the GMMOs are being monitored. GMMOs may be identified by isolation on media supplemented with chromogenic substrates; Winstanley *et al* (1989) showed that bacterial colonies carrying the *xyl*E gene coding for catechol 2,3-dioxygenase can be identified by the conversion of colourless catechol to yellow 2-hydroxymuconic semialdehyde. Similarly Drahos *et al* (1986) constructed broad host range plasmids containing the *E. coli lac* operon genes *lacZ* and *lacY* (encoding β-galactosidase and lactose permease respectively) and inserted these into fluorescent pseudomonad strains. The expression of the genes allowed the recombinant strains to grow on minimal medium plus lactose and to breakdown the chromogenic substrate X-Gal (5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside) causing the formation of distinctive blue/green colonies. Using this method it was possible to detect 1 colony-forming GMMO in 0.04 g of soil. Over 500 isolates of fluorescent pseudomonad strains were shown to lack β-galactosidase activity and the ability to grow on minimal lactose medium. Drahos *et al* suggest, therefore, that this marker system is useful due to its rarity in the soil environment and because the gene products are well characterised and are already present in the human and animal

gastrointestinal tracts.

Morgan and co-workers (1989) used a direct method to detect recombinant pseudomonads that were released into lake water samples. Using the plasmid constructs encoding a *xylE* marker gene that were previously used in a culture based method (Winstanley *et al*, 1989), they showed that by direct, enzymic assay of catechol 2,3-dioxygenase it was possible to detect 5×10^5 recombinant cells in a 100 mL sample.

The use of *gusA* (formerly denoted *uidA*), the gene encoding β-glucuronidase in *E. coli*, as an inserted marker gene allows highly sensitive fluorescence detection of the GMMO when presented with the appropriate fluorogenic substrate (Jefferson, 1989). A key advantage of β-glucuronidase as a marker enzyme is the absence of its activity in many organisms other than vertebrates and their attendant microflora. Lower and higher plants and most bacteria and fungi are largely if not completely lacking in β-glucuronidase activity and minute amounts of the enzyme can therefore be accurately measured.

The *lux* operon, coding for bacterial luciferase (*luxAB*) and fatty acid reductase (*luxCDE*) of *Vibrio fischeri*, has been introduced into a range of bacteria allowing the detection of a bioluminescent phenotype (Shaw and Kado, 1986; Rattray *et al*, 1990; Stewart, 1990; Shaw *et al*, 1992; Blackburn *et al*, 1995). Rattray *et al* (1990) used strains of *E. coli* containing a plasmid encoding *lux AB* or *luxABCDE* and monitored the luminescence produced after the cells had been inoculated into soil samples. The method used involved an extractive step but did not involve culture and a detection limit of 6×10^3 cells per gram of soil was possible. Shaw *et al* (1992) went one stage further by using a cooled charge-couple device (CCD) camera to detect and enumerate *in planta* bioluminescent strains of black rot causing *Xanthomonas campestris*. Using this method it was possible to detect 1.5×10^4 cfu/leaf and there was reasonable correlation between quanta/leaf/min and cfu/leaf up to 10^7 cfu/leaf.

Because bioluminescence is dependant on cellular integrity, then it can be seen as a measure of viabilty in organisms that contain the appropriate genes. Moreover, Jassim *et al* (1990) have shown that a significant proportion of freeze injured *Salmonella typhimurium* cells retain the ability to bioluminesce but not the ability to replicate. Bioluminescence marker genes may thus be used as a measure of cell viability *per se*, rather than of culturabilty, this has important implications for the detection of viable but non-culturable cells (Colwell *et al*, 1985).

1.5.1.3 Immunological methods

Immunological methods for the detection of organisms rely on the binding of an antibody to a specific antigen that is expressed by the organism (to which the antiserum has been raised) and the measurement of that binding by some means. For GMMOs it is therefore necessary to select and purify an antigen that is specific to the GMMO and not the host, or other, strains. The purified antigen can then be used to raise a specific, that is a non cross-reactive, antiserum which may be mono- or polyclonal.

One of the more established immunological methods for the detection and enumeration of micro-organisms is the fluorescence antibody (FA) technique (Bohlool and Schmidt, 1968; Brayton and Colwell, 1987; Brayton *et al*, 1987; Levasseur *et al*, 1992; Swaminathan and Feng, 1994). An advantage of immunological techniques is that a direct count of all target organisms can be achieved, rather than a count of only culturable organisms which may represent a small proportion of the total viable population (Colwell *et al*, 1985; Brayton and Colwell, 1987).

Brayton and Colwell (1987) devised a method that combined an FA technique (Xu *et al*, 1984) with the direct viable counting (DVC) procedure of Kogure *et al* (1979). This hybrid technique allows the direct determination of specific viable organisms; viability is determined by the ability of the organisms under study to elongate in the presence of nutrient (yeast extract) and nalidixic acid, an inhibitor of DNA gyrase (Goss *et al*, 1965). Brayton *et al* (1987) used the FA-DVC method to enumerate *Vibrio cholerae* 01, the causative agent of epidemic cholera, in Bangladesh waters. They showed the presence of *V. cholerae* 01 in several water samples where the traditional culture based most probable number (MPN) technique showed its absence. In addition, the estimate of numbers of *V. cholerae* 01 in all 16 samples was invariably greatest by the FA technique, and lowest by MPN, with the FA-DVC technique results lying in between. The FA-DVC technique takes up to 24 hours compared to 3 hours for the FA method.

Possible drawbacks of this technique are the inability of certain viable cells to elongate under the given conditions due to either their advanced state of dormancy (Peele and Colwell, 1981) or their morphology, for example species that exist as cocci (Pickup, 1991). Furthermore, some bacteria may be resistant to the action of nalidixic acid (Brayton *et al*, 1987) although piromidic acid can also be used (Fry, 1990). However, despite some limitations, this method represents a bridge between counting culturable bacteria and direct counts.

One obvious limitation of counting procedures based on microscopy is the labour

intensive nature of the technique; the ability to process large numbers of cells may be impractical. In contrast to fluorescent microscopy, flow cytometry (FCM) allows the analysis of up to 5000 bacteria per second (Sayler, 1992) and several fluorescent and light scattering characteristics of a single cell can be analysed simultaneously. In this technique a suspension of particles (micro-organisms in this instance), that have been labelled with a fluorochrome, is passed through a fine laser beam in such a way that only one cell passes through the beam at a time. By using a range of fluorochromes it is possible to analyse several different cell types, or properties, at once. For instance, Volsch *et al* (1990) have used FCM to determine the concentration of two serotypes of nitrifying bacteria of the genus *Nitrosomonas* in activated sludge of sewage plants. Additionally, Phillips and Martin (1988) have established FCM methods for the specific detection of *E. coli, Legionella pneumophila* and *Bacillus anthracis* spores. However, they found that this method was unable to detect low numbers of bacteria (i.e. less than 5×10^4 organisms mL^{-1}). In order to measure low numbers of bacteria by FCM an amplification of the number of cells per mL was required, either by culture or by physical concentration (for example, centrifugation).

An important factor in the development of immunological methods for the detection of micro-organisms is the constant expression, and availability, of the antigen to which the antisera that forms the basis of the detection system has been raised. If cells were collected in a manner which gave rise to sublethal damage, then it is possible that a failure to detect the specific organism might occur if the target antigen were not expressed. However, Mason and Burns (1990) produced a monoclonal antibody that was highly specific to a *Flavobacterium* isolate from soil, and was shown to recognise the isolate when it was grown under low nutrient or stored under starvation conditions, suggesting that the antigen is a constitutive component of the cell. For GMMOs it is possible that there will be no such antigen that distinguishes the recombinant organism from the host or other closely related species and is also expressed under stressful conditions. Immunological methods can therefore be used as a monitor of gene function, rather than absolute presence. However, one idea might be to use the induction system of the GMMO, by, for example, collecting organisms in a solution containing an inducer and then assaying for the presence of the gene product.

An interesting aspect of the work of Winstanley and colleagues (Morgan *et al*, 1989; Winstanley *et al*, 1989) is that plasmid constructs were made where the *xylE* gene was expressed from bacteriophage lambda promoters under the control of the temperature sensitive lambda repressor cI857. It was found that in these constructs *xylE* induction was readily achieved by elevation of the temperature from 28 to 37°C. In this study the

aim was to minimize the potentially deleterious metabolic burden imposed on the recombinant organism, but this type of system might also be interesting in terms of assessment of cell viability, since the population of cells that are viable might be expected to be inducible. Mahbubani *et al* (1991) (Section 7.1.1.3) showed in a similar manner that live and dead Giardia cells could be distinguished by the ability to produce a positive PCR result for the giardin mRNA gene after induction.

Some of the problems associated with the use of immunological methods have already been mentioned. Perhaps the greatest drawback for the detection of GMMOs is the need to produce an antiserum to the GMMO that would not be cross reactive with either the host strain or other strains that might be encountered in the sampling environment. Moreover, the level of expression and availability to antibody of the specific antigen would need to be constant so that accurate quantitation could be effected.

Colwell *et al* (1988) have noted that antibody based techniques, such as fluorescent antibody-microscopy, are useful in tracking host organisms in the environment, and can be adapted to detect only viable organisms, but can not monitor the presence or absence of specific gene sequences. They therefore suggested the use of fluorescent antibodies and gene probe methods to monitor the fate of the host organism and the introduced genetic sequence in the environment.

1.5.1.4 Bacteriophage sensitivity

Phage specific for bacterial species have been widely used to identify pathogens of plants and animals (Cherry *et al*, 1954; Hirsh and Martin, 1983; Pitt and Gaston, 1995). Cherry and co-workers (1954) showed that by dropping a suspension of *Salmonella* specific phage onto a lawn of bacteria on agar, *Salmonella* could be identified by the inhibition of growth around the added phage after an appropriate incubation time (6-30 hours). Hirsh and Martin (1983) used the same phage, which detected over 98 % of *Salmonella* strains tested against 0.3 % of non *Salmonella* strains, to develop a more rapid method with detection by HPLC. This technique takes less than 3 hours and was shown to have a limit of detection of 3×10^6 *Salmonella* per mL, if enhanced sensitivity is required, an enrichment step could be included.

Ulitzer & Kuhn (1987) developed a novel method involving introduction of the *lux* genes from *Vibrio fischeri* into specific bacteria via a recombinant bacteriophage. In this system, bacteriophage genetically modified to contain the *lux* genes are dark because they lack the intracellular biochemistry necessary for light production. Infection of host bacteria by phage, however, leads to the expression of host phage genes and, within 30-

50 minutes, the additional lux genes (Dodd *et al*, 1990). Other workers (Stewart *et al*, 1989) have used different phage constructs to detect *Salmonella typhimurium* cells using a simple luminometer.

The main limitation of the bacteriophage sensitivity approach is the availability of a virulent phage specific to the given organism. This problem is particularly significant in the case of the application of this method for the detection of GMMOs, since the difference from the wild-type host organism may be minimal. Lenski (1984) has suggested the use of bacteriophage as a means of limiting the spread of GMMOs in the environment, a method that is dependant on the isolation of a bacteriophage whose site of adsorption is a cell surface antigen unique to the GMMO. In cases of mutation or low nutrient conditions, however, outer membrane surface properties of bacteria can be significantly affected (Chai, 1983; Brown and Williams, 1985), such changes may confer phage immunity on the organism (Chai, 1983). The practicality of such a strategy for the detection of GMMOs remains to be proven.

1.5.2 Genotypic methods

Methods of detection of GMMOs that rely solely on the expression of a target protein are open to criticism since the physiological status of the organism may adversely affect the expression of the protein. Non expression of target protein would therefore lead to a negative result despite the fact that the GMMO, with intact inserted DNA, is present. This is demonstrated by the work of Green and co-workers (1991) who have shown that environmental isolates of *E. coli* can be detected by using a gene probe to the β-glucuronidase gene *gusA*, even when the enzyme is not actively being produced. Some workers (Colwell *et al*, 1988; Ford and Olson, 1988) have suggested that in order to track the gene and the host organism in the environment, a combination of phenotypic and genotypic methods is advisable. However, since there are genotypic methods for specifically detecting host strains (Bej *et al*, 1990; Green *et al*, 1991; Kuhnert *et al*, 1995) monitoring of gene and host organism may be achieved by a combination of genotypic methods aimed at different nucleotide sequences. Whether it is necessary to monitor the host organism as well as the inserted DNA in the bioprocessing environment, where it is unlikely that there has been much chance for genetic transfer, should be considered. This is referred to at greater length in Section 1.3.1.

1.5.2.1 Nucleic acid probes

Methods for the specific measurement of micro-organisms based on the annealing of two complimentary strands of DNA or RNA (one a labelled probe molecule, the other being the target DNA/RNA sequence) are numerous and have application in a wide variety of

fields. For example, evaluation of the microbiological safety of foods has become an important concern of consumers, industry and regulatory agencies. Among all the foodborne pathogens, three organisms (*Listeria monocytogenes, Escherichia coli and Salmonella* spp.) have caused a great deal of concern (Datta, 1990; Tietjen and Fung, 1995). Hence there has been a significant effort put into the development of gene probe based methods for detection of these organisms (Fitts, 1985; Klinger and Johnson, 1988; Romick *et al*, 1989; Huck *et al*, 1995). Much of this effort has been concentrated on the search for sequences of DNA that are specific to the organism being analysed. This is often achieved by identifying specific genes that are involved in the virulence mechanism. Because different pathogens use different strategies and toxins to establish infection, the genes controlling these functions will be specific (Hill, *et al*, 1983; Nataro *et al*, 1985; Ruben *et al*, 1985). Alternatively probes have been constructed from cloned genes encoding resistance to antibiotics (Tenover, 1988) or heavy metals (Barkay *et al*, 1985), genes encoding degradative phenotypes (Sayler *et al*, 1985; Pettigrew and Sayler, 1986) and nitrogen fixation determinants (Smith, and Tiedje, 1992).

Probes that have been developed using a targeted strategy can be employed as function specific probes to detect similar genes in different bacteria, or they can be used as species specific probes if the cloned gene sequence is conserved within a species but highly variable between species (Ogram and Sayler, 1988). Function specific probes are useful tools in the study of microbial ecology and have been used both to assess the potential of a particular community for expressing a given phenotype and for detecting and culturing isolates possessing genes encoding given functions. Alternatively, the 'shotgun' approach can be used for the generation of probes to specific bacteria. An example of this strategy is the development work that led to the first commercially available DNA probe kit for *Salmonella* spp., used to detect the micro-organism in foods (Fitts *et al*, 1983). The probe, marketed by GeneTrak Systems was produced by constructing a genomic DNA library from *Salmonella typhimurium* and testing the cloned restriction fragments for cross-hybridization with radiolabelled *E. coli* chromosomal DNA. The clones which did not cross-hybridize were then tested for cross-hybridization with a battery of strains other than *Salmonella* spp. and those which did not cross react were tested for specificity using hundreds of *Salmonella* strains. Those clones which hybridized with the largest number of strains were used to prepare the probe.

For the preparation of probes to GMMOs, the extensive screening of the shotgun approach is not necessary, nor is knowledge of the molecular basis for pathogenicity or other traits of the organism a prerequisite. This is because the sequence of the inserted,

or foreign, DNA will be easily available. Assuming that a sequence of the inserted DNA (or a sequence that spans the site of insertion into the vector) can be identified that is specific to that organism, then the design of an appropriate DNA probe method, or any method that exploits complimentary base pairing, should be theoretically quite straightforward. This is a distinct advantage of the genotypic methods over, say, an immunological method, where a specific antiserum that does not cross react with other strains, must be raised to the GMMO. Although such an antiserum might be produced, any method centred around this would entail a great deal of validation work.

In addition to the use of probes that are derived from cloned restriction fragments, it is also possible, once a DNA sequence is known, to use synthetic oligodeoxynucleotide probes. Such probes are generally from 15 to 50 base pairs long and can be more specific than longer restriction fragment probes. This is because instability introduced into a hybrid by a mismatched base pair is much higher for short hybrids than for long ones. It is possible to design an oligodeoxynucleotide probe which will discriminate between two targets differing by only one base (Wallace *et al*, 1979), provided that the hybridization is carried out under sufficient stringency. Moreover, longer probes containing the sequences of long stretches of, or entire, genes may show some homology with gene sequences of other organisms. A disadvantage of using oligodeoxynucleotide probes is that they are generally less sensitive than restriction fragment probes because less label can be incorporated per molecule.

One of the simplest procedures for using DNA probes to identify specific micro-organisms is colony-blot hybridization (Grunstein and Hogness, 1975; Sayler *et al*, 1985). In this technique, bacterial colonies, or phage plaques, are transferred from primary cultivation media (i.e. agar) to a hybridization filter. The filter membrane is treated with a high concentration of NaOH which lyses the cells and denatures the DNA, which then binds to the membrane. The membrane is then neutralised and the DNA is linked to the filter for subsequent hybridization analysis. Sayler and co-workers (1985) used DNA-DNA colony hybridization to study the distribution of catabolic genotypes in environmental samples. A whole-plasmid probe of *TOL* (the toluene catabolic plasmid from *Pseudomonas putida*) was used as a general indicator of aromatic hydrocarbon catabolism in microcosm sediments exposed to synthetic oils. Sayler *et al* (1985) noted that the enumeration of specific bacterial populations in environmental samples is hampered by the uncertainties of selective enrichment procedures. Although the colony-blot hybridization procedure may overcome this particular hurdle, it is still dependant on the target organism being culturable; a strategy that has limitations in terms of the time required for the feedback of results and the occurrence of viable but

non culturable organisms (see Section 1.5.1.1).

An approach that does not necessitate the culture of target organisms is the use of dot or slot blot methods (Morgan *et al*, 1989; Holmes *et al*, 1992). Using this technique bacterial suspensions are collected (or dotted) onto a membrane by means of filtration using a vacuum manifold. DNA of membrane bound cells can then be released by alkaline treatment and subsequently fixed to the hybridization support. Hybridization of DNA probes to the immobilized, captured, DNA can then take place. Morgan et al (1989) used a *Pvu II* restriction fragment of the *xylE* gene to monitor recombinant *Pseudomonas* populations in lake water microcosms. Dot and slot blot procedures are sometimes used after an enrichment step, in order to increase the overall sensitivity of the technique. In the food industry a number of probes for Salmonellae have been developed, but for all probes, in the order of 10^8 Salmonellae are required for a positive assay (Dodd *et al*, 1990).

Interestingly, Neef and co-workers (1995) have recently described the use of nucleic acid probes to detect microbial cells collected from bioaerosols by filtration. In this report, colony hybridization was compared to a direct whole cell hybridization method. It was found that genetically modified cells could be detected in the aerosol by both methods, but the superior sensitivity of colony hybridization was necessary to detect GMMOs derived from less concentrated aerosols. A notable finding of this work was that less than 1 % of the cells collected by filtration were culturable compared with over 90 % culturability of cells filtered directly from the cell suspension used to make the aerosol.

The sensitivity of DNA probe methods is largely dependant on the hybridization format and the labelling and detection scheme employed in the assay (see Section 1.5.2.3). However, Holmes *et al* (1992) showed that in certain cases, careful choice of target sequence can have a significant effect on sensitivity. This group was interested in the detection of *Candida albicans* in human blood. The probe that had previously been used to identify *C. albicans* in clinical specimens was directed against the P_{450} gene sequence which was present as a single copy locus. The approach of Holmes and co-workers was to use a probe directed at a repetitive sequence in the genome of *C. albicans* using a dot blot format. It was shown that there were around 100 copies of the target sequence per cell, and the detection limit of this method was as low as 500 *C. albicans* cells in 1 mL of human blood.

Results of hybridization of probes with dot or slot blots can be quantitative as well as

qualitative. By applying a dilution series of known target DNA to the membrane and measuring the amount of hybridized probe to each dot, one can generate a standard curve for estimating the amount of hybridizable nucleic acid in an unknown sample from the amount of probe which is bound (Kafatos *et al*, 1979; Sambrook *et al*, 1989). Southern hybridization is another mixed phase format that can be used in conjunction with DNA probes to identify specific bacteria (Southern, 1975; Kaper and Levine, 1981; Kaper *et al*, 1982). More recently, a lot of research effort has been directed at the development of formats that either do not require a separation step or the separation is simple, for example by use of a dipstick. Examples of some of these methods are outlined in Section 1.5.2.3.

So, detection of sequences of DNA is a promising strategy for the detection of GMMOs. However, the sensitivities of DNA probe methods are relatively poor (generally $>10^4$ copies of target DNA are required for a positive reaction). In order to use DNA probe methods in real applications, many workers have resorted to incorporating a culture step to provide a biological amplification of the target. However, it has previously been noted that there are serious limitations inherent in culture based methods. An alternative approach is to use probes to ribosomal RNA (rRNA) molecules which are naturally amplified in the cell.

Several workers (Giovannoni *et al*, 1988; DeLong *et al*, 1989; Amman *et al*, 1990b, 1992a, b) have used oligonucleotide probes to rRNA sequences to identify specific organisms or groups of related organisms. Because of their large size (1500-2000 nucleotides), the 16S-like rRNAs have been particularly useful. Some segments in the 16S rRNA are invariant in all organisms and therefore are useful as binding sites for oligonucleotide primers for sequencing protocols and for the design of universal probes which will bind to all cellular 16S-like rRNA (Giovannoni *et al*, 1988). Other portions of the 16S rRNAs are unique to particular organisms or related groups of organisms and hence offer targets for hybridization probes with various specificities. The ubiquity of the rRNAs ensures that probes can be designed to identify virtually any organism or group of related organisms. Giovannoni and co-workers (1988) illustrated this by designing oligonucleotides that were diagnostic for each of the 3 primary lines of descent; the eubacteria, the archaebacteria and the eukaryotes. Amman *et al* (1990b) used fluorescent-dye-conjugated oligonucleotide probes, with visualization by epifluorescent microscopy, to identify single cells of 14 different *Fibrobacter* strains. A method involving flow cytometry has also been devised (Amman *et al*, 1990a).

The advantages of using rRNA sequences to devise probes are the control of probe

specificity (from strain specific to kingdom specific to universal probes) and the number of ribosomes per cell. Actively growing cells may contain 10^4 ribosomes (Giovannoni *et al*, 1988) and this high number of target sequences is responsible for the sensitivity of the technique. However, the specific ribosome content is known to be inversely proportional to doubling time over a wide range of growth rates (Ingraham, *et al*, 1983). DeLong *et al* (1989) have shown a direct correlation between growth rate and the strength of the fluorescent hybridization signal for *E. coli*. Because in natural settings micro-organisms may exist in sub optimal conditions and correspondingly contain fewer ribosomes and there is a certain amount of background fluorescence from various sources (Amman *et al*, 1992b), then the sensitivity of this technique may be compromised.

A considerable amount of work has been carried out on the characterisation of rRNA sequences for the identification of microbial community structure in environmental populations. The fact that this technique harnesses the natural amplification provided by the high number of ribosomes in a growing cell means that it is an interesting technique with respect to sensitivity enhancement. However, for the monitoring of GMMOs it is unlikely that this technique can be used as the rRNA sequences of a host organism are unlikely to be altered upon insertion of foreign DNA.

1.5.2.2 Polymerase chain reaction (PCR)

The advent of PCR (Mullis *et al*, 1986; Mullis, 1990) allows selective amplification of specific DNA sequences which, then at high concentration, can be easily detected. Additionally, the use of a reverse transcriptase activity (which may reside on the same protein as the DNA polymerase activity) allows RT-PCR of RNA sequences (Singer-Sam *et al*, 1990).

Briefly, PCR is a method for the enzymic amplification of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite DNA strands and flank the region in the target DNA to be synthesized (Figure 1.2).

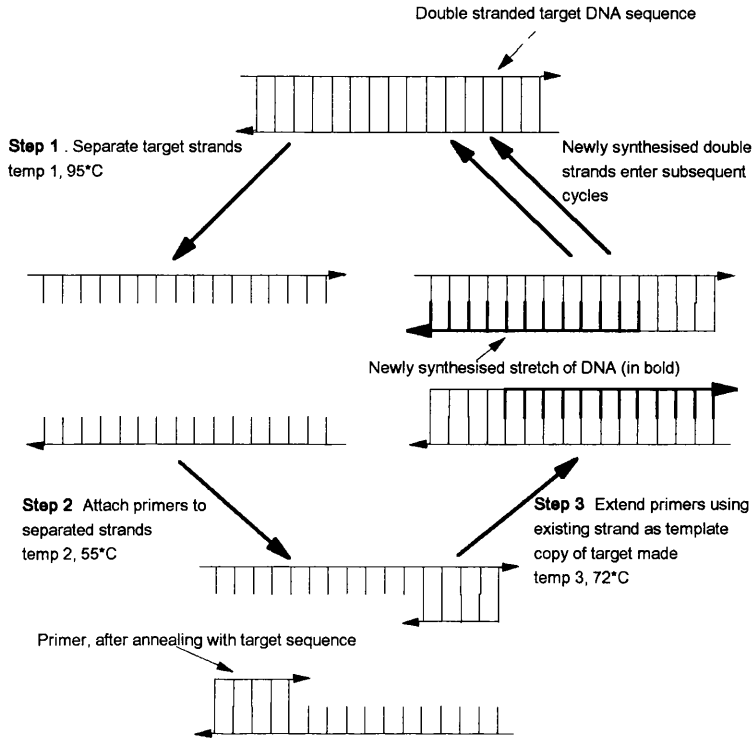


FIGURE 1.2 Schematic representation of the cyclic PCR process. Scheme shows the cyclic nature of the PCR and the requirement for temperature cycling. Note that for each cycle, two strands are produced from one template, therefore there is an exponential increase in the number of target DNA strands as the reaction proceeds. Arrows on DNA strands denote the 5'-3' orientation.

A repetitive cycle of temperature changes produce template denaturation, primer annealing and extension of the annealed primers by DNA polymerase. The primer extension products synthesized in one cycle can serve as a template in the next and so the number of copies of target DNA theoretically doubles at every cycle. This leads to the exponential amplification of a specific fragment whose termini are defined by the 5' ends of the primers. Thus, 20 cycles of PCR theoretically yields a million fold (2^{20}) and 30 cycles a billion fold (2^{30}) amplification. The length of each phase of the PCR is typically 30 seconds to 2 minutes, hence a whole PCR of 25 cycles can take less than 3 hours.

Once PCR has been performed, there are a variety of ways of specifically identifying the amplified DNA (or amplicon). The simplest method is to separate DNA fragments by electrophoresis. Using this technique, the rate of migration of DNA in an agarose gel under an applied electric field is related to the size, in base pairs, of the fragment. To confirm the identity of the amplicon, a labelled DNA probe, directed at an internal sequence of the fragment, can be used in a Southern hybridization or dot blot format. Section 1.5.2.3 deals with the detection and measurement of PCR products.

PCR has application in the clinical diagnostic (e.g. HIV testing), food pathogen testing and environmental testing markets as well as being a powerful tool in molecular biology. Several workers have used PCR based methods for the detection of bacteria in a variety of fields. Examples of methods that have been developed include the detection of *Legionella pneumophila* (Bej *et al*, 1991b; Mukoda *et al*, 1994), *Giardia* cysts (Mahbubani *et al*, 1991; Mayer and Palmer, 1996), enterotoxigenic *E. coli* (Olive, 1989; Deng *et al*, 1996), *Salmonella* spp. (Cano *et al*, 1993) and *Vibrio cholerae* (Bej *et al*, 1996). Chaudry *et al* (1989) have used PCR for the detection of GMMOs in environmental microcosms. In this work a 0.3 kb sequence of eukaryotic DNA (from *Pennisetum purpureum*, napier grass) was inserted into a plasmid that was transferred into an *E. coli* strain. The eukaryotic DNA was chosen as a marker because it was a unique sequence in the test environment; there was no hybridization expected with DNA from prokaryotes of the ecosystem. Using PCR, Chaudry and co-workers showed the presence of the GMMO in the test environments 14 days after seeding. Culture based methods, however, failed to show its presence after 6 days in one instance and 10 days in another. The PCR method that was used, involving dot blotting and radioactive oligonucleotide probing, was simply able to show presence or absence of the GMMO, no indication of the number of organisms present was obtained, although the limit of detection was found to be at 1000 copies of the plasmid.

The work of Cano *et al* (1993) illustrates the extent to which some PCR based assays for bacteria, in this case a food pathogen, have now been designed with automation in mind. PCR was used to amplify a 206 bp segment of IS200, an insertion sequence present in most Salmonellas, the amplified sequence was then selectively separated by means of a capture oligonucleotide that was covalently bound to the well of a microtitre plate. An alkaline phosphatase labelled probe (complimentary to an internal sequence of the PCR product) was added and a fluorogenic substrate allowed fluorescent detection of the amplicon. The level of sensitivity of this assay was found to be 1-10 cfu of *Salmonella enteriditi*s. It was noted that this high level of sensitivity may be due to the presence of multiple copies of the IS200 gene in the *Salmonella* genome as well as the highly sensitive detection method employed. However, other workers have reported similar sensitivities in different applications; the limit of detection that has been achieved is 1 to 10 fg of genomic DNA and 1 to 10 cells of target organism per 100 mL of sample (Bej *et al*, 1991a, c, 1994; Mahbubani *et al*, 1991).

Although the PCR cycling conditions are of great importance with respect to the sensitivity of the reaction, other factors such as sample preparation and detection of product are equally significant. Bej *et al* (1991c) have developed a method of filter

concentrating aqueous samples and subsequently using the PCR reaction in the presence of the filter. Cano *et al* (1993) have reported that detection of alkaline phosphatase labelled oligonucleotides requires up to 1×10^8 target genomes with *p*-nitrophenyl phosphate as the (colorimetric) substrate, but as few as 1×10^3 target genomes with a fluorimetric substrate.

With regard to PCR detection of bacteria captured from aerosols, a few reports have recently come to light (Alvarez *et al*, 1994, 1995; Mukoda *et al*, 1994; Roll and Fujioka, 1995). In most cases, it has been pointed out that, relative to culture, the PCR overcomes the problem of nonculturable cells that arise from the stresses of aerosolization and aerobiological sampling. This is an issue that has previously been raised in relation to detection using nucleic acid probes ((Neef *et al*, 1995), Section 1.5.2.1). An important application in airborne pathogen detection is the identification of *Legionella pneumophila*, this is investigated by both Mukoda *et al* (1994) and Roll and Fujioka (1995).

1.5.2.2.1 Quantitative PCR

Much of the work on detection of specific micro-organisms by PCR has been concerned with presence/absence tests, i.e. non-quantitative PCR. The main constraint in obtaining quantitative data is inherent in the amplification process. Because amplification is (at least initially) an exponential process, small differences in any of the variables that influence the rate of reaction will dramatically affect the yield of PCR product. These variables include just about any chemical or physical parameter that is involved in the reaction. Even with carefully controlled reaction conditions, tube-to-tube variation may be significant (Gilliland *et al*, 1990b). Additionally, the well documented plateau phase in PCR (Gause and Adamovicz, 1994; Morrison and Gannon, 1994), where the rate of product accumulation decreases with increasing cycle number, prevents direct correlation between the amount of PCR product and the starting number of copies of the target. This can be seen more clearly by looking at the relationship which describes product accumulation during PCR:

$$\log y = \log x + n.\log (1+E)$$

where y is the yield, x the starting number of target DNA molecules, n the number of cycles performed and E the efficiency of the reaction. In an ideal PCR, where the number of copies of the amplicon doubles at each stage, the efficiency of amplification is equal to 1. However, in reality efficiency values will typically be in the range 0.7-0.8 (Ferre, 1992). In itself, this does not present a problem in the quantitation of the starting

number of targets since E can be measured. So, if E and y can be measured and n is known then calculation of the starting number of target molecules, x, is possible. A problem arises when the plateau is reached as at this point, E falls dramatically. Hence, the relationship shown above only applies in the portion of the PCR before the onset of the plateau phase.

There are three basic strategies that are used to quantify the starting number of target copies in the PCR: i) measurement of products in the linear (pre-plateau) portion of the reaction; ii) limiting dilution or MPN based PCR; and iii) the use of a competitive internal standard in the PCR (Jansson, 1995; Reischl and Kochanowski, 1995).

Measurement of products in the linear portion of the PCR is clearly a strategy designed to avoid the problem of variable efficiency caused by the plateau phase. Using this approach, it is necessary to define the linear portion of the reaction and to measure reaction products during that phase. This normally entails the use of highly sensitive detection methods since there may be insufficient product accumulation to allow measurement by ethidium bromide staining (Gause and Adamovicz, 1994). Such methods have been developed and are now marketed specifically for quantitative PCR (see Section 1.5.2.3). However, a further problem that has been identified is the variation between tubes (variable efficiencies of amplication) that might occur when samples are derived from environmental origins and may contain inhibitors of the PCR (Jansson, 1995).

The use of the MPN principle in conjunction with PCR has been illustrated by the work of Picard *et al* (1992). This group used an MPN approach to estimate the indigenous population of *Frankia* species in soil. DNA extracted from soil solutions was serially diluted by a factor of three and amplified in triplicate according to a PCR protocol that was capable of amplifying the DNA equivalent of a single cell. The number of amplifiable target DNA sequences corresponding to the number of bacterial cells was determined according to most probable number statistics. A similar approach was used by Sykes and co-workers (1992) to detect and quantitate leukaemic cells within a large population of normal cells. The major drawback of this method is the necessity for a large number of PCR amplifications per sample.

The third method of quantitation, the use of a competitive internal standard in the PCR, overcomes many of the problems of the other methods (Becker-Andre and Hahlbrock, 1989; Gilliland *et al*, 1990a; Jansson, 1995). In this approach, a known quantity of an internal standard DNA sequence is co-amplified with the target. The ratio of standard to

target product at the end of the PCR can then be used to deduce the starting ratio and hence the absolute amount of target DNA in the sample. Ideally, an internal standard used in the quantitation of PCR should be a stretch of DNA, or RNA, that is very similar to the target sequence. Most importantly, in order to minimize differences in amplification efficiency, the target and standard DNA sequences should use the same set of primers. There are presently a series of methods for the generation of such internal standards (McCulloch *et al*, 1995). It is important, of course, that the internal standard and target amplicons can be distinguished after PCR and this can be achieved on the basis of size or minor sequence difference, for example the incorporation of a restriction site (Gilliland *et al*, 1990a, b). Since this is the method of quantitation used in this thesis, there is further discussion of its application in Section 7.1.1. It is worth noting here that there are now published examples of its use in the measurement of bacteria from environmental samples (Mahon and Lax, 1993; Leser, 1995).

1.5.2.2.2 Distinguishing viable from non viable cells in PCR

It has already been mentioned on several occasions that one of the advantages of using PCR to detect bacteria isolated from the environment is that it enables the measurement of that proportion of cells that are viable but not culturable. However, because PCR simply detects specific DNA sequences, there are instances when non-viable cells will be detected and this may cause problems in the interpretation of results (Masters *et al*, 1994). Several reports (Mahbubani *et al*, 1990, 1991; Bej *et al*, 1991b, 1996; Pichard and Paul, 1991) have described the detection of specific mRNA molecules in cells, by direct gene probes or PCR amplification, in order to gain information about the physiological activity of the target micro-organism in the environment. The underlying principle of this approach is that most bacterial mRNAs have half lives of less than 2 minutes. Examples of approaches to the distinction between viable and non-viable cells using PCR are given in Section 7.1.1.3.

1.5.2.2.3 *In situ* PCR

A recent development of PCR is a technique which combines its extreme sensitivity with the cell localizing ability of in situ hybridization (Haase *et al*, 1990; Bagasra *et al*, 1993; Hofler, 1993; Long *et al*, 1993; Patterson *et al*, 1993). This technique is known as 'in cell PCR' or, more widely, as '*in situ* PCR'. The method of *in situ* PCR is relatively straightforward; fixation of cells or tissue, generating semipermeable cell membranes allowing the primers and enzymes to enter the cell but avoiding the loss of the generated amplicons, in cell PCR amplification, and finally direct or indirect detection of amplicons. To date, much of the work on this technique has been for the detection of rare DNA sequences (viral DNA, single copy genes and gene rearrangements) in human cell lines.

Although rare target sequences can be detected *in vitro* with quantitative polymerase chain reaction, the product DNA signal is averaged for the number of cells that are lysed; thereby the association with individual cells is lost. It has been shown that conventional *in situ* hybridization can unambiguously identify target sequences in a single cell, however, a low copy number target sequence may not be detected. The combination of PCR with *in situ* hybridization allows the target sequence to be amplified (typically by less than one hundred fold (Long *et al*, 1993)) above the limit of detection while maintaining the cellular architecture.

Patterson *et al* (1993) have developed an *in situ* PCR assay for HIV-1 DNA that was highly sensitive, specific and quantitative. This group used fluorescein labelled oligonucleotides and counted the number of cells in a population that contained the target HIV-1 DNA by flow cytometry. Interestingly, this work showed that in the HIV positive patients tested, the proportion of blood cells infected with HIV was as high as 1 in 10 where previous estimates had been only as high as 1 in 1,000 to 1 in 10,000.

To date, there has been little, if any, application of *in situ* PCR to the detection of specific DNA sequences in bacteria. However, should this prove possible in a predictable, reliable way, then the possibility of counting single GMMOs may become real. It is envisaged that in-situ PCR could be followed by flow cytometry analysis to quantify the number of bacteria containing amplified products. The advantage of *in situ* PCR with respect to counting bacteria is that there is no requirement to relate the strength of signal in an arbitrary way to the number of cells present.

1.5.2.3 Detection and quantitation of PCR products

The amplification of a target nucleic acid sequence requires quantitative detection of the products in order to constitute a quantitative analytical procedure. Many of the approaches used for labelling and detecting PCR products stem from methods used in nucleic acid probe applications. Indeed, after PCR amplification it is often advisable to use a DNA probe to detect the appropriate amplicon as this confers an additional layer of specificity (Reischl and Kochanowski, 1995).

Non specific amplicon detection methods include electrophoresis, using agarose or polyacrylamide gels (Gebhardt *et al*, 1994), high performance liquid chromatography (HPLC) (Chan *et al*, 1994), solid phase capture assays and the scintillation proximity assay (SPA) of Amersham International plc (Reischl and Kochanowski, 1995).

The use of traditional agarose and polyacrylamide gel electrophoresis is widespread in

molecular biology laboratories. Normally, the analysis is simply qualitative, but the incorporation of a subsequent densitometry (or image analysis) step allows quantitation of the nucleic acid bands (Sundfors and Collan, 1996). A newer format for electrophoretic separation of DNA molecules that is readily applicable to quantitation is capillary gel electrophoresis (Section 3.2.9). This is based on the same separation principles as as conventional horizontal slab gel electrophoresis but is quicker, automated and allows direct quantitation of products without any additional steps. HPLC methods are similar to capillary electrophoresis in this regard, but the method of separation is different (Chan *et al*, 1994). Solid phase capture assays frequently employ streptavidin coated microtitre plates which bind biotin labelled amplicons (Reischl and Kochanowski, 1995). Amersham's SPA technique is based on a similar principle. In this case, PCR primers are biotin labelled and the deoxynucleotides are tritiated. After PCR, the biotinylated amplicons are captured by streptavidin coated beads which are linked to a fluorophore. The proximity of the fluorophore and the tritium causes a fluorescent signal to be emitted. This assay can also be configured to be specific by use of a biotin labelled DNA probe.

There are numerous approaches to the labelling and detection of nucleic acid probes (Matthews and Kricka, 1988; Forster *et al*, 1991; Kricka, 1995) which can be used for specific detection of PCR products. In principle, there are two basic strategies that can be used in this context; direct and indirect labelling. In direct labelling techniques the label is directly attached to the probe molecule via a covalent bond. Direct techniques include the use of chemiluminescent (Arnold *et al*, 1989), fluorescent (Richardson and Gumport, 1983; Kamur *et al*, 1988) and enzyme labelled (Jablonski *et al*, 1986; Li *et al*, 1987) oligonucleotides. Techniques of particular note include the recently introduced the ABI PRISM™ 7700 Sequence Detector System of Perkin Elmer (Foster City, CA). The principle of quantitation is that during PCR a fluorogenic probe, consisting of an oligonucleotide with both a reporter and a quencher dye attached, anneals specifically between the forward and reverse primers. When the probe is cleaved by the 5' nuclease activity of the DNA polymerase, the reporter dye is separated from the quencher dye and a signal is generated. With each cycle, additional reporter dye molecules are liberated and fluorescent intensity is monitored during the PCR. The advantages of this system are that it is a single, closed tube assay and that the detection is real-time (no need for post-PCR analysis). However, there is a need to synthesise a new reporter probe for each sequence to be quantitated and clearly the instrumentation is expensive. Another notable system is the QPCR System, again from Perkin Elmer, which is based on electrochemiluminescent detection and provides sensitive and reproducible DNA quantitation at the attomole level.

Indirect techniques involve the attachment of a 'tagging molecule' to the nucleic acid probe and subsequent detection by use of a labelled molecule with specific affinity for the tag. Examples of this include the recognition of biotin by avidin and its bacterial counterpart, streptavidin (Leary *et al*, 1983), (Tijssen, 1993), antigens by antibodies (Syvanen *et al*, 1986), (Tijssen, 1993), (Dooley *et al*, 1988) and mercurated nucleic acids by sulfhydryl ligands (Tijssen, 1993). An advantage of indirect procedures is the ability to build amplification steps into the assay.

The choice of labelling and detection strategy is complicated by the variety of detection formats available. Since Southern (1975) first demonstrated the use of DNA probes in gene analysis on a solid phase other techniques such as dot or slot blotting (Schuster *et al*, 1986), in situ hybridization (Tijssen, 1993) and colony blot hybridization (Grunstein and Hogness, 1975) have been developed. In addition a number of strategies have evolved that have departed completely from hybridization on a solid support. This is largely because solution hybridization is inherently faster than hybridization on a solid support, which frequently proceeds overnight (Matthews and Kricka, 1988). Examples of this newer generation of hybridization formats include: fluorescence resonance energy transfer methods (Morrison, 1995) where two probes directed at adjacent sequences on the target each containing a different label need to be brought into close proximity before a signal is emitted; molecular beacons that fluoresce on hybridization with their target due to a conformational change in the probe molecule which moves fluorophore and quencher apart (Tyagi and Kramer, 1996); and the hybridization protection assay of GenProbe (Arnold *et al*, 1989; Nelson *et al*, 1995) (see Figure 1.3).

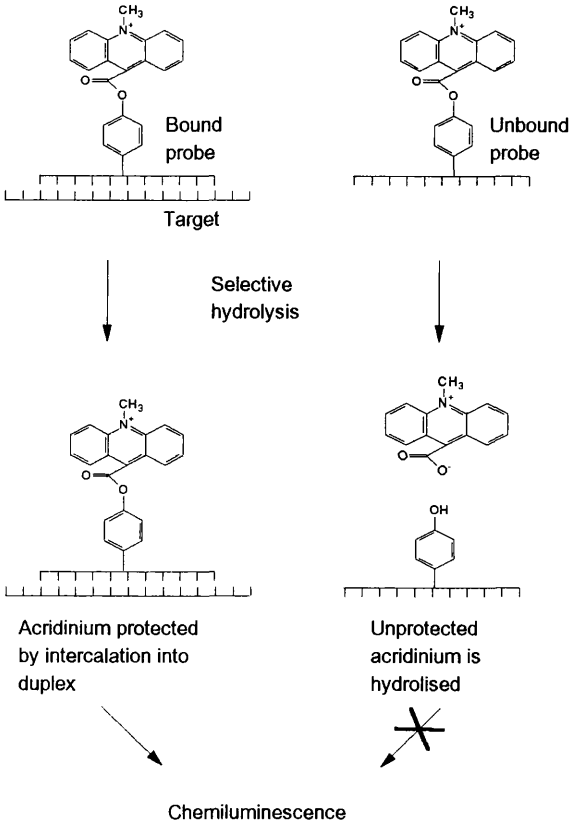


FIGURE 1.3 Schematic representation of GenProbe hybridization protection assay (Tijssen, 1993). In this assay, bound and unbound label (in this case an acridinium ester, a chemiluminescent substrate) are distinguished by their relative sensitivity to hydrolysis. Under certain conditions it is possible to hydrolyse the unbound probe-acridinium ester complex, rendering it non-chemiluminescent, whilst the bound probe-label complex remains intact. The amount of chemiluminescence observed, after initiation of the reaction by addition of hydrogen peroxide, is therefore proportional to the amount of target nucleic acid present.

Traditionally, DNA probes have been labelled by the use of radioisotopes, most commonly ^{32}P. However, this label has the major disadvantage of a relatively short half life (14.2 days). Thus, nucleic acid probes have a very short shelf life. Add to this the problems of safety in use and disposal when working with radioisotopes and it is not difficult to understand the reasons behind the great body of work devoted to the establishment of non-isotopic labelling methods. The possibilities of non-radioactive labelling of nucleic acids are numerous and so will only be mentioned briefly here, Table 1.3 shows some of the labelling methods that have been used and their detection limits. Similarly, the range of techniques involved in the incorporation of label into the probe is extensive and will not be covered here, there are several references dealing with this subject (Tijssen, 1993; Kessler, 1995).

TABLE 1.3 Detection limits (in attomoles) for selected nucleic acid labels (from Kricka, (1995))

Detection Method	CL	BL	FL	COL	RL	EL
Acridinium ester	0.5	--	--	--	--	--
Alkaline phosphate	0.001	0.01	0.1	50	--	--
B-D-galactosidase	--	5	--	--	--	--
Europium chelate	--	--	10	--	--	--
Glucose 6-phosphate dehydrogenase	--	0.1	--	--	--	--
Horseradish peroxidase	25	0.4	10	100	--	--
Isoluminol	1000	--	--	--	--	--
^{32}Phosphorus	--	--	--	--	50	--
Rhodamine	5000	--	--	--	--	--
Ruthenium *tris* (bipyridyl)	--	--	--	--	--	20
Texas Red	20000	--	--	--	--	--
Xanthine Oxidase	3	--	--	--	--	--

BL; bioluminescence: CL; chemiluminescence: COL; colorimetry: EL; electro-chemiluminescence: FL; fluorimetry: RL; radioluminescence.

1.6 Aims of this study

The need to identify and develop methods that can be used for the measurement of release of GMMOs from bioprocesses has been identified. Any such method will consist of two, inter-related parts: the capture of the organism by an appropriate air sampling method (see Section 1.4.4); and the analysis of the entrained organisms by a method that will specifically identify the released process GMMO in a background of environmental micro-organisms (Section 1.5). Quantitative sampling of aerosols has been extensively studied by Ferris (1995), who has shown that an Aerojet General cyclone can be used to efficiently and repeatedly sample aerosols of suspended micro-organisms. This study aims to take the quantitative air sampling methodology further by developing a specific, sensitive and quantitative detection method that will allow application to real bioprocess environments.

To develop a method to fulfil the aim stated above, it is necesary to lay down a series of criteria against which the technique can be judged. These criteria are listed below:

(i) Selectivity - the ability to measure target GMMOs against a background of

other, non process organisms, cellular debris (e.g. DNA and cell wall components) and airborne particulate material.

(ii) Sensitivity - the ability to measure released GMMOs in the environment at levels encountered in real bioprocessing situations. The sensitivity (capture efficiency) of the sampling methods will be taken into account.

(iii) Quantitative - the ability to determine the scale of release of target GMMOs into the environment. Again, capture efficiency of the sampling method will be an important factor.

(iv) Physiological status - the developed method should be able to distinguish viable GMMOs including those that are non culturable. However, it is also important that non-viable, killed cells can be distinguished from viable ones.

(v) Speed - in order to function as a monitor of bioprocess release, rather than post process verification, any method developed should allow correction/containment of any process that is operating incorrectly. It is therefore important that results of analysis are available to the operator within a relatively short space of time.

(vi) Automation - for any new technique to be widely used in industry, it is necessary that there is a high degree of automation. The possibility of automating the technique will therefore be considered throughout the development.

A survey of available methods has indicated that an approach based on quantitative PCR, using a competitive internal standard is the most promising. Quantitative PCR (QPCR) methods will therefore be developed for two different recombinant *E. coli* strains: JM107 pQR701, harbouring a plasmid encoded transketolase gene; and JM107 pQR126, harbouring a plasmid encoded α-amylase gene. *E. coli* strain JM107 pQR701 is used within the Depatrment of Chemical and Biochemical Engineering at UCL for carbon-carbon bond forming biotransformations (French and Ward, 1995). *E. coli* strain JM107 pQR126 secretes a thermostable α-amylase into the periplasm, and as such is used as a model system to study the purification of periplasmically secreted proteins. The aim is to use the developed QPCR methods for validation of biocontainment in a series of unit operations using either of these *E. coli* strains.

2. MATERIAL AND METHODS

2.1 Microbiological methods

2.1.1 Micro-organisms used in this study

The following strains of micro-organism have been used either as model strains which are specifically detected by the PCR methods subsequently described (denoted by *), or as competitive strains, used to test the selectivity of the PCR methods developed.

TABLE 2.1 Micro-organism strains used in this study

	Strain	Characteristics	Strain donated by:	Reference
1	*E. coli* JM107 pQR701*	Km^R, transketolase	Drs J. Ward & C. French[a]	(French and Ward, 1995) (Yanisch-Perron *et al*, 1985)
2	*E. coli* JM107 pQR126*	Km^R, α-amylase	Dr J. Ward[a]	(Bahri and Ward, 1990) (Yanisch-Perron *et al*, 1985)
3	*E. coli* JM83 pQR187	Km^R, α-amylase	Drs J. Ward & C. French[a]	(Yanisch-Perron *et al*, 1985)
4	*E. coli* JM107 pQR752	Ap^R, T4 lysozyme	Drs J. Ward & R. Sloane[a]	(Yanisch-Perron *et al*, 1985)
5	*E. coli* JM107 pQR150	Km^R, TOL *meta*-cleavage	Dr J. Ward[a]	(Yanisch-Perron *et al*, 1985)
6	*E. coli* JM107	unmodified	-	(Yanisch-Perron *et al*, 1985)
7	*S. cerevisiae wt*	unmodified	-	-
8	*S. cerevisiae 7d*	osmotically fragile	Prof. S.G. Oliver[b]	(Stateva *et al*, 1991)

Ap^R; ampicillin resistance: Km^R; kanamycin resistance: [a]; Department of Biochemistry and Molecular Biology, UCL, Gower Street, London, WC1E 6BT: [b]; Department of Biochemistry and Applied Molecular Biology, UMIST, Manchester, M60 1QD.

2.1.2 Culture and maintenance of micro-organisms

2.1.2.1 Culture on solid media

E. coli strains were grown at 37°C on 28 g L^{-1} nutrient agar (Oxoid) with the addition of 20 μg mL^{-1} kanamycin (Sigma) for strains 1, 2, 3, and 5 and 100 μg mL^{-1} ampicillin (Sigma) for strain 4. Antibiotic was filter sterilised and added to the molten agar after autoclaving. Yeast strains were grown on 50 g L^{-1} malt extract agar (Oxoid) at 28°C.

2.1.2.2 Culture in liquid media (shake flasks)

E. coli strains were grown at 37°C in 25 g L^{-1} nutrient broth (Oxoid) with the addition, after autoclaving, of 10μg mL^{-1} kanamycin (strains 1, 2, 3 and 5) or 100μg mL^{-1} ampicillin (strain 4). Seed cultures were grown by inoculation of a 5 mL volume of the appropriate culture medium in a sterile universal tube with a single colony. The universal tube was then loosely capped and placed in a reciprocal shaking incubator at 200 rpm (New Brunswick). After approximately 4 hours, or sooner if turbidity was noted, 0.5 mL of the seed culture was aseptically transferred to a 250 mL shake flask containing 50 mL of the sterile medium. The culture was grown overnight in an orbital shaker (Adolf Kuhner Ag) at 200 rpm.

Yeast strains were grown up in 20 g L^{-1} malt extract broth at 28°C, the seed time was extended to 8 hours, otherwise the same procedure as for *E. coli* was used.

2.1.2.3 Fermentation medium

For fermentation studies the medium used was a modified version of T-broth (Tartof and Hobbs, 1987) made up according to Table 2.2.

Essentially this medium is 2/3 × T-broth, the reduction in nutrients being necessary to allow a more controlled fermentation in the 2 L flask (Section 5.3.1). The seed was produced by inoculating 100 mL of this medium in a 500 mL shake flask with a loopful of culture from a fresh agar plate. The shake flask was incubated for 12-14 hours in a reciprocal shaking incubator at 37°C and 150 rpm. The entire contents of the seed flask were used to inoculate the 2 L fermenter (1.5 L working volume), hence the inoculum represented 6 % of the final volume in the fermenter.

TABLE 2.2 Composition of modified T-broth

Component	Concentration ($g\ L^{-1}$ unless otherwise stated)
KH_2PO_4	2.3
K_2HPO_4	3.8
Bactotryptone	8
Yeast Extract	16
Glycerol	3 mL L^{-1}
Polypropylene glycol (PPG, antifoam, BDH)	0.2 mL L^{-1}
Kanamycin	20 mg L^{-1}*

* Sterile filtered and added to broth after autoclaving

2.1.2.4 Maintenance of micro-organisms

Stock cultures of all *E. coli* strains were maintained on 20 % (w/v) glycerol solution at -70°C. Glycerol stocks were prepared by pipetting 3 mL of sterile 20 % glycerol solution onto an agar plate on which the culture had grown (without contamination). Using a sterile inoculation loop, colonies were lifted off the agar into suspension. Finally, using a sterile pipette tip, the suspension of micro-organisms in glycerol solution was returned to the sterile glass bijoux, which was then stored at -70°C. Stock cultures of the yeast strains used were stored on slopes at 4°C. Slopes were prepared using 50 g L^{-1} malt extract agar.

Working cultures of all micro-organisms were maintained on agar at 4°C. Cultures were replated every 2 weeks. Stock and working cultures were stored in duplicate.

2.1.3 Optical density (A_{600}) measurement

Optical density of broth cultures was measured at 600 nm using a Beckman DU-64 spectrophotometer (Beckman Instruments). Cultures were diluted in reverse osmosis (RO) water to ensure that the A_{600} reading was in the range 0.05-0.3 absorbance units. Sterile broth diluted in RO water was used as the blank.

2.1.4 Microscopic cell counting

2.1.4.1 *E. coli*

The concentration in suspension of *E. coli* cells in a sample was determined by microscopic counting using a cell counting chamber. A small drop of sample was pipetted onto a Helber Bacteria Counting Chamber with Thoma rulings (Weber Scientific International Ltd) and a cover slip was carefully but firmly slid into place. In

order to ensure that the film between the grating and the cover slide was of the required thickness, the presence of a rainbow pattern or 'Newton's rings' around the sample chamber was used to confirm an adequate seal. If this was not seen, the chamber was rinsed with RO water and the procedure re-started. The chamber was viewed using phase contrast and ×400 magnification (×40 objective, ×10 eyepiece, Nikon Optiphot Microscope, Nikon). The number of cells in each of the 16 squares of the grid was counted and recorded. The sample was diluted so that the average number of cells per square was in the range 1-50. In order to convert the number of cells counted per grid to number of cells per mL a multiplication factor of 7.8×10^4 was used, hence the limit of detection is equivalent to 1.25×10^6 cells mL^{-1}.

2.1.4.2 Yeast cells

Yeast cell concentration was measured using a haemocytometer with improved Neubauer ratings (Weber Scientific). Each slide has two gratings allowing the determination of two samples. The cover slide is applied to the haemocytometer before application of the sample by wetting of its edges. Once it has been firmly applied, a small drop of sample is pipetted onto the edge of the gap provided by the cover slide and grating. The sample is drawn into the central well by capillary action. The 'slide' is viewed at ×100 magnification (×10 objective, ×10 eyepiece) using normal phase illumination. Samples were diluted to produce an average count of between 1-50 per square; there are 25 squares per grid. In order to express the number of cells per mL, the number of cells counted per grid was multiplied by 1×10^4, giving a limit of detection of 2.5×10^5 cells mL^{-1}.

2.1.5 Enumeration of colony forming units (CFU) by agar plate counts

The number of CFU in a sample was estimated by use of spread plates. Between 10 - 100 μL was spread onto the surface of an agar plate comprising the appropriate growth medium. Agar plates were thoroughly dried before use to prevent spreading of colonies. The number of colonies was counted after 1-2 days incubation at 37°C.

2.1.6 Determination of plasmid stability

The proportion of CFU in a sample that express the plasmid of interest (referred to as plasmid stability) was determined by comparing the numbers of CFU mL^{-1} using nutrient agar as the plate count medium (N_T) with the CFU mL^{-1} using nutrient agar supplemented with the appropriate antibiotic (N_A). Plasmid stability was calculated thus:

Plasmid stability (%) = $N_A / N_T \times 100$

2.2 Polymerase chain reaction (PCR)

The principle of the PCR is outlined in Section 1.5.2.2 along with several examples of its use in diagnostic microbiology.

2.2.1 General precautions taken to prevent PCR product carry-over

The sensitivity of PCR is one of the main benefits of the technique, however it can also lead to problems of sample contamination due to product carryover from a previous PCR. Whilst PCR can detect less than 10 copies of target DNA, after amplification the number of copies of DNA can reach 10^{12} in a 0.1 mL reaction (Mullis and Faloona, 1987). Several workers (Kwok and Higuchi, 1989; Sarkar and Sommer, 1990; Kitchin and Bootman, 1993) have therefore made recommendations on how best to avoid carry-over of PCR products causing subsequent false positive results. For all PCR work carried out here a series of measures was taken to reduce the chances of product carry-over.

1. PCR preparation and product analysis was physically separated. Sample preparation was carried out within a Class II microbiological safety cabinet (Gelman), whilst analysis of PCR products was carried out in a separate lab. No pipettes or consumables were ever shared between the two areas. Disposable gloves were worn at both locations and were changed on moving between the two.
2. Stock solutions were aliquoted. Primers, sterile RO water, deoxynucleotide triphosphate solutions, buffers and enzyme preparations were made up in batches in the Class II safety cabinet and aliquoted into clean eppendorf tubes and stored at -20°C. During experimentation the batch reference number of each reaction component was noted in case of any contamination, the source of which could then be traced. Each aliquot was sufficient for between 20 and 40 PCRs.
3. Sterile consumables were used. Consumables were either purchased sterilised or were autoclaved prior to use.
4. Pipettes were contamination free. All pipette tips used in the setting up of a PCR possessed a filter (Anachem), which prevented aerosols formed during pipetting from reaching the barrel of the pipette. This should prevent pipette contamination.
5. Reagents were dispensed as a premix and sample DNA added to the reaction tube last. The use of a premix reduces the number of pipetting steps and hence the chances of sporadic contamination. Addition of sample DNA to the reaction tubes after all other components reduces the risk of inadvertent transfer of DNA.
6. Negative and positive controls were used. Each PCR experiment contains a 'no DNA' control, i.e. a tube containing all necessary ingredients of the PCR except the template. This tube was the last to be prepared so that any inadvertent

contamination of pipette tips or drip formation from previous tubes would be detected.

2.2.2 Preparation of samples for PCR

Purified plasmid DNA samples were made from an overnight culture of the relevant micro-organism using a Wizard Miniprep Kit (Promega) and following the manufacturer's instructions. Plasmid DNA was stored at -20°C and was used in the PCR without any further pretreatment. Dilutions of these DNA preparations were made using sterile RO water (SROW) as the diluent.

For the PCR of 'whole cells' during the development and validation stages of the PCR methods, cells were grown up as previously described (Section 2.1.2.2). A 0.8 mL sample of culture was taken 15-20 hours after inoculation. The A_{600} of this sample was measured and the cells in the sample were pelleted by centrifugation at 7000 × g for 2 minutes (Sigma Laboratory Centrifuges). Cells were resuspended by whirlimixing in sterile thiosulphate ringers solution (TRS, Oxoid) using the appropriate volume to achieve a suspension of A_{600} of 2.5. The aim of this 'standardisation' step was to provide a relatively consistent sample of whole cells in the developmental stage of the project. The resuspended cells were serially diluted by 1/10 in sterile TRS to achieve the desired concentration. In order to determine the number of whole cells per mL of the samples, the second 1/10 dilution (10^{-2}) was routinely counted microscopically (Section 2.1.4) and the 10^{-6} sample was subjected to agar plate count (Section 2.1.5) to determine CFU mL^{-1} and to ensure that no errors had occurred on dilution.

2.2.3 Lysis of samples prior to PCR

Unless otherwise stated, cell suspensions derived from culture and samples derived from cyclone operation were used directly in the PCR without any pretreatment. However, in some instances, samples were first subjected to a boiling step to effect lysis prior to PCR. This was carried out by pipetting 100 µL of sample into a clean, sterile, screw cap 1 mL Eppendorf tube and then placing this tube in a boiling water bath for 30 minutes. At the end of this time, the Eppendorf tube was briefly centrifuged so that any condensate returned to the bottom of the tube. When the tube had cooled, the contents were used in the PCR.

2.2.4 Preparation of stock solutions for PCR

The following stock solutions were made up and aliquoted according to the precautions described in Section 2.2.1.

TABLE 2.3 Stock solutions used in the PCR

Component	Stock Concentration	Made up in;
SROW	-	-
Deoxynucleotide triphosphate mix (dATP, dTTP, dCTP, dGTP)	Each dNTP at 1.25 mM	SROW
Primer	20μM	SROW

2.2.5 Primers used in this study

There are several informal rules for the choice of primers to be used in PCR (Steffan and Atlas, 1991). Adherence to these guidelines should prevent the formation of primer-dimers and other non-specific amplification products. In addition, it is important that the primers chosen will not cross-react with sequences other than the target, otherwise false positive results might occur in diagnostic applications. The specificity of primers is determined by the number of nucleotides in the sequence: too few means that the probability of the primer recognising non-target sequences might be high; too many means that any base pair mis-matches between the primer and target sequences might be compensated for by the stability of the numerous other pairings. The recommended length for primers in PCR is 18-20 base pairs (bp). It should also be remembered that since some genes are highly conserved between different species, the choice of a primer that is specific to a certain gene might still tend to cross-react. In this study, this problem has been avoided by choosing one primer that is complimentary to a sequence on a gene inserted into a plasmid and the other that is complimentary to a section on the vector itself. For PCR of a sequence of *E. coli* chromosomal DNA, primers have been chosen that have been shown to specifically detect this species (Bej *et al*, 1991). For further discussion of the choice of primers used in this study, see Section 3.1. It is also worth noting that the linker primers used in this study (chosen according to the method of Forster (1994)) are not of the optimal length for PCR, but are used solely in the preparation of the internal standards that are required for quantitative PCR.

TABLE 2.4 Primers used in this study

Primer	Sequence 5'-3'	Anneals to;
M13R1	GGA CCA AGC TAT GAC CAT G	M13 sequence in pBGS18, pBGS19 vectors (Spratt *et al*, 1986)
CMTA1	CGT CAA AGA GTG TAT TGA GG	*CmtA* gene in transketolase insert in pQR701
TK-LINKER	GTG TAT TGA GGG ATC GAT CAG GGC GTC TAT	*CmtA* gene in transketolase insert in pQR701
AMY-2	GCG TAG TCC CAC TCG AAG A	*Amy* gene in α-amylase insert in pQR126
AMY-LINKER	CAC TCG AAG AAG CGC GCC CGA GAG CGT TCT	*Amy* gene in α-amylase insert in pQR126
URL-301	TGT TAC GTC CTG TAG AAA GCC C	*gusR* (regulatory gene of *gusA*, β-glucuronidase) on *E. coli* chromosome
URR-432	AAA ACT GCC TGG CAC AGC AAT T	*gusR* (regulatory gene of *gusA*, β-glucuronidase) on *E. coli* chromosome

2.2.6 PCR Protocols

The standard conditions for PCR of target plasmid pQR701 and E. coli *gene* gusR *were as follows;*

The PCR was carried out in 0.6 mL reaction tubes with a 25 μL reaction volume containing 0.25 μL (1.25 units) *Taq* polymerase (Gibco BRL Life Technologies), 2.5 μL 10× *Taq* polymerase buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 0.75 μL 50 mM $MgCl_2$ (giving a final concentration of 1.5 mM), 4 μL dNTP solution (1.25 mM each: dATP; dTTP; dCTP; dGTP, Pharmacia), 2.5 μL (20 pmoles μL^{-1}) of each primer M13R1 and CMTA1, 2.5 μL SROW and 10 μL of sample. Each reaction mixture was overlaid with 25 μL light mineral oil (Sigma). PCR was carried out by placing the

reaction tubes into an OmniGene temperature cycler (Hybaid) which was programmed for 94°C for 5 minutes followed by 30 cycles of: 94°C for 30 sec; 55°C for 1 min; and 72°C for 1 min. A final 10 min extension step at 72°C then followed. The total time taken to complete the PCR was approximately 3 h.

The standard conditions for PCR of target plasmid pQR126 were as follows;
The PCR was carried out in 0.6 mL reaction tubes with a 25 μL reaction volume containing 0.5 μL (2.5 units) *Taq* polymerase , 2.5 μL 10× *Taq* polymerase buffer, 4 μL dNTP solution (1.25 mM each), 2.5 μL (20 pmoles μL^{-1}) of each primer M13R1 and AMY-2, 1 μL SROW, 1.25 μL formamide and 10 μL of sample. Each reaction mixture was overlaid with 25 μL light mineral oil. PCR was carried out according to the protocol described above.

In experiments to determine an optimum set of conditions for PCR of the segment of the pQR126 target the co-solvents formamide and dimethyl sulphoxide (DMSO) were added to the reactions at concentrations of 5 % (1.25 μL/PCR) and 10 % (2.5 μL/PCR), with the SROW volume reduced accordingly. *Pfu* polymerase (Stratagene), *Taq* polymerase and Vent polymerase (New England Biolabs) were each used under these conditions.

For all experiments where an internal standard was co-amplified, it was added in a 1 μL volume, the volume of SROW was reduced accordingly. In developing an appropriate PCR protocol for each target, annealing temperature, extension time, the source of the polymerase enzyme and magnesium concentration were all varied in an effort to produce a reliable and sensitive PCR. Details of individual experiments are not given.

2.2.7 Preparation of competitive internal standards for quantitative PCR

The principle of the quantitative PCR (QPCR) assays used in this study is that the target DNA sequence is co-amplified with a known amount of a competitive DNA sequence (Becker-Andre and Hahlbrock, 1989; Gilliland *et al*, 1990). In order for the assay to work, the competitor DNA sequence must have the same primer binding sites as the target, and the products of the target and competitor sequences must be distinguishable after PCR amplification. In the method used here, the products can be distinguished by their difference in migration rate under agarose gel electrophoresis. For a further discussion of the generation of the internal standards and the QPCR method see Sections 3.2.1 and 7.1.1.2, respectively.

2.2.7.1 Generation of the internal standards

The competitive internal standard (IS) DNA fragments used in the QPCR assays were generated according to the method of Forster (1994). The production of IS(T) - the competitive internal standard for the transketolase construct pQR701- is shown below. IS(A), the competitive internal standard for the amylase construct pQR126, was produced in the same way. Note that each PCR round was carried out using the standard conditions for the original primer pair.

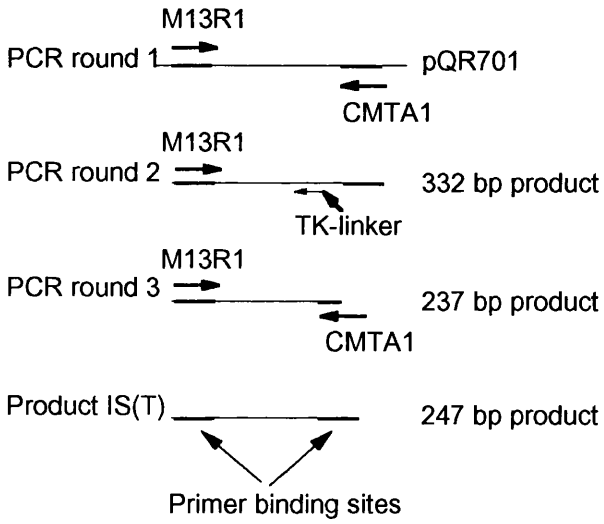


FIGURE 2.1 Schematic showing construction of IS(T). PCR round 1: 'normal' PCR of the template pQR701 using the primers M13R1 and CMTA1. The products of the PCR are checked on an agarose gel and 5 μL of the post-PCR mix was diluted by a factor of 10^4 in SROW for the subsequent round of PCR. PCR round 2: diluted product from PCR round 1 is amplified using primers M13R1 and TK-linker. The TK-linker primer anneals to a section in the DNA sequence between the 2 original primer binding sites. Because the TK-linker has a section of the primer CMTA1 at its 5' end, the compliment of this sequence becomes incorporated into the PCR product. Products were checked on an agarose gel and again diluted by 10^4 for the subsequent round. PCR round 3: diluted product from PCR round 2 was amplified using primers M13R1 and CMTA1. The primer CMTA1 anneals to its complimentary sequence that was incorporated into the DNA segment on round 2. Product (IS(T)) was run on an agarose gel and purified (see next section).

2.2.7.2 Isolation of DNA from agarose gels

Bands were excised from agarose using a scalpel under incident UV light. DNA was isolated from the excised bands by use of a Costar Spin-x tube (Costar) and the method of Polman and Larkin (1989).

2.2.7.3 Purification of DNA isolated from agarose gels

IS DNA isolated from agarose was further purified by use of a QIAquick nucleotide removal kit (Qiagen). DNA solutions were stored at -20°C. DNA purified in this way was sufficiently pure to allow accurate quantitation by optical absorption measurement.

2.2.7.4 Quantitation of DNA by optical absorption measurement

The IS DNA solutions prepared as described above were quantified by measurement of A_{260} and A_{280} using a Beckman DU-64 spectrophotometer (Beckman Instruments). The ratio of A_{260} /A_{280} was used as a measure of purity of DNA, if this value was found to be less than 1.7 the DNA solution was deemed to be insufficiently pure for quantification without further purification. DNA concentration was calculated using the approximation that dsDNA at 50 mg mL^{-1} has an A_{260} of 1 (Sambrook *et al*, 1989).

2.2.8 Differentiation between extracellular and intracellular DNA

For a full account of the development of these methods and their purpose, see Section 4.2.

2.2.8.1 Treatment with DNase I

In this method, extracellular DNA is degraded with DNase I. Prior to PCR, samples of micro-organisms or DNA diluted in TRS were mixed at a 5:1 ratio with a solution of DNase I (Sigma) in 0.15 M NaCl to give a final DNase concentration ranging between 250-25,000 U mL^{-1} in a 20 µL sample volume in sterile 0.6 mL reaction tubes. The reaction tubes were then incubated at 37°C for 30 minutes, followed by 95°C for 10 minutes. After cooling, a 10 µL aliquot was used in the PCR in the usual way. The results were compared with those from an untreated sample.

2.2.8.2 Microfiltration

In this method, intracellular DNA is removed when the cells are retained on the microfilter. Where appropriate, a 0.5 mL aliquot of a micro-organism or DNA sample was taken and was microfiltered by placing in a Costar spin-x tube (0.2 or 0.45 µm pore size, low DNA binding cellulose acetate membrane, Costar) which was then centrifuged for 5 minutes at 7000 × g. A 10 µl aliquot of the filtrate was then used in the PCR and the results compared with those from an unfiltered sample.

2.3 Analysis of PCR products

2.3.1 Gel electrophoresis

Agarose gel electrophoresis of DNA (PCR products) was performed as described in Sambrook *et al* (1989). The running buffer used was 0.5 × TBE (Tris borate EDTA, working concentrations; 45 mM Tris base, 45 mM boric acid, 1 mM EDTA) with 0.25 μg mL^{-1} ethidium bromide. Gels were made up in 100 mL of 0.5 × TBE and cast in the gel tray (12 × 14 cm) provided with the electrophoresis tank used (Midi system with buffer puffer, Hybaid). For PCR fragments of < 500 bp, 2 % agarose gels were typically run at 80 V for 2-3 hours at room temperature. This was sufficient to allow separation of IS and template DNA. PCR samples were run by mixing 7.5 μL with 2.5 μL of gel loading solution (0.05 % (w/v) bromophenol blue, 40 % (w/v) sucrose, EDTA (0.1M, pH 8.0) and 0.5 % (w/v) SDS) and loading the resulting mixture.

The molecular weight marker used in these studies, φx-174 RF DNA Hinc II digest (Pharmacia), was stored at -20°C as a 0.1 μg μL^{-1} solution in 10 mM tris-HCl (pH 7.5), 1 mM EDTA and 20 % (v/v) gel loading solution. A 4 μL aliquot of this solution was run on the gel.

2.3.2 Gel documentation and densitometry

Gels were routinely documented using a UV transilluminator and Gel Documentation System with ImageStore 5000 software (UVP Ltd), which produces a thermal print of the image. On some occasions, gels were additionally photographed using a Polaroid Land Camera and HP5 negatives (Ilford). The film was developed with Ilford Microphen developer and fixer according to the manufacturer's instructions. Several prints of these negatives were made if desired.

The UVP Gel Documentation System was also used for densitometry of gels by downloading the gel image onto a disk then analysing the data using Gelbase software (UVP Ltd). In order to ensure that the downloaded data did not comprise of overloaded images (which would affect the quantitation procedure but which would be detected by the software), up to 3 images of each gel were taken and downloaded using different aperture settings on the lens. For maximum sensitivity (i.e. weak bands) a wide aperture was used, but this increases the likelihood of image overload from adjacent intense bands. Since the quantitation method used in this study involves measurement of the peak area ratios of one band to another, it is necessary that the intensity of fluorescence emitted by each band is within the linear range of detection. However, if one band, say the IS, was so weak that it required the use of a wide aperture to be visible and the other

band in the same lane, in this case the target product band, was very intense, then it would be impossible to accurately measure the ratio of the peak areas. This is what limits the dynamic range of detection of this densitometry method. In practice it was found that the ratio of intensities of one band to another within the same lane must be less than 20, both peak areas must be greater than 100 units, and that, of course, neither band must be saturating. Peak area were scored according to these rules, which are noted below in point form:

Rule 1 - bands recorded must be within the linear range of detection of the system (peak areas must be > 100, but not saturated)
Rule 2 - bands recorded at a certain aperture setting must have peak area ratios of between 0.05 - 20.

2.3.3 Capillary electrophoresis

Separation and quantitation of PCR products was also attempted by capillary gel electrophoresis using a P/ACE 2000 system with an eCAP dsDNA 1000 kit (Beckman Instruments). The kit contains gel buffer (polyacrylamide solution in tris-borate buffer), a standard test mix containing fragments of DNA of known length and the coated capillary. The PCR mixtures were separated from the mineral oil layer, but otherwise no sample pre-treatment was carried out. Separation of DNA fragments was attempted using the conditions listed in Table 2.5.

TABLE 2.5 Conditions used for capillary electrophoresis separation of PCR products

Parameter	Condition
Capillary length	37 cm (30 cm to detection window)
Voltage applied	7.4 KV (200V/cm field strength)
Current limit	100μA
Typical current	16-22μA
Detection wavelength	254 nm
Injection mode	Pressure, 10 seconds
Rinse time between runs	3-5 minutes, pressure

2.4 Aerosol Sampling

2.4.1 Operation of the Aerojet General Cyclone.

The air sampling device used in theses studies was an Aerojet General Cyclone (Soham Scientific) (Decker *et al*, 1969; Upton *et al*, 1994), a modified version of an Errington-Powell cyclone (Errington and Powell, 1969) and is shown schematically in Figure 2.2. The principle of operation is that airborne micro-organisms drawn into the cyclone are deposited on the inner walls of the glass device due to the fast rotation of the air and are subsequently washed off by the circulating liquid. At the end of operation, all circulating liquid is collected, the volume is measured and the liquid sample is used for subsequent analytical techniques (PCR, cell and CFU counts).

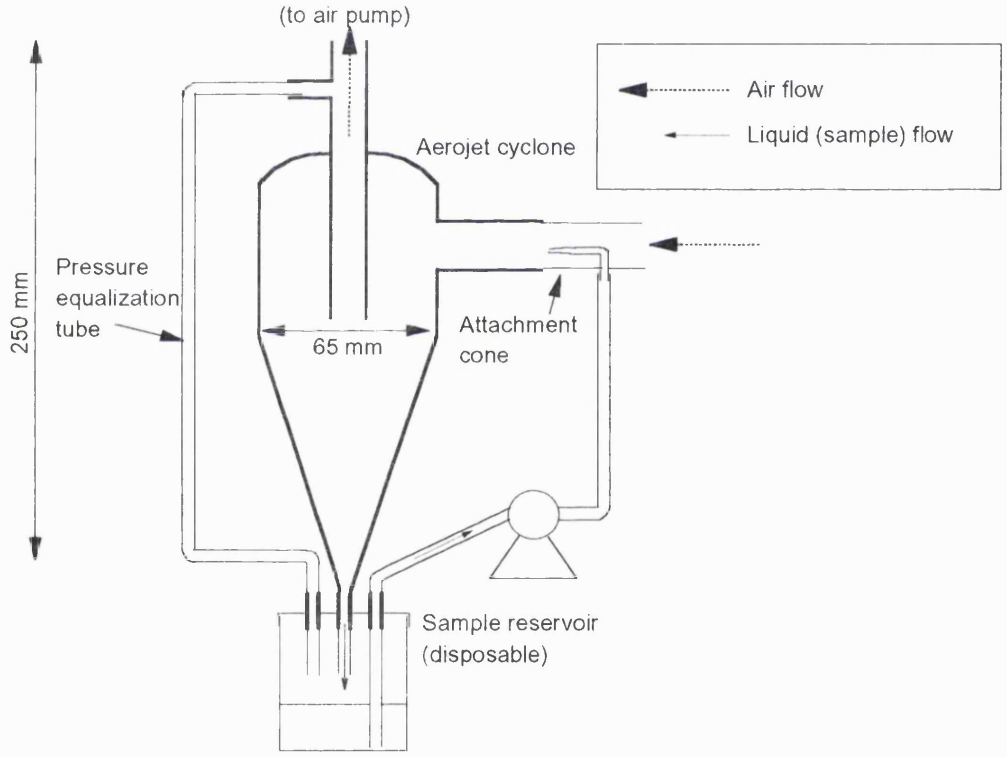


FIGURE 2.2 Schematic representation of an Aerojet General Cyclone. The cyclone and attachment cone are made of borosilicate glass. All tubing is silicone rubber (7.5 mm OD, 4 mm ID). The sample reservoir is made of polypropylene and has a metal lid.

A number of modifications have been made to the cyclone described in an earlier publication (Ferris *et al*, 1995). The sample reservoir is now situated immediately beneath the cyclone and the scrubbing liquid is recirculated directly from here to the cyclone inlet. This eliminates the need for a second sample reservoir and and an additional pump head. The sample reservoir used in these studies was a 140 mL sterile disposable pot with a metal lid. A series of metal lids were made up with holes drilled

through and small lengths of rigid plastic piping were pushed through the holes creating a tight seal. The tubing of the cyclone was attached to this piping creating a lid to which new sample pots could easily be screwed on. This greatly minimised the handling of tubing between runs and allowed a sample pots to be interchanged without excessive manipulation that might lead to cross-contamination.

The other major alteration was that liquid was injected into the cyclone by means of a cone attachment which is illustrated below in Figure 2.3.

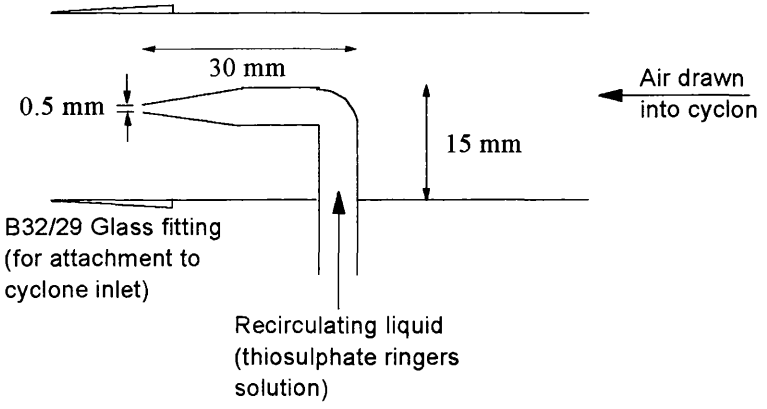


FIGURE 2.3 Cross sectional schematic view of cyclone attachment cone

The dimensions of this attachment were established empirically to optimise the washing efficiency of the cyclone and to minimise the loss of liquid through the central pipe of the cyclone (a phenomenon known as 'precession' that is common to irrigated cyclones (Swift, 1986)). It should be noted that the attachment has a ground glass fitting by which it is attached to the cyclone inlet arm (which was made up with a corresponding female fitting). The reason for this modification to the cyclone was that the previous means of introducing liquid into the cyclone (via a hypodermic needle attached to the cyclone arm by cable tie grips) was found to be very prone to incorrect set-up. Ferris (1995) has found that the angle of injection of the liquid into the cyclone is very influential on the recovery efficiency and the amount of precession that occurs. The benefit of using an attachment cone was that the liquid would be introduced into the cyclone in a consistent fashion and that setting up the cyclone was simplified.

In the experiments described here, air was drawn into the cyclone by an air pump (Air Control Installations) at 360 L min^{-1}, and the collecting liquid, 80 mL TRS, was recirculated at 20 mL min^{-1} using a peristaltic pump (Watson-Marlow). Sampling was carried out in batch-mode for 10-30 minute periods. At the end of sampling the volume of collection liquid remaining was measured. Loss of volume by evaporation and escape

of small streams of liquid up into the air pump normally amounts to approximately 0.5 - 1 mL min^{-1} of sampling. In experiments where the cyclone was connected to a contained cabinet into which air was only allowed to enter via HEPA filters, air flow rate through the cyclone was verified by measuring the air filter exhaust flow rate using a mass flow meter (Laminar Flow Element, Teledyne Hastings-Raydist).

2.4.2 Cleaning the cyclone.

The cyclone and associated tubing have inaccessible surfaces which can not be scrubbed clean, so all cleansing was carried out by immersion into a 5 L container containing 1 % Tego solution (Th. Goldschmidt Ltd) and allowing a 15 minute period of soaking. To ensure that all surfaces of the cyclone were thoroughly wetted, the cyclone was inverted in the container half way through the soaking the period. During this time all tubing was thoroughly wetted by squirting through the cleaning solution using a wash bottle. After the soaking stage, the cyclone and tubing were thoroughly rinsed out with tap water and were allowed to dry by standing for 5-10 minutes. Fresh disposable containers were used for each sample and great care (and gloved hands) were used to transfer tubing into the fresh sample pots.

2.4.3 Sampling aerosols from within the Bassaire cabinet

The Bassaire cabinet (Bassaire Ltd) is a sealed laminar flow cabinet of 0.36 m^3 volume with inlet and outlet fan-assisted HEPA filters (Ferris *et al*, 1995). There are ports on the side of the cabinet which allow the connection of a cyclone and an atomizer via drilled bung adaptors (see Figure 2.4). This piece of equipment was used for sampling aerosols of known bacterial concentration within a contained, fixed volume.

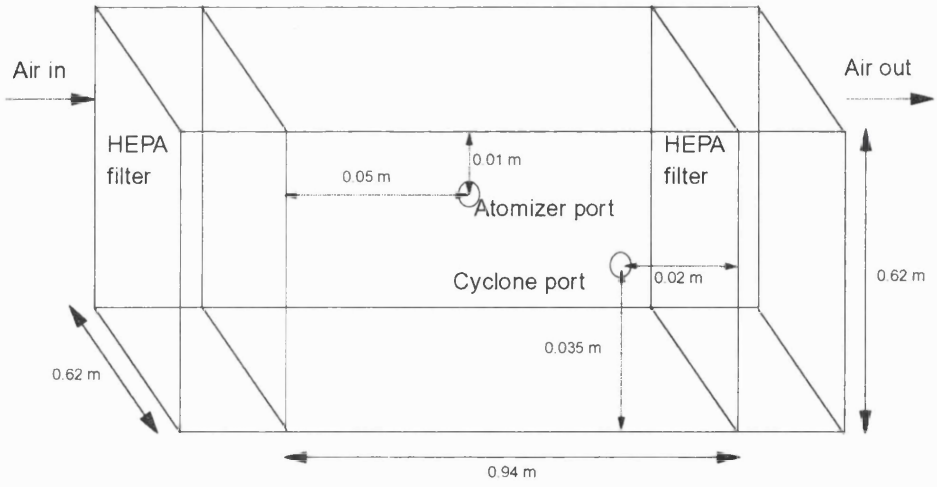


FIGURE 2.4 Bassaire cabinet. The walls of the cabinet are constructed of clear perspex. The front panel can be removed for cleaning (see Section 2.4.3.2).

2.4.3.1 Production of aerosols using an atomizer

Aerosols were generated by use of a glass atomizer (Warren Spring Laboratories) consisting of two concentric tubes (Figure 2.5) (Ferris *et al*, 1995). A suspension of bacteria was pumped up through the inner tube at 1 mL min^{-1}, whilst compressed air at 68 Kpa was passed through the outer tube. Liquid reaching the end of the inner tube is subject to rapid air flow causing aerosolization. The particle size distribution of the aerosol formed in this way has been shown to be quite broad (Ferris, 1995), and can be described as a plateau ranging from 1 to 15 μm diameter.

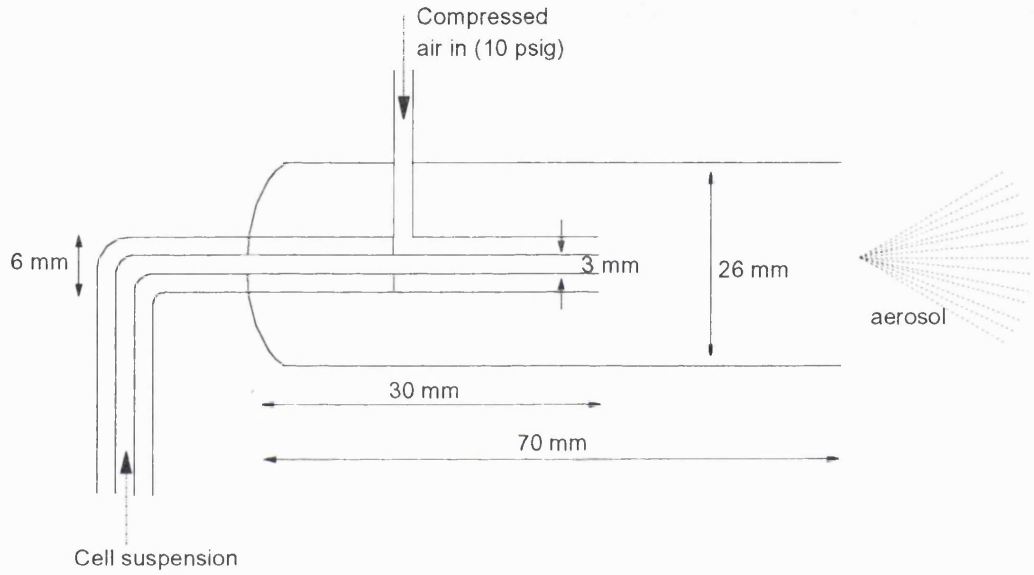


FIGURE 2.5 Schematic cross section of atomizer

Overnight cultures of *E. coli* JM107 pQR701 were serially diluted into either sterile TRS or sterile nutrient broth immediately before spraying. A volume of 18 mL was aerosolized and then the spray line was flushed by passing 5 mL of sterile TRS through at the same rate.

2.4.3.2 Operation of the Bassaire cabinet

Before spraying, the Bassaire cabinet was washed inside with 1 % Tego solution and then the HEPA filter fans were turned on for 30 minutes to flush out the cabinet. Spraying took place with the fans turned off so that the only air movement inside the cabinet was caused by the spraying (mass flow = 23 L min^{-1}) and the cyclone air sampling (at 360 L min^{-1}). Throughout the spraying, the cyclone was operating and was left to run for a further 5 minutes to bring the total sampling time to 30 minutes. When the experiment was finished, 30 mL of 0.5 % Tego was sprayed into the cabinet and then the atomizer, cyclone and all tubing were washed out by immersion in 1 % Tego for 30

minutes followed by rinsing with tap water. The inside of the cabinet was wiped clean and then the HEPA fans were started in preparation for the next experiment. In order to account for any carryover from previous experiments, 18 mL of sterile thiosulphate ringers solution was typically sprayed and collected by the cyclone to give a background reading before any cells were sprayed. Additionally, the lower cell concentrations were always used before the higher concentrations.

2.5. Operation of process equipment

2.5.1 APV 30CD homogeniser

The 30CD (APV (UK) Ltd) is a positive displacement high pressure reciprocating pump homogeniser. Homogenisation is achieved by means of a triple action pump which can create pressures of up to 100 MPa on the process liquid as it passes through the cell disruption (CD) valve. The pistons that provide the pumping action are supplied by lubricating/cooling water from a dedicated source. For contained operation, it is recommended that the homogeniser is used within an isolator which provides secondary containment and that the lubrication/cooling water is run to a kill tank (Kastelein *et al*, 1992).

In order to monitor release of micro-organisms from the 30CD, the homogeniser was placed inside a soft film cabinet of 8.37 m^3 volume and fitted with inlet and outlet HEPA filters (Elwyn E. Roberts Isolators Ltd). The efficiency of microbial aerosol capture from this cabinet when the cyclone is attached to the side (Figure 2.6) has previously been determined (Ferris *et al*, 1995).

Prior to use, the homogeniser was cleaned in place (CIP) by recirculating 5 L of 1 % w/v NaOH solution through the process pipework at 56 L h^{-1} for 30 minutes, followed by thorough rinsing with RO water. The lubrication circuit was cleaned in similar fashion, the flow rate being 10 L h^{-1}.

To monitor release of *E. coli* cells during homogenisation, 5 L of 10 % w/v *E. coli* JM107 pQR701 cells in 0.05 M sodium phosphate buffer, pH 7.0 was used as the process fluid. Frozen *E. coli* JM107 pQR701 cell paste (1 Kg, harvested from a 500 L fermentation at Glaxo Wellcome Medicines Research Centre using a using a Sharples AS 16 V:B tubular bowl centrifuge (Alfa-Laval) operating at 17,000 rpm (15,000 × g)) was resuspended into 10 L of cold 0.05 M sodium phosphate buffer, pH 7.0, using a high speed mixer (Silverson Machines).

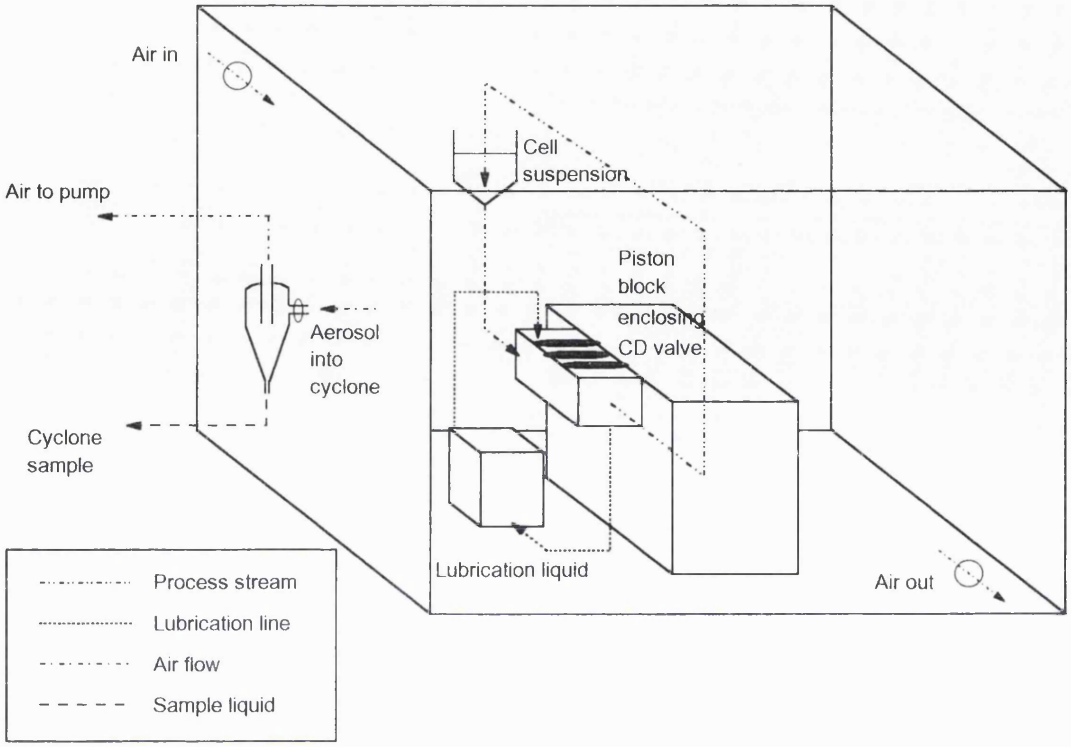


FIGURE 2.6 Air sampling from CD30 homogeniser within the soft-film cabinet using the Aerojet-General Cyclone

The cell suspension (5 L) was pumped into the hopper of the homogeniser in an attempt to avoid spillages or aerosol generation. The cell suspension in the hopper was mixed throughout the experiment and was cooled by 6°C water passing through a surrounding jacket. After filling the hopper, the cabinet was sealed and all subsequent operations were carried out from outside. The inlet HEPA filter fan was turned on causing air to enter the cabinet at 40 L min^{-1}, air flowed passively out of the exit filter.

Sampling of the air within the cabinet by operation of the cyclone (at 360 L min^{-1}) took place over 30 minute periods. At the end of each period the cyclone was washed and a sample taken from the recirculating lubrication liquid. A background sample of the air in the cabinet was taken before the homogeniser was operated.

The homogeniser was operated according to S.O.P. no. 37 (ACBE, UCL). Lubrication liquid (RO water) was circulated at 10 L h^{-1}. Air samples were taken during operation of the homogeniser at a flow rate of 113 L h^{-1} without pressure applied by restricting the CD valve; and at 113 L h^{-1} flow rate with a pressure of 45 MPa created by partial closing of the CD valve by operation of a handwheel. In all instances, the product temperature in the hopper remained at around 12°C, although immediately downstream of the CD valve, the temperature approached 30°C when the valve was partially closed.

After operation, the homogenate was sterilised and discarded. A CIP routine was carried out on the homogeniser. Cyclone and lubrication liquid samples were analysed by QPCR. A sample of the feed and homogenate process streams were also analysed by QPCR and cell counting.

2.5.2 2 L Fermenter

The fermenter used in these studies was a 2 L glass vessel (1.5 L working volume) with a 6 blade Rushton turbine impeller (Incelltech (UK) Ltd). Temperature was controlled thermostatically by means of a heating element and probe. Control of pH was achieved by addition of 0.5 M NaOH solution, using a steam sterilisable pH probe (Broadley James Corporation). No acid additions were made to prevent the pH from rising. The sparge rate used in all experiments was 1.5 L min^{-1} (1 v.v.m.). Inlet and outlet air lines were both 0.2 μm filtered using Gelman Acro50 filters (Fisons) The dissolved oxygen tension (DOT) was maintained at above 20 % by automatic variation of the stirrer speed between 300-1100 rpm. Temperature, pH and DOT were all logged throughout the fermentation. Exit-gas was analysed by mass spectrometry (Prima 600, Fisons Scientific Instruments) to determine the oxygen uptake rate (OUR) and the carbon evolution rate (CER). A real time data acquisition system (RTDAS) was used.

Release of cells into the exit gas stream was measured by disconnecting the exit gas filter and rapidly connecting the exit gas line to the cyclone air intake by means of an adapter (Figure 2.7). Additionally, some experiments were conducted where exit-gas was monitored downstream of the exit gas filter. In both cases, exit gas is introduced directly into the cyclone where it is greatly diluted by incoming air from the room in which the fermenter is placed (exit gas air flow is 1.5 L min^{-1}, whilst cyclone air intake is 360 L min^{-1}).

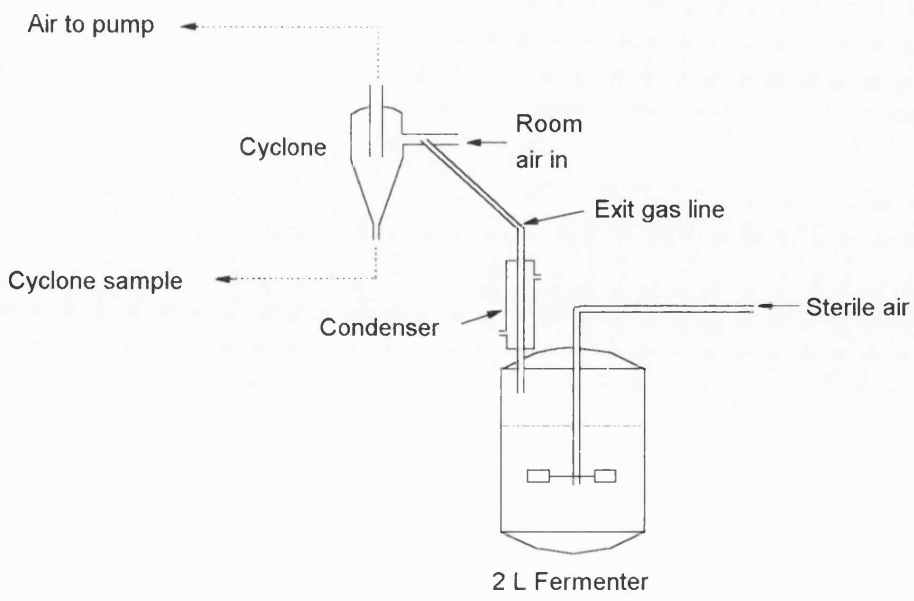


FIGURE 2.7 Sampling fermenter exit-gas using the Aerojet-General Cyclone. Exit air flows at 1.5×10^{-3} m^3 min^{-1} whilst the air pump in the cyclone draws air in at 0.36 m^3 min^{-1}. Air flow through the cyclone is maintained by allowing unfiltered room air to enter. The distance travelled by exit gas from the fermenter to the cyclone is 1 m.

Since the incoming room air is unfiltered, it is essential that careful background measurements are made to determine whether the room air itself contains target *E. coli* JM107 pQR701 cells. For each measurement the cyclone was therefore run for 10 minutes before connection to the exit-gas line. After this time, a 1 mL sample was aseptically taken from the sample pot. This sample was subjected to QPCR and the results were used to determine the cleanliness of the cyclone (i.e. how efficiently it had been cleaned since a previous sample) as well as any background level of *E. coli* JM107 pQR701 cells that may be present in the air. On connection to the exit-gas supply, the cyclone was run for a further 15 minutes. Therefore, for each sample there were 2 measurements; a background measurement and an exit-gas measurement. On some occasions, the cyclone was run for the entire 25 minute sampling period without connection to the exit-gas. This was to provide information about any background levels of *E. coli* JM107 pQR701 that might be present in the air. The rationale was that if the background was entirely due to poor cleaning, then the level after 25 minutes would be the same as that after 10 minutes and if the background was due to airborne *E. coli* JM107 pQR701 whole cells then the level would be increased at the end of the complete sampling period.

The overall turnaround time of the cyclone was 45 minutes, consecutive samples were therefore taken at least this time apart.

3. DEVELOPMENT OF QPCR ASSAYS FOR *E. coli* JM107 pQR701 AND *E. coli* JM107 pQR126

This chapter will deal with the development of PCR methods for the detection of two different plasmids and *E. coli* chromosomal DNA. The selectivity and limit of detection of the PCRs will be determined. For the plasmids, pQR701 and pQR126, quantitative PCR methods have been developed based on the use of competitive internal standard DNA segments (Becker-Andre and Hahlbrock, 1989; Gilliland *et al*, 1990). The properties of these quantitative assays, particularly the one for pQR701 measurement, will be examined. Subsequent chapters will describe the application of the QPCR assay to the measurement of *E. coli* whole cells in aerosols.

3.1 PCR assay development

For the plasmids pQR701 and pQR126, PCR methods have been developed where the amplified section of DNA crosses the point of insertion of the overexpressed gene into the vector (see Figures 3.1 and 3.2). In order to achieve this, one primer must anneal to a section of DNA on the vector, whilst the other anneals to a section on the insert. The primers must, of course, anneal to complimentary strands of the DNA and have their 3' ends pointing towards each other.

For *E. coli* chromosomal DNA the two primers chosen were those used by Bej *et al* (1991) and bind to the *gusR* gene, the regulatory gene for *gusA*, β-glucuronidase. The expression of β-glucuronidase has been used as a definitive diagnostic test for the presence of *E. coli* (Edberg and Edberg, 1988; Edberg *et al*, 1989), however some *E. coli* strains have been found not to express this activity. Bej *et al* (1991) have shown that amplification of a portion of the *gusR* gene by PCR detects all *E. coli* strains, including those that do not express the gene. Moreover, there is little cross reaction with closely related species: only 4 strains of *Shigella* species out of 100 tested species gave a positive result.

Because the extension time in PCR is dependant on the length of the amplified sequence (generally around 1 min/kb is allowed (Saiki, 1991)), then relatively short DNA sequences of 100-500 bp were chosen to be amplified. The lower limit of 100 bp reflects the fact that larger fragments are easily separated by agarose gel electrophoresis; smaller fragments may necessitate the use of polyacrylamide gels.

In all cases in this chapter, no distinct lysis or DNA extraction step has been included.

Other workers have used whole cells in PCRs without a lysis step on the basis that the initial 94°C heat denaturation step is sufficient to lyse the cells (Mahon and Lax, 1993). However, in Section 4.1 it is shown that this is an inefficient way of releasing plasmid into solution and so for some experiments in subsequent chapters, a lysis step is incorporated. Notably, one of the aims of developing a PCR for chromosomal DNA was to measure absolute lysis efficiency (see Section 4.1), however this has not been achieved at present.

3.1.1 PCR of 332 bp fragment of pQR701

The primers M13R1 and CMTA1 (Section 2.2.5), chosen for the detection of plasmid pQR701, are shown schematically below in relationship to the target plasmid (Figure 3.1).

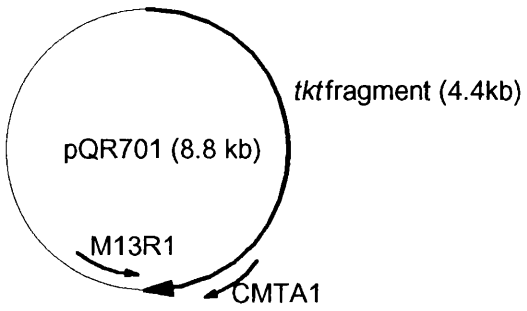


FIGURE 3.1 Schematic showing primer binding sites on pQR701

The primer M13R1 is similar to the M13 reverse sequencing primer (New England Biolabs), however an additional 3 bp are used in this case to make the primer more suitable for PCR (Saiki, 1991). The primer situated on the insert, CMTA1, anneals to the *cmtA* gene (Sprenger, 1993) on the chromosome derived transketolase fragment of *E. coli* at position 984-1004.

The idea of amplifying by PCR across the point of insertion, thereby only detecting a specific gene on a specific vector, is particularly pertinent in this instance. This is because the inserted transketolase gene (*tkt*) is actually derived from the *E. coli* chromosome (French, 1993; Sprenger, 1993). If two primers were chosen that were both internal to the 'foreign' gene, then the PCR method would detect wild type (unmodified) *E. coli* strains as well as the modified organism *E. coli* JM107 pQR701. Conversely, if both primers were situated on the vector (pBGS18/19) segments of the plasmid, then other constructs using the same vector would be detected.

It is worth noting that if an *E. coli* strain carrying the pBGS18 or pBGS19 vector

without an insert were subject to PCR using this primer set, then some amplification would occur. The primers would anneal to their respective targets and would act as starting points for replication, but because the primers are on different DNA molecules, accumulation of DNA fragments of undefined length would occur. These fragments would not be detected after PCR because the accumulation would be linear (arithmetic) rather than exponential (geometric).

The conditions used in this PCR are relatively standard (Saiki, 1991; Steffan and Atlas, 1991), no difficulty was seen in amplifying the desired fragment.

3.1.2 PCR of 237 bp fragment of pQR126

The primer pair chosen for this amplification, M13R1 and AMY-2, are depicted in relation to the target plasmid in Figure 3.2.

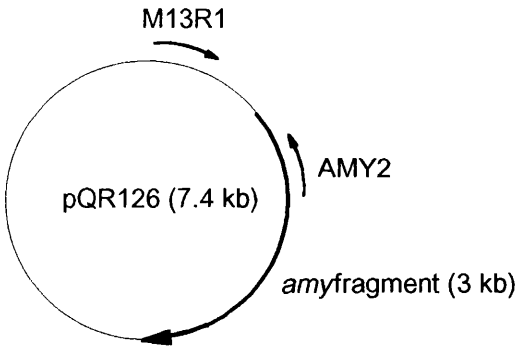


FIGURE 3.2 Schematic showing primer binding sites on pQR126

Notably, the same vector based primer (M13R1) was used as in amplification of a fragment from pQR701. The primer that binds to the amylase gene, *amy*, was carefully chosen so that the 3' end was not too GC rich as this can cause mispriming in GC rich sequences such as this gene (Steffan and Atlas, 1991).

PCR of a fragment crossing the point of insertion of pQR126 was found to be not straightforward. It was noted that PCR was not consistently successful using the "standard" conditions used for pQR701 amplification. The gene that is inserted in pQR126 codes for a thermostable α-amylase (Bahri and Ward, 1990) and is derived from *Streptococcus thermoviolaceus*. Analysis of the sequence of this gene shows that it is highly GC rich. Several authors have attributed difficulty in amplifying such sequences to: the presence of stable secondary structure (Cusi *et al*, 1992) allowing only poor access of the primer and slowing elongation (Dutton *et al*, 1993); poor denaturation at 94°C (Dutton *et al*, 1993; Agarwal and Perl, 1993); and rapid renaturation of the

template (Sun *et al*, 1993). In order to overcome these problems the use of denaturants DMSO or formamide in the PCR in combination with one of a choice of polymerases has been recommended (Dutton *et al*, 1993; Sun *et al*, 1993; Moreau *et al*, 1994; Sarkar *et al*, 1990). Other researchers (Cusi *et al*, 1992; Agarwal and Perl, 1993) have suggested using an alkaline denaturation step prior to the PCR to enhance template denaturation.

In this study, the denaturants DMSO and formamide were used at 5 % and 10 % (v/v) in combination with one of the three polymerases *Taq*, Vent and *Pfu*. The template DNA in each case was *E. coli* JM107 pQR126 cells at 3 different concentrations (microbial DNA rather than miniprep was used because earlier experiments had shown that this is a more difficult target). The combinations attempted are summarised in the legend of Figure 3.3 which shows the products of each PCR run on an agarose gel. The gel image shows that, of the conditions tested, the following were potentially useful for amplification of the desired fragment:

TABLE 3.1 Potentially useful formulations for amplification of pQR126 fragment

Enzyme (units/PCR)	Co-solvent
Taq (2.5)	5 % Formamide
Taq (2.5)	5 % DMSO
Taq (2.5)	10 % DMSO
Vent (1.25)	5 % Formamide
Vent (1.25)	10 % Formamide
Pfu (1.25)	none
Pfu (1.25)	5 % DMSO
Pfu (1.25)	10 % DMSO

Of the combinations above, those that produced the band of strongest intensity were 2.5 units *Taq* polymerase + 5 % formamide and 1.25 units *Pfu* polymerase with no co-solvent. In all cases Vent polymerase amplified reactions produced a smear on the gel, although moderate intensity bands were visible above this background when co-solvent was used.

Further investigation of the two experimental conditions (*Taq* polymerase + 5 % formamide; *Pfu* polymerase alone) showed that the limit of detection with respect to

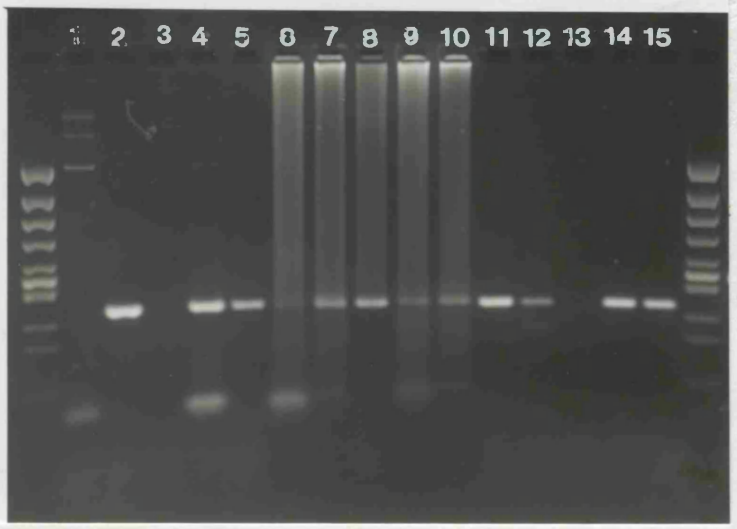


FIGURE 3.3 Gel showing results of varying conditions in PCR of pQR126 fragment. Table below describes PCR conditions for each lane number (in boxes).

Enzyme (units/PCR)	no co-solvent	5 % formamide	10 % formamide	5 % DMSO	1 % DMSO
Taq (2.5)	1	2	3	4	5
Vent (1.25)	6	7	8	9	10
Pfu (1.25)	11	12	13	14	15

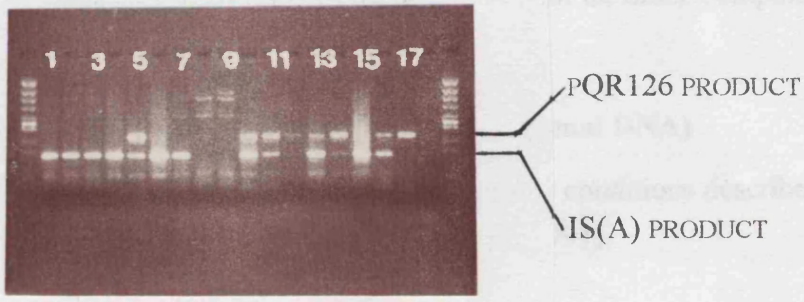


FIGURE 3.4 Co-amplification of *E. coli* JM107 pQR126 DNA with IS(A) using two different sets of conditions. Various IS(A)/pQR126 concentration combinations in PCR with *Pfu* polymerase and no co-solvent (lanes 1-9); same combinations in PCR with *Taq* polymerase + 5 % formamide (lanes 10-18). Note that when using *Pfu* polymerase, IS(A) is often amplified to exclusion of pQR126 fragment. With *Taq* polymerase the ratios of the two products are related to their relative starting concentrations.

whole cells of *E. coli* JM107 pQR126 was similar, being 5-50 cells/PCR (Section 3.1.5). However, co-amplification of *E. coli* JM107 pQR126 DNA together with the standard fragment IS(A) showed that with *Pfu* polymerase alone, IS(A) was preferentially amplified to the exclusion of target fragment amplification (Section 3.2). This was not the case using *Taq* polymerase + 5 % formamide where the intensity of the IS(A) product band on the gel was less than that of the target product band in some, expected cases (Figure 3.4).

The reason for the biased co-amplification when using *Pfu* polymerase alone is not apparent from these experiments. It is possible that secondary structure of the target DNA is stabilised by the presence of cellular protein. Such stable structures are unlikely to be present in the short linear strand of DNA which is IS(A). This would mean that although the target and competitive internal standard DNA share the same primer binding sites and are of similar length, their amplification efficiencies under these conditions are quite different. The presence of stabilised secondary structure in the target sequence makes a significant distinction between the two stretches of DNA and hence the requirement for quantitative PCR that the target and standard DNAs are co-amplified with similar efficiencies (Gause and Adamovicz, 1994; Leser, 1995) is not upheld. In the presence of a co-solvent (in this case formamide), secondary structure is destabilised (Moreau *et al*, 1994) and so the difference in amplification efficiencies is minimised.

The standard conditions used for amplification of DNA from *E. coli* JM107 pQR126 were therefore 2.5 units *Taq* polymerase and 5 % formamide with all other components as stated in Section 2.2.6.

3.1.3 PCR of 153 bp fragment of *gusR* gene (*E. coli* chromosomal DNA)

Successful PCR of this fragment was achieved using the standard conditions described in Section 2.2.6 using primers URR-432 and URL-301 (Bej *et al*, 1991).

3.1.4 Choice of microbial diluent for PCR assays

Having established methods for the PCR detection of plasmids and whole cells diluted in sterile RO water, it was necessary to determine which commonly used microbial diluent solution could be used in conjunction with the PCR. This was important because in several subsequent experiments a particular cellular concentration was used as a template for a PCR. Such cellular suspensions were derived from overnight cultures of the relevant organism by dilution. In order to produce a suspension close to a predicted concentration of cells mL^{-1} it is helpful to use a diluent in which the micro-organisms are

relatively stable in terms of lysis and viability. Additionally, although the number of micro-organisms in suspension can be checked at low dilutions of culture by microscopy (when cells mL^{-1} exceeds 1.25×10^6), at higher dilutions it is necessary to check the numbers of CFU mL^{-1} by plate counting. Although, as widely reported (Colwell *et al*, 1985), the numbers of CFU mL^{-1} and cells mL^{-1} may not equate (experience has shown that CFU mL^{-1} is approximately 25 - 50 % of total cells mL^{-1} when diluted from an overnight culture), it is a useful way of checking the higher dilutions of cells to assure that no gross errors have occurred during dilution.

In subsequently described experiments where released micro-organisms are collected by cyclone sampling and enumerated by QPCR, it is necessary to differentiate extracellular DNA from total DNA (see Section 4.2). Again, the use of a diluent in which the captured micro-organisms are unlikely to lyse is important. The following solutions were tested for inhibition of the PCR:

1. Sterile RO water (SROW)
2. Ringer's solution (quarter strength, Oxoid)
3. Thiosulphate Ringer's solution (TRS, quarter strength, Oxoid)
4. Phosphate buffered saline (PBS; 66 mM Na_2PO_4, 0.85 % (w/v) NaCl, pH 7.0)
5. Saline (0.85 % NaCl)
6. Tris-HCl (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

A sample of miniprep purified plasmid pQR701 DNA was diluted into each diluent to produce a final concentration of 0.15 pg μL^{-1}. A 10 µL sample of this solution was then used in the PCR, the results are summarised below.

TABLE 3.2 Choice of microbial diluent for PCR

Diluent	Intensity of Product Band
1. SROW	+++
2. Ringer's solution	+++
3. TRS	+++
4. PBS	-
5. Saline	++
6. Tris-HCl	+++

Number of +'s indicates intensity of bands observed (judged by eye); +++ strongest band intensity;
- indicates PCR was inhibited.

Therefore apart from SROW, there are a number of possible diluents that could be used. TRS was chosen for all subsequent experiments as i) it is a recognised microbial diluent and ii) the presence of thiosulphate neutralises any ClO^- anions that might pass into the sample from cleaning of the cyclone using sodium hypochlorite solution, Chloros (see Section 4.4).

3.1.5 Limit of Detection of the PCR

In order to determine the limit of detection of the PCR methods developed, serial dilutions of an overnight culture of the relevant micro-organism were made in TRS and each dilution was subject to PCR. The limit of detection (minimum number of cells required for a positive PCR) was the highest dilution giving a positive PCR. The number of cells/PCR was determined by cell counting (Section 2.1.4) and plate counting (Section 2.1.5). The gel from the limit of detection determination for *E. coli* JM107 pQR701 cells in TRS is shown in Figure 3.5.

The gel shows that at 50 cells/PCR there is a product band but at the next dilution, 5 cells/PCR, no band is apparent. The limit of detection of *E. coli* JM107 pQR701 cells using this PCR method can therefore be said to be 5 - 50 cells/PCR (equivalent to 5×10^2 - 5×10^3 cells mL^{-1}). Using *E. coli* JM107 pQR126 cells as the target, the limit of detection was again determined as 5 - 50 cells/PCR. For amplification of a segment of the *E. coli* chromosomal gene *gusR*, the limit of detection, regardless of strain type, was found to be 3×10^3 cells/PCR. This higher value probably reflects the fact that for each cell there is a single copy of the target gene per chromosome whereas the genes *tkt* and *amy* are expressed on multi-copy plasmids (pQR701 and pQR126 respectively).

Although these measurements of limit of detection are strictly estimates and accurate determination of the values was not made, it was clear that the PCR methods offered sufficient sensitivity for the measurement of release of micro-organisms from bioprocesses. Notably, the higher estimate of the limit of detection for the specific PCR methods of 5×10^3 cells mL^{-1} is considerably less than that for cell counting by microscopy, where the limit of detection is 1.25×10^6 cell mL^{-1}.

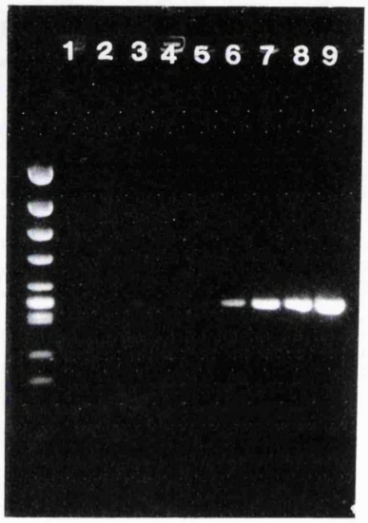


FIGURE 3.5 Gel showing limit of detection of *E. coli* JM107 pQR701 cells by PCR. Table below describes the number of cells in each PCR (PCR numbers correspond to lane numbers). Lanes 1 and 2 are negative controls containing SROW and TRS respectively.

Lane	1	2	3	4	5	6	7	8	9
No. cells	0	0	0.5	5	50	5×10^2	5×10^3	5×10^4	5×10^5

3.1.6 Selectivity of the PCRs

The selectivity (that is the ability to detect only the target strain) of the PCR methods was determined by carrying out the appropriate PCRs using a range of other, sometimes closely related strains of *E. coli* and yeast strains as the sample.

3.1.6.1 Selectivity of PCR for *E. coli* JM107 pQR701

The following figures show the results of PCRs using *E. coli* JM107 pQR701 cells and/or cells from other *E. coli* strains (Figure 3.6) or yeast strains (Figure 3.7) as the sample in the reaction.

The results are summarised in the following table, where the figures shown denote the number of cells present in the PCR.

TABLE 3.3 Summary table showing selectivity of PCR for *E. coli* JM107 pQR701

Non Target Strain		Target strain (*E. coli* JM107 pQR701)	PCR Result[c]	Lane *(Figure)*
Designation[a]	Cells/PCR[b]	Cells/PCR[b]		
none	-	5.0×10^4	+	2 *(3.6)*, 1 *(3.7)*
	-	51	+	5 *(3.6)*, 4 *(3.7)*
JM83 pQR187	5.6×10^4	0	–	4 *(3.6)*
	"	51	+	8 *(3.6)*
JM107 pQR150	6.1×10^4	0	–	3 *(3.6)*
	"	51	+	7 *(3.6)*
JM107	4.7×10^4	0	–	1 *(3.6)*
	"	51	+	6 *(3.6)*
S. cerevisiae wt	4.5×10^3	0	–	2 *(3.7)*
	"	47	+	5 *(3.7)*
S. cerevisiae 7d	4×10^3	0	–	3 *(3.7)*
	"	47	+	6 *(3.7)*

[a] *E. coli* strains unless otherwise stated.
[b] Cell counts by microscopy.
[c] PCR result is positive if a band of the correct size (332 bp) is visible (by ethidium bromide staining) on agarose gel.

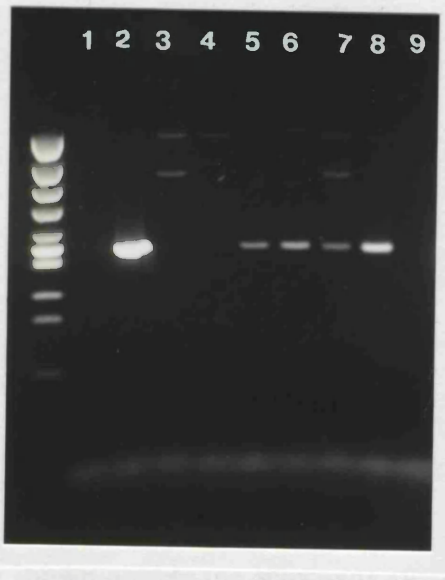


FIGURE 3.6 Selectivity of PCR for *E. coli* JM107 pQR701 compared to other *E. coli* strains. For key to lanes see Table 3.4. Note that lane 9 is a negative control PCR containing SROW.

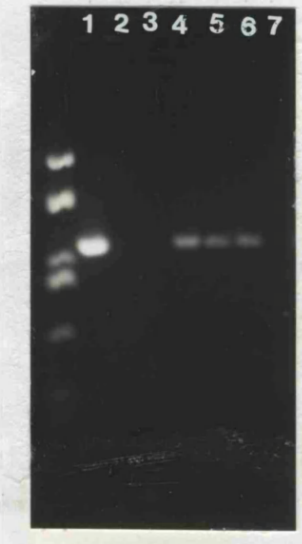


FIGURE 3.7 Selectivity of PCR for *E. coli* JM107 pQR701 compared to two yeast strains. For key to lanes see Table 3.4. Note that lane 7 is a negative control PCR containing SROW.

There are several points that can be made about the results shown in Table 3.3: i) around 5×10^4 cells /PCR of all the *E. coli* strains tested (one 'wild type K-12' and 2 strains that are closely related to the target in terms of vector and host strain) do not give a band of similar size (in terms of bp) to the target strain when subjected to PCR under the same conditions; ii) approximately 4×10^3 cells of a wild type and a genetically modified *Saccharomyces cerevisiae* strain do not give a band of similar size to the target when subjected to PCR under the same conditions; and iii) low numbers (25-50) of target cells will produce a positive PCR even in the presence of a 1000 fold excess of other *E. coli* cells or a 100 fold excess of *S. cerevisiae* cells. Although there is no reason to expect a positive response from the *S. cerevisiae* strains used, it was thought that the additional amount of genetic material present (*S. cerevisiae* has a genome size of 1.8×10^4 kb compared to the 4×10^3 kb genome of *E. coli* (Sambrook *et al*, 1989)) may have interfered with the desired primer binding reactions or amplification steps.

In addition to the results shown above, the selectivity of the PCR was tested at more extreme levels using the strain (*E. coli* JM107 pQR150) that gave some weak bands at around 700 and 1000 bp in the original experiments (Figure 3.6, lane 3). It was thought that this might cause some competition for primers if it were present at a very significant excess. It was found that 4.6×10^6 cells of *E. coli* JM107 pQR150 in a PCR do not give rise to a band of similar size to the target (332 bp) and that 50 cells of *E. coli* JM107 pQR701 in a 'background' of 4.6×10^6 *E. coli* JM107 pQR150 cells can still be easily detected. The target strain can therefore be detected in the presence of a 1×10^5 excess of other *E. coli* cells.

3.1.6.2 Selectivity of PCR for *E. coli* JM107 pQR126

Experiments to determine the selectivity of this PCR assay were carried out in the same manner as those for *E. coli* JM107 pQR701. However, in this series of experiments each test strain was also used in a PCR with the *gusR* primer set. This was to demonstrate that *E. coli* strains that may give a negative PCR with the *amy* primers should be positive for the chromosomal gene primers. This is a way of showing that negative results for the *amy* primer pair PCR are not due to inhibition of PCR or sample mis-handling, but are genuinely due to the selectivity of the *amy* assay. In addition, these experiments should reveal the selectivity of the chromosomal PCR; it would be expected that all *E. coli* strains give a positive response (i.e. a band of 153 bp), whereas the yeast strain tested should give a negative result in this assay. Results are shown in Figure 3.8 and are summarised in Table 3.4.

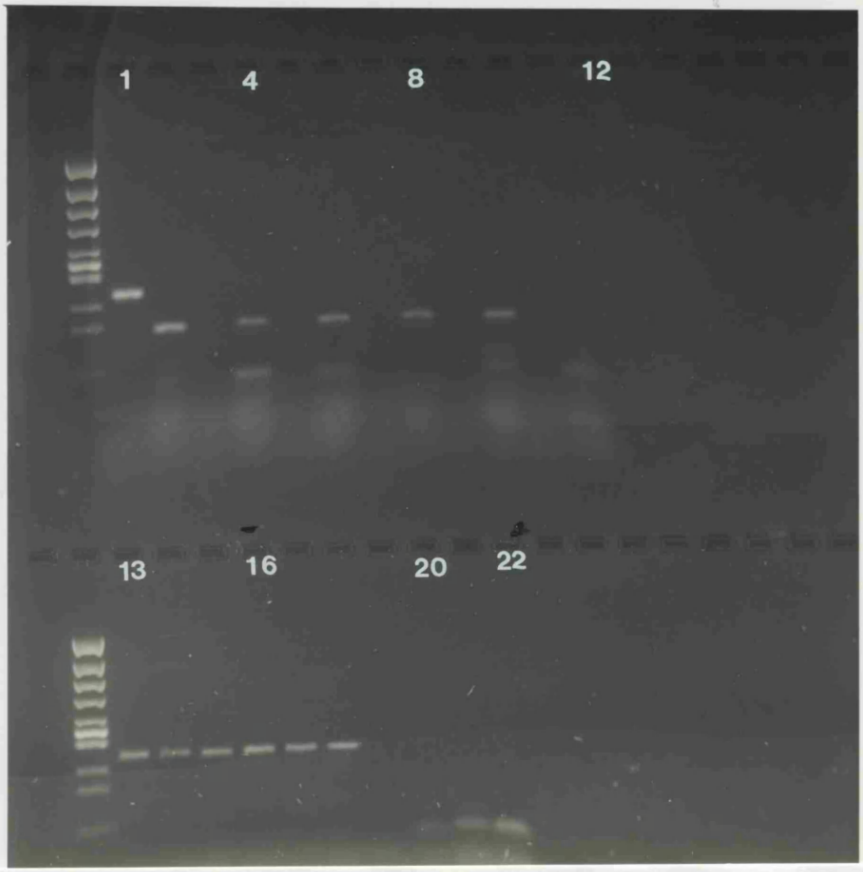


FIGURE 3.8 Selectivity of PCR for *E. coli* JM107 pQR126 compared to other *E. coli* strains and a *S. cerevisiae* strain. For key to lanes see Table 3.5. Note that lanes 19-22 are negative control PCRs containing SROW or TRS.

TABLE 3.4 Summary table showing selectivity of PCR for *E. coli* JM107 pQR126

Non Target Strain		Target strain (*E. coli* JM107 pQR126)	PCR Result[c]			
Designation[a]	Cells/PCR[b]	Cells/PCR[b]	*amy*	Lane[d]	*gusR*	Lane[d]
none	-	3.8×10^4	+	1	+	2
	-	38	+	13	n/t	
JM83 pQR150	5.4×10^4	0	–	3	+	4
	"	38	+	14	n/t	
JM107 pQR701	2.6×10^4	0	–	5	+	6
	"	38	+	15	n/t	
JM107 pQR752	4.5×10^4	0	–	7	+	8
	"	38	+	16	n/t	
JM107	4.2×10^4	0	–	9	+	10
	"	38	+	17	n/t	
S. cerevisiae wt	1.1×10^3	0	–	11	–	12
	"	38	+	18	n/t	

[a] *E. coli* strains unless otherwise stated.
[b] Cell counts by microscopy.
[c] PCR result is positive if a band of the correct size (237 bp for *amy*, 153 bp for *gusR*) is visible (by ethidium bromide staining) on agarose gel.
[d] Lane numbers refer to Figure 3.8.
n/t: not tested

It can be concluded, therefore, that this PCR assay is selective for *E. coli* JM107 pQR126 against the range of other *E. coli* strains and the yeast used here. Like the PCR for *E. coli* JM107 pQR701 this PCR assay is not affected by the presence of a large excess of non target cells of closely related strains. In this case, the maximum ratio of background cells to target cells tested was 1000, at which point the detection of *E. coli* JM107 pQR126 was unaffected.

It can also be noted that the *E. coli* chromosomal PCR (*gusR* gene) is positive for all *E. coli* strains tested but is negative for the *S. cerevisiae* strain.

3.2 Development of QPCR assays

3.2.1 Generation of internal standard DNA preparations

The generation of the purified and quantified IS DNA preparations is described in Section 2.2.7. For the production of IS(T), the internal standard for the *tkt* encoding plasmid pQR701, the procedure was exactly as described, in that subsequent PCR rounds

were carried out using a $1/10^4$ dilution of the previous PCR product mixture. However, for IS(A), the internal standards for the *amy* gene on pQR126, there was a significant impurity band that was smaller than the intermediate product after the first round of PCR. It is possible that this may be a primer-dimer product as it was also present in the negative controls where no target was present. Since it was thought possible that this impurity might carry through to the final IS product, then a purification from gel step (Section 2.2.7.2) was introduced at this stage. Subsequent PCR rounds were then carried out using 1/100 dilutions of the DNA that was purified from the gel, since it was calculated that the dilution of PCR product introduced by the purification step was approximately 1/50.

Details of the purified IS DNA preparations are shown below in Table 3.5.

TABLE 3.5 Purified IS DNA preparations

Preparation	Concentration ($\mu g\ mL^{-1}$)	Length (bp)	M.W. (Daltons)	Molarity (μM)	Molecules /10 μL
IS(T)	33.6	247	1.61×10^5	0.21	1.25×10^{12}
IS(A)	5.8	136	8.84×10^4	0.066	3.94×10^{11}

Yields of these preparations was typically 1.5 - 2.0 μg per 50 μL final round PCR.

The number of molecules of DNA in a 10 μL volume (the sample volume used in the PCR) was used to calculate appropriate concentrations of IS DNA preparations for use with the target DNA preparations in construction of the calibration curves. Miniprep purified samples of the target plasmids (pQR701 and pQR126) were also quantified by absorbance and the same calculations were made in preparation for calibration curve construction.

3.2.2 Quantitation of target plasmids using the constructed internal standards

Once the concentrations of the competitive internal standards IS(T) and IS(A) and the purified plasmid preparations of pQR701 and pQR126 were determined, it was possible to set up PCRs in which the numbers of molecules of standard and target were approximately equal. It was hoped that in these experiments the intensity of each band on an agarose gel of the PCR products would also be approximately equal. Figure 3.9 shows a gel from such an experiment using pQR701 as the target and IS(T) as the competitive internal standard.

In this gel the lanes of interest are 6, 7 and 8. These lanes show the results of

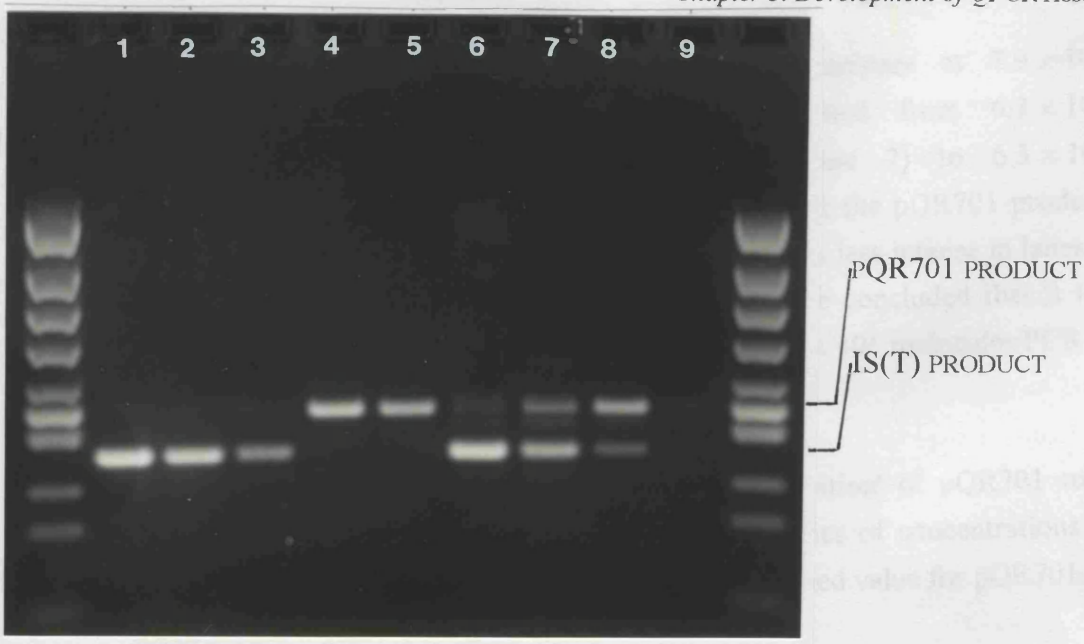


FIGURE 3.9 Gel showing the co-amplification of pQR701 and IS(T) in the PCR. Table below describes the number of molecules of pQR701 and IS(T) in each PCR (PCR numbers correspond to lane numbers). Lane 9 is a negative control containing SROW.

Lane	1	2	3	4	5	6	7	8
pQR701	0	0	0	1.6×10^4	7.9×10^3	7.9×10^3	7.9×10^3	7.9×10^3
IS(T)	1.3×10^6	1.3×10^5	1.3×10^4	0	0	6.3×10^5	6.3×10^4	6.3×10^3

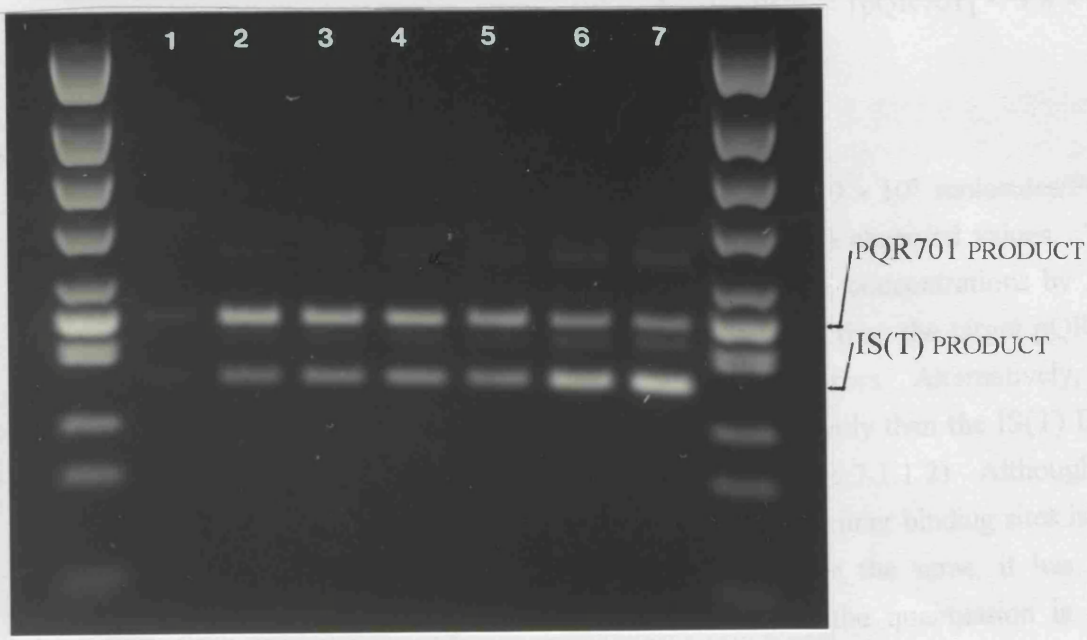


Figure 3.10 Determination of pQR701 concentration by use of a series of IS(T) concentrations. Table below describes the number of molecules of pQR701 and IS(T) in each PCR (PCR numbers correspond to lane numbers). Lane 1 is a negative control containing SROW.

Lane	2	3	4	5	6	7
pQR701	7.9×10^3	7.9×10^3	7.9×10^3	7.9×10^3	7.9×10^3	7.9×10^3
IS(T)	6.3×10^3	8.8×10^3	1.1×10^4	1.3×10^4	3.8×10^4	6.3×10^4

experiments where the pQR701 concentration was kept constant at 7.9×10^3 molecules/PCR whereas the IS(T) concentration was varied from 6.3×10^5 molecules/PCR in lane 6 to 6.3×10^4 molecules/PCR (lane 7) to 6.3×10^3 molecules/PCR (lane 8). Visual inspection of the gel shows that the pQR701 product band is more intense than the IS(T) product band in lane 8 and is less intense in lanes 6 and 7. If the pQR701 concentration was unknown, it could be concluded that it lay between the concentrations of IS(T) in lanes 7 and 8, i.e. 6.3×10^3 molecules/PCR < [pQR701] < 6.3×10^4 molecules/PCR, and indeed this is the case.

However, if one wanted to determine the 'unknown concentration' of pQR701 to a greater degree of accuracy, it would be necessary to use a series of concentrations of IS(T) in the PCRs that are clustered around the roughly determined value for pQR701.

The gel in Figure 3.10 shows that as the IS(T) concentration decreases (going from right to left) the ratio of intensities of pQR701 product to IS(T) product increases. Visual inspection shows that in lane 6, IS(T) band intensity is greater than pQR701 ,whereas in lane 5 the converse is true. The concentration of pQR701 can therefore be placed between those of IS(T) in each lane;. 1.3×10^4 molecules/PCR < [pQR701] < 3.8×10^4 molecules/PCR.

There several points to note about this finding:

1. [pQR701] in these experiments is in fact known to be 7.9×10^3 molecules/PCR, therefore there is a discrepancy between the known and the observed values. This difference may be due to inaccurate determination of DNA concentrations by A_{260} measurements (due to contaminating protein or DNA other than the target pQR701 or the standard IS(T)) or to an accumulation of dilution errors. Alternatively, it is possible that the pQR701 fragment is amplified more efficiently than the IS(T) DNA fragment by the PCR under the conditions used (see Section 7.1.1.2). Although the idea of using competitive internal standards with common primer binding sites is that the amplification efficiency of target and standard will be the same, it has been pointed out that even if the efficiencies are not equal, the quantitation is valid assuming that the ratio of amplification efficiencies is constant and the amplification is in the exponential phase (Zachar *et al*, 1993). Other workers who have used this competitive internal standard approach have constructed standard calibrations using a range of concentrations of analyte and/or standard to correct for differences in amplification efficiencies (Mahon and Lax, 1993; Gebhardt *et al*, 1994; Leser, 1995; McCulloch *et al*, 1995).
2. Visual inspection of the gel is somewhat misleading in that fluorescent intensity is a

function of fragment length as well as concentration. Because the IS(T) fragment (247 bp) is significantly shorter than the pQR701 fragment (332 bp), it binds fewer molecules of ethidium bromide per DNA molecule and hence the amount of fluorescence per molecule is reduced accordingly. This should be allowed for in determining the concentration of the 'unknown' pQR701 concentration.

3. Visual analysis of the relative intensities of the bands can only give a range of concentrations for the unknown. The range depends on the closeness of IS(T) concentrations used. In this instance the pQR701 concentration is defined as lying between 1.3×10^4 and 3.8×10^4 molecules/PCR. For a more accurate determination, another PCR would need to be set up where the IS(T) concentrations differ by, say, 1000 molecules/PCR over this range. This is obviously time consuming and expensive. The best strategy would be to do a series of increasingly narrow ranges of IS(T) in order to home in on the actual [pQR701]. However, this would require one to wait for the results of one PCR before embarking on the next.

Because of the disadvantages of this approach it was decided that measurement of the actual band intensities by densitometry would be a more effective method.

3.2.3 Construction of calibration curve for pQR701

Densitometry of ethidium bromide stained agarose gels using Gelbase software is described in Section 2.3.2. Close attention has been paid to the densitometry since this step is very important in determining final quantitative results. Empirical means of ensuring consistency and low amounts of variance are described in Section 3.2.8.

A series of experiments were carried out in order to construct a calibration curve for a range of purified plasmid pQR701 concentrations when used in the PCR with different IS(T) concentrations.

Preliminary experiments showed that if an IS(T) concentration is co-amplified with a pQR701 concentration giving bands of equal intensity (denoted $[pQR701]_0$), then the same [IS(T)] can be used to quantify pQR701 within a concentration range of $[pQR701]_0 \pm 1.5$ logs (see, for example, Figure 3.9). If [pQR701] falls outside of this range, then normally only one band, IS(T) or pQR701, will be detected on the agarose gel. This is thought to be due to a combination of the limited dynamic range of the video monitor/densitometry system and the likelihood that when the minority species is present at a very low ratio compared to the majority species it will be out-competed in the PCR. Similarly, other workers have found that the ratio of target fragment/internal standard is limited to up to $\pm$ 2 logs (Simon *et al*, 1992; Chan *et al*, 1994).

Since a single IS(T) concentration can be used in the quantification of pQR701 over a range of 3 orders of magnitude, and the feasible range of [pQR701] could extend for 6-7 orders of magnitude (500 cells mL^{-1}, the limit of detection of the technique, up to 5×10^9 cells mL^{-1}, an approximate broth cellular concentration), then it was decided to use 3 different, fixed IS(T) concentrations that would overlap and cover the whole range of possible target concentrations (Chan *et al*, 1994). This meant in practice that each unknown sample was divided into 3 aliquots, each aliquot being co-amplified with one of the IS(T) concentrations. The IS(T) concentrations used were decided upon as outlined below in Table 3.6.

TABLE 3.6 IS(T) concentrations used in construction of calibration curve for pQR701

	Concentration (molecules/μL)	Anticipated linear range (molecules pQR701 per PCR)$^+$	Approx. range *E. coli* JM107 pQR701 (cells per PCR)*
IS(T)1	1.3×10^4	2.0×10^2 - 2.0×10^5	2 - 2.0×10^3
IS(T)2	1.3×10^6	2.0×10^4- 2.0×10^7	2.0×10^2- 2.0×10^5
IS(T)3	1.3×10^8	2.0×10^6- 2.0×10^9	2.0×10^4 - 2.0×10^7

$^+$; Densitometry has shown that 2 molecules IS(T) ≡ 1 molecule of pQR701 in terms of band intensity ;
*; Rough estimate based on 100 plasmids per cell (Section 5.1).

The IS(T) concentration standards IS(T)1, IS(T)2 and IS(T)3 were diluted from the IS(T) preparation that was obtained as described earlier (Section 2.2.7). Portions of these IS(T) standards were dispensed in batches and stored at -70°C. The long term stability of these standards stored under these conditions has been verified (Section 3.2.11). Notably, results from a subsequent section (3.2.8) suggest that if more precision was required, it could be achieved by using more IS(T)s and measuring pQR701 concentrations within only, say, ± 0.5 logs, instead of the range of ± 1.5 logs around each IS(T) used in these experiments. The cost of this enhanced precision would be the necessity to perform more assays.

These 3 set IS(T) concentrations (in 1 μL volume) were co-amplified in the PCR with pQR701 concentrations ranging from 2×10^2 - 2×10^9 molecules (in 10 μL volume), in gradations of 0.5 logs. Three replicates for each [IS(T)]/[pQR701] combination were obtained. Each replicate was derived from a separate PCR using the same samples. A gel showing the generation of one set of points is shown in Figure 3.11.

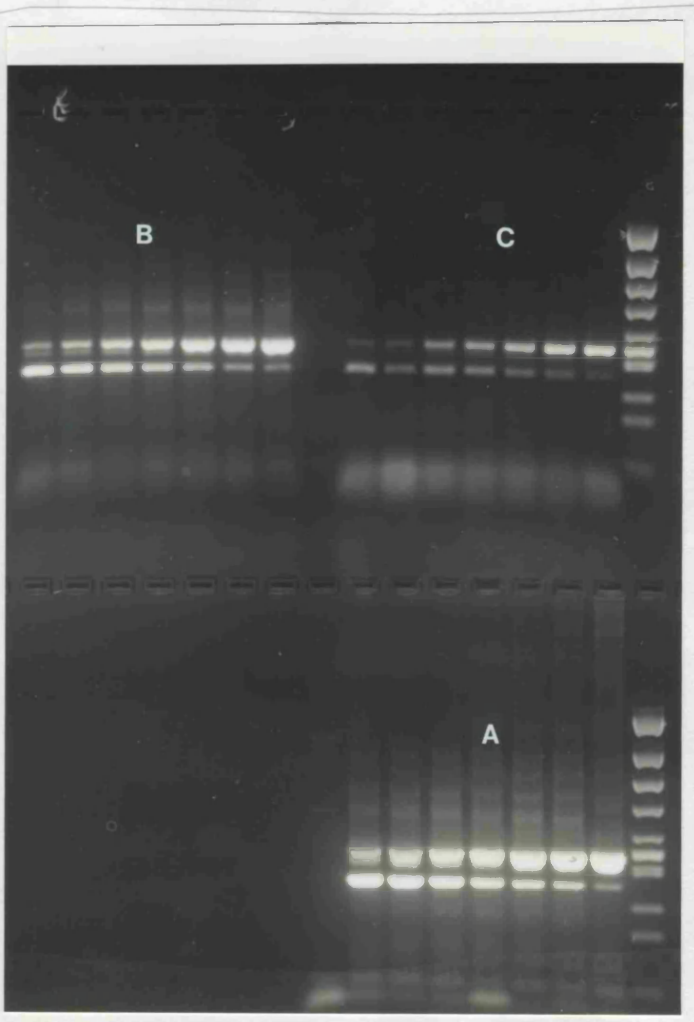


FIGURE 3.11 Gel showing results of co-amplification of each [IS(T)]/[pQR701] pair for construction of calibration curve. Figure shows three sets of [pQR701]/[IS(T)] pairs. A; range of [pQR701] + IS(T)3: B; range of [pQR701] + IS(T)2: C; range of [pQR701] + IS(T)1. In all sets [pQR701] increases from left to right.

Analysis of this and two other similar gels by densitometry was used to generate the calibration curve which is shown in Figure 3.12.

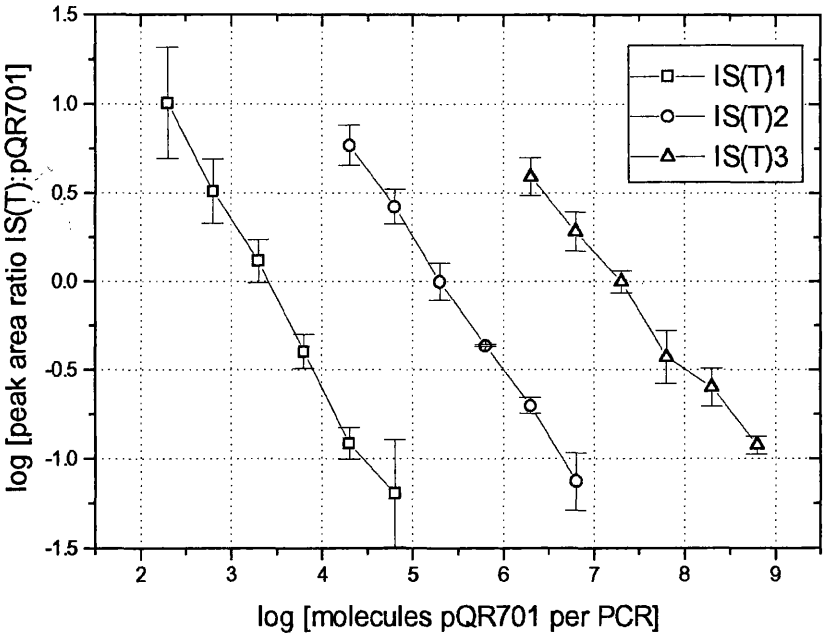


FIGURE 3.12 Calibration curves for quantitation of pQR701. Error bars shown represent the standard deviation of the 3 replicate points used for each data point.

The graph shows that:

1. For each IS(T) standard there is a distinct calibration curve covering around 3 orders of magnitude of pQR701 concentrations.
2. The curves for adjacent IS(T)s overlap such that over the range of pQR701 concentrations from 2×10^2 - 6.3×10^8 molecules $10\mu L^{-1}$ all pQR701 concentrations give a valid response (two distinct scorable bands) with at least one IS(T).
3. The calibration curves for each IS(T) are parallel and reasonably linear. Error bars indicate that the measurements are relatively precise. The IS(T)1 curve has the highest degree of error, this may reflect the finding that, especially in the least concentrated samples, the bands seen on the gel were quite faint and hence background 'noise' is likely to play a more significant role. At the highest concentration attempted (2×10^9 molecules 10 μL^{-1}) the bands were so intense that adequate resolution was lost, hence this point is not shown in the graph.

Plotting a line through each of the calibration curves will allow determination of an unknown pQR701 concentration by measurement of the ratio of band intensities (peak areas) and then fitting that ratio into the equation that describes the appropriate calibration curve line. Using a linear regression method a line was plotted for each IS(T) calibration curve. The results are shown in Figure 3.13.

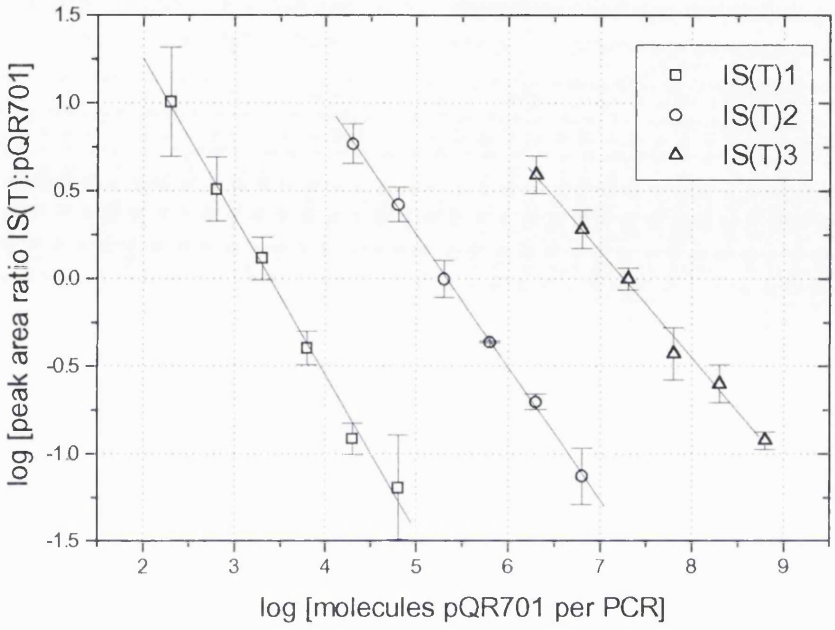


FIGURE 3.13 Calibration curves for quantitation of pQR701 with linear regression lines. Error bars shown represent the standard deviation of the 3 replicate points used for each data point. The line is drawn using a linear fit approximation.

Table 3.7 shows the appropriate parameters of the plotted lines.

TABLE 3.7 Parameters of IS(T) linear regression lines

	A	(s.d.)	B	(s.d.)	r	n
IS(T)1	2.97	0.11	-0.87	0.028	-0.998	6
IS(T)2	4.03	0.067	-0.76	0.012	-1.00	6
IS(T)3	4.42	0.19	-0.61	0.026	-0.996	6

Where y=A+Bx, r is the regression coefficient and n the number of points.

These formulae were input into a Microsoft Excel spreadsheet so that to determine an unknown [pQR701], the peak areas and relevant IS(T) were simply input. Appendix 2 gives an example of the calculation of a [pQR701] from peak area data.

3.2.4 Quantitation of a dilution series of *E. coli* JM107 pQR701 whole cells

So far, all quantitation experiments described have been carried out using a purified

preparation of plasmid pQR701. The calibration was set up using purified plasmid rather than whole cells because the number of plasmids per cell may not be constant between different experiments. Plasmid copy number can vary considerably depending on the growth conditions (Margaritis and Singh Bassi, 1991). Factors such as nutrient availability, aeration, temperature (and hence specific growth rate) could be reasonably controlled by using the same growth conditions each time, however, it is still possible that there would be some variation that would be indistinguishable from assay imprecision. In addition, the shake flask conditions used to grow cells for these experiments would be different from those in subsequent fermentations. Therefore, the approach that was used was to calibrate against known concentrations of the pQR701 plasmid and to correct for the number of plasmids per cell in each individual experiment according to the method described later (Section 5.1).

To determine whether the quantitative method could be used to measure the number of cells of *E. coli* JM107 pQR701, a shake flask culture of the micro-organism was grown and a sample from this was serially 10 fold diluted. Each dilution was subjected to PCR with IS(T)1, IS(T)2 and IS(T)3. The results are shown in Figure 3.14.

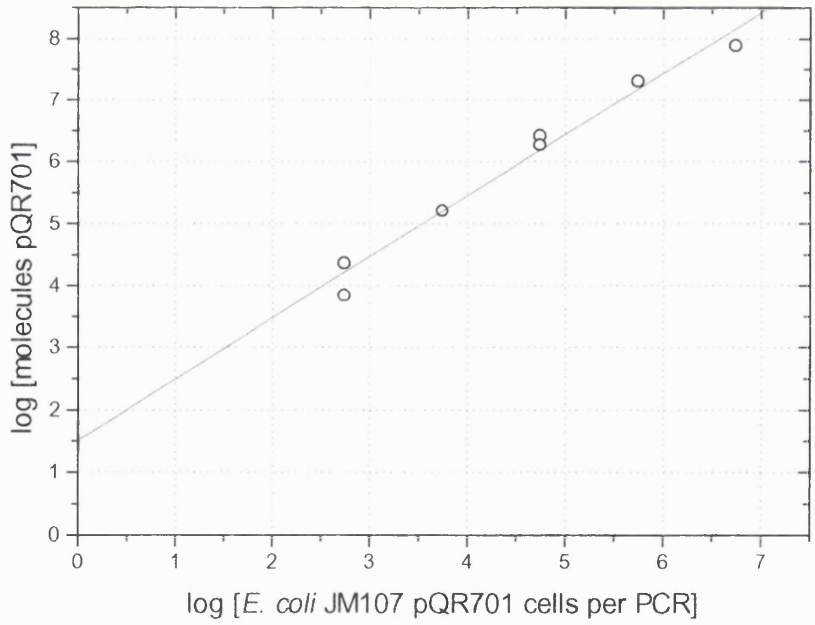


FIGURE 3.14 Measured [pQR701] in a dilution series of *E. coli* JM107 pQR701 cells. Figure shows measured number of pQR701 molecules per PCR assay versus number of cells/PCR (derived from microscopic cell counting). The intercept of 1.51 indicates that the number of plasmids per cell is approximately 32. If measured pQR701 was plotted against CFU/PCR the intercept indicates that the number of plasmids per CFU is 80. The measurement of plasmid copies per cell is dealt with in Section 5.1.

The gradient of the line drawn through the points is 0.987 (r = 0.988), indicating that there is a good correlation between the number of cells present in the assay and the number of molecules of pQR701 measured. This implies that the QPCR assay can be used for quantitation of *E. coli* JM107 pQR701 cells.

3.2.5 Construction of calibration curve for pQR126

The calibration curves for the α-amylase encoding plasmid pQR126 were constructed in the same fashion as those for pQR701. The details are shown below in the following Tables (3.8, 3.9) and Figure (3.15).

TABLE 3.8 IS(A) concentrations used in construction of calibration curve for pQR126

	Concentration (molecules/μL)	Anticipated linear range (molecules pQR126 per PCR)$^{+}$	Approx. range *E. coli* JM107 pQR126 (cells per PCR)*
IS(A)1	2.0×10^4	1.1×10^2 - 1.1×10^5	1 - 1.0×10^3
IS(A)2	2.0×10^6	1.1×10^4- 1.1×10^7	1.0×10^2 - 1.0×10^5
IS(A)3	2.0×10^8	1.1×10^6- 1.1×10^9	1.0×10^4 - 1.0×10^7

$^{+}$; Densitometry has shown that 5.8 molecules IS(A) ≡ 1 molecule of pQR126 in terms of band intensity.
*; Rough estimate based on 100 plasmids per cell (Section 5.1).

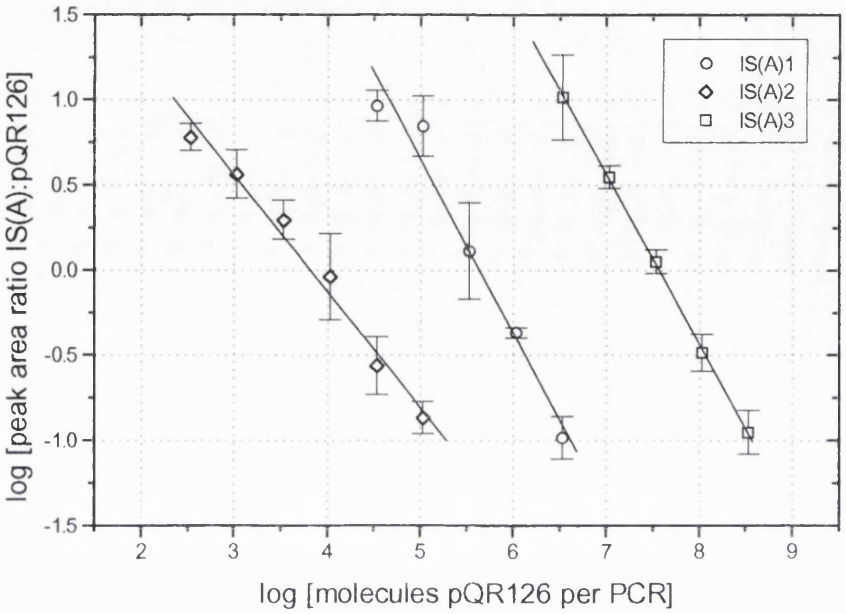


FIGURE 3.15 Calibration curves for quantitation of pQR126 with linear regression lines. Error bars shown represent the standard deviation of the 3 replicate points used for each data point.

Table 3.9 shows the appropriate parameters of the plotted lines.

TABLE 3.9 Parameters of IS(A) linear regression lines

	A	(s.d.)	B	(s.d.)	r	n
IS(A)1	2.4	1.19	-0.68	0.048	-0.990	6
IS(A)2	5.8	0.59	-1.02	0.11	-0.984	5
IS(A)3	7.5	0.10	-0.99	0.013	-0.999	5

Where y=A+Bx, r is the regression coefficient and n the number of points.

3.2.6 Quantitation of a dilution series of *E. coli* JM107 pQR126 whole cells

Again the methodology was the same as the *E. coli* JM107 pQR701 dilution series quantitation. Results are shown in Figure 3.16.

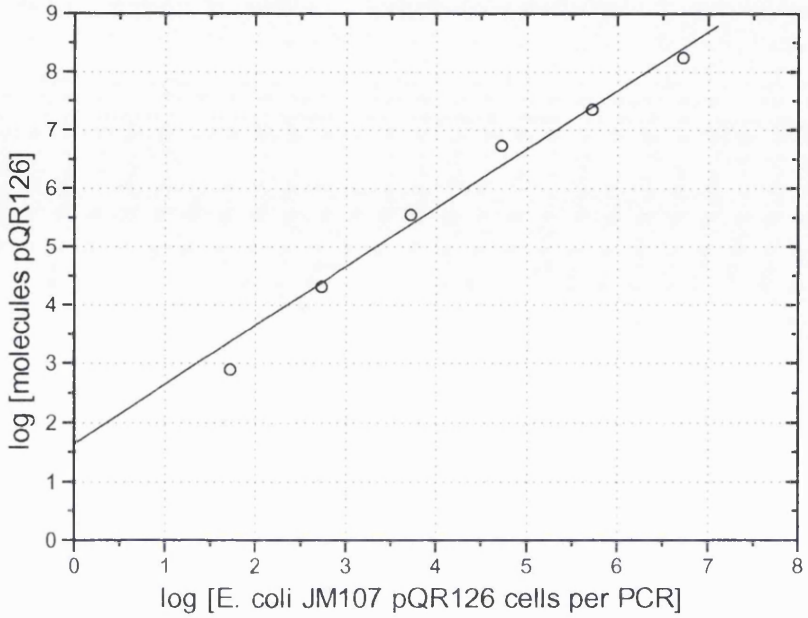


FIGURE 3.16 Measured [pQR701] in a dilution series of *E. coli* JM107 pQR701 cells. Figure shows measured number of pQR701 molecules per PCR assay versus number of cells/PCR (derived from microscopic cell counting). The intercept of 1.64 indicates that the number of plasmids per cell is approximately 44. The measurement of plasmid copies per cell is dealt with in Section 5.1.

The gradient of the line drawn through these points (by linear regression) is 1.008 ($r = 0.991$), indicating a good correlation.

3.2.7 Precision of the QPCR assays for *E. coli* JM107 pQR701 and *E. coli* JM107 pQR126

In order to aid in subsequent interpretation of results from QPCR assays, the precision of the assays for both purified plasmid and whole cells was measured. This was achieved by making a dilution of plasmid preparation or whole cells where the anticipated number of copies of plasmid would give a band of approximately equal intensity to IS(T)2 or IS(A)2. Each whole cell or plasmid sample was co-amplified with the appropriate IS in 5 separate PCRs. The results from each set of PCRs were averaged and the standard deviations calculated.

The standard deviation of the estimated pQR701/126 concentrations was calculated from the ratios of the peak areas for each PCR set using the relevant equation. The range of the estimate for the number of plasmids present in the sample was then calculated for the average ratio of the peak intensities ± the standard deviation. An example is given below (Table 3.10) for measurement of pQR701 in whole cells of *E. coli* JM107 pQR701,

where the average log (peak area ratio) was -0.986 and the standard deviation was 0.086;

TABLE 3.10 Example of precision calculation

	log (peak area ratio)	Calculated [pQR701]	log [pQR701]	%
Average	-0.986	4.31×10^6	6.63	(100)
Average + S.D.	-0.900	3.31×10^6	6.52	77
Average - S.D.	-1.072	5.60×10^6	6.75	130

Therefore in this case the standard deviation, in terms of final calculated [pQR701], is equal to ± 0.11 logs. Using this approach the standard deviation of the assays with each sample type was calculated (Table 3.11). In all cases except the one shown above the band intensity of target and IS was similar, being within a factor of 2.

TABLE 3.11 Precision of QPCR assays for pQR701 and pQR126 in purified form and in whole cells

Sample type	Precision (± logs)
Purified pQR126	0.10
E. coli JM107 pQR126 whole cells	0.50
Purified pQR701	0.09
E. coli JM107 pQR701 whole cells	0.11

It is evident then that measurement of *E. coli* JM107 pQR126 cells is far less precise than that of *E. coli* JM107 pQR701 cells. Notably, the measurement of pQR126 within the whole cells of *E. coli* JM107 pQR126 is substantially less precise than the measurement of the purified plasmid. In this PCR it has been found that the addition of 5% formamide as denaturant was necessary to consistently produce a product band, presumably due to stabilised secondary structure within the segment to be amplified (see Section 3.1.2). It seems that the problem is compounded when the plasmid is not free of cellular components and hence an inconsistent quantification results.

The quantification of pQR701 as a purified preparation or within whole cells is acceptably precise. An error of ± 0.11 logs for measurement of the whole cell preparation represents approximately 30 % error. For these reasons, all subsequent experiments are carried out using the more consistent pQR701 QPCR system. This will allow easier interpretation of results where small changes in the concentration of

plasmids or whole cells may be observed.

In these experiments, precision was quantified using a single target concentration point, where the band intensities were relatively close. The subsequent section shows that assay precision is greatest under these conditions, as opposed to when one band is significantly more intense than another. However, the difference in precision over the range of each calibration curve is relatively small. In fact, determination of the precision by averaging the SD values of the entire calibration curves (i.e. the average error bar magnitude, Figure 3.13) yields a similar figure for the assay precision.

3.2.8 Quantification using densitometry

It has previously been noted that densitometry, where peak area ratios are derived from an agarose gel, is an important step in terms of consistency of quantitation of plasmid DNA. This is because densitometry requires an element of judgement on the part of the analyst. In order to minimise the subjective nature of the analysis, a good deal of emphasis has been placed on the setting of rules by which peak areas are scored (Section 2.3.2).

It was noted in Section 2.3.2 that it is possible to overload the detection system and hence lose linearity of detection. The software can detect saturation of this type and displays this in the analysis. Figure 3.17 shows a 3 dimensional image of a gel analysed by the software.

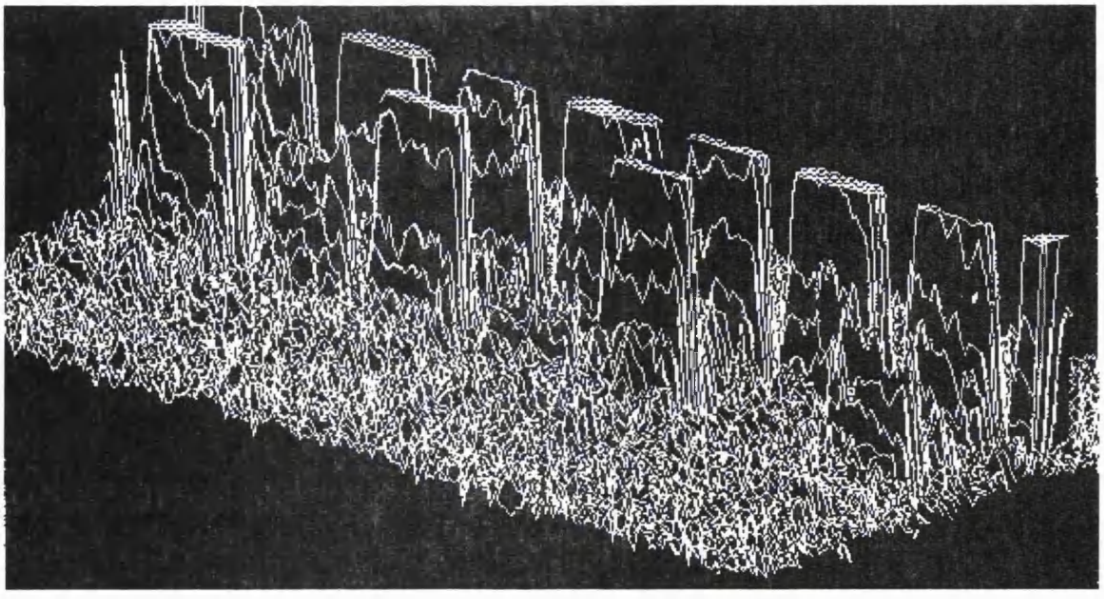


FIGURE 3.17 3-D image of ethidium bromide stained gel using Gelbase software. Figure shows 3-D image of IS(T) and pQR701 product bands in 7 gel lanes. The detection system is overloaded in several cases.

The truncated peaks are those that saturate the detection system. If the area of such a peak was measured, it would therefore be significantly underestimated. Saturation of images was accordingly looked for very carefully. The 'rules' for scoring peak areas that were laid down in the Section 2.3.2 were devised by analysing 3 different samples, each comprising a different ratio of IS(T) to pQR701 product. In this experiment, different volumes of each sample were run on a gel and the gel image was taken using 3 different aperture settings. The result was that for each sample there were a series of scores for the ratios of the peak areas, some of which were ruled invalid either through saturation (fluorescence level too high) or insufficient peak area (fluorescence level too low) for one of the bands. Figure 3.18 demonstrates how the application of these rules leads to replicates with a smaller standard deviation than would otherwise be seen. In this experiment, the net effect is to reduce the average standard deviation from ± 0.15 logs to ± 0.1 logs.

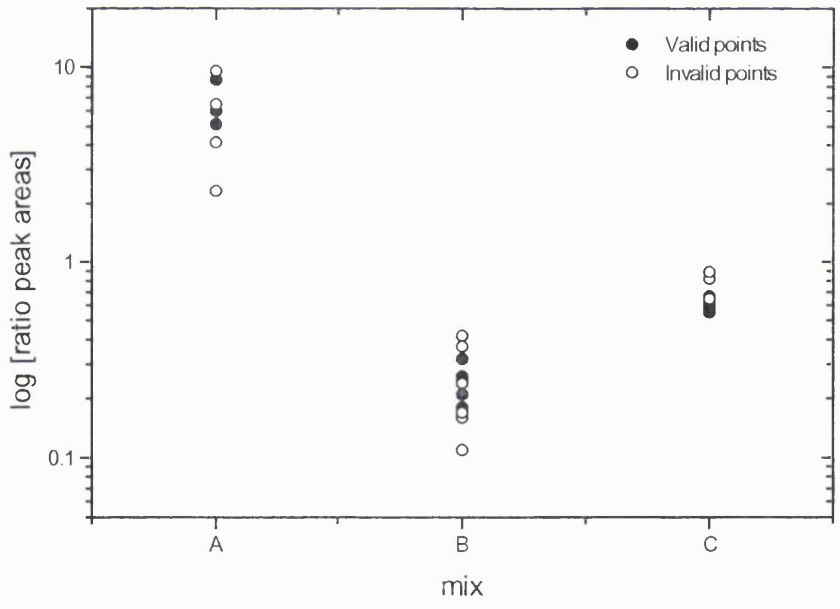


FIGURE 3.18 Effect of applying scoring rules on variance of replicate measurements of peak area ratios. Mixes A, B and C were loaded onto a gel at 20, 10, 5 and 2 μL per lane and images taken at 3 aperture settings (f2, f3 & f6), giving a total of 12 peak area ratio scores. For mix A, only 8 scores were measured due to 1 lane running very poorly. Mixes resulted from co-amplification of 1.25×10^6 IS(T) molecules with: A; 1.7×10^4 molecules pQR701: B; 1.4×10^6 molecules pQR701: C; 3×10^5 molecules pQR701.

It is also worth noting that the lowest variance is seen with sample C, where the band fluorescent intensities are approximately equal. This has implications for the precision that can be achieved by the assay.

A typical lane profile is shown in Figure 3.19. It is evident that the two peaks of interest, the IS(T) and pQR701 bands, are resolved and peak areas can be accurately measured. However, it became apparent that in many instances a third band appears between the 2 bands of interest. This has been noted by other workers (Gebhardt *et al*, 1994; McCulloch *et al*, 1995) and is thought to be due to heteroduplex formation where, due to their similar compositions, strands of IS product and pQR701/126 product will pair with each other in the latter stages of the PCR. The result is a band of intermediate size which appears between the IS and pQR701/126 product bands.

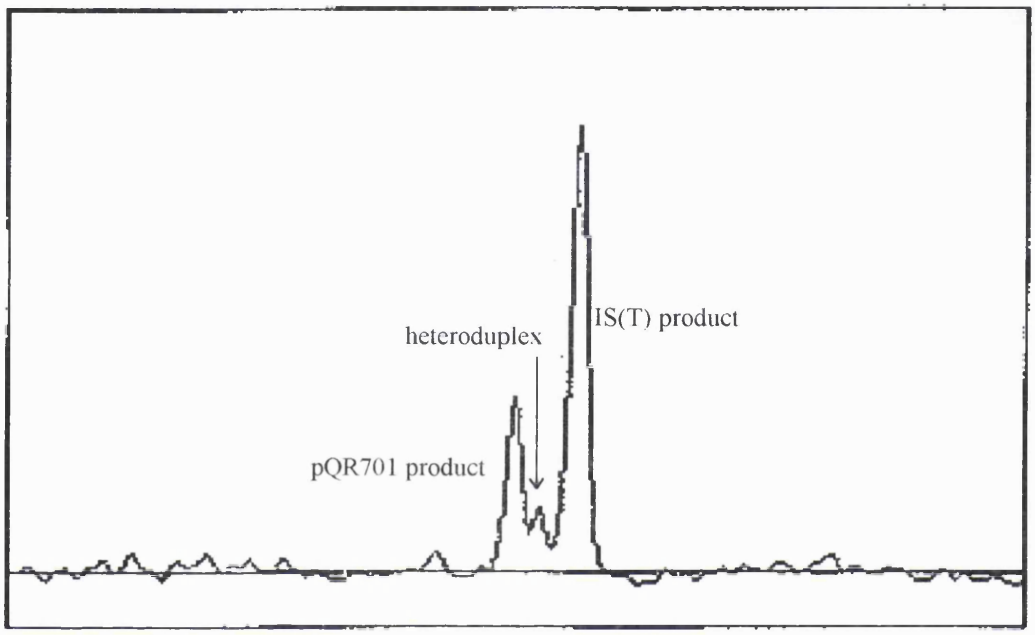


FIGURE 3.19 Typical lane profile for densitometry analysis of agarose gel. Figure shows cross sectional profile of a lane analysed by Gelbase. From left to right, first peak is pQR701 band (332 bp), second smaller peak is heteroduplex (see text), and third peak is IS(T) (247 bp).

Despite the occasional appearance of this band, the two peaks of interest can still be resolved. Because the heteroduplex band is always relatively insignificant and is composed of one strand from each main product, it is not quantitated and is ignored in analysis of peak areas.

3.2.9 Capillary gel electrophoresis for measurement of PCR products

Capillary gel electrophoresis (CGE) is a technique that has recently begun to be used for the analysis of PCR products (McCord *et al*, 1993b; Srinivasan *et al*, 1993; Lu *et al*, 1994). Many of these applications are based on the fact that CGE separations of nucleic acids can have very fine resolution (4 bp resolution has been reported (McCord *et al*, 1993a)). Additionally, CGE can be used in conjunction with a very sensitive detection

system such as laser induced fluorescence (LIF) to detect very low amounts of DNA (McCord *et al*, 1993b; Srinivasan *et al*, 1993). The advantage of CGE-LIF with respect to PCR quantitation is that its sensitivity is such that PCR products can be measured whilst the PCR is still within the linear portion of the reaction. With adequate controls this allows direct quantitation of the starting number of copies of template in a PCR.

In these experiments CGE was investigated as an alternative to standard agarose gel electrophoresis for quantitation of PCR products. The samples used for analysis by CGE were the same as those used in construction of the calibration curves (Sections 3.2.3 and 3.2.5), the principle of quantitation being the same, that is, based on the use of a competitive internal standard. However, it was hoped that CGE, once a method was established, would offer certain advantages over agarose gel electrophoresis (see Figure 3.20): i) analysis by CGE allows direct quantitation of DNA fragments in solution without the additional densitometry step; ii) CGE analysis can be readily automated since there is no need to pour fresh slab gels for each series of samples and because the instrument used (see Section 2.3.3) has an autosampler allowing up to 40 samples to be analysed at a time; and iii) CGE analysis uses UV detection of DNA fragments, hence there is no requirement for ethidium bromide.

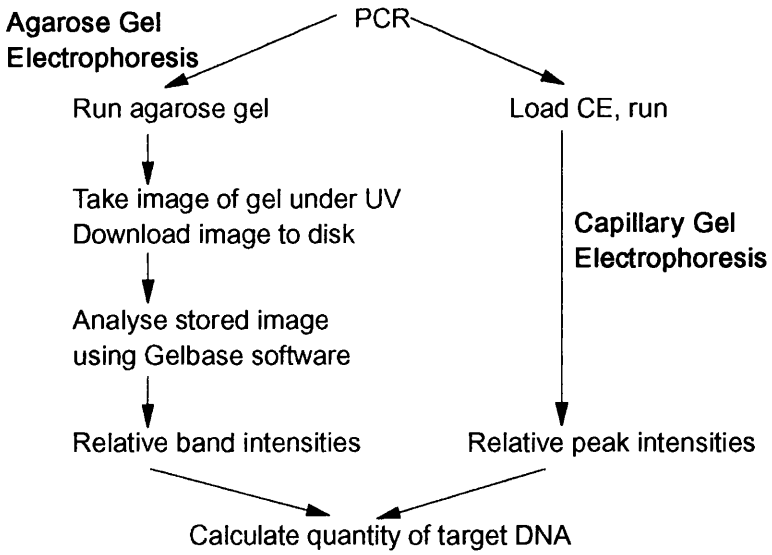


FIGURE 3.20 Comparison of agarose gel electrophoresis and CGE for quantitation of PCR products

The results of CGE analysis of PCR product samples were promising but ultimately frustrating. Although it was found that all of the IS and pQR701/126 fragment bands could be adequately resolved from each other and from the components of the PCR mixture (which do show up on a chromatogram due to their UV absorbance), it was not

possible to achieve enough data to be truly convincing because of equipment failings. The main problem was that the capillary used (at a cost of £400) was liable to breakage, which occurred as the capillary entered the instrument. Other problems such as an increase in the baseline noise as the running buffer ages and as the number of samples analysed increased were overcome by the use of fresh buffer and the introduction of a 5 minute pressure rinse step between runs. Some separations achieved by CGE are shown in the Figures 3.21 and 3.22.

Analysis of the peak area ratios (IS/pQR) obtained by agarose gel electrophoresis and CGE indicate that the two techniques gave similar results (within 10%). However, it was not possible to fulfil the aim of constructing a calibration curve using CGE, due to the problems mentioned earlier. The sensitivity of the two techniques were also found to be approximately equivalent, although with CGE the sample volume loaded is much less (<1μL) than that used in agarose gel electrophoresis (7.5μL). The smaller sample volume required in CGE allows more re-runs in the event of an inconclusive result or alternatively introduces the possibility of running smaller volume (and cheaper) PCRs (see Section 7.1.4).

Comparison of the two techniques in terms of the time taken to give a final result is not straight forward. This is because CGE runs samples consecutively whereas up to 40 samples can be run in parallel on an agarose gel. Therefore although it takes only 20 minutes to run one sample by CGE and there is no time required for subsequent analysis, whilst a gel takes 2 hours plus analysis time, CGE might not always be faster.

TABLE 3.12 Comparison of time taken from sample loading to results availability using CGE and agarose gel electrophoresis (AGE)

No. of samples	Turnaround time - CGE (min)	Turnaround time - AGE (min)
1	20	135
5	100	145
10	200	170
40	800	320

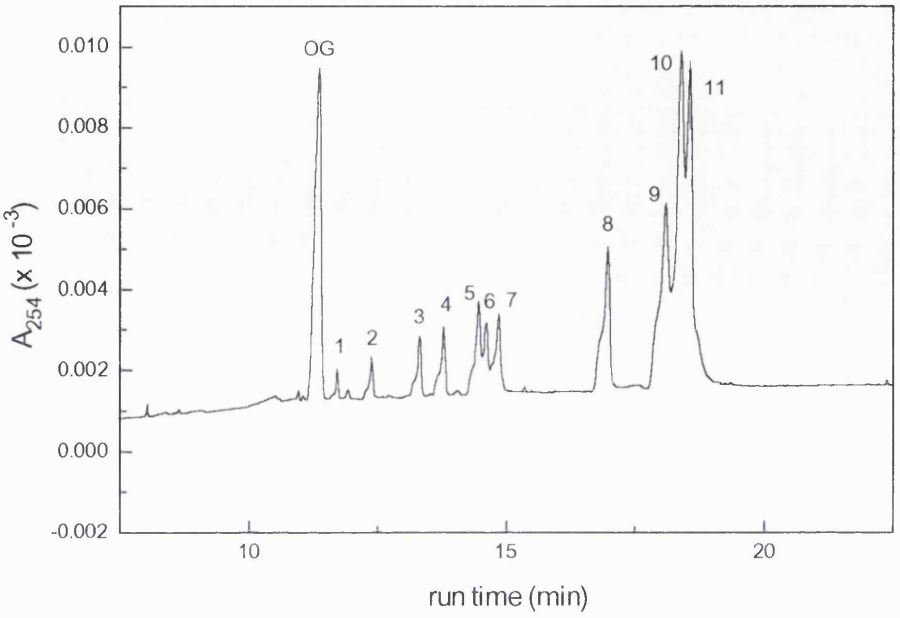


FIGURE 3.21 CGE Separation of Phi-X 174 RF DNA Hae III Digest (Test Mix). Peaks identified: OG; orange G (marker dye): 1; 72 bp: 2; 118: 3; 194: 4; 234: 5; 271: 6; 281: 7; 301: 8; 603: 9; 872: 10; 1078: 11; 1353.

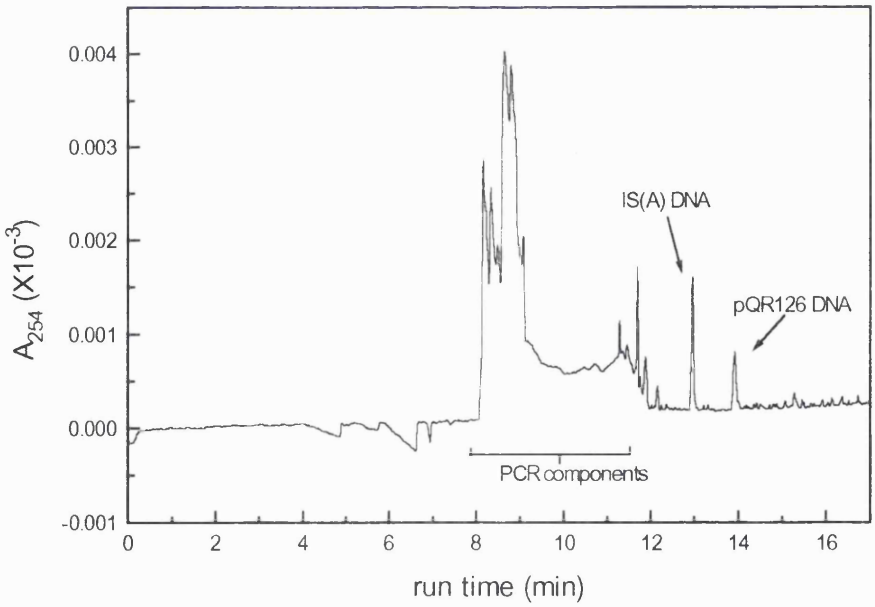


FIGURE 3.22 CGE Chromatogram of PCR of pQR126 and IS(A). Unpurified PCR products loaded onto CGE. PCR components that absorb at 254 nm include proteins, primers and primer/dimer products.

However, since the CGE has an autosampler, it is possible to leave samples running overnight. In addition the 'hands-on' time for CGE is virtually nil irrespective of the number of samples, whereas for agarose gel electrophoresis the hands-on time for 10 samples is estimated at 50 minutes and for 40 samples is 200 minutes.

In conclusion it can be said that CGE is potentially a very useful analysis technique for this type of quantitation. However, it has not been possible to validate the quantitation to any great extent, so all subsequent experiments were carried out using agarose gel electrophoresis and densitometry.

3.2.10 Effect of simulated inhibition on quantification

QPCR methods that rely on measuring the quantity of PCR product and then relating this amount to the original number of copies of target are sensitive to inhibition of the amplication which can lead to variable results (Jansson, 1995). In order to test whether inhibition of the amplification affects the QPCR method developed here, a series of PCR experiments was set up with different levels of *Taq* polymerase and different numbers of cycles to simulate inhibition and enhancement of the amplification reaction. Results are shown below in Figure 3.23.

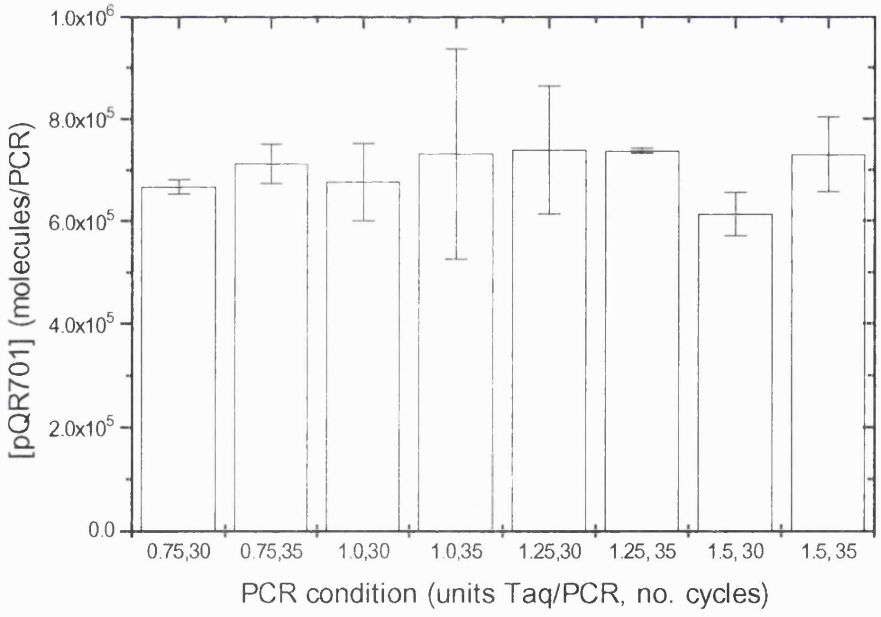


FIGURE 3.23 Effect of varying *Taq* polymerase concentration and cycle number on quantification. Figure shows average (n = 2) values with error bars representing SEM. The x-axis labels indicate the number of units of *Taq* polymerase per assay (ranging from 0.75 to 1.5), and the number of cycles carried out in the PCR (either 30 or 35).

For all conditions, there is no significant difference (measured using a 2 sample t-test) relative to the control; 1.25 units *Taq* polymerase, 30 cycles. Therefore over a twofold range in *Taq* concentration, simulating up to 40% inhibition and 20% enhancement, the quantitation is not affected.

3.2.11 Long term stability of IS(T) preparations

Since the basis of measurement of pQR701 concentrations is a comparison of the unknown concentration of the plasmid with a fixed, known concentration of the internal standard (IS(T)), then it is important to ensure that IS(T) itself is invariant in its concentration. This can be achieved either by making up fresh IS(T) for each experiment or by determining the stability of IS(T) under appropriate storage conditions. The stability of IS(T) was therefore investigated over a 5 month period. The principle of the stability tests was that each IS(T) (i.e. IS(T)1, 2 and 3) was co-amplified with a freshly prepared and quantified pQR701 solution of known concentration. The response in the assay was compared with that at the time of making up the IS(T) batch (t = 0), the results are shown in Table 3.13.

TABLE 3.13 Results of stability trial of internal standards IS(T)1, 2 and 3

Standard	Storage Temp. (°C)	Time		
		2 weeks	1 month	5 months
IS(T)3	-20	98 ± 34	132 ± 32	108 ± 27
	-70	104 ± 7	73 ± 4	126 ± 26
IS(T)2	-20	119 ± 13	27 ± 5	11 ± 1
	-70	111 ± 16	76 ± 15	87 ± 18
IS(T)1	-20	98 ± 2	n/d	n/d
	-70	99 ± 6	58 ± 6	66 ± 12

'n/d' indicates no band detected on gel. Figures are expressed as percentage recovery compared to t = 0 values. Each result is the average stability figure derived from two pQR701 concentrations per IS(T), 2 duplicates per concentration (n = 4, ± SEM). Results are calculated by comparing observed pQR701 concentration (determined by QPCR) with calculated pQR701 concentration (from quantification by A_{260}). If observed pQR701 is greater than the calculated value, then it is inferred that the standard is at a lower concentration than anticipated (i.e. it has lost stability). For instance, if observed pQR701 concentration of 150 %, then IS(T) would be said to be 67 % of it's anticipated value. Basically, this assumes that the response of the QPCR assay to varying IS(T) concentration is the same as that to varying pQR701 concentration. This method is used, as otherwise it would be necessary to characterise the response of the QPCR assay to varying IS(T). It is thought that the relatively high degree of scatter on these data is a consequence of the fact that each assay point involved the preparation of a fresh mini-prep of pQR701, followed by quantitation and dilution by a factor of up to 10^8.

The results indicate that IS(T)3 is stable over 5 months at both storage temperatures, but that otherwise stability falls with decreasing concentration (IS(T)1 less stable than IS(T)2) and increasing storage temperature (-20°C more destabilising than -70°C). This pattern would suggest that there is a concentration independent process (first order decay), in which the standard concentration decreases with time. This process is slower at the lower storage temperature. It is possible that the effect is at least partly related to the freezing and/or thawing, although no significant difference in the response was noted after an additional (-20°C) freeze/thaw cycle.

The experimental implication of this finding was that IS(T) should be stored at -70°C and that fresh IS(T)1 and 2 were prepared at monthly intervals by dilution from IS(T)3.

4. ADAPTATION OF THE QPCR METHOD FOR AIR SAMPLING

The QPCR method, whose development was outlined in the previous chapter, could be applied to the measurement of the relevant target micro-organisms in, say, sea water or, given a DNA extraction technique (Ogram *et al*, 1987), soil samples. However, for the purposes of this project, it is necessary to adapt the technique to the measurement of samples of airborne micro-organisms derived from an aerojet cyclone. One of the advantages of using the cyclone is that aerosolized bacteria impinge into liquid that can be directly analysed. With impactors and filter based techniques it would be necessary to perform some sample pre-treatment before PCR.

There are some specific requirements of the methodology that need to be considered before measurement of releases of aerosols from bioprocesses into the air can be achieved. These requirements can be expressed as the following questions: how efficient is the lysis of *E. coli* cells prior to PCR?; how can release of sub-cellular debris (including plasmid DNA) be distinguished from the release of whole cells?; once a sample has been collected, for how long and under what conditions can it be stored until assayed?; and is it possible to adequately clean the aerojet cyclone between uses so that cells/DNA from a previous sample will not contaminate the subsequent one? These issues will be addressed in this chapter.

4.1 Optimization of lysis prior to PCR

In experiments described so far and in some subsequent sections, the lysis of *E. coli* cells that is required to allow primers to gain access to the target plasmid has been assumed to occur in the initial heat denaturation step of the PCR (Mahon and Lax, 1993). Although this method has allowed a good correlation between the number of cells in a PCR and the number of pQR701 copies measured (Section 3.2.4), there are potential drawbacks involved in not having a good understanding of the lysis process. These drawbacks are: i) that the lysis may be inefficient, compromising the sensitivity of the PCR; and ii) that the lysis efficiency may be variable, depending on the influence of certain factors. Variable lysis efficiency might be encountered after storing cells at low temperatures (for example, see Ingraham (1987)), leading to apparent sample instability. It was therefore decided to look at a variety of different methods in an attempt to optimise the lysis and to minimise any artefacts that might otherwise be seen.

Several methods for the lysis of bacterial cells present in simple aqueous systems prior to PCR have been reported in the literature. These methods (which are distinct from those

used to isolate DNA from more complex systems such as soil, see for example Leff *et al* (1995)) can be classified into those depending on physical, chemical or enzymic methods. Examples of physical methods include the use of repeated freeze/thaw cycles (Bej *et al*, 1991b; Grant *et al*, 1992), boiling (Josephson *et al*, 1993; Zwadyk *et al*, 1994; Madico *et al*, 1995), sonication (Kirk and Rowe, 1994; Sparagano *et al*, 1994) and mechanical disruption using glass or zirconia beads (Johns *et al*, 1994), the latter being used to lyse spores of *Bacillus anthracis*. Chemical methods are illustrated by the use of detergents (Sparagano *et al*, 1994; Alvarez *et al*, 1995; Sakallah *et al*, 1995) or formamide (Sparagano *et al*, 1994). Enzymes that have been used in lysis treatments include proteinase K (Atlas and Bej, 1990; Grant *et al*, 1992; Goldenberger *et al*, 1995) and lysozyme (Grossman and Ron, 1975). Several of the above methods are not easily classified as they may use a combination of techniques; for instance, the use of proteinase K and SDS detergent (Goldenberger *et al*, 1995).

Several workers have compared methods side by side in an attempt to determine which is best suited to the particular application. For example, in a study by Sparagano *et al* (1994) sonication, freeze/thawing and formamide treatment all produced consistent positive PCR results, whilst chemical lysis and disruption using glass beads were not successful. However, this and other studies have not used truly quantitative methods to assess the relative recoveries effected by the different methods. The aim of this series of experiments was therefore to compare a cross section of different methods using QPCR to determine which gives the optimal recovery of plasmid pQR701. The methods used are summarised in Table 4.1.

TABLE 4.1 Summary of lysis methods attempted on *E. coli* JM107 pQR701 cell suspension

Method type	Comments	Reference
A. No lysis step	Control	(Mahon and Lax, 1993)
B. Boiling	10 minutes in boiling water bath	(Josephson *et al*, 1993)
C. Freeze/thaw	6 × 1 min cycles between ethanol/dry ice and 50°C.	(Bej *et al*, 1991b)
D. Sonication	6 cycles: 30 s at 8 μm amplitude, 50 Hz, 10 sec off (Soniprep, MSE); sample cooled on ice	(Kirk and Rowe, 1994)
E. Detergent	4mM SDS, 10 minute incubation at RT.	(Alvarez *et al*, 1995)
F. Detergent	ATP releasing agent (Celsis Ltd) at 1:1, 10 minute incubation at RT.	-
G. Lysozyme	0.1 mg mL^{-1} lysozyme (Sigma), 1 hour incubation at 37°C.	(Sparagano *et al*, 1994)

The results of the comparison of lysis methods are shown in Figure 4.1. Note that no results are shown for lysis methods E (SDS treatment), F (ATP releasing agent) and G (lysozyme) since, in all these cases, the PCR was inhibited. It was therefore not possible to determine how efficient lysis was on each of these occasions. Sparagano *et al* (1994) have similarly found that lysozyme and SDS methods of lysis did not provide any detectable PCR product. The idea of trying ATP releasing agent was that this solution, which contains chlorhexidine digluconate, would be effective at releasing plasmid as it is formulated to release ATP from bacteria into solution whilst not interfering with subsequent luciferase based assay.

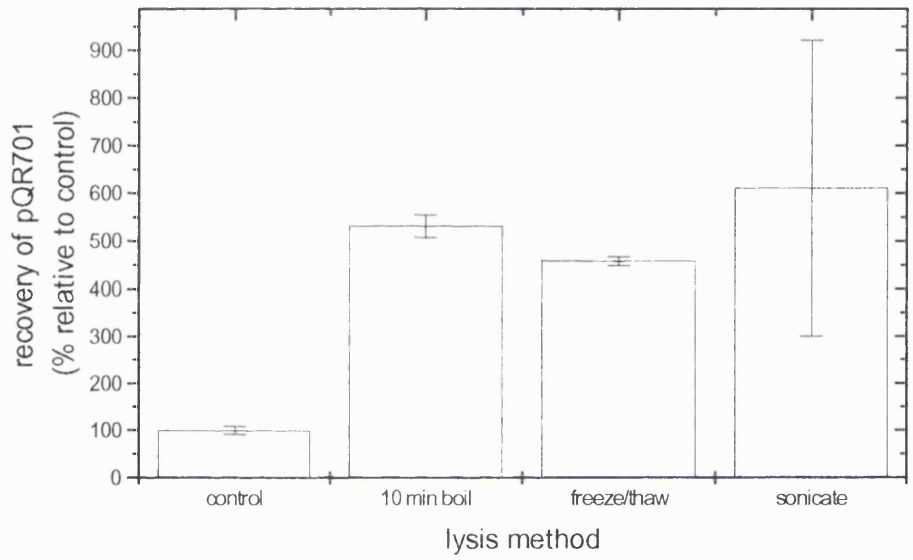


FIGURE 4.1 Comparison of recoveries of pQR701 from whole cells using different lysis methods. Figure shows recoveries of plasmid pQR701 relative to control value (assigned 100 %). Values shown are averages (n = 2) ± SEM. All lysis methods were conducted using 5×10^4 *E. coli* JM107 pQR701 cells in in 100 µL TRS, of which 2×10 µL was assayed by PCR.

Since boiling is a very convenient and effective method to use, it was further investigated. This was achieved by carrying out an experiment where the same cell suspension was subjected to boiling for different lengths of time. Results are shown in Figure 4.2. It is apparent from this figure that lysis efficiency is a function of boiling time. There is an indication that the relationship levels off between 30 to 45 minutes of boiling. Considering this trend in conjunction with the need for rapid assay turnaround, it was decided to use a 30 minute boiling step to effect lysis for certain subsequent

experiments (the 'no lysis' method was used unless otherwise stated). Notably, in this experiment the ratio of the recovery of pQR701 after 30 minutes of boiling compared to the 'no lysis' control method is 20:1 (in other experiments the ratio has varied from 3:1 to 20:1). This suggests a substantial improvement in the sensitivity of the PCR.

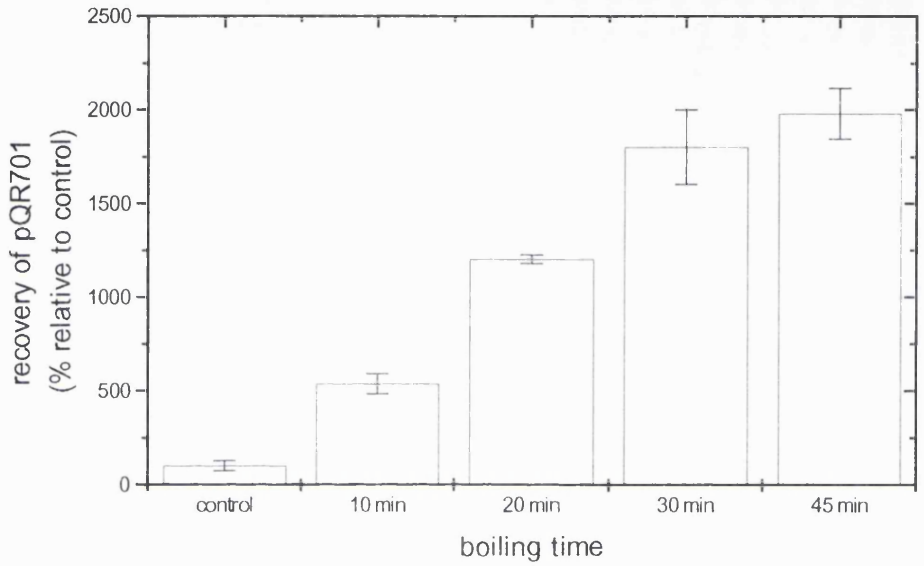


FIGURE 4.2 Effect of varying boiling time on lysis efficiency. Figure shows recoveries of plasmid pQR701 relative to control value (assigned 100%). Values shown are averages (n = 2) ± SEM. Boiling was carried out using 2.5×10^4 *E. coli* JM107 pQR701 cells in 100 µL thiosulphate ringers solution, of which 2×10 µL was assayed by PCR.

It should also be noted that the 30 minute boiling step has an insignificant effect on the concentration of extracellular (filtered) pQR701. This implies that heat does not cause excessive DNA degradation, a point noted by Zwadyk *et al* (1994) who found that the effect of heating on *Mycobacterium* genomic DNA is to produce strands which are suitable for PCR amplification. Additionally, this result suggests that the proportion of pQR701 that is extracellular in a culture would be overestimated if the 'no lysis' method is used (Section 5.3.2.1).

It is also worth noting at this point that the PCR developed for a chromosomal *E. coli* gene, *gusR*, was intended to be used to determine absolute lysis efficiency effected by a variety of treatments. It was envisaged that since it is possible to determine the number of chromosomes per cell under various growth conditions (Bremer and Dennis, 1987) and the number of cells in a sample, then it would be possible to calculate the total

number of copies of the *gusR* gene that should be made available to PCR after a theoretical 100 % efficient lysis procedure. Absolute rather than relative lysis efficiencies could then be determined. Unfortunately, it has not been possible to pursue these experiments in the time allowed.

4.2 Distinguishing between intracellular and extracellular DNA

Because of the nature of the QPCR method used here, where the segment to be amplified crosses the point of insertion of the 'foreign' DNA (Section 3.1.1), it is not likely that a positive PCR would result from any other organism or *E. coli* strain, irrespective of whether it may be the originator of the 'foreign' DNA. It is also highly unlikely that the segment to be amplified has transferred to another organism by one of the mechanisms of conjugation, transformation or transduction (Section 1.3.1). It can be concluded, therefore, that if there is a positive PCR when assaying a sample collected from the air, then the target plasmid pQR701 is indeed present in the air sample (cross-contamination notwithstanding).

However, the QPCR method in itself can not distinguish between airborne plasmid pQR701 and airborne *E. coli* JM107 pQR701 cells. This is an important point because the aim of this project is to measure release of whole cells, and the release of plasmid in solution may occur in a different manner under different conditions. In order to distinguish whole cells from extracellular plasmids, two contrasting methods have been attempted (Figure 4.3): i) the selective degradation of free plasmid by a DNase and ii) the removal of whole cells by filtration (for details of both methods see Section 2.2.8). In both cases subsequent QPCR will allow the determination of quantities of intracellular and extracellular plasmid.

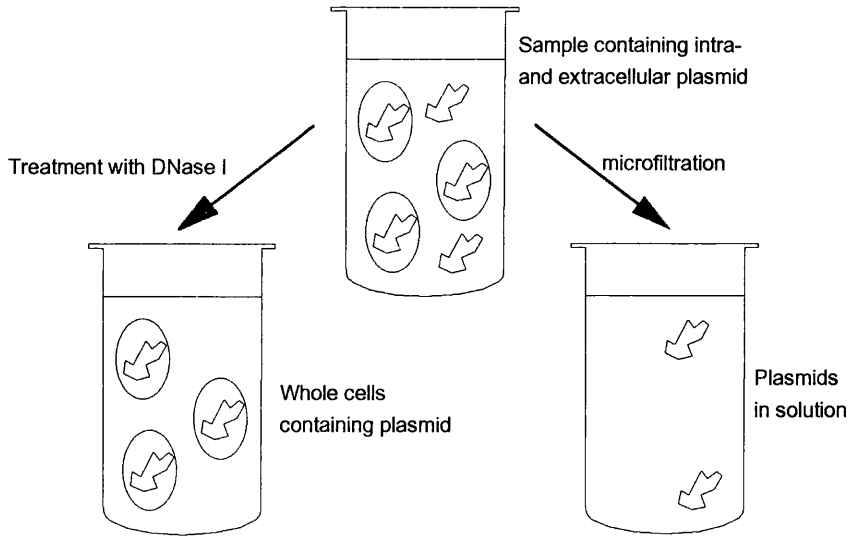


FIGURE 4.3 Schematic of two strategies for differentiation of intracellular and extracellular plasmid. Figure shows alternative pre-treatment methods to allow distinction between intra- and extracellular plasmid in a sample. Treatment with DNase I removes extracellular plasmid, so that only intracellular plasmid is measured by the QPCR. Microfiltration allows the plasmid but not whole cells to pass through the membrane, so that the QPCR measures only extracellular plasmid. Intracellular plasmid concentrations can be determined by subtraction of extracellular from total plasmid concentration.

4.2.1 Removal of extracellular DNA by use of DNase I

The assumption underlying this method is that the DNase used will degrade plasmid DNA that is free in solution to an extent where PCR of the target segment is not possible, and that no degradation of intracellular plasmid will occur. Maruyama *et al* (1993) have used DNase to distinguish viral DNA from free DNA in sea water, on the basis that virus-sized non-DNase digestible (and therefore coated) DNA is likely to represent marine viruses. Other workers (Furrer *et al*, 1990; Rochelle *et al*, 1992) have used DNase to 'clean up' PCR preparations and have then heat deactivated the DNase prior to addition of target DNA in solution. It is unlikely that under the near physiological conditions used in the incubation step that DNase I will penetrate the *E. coli* cell wall and degrade the intracellular DNA pool.

Initial experiments involved making up the PCR mix as normal with a known pQR701 concentration in the absence of IS(T), and with addition of DNase I at concentrations ranging from 0.005 - 50 units/PCR. The PCR mix was then incubated at 37°C for 30 minutes followed by 10 minutes at 94°C (to inactivate the DNase) prior to the PCR. IS(T) was then added before the initial 94°C step in the PCR. In terms of ease of set up, this was considered the best option for a DNase step. However, although it was shown that in the absence of DNase the QPCR is unaffected by the pre-incubation step, it was found that the higher DNase concentrations (above 5 units/PCR) did not allow the PCR

of IS(T), added after the 94°C incubation step, to proceed. This may be due to degradation of the primers by the DNase during the incubation or to proteolytic activity associated with the DNase preparation (suppliers state that there is less than 0.005 % protease activity in this preparation). Using 0.5 units DNase/PCR, the pQR701 concentration was reduced from 1.7×10^6 molecules per PCR to 1.0×10^4 molecules/PCR, and with 0.05 units/PCR the reduction was to 3.8×10^5 molecules/PCR. Similarly, Furrer *et al* (1990) have used 0.5 units DNase/PCR and shown no primer degradation and a 1000 fold reduction in the quantity of a PCR contaminant.

In this experimental set up, the useful DNase concentration is therefore limited on the one hand by the need to avoid primer degradation and on the other by the requirement for adequate degradation of target. The best concentration found (0.5 units/PCR) only reduced the [pQR701] by a factor of 170 and even in this case the product bands were quite faint, suggesting some primer degradation. It was therefore decided to use a different protocol where sample and DNase are pre-incubated and, following heat denaturation of the DNase (94°C, 10 minutes), the sample/DNase mix is added to the PCR in the normal way. Initially, a range of DNase I concentrations were tested with samples of purified plasmid DNA, the results are shown in Table 4.2.

TABLE 4.2 Effect of [DNase] in pre-incubation step on measured [pQR701]

[DNase] (units/PCR)	log [pQR701] (molecules/PCR)	Recovery (%)
0	7.2	(100)
0.5	4.7	0.29
5	4.4	0.14
50*	-	-

* At 50 units DNase/PCR there was a hazy precipitate in the reaction tube after the 94°C heat denaturation step. Subsequent PCR was inhibited.

The next step was to determine the effect of pre-incubation of DNase on the pQR701 concentration of whole cell preparations. The results of this experiment are shown in Table 4.3.

TABLE 4.3 Effect of pre-incubation with DNase on pQR701 concentration of whole *E. coli* JM107 pQR701 cell preparations

[DNase] (units/PCR)	Sample type	log [pQR701] (molecules/PCR)	Recovery (%)
0	Whole cells	6.9	(100)
	Plasmid prep	5.7	(100)
0.05	Whole cells	5.5	3.55
	Plasmid prep	3.9	1.5
0.5	Whole cells	5.7	9.55
	Plasmid prep	3.9	1.38

The results show that the DNase pre-incubation method is only marginally selective. That is, intracellular pQR701 is reduced to a similar extent as a purified preparation of (extracellular) pQR701. In this experiment the whole cell preparations were derived from an overnight shake flask culture (Section 2.2.2) and there is no way of knowing what proportion of the total pQR701 DNA is extracellular. However, it is very unlikely that greater than 95 % of the total pQR701 population is extracellular, subsequent filtration experiments suggest that extracellular pQR701 in a preparation such as this represents less than 20 % of the total. It can therefore be concluded that this DNase I method can not adequately distinguish extracellular and intracellular pQR701.

The reason for this is thought to be that lysis of *E. coli* cells occurs at elevated temperatures whilst DNase is still active. Determination of the thermostability of DNase I (data not shown) has indicated that the enzyme retains significant activity at 70°C. It is not known at what temperature *E. coli* cells lyse under these conditions; although it is known that cell death occurs quickly at 60°C (Bailey and Ollis, 1986). In the PCR it is known that 5 minutes at 94°C is sufficient to achieve some lysis (Mahon and Lax, 1993). It is quite possible, therefore, that *E. coli* cells are lysing, and hence releasing pQR701 molecules into solution, at temperatures where DNase retains some activity. It should be noted that DNase preparations with less thermostability may be available and a different preparation may be more suitable for this application. Additionally, the DNase/lysis effect may be useful in delineating the high temperature lysis of *E. coli* cells prior to PCR.

4.2.2 Removal of whole cells by filtration

Since it is widely accepted that *E. coli* cells can be efficiently removed from solution by micro-filtration using a 0.2 μm or 0.45 μm pore size membrane (Cartwright *et al*, 1993),

the parameter that must be looked at closely in developing a filter method for the removal of whole cells is the tendency of free DNA to bind to or block the filter membrane or housing. To this end, a variety of 0.2 μm filter types were tested for their binding capacity by passing through 1 mL of a known concentration of pQR701 solution. The results are shown below in Table 4.4.

TABLE 4.4 Filtration of plasmid pQR701 solution by various filters

Filter Type	log [pQR701] in filtrate (molecules/PCR)	Recovery (%)
None	4.4	(100)
Sartorius Minisart	4.0	41
Nalgene (cellulose acetate)	3.9	30
Whatman (polysulfone)	4.3	76
None	4.8	(100)
Costar spin-x (cellulose acetate)	4.8	101

Note; the Costar spin-x column was tested in a separate experiment. All other filters are syringe tip variety.

There may be no real difference between the binding capacity of the Whatman polysulfone and the Costar cellulose acetate filters as the two recoveries are within the technique's measurement error, but the Costar method is simpler and involves less sample loss. In order to test the transmission of a wide range of concentrations of plasmid DNA solutions through the Costar cellulose acetate spin-x filters, solutions of pQR701 were made up at 3 concentrations and the recovery after filtration was tested using 0.2 μm and 0.45 μm pore size membranes. The results are shown in Table 4.5.

TABLE 4.5 Transmission of pQR701 solutions through Costar spin-x cellulose acetate filters: effect of membrane pore size

pQR701 concentration (molecules/10 μL)	Membrane pore size	
	0.2 μm	0.45 μm
10^8	62 ± 7	92 ± 7
10^6	73 ± 8	87 ± 16
10^4	94 ± 7	91 ± 5

Figures are expressed as average (n= 2) percentage recovery ± SEM.

It is apparent that at higher pQR701 concentrations, transmission is reduced with 0.2 μm pore size membranes. If this were due to binding, a fall in transmission would probably

be more apparent at the lower concentrations. The more likely explanation is blocking of the pores by the plasmid molecules; this would be consistent with reduced transmission with higher concentrations and smaller pore sizes.

The 0.45 μm filter columns were subsequently tested using the following feed solutions: diluted whole cell preparations; resuspended cell pellets; and a cell free preparation of the plasmid pQR701, in an attempt to determine the selectivity of the method for removal of whole cell pQR701. The results are shown in Table 4.6.

TABLE 4.6 Filtration of *E. coli* JM107 pQR701 whole cell preparations by Costar spin-x filter columns

Filter method	Sample type	log [pQR701] in filtrate (molecules/PCR)	Recovery (%)
None	cell free pQR701 prep	4.8	(100) ± 15
	Whole cells	6.0	(100) ± 12
	Resuspended cell pellet	5.2	(100) ± 4
Costar spin-x	cell free pQR701 prep	4.8	101 ± 11
	Whole cells	5.3	18 ± 6
	Resuspended cell pellet	n/d	0 ± 0

All figures shown are averages (n = 2) ± SEM. 'n/d' indicates none detected.

The results show that when a suspension of whole cells is filtered by this method, 18 % of the total DNA passes through the filter into the filtrate. This suggests that over 80 % of the total pQR701 is found within the cells. Again, this figure can not be verified, and may well be an underestimate due to poor lysis of whole cells (cells were not boiled in this experiment, see Section 4.1), but it seems a far more acceptable estimate of the ratio of extracellular to intracellular plasmid DNA in a stationary phase culture than that provided by the DNase method. Additionally, the finding that no pQR701 from the resuspended cell pellet passed through the filter indicates that cells are filtered out by the membrane and that intracellular plasmid can be excluded from the assay of a sample. Consequently, 0.45 μm pore size, cellulose acetate Costar spin-x filters were routinely used for all experiments where distinction between intra- and extracellular DNA was required (Section 2.2.8.2).

4.3 Stability in solution of microbially derived plasmid pQR701

Generally, DNA solutions are thought to be stable if stored free from microbial contamination at -20°C. In experiments where micro-organisms are collected from the air into a liquid sample it is possible that instability might arise due to: i) the presence of microbial DNase activity; and ii) lysis of the cells altering the ratio of concentrations of extra- to intracellular DNA. Because of the need to take successive samples at times where process release is being monitored, it was necessary to store samples before analysis. The stability of samples collected from an aerosol and stored in TRS at a series of different temperatures was therefore investigated. The method of lysis used, boiling for 30 minutes (Section 4.1), was carried out immediately before assay; samples were simply stored as collected from the cyclone.

TABLE 4.7 Stability of aerosol-collected microbial plasmid DNA at different temperatures

Storage temp. (°C).	Time		
	24 hours	4 days	7 days
-20	79 ± 11	74 ± 11	68 ± 3
4	80 ± 28	69 ± 6	75 ± 8
RT	95 ± 14	69 ± 5	52 ± 12

Values shown are average (± SD, n = 2) percentage recoveries compared to the t = 0 value (100%). Each sample was stored as a 100 μL aliquot in a sterile Eppendorf tube and was taken directly from storage before assay. The Eppendorf tube was then placed into a boiling water bath for 30 minutes to achieve cell lysis, and two 10 μL samples were taken for assay.

Considering the assay imprecision (± 0.11 logs, approximately ± 30%, Section 3.2.7), it appears that storage at -20°C or 4°C does not cause a significant change in the quantity of pQR701 measured in cell suspensions collected from aerosols. In all experiments, cells were therefore aliquoted and stored at 4°C if assayed within 24 hours or at -20°C when assayed within 7 days of collection of the sample.

It was also found that freezing and storage of cells collected from an aerosol does not increase the extracellular plasmid concentration by causing lysis. In one experiment, where the sample showed no extracellular plasmid immediately after collection (t = 0), there was no appearance of pQR701 in the filtrate after the sample had been frozen and stored at -20°C for 7 days.

4.4 Efficiency of Aerojet Cyclone cleaning

Since the QPCR method developed for the detection of *E. coli* JM107 pQR701 cells is a highly sensitive technique and the aerojet cyclone device used for air sampling is made of glass and therefore not intended for 'one-off' use like most PCR consumables, then it is essential that an effective method be developed for its cleaning. The cyclone is a relatively complex piece of glassware with attached tubing (see Figure 2.2). In an effort to ease the task of cleaning out the cyclone and attached tubing between uses, the collection vessels used were disposable plasticware.

Several cleaning methods were tested by circulating a 50 mL sample with a known, relatively high concentration of plasmid pQR701 or *E. coli* JM107 pQR701 cells through the cyclone and tubing for 10 minutes then emptying, washing by the appropriate method (Section 2.4.2) and finally passing a clean, sterile thiosulphate ringers solution sample through the cyclone for a further 10 minutes. If a residual signal was seen in the sample after washing, then the wash method was less than 100 % efficient. The wash methods and their efficiencies when challenged with 50 mL of pQR701 preparation at 3.5×10^8 molecules mL^{-1} are shown below in Table 4.8.

TABLE 4.8 Wash methods used to clean cyclone between samples

Wash solution/method	Post wash [pQR701] (molecules mL-1)	Recovery (%)	Wash efficiency (%)
1 % Tego solution	8.3×10^4	0.023	99.98
0.4 M HCl	2.0×10^5	0.057	99.94
UV light*	7.8×10^5	0.22	99.78
10 % Chloros (Hays)	1.6×10^5	0.046	99.95

* In this method, the cyclone was placed under UV light for 20 minutes, then rinsed out with tap water.

Looking at the washing efficiency figures, all methods seem very effective and this is most likely due to the extensive dilution that occurs in all the rinsing steps. However, of the methods used, placing the cyclone under UV light is least effective. UV irradiation has been suggested as a method of decontaminating reaction mixtures prior to PCR (Sarkar and Sommer, 1990), and so it was thought that it might be useful in cleaning the glass cyclone. In this case it is not very effective, probably because although the cyclone itself is made of transparent glass, the tubing is only translucent and some connecting joints are made of opaque plastic - UV light will not penetrate inside and so the only washing achieved here is by rinsing. Of the other methods, there is little to choose on the basis of washing efficiency but 1% Tego is the least aggressive solution and was

therefore used in subsequent experiments. Prince and Andrus (1992) have advocated the use of 10 % Chloros solution for washing of surfaces that may be used for PCR preparation. Although this was used in some early experiments and found to be effective for the cleaning of low amounts of DNA, it has a corrosive effect on the glass of the cyclone leaving the surface pitted, this is due to reaction between hypochlorite and borosilicate glass.

Further experiments looking into the cleaning efficiency of 1 % Tego were carried out. Table 4.9 shows that there is no accumulation of DNA through sequential challenges with 50 mL of a moderate concentration of pQR701.

TABLE 4.9 Wash efficiency of 1% Tego after sequential challenges with pQR701

	Wash 1	Wash 2	Wash 3
Before wash	5.9×10^6	4.5×10^6	5.7×10^6
After wash	n/d	n/d	n/d

Figures shown indicate pQR701 concentration expressed in numbers of molecules mL^{-1} in the cyclone, n/d indicates none detected.

Washing efficiency was also tested with *E. coli* JM107 pQR701 whole cells by challenging with increasing concentrations. The results of this experiment are shown in Table 4.10.

TABLE 4.10 Wash efficiency of 1% Tego with increasing concentrations of *E. coli* JM107 pQR701 cells

Challenge (cells mL^{-1})	Before wash [pQR701] (molecules mL^{-1})	After wash [pQR701] (molecules mL^{-1})	Recovery (%)	Efficiency (%)
5×10^3	1.0×10^6	n/d	0	100
5×10^5	1.3×10^8	1.8×10^4	0.014	99.99
5×10^7	6.2×10^9	3.2×10^7	0.51	99.49

The results show that at low to moderate concentrations of *E. coli* JM107 pQR701 the cleaning is highly efficient. At higher levels the cleaning becomes less efficient and a significant residue remains. To check the efficiency of cleaning during sampling experiments, the protocol that was adopted was therefore to circulate the sampling liquid (sterile TRS) around the cyclone for a 10 minute period (in a clean environment) after cleaning and to use this 'pre-sample' as an indicator of background levels. Although cleaning efficiency falls as the concentration of the cells in the cyclone increases, this was

not deemed a great problem as it was not expected that airborne cellular concentrations would ever be very high. One point to note is that cleaning efficiency of whole cells is roughly equivalent to that of the plasmid preparation. This is presumably because Tego is an amphoteric disinfectant and is likely to be very efficient at cell disruption.

The final experiment carried out to check the use of 1 % Tego was to determine whether any residues remained after cleaning which might subsequently inhibit the PCR. This was achieved by alternately washing and challenging the cyclone with a fixed concentration of *E. coli* JM107 pQR701 cells (giving a pQR701 concentration of 1×10^9 molecules mL^{-1}). It was found that, after washing, the measured pQR701 concentration was unaltered, within the limits of the assay, indicating that no inhibition was taking place.

5. MONITORING THE RELEASE OF MICRO-ORGANISMS FROM SELECTED BIOPROCESS OPERATIONS

Having established that it is possible to measure *E. coli* JM107 pQR701 cells with a reasonable degree of precision by the QPCR method developed, and that application of this method to the analysis of samples derived from an aerojet cyclone can be achieved, attention was then turned towards monitoring the release of cells from selected, key bioprocess operations. The unit operations chosen were batch fermentation at 2 L scale, and high pressure homogenisation. However, before this was attempted, experiments were carried out to determine the relationship between the number of cells aerosolised in a fixed, contained volume (the Bassaire cabinet) and the number captured by the cyclone and enumerated by QPCR. This is a key step in demonstrating the ability of the methodology to quantitate the release of micro-organisms via the aerosol route.

For fermentation, release of micro-organisms into unfiltered fermenter exit-gas was measured. The rationale behind this was: i) to demonstrate the application of the QPCR method to bioprocess release monitoring; and ii) to determine the number of micro-organisms that are released into the exit-gas stream and normally filtered out by the exit gas filter. This will allow consideration of the suitability of normal practice with regard to exit gas filtration and will give a baseline against which putative microbial scrubbing systems for exit gas (e.g. the Turbosep system of Domnick Hunter (Rollinson, 1988)) can be measured. Notably, recommendations for the optimum design with regard to exit gas filtration of fermentations of recombinant organisms have been made (Hambleton *et al*, 1991).

Monitoring of release from a high pressure homogeniser was carried out in recognition of the view that downstream processing operations may pose the greatest risk of release of micro-organisms from bioprocesses (Dunnill, 1982). The high pressures involved in this operation suggest that aerosolisation of microbial suspensions is a possibility (Foster, 1992). The release of micro-organisms into the air surrounding the homogeniser (enclosed within a soft-film cabinet) was therefore measured.

In experiments described so far, QPCR has been used to determine the number of plasmid molecules in samples. In order to distinguish plasmids that are extracellular from those that are located within an intact cell, a filtration pre-treatment step can be incorporated (Sections 2.2.8.2 and 4.2.2). However, there is clearly a need to relate the number of intracellular plasmids measured to the number of whole cells in a sample. This has been achieved by determining the average number of plasmids per cell for cultures in

each individual experiment. The determination of the number of plasmids per cell is explained in the following section.

5.1 Determination of the number of copies of plasmid pQR701 per *E. coli* JM107 pQR701 whole cell

In essence, this section is concerned with the measurement of plasmid copy number. Published methods for the determination of plasmid copy number involve making a preparation of the total cellular DNA, restriction digesting it and then running the preparation on a gel and determining the relative amounts of plasmid and chromosomal DNA by densitometry (Lewington and Day, 1986; Wrigley-Jones *et al*, 1992). The appropriate calculations therefore express the plasmid copy number in terms of number of plasmids per chromosome.

The average number of chromosomes per cell may well exceed 1 and is, in fact, variable, depending on the growth rate of the cell (Bremer and Dennis, 1987). Therefore the plasmid copy number is likely to be very different from estimates made of the absolute number of copies of plasmid in a preparation divided by the number of whole cells counted by microscopy, which is the method used here. For this reason, the number of plasmids per cell calculated by QPCR and cell counting will not be referred to as the plasmid copy number and will be used simply as a correction factor for subsequent experiments involving the measurement of release of whole cells from bioprocess equipment. One might expect, however, that observed trends would be the same and experiments to clarify this are being planned (see Section 7.2.1).

In these experiments it is assumed that plasmid loss occurs mainly as a result of segregational instability (Margaritis and Singh Bassi, 1991; Summers, 1991). Therefore, the number of plasmids per cell determined within the process stream at any given time will be the same as the number of plasmids per cell in a sample that has been collected from an aerosol derived from that process. Within any given sample, there will be a range of values for plasmids per cell, in all experiments here only the mean value is determined. However, from a measurement of the intracellular pQR701 concentration in the collected sample and knowledge of the number of plasmids per cell in the process stream at that time, it is simple to calculate the number of whole cells released.

The number of copies of plasmid per whole cell in the process stream was therefore determined at the same time as containment monitoring measurements were made. This was achieved by measuring, in the same sample, the concentration of intracellular

pQR701 and the concentration of whole cells mL^{-1} by microscopy. An example of the calculation of the number of copies of plasmid per cell is shown below in Table 5.1.

TABLE 5.1 Example of calculation of number of copies of plasmid pQR701 per cell

Total [pQR701] (molecules mL^{-1}) (1)	Extracellular [pQR701] (molecules mL^{-1}) (2)	Intracellular [pQR701] (molecules mL^{-1}) (3)	Cell concentration (cells mL^{-1}) (4)	No. of copies of plasmid per cell (5)
8.3×10^{10}	5.8×10^{9}	7.7×10^{10}	8.2×10^{8}	94
(measured)	(measured)	(1) - (2)	measured	(3)/(4)

An alternative method is to set up a dilution series of the culture in which the plasmid per cell number is to be measured and to determine the number of copies of plasmid in each dilution. The gradient of the line describing the relationship between the number of cells and the number of copies of plasmid should be equal to 1, and in practice it is very close to this value (Section 3.2.4). The number of plasmids per cell could then be calculated by extrapolation of this line to the point where there is 1 cell per PCR (i.e. log[no. cells/PCR], x = 0). However, the double logarithmic plot will magnify any small inaccuracy in the the gradient of the extrapolated stright line so that the intercept may not truly reflect the plasmid per cell value in the culture. For this reason, the mean plasmid per cell value was determined from the average of the number intracellular pQR701 molecules measured in samples whose cellular concentration was known.

5.2 Sampling microbial aerosols from within a 0.36m^3 Bassaire cabinet

The aim of this series of experiments was to determine the relationship between the number of cells sprayed as an aerosol into a fixed, contained volume and the number of cells captured and enumerated using the cyclone and QPCR method. Data of this kind can be used to develop models to predict collection efficiencies, defined as the proportion of aerosolised cells in a sampled area that are collected and enumerated, in areas where it is not possible to perform such a calibration. This type of experiment has previously been reported (Ferris *et al*, 1995), but in this case the detection and enumeration method was microscopic cell counting, hence the range of aerosol concentrations that could be detected was limited by the relatively poor limit of detection. Additionally, Mukoda *et al* (1994) have aerosolised *Legionella* cells within a contained cabinet and, after sampling using an AGI-30 impinger or membrane filter, have

detected cells by PCR. However, in this report true quantification was not achieved (the level of PCR product was used to estimate the number of starting target copies of a Legionella 5S rRNA gene and it was stated that there was no consistent relationship between the intensity of the bands and the estimated recovery concentrations) and there was no attempt to relate the number of cells collected with the number aerosolised. Now that a sensitive QPCR method is available, these experiments have been extended to allow measurement of low concentrations of aerosols within the cabinet and to determine whether there is a good correlation between cells released and cells collected over several orders of magnitude. It should be noted that at the lower end of the concentration range, the cell suspensions used for producing aerosols in the cabinet were very dilute and in no way representative of a process stream. However, since any release from a process is likely to be highly diluted by the surrounding air, and that cyclone sampling may be well removed spatially and temporally from the point of release, then such low aerosol concentrations are thought to constitute a realistic air sample that might be encountered.

5.2.1 Experimental design

The basic principle of these experiments was that different concentrations of *E. coli* JM107 pQR701 cells were sprayed using an atomizer into a Bassaire cabinet of 0.36 m^3 volume and the aerosol formed was sampled by a cyclone which was attached to the side of the cabinet (Section 2.4.3). Since the volume of *E. coli* suspension that was aerosolised (constant at 18 mL) and the cell concentration were known, then it was possible to determine what proportion of the total cells were collected.

Two series of experiments were carried out, the first where cells were sprayed from nutrient broth and the second where cells were sprayed from TRS. The reason for this was that in some unit operations (e.g. fermentation or primary cell recovery by filtration or centrifugation) cells that are released would originate from a nutrient medium. In operations such as homogenisation the cell paste would most likely be resuspended in a buffer solution. Since it is has been shown (Marthi *et al*, 1990) that the medium from which an aerosol has been produced is influential on the properties of the aerosol, then both nutrient broth and TRS were investigated.

5.2.2 Cells sprayed from nutrient broth

Figure 5.1 shows the relationship between the number of cells sprayed into the 0.36 m^3 Bassaire cabinet and the number collected by the cyclone and enumerated by the QPCR method. The number of cells collected is determined by measuring the number of plasmid pQR701 molecules in a sample and then dividing that number by the number of

plasmids per cell as determined in the culture that was used for creating the aerosol (see Section 5.1).

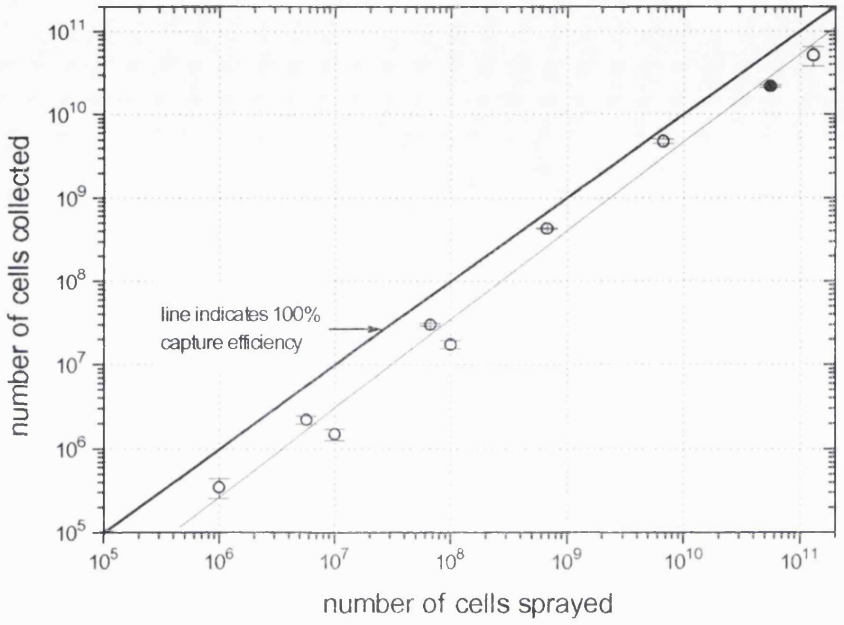


FIGURE 5.1 Relationship between cells aerosolised from nutrient broth into the Bassaire cabinet and cells collected and enumerated by QPCR. All data are average values of 2 determinations. Error bars represent the SEM. All data gained using 30 minute boiling lysis step. The single solid data point represents the data of Ferris *et al* (1995) (sprayed from TRS).

The figure shows that there is a strong correlation (gradient = 1.06, r = 0.999) between the number of cells sprayed and the number collected and enumerated. The linearity extends over 5 orders of magnitude. Experiments with lower concentrations of cells suspended in nutrient broth were not performed because the TRS that circulates within the cyclone gradually became discoloured as a significant quantity of nutrient broth accumulated from the aerosol. PCR was inhibited by the TRS/nutrient broth mixture, necessitating a 10 fold dilution of the sample.

The average recovery of sprayed cells in these experiments is 41% (± 7% SEM). Since the gradient is close to a value of 1, there does not appear to be any marked change in recovery as the bacterial concentration in the aerosol varies. In the earlier work of Ferris and co-workers (1995), it was found that for *S. cerevisiae*, the proportional recovery of cells from this cabinet fell from 64 % for 1×10^8 cells aerosolised to 38 % for 1×10^{10} cells aerosolised. This variation in collection efficiency might be related to a difference in

the aerosol particle distribution between the different concentrations of *S. cerevisiae* (for example, an aerosol created from a more concentrated suspension might be more likely to contain large clumps of cells which would quickly fall to the floor of the cabinet). Alternatively, the explanation might lie in the fact that the LOD of the enumeration method was being approached and so inaccuracy would be expected to be increased at the lower concentrations.

5.2.3 Cells sprayed from TRS.

The experimental protocol was similar to that used for spraying cells from nutrient broth with the exception that cells were diluted into sterile TRS before spraying. The range of concentrations sprayed was generally lower than that used in the nutrient broth experiments for two reasons: (i) it was hoped that by using lower concentrations it would be possible to determine the limit of detection of the technique in terms of the number of cells per litre of incoming air that can be detected; and (ii) the high concentrations used in the nutrient broth experiments caused great difficulty in cleaning out the cyclone and cabinet. The results of this series of experiments are shown in Figure 5.2.

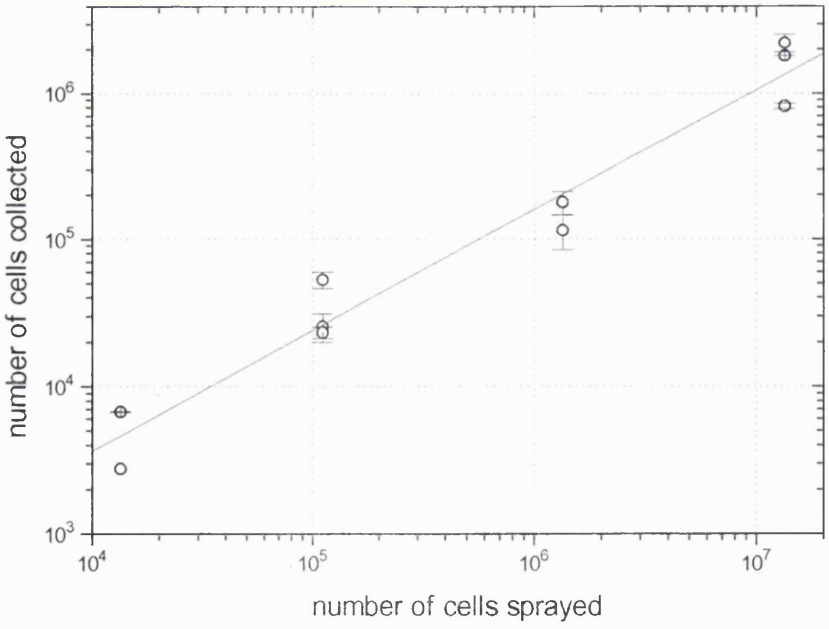


FIGURE 5.2 Relationship between cells aerosolised from TRS into the Bassaire cabinet and cells collected and enumerated by QPCR. All data are average values of 2 determinations. Error bars represent the SEM. All data gained using 30 minute boiling lysis step.

The relationship between cells sprayed and cells collected shows a less strong correlation (gradient = 0.82, r = 0.91) than when spraying cells at high concentration from nutrient broth. It is thought that there is a "levelling off" effect at lower concentrations as the number of cells detected approaches the limit of detection of the technique.

Since some 'background' samples (n = 9) showed a PCR signal (arising from an unknown source), then it is necessary to set an LOD above which any signal can be considered to be truly differentiated from the background (see Section 7.1.3 for further discussion). The LOD was therefore calculated as follows;

$$\begin{aligned} \text{LOD} &= \text{average background measured} + (3 \times \text{S.D.}) \\ &= 1.5 \times 10^4 + (3 \times 3.5 \times 10^3) \\ &= 2.6 \times 10^4 \text{ cells} \end{aligned}$$

In experiments where less than this number of cells are collected, the average recovery is 32.6% (± 6.7 SEM, n = 5) whereas above the LOD the average recovery is 11.2% (± 1.8 SEM, n = 5). The former, higher figure may arise from the increase in relative error that occurs when the measurement technique approaches its LOD. More specifically, it could be due to small amounts of DNA that carry over from one experiment to the next but which are insignificant when measuring higher levels.

5.2.4 Comparison of recoveries of cells sprayed from TRS and from nutrient broth.

Because the series of experiments carried out on cells sprayed from TRS and from nutrient broth did not use the same concentration range, the results are not directly comparable. However, there is some overlap of concentrations and the results from spraying from nutrient broth over a wide concentration range suggests that recovery is not markedly affected by concentration. The recovery seen with cells sprayed from TRS is lower than that seen with cells sprayed from nutrient broth (41 %). This is the case if all TRS data are included (average recovery = 22 %, difference significant at $p < 0.05$) or if those data falling below the LOD are excluded in which case the recovery is 11 % and the difference significant at $p < 0.005$.

There are two reasons why the recovery might be greater with cells sprayed from nutrient broth. The first is based on the observation that nutrient broth that accumulates in the recirculating liquid of the cyclone has the effect of improving the wetting properties of the scrubbing liquid. Hence, the inner body of the cyclone appears to be more efficiently rinsed, contributing to enhanced capture efficiency. This possibility could be investigated in future work (Section 7.1.4). The second reason lies in the different

properties of the aerosol generated in each case. In general, for aerosols generated from nutrient broth, the average particle size will be larger (Bennett and Norris, 1989; Hambleton *et al*, 1992; Ferris, 1995) than that for aerosols generated from TRS. Since the efficiency of capture of the cyclone increases with increasing particle size (Beeckmans and Kim, 1977; Iozia and Leith, 1990; Konig *et al*, 1991) then this might well explain the observed difference in collection efficiencies.

In these discussions, recoveries are calculated for cyclone sampling by determining the proportion of cells released into the cabinet that are collected by the cyclone. This does not yield any direct information about the capture efficiency of the cyclone (i.e. the proportion of cells entering the cyclone that are entrained into the circulating liquid), as it is not possible to say what proportion of the cells within the aerosol enter the cyclone rather than come to rest on the walls of the cabinet. A different experimental set up would be required to determine capture efficiency in this way (Beeckmans and Kim, 1977; Iozia and Leith, 1990; Konig *et al*, 1991; Thompson *et al*, 1994; Upton *et al*, 1994)

However, the experimental set up used here shows that there is a good correlation between the number of cells within an aerosol inside a fixed, contained volume and the number that are captured and enumerated. This is an important stage in the validation of the methodology and illustrates an important advance over similar work previously reported (Mukoda *et al*, 1994). Additionally, the recoveries calculated are useful in helping to build up a picture of the relationship between the volume of air into which an aerosol is released and the collection efficiency from it. In these experiments, recoveries of 11 - 40 % have been measured for an *E. coli* laden aerosol released into a 0.36 m^3 volume. Ferris *et al* (1995) have noted a similar recovery using the same cabinet, but when cells are released into an 8.37 m^3 volume soft-film cabinet, the recovery was found to be 17 %. These data are useful in predicting likely recoveries in areas in which it is not possible to perform such a calibration (for example, areas within Glaxo Wellcome Medicines Research Centre bioprocessing pilot plant, see Chapter 6) and for validating air flow models generated by Computational Fluid Dynamics (CFD). Such CFD models are being developed at UCL. Notably, in the case of the 0.36 m^3 Bassaire cabinet, CFD models predict the proportion of cells that would enter the cyclone as being in the region of 20 % (personal communication; P. Agutter, Dept. of Chemical and Biochemical Engineering, UCL). This would suggest that the capture efficiency of the cyclone operating under these conditions is indeed very high. Upton *et al* (1994) have shown that the capture efficiency of the cyclone for particles of greater than 2 μm diameter is close to 100 %.

5.3 Fermentation exit-gas sampling

Although several workers have measured particle concentration (Pilancinski *et al*, 1990; Szewczyk *et al*, 1992), little work has been reported on the measurement of concentrations of bacterial cells in fermenter headspace and exit-gas. Winkler (1987) has investigated the number of 'contaminated particles' in exit-gas, but since this report relied on measurement of culturable cells, and in the aerosol state the culturable portion of viable cells is very low (Neef *et al*, 1995), then it might be inferred that there was an underestimate of the number of viable cells present in the exit-gas. Ferris (1995) has shown that release of micro-organisms into fermentation exit-gas can be detected by use of an aerojet cyclone sampler. In these experiments, it was shown that *E. coli* cells are detected, but the quantity of cells was not known precisely since the enumeration method used (microscopic cell counting, Section 2.1.4.1) was not sensitive enough. However, a diagnostic (non-quantitative) PCR did show that target *E. coli* cells were present in the exit-gas.

In these experiments, the aim is to use the QPCR method and to apply it to exit-gas monitoring. In order to achieve this in a systematic way it is necessary to use an appropriate growth medium and to understand what is happening within the fermentation, in terms of pQR701 and cellular concentrations, whilst exit-gas release is being monitored.

5.3.1 Choice of medium

Since it was the aim of these experiments to monitor release of micro-organisms through the exit-gas throughout the course of a fermentation (i.e. from inoculation through to stationary phase), then a medium in which the fermentation would be complete within 12 hours was sought. The aim, therefore, was to set up the seed flask on the evening prior to fermentation, inoculate early in the morning and then sample the fermentation throughout the day.

Initially, the following defined medium, based on a recipe used at UCL for fed batch fermentations of *E. coli* K-12 strains (personal communication; Dr R. Sheridan, Dept. of Chemical and Biochemical Engineering, UCL) was tested (Table 5.2).

TABLE 5.2 Defined medium used for 2 L *E. coli* JM107 pQR701 fermentation

Medium component	Concentration (g L^{-1}, unless otherwise stated)
Yeast extract	1.0
NaCl	0.5
$Na_2HPO_4.12H_20$	15.12
K_2HPO_4	3.0
$(NH_4)_2SO_4$	7.4
Glycerol	10
$MgSO_4$ (100 mM)	10 mL
$CaCl_2$ (10 mM)	10 mL
FeIII/Citrate	0.66 mL
Kanamycin (20 mg mL^{-1})*	1.0 mL

*Added after autoclaving as filter sterilised solution.

However, using this medium growth of the culture was relatively slow. A 6 % inoculum (A_{600} of 2) reached stationary phase in the fermenter (A_{600} of 5) after between 12-15 hours. It was therefore decided to use the complex medium T-broth (Tartof and Hobbs, 1987) in an effort to speed up the growth and allow a higher cell density to be reached. In this case the culture in the fermenter reached an A_{600} of 15 within 10 hours. However, since the medium is so rich the growth rate was maintained at near maximum value for a prolonged period so that in order to keep the DOT above 10 % it was necessary to turn the stirrer up to well above 1000 rpm. This in turn meant that foaming was excessive and that large volumes of antifoam (up to 50 mL of 1 % PPG) had to be added.

The medium finally chosen was therefore a less rich version of T-broth (Section 2.1.2.3). Using this medium the growth curve was as shown in Figure 5.3. The maximum growth rate (μmax) here is 0.73 h^{-1}. This medium allowed the fermentation DOT level to be controlled at 20 % by variation of stirrer speed between 300 - 1100 rpm. The whole fermentation therefore took place within a day allowing sampling through the different growth phases.

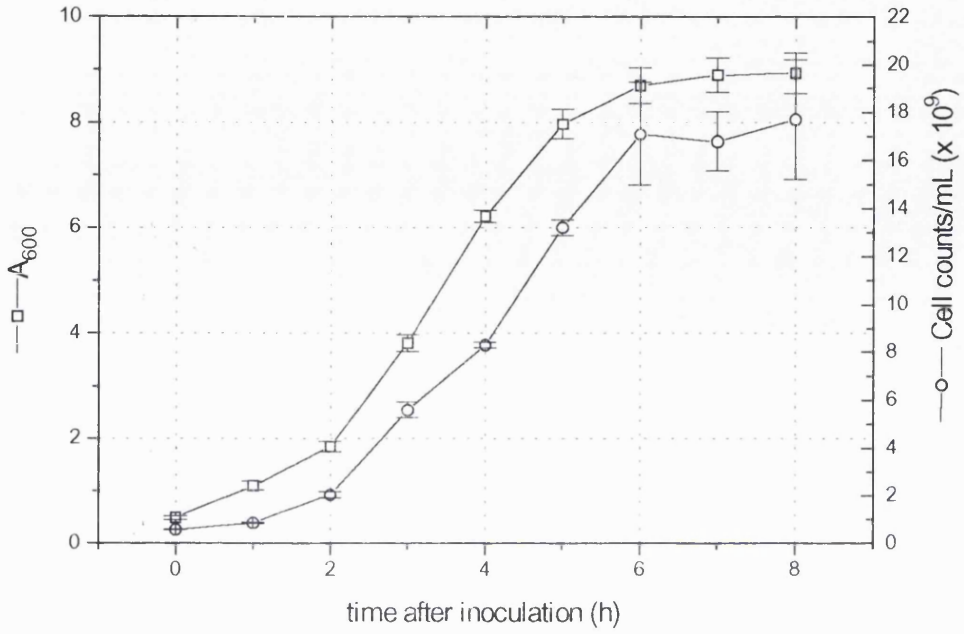


FIGURE 5.3 2 L Fermentation growth curve for *E. coli* JM107 pQR701 using modified T-broth. Points shown are the means obtained from 6 fermentations for A_{600} measurement, 3 fermentations for cell count measurements. Error bars represent the SEM. Note that Figure 5.7 shows the OUR and CER data obtained from a typical fermentation.

5.3.2 Measurement of pQR701 concentrations within the broth

In order to interpret the results of exit-gas sampling, it is necessary to have knowledge of the condition of the culture within the fermentation. Specifically, at any given sampling time, what proportion of the total pQR701 pool is extracellular and how many copies of pQR701 are there for each whole cell.

5.3.2.1 Total and extracellular pQR701 measurement

Total and extracellular pQR701 concentrations were determined by the method previously detailed (Section 2.2.8.2) from samples taken from the fermentation at 1 hour intervals. The samples were diluted by a factor of 10^4 in sterile TRS before pQR701 measurement by QPCR. The A_{600} and cell density of the same samples were determined immediately after they were taken. Three fermentations were carried out under the same conditions to build up a reliable picture of the variation in total and extracellular pQR701 within the medium throughout a fermentation. Figure 5.4 shows the average data obtained with the growth curve (in terms of cell density) superimposed.

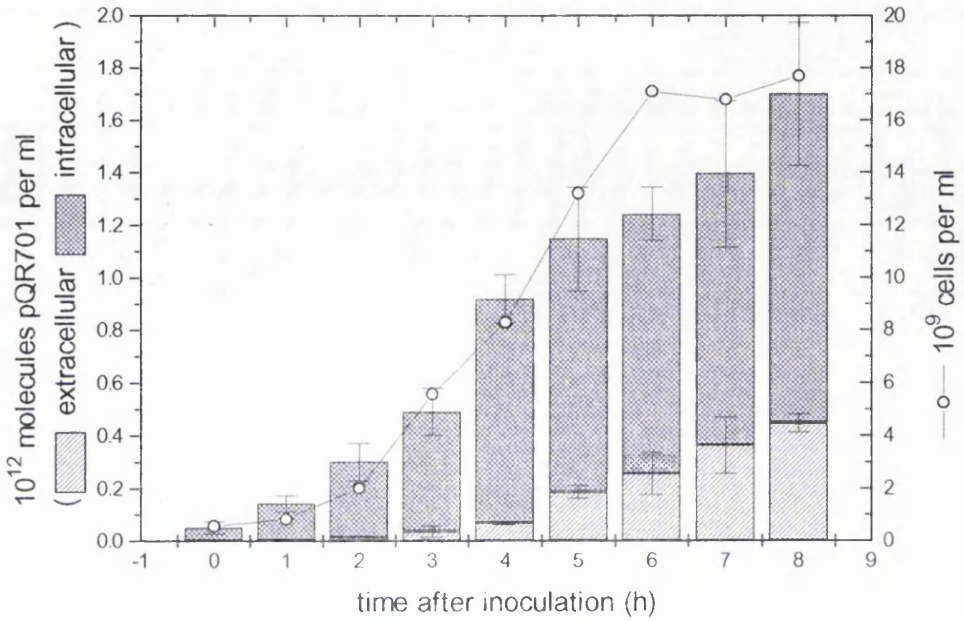


FIGURE 5.4 Total and extracellular pQR701 concentrations throughout *E. coli* JM107 pQR701 fermentation. Error bars on plasmid concentration determinations (taken from 3 fermentation runs) represent the SEM.

This figure shows that the pQR701 concentration in the broth increases in a manner similar to the cell density, and that both curves show a short lag, an exponential and a stationary phase, although this is less pronounced for the increase in pQR701 concentration. An increase in the concentration of pQR701 would be expected to mirror an increase in cell density, provided that the *E. coli* JM107 pQR701 culture remained uncontaminated by the presence of other strains not bearing the same plasmid and that the plasmid stability and copy number was fairly constant. Notably, plasmid stability of fermentation cultures was routinely found to be in excess of 90 %.

The proportion of total pQR701 that is extracellular increases as the fermentation proceeds. This might be expected towards the end of the fermentation due to a build up of material released from lysed cells. However, the proportion of plasmid that is extracellular is likely to be overestimated because lysis of whole cells was inefficient (samples were not subjected to a boiling-lysis pre-treatment, see Section 4.1). The average percentage values for extracellular plasmid pQR701 as a proportion of the total are given in Table 5.3. The figures are used to determine the number of plasmids per whole cell since extracellular pQR701 is not included in the calculation.

TABLE 5.3 Extracellular pQR701 as a percentage of total pQR701 throughout the fermentation

Time (h)	Extracellular pQR701 (%)
0	0
1	5
2	6
3	8
4	8
5	19
6	22
7	28
8	29

5.3.2.2 Variation in the mean plasmid per cell value during fermentation

The number of copies of plasmid per cell was determined at hourly intervals during 3 separate fermentations. Each fermentation was carried out under the same conditions. The mean values for each time point are plotted in Figure 5.5, with the growth curve superimposed.

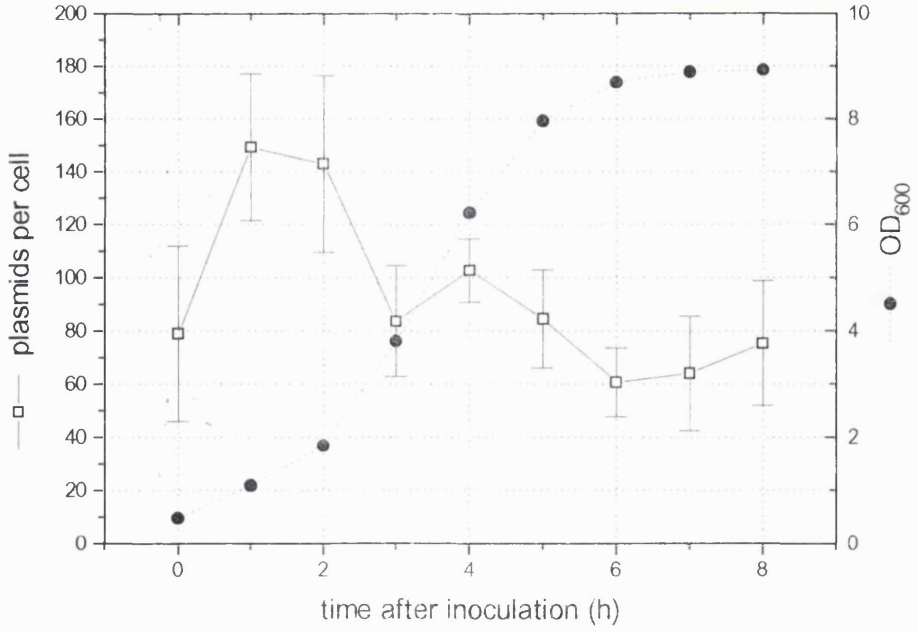


FIGURE 5.5 Variation of the number of plasmids per cell throughout *E. coli* JM107 pQR701 fermentation. Error bars on plasmid per cell determinations (taken from 3 fermentation runs) represent the SEM.

It is notable that the error bars in these measurements are larger than those expected from the QPCR measurement. This is because, in addition to QPCR imprecision, the data is derived from 3 separate fermentations which, although growth conditions remain the same, inevitably show some variation. Since data are determined at pre-set time intervals, rather than at pre-set values of A_{600}, variations in the rate of growth will be incorporated into the plasmid per cell measurement error. This was thought to be the most practical method of defining plasmid per cell variation since monitoring of release of micro-organisms into the exit-gas was carried out at pre-set time points and not at pre-set A_{600} values. The plasmid per cell trend therefore reflects the variation with time after inoculation, and not directly with the growth rate or cell biomass of the culture. However, Figure 5.3 demonstrates that cell biomass does increase predictably with time, hence some inferences can be drawn about the relationship between plasmid per cell value and growth phase.

Figure 5.5 shows the following trend in the number of plasmids per cell throughout fermentation: i) a sharp rise immediately after inoculation to a maximum value of approximately 140 plasmids per cell; ii) the maximum value is seen during the lag phase; iii) as exponential phase begins the number of plasmids per cell falls sharply from its lag phase value; and iv) a fairly steady state throughout the late exponential and stationary phases.

The following hypothesis is put forward for the variation in the number of plasmids per cell throughout the fermentation. The inoculum is a stationary phase culture (12-14 hours old) and when it is introduced into the fresh fermentation medium the increase in nutrient availability allows the cells to grow in preparation for division. This increase in cell size is mirrored by an increase in the number of plasmids per cell which may occur due to the increase in cell volume which would dilute any negative regulatory elements in the cell (Thomas, 1988). At the onset of exponential phase, the number of plasmids per cell falls as the rate of cell division outstrips the rate of synthesis of plasmid DNA (segregational instability). During exponential phase, a steady state is reached and at stationary phase, where cell division and DNA synthesis are much reduced, the number of plasmids per cell stabilises. Interestingly, the plasmid per cell value during stationary phase is similar to that of the inoculum. The inoculum is also a stationary phase culture, but since it is grown in a shake flask, where aeration is very different to that in a fermenter, it can not be assumed that the plasmid per cell trend would be similar.

The mean number of plasmids per cell were used to convert the number of pQR701 molecules captured from the exit-gas and measured in the cyclone into the number of

whole cells released. The plasmids per cell values used, and the exit-gas sampling times, are shown below in Table 5.4.

TABLE 5.4 Plasmid per cell values and exit gas sampling times

Sampling time (hours after inoculation)	Plasmids per cell
1.5	146
3.0	84
5.0	84
7.0	64

5.3.3 Measurement of pQR701 released into unfiltered fermenter exit-gas

In Section 2.5.2., it is detailed how for each exit-gas cyclone sample there is an additional background sample taken (before connection to the exit-gas line) and that on some occasions an air sample is taken where there is no connection to the exit-gas line in order to provide information about the nature of any background pQR701 levels that might be detected. In the first experiments on exit-gas sampling, a significant background level was always seen within the cyclone and this background generally decreased throughout the day as the fermentation proceeded. Figure 5.6 shows the results of background sampling during one fermentation.

In this figure, some samples (1, 4 and 7) are shown where after the 10 minute background measurement, the cyclone was left unconnected to the exit-gas line, and hence it was sampling only room air for the subsequent 15 minute period. In these cases comparison of the pQR701 concentration measured after 10 and 25 minutes of sampling of the room air shows that there is little, if any, increase after the second time period. This infers that any background measured in the cyclone originates from contamination of the cyclone itself, rather than from contaminated room air. If the latter were the case, then the background level would be expected to increase significantly between 10 and 25 minutes of air sampling. The theory of background contamination arising from within the cyclone itself is supported by the (generally) decreasing background levels as the fermentation proceeds. If the fermenter was releasing cells into the surrounding environment, then one expect background levels to increase as time passes.

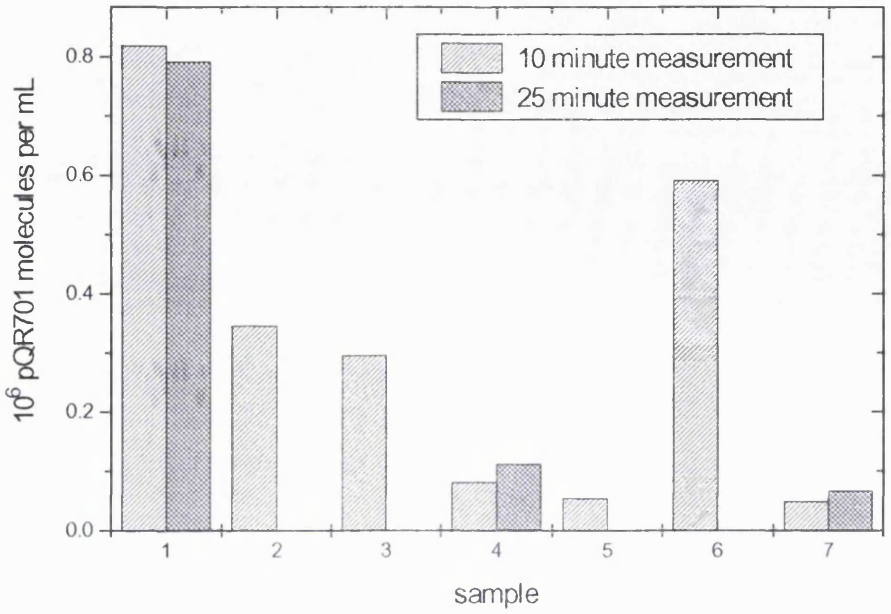


FIGURE 5.6 Cyclone sample background measurements in the fermenter vicinity. Note: sample 1 taken at 0.67 hours; 2 at 1.5 h; 3 at 3 h; 4 at 4 h; 5 at 5 h; 6 at 7 h; and 7 at 8h. Full (25 minute) background samples were taken only for samples 1, 4 and 7.

However, the cleaning efficiency of the cyclone has been shown to be acceptable (Section 4.4), especially at these relatively low levels of pQR701 concentration. In this case, it was found that the contamination problem was arising from the sink in which the cyclone was being cleaned. This sink was also used to dispose of autoclaved cell cultures, including *E. coli* JM107 pQR701 cultures. Since autoclaving does not totally degrade DNA (Masters *et al*, 1994), it is thought that pQR701 DNA fragments were present in the sink and during cleaning it was inevitable that some of this DNA found its way into the cyclone. The procedure was therefore changed and the cyclone was washed in a different sink in another room where *E. coli* JM107 pQR701 cells had never been handled. Subsequently, no background contamination was ever found in the cyclone (or originating from the room air). This illustrates the care which must be taken when using a detection method which is as sensitive as PCR.

The exit gas from the 2 L fermenter was measured downstream of a condenser at pre-determined sampling times for a series of fermentations (see Section 2.5.2, Figure 2.7). The sampling times are shown below in relation to the fermentation growth characteristics (Figure 5.7).

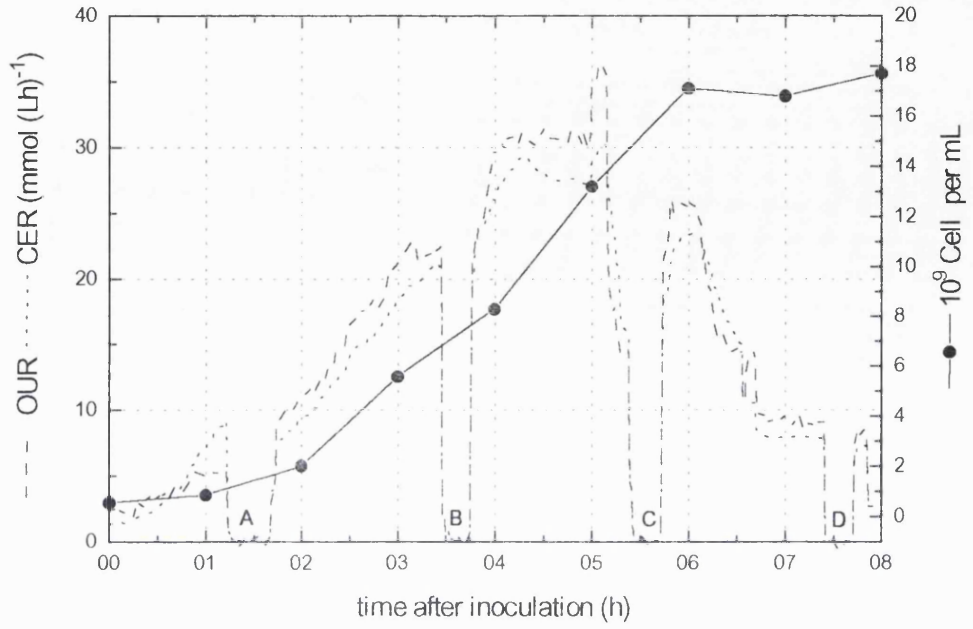


FIGURE 5.7 2 L fermentation of *E. coli* JM107 pQR701. Growth characteristics and exit gas sampling times. Note that the sudden falls in OUR and CER occur when exit gas samples are taken (at times marked A - D) as the exit gas is not analysed by mass spectrometry at this time.

Sampling at approximately 1.5, 3, 5 and 7 hours after inoculation allows the release of micro-organisms into the exit gas to be monitored over most of the time course of the fermentation. In addition, there is a sampling point in each of the growth phases of the culture: lag (1.5 hour sample); exponential (3 hour sample); and stationary (7 hours). This will enable the determination of release patterns and their relationship if any, to the growth phase of the culture. Other parameters to which release rates might be related include the sparge rate, stirrer speed and the extent of foaming (Pilancinski *et al*, 1990). In these experiments, sparge rate was held constant at 1.5 L min^{-1} (1 v.v.m), but stirrer speed was variable between 300 - 1100 rpm. The stirrer speed was automatically varied to maintain a DOT of 20 % in the culture, unfortunately there was no facility for monitoring the change in stirrer speed throughout the fermentation. However, once exponential phase was reached, it was observed that stirrer speed was always within the range 800 - 1000 rpm. The amount of foaming was observed and noted throughout the fermentations.

Samples obtained from the cyclone were stored overnight at 4°C and were assayed by QPCR within 24 hours. For each exit-gas sample, the extracellular pQR701 concentration was measured (Section 2.2.8.2) and 3 replicate total plasmid concentration

measurements were made. The means of the 3 values obtained were calculated. Extracellular pQR701 concentration in all cases represented a low proportion (<5 %) of the total plasmid concentration measured in the cyclone samples. Room air background samples were taken throughout the course of a fermentation and, in the experiments whose data is collated below, no background was seen. An example of a calculation of the number of cells released at a given point into the exit-gas is shown below.

TABLE 5.5 Example calculation of number of *E. coli* JM107 pQR701 cells released into exit-gas

Sample time (h)	Total [pQR701] (molecules mL^{-1})	Extracellular [pQR701] (molecules mL^{-1})	Plasmids per cell	Sample volume (mL)	Total cells released	Cell release rate* (min^{-1})
(a)	(b)	(c)	(d)	(e)	(f)	(g)
3	2.3×10^6	2.9×10^4	84	55.6	1.2×10^6	1.0×10^5
	(measured)	(measured)	(measured)	(measured)	((b-c)/d) × e	f/15

* Note assumption that cells released at constant rate over the 15 minute sampling period.

Four time points were measured for 3 different fermentations run under the same conditions. The average number of cells released at each time point is shown in Figure 5.8.

In general, there is an increase in the rate of release of cells into the exit-gas as the fermentation proceeds. However, there is no simple relationship between cell density in the fermentation broth and the number of cells released into the exit-gas. This can be seen more clearly if the release rate is plotted against cell density (Figure 5.9).

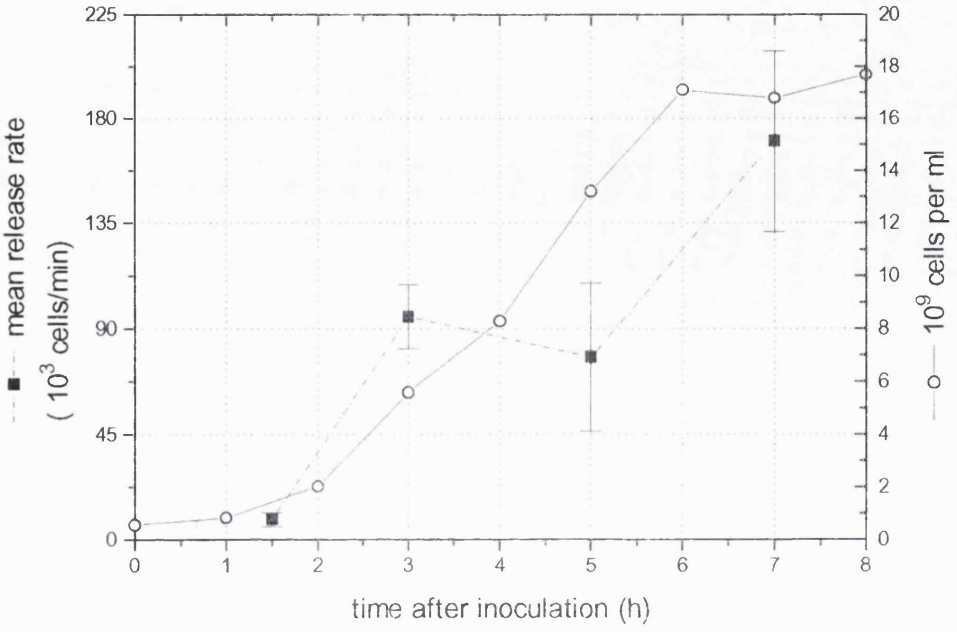


FIGURE 5.8 Release of *E. coli* JM107 pQR701 cells into unfiltered exit-gas throughout fermentation. Error bars on mean release rate determinations (taken from 3 fermentation runs) represent the SEM. Integration of the curve describing the mean release rate between 1.5 and 7 hours after inoculation allows estimation of the total (cumulative) number of cells released into the exit gas during this period. This value is 3.0×10^7 cells.

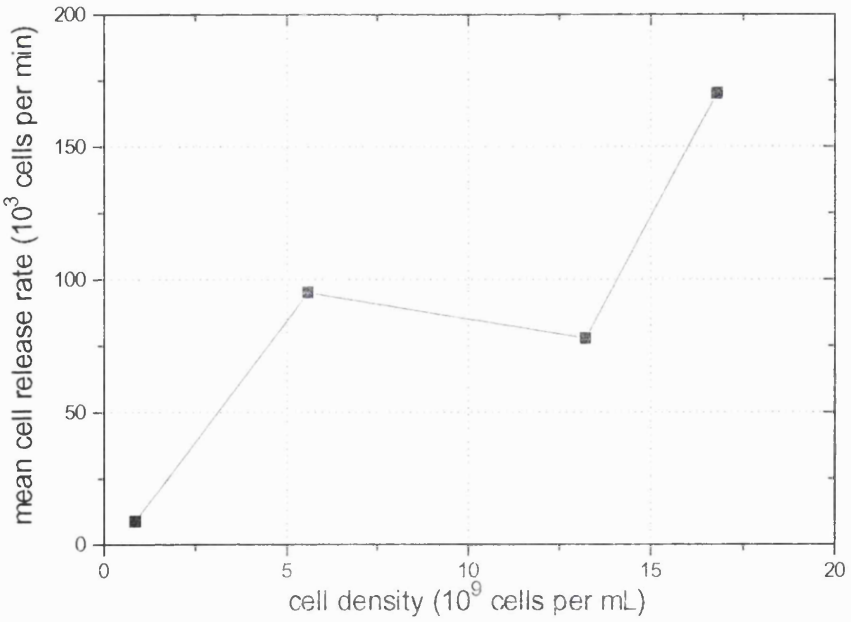


FIGURE 5.9 Exit-gas cell release rate as a function of cell density

For the first two sampling points, at 1.5 and 3 hours, the release rate correlates reasonably well with the cell density in the broth. However, at the third sampling point, this relationship breaks down (overall the correlation is fairly poor, r = 0.849).

There are a series of factors that might be implicated in the in the lack of a simple relationship between cell density and release rate. Pilancinski and co-workers (1990) have shown that aerosol formation from a fermentation broth is influenced by several factors such as air flow rate, agitation rate and the rheological properties of the liquid. Notably, the fraction of particles large enough to potentially carry micro-organisms was found to increase with agitation rate and air flow rate. This work was extended by Szewczyk *et al* (1992) who also looked at aerosol generation in an industrial pilot scale fermenter but, unlike the Pilancinski work, studied the effects of growth of the micro-organisms (an *E. coli* K-12 strain) on the change in aerosol properties. It was found that aerosol particle concentration decreased significantly with increasing cell density and that the change in particle concentration was more pronounced in the size range above 2 μm. Huang *et al* (1994) attempted to monitor aerosol generation and properties as a method for on-line biomass monitoring of an *E. coli* fermentation using T-broth as the growth medium. Since there were relatively few particles of greater than 1 μm diameter detected, the aerosol number concentrations in the size range 0.1 -1.0 μm diameter was measured and compared with the bacterial growth rate and the rheological properties of the broth. It was found that effluent aerosol number concentration increased during the growth phase and subsequently decreased after bacterial cell concentration had reached a stable level. Metabolic changes in the composition of the fermentation medium subsequently caused the surface tension to decrease, leading to an increased foaming tendency which, in turn, caused greater aerosol release.

An additional factor is that, as cell density increases, it is more likely that clumping of cells will occur in the broth. Larger clumps of cells may not be lifted into an aerosol due to their size not being compatible with the particle size distribution of the aerosol formed or due to the increase in settling velocity (Ferris, 1995). However, the final sampling point shows a significant increase in the release rate. Presumably, clumping would be at least as likely at this stage (7 hours after inoculation). Observation of the fermentation shows that foaming begins to occur at 5 - 6 hours after inoculation, at the end of the exponential growth phase. This means that at the final sampling point there is significant foaming, which is not the case after 5 hours (the third sampling point). It is thought that the presence of foam might increase the rate of release of cells, since the formation of aerosols is enhanced under these conditions (Pilancinski *et al*, 1990; Huang *et al*, 1994). Huang *et al* (1994) used a mechanical foam breaker to control the level of foaming in

their fermentations, in this study foaming level in the fermentations is controlled by the addition of antifoam. This would clearly have an effect on the surface tension and might explain why the increase in aerosol release due to foaming was delayed for some time after the maximum growth rate.

The number of cells released from the fermentation can be expressed in terms of the volume of exit gas sampled, which was flowing at a rate of 1.5×10^{-3} m^3 min^{-1} (Table 5.6).

TABLE 5.6 Cell release rates expressed in terms of exit gas volume

Sampling point (hours after inoc.)	Release rate	
	Cells min^{-1}	Cells m^{-3} exit gas[a]
1.5	8.7×10^3	5.8×10^6
3	9.5×10^4	6.3×10^7
5	7.8×10^4	5.2×10^7
7	1.7×10^5	1.1×10^8

[a] Exit gas flow rate 1.5×10^{-3} m^3 min^{-1}

Expression of the rate of release in terms of the exit gas volume allows extrapolation of results to larger scale fermenters and the comparison of results with other workers who have used different methods to measure cell numbers in fermenter head space. Winkler (1987) has reported that in the fermenter head space there are about 10^6 contaminated particles per m^3 gas. Since each 'contaminated particle' contains at least one viable cell then this describes only the minimum number of culturable cells present. Additionally, it is well known that a large proportion of viable cells are not culturable (Colwell *et al*, 1985) and that in the aerosolised state the culturable portion is likely to be less than 1 % (Neef *et al*, 1995).

The detection method used here is PCR which detects both viable and non-viable cells (Masters *et al*, 1994) and so comparison of levels of contamination with those from Winkler's findings (1987) are not straightforward. However, one might calculate that the total number of cells, as opposed to 'contaminated particles', in the head space of the fermenter as measured by Winkler (1987) is in excess of 10^8 m^{-3}. There would therefore be a degree of correlation with the results from these studies.

Other studies on aerosols produced by fermentation have concentrated on aerosol particle characteristics rather than cellular concentration in the aerosol. For instance, Pilancinski *et al* (1990) measured over 10^8 particles m^{-3} of which 30 - 40 % exceeded

2 μm in diameter above a complex broth stirred at 130 rpm. Szewczyk and co-workers (1992) measured the particle concentration at approximately 15 cm above the fermentation liquid and found that the level decreases from greater than 6×10^8 particles m^{-3} at inoculation to 2.5×10^8 particles m^{-3} after microbial growth (using a stirrer speed of 450 rpm). Again, it is not possible to compare figures as there is no direct correlation between the particle concentration in the aerosol and the number of whole cells which it contains, since the latter depends on the particle size range of the aerosol and the fermentation conditions.

The total number of cells released throughout the fermentation (between 1.5 and 7 hours) can be calculated by integration of the curve in Figure 5.8. This gives a value of 3.0×10^7 cells. Comparison of this figure with the total number of cells in the broth, 2.7×10^{13} cells at stationary phase (1.5 L volume), reveals that the number of cells released is a very small proportion of the total. Indeed the total number of cells released into the exit-gas is the same as that contained within 1.7 μL of the final broth. A final point to note is that in experiments where exit-gas was sampled downstream of the exit gas filter, no cells were detected, indicating that in normal operation, the exit-gas is adequately treated. The implications for current practice with regard to exit gas containment for fermentations involving genetically modified micro-organisms is discussed in Section 7.3.

5.4 Measuring the release of process organisms from a high pressure homogenisation operation

Homogenisation of cell suspensions is a key operation in the downstream processing of products that are located within the cell of the production organism (Middleberg, 1995). Since *E. coli* and *S. cerevisiae* are currently the hosts of choice for many first generation large-scale processes and these organisms generally do not excrete proteins into the medium, there is often a necessity to disrupt the production cell to release the product. There are several methods that can be used to homogenise bacterial cells at large scale (Middleberg, 1995). One widely used mechanical disruption technique is high pressure homogenisation where cells are efficiently disrupted by being subjected to high pressures within a cell disruption (CD) valve. Disruption of cells occurs as the suspension is pumped by positive displacement through a valve seat and then radially across the valve face so that it strikes an impact ring surrounding the valve. The mechanism of disruption may rely on the rapid transfer of sample from a region of high pressure to a region of low pressure or be a result of the impact of the cells onto the impact ring (Foster, 1992). Whatever the mechanism, it is apparent that a significant amount of heat is generated during homogenisation (Foster, 1995).

The APV 30CD is a moderately high throughput homogeniser (up to 120 L h^{-1}) that has been adapted for cell disruption from use in the dairy industry. Pandolfe (1989) has described several features which were specifically incorporated into this homogeniser to meet the needs of the biotechnology industry. However, Foster (1995) has noted that the containment afforded by homogenisers derived from non-biotech industries is not absolute but can be enhanced by the provision of secondary containment, such as the use of a flexible isolator. Clearly, subjecting concentrated process streams to high pressures (up to 100 MPa) has the potential to cause organism bearing aerosols that may be hazardous to the operator or environment (Foster, 1992).

The 30CD homogeniser in the ACBE at UCL is housed within a flexible soft-film cabinet (Section 2.5.1). Earlier work (Ferris *et al*, 1995) using process streams of *S. cerevisiae* and *E. coli* suspensions have shown that organisms can not be detected within the cabinet during homogeniser operation. In this report, air was sampled using a cyclone and the enumeration method used was microscopic cell counting. However, it was found that there was release of the process organism into the lubrication fluid of the homogeniser.

In these experiments, the homogeniser has been operated using a process stream consisting of 10 % (w/v) *E. coli* JM107 pQR701 cell paste in sodium phosphate buffer, pH 7.0. The air within the soft-film cabinet surrounding the homogeniser has been sampled using the cyclone. Liquid samples of the lubrication fluid, unhomogenised cell suspension and homogenate were also taken (see Section 2.5.1 for experimental details). All samples were subjected to QPCR and the results are summarised in the following tables and figures.

In order to express results in terms of numbers of cells as opposed to plasmid numbers, it is first necessary to determine the average number of copies per cell within the unhomogenised cell suspension (Section 5.1). In this case it was found that the number of pQR701 plasmids per mL of process stream was 3.5×10^{12} and that there were 4.3×10^{10} cells mL^{-1}, giving a plasmid per cell average of 80 (in these experiments samples were not subjected to a boiling lysis step). Notably, the proportion of total plasmid concentration that was found to be extracellular in the unhomogenised sample was 3 %. For the homogenate the proportion of extracellular plasmid was measured at 153 %. It is likely that this figure reflects some inefficiency in lysis of whole cells in the PCR compared to the cell lysis that is achieved by the homogeniser.

5.4.1 Monitoring of air within the cabinet during homogenisation

Table 5.7 shows the number of *E. coli* JM107 pQR701 cells enumerated in samples taken from the cyclone.

TABLE 5.7 *E. coli* JM107 pQR701 cells enumerated in cyclone samples from soft-film cabinet during operation

Sample	Number of cells measured in cyclone sample*	Equivalent process fluid volume (mL)$^{+}$
1. Background: after cell suspension loaded into hopper	$8.5 \pm 0.3 \times 10^{5}$	2.0×10^{-5}
2. Homogeniser run at 113 L h^{-1}, no pressure applied	$5.4 \pm 3.2 \times 10^{5}$	1.2×10^{-5}
3. Homogeniser run at 113 L h^{-1}, 45 MPa pressure applied, first 30 min.	$4.5 \pm 1.8 \times 10^{6}$	1.0×10^{-4}
4. Homogeniser run at 113 L h^{-1}, 45 MPa pressure applied, second 30 min.	$6.9 \pm 2.8 \times 10^{5}$	1.6×10^{-5}

Results expressed as total number of cells in cyclone sample ± SEM (n = 2). This is calculated by multiplying the number of cells detected in a PCR reaction (10 µL) by [cyclone sample volume (in mL) × 100]. Results shown are corrected for extracellular pQR701 concentration in the cyclone. $^{+}$ Calculated by dividing the number of cells detected into the cell density (cells mL^{-1}) of unhomogenised cell suspension.

The results indicate that process cells are detected in all cyclone air samples, including the background taken before the homogeniser was operated. This is not a totally surprising result if one considers the equivalent process volume and compares this to the 5 L of unhomogenised cell suspension that was pumped into the hopper 30 minutes before the first, background, sample was taken. Although all efforts were made to minimize any aerosol generation, it is still possible that this might have occurred. Alternatively, the background may have arisen due to the fact that the cell suspension was loaded into the hopper and the cyclone was operated and washed out by the same person (myself). Disposable gloves were used and were changed between operation, but is possible that such a low level of contamination can be transmitted by clothing or other means (Collins, 1992). Ideally, the air sampling should be handled by a different person than the process operator. Issues of this type are raised in the discussion chapter (Section 7.1.2.1).

The number of cells detected in all samples is consistent with the background level except for sample 3, the first sample taken during high pressure homogenisation. This is perhaps the time when release of whole cells is most likely since not only is high pressure applied, but a significant proportion of the cells would be intact in these early stages. After 30 minutes of homogenisation, the process stream would be subject to around 10 passes, which is normally sufficient for effective lysis of *E. coli* cells (Harrison, 1991; Foster, 1995).

Previous work has shown that the efficiency of collection of airborne cells within the soft-film cabinet is 17 % for high concentrations of *E. coli* cells (Ferris *et al*, 1995). If this collection efficiency were taken into account, it would imply that the total number of cells released by the homogeniser into the air is 2.1×10^7 (or 2.6×10^7 if the background level is not subtracted) during the first 30 minutes of homogenisation. Either way these levels are fairly insignificant in terms of the total volume of the process stream and tally with earlier findings that less than 5×10^7 *E. coli* cells are released under these conditions of homogenisation (Ferris *et al*, 1995). Notably, Kastelein and co-workers (1992) have found that when operating a 30CD homogeniser, airborne particle emission increases with time and operating pressure. This group also noted that when the primary packing in the piston assembly failed, there was an increase in emission together with contamination of the lubricating water. From this account, it is not possible to determine the scale of the emissions detected or whether such emissions consisted of viable process cells.

Experiments with a different type of high pressure homogeniser, a Constant Systems disrupter, has shown a similarly low level of detectable release (Bennett, 1991). In this instance, 10^{12} *Bacillus subtilis* spores were processed by the disrupter at 170 - 306 MPa and only a 'background count' of 5 CFU per test was detected.

5.4.2 Monitoring lubrication liquid during homogenisation

The results of sampling from the 5 L of recirculating lubrication liquid after each operation are shown below in Figure 5.10.

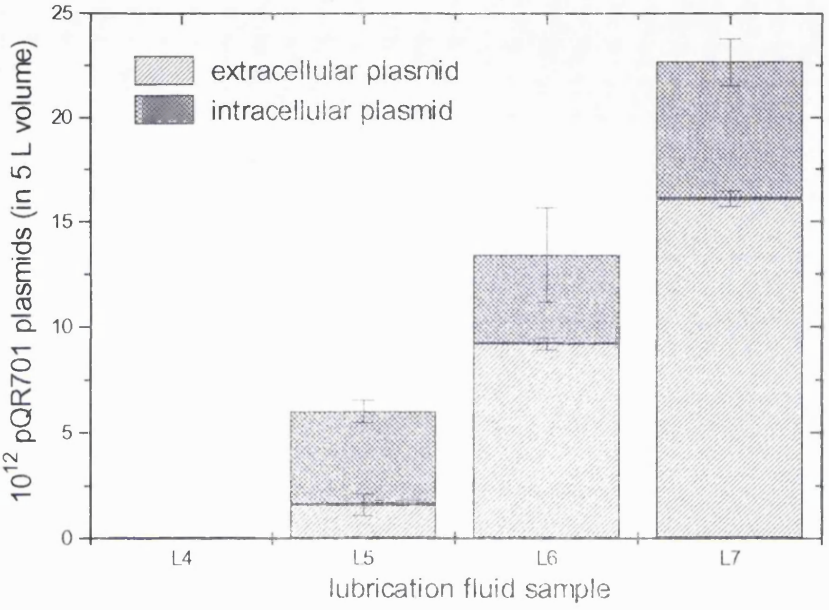


FIGURE 5.10 Plasmid levels found in homogeniser lubrication liquid after homogeniser operations. Figures shown are cumulative and are calculated for the whole 5 L of lubrication liquid, error bars represent the SEM (n = 2). Error bars are greater for the intracellular pQR701 concentrations since these values are derived from subtraction of extracellular from total plasmid concentrations. Samples were taken after the following operations: L4; cell susupension loaded into hopper: L5; homogeniser run for 30 minutes at 113 L h^{-1}, no pressure applied: L6; homogeniser run for 30 minutes at 113 L h^{-1}, 45 MPa applied: L7; homogeniser run for further 30 minutes at 113 L h^{-1}, 45 Mpa applied.

The figure shows a fairly steady rise in total pQR701 concentration after each homogeniser operation. The concentration in sample L4 was measured at 3.9×10^8 cells of *E. coli* JM107 pQR701 in 5 L, or 7.8×10^4 cells mL^{-1} (extracellular plasmid level was negligible in this sample). This 'background' level is most likely due to the fact that the homogeniser had previously been used to disrupt *E. coli* JM107 QR701 cells and that despite a full CIP routine, cells remained in the lubrication line from the previous experiments.

Since samples L5, L6 and L7 are each taken after 30 minutes of homogeniser operation, the x-axis of Figure 5.10 can be thought of as being proportional to elapsed time since the process flow was begun in the homogeniser. Consequently, the rise in the level of

plasmid within the lubrication fluid can be seen to increase as a function of the time for which the homogeniser is operated. This is noteworthy as sample L5 was taken after 30 minutes at 113 L h^{-1} with no pressure applied, whilst the subsequent samples were taken after the application of 45 MPa pressure in the CD valve. It would seem that the application of pressure does not increase the rate of leakage of process stream into the lubrication liquid. This is consistent with the leakage being caused simply by the lubrication liquid contacting the piston that, in turn, comes into contact with the process stream. This would imply that there is no containment failure, which might be exacerbated by the application of pressure, but that this constant level of release is an incidental feature of operation of the homogeniser. The rate of leakage of process liquid into the lubrication liquid can be calculated at 73 μL min^{-1}.

The changing ratio of intra- to extracellular plasmid measured in the lubrication liquid most likely reflects the extent of homogenisation of the cell suspension. Values shown in Figure 5.10 are cumulative, so the presence of whole cells in sample L7, after 1 h homogenisation at 45 MPa, can be largely put down to the release of whole cells in the pre cell disruption sample, L5. However, it is interesting to note that not only extracellular plasmid, but whole cells can pass into the lubrication line. This has also been seen by Ferris *et al* with *E. coli* cells but not with larger *S. cerevisiae* cells (Ferris *et al*, 1995). In that report, the concentration of *E. coli* cells in the lubrication fluid was 0.015 % of that in the process stream, whilst here it is between 0.025 - 0.038 %. This decrease in containment efficiency could be related to (partial) failure of a seal, although it should be noted that the homogeniser was deemed to be working satisfactorily by an experienced operator during pre-operational tests.

There is acknowledgement on the part of the manufacturers of this homogeniser, APV, that release into the lubrication line is likely since the pistons move back and forth through the seals. In more recent designs, this has been addressed by allowing a tightening of the stuffing box into which the pistons seat (Ferris *et al*, 1995). For the design tested, it is recommended that the lubrication line itself is contained and that the cooling water supply to the homogeniser is dedicated to this instrument (Kastelein *et al*, 1992). Clearly, these additional containment precautions are necessary, judging from the results obtained here. It is interesting to note that whilst the equivalent of 6.6 mL of process fluid is released into the lubrication liquid over the entire 90 minutes of homogeniser operation, a release of only the equivalent of 0.6 μL of process fluid can be detected into the air surrounding the homogeniser.

6. VALIDATION OF CONTAINMENT IN A BIOPROCESS PILOT PLANT: A CASE STUDY AT GLAXO WELLCOME MEDICINES RESEARCH CENTRE

In the commissioning of a new bioprocess pilot plant, one of the key performance criteria is the effectiveness of its containment. This is of particular importance where the hazards associated with the product or the production organism require the use of higher containment levels.

It has been noted (Section 1.2.3) that increasing the complexity of bioprocess design does not necessarily provide an enhanced containment effectiveness. So, in design of a new plant whilst it is useful to draw on existing good practice, it is also helpful to be able to validate the appropriate level of containment. One method of attempting this is to use a tracer gas such as sulphur hexafluoride (SF_6) or helium (Hesselink *et al*, 1990; Bowes *et al*, 1993; Tsou *et al*, 1993). Tracer gas testing is carried out by filling the vessel to be tested with the gas and then detecting any leakage to the environment using a sensitive detector. However, this method is problematic in that, since SF_6 and He are gases of low molecular mass, they can escape from the process in situations where micro-organisms would be adequately contained (Hesselink *et al*, 1990). For example, a filtration cassette with silicon rubber seals at Glaxo Wellcome Medicines Research Centre (GWMRC) has consistently failed SF_6 tests because the gas can pass into and through the silicon rubber (personal communication; John Piercey, Glaxo Wellcome). An additional problem in this instance is that the silicon rubber can absorb large quantities of SF_6 which is emitted slowly over a long period of time.

It is preferable then to attempt to validate new plant using a test that more closely monitors the effectiveness of containment of micro-organisms. Therefore it was decided to carry out a series of unit operations at GWMRC bioprocess pilot plant using *E. coli* JM107 pQR701 as the process organism and to monitor release using the cyclone air sampling-QPCR methodology. The aim of this case study at GWMRC was to assess the air sampling- QPCR technology within a newly commissioned, high containment specification bioprocess pilot plant. It would also enable Glaxo Wellcome to measure the level of containment in a series of unit operations.

The following plan of work was carried out:

- The target strain, *E. coli* JM107 pQR701, was deposited at the GWMRC culture collection.
- *E. coli* JM107 pQR701 was grown in the fermentation pilot plant, which provides complete primary containment, at 500 L scale.

- Cell mass was harvested using a Sharples tubular bowl centrifuge. Secondary containment is provided by the room housing the centrifuge and the safety cabinet in which cell paste is removed from the bowl of the centrifuge.
- Cell paste was stored at - 20°C, and a portion was subsequently homogenised using a Dyno-Mill bead mill. The Dyno-Mill is used within a flexible isolator which provides secondary containment.

At each unit operation (fermentation, centrifugation, homogenisation) a series of air samples was taken using the cyclone. These samples were analysed by QPCR. In addition, a number of measurements of the air movements were made to provide data for CFD modelling.

6.1 Sampling regime

The three unit operations carried out at GWMRC with the target micro-organism *E. coli* JM107 pQR701 and the sampling regimes are summarised below.

6.1.1 Fermentation.

The culture was grown at 37°C on modified T-broth (Section 2.1.2.3) supplemented with 20 mg L^{-1} kanamycin. The pH was controlled at 7.0 by addition of 0.5 M NaOH and DOT was maintained at above 20 % by variation of the stirrer speed between 300 - 1000 rpm. PPG antifoam was added to the broth to control the extent of foaming.

The seed culture was inoculated into two 10 L fermenters and the contents were subsequently transferred to 50 L then 500 L scale. Air sampling was carried out in the vicinity of the 10 L fermenter before and after inoculation. The contents of only one 50 L fermenter were transferred to the 500 L scale which was eventually harvested. The remaining 50 L fermenter was used to test the contained sampling procedure, before and after steam sterilisation, and the air around this vessel was sampled at appropriate times. For air sampling, the cyclone inlet was positioned in the open room within 2 m of the sampling port at 1.1 m above ground level.

6.1.2 Tubular bowl centrifugation

The 500 L fermenter was harvested using a Sharples AS 16 V:B tubular bowl centrifuge (Alfa-Laval, Figure 6.1) operating at 17,000 rpm (15,000 × g). The entire volume of the fermenter was harvested in two batches yielding a total of approximately 5 Kg of cell paste. After centrifugation the bowl is removed and transferred to a safety cabinet in which the acetate sheet with its cover of cell paste is removed (known as the 'dig-out').

The cell paste is scraped off the sheet in the cabinet and is subsequently placed into a bag which is then sealed before removal to a -20°C store.

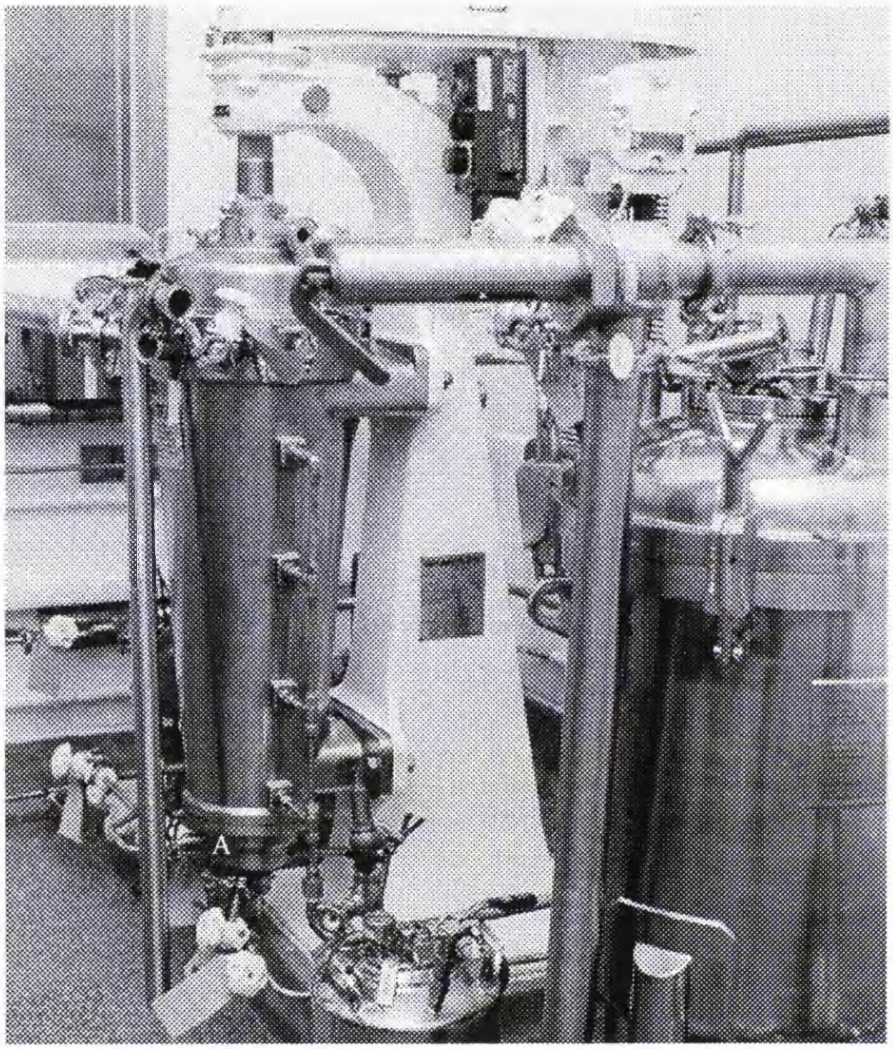


Figure 6.1 Sharples AS 16 V:B tubular bowl centrifuge (Alfa-Laval). The process stream enters through the bottom of the bowl (at A) and residence time depends on the flow rate through the centrifuge. Cell paste is deposited on the side walls of the tubular bowl, which is covered by a removable acetate sheet. After the process stream has stopped being pumped through the centrifuge, the bowl decelerates during a 15 minute locking step which prevents access to the bowl. During this time a 1 % hycolin disinfectant solution (William Pearson Chemicals) is sprayed onto the outside of the bowl so that any organisms that have escaped the primary containment will be killed before the bowl is removed and opened. The bowl is removed after a blanking plate is fitted to the top (the bottom is self-sealing). The bowl is then transferred to a safety cabinet where the acetate is removed and the cell paste scraped off.

The Sharples centrifuge is contained within its own room, which is at a negative pressure with respect to the surrounding area. Air sampling was carried out within the Sharples room prior to any operation, during centrifugation and during the dig-out procedure.

The position of the cyclone inlet in relation to the centrifuge and the safety cabinet is shown in Figure 6.2.

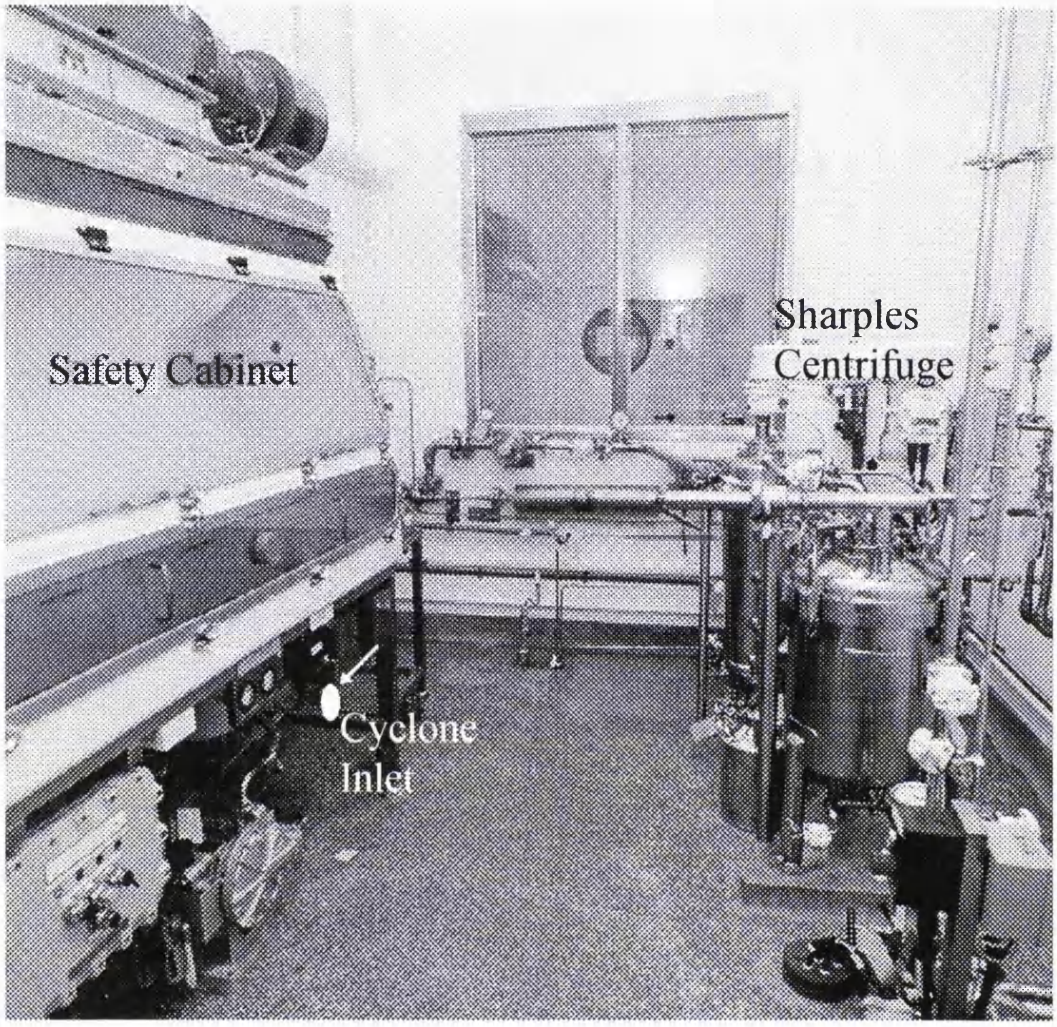


Figure 6.2 Sharples room layout. Safety cabinet is situated on the left hand side and centrifuge on the right at the rear of the room. The cyclone intake was positioned adjacent to the safety cabinet at the position marked, the arrow indicates the direction of air flow into the cyclone. Note that there is an air outlet duct on the RHS wall, adjacent to the centrifuge. The air inlet is on the ceiling at the front LHS of the room (above the safety cabinet). Room dimensions: 3.70 m × 3.00 m × 3.97 m (h × w × d). Cyclone inlet: 1.07 m from floor; 1.22 m from LHS wall; 2.35 m from back wall.

6.1.3 Bead mill homogenisation

Bead mills provide simple and effective means for disrupting micro-organisms, although they were originally designed for grinding purposes in other industries (Middleberg, 1995). The basic design is to have a jacketed chamber with a rotating agitator shaft through its centre. Glass beads are retained in the grinding chamber by means of an axial slot which is smaller than the bead diameter. Virtually all energy input is dissipated as

heat, necessitating cooling of the chamber. Cells are believed to disrupt as a result of the compaction and shearing by the beads and by energy transfer from bead to cell.

In these experiments 2 Kg of *E. coli* JM107 pQR701 cell paste was resuspended at 10 % (w/v) in 20 L of 0.1 M sodium/potassium phosphate buffer, pH 7.0. This suspension was fed into the Dyno-Mill KDL bead mill (Glen Creston Ltd) at a rate of 20 L h^{-1}, using a continuous flow chamber (600 mL capacity). The agitation speed was 4200 rpm, 0.2 - 0.5 mm diameter glass beads were used, and the separator gap was set at 0.05 mm. The chamber and bearings were cooled by recirculating glycol at -5°C.

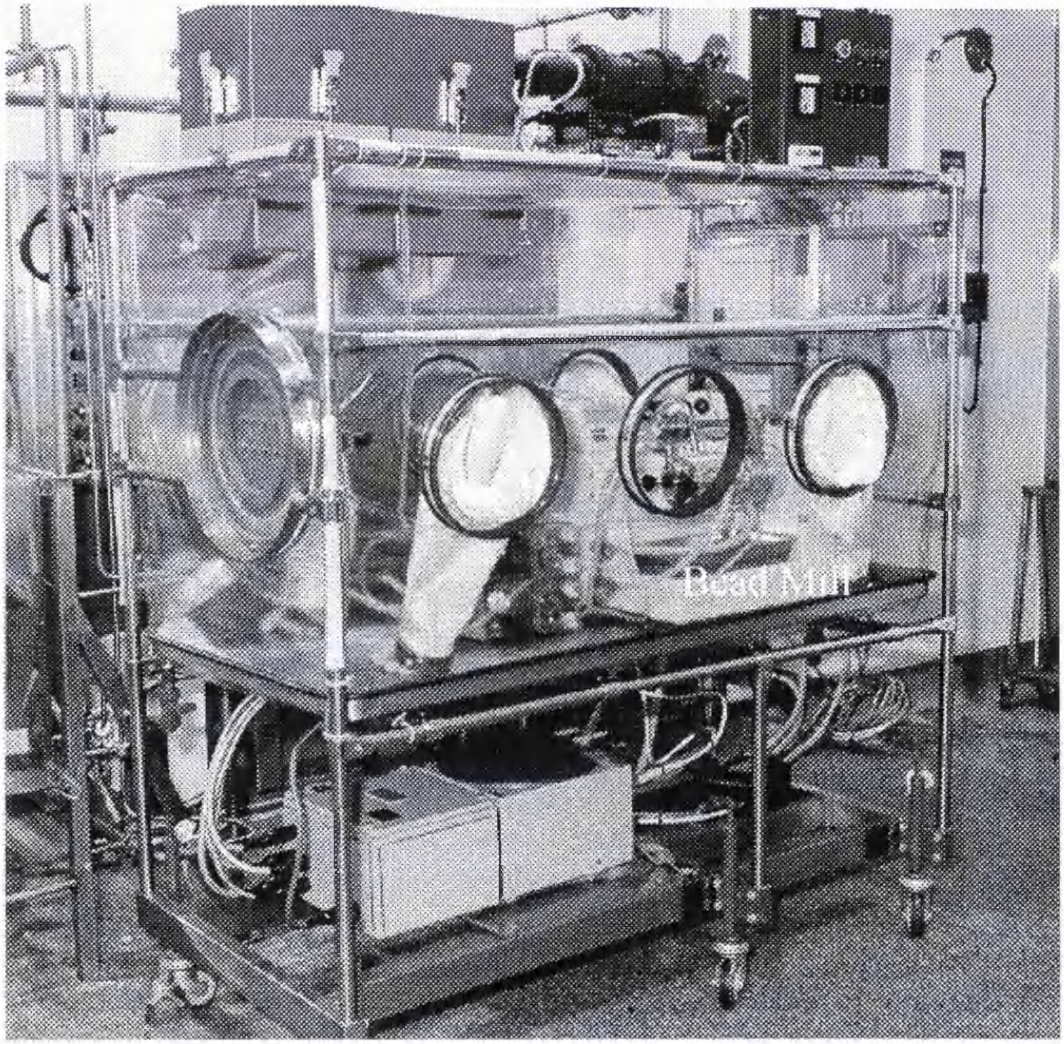


Figure 6.3 Flexible isolator housing Dyno-Mill KDL bead mill homogeniser. The isolator (1.6 m × 0.9 m × 0.77 m (w × h × d)) normally operates at a negative pressure relative to the room of - 75 Pa. The Dyno-Mill is operated via the sleeve ports. For sampling within the isolator, the cyclone was attached via flexible hosing to a modified glove port on the far side of the isolator (see Figure 6.4).

The bead mill is contained within a flexible film isolator with double HEPA inlet and exhaust filters (MDH Ltd) which is normally operated at a negative pressure of -75 Pa relative to the surrounding environment (Figure 6.4). Air sampling was carried out outside the isolator before and during operation of the bead mill. In addition, the cyclone was attached to the side of the isolator, with the isolator's air extraction turned off and the bead mill running, to sample the air within the isolator during operation.

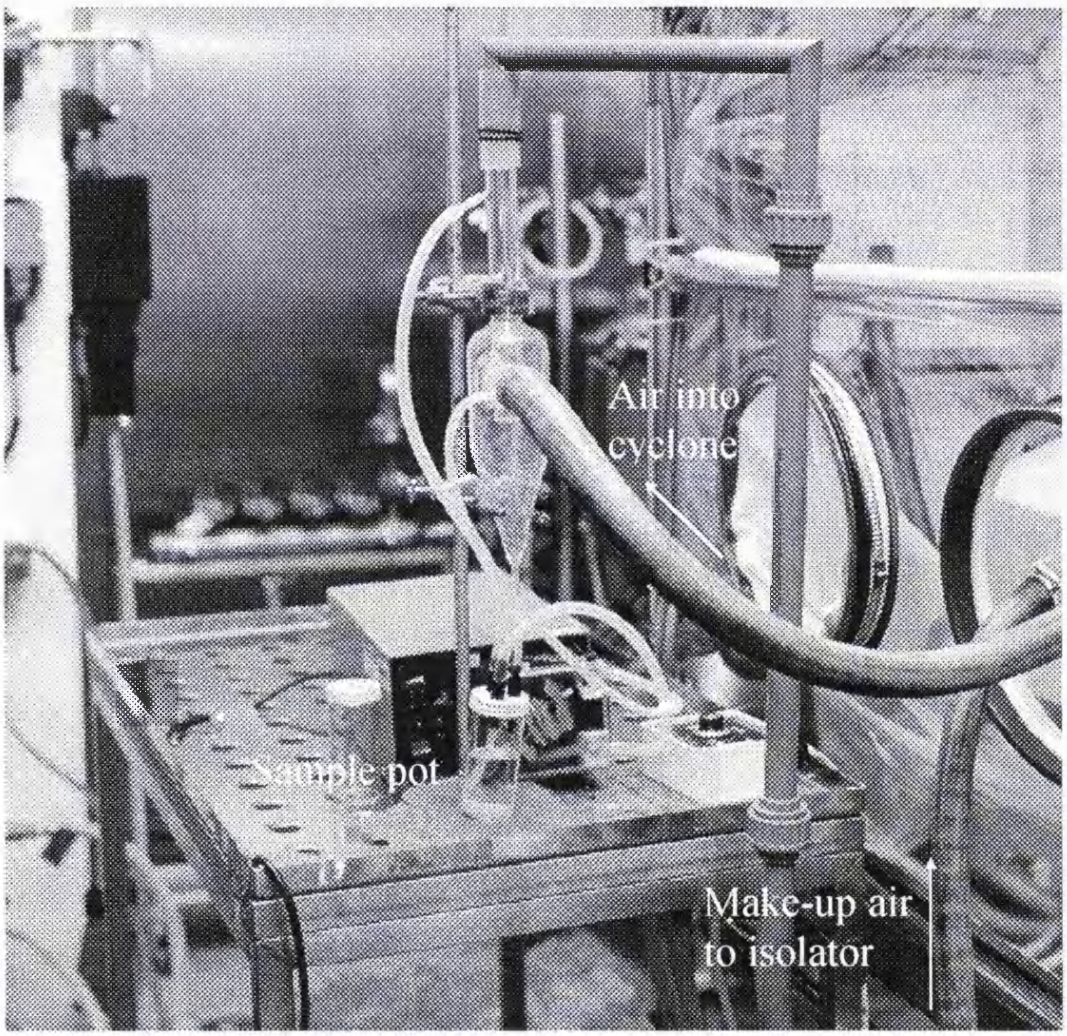


Figure 6.4 Cyclone attached to side of Dyno-Mill isolator. The cyclone is attached via a flexible hose and a modified sleeve port (a circular perspex plate with 2 holes drilled through for the cyclone inlet and supply of 'make-up' air to the isolator). The air flow through the cyclone was verified using a mass flow meter (Hastings Laminar Flow Element, Teledyne Hastings-Raydist).

6.1.4 Background samples

In order to determine the background level of target plasmid that might be present in the cyclone after cleaning, the cyclone was on occasion run for 15 minute periods in the media prep. lab, an area where there had been no known handling of the target strain.

6.2 Results of case study

In order to calculate the number of cells collected by the cyclone in a particular experiment, the mean plasmid per cell number was determined (Section 5.1). Microscopic cell counting showed that the concentration of cells in a sample from the harvested fermenter was 1.27×10^{10} cells mL^{-1}. The number of plasmids per cell was found to equal an average of 139 (cell samples were boiled for 30 minutes to achieve lysis before PCR in all experiments in this chapter, see Sections 2.2.3 and 4.1). This figure is therefore used in all subsequent calculations when converting the number of plasmids in a sample to the number of cells.

6.2.1 Background levels

Table 6.1 shows the results of background sampling within the clean area chosen.

TABLE 6.1 Summary of background levels found on sampling in media prep. area

Sample ID	Comments (date)	Cells collected (in cyclone)
P1	Media prep lab (19/3)	$6.3 \pm 1.0 \times 10^4$
P2	Media prep lab (21/3)	0
P3	Media prep lab (21/3)	$6.0 \pm 0.2 \times 10^4$
P4	Media prep lab (21/3)	0
P5	Media prep lab (21/3)	$1.5 \pm 0.02 \times 10^5$
P6	Media prep lab (27/3)	0
P7	Media prep lab (28/3)	0
P8	Media prep lab (28/3)	0
Avg.		3.4×10^4
SD		5.5×10^4

All data shown are calculated for the total number of cells collected by the cyclone (in a volume of approximately 60 mL) from the number of cells detected in a 10 μL PCR sample. Where cells were detected, data are shown ± SEM (n = 2).

Before discussion of the findings of this survey, it is important to mention the results of the background sampling experiments as this has an impact on the limit of detection of the methodology (see Section 7.1.3). Clearly, there is a variable degree of background contamination present in the samples derived from the cyclone despite the fact that the cyclone was operated in a clean area. Whatever the cause of the background levels (Section 7.1.3), the effect is to reduce the overall sensitivity of the assay. The limit of detection of the assay is calculated as being equivalent to the average background level plus 3 times the standard deviation:

$$\begin{aligned}\text{Limit of detection (L.O.D)} &= \text{Background average} + (3 \times \text{S.D.}) \\ &= 3.4 \times 10^4 + (3 \times 5.5 \times 10^4) \\ &= 2 \times 10^5 \text{ cells}\end{aligned}$$

Considering that 1 mL of broth at the point of harvest contains 1.27×10^{10} cells, then the calculated background level is equivalent to the number of cells contained within approximately 16 nL. So, although there is some background, it is at a very low level in terms of the number of cells that might be detected in the event of a significant release. For all subsequent results, any detected level of less than 2×10^5 cells in the cyclone is considered as a negative result and is reported as not detected (n/d). It should be noted at this point that the validity of using 3 standard deviations to set the limit of detection (LOD) assumes that the background data are normally distributed around the mean value. In this experiment there is insufficient data to verify this assumption. In practice, this means that it is not possible to determine that measured levels of release are caused by genuine events and are not merely scattered background measurements. However, in all operations where a release (above calculated LOD) was detected, it is notable that the level was greater than 10 times higher than the highest background level observed.

6.2.2 Fermentation samples

The results of sampling in and around the fermenter area are summarised in Table 6.2.

TABLE 6.2 Summary of results of sampling in fermentation area

Sample ID	Comments	Cells collected (in cyclone)
F1	Background, before inoculation	n/d*
F2	2 h after inoc.	n/d*
F3	5 h after inoc.	n/d*
F4	During sampling of 'live' fermenter	$4.7 \pm 1.1 \times 10^6$
F5	After fermenter contents steam sterilised	n/d*
F6	During sampling of sterilised fermenter	$2.7 \pm 0.3 \times 10^5$
F7	1.5 h after sampling from sterilised fermenter	n/d*
F8	3.5 h after sampling from sterilised fermenter	n/d*

*n/d (none detected, i.e. $<2 \times 10^5$ cells in the cyclone). Where cells detected, data shown ± SEM (n = 2). All samples were taken at a position 2 m from the fermenter, with the cyclone inlet situated 1.1 m above ground level.

The results indicate that during fermentation to a high cell density, there is no evidence of any release of process organisms to the air surrounding the fermenter. This is an expected result considering that the fermenters used are designed to operate to a high degree of containment and incorporate double mechanical seals with steam traces. However, there is evidence of a release of plasmid material during the use of the contained sampling array on two occasions. The amount of material captured equates to the equivalent of less than 0.5 μL of live cell broth in both instances. It was observed during both sampling events that after decoupling of the sampling array (Figure 6.5) a small amount of liquid leaks out. This liquid is likely to comprise mainly condensed steam, but there is a possibility that there is also a minuscule amount of material derived from the broth. Temperature mapping studies carried out as part of the commissioning program for the plant suggest that any cells that remained in the pipe work of the sampling array would be steam sterilised by the time of decoupling.

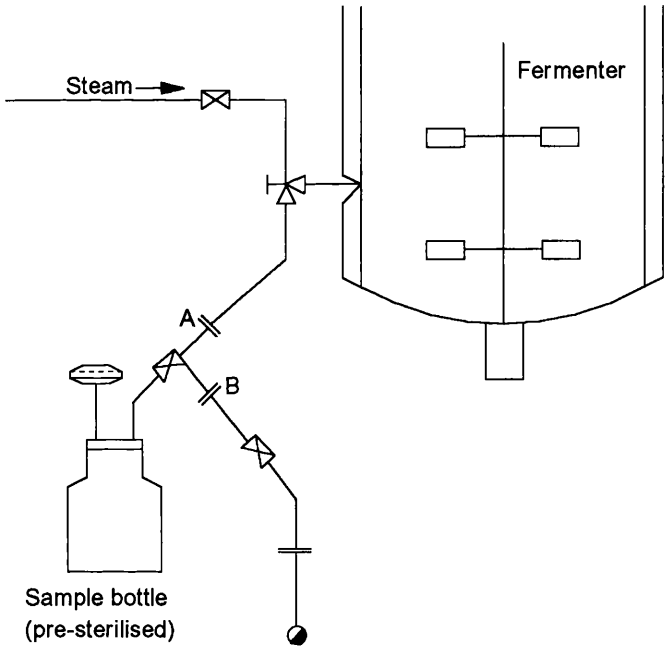


FIGURE 6.5 Schematic representation of the contained sampling array using at GWMRC Bioprocessing Pilot Plant. The pre-sterilised sample bottle and piping are connected to the sampling line via the couplings A and B. The line is steamed through before and after taking the sample. It is thought that there is a possibility that after steaming after collection of the sample a small quantity of the sampled broth might remain in the pipework, particularly in the vicinity of the valve to the sampling bottle. Glaxo Wellcome have temperature mapped the pipework during steam sterilisation right up to the 'weir' of the diaphragm valve to validate that any residual cells would be heat deactivated.

On the second occurrence of a release during sampling, the contents of the fermenter itself had been sterilised. Analysis of a sample of the sterilised broth indicates that per mL there is 1000 fold less pQR701 detected than there was in an unsterilised broth sample. The PCR method is therefore less sensitive to cells that are steam sterilised

within the bulk of the fermenter. This may be due to enzymatic 'nicking' of DNA occurring as the temperature slowly rises within the media. It is thought possible that cells that are killed by a sudden temperature rise, for example within the sampling line, are less susceptible to nicking as cellular proteins are rapidly denatured. In this case, the PCR might be equally sensitive to these killed cells as it is to viable cells.

The use of a microfiltration step before PCR to distinguish plasmid that is contained within cells from plasmid that is extracellular showed that with both samples being discussed here, target plasmid was not detected in the filtrate. It was hoped that the ratio of intra- to extracellular pQR701 plasmid concentration measured in the samples might indicate the condition of the cells which are released into the air and detected. For instance, steam sterilisation might have caused the intra/extracellular ratio to rise as lysis occurs in parallel to cell death. Since the pQR701 concentration in each sample was found to be entirely intracellular (or at least non-0.45 μm filterable) then it can be concluded that this method is unable to distinguish steam killed cells from those that are viable. This is a failing of the currently available methodology as it is envisaged that there will be occasions when killed cells are present in the environment which could give rise to a false positive result for the presence of live cells. Notably, however, the concentration of killed cells in the air surrounding the sampling port is reduced to background levels within 1.5 h after the original sample (Table 6.2, samples F6, F7, F8).

Since it is recognised that sampling from fermenters is an operation that might give rise to breach of containment, the design of sampling ports has received some consideration (Cameron *et al*, 1987; Hambleton *et al*, 1991; Leaver and Hambleton, 1992). Cameron *et al* (1987) have in fact monitored the containment effectiveness of a sampling port with a *B. subtilis* spore suspension in a fermenter using a cyclone as the air sampling device. In this study, bacteria were detected (by culture) at times coincidental with the operation of the sample valve. It should be noted that the port was not steam sterilised and it was thought that some of the seals were subject to wear, which may have accounted for the release of culturable cells. However, Hambleton and co-workers have suggested that where high containment is required, the use of secondary containment features may be necessary (Hambleton *et al*, 1991). A contained sampling device that is of interest with regard to this study is supplied by Bioengineering AG (based on European patent 0172838) and is described as an aerosol-free high containment sampling mechanism. In this device, a needle that pierces the sample container and retracts after sampling is enclosed and steamed before the next sample container is attached. It would seem that the propensity for aerosol generation (even of steam-killed cells) is reduced relative to that for the sampling array used at GWMRC (Figure 6.5). The use of sampling devices

such as these in a fermentation plant might solve the problem of false positives that would arise by QPCR detection of aerosol release.

In summary, it seems most likely that non-viable cells were detected during fermenter sampling. However, at present, it is not possible to distinguish between viable and non-viable cells. This is a refinement that will need to be addressed if sampling is likely to occur in areas where viable and non-viable cells may be released and the former present a hazard whilst the latter is, to a certain degree, acceptable. There is some discussion of possible future work in this regard in Section 7.1.1.3.

6.2.3 Sharples samples

The results of sampling around the Sharples centrifuge are shown in Table 6.3.

Table 6.3 Summary of results of sampling in Sharples pen

Sample ID	Comments	Cells collected
S1	Background, day before use	n/d*
S2	Background, am before use	n/d*
S3	during centrifugation	n/d*
S4	during bowl removal and dig-out	$7.0 \pm 0.1 \times 10^6$

*n/d (none detected, i.e. $<2 \times 10^5$ cells in the cyclone). Where cells detected, data shown ± SEM (n = 2).

The results of sampling in the Sharples pen are clear cut. There is no detectable release of process cells during operation of the centrifuge, but there is a significant release (35 fold above background) during the 'dig-out' procedure. This is despite the fact that dig-out occurs within a safety cabinet and the sampling point was outside. Again, the microfiltered sample showed an absence of free plasmid.

Observation of the procedure suggests that contamination of the operator's clothing with cell debris may provide a route for escape of the cells from containment. It should be noted, however, that the cyclone inlet, which draws air in at 0.36 m^3 min^{-1}, was placed close to the front of the cabinet and it is possible that the anticipated curtain of air flow that protects the operator and external environment may have been severely disrupted. In addition, when the operator places his arms into the cabinet this can disrupt the protective air curtain and indeed it is possible that the arms actually draw air from the cabinet up and around them, thereby causing the potential for release. Although the result in this instance might seem to be an artefact, the test could be said to be analogous to pressure testing of vessels where pressures in excess of those used in normal operation

are applied in an attempt to uncover potential sources of sterility failure or containment breach. Whatever the cause of the release, there are implications for the use of this particular unit operation with organisms that pose a high risk.

Notably, the operation causing the release in this instance is the dig-out. The containment afforded by the centrifuge during its operation appears to be satisfactory (although the air flow in the room suggests that sampling efficiency might be poor, see Section 6.3.1). Carr Separations Inc. have recently introduced the Powerfuge, which reputedly can attain the same high separating forces and dewatering capacity as traditional tubular bowl centrifuges but has the additional capability of automatic desludging (i.e. removal of paste from within the centrifuge). This obviates the need to manually scrape cell paste off the acetate. Judging from the results gained here, this auto desludging feature might well provide advantages in terms of containment.

6.2.4 Bead mill samples

The results of sampling in and around the bead mill isolator before and during operation are summarised below in Table 6.4.

TABLE 6.4 Summary of results from sampling around bead mill

Sample ID	Comments	Cells collected
B1	Background, day before use, outside isolator	n/d*
B2	During use, outside isolator	n/d*
B3	During use, inside isolator	n/d*

*n/d (none detected, i.e. $<2 \times 10^5$ cells in the cyclone). Where cells detected, data shown ± SEM (n = 2).

Sampling around the bead mill before and during operation indicated that there was no detectable release. However, when the cyclone was connected up to the side of the flexible isolator, thereby sampling the air inside, a low level release (just below the LOD, 1.5×10^5 cells collected) was detected. Because of the very high flow rate inside the isolator when the fan was turned on, sampling took place with the fans off. It is likely therefore that the cyclone aerosol collection efficiency would be relatively high in this instance. In fact it has been estimated at 10 % by CFD studies (Section 6.3.1)

There was no noticeable event that might have given rise to a detectable release, which probably explains the low level that was actually found. One conclusion that could be drawn from these experiments is that the secondary containment (the flexible isolator)

plays a necessary role in containing low levels of incidental release that occur on operation of this piece of equipment.

6.3 Findings of the case study at GWMRC

The intention of the study at GWMRC was to provide data in assessment of the containment effectiveness of a series of unit operations as well as to gain experience in real problems encountered 'in the field'. With regard to assessing the containment effectiveness of the plant it is important to remember that any findings are of questionable statistical validity due to the limited number of data points gathered (see Section 6.2.1). However, bearing in mind this note of caution, some tentative conclusions can be drawn:

Fermentation

- No cells were detected during the normal course of the fermentation.
- On sampling from the fermenter on two occasions, a small release was detected.
- The scale of release was low; the equivalent of less than 0.5 μL of broth was detected in each instance.
- It is likely that the detected 'cells' were not viable, although the method can not provide this information.

Tubular bowl centrifugation

- No cells detected during centrifugation.
- During the 'dig-out' procedure, which took place within a safety cabinet, 7.0×10^6 cells (equivalent to 0.7 μL fermenter broth at harvest), was detected by the cyclone outside the secondary containment.

Bead-mill homogenisation

- No cells detected outside the flexible isolator during homogenisation.
- When the cyclone was sampling within the isolator, with the fan turned off, a small number of cells were detected (1.5×10^5, 0.01 μL broth), this level is below the calculated LOD of the method.

However, to truly gauge the effectiveness of containment, it is necessary to estimate the number of cells released from a process rather than simply the number detected.

6.3.1 Relationship between capture and release.

The number of organisms captured from the process environment is only a proportion of the number released. When aerosols are released into a small Bassaire cabinet (0.36 m^3)

between 11 - 40 % of the cells are subsequently captured in the cyclone (Section 5.2). In a larger soft-film cabinet (8.35 m^3) the corresponding figure lies between 10 and 20 % (Ferris *et al*, 1995), and it could be reasonably assumed that a similar relationship holds for the flexible isolator (1.1 m^3) which contained the Dyno-Mill. Presently there is little understanding of how release and capture are related in the open environments which surround the fermenter or the Sharples centrifuge.

It is hoped that the simulations of air-flow and particle release which are possible with computational fluid dynamics (CFD) will allow estimation of the relation between the two figures, at least under circumstances where the point of release is known and the patterns of air flow are simple. Indeed, an analysis of the small Bassaire cabinet predicts an efficiency of about 20 %, which is within the range of the observed values, and of about 10 % for the cabinet which holds the Dyno-Mill (Figures 6.3 and 6.4).

Measurements of air-flow that were taken during this series of experiments, particularly in open areas such as the Sharples room (Figure 6.2) have highlighted how much information is required before the computer models generated by CFD can be validated. These CFD studies are the subject of ongoing research which will be reported separately at a later date (personal communication; P. Agutter, Dept. of Chemical and Biochemical Engineering, UCL). However it already seems that a release may be confined to a very small volume of the room, and may not be detected unless the cyclone is correctly sited. In the case of the Sharples room, air is rapidly drawn into the vent behind the centrifuge (Figure 6.2). In this instance, the cyclone was effectively positioned 'upwind' of a potential release, so the inability to detect any cells in the air can not be considered to be a definitive result. This highlights the importance of assessing the air flows within a room before air sampling is undertaken. In addition, it draws attention to the bulkiness of the cyclone air sampling apparatus (Figure 6.4) as it would not be possible, at least within the Sharples room, to site the cyclone where a release would be most likely to be detected.

In the open environments it could be estimated that the release is at least five or ten-fold greater than the amount captured, since this is the relationship that has been measured in a confined space of about 8 m^3. On the other hand were this relationship as large as 100-fold, then the total release from the sampling valve would be 4.7×10^8 cells (see Table 6.2, sample F4), which is equivalent to about 40 μL of fermentation broth. Given that the sampling line was washed through before being broken apart, this would seem a rather high figure.

6.3.2 Other observations from case study

As well as realising the importance of relating the number of cells released to those captured and of siting the cyclone in the optimum place for capture, a number of other observations were made with regard the practicability of the cyclone air sampling - QPCR technique.

One important factor is the way in which results are used and what they imply. Since this requires lengthy discussion, it will be covered in Chapter 7. A related point however, is the inability of the method to distinguish between release of viable cells and steam-killed (inactivated) cells. One of the perceived advantages of PCR over culture-based techniques of microbial enumeration is the ability to detect cells which are viable but not culturable (Section 1.5.1.1). However, in this instance the PCR is also detecting cells that are killed by steam (evidenced by the temperature profile in the sample port during steam sterilisation). Masters *et al* (1994) have similarly noted that autoclaving *Listeria monocytogenes* and *E. coli* cells for 15 minutes does not abolish PCR detection completely. This report also suggests that there is no simple relationship between viability and detectability by PCR and that detection of pathogens by PCR in environmental monitoring requires additional evidence of viability before risk can be properly assessed. It was hoped that measurement of extra/intra cellular plasmid ratios might provide this additional evidence of viability, but this has not proved to be the case. In many cases it may not be necessary to distinguish viable from non-viable cells since the release of either could be thought to indicate that containment is compromised. However, in the instance in this case study, where steam-killed cells are released from a 'contained' sampling port, the inability to make the distinction is unsatisfactory. It is hoped that methods will be developed to run alongside the PCR to enable the distinction to be made (see Section 7.1.1.3).

The existence of a background signal in some cyclone samples taken in an area thought to be free from exposure to the target organism has been mentioned (Section 6.2.2). In general the existence of background levels is not thought to be too serious as the levels are always low and insignificant compared to levels of plasmid that exist within the process stream. Background levels and their effect on the limit of detection of the technique are discussed in Section 7.1.3. One final point is the labour intensive and time consuming nature of the cyclone cleaning process. It is difficult to turnaround the cyclone within 30 minutes, so that subsequent samples are taken 30 minutes plus sampling time apart. In some instances this can mean that successive operations that follow closely may not be able to be monitored.

7. DISCUSSION

In this final chapter, the findings and issues raised throughout the thesis are drawn together. In the course of the discussion, areas for future work are identified. In essence, the thesis consists of two major parts: the development of methods which can validate biocontainment; and the application of these methods in model and real bioprocess situations. Points arising from the method development are dealt with in Section 7.1, whilst findings from the application of the the methods are used in a discussion on implications for current engineering practice (Section 7.3). Additionally, since it is recognised that a method for the measurement of airborne bacteria has applications outside of biocontainment validation, a section (7.2) is devoted to this subject.

7.1 Cyclone air sampling - QPCR methodology

The preceding result chapters have raised several issues which concern the cyclone air sampling - QPCR (CAS - QPCR) methodology. The two aspects of the methodology, QPCR and cyclone air sampling, will be discussed in turn.

7.1.1 QPCR

An important point in these discussions is the desire for simplicity in the method. This is primarily because it is envisaged that any method that is developed will have to be adaptable so that it can be used with a series of different bacterial strains. It is also recognised that any widely used technique should be essentially straightforward so that automation can be achieved and operation can be easily performed.

7.1.1.1 Choice of QPCR method

There are essentially three strategies for achieving quantitative PCR amplification: (i) extrapolation from a standard curve during the linear range of amplification; (ii) competitive PCR; and (iii) most probable number PCR (Jansson, 1995). In these studies, a competitive PCR was used since it is now widely accepted that accurate quantitation can be achieved using this method (Jansson, 1995; Reischl and Kochanowski, 1995). Additionally, this method does not require the use of sensitive and costly instrumentation and has been successfully applied to the measurement of bacteria in environmental samples (Mahon and Lax, 1993; Leser, 1995). Morrison and Gannon (1994) have shown that the quantitation afforded by a competitive PCR does not require detection within the exponential phase of the reaction. An important advance in competitive PCR

is the abundance of reported methods for constructing the internal standard (McCulloch *et al*, 1995). There are a series of methods for producing internal standards that differ from the target sequence in a variety of ways. The method used in this study (Forster, 1994) was chosen because of the relative ease of both the construction of the internal standard and the differentiation of target and standard products after PCR. A disadvantage with the internal standard used was the formation of hybrid molecules of internal standard and target fragment product. The formation of these 'heteroduplexes' can be reduced if the sequence similarity between the standard and the target is reduced (Ross *et al*, 1995).

It should be noted that other QPCR methods could be used in this context (see Section 1.5.2.3).

7.1.1.2 Comments on the QPCR method

The principle of competitive PCR is that the competitive internal standard and the target sequence compete in the PCR for enzyme, nucleotides and primer molecules. Since target and standard share the same primer binding sites, the efficiency of amplification of each is generally thought to be approximately equal (Reischl and Kochanowski, 1995). However, differences have been observed in the amplification efficiencies of standard and target (Chan *et al*, 1994; McCulloch *et al*, 1995) and there is some disagreement over whether this disallows absolute quantitation of target sequences (Raeymaekers, 1993; Chan *et al*, 1994; McCulloch *et al*, 1995; Reischl and Kochanowski, 1995). In the experiments reported here, it has been found that to achieve equivalent product band intensity, two molecules of IS(T) are required for each pQR701 plasmid molecule in the PCR (Section 3.2.3). It is not possible to say whether this ratio is a result of inaccurate estimation of the starting quantities of the plasmid and/or the IS(T) or due to a differential amplification efficiency. The former is likely to be a contributory factor since both plasmid and IS(T) were quantified by absorbance at a concentration of up to 10^8 fold higher than that at which they were used. The possibility of cumulative dilution errors giving rise to a two fold difference in apparent amplification efficiencies cannot therefore be ruled out. One improvement to the method that is worth mentioning here is the use of fluorescent DNA intercalating dyes which allow the accurate quantitation of very low levels of DNA; this would reduce the potential for dilution errors of the standard. However, assuming that the starting quantities of plasmid and IS(T) were accurate, then the 2:1 required starting ratio for equal amounts of product suggests a difference in amplification efficiency of 3 %. To accurately measure the amplification efficiencies of the two sequences, it would be necessary to quantitate the amounts of product accumulated after successive rounds in the exponential phase of the PCR. This

would be time consuming and would require access to instrumentation capable of detecting low levels of DNA (e.g. CGE-LIF). McCulloch *et al* (1995) and Chan *et al* (1994) have both corrected for differences in apparent amplification efficiencies by constructing calibration curves to standardise the competitor against the target. This is the approach that has been used here and the straight lines describing the calibration curves all have good correlation coefficients, suggesting that differences in amplification efficiency are consistent over the range of each curve. Moreover, linear dilution series of cells added to the PCR gave good correlation ($r = 0.988$) with the number of cells observed by QPCR analysis.

The use of gel electrophoresis and densitometry of bands of ethidium fluorescence is a relatively straightforward method for analysing PCR product ratios and can be performed in most molecular biology labs. This method of analysis has been used by other workers (Mahon and Lax, 1993; Gebhardt *et al*, 1994). However, a significant improvement on the method would be provided by the use of other analytical techniques such as HPLC (Chan *et al*, 1994) or CGE (Section 3.2.9). Some workers have used a labelled probe to detect and quantitate target species (see Reischl and Kochanowski (1995)) and this has the advantage of adding a layer of specificity to the method since the sequence of the product (not just the molecular weight) determines the response.

The PCR for target *E. coli* strain JM107 pQR701 is a specific detection method that shows no cross-reactivity with other *E. coli* strains or *S. cerevisiae* strains tested. This is despite the fact that the *E. coli* strains are closely related to the target strain in terms of host organism and vector. Additionally, the target strain has a DNA sequence inserted into the plasmid that is present in the chromosome of the *E. coli* JM107 strain (Sprenger, 1993; French and Ward, 1995). Amplification across the point of insertion of the *tkt* gene into pBGS18 has the advantages that the PCR is specific for the target construct and that assays developed for constructs based on the same vector but with a different insert, will also be able to use the vector primer (M13R1). This approach has been used for the strain *E. coli* JM107 pQR126; the plasmid pQR126 is a construct based on the vector pBGS19, which has an inverted multiple cloning site relative to the vector used in the construction of pQR701, pBGS18 (Spratt *et al*, 1986). Kuhnert *et al* (1995) have recently described a PCR assay that is specific for all *E. coli* K-12 strains which contrasts to the method presented here which is specific for a particular construct. In biocontainment validation it is envisaged that monitoring for the target strain will be carried out in environments where other *E. coli* strains are or have been used. It is therefore necessary to use a technique that could identify the specific process organism being monitored. Although it is conceivable that detection of a particular strain based on

the presence of a plasmid borne sequence may give false results due to loss or transfer of the plasmid, in the immediate environment of the bioprocess stream plasmid loss or transfer is highly unlikely.

One very important aspect of the QPCR methodology is the ability to apply it specifically to a variety of process organisms. In this thesis, two strains have been studied, *E. coli* JM107 pQR701 and *E. coli* JM107 pQR126 and a competitive PCR has been developed for each. With the strain *E. coli* JM107 pQR126 the QPCR was found to have relatively poor precision (± 0.5 logs, compared to ± 0.11 logs for *E. coli* JM107 pQR701) but this may still be sufficient for monitoring release of process micro-organisms. It is thought that by alteration of the PCR conditions (and possibly by utilising more than 3 internal standard concentrations) the precision of this assay could be improved. The ability of the method to be applied to other process strains has recently been shown for a further, BST encoding *E. coli* strain (personal communication; M. Bradley, Dept. of Chemical and Biochemical Engineering, UCL).

An interesting idea for identification of a variety of different strains is the incorporation of a specific sequence into the production strain at the point of its genetic modification. Amici *et al* (1991) have used a synthetic DNA 'number plate' to detect the fate of a genetically modified *Pseudomonas* strain in a freshwater environment. It is possible that such a number plate, or bar-code, could identify several characteristics about the strain including its origin. This might have useful implications in enforcement of environmental legislation, should an organism that is licensed for contained use be released into the environment.

As far as containment validation is concerned, it may not be necessary to develop a new QPCR system for each construct used in a bioprocess. For instance, the containment of the bioprocess plant at GWMRC was tested using the *E. coli* strain for which the QPCR has been developed at UCL. It may therefore be possible to validate equipment and plant using a series of standard strains; say, an *E. coli*, a yeast and possibly a mammalian or insect cell line. These standard strains chosen would be generally regarded as safe so that, were any release to be detected, the environmental and health consequences would be minimal. To monitor the day to day effectiveness of plant containment during operation, it would of course be necessary to have a QPCR method available that can measure the particular process organism being used.

7.1.1.3 Distinguishing viable from non-viable cells

The containment validation case study at GWMRC highlighted one of the limitations of the existing CAS-QPCR methodology; namely, the inability to distinguish steam-killed from viable cells. The use of microfiltration to distinguish intracellular from extracellular located plasmid was not sufficient to reveal information about the viability of the cells detected. One of the main advantages of PCR when compared to culture based techniques is its ability to detect viable but non culturable cells (Section 1.5.1.1). This is particularly important in the detection of cells derived from aerosols (Neef *et al,* 1995). Additionally, PCR detection is less sensitive to sampling stress caused by the collection of viable cells from the air. Alvarez *et al* (1995) have noted that when using an AGI-30 sampler (Section 1.4.4.2.2) no decrease in sensitivity of the PCR assay was obtained as a result of sampling stress whilst a 10 fold decrease in culturability was observed. For containment validation, it could be argued that any whole cells collected by the air sampling method and enumerated by the PCR represent a failure of containment, whether they are viable or not. However, the experience at GWMRC when monitoring the air around a sampling port shows that it is necessary, at least in some cases, to be able to distinguish viable from non-viable cells (Section 6.2.2). In fact, if one considers the frequent use of steam traces in static and mechanical seals and in sampling and addition ports, it becomes apparent that there might be numerous instances when containment functions by killing cells, but non-viable cells may still be released. The centrifugation operation at GWMRC used a 1% hycolin solution to decontaminate the outside of the tubular bowl after operation. Here, the challenge might be slightly different; to differentiate hycolin killed cells from viable ones. Since the mechanism of cell inactivation will be different when using hycolin (which is a mixture of chlorinated phenol compounds) to steam, then the method(s) for distinction of non-viable from viable will have to take this into account.

There are several reported methods for adapting PCR technology to the detection of viable cells only. It has already been noted (Masters *et al* (1994) and Chapter 6) that there is no simple relationship between viability and detectability by PCR. Most of the approaches have therefore used an additional test of viability in conjunction with the PCR. For instance, to detect only live *Giardia* cysts, Mahbubani and co-workers (1991) developed a method where the levels of giardin mRNA were detected (by RT-PCR) before and after induction of excystation. Notably, this study also found that detection of giardin mRNA by PCR in dead cysts depended on the mode of killing. The same group (Bej *et al*, 1996) have used a protocol based on the detection of mRNA by RT-PCR to specifically detect the viable pathogen *Vibrio cholerae* whilst a pre-enrichment step was used to specifically detect viable *Salmonella* species (Bej *et al*, 1994). A

different approach relying solely on PCR has been developed by McCarty and Atlas (1993). In this study, it was found that viability of *Legionella pneumophila* after chlorine inactivation correlated better with PCR using larger amplicons (650 bp) than when using smaller amplicons (168 bp). This is interesting as it suggests that using QPCR, there might be some value in determining the ratios of, say, 1 kb amplicon to 100 bp amplicon and this would reveal information about viability. Where cells are inactivated by detergent induced lysis, then it is possible that the filtration method developed in this thesis will be useful, possibly in conjunction with one of the other techniques, in determining viability. This is an area for further work.

7.1.2 Cyclone air sampling

7.1.2.1 Comments on operation of the cyclone

The Aerojet General cyclone is a high efficiency air sampling device for particles above 2 μm in diameter (Upton *et al*, 1994) and has the advantage that it samples air at a relatively high rate. In addition, since cells are impinged into liquid, then a sample can be taken directly and analysed in the PCR; this would not be the case with air sampling using filters for collection of cells.

It has been pointed out (Ferris, 1995) that the cyclone is not an easy device to use and that a good deal of experience is required before it can be operated effectively and reproducibly. Some important modifications have been made to the cyclone however, in an effort to make its operation more straightforward and to reduce sample loss via precession (Section 2.4.1). However, there are still some facets of cyclone operation which are time-consuming or which may give rise to poor reproducibility. One of the major problems is the difficulty in cleaning the device between runs. A 30 minute Tego soak/rinsing procedure has been adopted but this is labour intensive, time consuming and may not always be totally effective. Clearly, the task of cleaning the cyclone to cause a reduction in plasmid levels of up to 10^6 fold is difficult considering the comparative complexity of the device and its associated tubing. Nevertheless, it is very important that cleaning is effective since a failure to remove all the plasmid from within the cyclone from one sample to the next might result in false positives. Ideally, the cyclone would be disposable so that a clean, sterile, pre-packaged one could be used for each sample. Alternatively a series of cyclones (each with their own associated tubing) could be available and each would be subject to extensive washing after use. In theory, there is no reason why the cyclone could not be adapted so that services could be attached (e.g. steam, disinfectant) and a clean-in-place routine could be developed. This would be particularly useful if an effective method for degrading plasmid DNA was found - it is thought that viricidal agents might be useful in this respect. In all cases, it would be

necessary to validate the cleaning before next use. Redesign of the cyclone to allow the fitting of an absolute air filter to the inlet would allow the cyclone to be operated in conditions where any plasmid DNA present must have originated from within (i.e. due to inefficient cleaning) rather than from the surrounding air. Improvement of the cleaning procedure for the cyclone requires further work.

One other aspect that could benefit from some attention is the bulkiness of the cyclone's air vacuum pump set up. In the experiments reported here the cyclone is mounted on the top shelf of a trolley and the air pump is located beneath. The problem with this arrangement is the inability to position the air inlet of the cyclone in areas without easy access to a trolley of approximately 0.8 m width by 1 m height. In some instances, the most likely point of release and subsequent movement of an aerosol may indicate that the cyclone air inlet should be placed in a certain area to achieve optimal sensitivity of detection and reproducibility (see, for example, sampling of air around the Sharples centrifuge at GWMRC, Section 6.3.1). It is conceivable that flexible attachments could be designed for the cyclone that allow positioning of the inlet in the most telling area close to a particular operation. It would be necessary to determine the effect of changing the cyclone inlet characteristics on the capture efficiency of the device.

A further point on the operation of the cyclone that has been noted in the results chapters (Section 5.4.1) is the advisability of dividing between two operators the tasks of running the unit operation under study and sampling the surrounding air using the cyclone. Of course, this might not be desirable in modern companies where emphasis is placed on multi-tasking and bioprocess plants are thinly populated. However, the risk of cross-contamination is so great that the separation of the tasks is seen as being essential. Future developments of the cyclone, like those already outlined in this section, will tend to enclose the parts of the cyclone that are liable to contamination by the operator; this might then allow a single person to control both the bioprocessing and the sampling functions.

7.1.2.2 Relationship between capture and release

To achieve truly quantitative biocontainment validation, it is necessary to determine the number of cells released by a particular operation rather than simply to measure the number of cells collected by the air sampling method. The importance of determining the relationship between capture and release has already been noted (Section 6.3.1). In order to establish this relationship in a variety of different settings, an approach using CFD modelling of air flows is being used at UCL (personal communication; P. Agutter, Dept. of Chemical and Biochemical Engineering, UCL). To validate the CFD models

generated, a series of experiments has been carried out where the number of cells released into a fixed volume was known and the number captured determined (Section 5.2). It is envisaged that several further series of experiments of this kind in different environments will be carried out before confidence in CFD models can be built up.

One important aspect of the CFD work is its ability to predict the optimum sites for the cyclone air inlet. It is now apparent that to conduct a containment validation programme in a plant, it is essential to model air flow within rooms by CFD before the actual sampling takes place. The continued development of CFD models is a key step in developing a truly quantitative method of containment validation.

7.1.3 Limit of detection of methodology

At several points throughout this thesis the limit of detection (LOD) of CAS-QPCR has been mentioned. This is affected by a number of factors including: the LOD of the QPCR itself; the occurrence of detectable background levels; the duration of air sampling; and the sample processing steps that occur before PCR. These factors will be considered in turn.

The LOD of the QPCR technique has been found to be 5-50 cells per PCR (Section 3.1.5). This range was determined by observing the point at which a negative PCR was obtained on serial dilution of a cell suspension. In this case, the PCR was carried out without a boiling lysis step (Section 4.1) which might lower the LOD by up to 20 fold by increasing the efficiency of the release of plasmid into solution. The value of 5 cells/PCR as LOD can therefore be considered to be a conservative estimate (other workers have stated that optimised PCR can detect single cells in a sample (Bej *et al*, 1991c)). An LOD for the PCR of five cells equates to a level of 500 cells ml^{-1}. Since the cyclone is operated with approximately 50 mL of circulating liquid (at the end of air sampling after losses due to evaporation and precession) then the theoretical LOD for the CAS-QPCR is 2.5×10^4 cells. This means that at least this number of cells must be captured by the cyclone in order to be detected by PCR.

However, if background samples reveal the presence of target cells (where none are expected) then this has to be taken into account in setting the LOD. There are several potential sources of background in CAS-QPCR in a bioprocess plant: poor cleaning of the cyclone between runs leaving residual whole or fragmented cells between one sample and the next; contamination of the cyclone and/or associated tubing by the operator; a periodic appearance of airborne target cells in the clean area used for taking background measurements; or cross contamination at the PCR stage. Whatever the cause of the

background, the effect is to undermine confidence in the calculated LOD of the technique, since a positive result does not necessarily imply that a release of cells has been detected. To account for this, the practical LOD has been defined for each set of experiments by taking into account the mean of background readings and the standard deviation. LOD values have therefore been determined as being equal to the mean background value plus 3 × S.D. For normally distributed data, this means that the probability of a background giving a false positive is $p = 0.0015$. In practice, it has been found that the background values tend to be scattered, if observed at all, and there are really too few data to be able to say whether the values are normally distributed. This is in part because pQR701 concentrations are calculated for cyclone volumes (approx. 60 mL) from PCR sample volumes (10 μL) so that even low background levels are scaled up and appear significantly above 'undetectable'. LOD values calculated in this way have been equal to 3×10^4 and 2×10^5 cells in the cyclone. Clearly, there is a need to determine the source of the background count and to eradicate it. However, even the highest LOD value determined, 2×10^5 cells, is equivalent to less than 0.1 μL of a typical fermentation broth at harvest, so in terms of process release, the raised LOD caused by occasional background readings is negligible.

Describing the LOD of CAS-QPCR in terms of the number of cells required in the cyclone to cause a positive result does not readily provide an operator with a figure that can be related to cell concentration in a sampled aerosol. To achieve this, it is necessary to express the LOD in terms of the number of litres of air sampled.

Since the cyclone was normally operated at a flow rate of 360 L min^{-1} for 30 minutes, then the total volume of air drawn in was 10,800 L. The LOD therefore represents 2.5 cells L^{-1} of incoming air, assuming 100 % capture efficiency. A negative result would therefore imply that there is less than an average of 2.5 target cells per litre of air entering the cyclone. However, the LOD has a time dependant element since sampling for a 15 minute period would have a higher LOD of 5 cells L^{-1} air. The LOD is therefore best defined as:

$$\text{LOD (cells L}^{-1}\text{ air)} = 2.5 \times 10^4/(v \times t)$$

where t is the sampling time in minutes and v is the sampling rate in L min^{-1}. As well as assuming 100% capture efficiency by the cyclone, this calculation assumes a constant cell concentration in the air entering the cyclone. This assumption may well be untrue, but the LOD merely reflects the average value. This is a factor that should be taken into account when planning a monitoring regime.

In all the above discussion, there has been no mention of means of achieving lower LOD values. One of the simplest methods to achieve this is by effecting a concentration step on the cyclone sample before measuring the plasmid concentration by QPCR. This could be carried out by filtration or centrifugation (Atlas and Bej, 1990; Bej *et al*, 1991c). If, for example, the cells within 50 mL of cyclone sample could be spun down and resuspended in 1 mL then this would have the effect of lowering the theoretical LOD of CAS-QPCR from 2.5×10^4 cells to 500 cells in the cyclone. However, it should be remembered that by increasing the number of sample manipulations, the potential for error or inadvertent sample contamination might increase. The effect of this would be to increase the practically observed LOD as background levels become more significant. In developing a sample concentration step, the potential for improved theoretical LOD should be weighed against the likelihood of enhancing background 'noise levels' and thereby increasing the observed LOD.

In conclusion, the LOD of the CAS-QPCR has been shown to be affected by several factors, some poorly understood. However, observed background levels are very low in comparison to the scale of release that might occur on breach of containment. If necessary, it is likely that methods could be developed to lower the LOD of the methodology.

7.1.4 Suggestions for further improvements of the methodology and future work

Experience of using the CAS-QPCR method for the validation of containment of bioprocesses has enabled the identification of possible improvements that might be incorporated in the future. At present, the technique requires a reasonable amount of experience and time to be correctly carried out. Ideally, the method should be simply operated, involve little hands-on time and the results should not be operator-dependent. In order to achieve this, areas for future development are put forward in Table 7.1.

Most of the possible improvements have already been alluded to or specifically mentioned. The possible use of wetting agents in the recirculating fluid is inspired by the observation that recovery of aerosolised cells from the Bassaire cabinet is enhanced when the cells are sprayed from nutrient broth as opposed to TRS (Section 5.2.4). It is observed that some of the nutrient broth enters the cyclone and improves the wetting properties of the recirculating liquid, this may be responsible for the improved recovery of cells.

TABLE 7.1 Possible improvements in the methodology

Step	Possible improvements
0. Cyclone design	i) Minimisation of liquid loss; less manipulation of tubing by operator; construction material less fragile (not glass)
	ii) Ability to fix micro-filter on air inlet allowing background measurement
1. Cyclone air sampling	i) Positioning of cyclone inlet in optimum position according to CFD prediction of air flow
	ii) Improving cyclone capture efficiency by use of wetting agents in recirculating liquid
2. Cyclone washing	i) CIP procedure desirable: viricidal agents might be most effective in removing DNA
3. PCR	i) Further optimisation of the cell lysis step
	ii) Differentiation between viable and killed cells
	iii) PCR can be automated; reduction of liquid handling steps
	iv) Fast thermal cyclers available; total PCR time can be reduced to less than 60 min.
	v) Reduced reaction volume: fast thermal cyclers require less total volume, giving reduction in consumables cost
4. Analysis of PCR products and densitometry	i) Automated analysis; CGE or HPLC methods allow analysis time to be reduced to < 20 min./sample; compatible with very small reaction volumes (see 3.iv)
	ii) Automatic calculation of relative peak areas: removes element of subjectivity from densitometry of gel
5. Interpretation of results	i) Validated CFD models for air flow within sampled areas enabling sound relationship between released and captured organisms to be established.

Developments in thermal cyclers include machines where the sample and reaction components are sealed within capillary tubes. The short distance from the centre of the capillary to the heating block means that heat transfer is very rapid and, for example, 30 cycles can be performed within 60 minutes (Black *et al*, 1995). Moreover, rapid cycling can be achieved using air thermal cyclers: the combination of rapid temperature transmission time and small sample volume (10 μL) allows cycles to be reduced to 5 seconds for denaturation and 10 seconds for extension and annealing. Hence an entire PCR can be completed in approximately 30 minutes (Buck, 1996). The small volume in the PCR would entail less consumables cost and would be sufficient for analysis by CGE or HPLC with sensitive detection. An interesting future development might be the use of DNA amplifiers based on silicone chips (Beard, 1994). These are predicted to allow very fast amplification in portable, hand-held devices.

The move from the currently used labour intensive and subjective PCR product analysis method of gel electrophoresis and densitometry to a more automated system such as CGE is seen as being essential. This is partly to build confidence in the methodology (as operator input becomes less significant) as well as to allow greater sample throughput.

In addition to improving the current methodology, it is envisaged that future experiments could extend the scope of the work presented here. Specifically, it would be useful to devise a QPCR method for different species of micro-organisms that might be used in bioprocesses. In particular, the development of a method for the detection and enumeration of *S. cerevisiae* cells would be useful since this organism is widely used in many large scale processes and its larger size, compared to *E. coli*, might lead to a difference in the effectiveness of its containment or the characteristics of any airborne release.

It has already been mentioned that the current methods could be used to validate the containment provided by a further series of unit operations: centrifugation using a tubular bowl centrifuge with an automatic desludging feature (Section 6.2.3); and the use of a Turbosep foam separator (Chapter 5) have been specifically mentioned. In theory, a ranking system could be drawn up to indicate the degree of containment provided by a range of operations. This would be useful in assisting the specification of plant that is appropriate for a particular process.

Finally, it is noted that adaptation of the methods used here to personal sampling could be achieved. The detection of disease causing airborne micro-organisms is discussed in the following section.

7.2 Potential applications of cyclone air sampling-QPCR methodology

In this thesis, the CAS - QPCR method has been developed for the specific purpose of validating the containment afforded by bioprocesses. However, there are a series of other uses to which all, or part, of the methodology could be applied. A brief survey of these applications follows.

7.2.1 Other applications in bioprocessing

There are two areas within bioprocessing where the developed techniques might be useful. These areas are the determination of plasmid copy number in cultures of micro-organisms and filter integrity testing (see Sections 5.1 and 7.3.2 respectively).

The estimation of plasmid copy number in growing cultures is an important measure since copy number is of economic significance in large scale operations where expression of the required phenotype must be optimised. In addition, where a culture is grown for production of the plasmid itself for use in gene therapy applications (Horn *et al*, 1995), increasing the copy number is clearly desirable. The idea of using QPCR to assess plasmid copy number in cultures stems from the need to calculate the number of cells detected in a particular sample, where it is the number of plasmids that is directly measured (Section 5.1). To convert results from plasmids mL^{-1} to cells mL^{-1}, it is necessary to know the average number of plasmids per cell. In this thesis, this has been achieved by simply dividing the number of plasmids measured in a calibration sample by the number of cells present (determined by microscopic enumeration). There are some significant differences between this method and the published methods for copy number determination (Lewington and Day, 1986; Wrigley-Jones *et al*, 1992). In the latter, the quantities of plasmid and chromosome in a preparation are determined by restriction digestion followed by electrophoresis and densitometric quantitation. This yields a result that is expressed in terms of plasmid copies per chromosome equivalent. In order to determine the number of plasmids per cell, the number of chromosomes per cell must be calculated. This is achieved by using an algorithm that involves input of the specific growth rate of the culture.

An approach for determining copy number in a manner akin to the 'traditional' method but using QPCR could be developed. This could be achieved by using QPCR to measure the number of copies of plasmid in a sample and also, using a QPCR assay specific for a chromosomal DNA sequence (see Sections 2.2.5 and 3.1 and Kuhnert *et al* (1995)), the number of copies of the chromosome. Determination of plasmid copy number, in terms of plasmids per chromosome, would then be straightforward. The potential advantage of using this method to determine copy number is that, once the appropriate QPCR assays

are developed, it will simply require comparison of the relative quantities of plasmid and chromosome. Notably, the QPCR assay for the chromosome of *E. coli* K-12 strains could be used for a number of different constructs. For each new construct, it would therefore only be necessary to develop a QPCR assay for the plasmid; this might also be used in containment validation. For the traditional method of copy number determination, it is necessary for each strain to find a restriction enzyme that cuts the plasmid at a single site whilst cutting the chromosome into discrete pieces, none of which are similar in size (in base pairs) to the plasmid. The reason for the latter requirement is that the plasmid and chromosomal fragment bands should not overlap in gel electrophoresis. In fact, densitometry of the gel is a key step in accurately determining copy number. Using the QPCR based approach, where two discrete bands are produced, the precision of the method could be superior.

The application of CAS - QPCR to filter integrity testing is a possible extension of the work of Kastelein and co-workers (1992b) who showed, using culture for detection, that *Pseudomonas diminuta* cells can pass through widely used exit-gas filters. It is thought that collection and enumeration of cells passing through a filter by using CAS - QPCR would give a more accurate estimation of the total transmission, given that a high proportion of aerosolised cells are likely to be non-culturable (Neef *et al*, 1995).

7.2.2 Monitoring airborne disease transmission

It has long been recognised that disease can be transmitted by the airborne route via bioaerosols (Collins, 1992). Consequently, there have been numerous reports in the literature of air sampling studies in different environments (for review, see Griffiths and DeCosemo (1994)). However, these studies are either not truly quantitative in that the methods used for enumeration of captured micro-organisms are usually based on culture, or use non-specific methods of cell enumeration such as chemi- and bioluminescence. In the light of recent findings on the proportion of non-culturable cells in aerosols and the pathogenicity of viable but non culturable organisms, it can be seen that it is preferable to use a method of cell enumeration that can specifically measure the number of viable cells of a particular pathogen that is present in an air sample. In recent years, PCR has been used to detect bacterial cells captured from aerosols (Alvarez *et al*, 1994, 1995; Mukoda *et al*, 1994; Roll and Fujioka, 1995), however, true quantitation was not achieved. It is thought, therefore, that CAS - QPCR could be used in measurement of airborne pathogens and that additionally, air-flow modelling (by CFD) could be used to determine the source and the patterns of spread of aerosols. A series of potential applications for this method are given in Table 7.2.

TABLE 7.2 Potential applications of CAS - QPCR in measurement of airborne pathogens

Application	Comments	Additional Information
Hospitals (nosocomial infections)	Invasive surgical techniques and routine activities in wards (sweeping, bed making) can give rise to aerosols. Incidence of multiple-antibiotic resistance micro-organisms is increasing.	(Griffiths and DeCosemo, 1994; McGowan, 1995; Overberger *et al*, 1995)
Dentist's Surgery	Drilling, scaling etc. can cause aerosols containing particles derived from saliva and blood.	(Grenier, 1995; Miller, 1995)
Occupational	Workers in sewage, agricultural and biopharmaceutical industries are at risk.	(Lenhart and Cole, 1993; Zedja *et al*, 1993; Laitinen *et al*, 1994; Rautiala *et al*, 1996)
Public health	Risk factors include proximity to sewage works and agricultural land, sick building syndrome.	(Ehresmann *et al*, 1965; Sawyer *et al*, 1993; Teeuw *et al*, 1994)
Defence	Detection of biological agents in battlefields.	(Titball and Pearson, 1993; Evans *et al*, 1994)
Food and pharmaceutical production	Food and beverage spoilage organisms, sterile production facilities.	(Lungqvist and Reinmuller, 1993; Sheehan and Giranda, 1994)

It is worth noting that although QPCR is readily applicable to the measurement of virus particles, their smaller size would necessitate the use of different air sampling techniques (see, for example, McCluskey *et al* (1996)).

A related application for CAS - QPCR, although not necessarily concerned with pathogens, is the assessment of survival and dispersion of deliberately released organisms. This could be applied to measurement of micro-organisms (Stetzenbach *et al*, 1992b) or to detection of pollen from transgenic plants (Kareiva *et al*, 1994). In either case, the goal would be to generate additional information regarding the fate of genetically modified organisms in the environment and this could be used in the risk assessment process for future releases.

7.3 Implications for current engineering practice

This section will deal with the potential applications of the CAS-QPCR technique in the area of containment validation and monitoring. It is hoped that, rather than simply being used as a method to determine the containment effectiveness of a constructed plant or assembled unit operation, the data provided by the method will affect design considerations. For instance, if a homogeniser is found to cause aerosol release of the process organism, then it could be suggested that this instrument is either modified or that secondary containment is provided. These changes would be best made before plant commissioning. However, before discussing the application of CAS-QPCR to biocontainment design and validation, it is first necessary to discuss how results that are generated can be expressed in a useful way. It should be noted that, at this stage, there is no discussion of detriment caused by release, or how different manifestations of release may vary in their effects on health and the environment.

7.3.1 Utility of results

Crook and Cottam (1996) have conducted a survey of UK biotechnology companies to establish the methods that are being used for monitoring release of process micro-organisms into the environment. One of the findings was that, because of the lack of available data, it is difficult for the companies to interpret the results of environmental monitoring that are gathered. Strategies used include comparison of levels of micro-organisms found during operation with background levels, and the use of trending. The problem with comparing data between different operations is that the sampling method often gives only representative (i.e. non-quantitative) results. Now that it is possible to generate quantitative results, it is pertinent to ask how best to present the data.

An important question for any new analytical technique is how might the results be used. In this instance, there are two major options that are not mutually exclusive: i) to use the data as part of the commissioning for a new process; and ii) to monitor the operation of the process on a day-to-day basis. This decision would have a significant impact on any development program, since it would be inadvisable to expend a great deal of effort in an attempt to reduce the assay running costs if the assay itself were only used sporadically. It is worth noting that the precision of the result of any assay can feasibly be tailored according to the needs of the operator. For instance, a simple threshold assay could be developed where the level of target detected might be considered negligible if it fails to match a particular, pre-set standard level.

The other important question in this context is how to express results. Possibilities include:

- Total number of cells released over the course of an operation (analogous to environmental emissions standards (Strauss, 1987)).
- Total process volume equivalent released over the course of an operation.
- 'Spray factor' of a particular operation. This is defined as the quantity of material released into the air per minute, divided by the quantity of the material per volume being handled (Hambleton *et al*, 1992). For instance, a release of 1.7×10^5 cells min^{-1} from a fermenter containing 1×10^{10} cells mL^{-1} would give a spray factor of 1.7×10^{-5} mL min^{-1}.
- Worker/environment exposure.

An important point to reiterate is the inability of the present methodology to distinguish between viable and non-viable cells. Whilst it is possible that future developments will allow this distinction, at present the utility of the results may be limited for this reason.

Expression of results as the total number of cells released over the course of an operation assumes that it will be possible to confidently assert the relationship between the number of cells captured and the number released within a particular environment. Although the establishment of this relationship is foreseeable, it is less likely that it will be possible to infer, on the basis of the results from a series of samples at different times throughout the operation, the total release during that operation. This has been recognised by Strauss (1987) who noted that the estimation of the total number of cells released from a research lab. is fraught with difficulties owing to the number of uncontrollable variables. In the case of exit-gas sampling, the total release throughout a fermentation has been calculated by drawing a line between four sampling points (over a 5.5 hour period) and then integrating the curve (Section 5.3.3). This approach has some validity in this instance as the rate of release of cells into the exit gas is thought to be reasonably well described by the curve. But, in the case of, say, homogeniser or centrifuge operation, releases of cells might be sporadic and there will not be an easily determined relationship between release and time of operation. The homogeniser study in Chapter 5 is interesting in that there appears to be both sporadic aerosol release and continuous time-dependent liquid release during operation. In order to determine the total number of cells released, it would therefore be necessary to sample throughout the entire course of the operation. It is also possible, that if one operation were to be studied in depth, then a series of key sampling points could be identified and monitoring at these points would reveal valuable information about the process as a whole. To achieve this satisfactorily, it would be necessary to identify likely failure points in a process. However, Kastelein and co-workers (1992a), using a Failure Mode and Effect Analysis approach, found that

the quantification of risks in biotechnology is far more complicated than for the nuclear or chemical industries.

The idea of expressing results in terms of the process volume equivalent released is to highlight the contributions to overall release from incidental and accidental modes. So, in the case of unfiltered fermenter exit-gas sampling (Section 5.3.3) the total number of cells released was found to be 3×10^7, which is equivalent to the number of cells found in 1.7 μL of the final broth. Since, in this small scale experiment, there was 1.5 L of broth in the fermentation, it can be clearly seen that the potential for release of cells from mis-handling the broth is far greater than that from the unfiltered exit gas. Expression of containment monitoring results as the total process volume equivalent released, first requires calculation of the total number of cells released, so clearly there are the same limitations in the calculation of these figures. However, the advantage is that there is a ready basis for balancing risk from different sources of release.

Attributing spray factors to specific unit operations is potentially useful in terms of design of bioprocess plant. Indeed, there is already an inventory of spray factors ascribed to some common bioprocess operations (Hambleton *et al*, 1992). Since different operations may contain different cell concentrations, the use of spray factors allows a standardisation of the containment provided. For instance, the cell density within the fermenter might be 1×10^9 cells ml^{-1} whereas in a homogeniser process stream this figure might rise to 5×10^{10} cells ml^{-1}. If each operation has a spray factor of 10^{-8} mL min^{-1}, then there would be 10 cells min^{-1} emitted into the air around the fermenter and 500 cells min^{-1} emitted into the air around the homogeniser. Expression of containment efficiency in this way also allows one to anticipate levels of release in processes where cell densities may differ. One apparent advantage of reporting results in this manner is that it clearly identifies operations where the containment will have to be improved upon; in the case above, the homogeniser operation might be subject to scrutiny. The problem with the idea of spray factors as the design standard is that the calculated figure will only apply to a single set of circumstances. There is no assurance that, say, a spray factor of 10^{-8} mL min^{-1} associated with a fermenter which contains *E. coli* in a complex medium will also apply when the organism is a filamentous bacteria, a yeast or an animal cell. However, it is possible that this concept could be used in the containment validation of a plant during commissioning and it would be helpful if spray factors were more widely available for biotechnology equipment (Hambleton *et al*, 1992).

The principle underlying the requirement for containment in bioprocesses involving genetically modified micro-organisms is that, should a release occur, a detriment might be caused to human health or the environment. It would seem wise therefore to monitor the exposure of workers, or of the environment around the plant, to the process organism. Although this is undoubtedly true, it is not really the purpose of the methodology developed here. Using CAS-QPCR the goal is to determine how effective bioprocess plant is at containing micro-organisms and to use the findings to improve upon the containment design, rather than to determine the levels of exposure.

A number of samplers which can be worn or carried by the operator (Hambleton *et al*, 1992) will monitor personal exposure. Similarly, protocols could be devised for monitoring environmental exposure in the vicinity of the plant. The adaptation of the QPCR method to personal sampling might not be straightforward as the most appropriate personal sampler might collect cells on a filter or a solid surface. Nevertheless, this could be achieved with a certain amount of development. The use of QPCR in conjunction with personal sampling would offer a significant advantage over current techniques which rely on a culture and hence do not enumerate viable but non culturable organisms (Section 1.5.1.1). Data generated in this manner would be readily expressed as personal or environmental exposure.

7.3.2 Biocontainment design and validation

Data generated in containment validation studies are useful in indicating to the plant operator which operations might be likely to give rise to release of micro-organisms. However, the development of an accepted method that truly measures the effectiveness of biocontainment would allow considered assessment of equipment and plant design. Existing biocontainment design is, in part, drawn from experience of designing for operations with sterility requirements; accumulative knowledge of how to keep extraneous micro-organisms out of a process has been instructive in designing processes where it is necessary to contain process micro-organisms (Brooks and Russell, 1986). But since recommendations for design of contained operations have often been made in the absence of any real data with regard to effectiveness, it is thought that, in certain cases, the suggested design is inappropriate. This is illustrated by an argument about the choice of static seal types for different levels of operation (Section 1.2.3).

One of the differences between traditional antibiotic producing fermentations and processes involving genetically modified micro-organisms is that in the latter, fermentation exit gas is treated to reduce microbial burden before discharge into the environment. This is normally achieved by using microporous membrane filtration

(Leaver and Hambleton, 1992; Miller and Bergmann, 1993), other methods such as using simple water sprays (Devine, 1989), cyclones or simple depth filters have been discouraged (Orchard, 1991). Hambleton *et al* (1991) have suggested that exit gas is treated by passing through double (in series) hydrophobic cartridge air filters to prevent release. Single exit gas filters are considered sufficient to minimise release and therefore comply with containment categories B1 and B2 (Leaver and Hambleton, 1992). However, Kastelein *et al* (1992b) has shown that viable *Pseudomonas diminuta* can penetrate through widely used exit gas filters. Moreover, Miller and Bergmann (1993) have noted that there is a potential operational difficulty with exit gas filters and that is their tendency to foul and plug when they are wetted by moisture carried over with the exhaust gasses or due to excessive foaming. In order to overcome this problem a condenser or cyclone separator can be placed between the fermenter and the filter housing.

The notable feature in this discussion is that, on the whole, recommendations are made without reference to direct integrity testing methods. In fact, the work of Kastelein has shown that 'absolute filters' used alone may not be effective at containing airborne micro-organisms in exit gas (Kastelein *et al*, 1992b). In Section 5.3 in this study, the number of cells released through unfiltered exit gas was quantified by CAS-QPCR. Additionally, exit-gas was monitored downstream of a 0.2 μm filter and in these samples there was an absence of process organisms. These studies therefore indicate a method for assessing the efficacy of exit gas filters and the potential scale of any release should the exit gas be discharged untreated from the fermenter. It is possible then to anticipate the scale of the hazard that might occur on failure of exit gas treatment. The numbers of cells released in such a case is very small compared with the total process volume. Further, should the provision of an exit gas filter contribute to a major accident then its use might be questioned. For instance, this might occur as a result of plugging by foam escaping from the fermenter causing an increase in the pressure within the fermenter leading to bursting disk rupture. It is prudent to ask whether limited resources are best expended on containment of exhaust gasses (where, at any rate, the release of process organisms is likely to be numerically of small scale). However, in assessing the contribution to overall release from different sources, the type of release (aerosol or liquid) as well as the scale, is of importance in determining potential detriment caused to health or the environment.
In addition to studies of the release of micro-organisms through fermenter exit-gas, release of process organisms has been monitored during the following operations: high pressure homogenisation; fermenter sampling; tubular bowl centrifugation; and bead mill homogenisation. In each instance, there has been some discussion of the implications of the results on improved design for containment. One notable finding is that downstream

processing operations have caused the largest recorded releases of organisms; in dig-out after tubular bowl centrifugation (Section 6.2.3) and into lubrication liquid during high pressure homogenisation (Section 5.4.2). This potential for greater release in the operational steps between fermentation and cell inactivation is related to the increased cellular concentrations that are handled at these stages (Dunnill, 1982). This potential for release is accentuated because many of the unit operations in downstream processing are derived from industries other than biotechnology, whereas there has always been a sterility requirement in fermentation. It is interesting to note then that the high pressure homogeniser released 73 µL of concentrated (10 % w/v) process fluid per minute into the lubrication liquid whereas unfiltered fermenter exit gas released a total of less than 2 µL equivalent process fluid over 5½ hours of fermentation. It has been recommended (Kastelein *et al*, 1992a) that the homogeniser lubrication line is run to a contained drain/kill tank, however, the pistons that are lubricated are exposed to the external environment.

7.3.3 Risk assessment in bioprocessing

The generation of quantitative data relating to the release of micro-organisms during bioprocesses enables the unit operations to be subjected to quantitative risk analysis (QRA). The aim of QRA is to determine the level of risk of any activity and to thereby enable management to decide whether to put more or less effort into minimising hazards or into improving hazard management and to see clearly where increased or decreased effort should be focused (Tweedale, 1992). In addition to the risk posed by incidental (routine operational) releases of process micro-organisms, the risk arising from accidental releases needs to be quantified. Kastelein *et al* (1992a) have noted that an understanding of the processes leading up to an accident, and their related contribution to the overall hazard, can aid in the design of systems to minimise the risk of accidents and their subsequent development. Balancing of risk derived from incidental and accidental sources is seen as a key outcome of the QRA procedure and this will be dealt with later in this section.

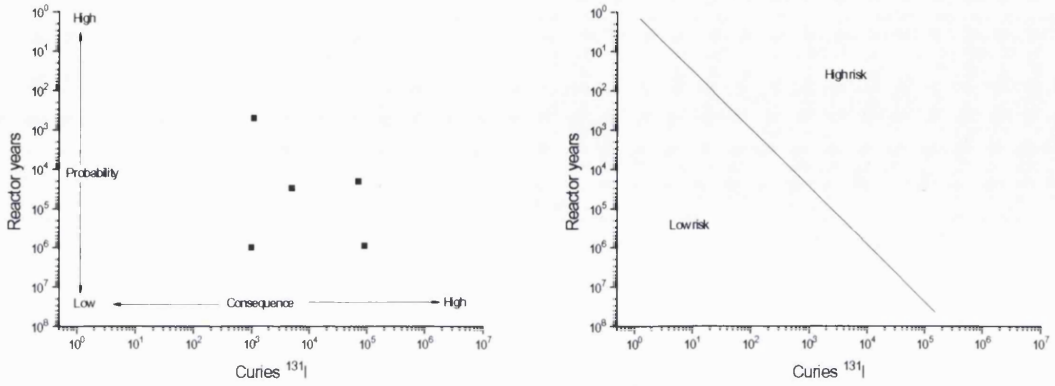
Firstly, the major features of QRA will be discussed. According to Tweedale (1992), risk assessment requires calculation of both the severity of the consequences of each postulated failure and the frequency of their occurrence (also known, respectively, as 'detriment' and 'probability' (Ferris *et al*, 1995b)). Suter (1985) has similarly identified the components in QRA as being effects assessment and exposure assessment, which are combined to arrive at an estimate of the risk. In this thesis, the sole concern has been to measure the release of micro-organisms from bioprocesses, this is a part of exposure assessment. However, in addition to release considerations, exposure assessment also

consists of models for transport and fate of released organisms. An example of an exposure assessment incorporating all of these facets is given by Winkler and Parke (1992). In this scenario it is calculated that the rate of release of micro-organisms into the environment from fermenter exit-gas, using a siphon to reduce the microbial burden, is less than 100 micro-organisms second^{-1} for a 50 m^3 fermentation. The dilution of the released aerosol in the atmosphere by horizontal and vertical diffusion was calculated according to a simple plume model. It was found that even at low wind speed the plume spread to a height of 300-400 m and a width of 800 m. The dilution that was calculated was sufficient to reduce releases of 1000 micro-organisms second^{-1} to a concentration of less than 1 micro-organism m^{-3} at ground level at a distance of less than 100 m. In addition, it was calculated that only a few micro-organisms sediment per m^2 per hour. Since continuous variations in wind speed and direction will prevent sedimentation at identical sites it was concluded that niches at ground level or on plants are generally only reached by single organisms. The likelihood of survival and multiplication in the environment is further reduced by the inability of many micro-organisms to survive aerosolisation and the competition that will be encountered from indigenous micro-organisms at the site of deposition.

The severity of consequences of a release (i.e. the effects or detriment) are related to the properties of the micro-organism released (pathogenicity, survivability and phenotypic traits) and the nature of the environment in which the micro-organisms locate. For instance, *E. coli* K-12 strains are unlikely to colonise the gut of healthy individuals, but immunu-compromised people might be subject to colonisation (see Section 1.3.2). This study has not revealed any new information about the detriment caused by a release of micro-organisms, but it is a prerequisite of effects assessment that the levels of release of micro-organisms likely to be encountered are known. Moreover, since there is no simple relationship between exposure and effect for micro-organisms (Suter, 1985), the scale and time frame of any release may well play an important role in determining the detrimental effect on health and the environment.

Earlier in this section it was mentioned that to complete a QRA it is necessary to determine the contributions to overall release from incidental and accidental sources. In this context, incidental releases can be defined as those with a probability of 1 per batch or per unit of time for continuous processes. Accidental releases will have a lower probability of occurrence. Since Farmer (1967) noted that "no engineering plant and no structure is entirely risk-free and there is no logical way of differentiating between 'credible' and 'incredible' accidents" it has been acknowledged that the most appropriate

format for capturing the results of a safety evaluation is a probability consequence diagram ((Ballard, 1993), Figure 7.1)



(A) (B)

FIGURE 7.1 Farmer diagrams showing probability consequence relationship for a hypothetical nuclear installation. (A) shows the results of a safety evaluation of the plant. Farmer suggested a line on such a diagram might be used as a safety criterion by defining an upper boundary of permissible probability for all fault consequences, shown in (B). (Adapted from Ballard (1993)).

Notably, in these Farmer diagrams the x-axis, the consequence measure, is plotted in easily determined units, in this case ground level release of ^{131}I. Although this is a suitable measure for the inherent safety of the plant itself, the current practice is to calculate consequence directly in terms of human or environmental harm, most commonly the number of human fatalities (Ballard, 1993). It is interesting to note that with the release of genetically modified micro-organisms the transformation of the x-axis scale from the number of micro-organisms released (analogous to ^{131}I ground level release) to human or environmental detriment is not yet possible. Indeed, in many instances it may be that there is no detriment caused even at high levels of exposure due to the inherent biological containment of genetically modified organisms used in contained facilities (see Section 1.3.3). The other major modification to Farmers original diagram that is currently employed is the use of frequency of events on the y-axis so that the expected consequence per unit time is simply the integral of the curve. These curves are commonly referred to as F-N curves since frequency (F) is plotted against number of fatalities (N).

A modified F-N curve can be used to quantify overall release of micro-organisms from bioprocesses and to thereby influence process design. For instance, the release of process *E. coli* cells via unfiltered exit-gas from a 1.5 L fermenter has been measured at 3.0×10^7 cells over a 5.5 hour period (Section 5.3). If this is considered to be normal

operation (usually exit-gas is treated in some way to reduce the microbial burden) then a point can be plotted on the frequency release curve at y (frequency) = 1 batch^{-1}, and x (release) = 3.0×10^7 cells. There is less certainty about the frequency and consequences of accidents, but there is some data in the literature. For instance, gross damage of fermenters due to large accidents such as explosion, earthquakes, terrorism and falling aeroplanes has been estimated at a frequency of 10^{-6} per fermenter per year (Cremer and Warner, 1982). Such an incident would release the whole contents of a fermenter, in this case equal to 2.7×10^{13} cells. However, there are more likely accidents that might occur that could lead to release of some of the contents of the fermenter. Bello and Colombari (1980) and Tweedale (1992) have noted that a high proportion of major accidents are not from predicted and assessed equipment (hardware) failures, but from human (software) errors such as poor plant isolation before maintenance, poor routine inspection, ill-considered plant modifications and poor operator training. The frequency of hardware and software failures depends on 2 factors; the inherent frequency for the operation; and the standard of management related to the hardware or software. In fact the frequency of accidents can be estimated using engineering and operational factors to modify the inherent hardware and human error generic failure rates (Tweedale, 1992). The provision of good management, operator training and the use of standard operating procedures in plant operation are clearly important tools in reducing the likelihood of accidents.

Bello and Colombari (1980) have developed a simple model, TESEO (Empiric Technique to Estimate Operators' Errors) in an attempt to evaluate the probability of human failure in plant operation. The model describes a plant control-room operator's failure probability as the product of 5 parameters linked to i) the type of activity to be carried out; ii) the time available to carry out the activity; iii) the human operator's characteristics; iv) the operator's emotional state; and v) the environmental ergonomic characteristics. Hesselink *et al* (1990) have used this model to assess the software induced failure probability during an industrial centrifugation harvesting operation. A failure probability of 0.04 - 0.18 per run was calculated suggesting that an undesirable event might happen per 5 - 25 batches. The scale of release caused by these events will vary. However, such occurrences fall between the unlikely catastrophes and the incidental releases that have already been discussed. A modified frequency consequence curve for the 1.5 L *E. coli* fermentation might therefore be constructed as in Figure 7.2.

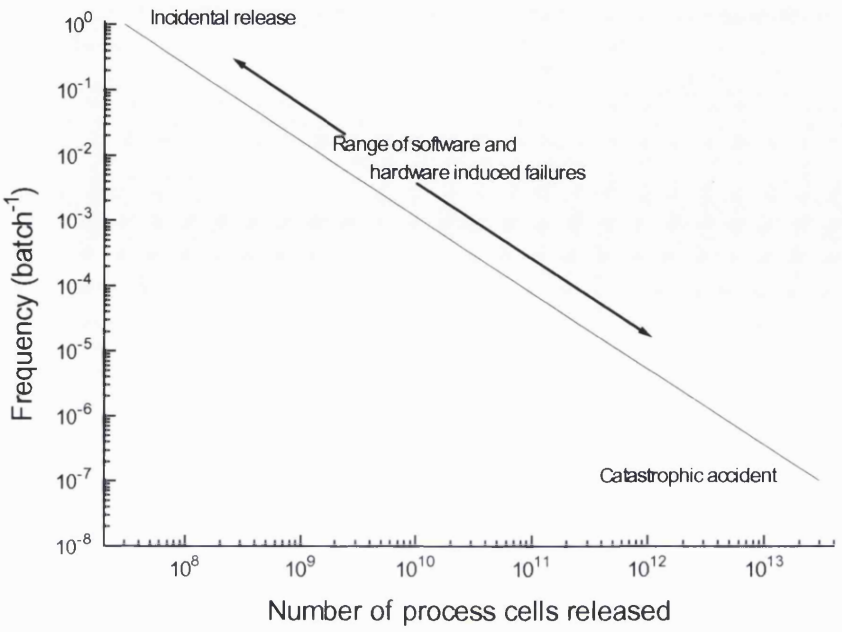


FIGURE 7.2 Possible frequency consequence plot for operation of 1.5 L *E. coli* fermentation with untreated exit-gas. The line drawn indicates a possible relationship between different levels of release and the frequency of their occurrence. In reality, the only point on the curve that is known is the level of incidental release and the relationship might not be a straight line. The plot assumes 10 batch fermentations per year.

It should be remembered in referring to this plot that if the x-axis were transformed to indicate actual detriment to health or the environment then the curve might have a different shape and gradient. This would be the case if a threshold number of cells were required to cause an effect and below this number a release would be harmless, or if a constant low level release of cells was more harmful than occasional significant releases. Assuming that the number of cells released is directly related to the detriment caused, then it can be said that both the magnitude of the releases represented on the x-axis and the gradient of the curve are important factors in determining process safety. Ballard (1993) has noted that, with regard to the nuclear industry, "most people would apply a relatively higher penalty against the possibility of a large scale release than a small release" and therefore the gradient of F-N curves should be greater than -1, in fact slopes of -1.5 to -2 are common.

In terms of process design, one implication of the above discussion is that when the incidental release of process micro-organisms is minimised or prevented it is important to ensure that the gradient of the frequency consequence curve is not lessened (see Figure 7.3). This might occur if the modification is such that operation of the equipment is

greatly complicated; 3 of the 5 parameters in the TESEO model could be affected by such a modification.

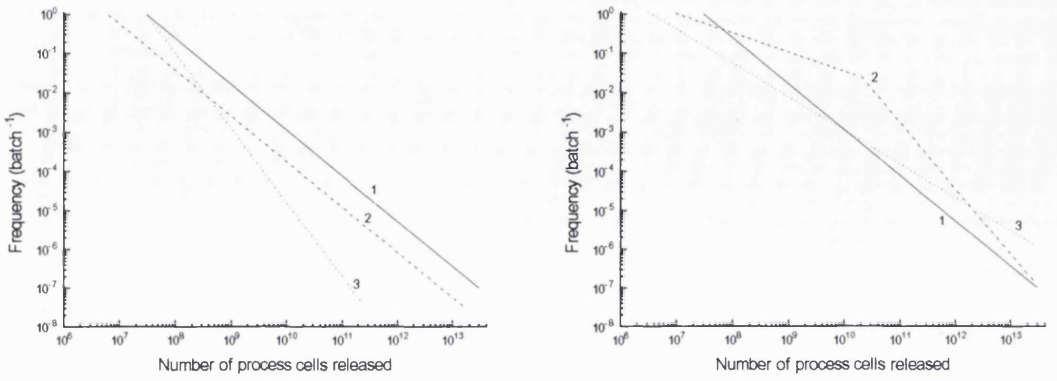


Figure 7.3 Possible effects on frequency consequence plot of modifying plant design. (A); Overall risk can be reduced relative to curve 1 by reducing the magnitude of the release at all frequency levels (curve 2) or by maintaining the level of incidental release (frequency = 1) whilst increasing the slope so that accidental releases are less likely (curve 3). (B); Overall risk can be increased, when incidental levels of release are lowered, by increasing the risk of software driven failures (curve 2) or by increasing the inherent failure rate of the plant (curve 3). Note that all figures are schematic.

A problem of the current legislation governing the contained use of genetically modified micro-organisms (Council of the European Communities, 1990; Commission of the European Communities, 1994) is that at containment levels B3 and B4 release of micro-organisms must be 'prevented'. Since there is always a risk of a credible accident, absolute prevention of release is impossible. If these risks are not acknowledged, then it is possible that processes will be designed where accidental releases are ill-considered and incidental (routine operational) releases are prevented by means of complex and costly engineering. In the chemical industry, where operating conditions are more hazardous than those used in bioprocessing, user friendly (forgiving) design has taken hold in the concept of inherently safer design (Kletz, 1996).

APPENDIX 1. LIST OF SUPPLIERS

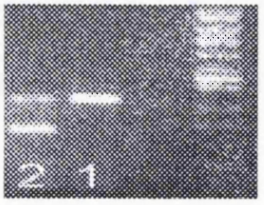
Adolf Kuhner Ag, Basel, Switzerland
Air Control Installations, Chard, Somerset, UK
Alfa-Laval, Camberley, Surrey, UK
Anachem, Luton, Bedfordshire, UK
APV, Crawley, West Sussex, UK
Bassaire Ltd, Swanwick, Southampton, UK
BDH Laboratory Supplies, Merck Ltd, Poole, Dorset, UK
Beckman Instruments, High Wycombe Buckinghamshire, UK
Bioengineering Ag, Wald, Switzerland
Biolog Inc., Hayward, California, USA
Broadley James Corporation, Santa Ana, California, USA
Carr Separations Inc., Easton, Massachusetts, USA
Celsis Ltd, Cambridge, UK
Constant Systems Ltd, Warwick, UK
Costar, Cambridge, Massachusetts, USA
Domnick Hunter, Birtley, Co. Durham, UK
Elwyn E. Roberts Isolators Ltd, Market Drayton, Shropshire, UK
Fisons Instruments, Middlewich, Cheshire, UK
Fisons Scientific Equipment, Loughborough, Leicestershire, UK
Gelman, Ann Arbor, Michigan, USA
GeneTrak Systems, Framingham, Massachusetts, USA
GenProbe, San Diego, California, USA
Gibco BRL Life Technologies, Uxbridge, Middlesex, UK
Glen Creston Ltd, Stanmore, Middlesex, UK
Hays Chemical, Leeds, UK
Hybaid, Teddington, Middlesex, UK
Ilford, Mobberley, Cheshire, UK
Incelltech (UK) Ltd, Reading, UK
MDH Ltd, Andover, Hampshire, UK
MSE, Crawley, Sussex, UK
New Brunswick, Edison, New Jersey, USA
New England Biolabs, Beverley, Massachusetts, USA
Nikon, Kingston upon Thames, Surrey, UK
Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK
Perkin Elmer, Foster City, California, USA

Pharmacia, Milton Keynes, Bedfordshire, UK
Promega, Madison, Wisconsin, USA
Qiagen, Chatsworth, California, USA
Sigma, Poole, Dorset, UK
Sigma Laboratory Centrifuges, Osterode, Harz, Germany
Silverson Machines Ltd., Chesham, Bucks, UK
Soham Scientific, Soham, Cambridgeshire, UK
Stratagene, La Jolla, California, USA
Teledyne Hastings-Raydist, Hampton, Virginia, USA
Th Goldscmidt Ltd, Milton Keynes, Bedfordshire , UK
UVP Ltd, Cambridge, UK
Warren Spring Laboratories, now at AEA Technology, Harwell, Didcot, Oxon, UK
Watson-Marlow, Falmouth, Cornwall, UK
Weber Scientific International Ltd, Teddington, Middlesex, UK
William Pearson Chemicals, Coventry, UK

APPENDIX 2. EXAMPLE OF CALCULATION OF pQR701 CONCENTRATION

Step 1. Agarose gel electrophoresis of PCR products

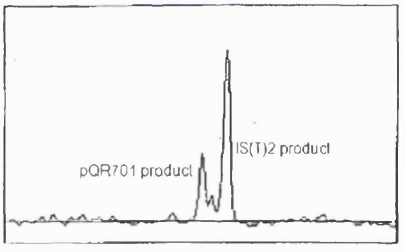
pQR701 product
IS(T) product



Gel shows the results of co-amplification of unknown [pQR701] with IS(T)2 (lane 2) and IS(T)1 (lane 1). The image indicates that the [pQR701] is in the range for IS(T)2, but the concentration is too high for IS(T)1. Lane 2 is therefore analysed by densitometry.

Step 2. Densitometry of lane 2

Densitometry allows measurement of peak areas of pQR701 and IS(T)2 peaks.



Peak I.D.	Peak area	log peak area ratio (IS(T):pQR701)
pQR701	333	0.59
IS(T)2	1300	-

Step 3. Calculation of [pQR701]

IS(T)2 regression line is described by:

$$\log(\text{peak area ratio (IS(T):pQR701)}) = 4.03 - (0.76 \times \log[\text{pQR701}])$$

$$\therefore \log[\text{pQR701}] = (4.03 - 0.59) / 0.76$$
$$= 4.53$$
$$\therefore [\text{pQR701}] = 3.4 \times 10^4 \text{ molecules/PCR}$$

(NB; the PCR sample volume is 10 μL).

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