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Optimization of degenerate oligonucleotide primed PCR for amplification of microdissected chromosome segments

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Optimization of degenerate oligonucleotide primed PCR for amplification of microdissected chromosome segments

A thesis submitted in fulfilment of the requirement of the award of the degree of

HONOURS MASTER IN SCIENCE

UNIVERSITY OF WOLLONGONG LIBRARY

by

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Bachelor in Genetics

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Master in Science

(The University of Wollongong, Australia)

from

SCHOOL OF BIOLOGICAL SCIENCES

THE UNIVERSITY OF WOLLONGONG

1994

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Summary

The degenerate oligonucleotide primer of sequence, 5' CCGACTCGAGNNNNNNATGTGG 3', contains a specific 3' hexanucleotide , a central random hexanucleotide and an *Xho*I site towards it's 5' end. This degenerate primer (Telenius *et al*, 1992) has been developed for the amplification and subsequent cloning of any source of target DNA, particularly the human chromosome, in a process termed degenerate oligonucleotide primed PCR (DOP-PCR). In the present study, bacteriophage lambda DNA was employed as a model target DNA to test a variety of DOP-PCR procedures. To achieve the highest efficiency, different concentrations of primer and Taq DNA polymerase and also a variety of PCR program cycles were tested. After achieving the optimal concentration of the DOP primer and Taq DNA polymerase, two different protocols were investigated. The first relies on low-temperature pre-amplification of the target DNA by modified T_7 DNA polymerase (Sequenase) and uses the amplified segments for a subsequent amplification catalysed by Taq DNA polymerase. With this protocol, amplified DNA was obtained with down to 0.2 pg DNA of template. The other procedure employs Taq DNA polymerase for all stages of amplification. We have developed a modified form of this strategy by boosting Taq DNA polymerase in the middle of the amplification program. This modification increases the efficiency and sensitivity of the procedure and enables us to amplify as little as 0.02 pg DNA.

The latter method should be of considerable value for amplification of DNA from a wide variety of sources, particularly human chromosomes, since it should reduce to a minimum the number of dissected chromosome fragments required. In fact, following the completion of this work, the method has been successfully applied to the analysis of a human chromosome translocation in collaboration with the Department of Cytogenetics, John Hunter Hospital, Newcastle.

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ABBREVIATIONS

ALL	acute lymphocytic leukemia
AP-PCR	arbitrary PCR
bp	base pairs
cM	centimorgan
CML	chronic myeloid leukemia
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOP	degenerate oligonucleotide primer
DOP-PCR	degenerate oligonucleotide primed PCR
dTTP	deoxythymidine triphosphate
EGTA	Ethyleneglycol tetraacetic acid
FISH	fluorescent *in situ* hybridization
IRS	interspersed repeat sequence
kD	kilodalton
Lambda DNA	λ DNA
M	molar
Min	minute
µl	microlitre
ml	millilitre
nm	nonometer
PCR	polymerase chain reaction

pg	picogram
RAWTs	RNA amplification with transcript sequence
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
TFA	trifluroacetic acid
Tm	melting temperature
UDG	uracil DNA glycosylase
UV	ultraviolet
°C	degrees Celsius

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1. Introduction

Section 1.1 The Polymerase Chain Reaction (PCR)

The standard polymerase chain reaction (PCR) uses two oligonucleotide primers which complement opposite ends of a target DNA segment, and which are oriented in such a way that DNA synthesis proceeds in the region between the primers. A standard reaction mixture contains the sample DNA, two primers, DNA polymerase, and four deoxynucleotide triphosphates (dNTPs) in a buffer solution. The template DNA is first denatured by heating. The reaction mixture is then cooled to a temperature that allows the primers to anneal to the target DNA after which the primers are extended by a DNA polymerase. The resulting extension products are complementary to, and capable of binding the two oligonucleotide primers. Through repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers, an exponential accumulation of the specific target fragment is achieved (Rose, 1991) (Fig. 1.1).

1.1.1 Reaction specificity

The initial PCR amplification with the Klenow fragment of *E. coli* DNA polymerase I were not highly specific. For example, Scharf and co-workers used this enzyme to amplify a 110 base pair segment of a human β-globin gene containing the Hb-s mutation. They modified the 5' end of the primer to provide convenient restriction sites for cloning directly into plasmid vectors (this modification did not effect the efficiency of PCR amplification of the specific β-globin segment). After amplification, the PCR products were cloned and colonies hybridized with a labelled oligonucleotide probe. The result showed that more than 80% of the clones contained DNA inserts containing the PCR primer sequences but only about 1% hybridized to the internal β-globin probe (Scharf *et al*, 1986).

By the introduction of a thermostable DNA polymerase, isolated from the thermophilic bacterium *Thermus aquaticus* (Taq DNA polymerase), the specificity of the reaction increased (Lawyer *et al*, 1989). Since it allows the primers to be annealed and extended at much higher temperatures than is possible with the Klenow fragment, its use eliminates much of the nonspecific amplification. Moreover, long PCR

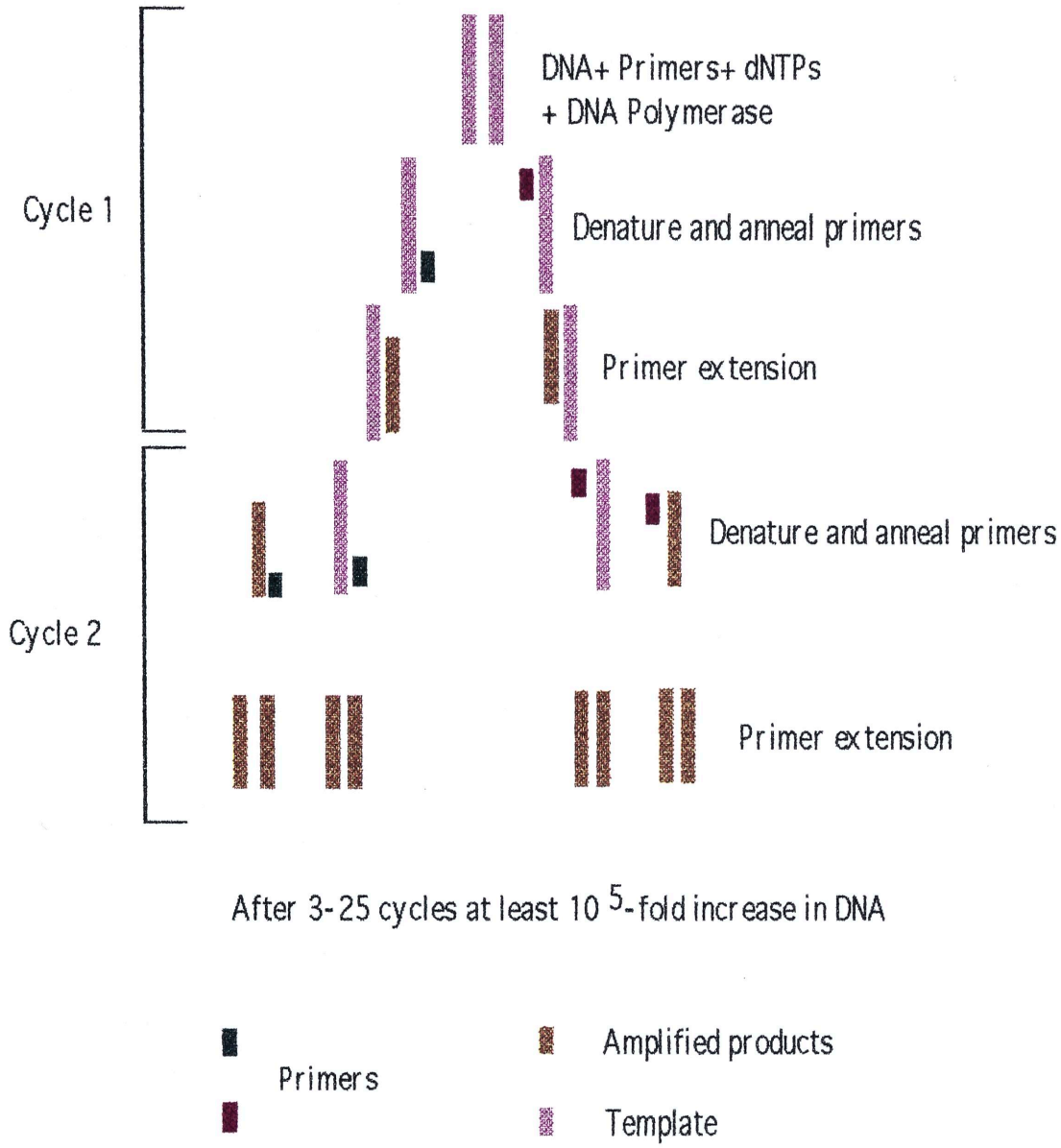

Figure 1.1 The polymerase chain reaction. DNA to be amplified is denatured by heating the sample. In the presence of DNA polymerase and excess deoxynucleotide triphosphates, oligonucleotides that hybridize specifically to the target sequence can prime new DNA synthesis. The first cycle is characterized by a product of indeterminate length; however, the second cycle produces the discrete "short product" which accumulates exponentially with each successive round of amplification. This can lead to a many million-fold amplification of the discrete fragment over the course of 20 to 30 cycles (Rose,1991).

products could be amplified, probably due to a reduction in the secondary structure of the template strands at the elevated temperature used for primer extension. The resulting products are long enough to be detected as a single ethidium bromide stained band on a electrophoretic gel (Saiki *et al*, 1988).

In addition, new approaches to improve specificity have been developed. These strategies are based on the recognition that the Taq DNA polymerase exhibits considerable enzymatic activity at temperatures well below the optimum for DNA synthesis. Thus in the heating step of the reaction, primers that anneal nonspecifically to a partially single stranded template region can be extended before the reaction reaches 72°C (Erlich *et al*, 1991). Some of these nonspecifically annealed and extended primers may be oriented with their 3' hydroxyl directed toward each other, resulting in the exponential amplification of a non target fragment. If the DNA polymerase is activated after the reaction has reached high (>70°C) temperature, non-target amplification can be minimized. This may be accomplished by addition of essential reagents (such as Taq DNA polymerase or $MgCl_2$) at elevated temperatures, an approach termed "hot start". Hot start not only improves specificity but minimizes the formation of so called "primer-dimer" (double stranded PCR products containing two primer molecules that have annealed with each another via complementary 3' sequences, followed by strand extension).

Another approach that can improve PCR specificity involves utilization of a two-step amplification employing single or double nested primers (Erlich *et al*, 1991). This approach utilizes sequence information internal to the two outer primers to define a subset of amplification products that corresponds to the target fragment (Mullis *et al*, 1987). In this procedure, two different pairs of specific primers (outer and inner) are used. In the first stage, outer primers amplify products that encompass the target sequence. In the second stage, the inner primers anneal within these products to give amplification of the actual target segment. However, a difficulty of this method is that it requires opening of the reaction tube, eliminating or decreasing the concentration of the original outer primers and adding the inner primers. This problem may be overcome if the outer and inner primers are all present in the initial

reaction mix and if the thermal profile is programmed to allow the outer primers, but not the inner primers to amplify initially and then to allow the inner but not the outer primers to amplify the target subset within the initial PCR products. This can be accomplished by using outer primers that have GC at the 5' end and with inner primers that are short or AT-rich. This strategy, termed "drop-in drop-out nested priming" can be aided by using limiting concentrations of outer primer (Erlich *et al*, 1991).

1.1.2 Parameters affecting the PCR

It is important to understand the factors that affect the experimental reproducibility of the PCR, before adoption of this technique. These parameters are:

1.1.2.1 The PCR buffer

Changes to the PCR reaction buffer will usually affect the outcome of the amplification. Particularly where large numbers of samples are to be handled, monovalent and divalent cation concentrations provide a means to control the stringency of the reaction.

Stringency generally falls sharply with an increase in KCl concentration up to a range inhibitory for the PCR (>0.2 M) due to stabilization of non specific double helical structures (Fig. 1.2). Doubling the potassium concentration will increase the DNA melting temperature by about 5°C. The underlying mechanism is the shielding of repulsion between charged phosphate ions of the DNA backbone, allowing the hydrogen bonding between bases to become the dominant force in double-strand formation. Thus, at higher concentrations of KCl an oligonucleotide can more easily bind to DNA sites of lower affinity. Furthermore, at potassium concentrations above 0.2 M, the PCR template-melting temperature is insufficient to form single-stranded DNA, and it is clear that amplification products cannot be formed (Blanchard *et al*, 1993).

The concentration of $MgCl_2$ can have a profound effect on the specificity and yield of an amplification. This parameter, which can vary even for different primers for the same region of a given template DNA, can have an enormous influence on the success of PCR. Generally excess Mg^{2+} will result in the accumulation of non-specific amplification

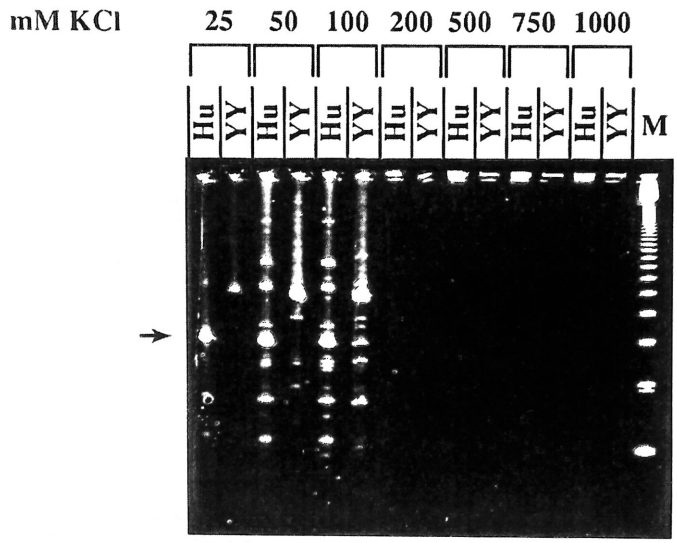

Figure 1.2 Effect of variation of potassium chloride concentration on PCR product formation. The potassium chloride concentration in the PCR was varied between 25 mM and 1 M as indicated. The template DNA included either 33 ng of total human DNA (Hu) or 33 ng of DNA from yeast strain AB1380 (YY). The arrow indicates the correct product size. The marker lane M shows a 123-bp ladder molecular size standard (Blanchard *et al*, 1993).

products and insufficient Mg^{2+} will reduce the yield (Fig. 1.3) (Saiki, 1989), due to effects on both DNA-DNA and DNA-protein interactions (Blanchard *et al*, 1993).

Specificity can sometimes be increased by lowering the magnesium concentration below 1.5 mM. Conversely, increasing magnesium concentrations above 4.5 mM can produce spurious amplification products (Bottema *et al*, 1993).

1.1.2.2 DNA polymerases

Among the advantages conferred by the thermostability of Taq DNA polymerase is its ability to withstand the repeated heating and cooling inherent in PCR and its ability to synthesize DNA at high temperatures that melt out mismatched primers and regions of local secondary structure. Usually 1 to 4 units of Taq polymerase per 100 µl reaction mixture is sufficient. Increasing the amount of enzyme beyond this level may result in greater production of non-specific PCR products and reduced yield of the desired target fragment (Saiki,1989). Moreover Taq DNA polymerase is very expensive.

Taq DNA polymerase has no 3' to 5' exonuclease ("proofreading") activity, but has a 5' to 3' exonuclease activity during polymerization (Erlich *et al*, 1991). The initial estimates of the misincorporation rate by Taq DNA polymerase during PCR were about 10^{-4} nucleotides per cycle based on the measuring frequency of nucleotide substitution by sequence analysis of cloned PCR products (Saiki *et al*,1988). However, changes in PCR conditions, such as lower $MgCl_2$ concentration have reduced the misincorporation rate to less than 10^{-5} nucleotide per cycle (Erlich *et al*, 1991). Moreover, other DNA polymerases like the bacteriophage T_4 DNA polymerase appear to have a very low misincorporation rate in PCR.

Genetically engineered variants of the Taq DNA polymerase such as AmpliTaq DNA polymerase and Stoffel Fragment exhibit increased processivity (i.e. the ability to proceed along the same chain without dissociating).

AmpliTaq DNA polymerase is a 94 kD thermostable recombinant DNA polymerase obtained by expression of a modified form of Taq DNA polymerase in *E.coli*. The thermal activity profile of this enzyme is

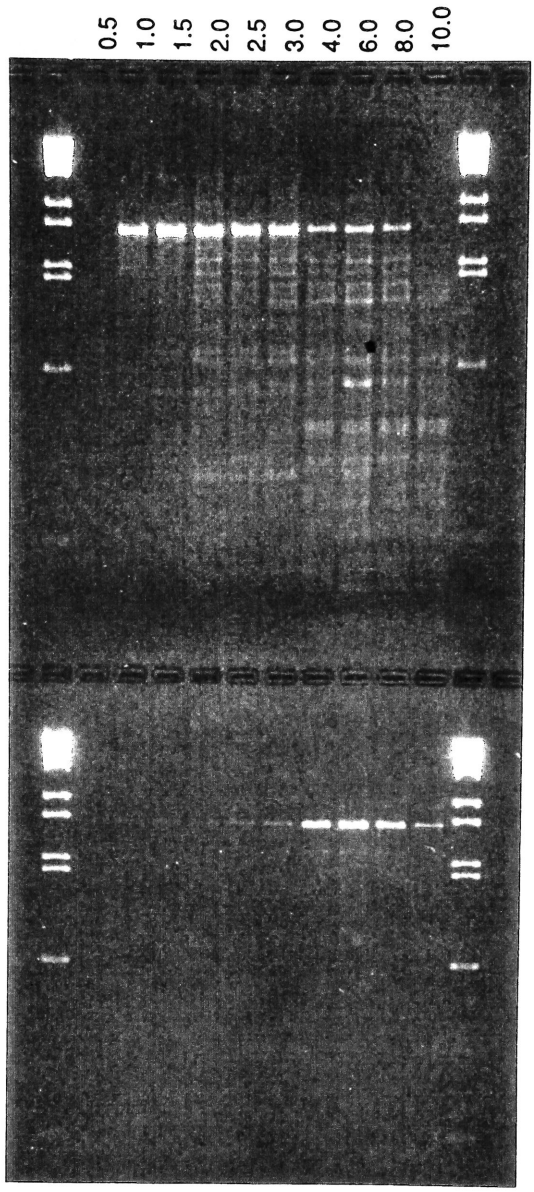

Figure 1.3 Effect of Mg^{2+} concentration on PCR specificity and yield. Two overlapping primer pairs that amplify a 1.8-kb fragment of the human β-globin gene were titrated with various concentrations of $MgCl_2$. Although similar in size and from the same region of β-globin gene, the primer pairs have very different magnesium optima (Saiki, 1989).

ideal for PCR applications because its optimal activity is in the range at which stringent annealing of primers occurs (55°C-75°C). The recombinant enzyme has a 5'-3' exonuclease activity and it lacks 3'-5' exonuclease activity (Wang *et al*, 1990).

Stoffel Fragment is a 61 kD highly thermostable recombinant DNA polymerase obtained by expression of a modified form of the Taq DNA polymerase gene cloned and expressed in *E. coli* (Wang *et al*, 1990). Stoffel Fragment differs from AmpliTaq DNA polymerase in that it is more thermostable (approximately two-fold at 97.5°C). It exhibits optimal activity over a broader range of magnesium ion concentration (2-10 mM) (Lawyer *et al*, 1993). In contrast with AmpliTaq DNA polymerase, it lacks any 5'-3' exonuclease activity. The enhanced thermostability of Stoffel Fragment enables the user to raise denaturation temperatures for improved amplification of templates known to be particularly G+C rich, or known to contain complex secondary structure. Stoffel Fragment generally requires higher magnesium concentrations and has optimal activity at lower ionic strength.

The ability to reverse transcribe RNA to cDNA and subsequently amplify the synthesized cDNA has become known as RNA PCR (Wang *et al*, 1989). A significant problem using RNA as a template, however, is the inability of standard reverse transcriptase to synthesize cDNA through stable RNA secondary structures. Elevation of the reverse transcription reaction temperature leads not only to destabilization of many RNA secondary structures, but also to increase specificity of primer hybridization and subsequent chain extension. Recombinant *Thermus thermophilus* (rTth) DNA polymerase is able to quickly and efficiently reverse transcribe RNA to cDNA in the presence of $MnCl_2$ at elevated temperatures (Myers *et al*, 1991), and act as a DNA polymerase for subsequent PCR amplification in the presence of $MnCl_2$, after chelation of the manganese ion with EGTA. rTth DNA polymerase can thus be used as both a thermostable reverse transcriptase and as a thermostable DNA polymerase in successive reactions in the same tube.

Increasing the processivity of the polymerase both aids the amplification of long sequences and minimizes the likelihood of occurrence of a rare artifact resulting from template strand switching. The potential for switching to another template molecule is greater when

amplifying sequences involved in multigene families (Paaba *et al*, 1990). The use of DNA polymerases with increased processivity, as well as conditions that increase processivity such as accessory proteins, may aid in achieving full primer extension. Thus using Tub polymerase with the capability of amplifying long DNA fragments (up to ca. 15 kb) could be beneficial (Kainz *et al*, 1992). Huber and co-workers have shown that a 12 kD *E. coli* protein (Thioredoxin) can act as an accessory protein to increase the processivity of T_7 polymerase up to 1000 fold, because it increases the half-life of the preformed primer-template-polymerase complex from less than a second to approximately 5 minutes (Huber *et al*, 1987).

1.1.2.3 Primer Selection

A. Primer concentration

Higher primer concentrations increase the yield of the amplification. In general, concentration ranging between 0.05 to 0.5 μM of each oligonucleotide should be acceptable (Saiki, 1989).

B. The primer length and sequence

The primer sequence complementary to the template should be 20 to 30 bases in length. It is unlikely that a longer complementary sequence will increase specificity significantly.

The distance between the primer annealing sites is rather flexible, ranging up to 10 kb (Ausbel *et al*, 1989). Sequences not complementary to the template can be added to the 5'-end of the primers. These exogenous sequences provide a means of introducing restriction sites or regulatory elements at the ends of the amplified target sequence.

The following conclusions were drawn by Bottema *et al* (1993):

a) When the 3' base of the primer mismatched a template, no amplification product could be detected.

b) When the mismatches were 3 or 4 bases from the 3' end of the primer, differential amplification was still observed, but highly dependent on concentration of magnesium chloride.

c) A primer as short as 13 nucleotides (Tm=36°C) was effective, although high magnesium concentrations were required (>4.5 mM).

The primer should have a G-C content similar to that of the template. Primers with unusual sequence distributions such as stretches of polypurines or polypyrimidines are to be avoided.

Primer-dimers are an amplification artifact often observed in the PCR product, especially when many cycles of amplification are performed on a sample containing very few initial copies of template. These are double-stranded fragments whose length is close to the sum of the lengths of the two primers and appears to occur when one primer is extended by the polymerase over the other primer. The exact mechanism by which primer dimer formation occurs is controversial. The observation that primers with complementary 3'-ends are predisposed to dimer formation suggests that transient interactions that bring the termini in close proximity provide the initiating event (Saiki, 1989).

Primer-dimer formation, can best be avoided by using primers without complementarity especially in their 3' ends. Furthermore, this problem can be minimized by booster PCR, a biphasic amplification strategy. During stage I, primers are in relatively low concentrations giving an initial 10^7-fold molar excess over template. At the beginning of stage II, primer concentration is brought to 0.1 µM (Fig. 1.4) (Ruano *et al*, 1989). This procedure differs from standard PCR methods which use 0.1-1.0 µM primers from the outset.

1.1.2.4 Deoxyribonucleotide triphosphates

Deoxyribonucleotide triphosphates (dATP, dTTP, dCTP, and dGTP) are usually present at 50 to 200 µM each. Higher concentration may tend to promote misincorporation by polymerase. Moreover, dNTPs at higher concentrations chelate magnesium ions and thereby change the effective optimal magnesium concentration. It has been demonstrated that decreasing the concentration of dNTPs to 25-50 µM can prevent spurious amplification (Bottema *et al,* 1993).

1.1.2.5 Template

The two main concerns regarding template are purity and amount. The DNA from a small number of tissue-culture cells was made accessible to PCR merely by the lysis of the cells during a heat-denaturation step. It is

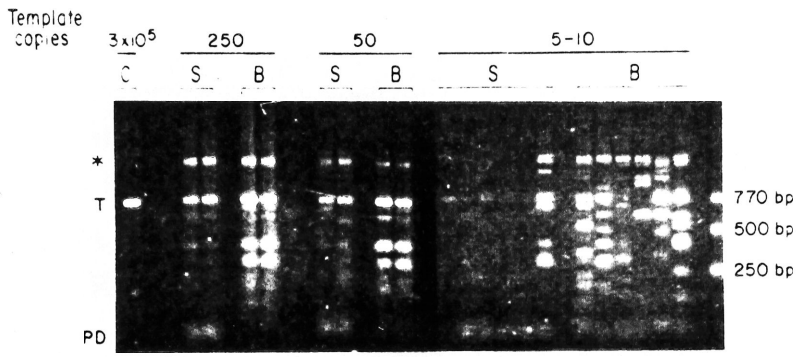

Figure 1.4 Comparison of Booster ("B") and Standard ("S") PCR. Stage I of "B": primers and template are diluted in the same proportion, 83 pM primers for 250 copies, 17 pM for 50 copies 2.5 pM for 5-10 copies; 20 cycles, 94°C denaturation (1 min), 45°C annealing (4 min), 72°C polymerization (3 min). Stage II of "B": primers brought up to 0.1 μM, 50 further cycles but annealing for 2 min. "S": same copy number as "B" but with 0.1 μM primers from outset; 50 cycles as in stage II of "B" (Ruano *et al*, 1989).

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obvious that the more template molecules available, the less likely are false results due to either cross-contamination between samples or "carry over"of analogous PCR products from earlier amplification. However, use of too much template may decrease efficiency due to contaminants (such as the detergent SDS, sodium acetate, etc) in the DNA preparation.

Sample preparation for PCR can be as simple and rapid as adding cells directly to the PCR reagent mixtures (Saiki *et al*, 1986). DNA and/or RNA molecules must be released from large numbers of cells in a form suitable for PCR while preserving the activity of DNA polymerase. Since Taq DNA polymerase activity is not significantly affected by certain non-ionic detergents, and since proteinase K may be inactivated by heat, cells can be added directly to a PCR mixture containing non ionic detergents and proteinase K but not containing Taq DNA polymerase. After residual proteinase activity is destroyed by incubation at 95°C for 1 min, Taq DNA polymerase is added and amplification cycles begin.

Another important strategy for template preparation involves a microdissection technique to physically excise the crucial chromosomal region, as will be explained later.

1.1.2.6 Thermal cycling parameters

It is critical that the DNA template denatures completely and the primers anneal stably to the template. Templates and primers with high G-C content require higher denaturation and annealing temperatures. Raising the PCR annealing temperature can increase specificity.

The time of extension depends mainly on the length of the sequences to be amplified. The number of cycles makes little difference to specificity (Bottema *et al*, 1993).

1.1.3 Contamination of PCR reactions

Because the PCR can generate 10^{12} of DNA copies from a template sequence, contamination of amplification reactions with products of a previous PCR reaction (product carryover), exogenous DNA, or other cellular material can create problems. Degenerate oligonucleotide primed PCR (see below) is more affected by traces of contaminating DNA than normal PCR.

Reagents suspected of being contaminated can be pre-irradiated with ultraviolet light, which causes dimerization of adjacent pyrimidines in DNA. Cyclobutane pyrimidine dimers (TT cyclobutane dimers in particular) are the most frequent form of UV-induced damage (Haseltine, 1983). These modified sequences can no longer act as a template for polymerization and, as a consequence, the DNA strand will not be amplified.

The amount of UV radiation needed for decontamination depends on many factors. These include:
a) the number of template copies,
b) the amount of contaminant DNA,
c) the percentage of adjacent pyrimidines per template molecule,
d) the presence and quantity of other UV absorbent molecules,
e) the DNA length (smaller sequences being less sensitive than longer ones),
f) and other parameters including tube colour (Sarker *et al*, 1990)

Care must be taken when deciding which PCR reagents should undergo UV pretreatment. For example, 10x reaction buffer and the water needed to dilute, should always be pretreated. Furthermore, Sarker and co-workers have demonstrated that the 5-min UV treatment reduced the efficiency of the Taq DNA polymerase (Sarker *et al*, 1990). Thus, the DNA polymerase should be added after the irradiation-inactivation step.

Moreover, it has been shown that absorption of UV light by dNTPs reduced the efficiency of PCR decontamination 16-fold and therefore PCR mixtures should be irradiated before dNTPs are added (Frothingham *et al*, 1992)). If dNTPs stocks became contaminated by DNA, centrifugal microcentrators are available that efficiently separate dNTPs from high molecular weight DNA.

Another approach for decontamination of PCR reagents is the use of Psoralen. Psoralens are known to intercalate into double-stranded nucleic acids and form a covalent interstrand cross-link after photoactivation with incident light of wavelength 320-400 nm.

Jinno and co-workers have shown that the Psoralen/UV treatment clearly eliminated the template activity of DNA (Fig. 1.5, Lane 1), while Psoralen or UV alone had no effect on the template activity of the DNA (Fig. 1.5, Lanes 2 &3). Fortunately the treatment did not seem to injure

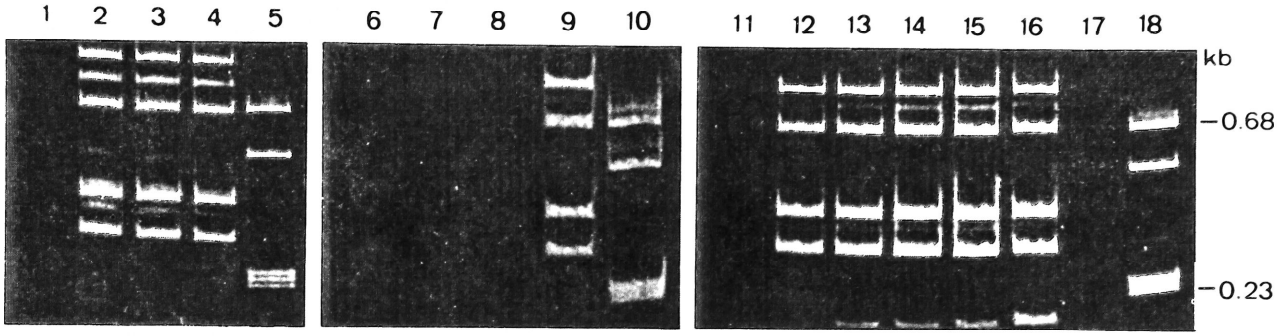


Figure 1.5 *Sau*3 A1 digest of pUC19 was treated with psoralen and UV under various conditions. Lanes 1, 6-8 and 11 are psoralen/UV-treated target DNAs; lanes 2 and 13, psoralen treatment alone; lanes 3 and 14, UV-irradiation alone; lanes 4, 9, and 15, positive controls; lane 12, psoralen/UV-treatment of PCR reaction mixture before adding the ligation mixture containing target DNAs and the primer alone 16, the addition of 10-fold reduced amount of the target DNAs and primer to the same pre-treated PCR reaction mixture as in lane 11 (Jinno *et al*, 1990).

Taq DNA polymerase and dNTPs (Fig. 1.5, Lane 12) and a primer oligonucleotide (Fig. 1.5, Lane 16)(Jinno *et al*, 1990).

An alternative method is treatment of individual reaction mixtures before adding template DNA and Taq DNA polymerase with DNaseI or restriction endonucleases that cut internal to the pair of amplification primer sites and prevent amplification of contaminating DNA. Furrer *et al* (1990) have shown that DNaseI treatment reduces contamination by a factor of 1,000 and the efficiency of PCR is not reduced by this method.

However, a problem encountered with enzymatic destruction is that preparation of DNaseI and restriction enzymes may themselves contain DNA. In addition, enzymes are powerless to remove DNA contaminants in preparations of proteinase K.

Another strategy suggested by Longo *et al* (1990), is that carry-over contamination can be controlled by the following two steps: (i) incorporating dUTP in all PCR products (by substituting dUTP for dTTP, or by incorporating uracil during synthesis of the oligodeoxyribonucleotide primers, and (ii) treating all subsequent fully preassembled starting reactions with uracil DNA glycosylase (UDG), followed by thermal inactivation of UDG. UDG cleaves the Uracil base from the phosphodiester backbone of the Uracil-containing DNA, but has no effect on natural DNA.

An alternative inactivation method involves the photochemical modification of the previously amplified DNA by isopsoralen, thereby blocking the Taq DNA polymerase from further extension after it encounters a modified base in the template strand (Issac *et al*, 1990).

1.1.4 Limitations of PCR

An apparent limitation of PCR is the requirement for specific sequence information to design the amplification primers.This limitation has been overcome by a variety of specific strategies.

The general approach has been to create primer binding sites by adding DNA of known sequences. It was initially illustrated by the amplification

of cDNA sequences inserted in the lambda phage gt11 cloning vector with primers that flank the *Eco*RI insertion site (Saiki *et al*, 1988).

One strategy for analysis of unknown sequences that flank a region of known sequence involves the digestion of the template with a restriction enzyme that cuts outside the known sequence. The resulting fragment is then circularized by ligation and amplification is carried out with primers that hybridize to the known sequence but whose 3' hydroxyls point away from each other. After circularization, the unknown sequences that had flanked the known region become joined between the 3' termini of the primers and can therefore be amplified and analyzed (Fig. 1.6).

Performing PCR with primers complementary to repetitive DNA families represents another approach to amplify unknown sequences. The overall distribution pattern of interspersed repeats in human DNA, for example, indicates that on average approximately 20% of the DNA in a given segment of human DNA corresponds to repetitive sequences (Moysis *et al*, 1989).

The *Alu* family is the most common short interspersed repeat sequence (IRS) in the human genome. *Alu* PCR has been used to obtain fingerprints of human DNA in hybrid cell lines for identification after amplifying the human sequences from human/mouse hybrid cells (Nelson *et al*, 1989).

The use of primers complementary to different repeated sequence families has also been reported. Primers with the combination of a single *Alu* primer and the L14 primer yield a larger numbers of distinct smaller products than those observed with either primer alone (Ledbetter *et al*, 1990).

Primers with repetitive sequences can be also used in cases where genomes are mixed such as in a human/rodent somatic cell hybrid. However, these repeats are not uniformly distributed. *Alu* elements, for example, are preferentially found in G-light bands of human chromosomes (Telenius *et al*, 1992). Furthermore, this strategy is only applicable to those species where abundant widely dispersed repeat families have been identified, whereas other species such as *Drosophila* are not amenable to this type of amplifcation

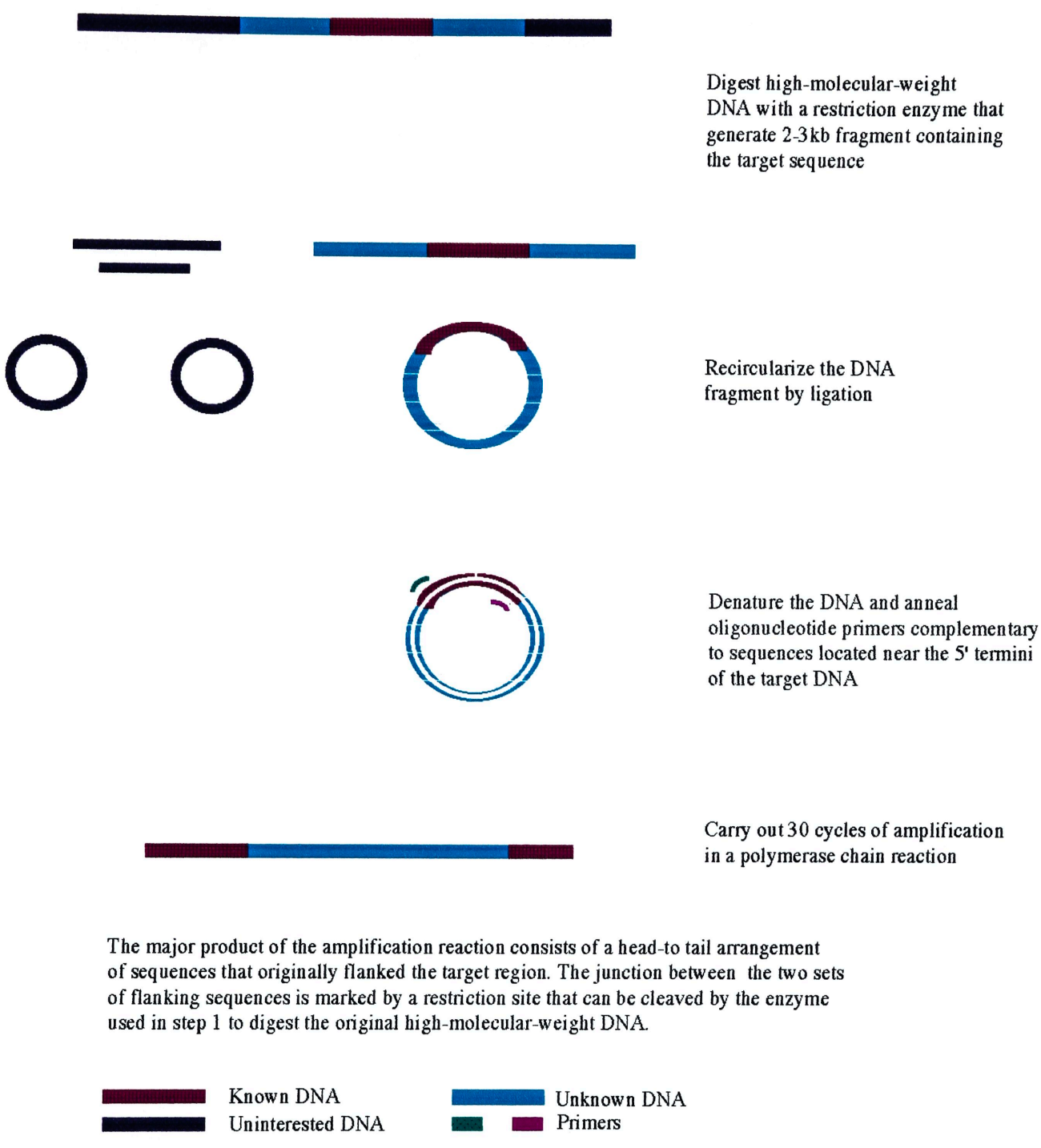


Figure 1.6 Amplification of unknown sequences that flank by a known sequence is conducted by digesting outside the known sequence, ligating, and amplifying with primers hybridising to the known sequence (Ausbel *et al*, 1989).

The use of transposons carrying unique primer binding sites which can integrate at random into the genome, represents a potentially powerful technique. This requires that a locus contains sites suitable for the insertion of a transposable element. After transposon integration, primers complementary to the transposon sequences can be used to amplify DNA fragment from the cloned inserts (Phadnis *et al*, 1989).

Another strategy involves employing oligonucleotides of partially degenerate sequence (DOP) to act as universal primers and offers advantages over the use of primers complementary to repetitive sequence (Telenius *et al*, 1992).

The surprising finding that amplification of genomic DNA can be detected using only one oligonucleotide primer of arbitrary sequences to produce a characteristic spectrum of short DNA products of varying complexity, termed Arbitrary PCR (AP-PCR) provides another approach. In Arbitrary PCR, instead of using a pair of highly specific and long oligonucleotide primers to amplify a specific target sequence, a single, short oligonucleotide primer five or more nucleotides in length, which binds to many different loci, is used to amplify sequences from complex DNA templates. Temperature cycling in the presence of thermostable DNA polymerase produces a range of short amplified DNA products of different length (Fig. 1.7).

The primer is generally of random sequences biased to contain at least 50% Gs, and Cs and to lack internal inverted repeats. The products are easily separated by standard electrophoretic techniques and visualized by UV illumination of ethidium bromide stained gels (Williams *et al*, 1990), or polyacrylamide gel electrophoresis and silver staining (Caetano *et al*, 1991).

1.1.5 Applications of PCR

1.1.5.1 *In vitro* expression

Regulatory elements such as promoters and translation initiation signals can be added to allow expression of the PCR products *in vitro*. One of these methods, known as RNA amplification with transcript sequencing (RAWTs), incorporates a phage promoter sequence into at least one of the PCR primers. RAWTs consists of four steps:

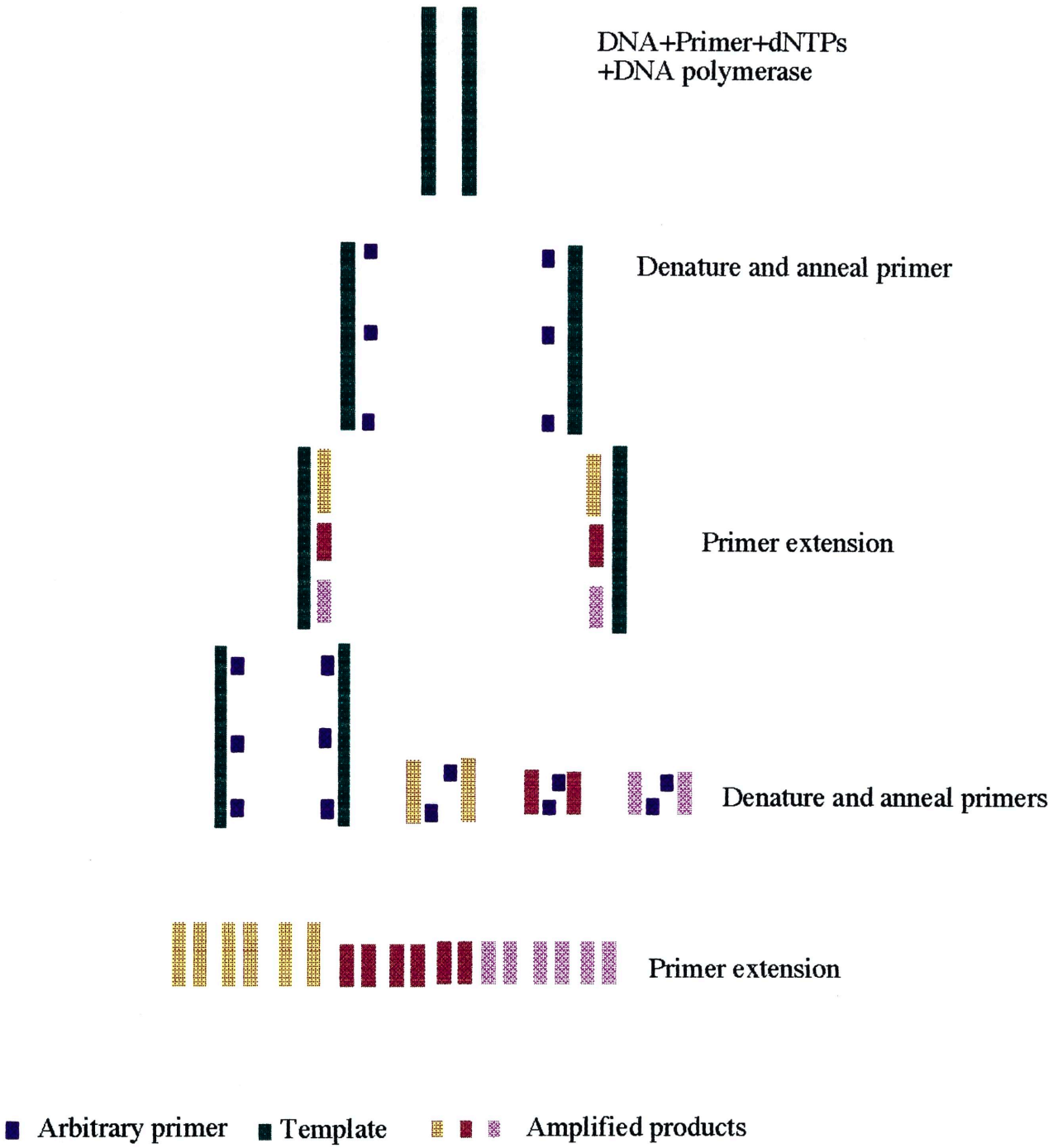
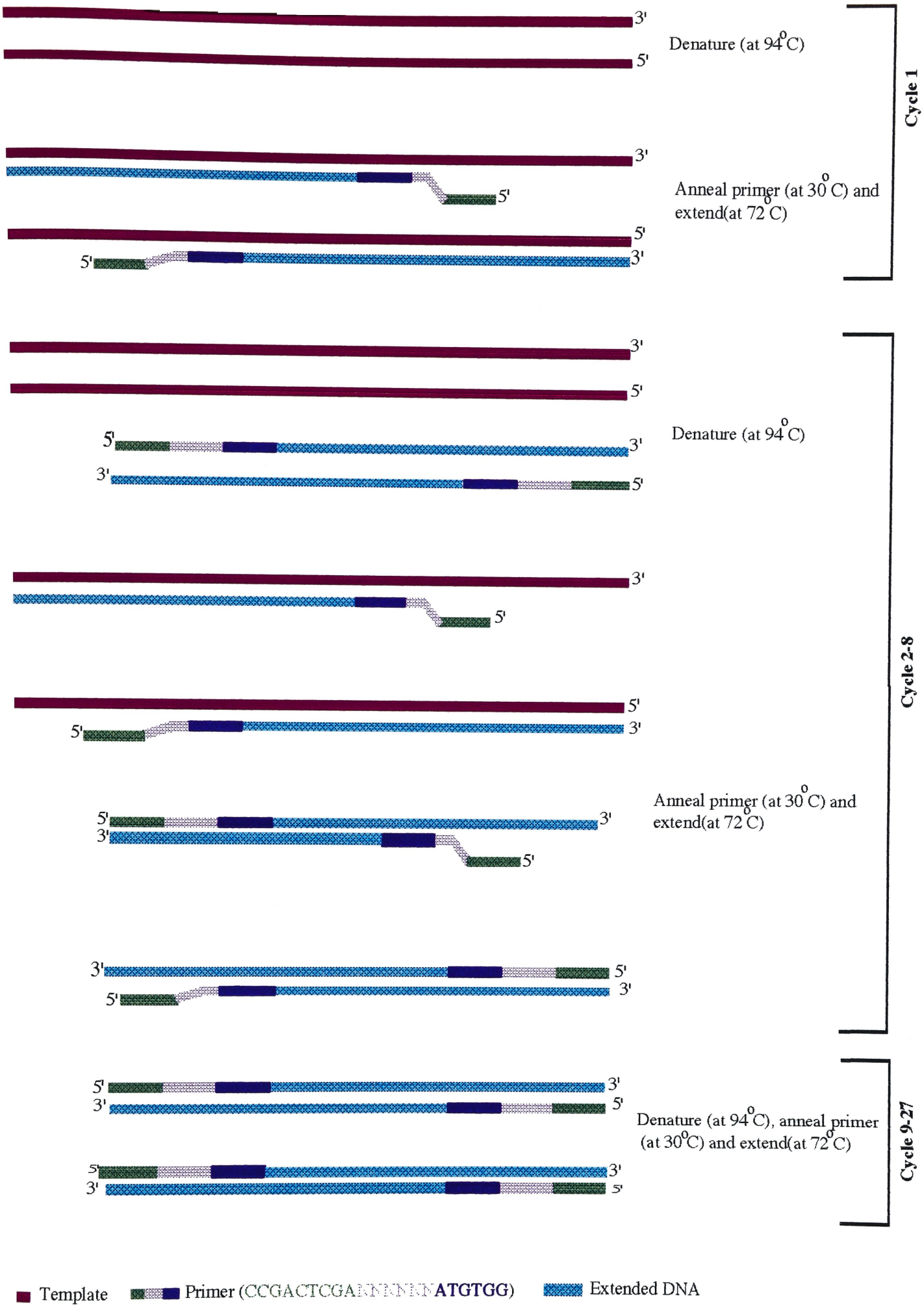


Figure 1.7 Arbitrary PCR. The arbitrary primer hybridizes to the different regions of the target sequence and produces many different DNA fragments.



igure 1.7 B. Diagrammatic representation of DOP-PCR. See text for details.

i) cDNA synthesis with oligo dT or a mRNA specific oligonucleotide primer,
ii) PCR where one or both oligonucleotides contains a phage promoter attached to a sequence complementary to the region to be amplified,
iii) transcription with a phage promoter,
iv) reverse transcriptase mediated dideoxy sequencing of the transcript which is primed with a internal (nested) oligonucleotide (Stoflet *et al*, 1988).

Once a mRNA has been amplified distal to a phage promoter, it is also possible to obtain the protein product by *in vitro* translation or by insertion into an appropriate expression vector. Sarkar and his colleagues added an eight-nucleotide translation initiation signal (CCACCATG) to the T7 promoter sequence of a PCR primer. When the PCR product was transcribed in the presence of Tm GpppG, a capped RNA containing a predicted 5' untranslated leader of only 11 bases and 3' untranslated region of 146 bases was obtained and could be translated *in vitro* (Sarker *et al*, 1989).

1.1.5.2 PCR and footprinting

The form of analysis of protein-DNA interactions, known as footprinting, is performed *in vitro* by the determination of protection from chemical or enzymatic cleavage conferred by a protein binding onto a specific nucleotide sequence. *In vivo* footprinting identifies the guanine base in a DNA segment protected from methylation by bound protein. Since only methylated guanosine can be cleaved by piperidine, this approach makes possible the determination of protein-DNA interaction sites within a cell. A modification of this *in vivo* procedure that utilizes PCR amplification with ligated primer sites to increase the amount of each cleaved genomic fragment has made genomic footprinting much more sensitive (Erlich *et al*, 1991).

1.1.5.3 PCR and construction of genetic maps

The use of restriction fragment length polymorphism (RFLP), has made a contribution to the construction of genetic maps. RFLP analysis gives a measure of genetic distances down to about 10 cM (Erlich *et al*, 1991).

RFLP and other markers combined with pedigree analysis have allowed the measurement of linkage down to about 1 cM (corresponding to about 1000 kilobases of DNA). The analysis of smaller distances requires such a large number of individuals as to be impractical (Erlich *et al*, 1991).

Sperm typing is beginning to make it possible to measure genetic recombination over shorter physical distances without family studies. Instead of comparing somatic DNA sequences of parents and their children, sperm typing relies on comparisons between the DNA of individual spermatozoa. The sperm donor must of course be heterozygous at each of the loci being studied. Following PCR, the allele at each locus is identified by analysing the amplified products. Thus the genetic recombination fraction between DNA polymorphisms can be estimated by dividing the number of recombinant sperm by the total number of typed sperm samples. Table 1.1 summarizes some of the features of sperm typing in comparison with traditional pedigree analysis (Arnheim *et al*, 1990).

1.1.5.4 PCR and molecular evolution

Nucleotide sequences in DNA provide the most informative set of characters for the reconstruction of the evolutionary history of species, as well as bacteria or virus phylogeny. Phylogenetic trees may be generated based on the nucleotide sequences of a given gene from different individuals in various contemporary species (Rose, 1991). PCR has been used to amplify mitochondrial DNA sequences from different human populations, allowing the construction of phylogenetic trees that confirm the African origins of humans (Vigiland *et al*, 1991). In addition, the MHC DNA sequences from a 7500-year-old mummified human brain have been amplified and sequenced. The sequences have been compared with those of modern humans and conceptually translated to give amino acids sequences of the proteins present in ancient humans (Lawlor *et al*, 1991).

1.1.5.5 Diagnostic applications of the PCR

The initial diagnostic application of PCR was in the prenatal diagnosis of sickle-cell anaemia through the amplification of ß-globin

sequences. Hybridization of labelled oligonucleotide probes or restriction site analysis of the amplified products allowed the distinction of normal and mutant alleles. In addition the amplification of specific loci by PCR made use of non-radioactive allele specific oligonucleotide (ASO) probes in a dot blot hybridization test, a rapid method for genetic typing. This approach depends on the stability of binding of a probe mismatched with the target sequence (Saiki *et al*, 1986).

Another method of allelic discrimination that uses PCR is based on the priming step (allele-specific amplification). This method utilizes primers which discriminate between alleles and allows direct detection of a normal or mutant allele in genomic DNA without probe hybridization, ligation or restriction enzyme cleavage. Two allele specific primers, one specific for the mutant allele and one specific for the normal allele, together with another primer complementary to both alleles are used in PCR with genomic DNA templates. The PCR products from the two alleles may be distinguished by length or by the differential labeling of the two primers (Wu *et al*, 1989).

In the area of cancer research, PCR has played an important role in the identification of chromosomal abnormalities and specific somatic mutation in oncogenes and tumour suppressor genes. PCR has been used, for example, for detecting leukemia using mRNA as the starting material for CML (Chronic Myeloid Leukemia) and ALL (Acute Lymphocytic Leukemia) diagnosis (Kawsaki *et al*, 1988).

The detection of specific pathogen sequences by PCR also promises to be important in infectious disease diagnosis and environmental testing, particularly when the pathogen is difficult to culture (Erlich *et a*l, 1991).

Finally, PCR has made prenatal testing of fetal amniotic fluid or chronic villus biopsy simpler and faster. The ability to amplify a DNA segment from a single cell has allowed genetic testing to be carried out on *in vitro* fertilized eggs before implantation.

Table. 1.1: Comparison between Human Pedigree Analysis and Sperm Typing (Arnheim *et al*, 1990).

	Family studies	**Sperm typing**
Usual available sample size of informative meiotic products	<500	Unlimited
Family structure required	Two or more generation	Single male
Measures recombination in both males and females	yes	No (male only)
Resolvable genetic distance	>1-2 cM	0.1 cM
Nature of polymorphic markers that can be mapped	Phenotypic traits, DNA polymorphisms	Only DNA
Requirement for sequence information at each polymorphic region	No	Yes
Chromosome interference can be measured directly	Not usually	Yes

Section 1.2. Microdissection

Molecular analysis and fine structure mapping of the human genome requires isolation of large numbers of DNA probes from defined region of the chromosomes. In addition, the effective use of genetic linkage analysis with polymorphic DNA probes from the human genome has localized more and more loci associated with inherited diseases of unknown etiology, as well as mapped specific forms of cancer to refined region of various chromosomes. Thus, the next major task is to isolate the genes underlying these diseases for better understanding, prevention, diagnosis and treatment.

A direct approach to this objective is to use the chromosome microdissection technique to physically excise the chromosomal region of interest and to clone the dissected chromosomal DNA by a microcloning procedure. Scalenghe *et al* (1981) first described how specific chromosomal regions that correspond to as little as 100 kb of the genome of *D. melanogaster* could be dissected from polytene chromosomes and cloned in a bactriophage lambda vector (Saunders, 1990). This approach has been applied later to mammalian metaphase chromosomes (Rohme *et al*, 1984).

Recently several groups have increased the power of chromosomal microdissection by applying the PCR technique to amplify DNA sequences dissected from chromosomes (Mackinnon *et al*, 1990; Davis *et al*, 1990 ; and Fiedler *et al*, 1991).

1.2.1 Description of Methodology

During microcloning, reactions are carried out in volumes of about one nanolitre (10^{-6} ml). All the manipulations are performed in an oil chamber to prevent evaporation of the reaction drop (Fig. 1.8). The chromosomes to be dissected and the reaction drops are located on the lower surfaces of coverslips bridging the groove of the oil chamber and the whole oil chamber is mounted on the stage of a compound microscope (Saunders, 1990). In Fig. 1.9 a schematic illustration of microcloning operations is presented.

Microneedles for dissecting the chromosomes are prepared by pulling out glass rods using a needle puller. Micropipettes are fashioned from

capillary tubing using a microforge. The desired fragment is cut from the chromosome with microneedles and transferred to the reaction droplet. Micropipettes are then used to add nanolitre volumes of proteinase K extraction buffer and phenol in order to purify the DNA from the chromosomal proteins. If microcloning is desired the DNA is then digested with a restriction enzyme, usually *Eco*RI. The enzyme is ultimately inactivated by heat treatment and the *Eco*RI cleaved vector DNA and T_4 DNA ligase are then added. Generally, a lambda insertion vector has been used. The ligated DNA is removed from the oil chamber, and packaged *in vitro*.

Microdissection can also be carried out using a laser microdissector. In this method, a laser beam is used to destroy all unwanted chromosomes as well as the unwanted segments within the relevant chromosome in a metaphase cell, leaving the selected fragment intact for isolation and subsequent cloning (Kao, 1993).

The chromosome regions to be excised are trimmed by irradiation with a finely focused argon ion laser beam. For precise trimming of the chromosome during laser microbeam treatment, the laser beam and a scanning area can be arbitrarily changed with the controlling computer program. This allows dissection of any particular region (down to 0.1 μm, approximately a few megabases). Dissection of chromosomes within a very short time after metaphase preparation is also possible. Furthermore, no physical manipulation of the desired fragment is required, which also decreases the chance for loss or breakage of chromosomal DNA sequences. A block diagram of the laser microdissector is shown in Fig. 1.10 (Hadano *et al*, 1991).

1.2.2 Microcloning and microamplification (micro-PCR)

There are several approaches for microamplification (micro-PCR). The first method was suggested by Ludecke *et al* (1989). In this procedure the purified DNA was cleaved with *Rsa*I, blunt-ligated into a *Sma*I-cut pUC vector, cleaved again with *Sma*I and amplified by PCR using pUC sequences as primers. The amplified sequences were then cloned into pUC13 (Fig. 1.11).

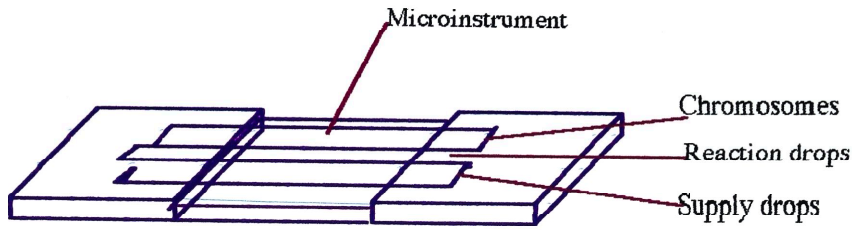


Figure 1.8 The oil chamber in which biochemical reactions on a microscale can be performed (Saunders, 1990).

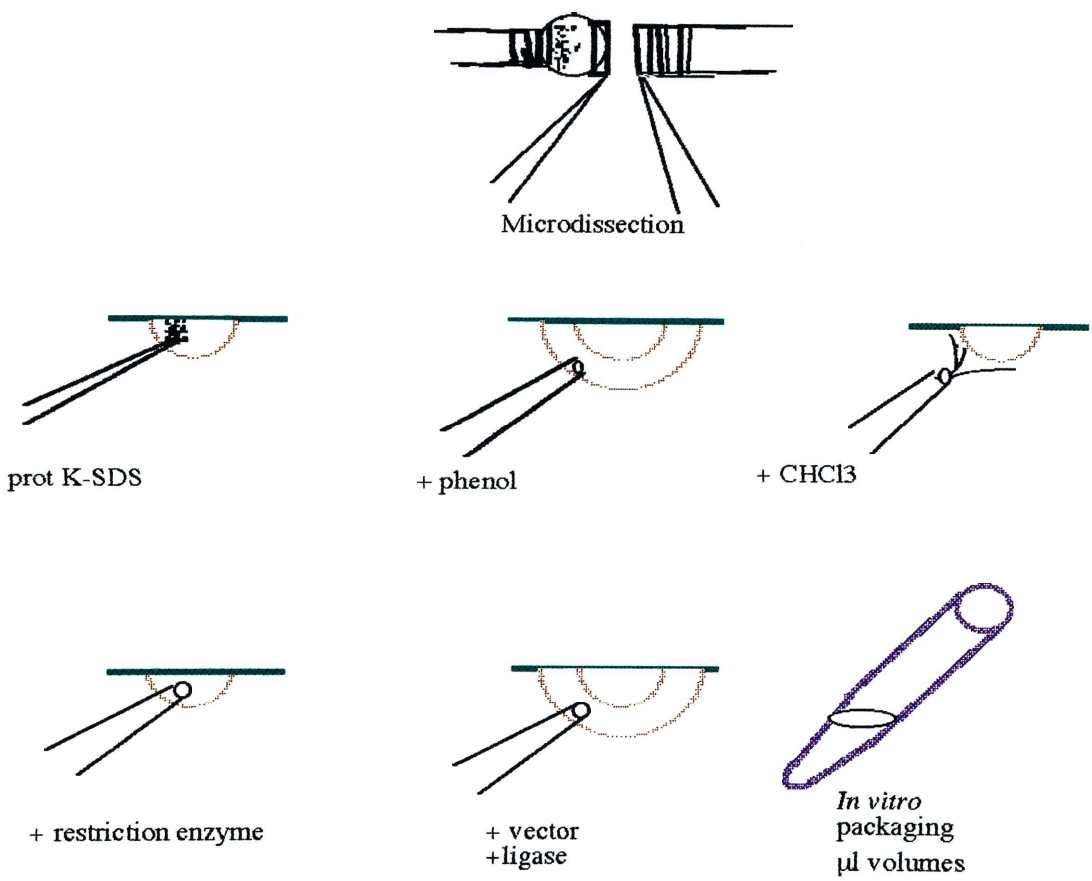


Figure 1.9 Schematic illustration of microcloning operations (Saunders, 1990).

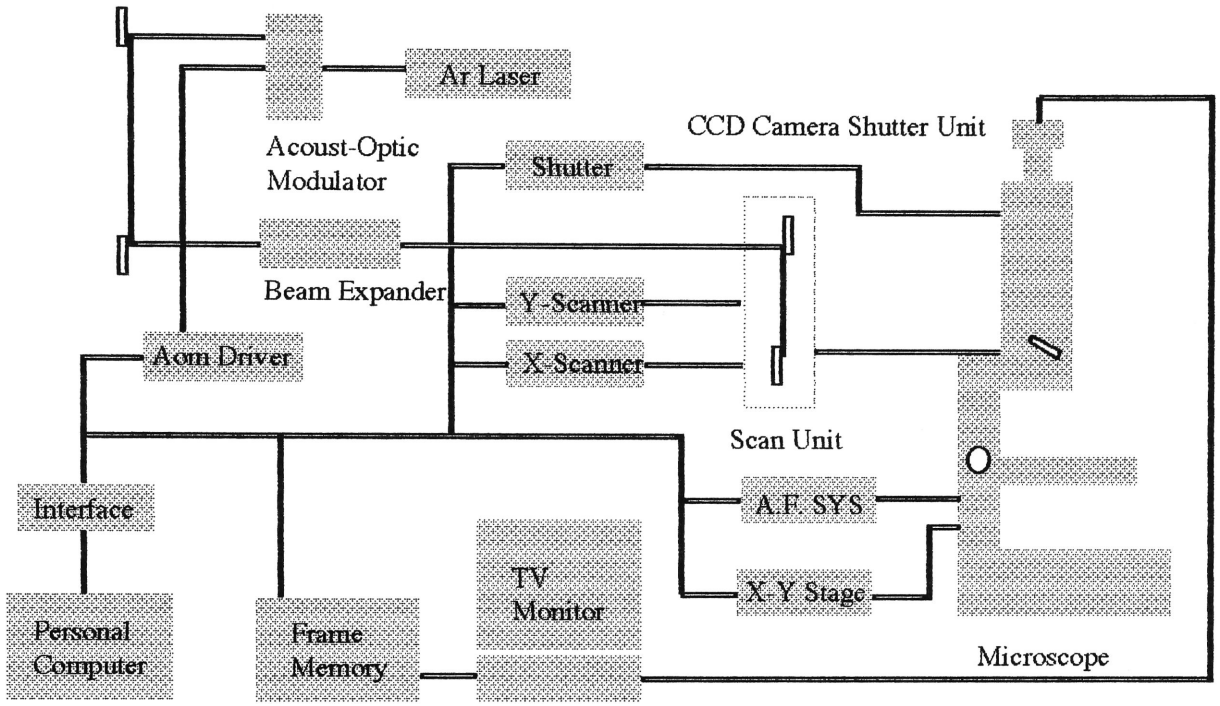


Figure 1.10 Block diagram of chromosome dissection system (Hadano *et al*, 1991).

Another PCR-mediated microcloning procedure has been described which uses sticky-end ligation of the dissected sequences to a linker-adaptor, followed by PCR amplification by using one strand of the adaptor as a primer. This method was first used to construct genomic libraries of segments of *Drosophila* polytene chromosomes. The entire procedure is summarized in Fig. 1.12 (Kao, 1993). This procedure has also been applied to human chromosome 21 (Kao *et al*, 1991).

Another strategy and one that the present investigation focuses on, is the application of universal degenerate oligonucleotide primers for direct PCR amplification of microdissected DNA. In this procedure a mixture of oligonucleotides varying in base sequence but with the same number of bases ("degenerate") is employed. This degeneracy coupled with a PCR protocol utilizing a low initial annealing temperature, ensure priming from multiple dispersed sites within a given genome (Meltzer *et al*, 1992; and Walsley *et al*, 1989).

1.2.3 Quality of the amplified DNA

It is very important to evaluate each library to ensure that it is of good quality, i.e, contains sufficient clones from the region of interest. This is particularly important because of the high efficiency of PCR, which may amplify any sequences of unknown origin introduced unintentionally during the microdissection and microamplifying processes.

The quality of the constructed library can be first monitored by examining the PCR products after gel electrophoresis and ethidium bromide staining.

Also, the microdissected libraries can be shown to derive from the dissected region by chromosome painting using the FISH (fluorescent *in situ* hybridization) technique in which the repetitive sequences in the library have been suppressed. Furthermore, a subset of the recombinant microclones from the library may be analyzed by hybridizing clones individually to Southern blots. When working with human chromosomes, specific cell hybrids containing individual human chromosomes provides a valuable source of DNA for checking the origin of amplified DNA.

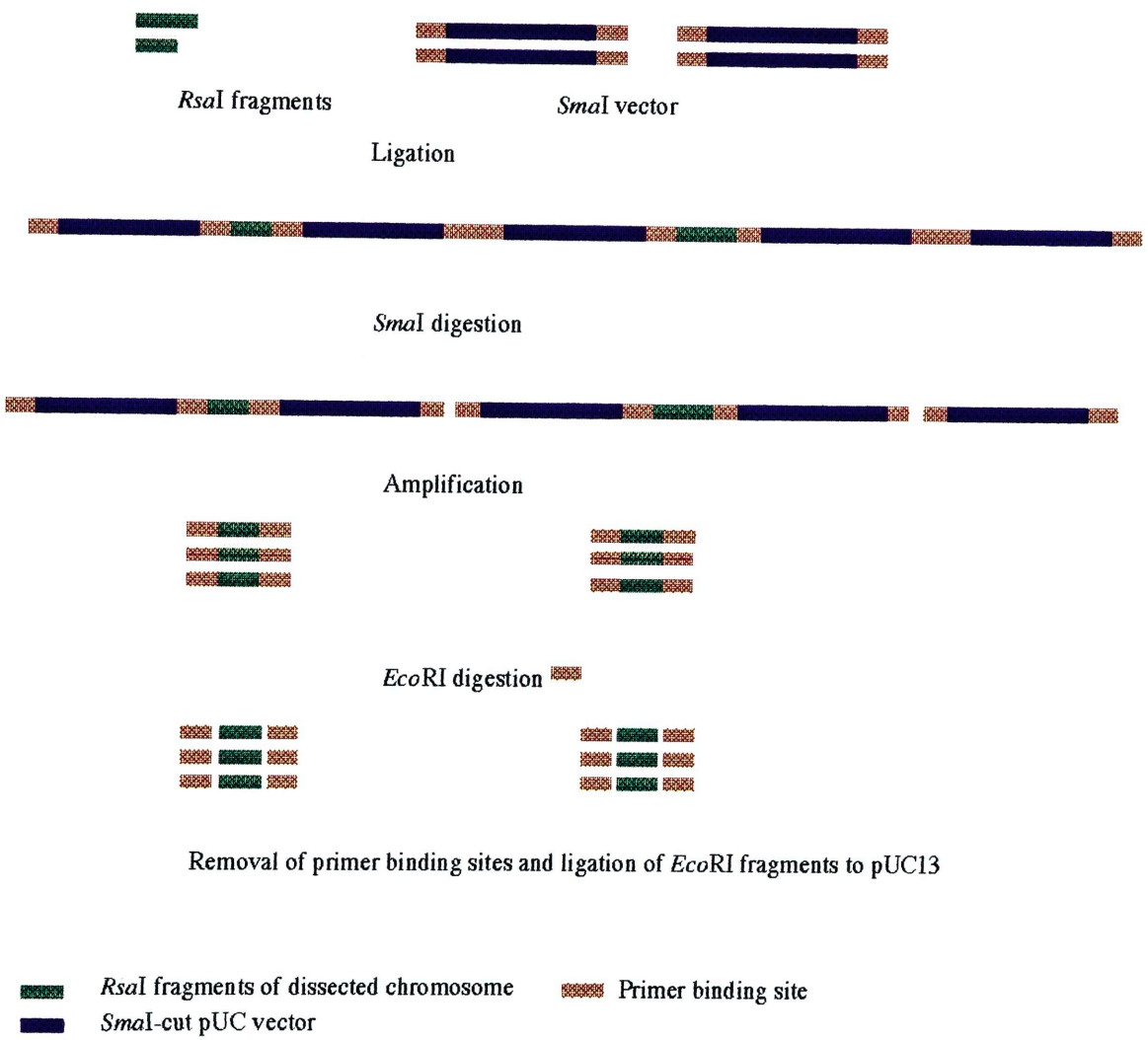


Figure 1.11 Universal DNA amplification procedure (Ludecke *et al*, 1989).

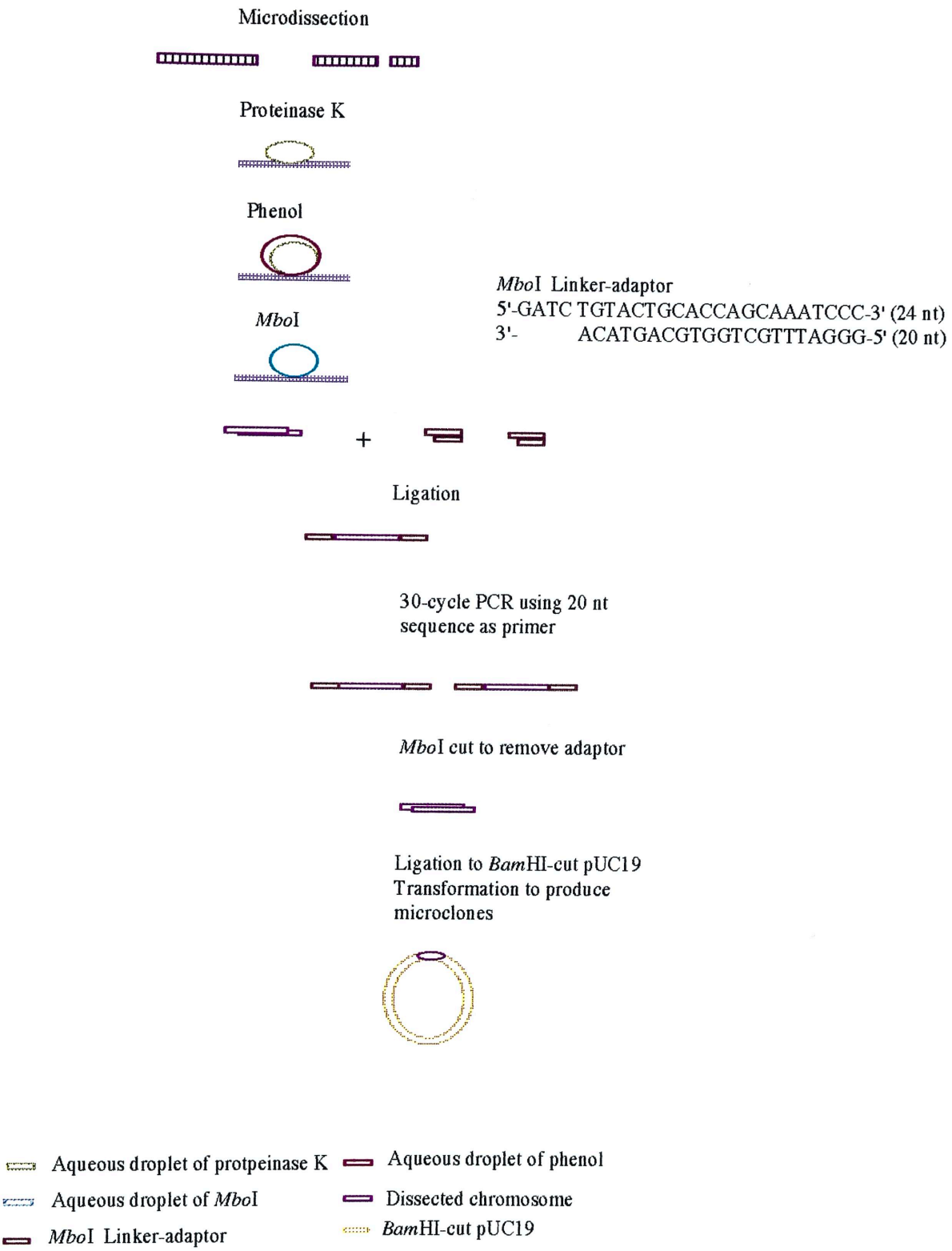


Figure 1.12 Diagram showing the procedure of microcloning of microdissected chromosomal sequences using the linker-adaptor technique (Kao, 1991).

The most important factor responsible for the quality of the library is the effective prevention of contamination by DNA during microdissection, microamplifying, and cloning steps.

Shortening the fixation time during acid treatment, storing the slides in liquid nitrogen, even using methanol instead of acetic acid (to fix the chromosomes), and avoiding nucleases present in certain reagents, such as trypsin for chromosome banding, can reduce DNA damage (Weber *et al*, 1990).

1.2.4 Comparisons between microcloning and micro-PCR

Following the successful application of microcloning to cloning *Drosophila* polytene chromosome bands (Scalenghe *et al*, 1981), this technique was then applied to a mouse chromosome (Rohme *et al*, 1984) and to human chromosomes (Bates *et al*, 1986).

Although clones obtained sufficed to act as starting points for chromosome walking and other applications, this method has the following drawbacks, especially when it is applied to mammalian chromosomes:

Firstly, the number of clones obtained is far from sufficient to fully represent the dissected region. The cloning of a single microdissected polytene chromosome fragment, for example, containing 100-200 kb can yield 30-50 recombinant clones, but more usually one obtains 5-10. One explanation is that the DNA molecules present in a band might be difficult to extract from chromatin or cleave with the restriction enzymes. As a consequence, certain regions within the chromosomal fragments might be less accessible. Also lack of clones can result from depurination of the DNA by acid conditions of fixation (Brown *et al*, 1987).

Secondly, the low average size of inserts in the recombinant clones can be problem (Scalenghe *et al*, 1981).

Thirdly, due to their small size, mammalian chromosomes are more difficult to dissect and many fragments need to be assembled for cloning (100 or more).

Finally, in microcloning, unbanded and unstained chromosome preparations were used.

The incorporation of PCR technology into the microcloning procedure has not only overcome the above problems, but has also made the method much more powerful, resulting in increased applicability. For instance, the number of fragments that need to be assembled is generally much lower for micro-PCR (ranging between 10-30) than required in microcloning (Table 1.2). In a recent experiment described by Guan *et al* (1993), it has been claimed that pretreating the microdissected chromosome fragments with topoisomerase I reduces the number of copies required to 1 to 5 fragments. Topoisomerase I, which catalyzes the relaxation of supercoiled DNA, is thought to increase access of primer and polymerase to the microdissected DNA template.

Furthermore, the libraries obtained by micro-PCR are more comprehensive than those obtained by microcloning and ranged between 50000-70000 microclones (Table 1.2).

Banded and stained chromosome preparations are used for micro-PCR.

Table 1.2 A representative list of micro-PCR experiments in the literature.

Region	No. of fragments pooled	Estimated length of inserted DNA	No. of recombinants clones	Ref.
Human 21	30	416 bp	70000	Kao (1991)
Human 21 8q23.3-q24	10	325 bp	50000	Hirota (1992)
Human 6 q21-23	30	500 bp	_	Meltzer (1992)
Human 2	20	270 bp	20000	Yu (1992)
Human 6 6q21	40	500 bp	20000	Guan (1992)
Human 10	-	200 bp	20000	Ohta (1993)

1.2.5 Applications

An obvious application of the Micro-PCR technique is to gain access to the vicinity of a locus which is well characterized genetically but for which no molecular probes are available.

When molecular probes are available, precise dissection is not essential. Microamplification then has the advantage of simplifying the screening and sometimes also eliminating ambiguities when sequences are present more than once in different parts of the genome or when the probe is not pure (Pirrotta *et al*, 1983).

Microamplification also provides an invaluable aid in the construction of complete physical maps of genomes.

The availability of large numbers of unique sequence probes from an important chromosomal region is also valuable in screening various cDNA libraries. This approach can be particularly useful because i) cDNA sequences have functional significance and are valuable material for early phases of genomic sequencing, and ii) cDNA clones isolated from relevant tissues can serve as candidate genes for an effective search for particular disease genes assigned to the region.

Isolation of sequences from translocation breakpoints and small deletions associated with genetic disease or specific forms of cancers is another application of this approach.

FISH chromosome painting with region-specific PCR amplified DNA allows the analysis of complex translocations, deletions, and other chromosomal abnormalities in patients.

Section 1.3. Objective of this investigation

Chromosome microdissection provides a direct approach for isolating DNA from any cytogenetically recognisable region. This material can then be PCR amplified for numerous applications. However, microdissection techniques are time consuming and extremely labour-intensive. Furthermore, with the addition of each copy to the collection drop, the probability of extraneous DNA contamination is increased. DNA

contamination is a critical problem in the PCR amplification of dissected DNA because the initial amount of dissected material is extremely small. Thus, even minute amounts of contaminating DNA can lead to irrelevant amplification products. For these reasons we have investigated the optimization of approaches designed to decrease the number of microdissected chromosome fragments required. Thus our aim was to develop a method which is simple, efficient, and with sufficient sensitivity to require only a small number of dissected chromosomal fragments. For the optimization of the procedure, we used bacteriophage λ DNA as a model. Owing to its relative simplicity, compared to segments of human DNA, it would be expected to give an easily recognisable, characteristic banding pattern after PCR amplification.

2. Materials and Methods

2.1 PCR Primers

Two specific primers with the following sequences were employed for the amplification of λ DNA:

Primer#1: 5' GATGAGTTCGTGTCCGTACAACTGG 3', complement of negative sequence from nucleotide 7131 to 7155 of λ DNA, and

Primer#2: 5' GGTTATCGAAATCAGCCACAGCGCC 3', complement of positive sequence from nucleotide 7606 to 7630 of λ DNA.

The following DOP primer was used in this study: 5' CCGACTCGAGNNNNNNATCTGG 3'. This primer has a fixed 3' hexanucleotide sequence, an internal degenerate hexanucleoide, and a 5' sequence incorporating a *Xho*I site as suggested by Telenius *et al* (1992).

2.2 Primer purification

Following synthesis on an Applied Biosystems Oligonucleotide Synthesiser, primers were cleaved from the support by three 20 min extractions with 1 ml volume of concentrated ammonium hydroxide. After 20 min the aliquot was removed. The combined extract was incubated at 55°C overnight.

For the purification of the primer, a polypropylene syringe (Aldrich Z11686-6) was connected to an OPC^{TM} cartridge. The cartridge was flushed with 5 ml HPLC grade acetonitrile followed by 5 ml 2 M triethylamine acetate. The primer solution was diluted with 1 ml of deionized water. 4 ml of this solution was slowly pushed through the cartridge. The eluted fraction was again gently passed through the cartridge which was then washed with 3 x 5 ml 1.5 M amonium hydroxide (freshly made) followed by 2 x 5 ml deionized water.

To detritylate the OPC^{TM} bound oligonucleotide, 1 ml of trifluroacetic acid (TFA) solution was gently pushed through the cartridge. After 5 minutes, 4 ml more was applied. The cartridge was washed with 2 x 5 ml deionized water. The detritylated oligonucleotide was eluted by slowly washing the cartridge with 1 ml 20% acetonitrile solution.

The yield of the primer in micromoles was calculated from the absorbence of the diluted sample by the following formula:

$$\frac{A260\ (sample) \times dilution\ factor \times 700 \times volume\ (ml)}{20 \times molecular\ weight\ of\ the\ primer}$$

The purified oligonucleotide was dried and stored at -20°C.

2.3 Polymerase Chain Reaction (PCR)

Amplification of the experimental DNA was performed in a 0.5 ml microcentrifuge tube in a total volume of 50 µl. A Corbett Research Thermal Sequencer was used for this study.

The experimental template was bacteriophage lambda DNA (λ DNA) stored in TE buffer. The reaction mixture used for the amplification by Taq DNA polymerase was prepared from the following reagents: 10X reaction buffer (containing 100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% Gelatin W/V), 10 mM of each dNTP, Taq DNA polymerase 5 U/µl, 20 mM specific primers or 20 µM DOP, 25 mM $MgCl_2$, and sterile distilled water.

DOP primer and $MgCl_2$ were prepared in our laboratory. Other reagents were taken from Perkin-Elmer/Cetus kits).

For pre-amplification of λ DNA with modified T7 DNA polymerase, Sequenase version 2.0 (United State Biochemical) was used.

All the reagents were stored at -20°C.

After addition of reagents, the reaction mixture was overlaid with 50 µl of paraffin oil.

After conclusion of the PCR, reactions were cooled to -20°C to facilitate the removal of the paraffin which remains in liquid form.

To avoid contamination, all reagents, except those from the kits, were prepared as stocks and autoclaved and stored in small aliquots for one use only. All the tubes were placed in beakers covered with foil and autoclaved. The autoclaved reagents and tubes were exposed to UV for at least 2 hours before using. Chemical manipulation were carried out under a sterile hood (Gelman Sciences). Gloves were worn for all manipulations. Aerosol barrier pipette tips were employed. The sterile tubes were taken by forceps and opened with flamed round-nosed pliers.

2.4 Analysis of PCR amplified λ DNA

PCR amplified λ DNA was detected and analysed by 2% agarose gel electrophoresis in 0.04 M Tris-acetate, 0.01 M EDTA buffer incorporating 0.5 µg/ml ethidium bromide. The gel loading buffer consisted of 0.25% bromophenol blue, 0.25% xylen cyanol FF and 15% Ficoll (type 400, Pharmacia) in water. Electrophoresis was performed at 60V for 2.5 hours in a Biorad Mini Sub Cell. A 123 bp ladder (G1BCO-BRL) was loaded on all electrophoretic gels to act as an internal control on DNA staining as well as a size marker (see Appendix I for the size marker). The molecular weight marker consisted of 2 µl 123 bp

ladder DNA, 6 µl TAE buffer and 2 µl ladder buffer mix (0.1 ml 10% SDS, 0.1 ml 1 mM EDTA, 0.05 ml 5 M NaCl, and 0.75 ml water) which was incubated at 65°C for 5 minutes before loading.

3. Results

3.1 PCR amplification from λ DNA using specific primers

In order to check the purity of the reagents, a 500 nucleotide target segment of bacteriophage lambda (between nucleotides 7131 and 7591) was amplified by the primers in the Gene Amp PCR Reagent kit. The reaction mixture is described in Table 3.1. The PCR reaction was taken through 25 cycles according to the program recorded in Table 3.2. A discrete band (corresponding to 500 bp) was detected upon agarose gel electrophoresis of the reaction product (Fig. 3.1). The smallest amount of the template giving detectable amplification was 5 pg (for 50 µl reaction mixture) (Fig. 3.2). Increasing the number of cycles to 35 did not offer any advantage as still no amplified product was seen with less than 5 pg of DNA template (Fig.3.3).

Table 3.1. The reaction mixture for the amplification of λ DNA using specific primers

Components	Volume	Final Concentration
Sterile distiled water	30.75 µl	
[10x] Reaction Buffer	5.0 µl	1x
dATP (10 mM)	1.0 µl	200 µM
dTTP (10 mM)	1.0 µl	200 µM
dCTP (10 mM)	1.0 µl	200 µM
dGTP (10 mM)	1.0 µl	200 µM
Primer#1 (20 µM)	2.5 µl	1.0 µM
Primer#2 (20 µM)	2.5 µl	1.0 µM
Taq DNA polymerase (5U/µl)	0.25 µl	1.25 Units/50 µl
Experimental Template (100 pg/µl)	5 µl	500 pg/50 µl
Total mix	50 µl	

Table 3.2. PCR Amplification cycle using specific primers

Step	Temperature	Time	No.of Cycles
1.1	94° C	0.30 Min	
1.2	95° C	0.30 Min	X1 cycle
2.1	94° C	1.00 Min	
2.2	37° C	1.00 Min	
2.3	72° C	1.00 Min	X25 cycles
3.1	94° C	1.00 Min	
3.2	37° C	1.00 Min	
3.3	72° C	10.00 Min	X1 cycle

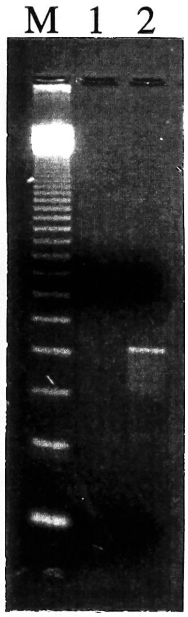


Figure 3.1 Segment of λ DNA amplified by specific primers. (M) 123 bp marker ladder; (1) negative control (no DNA); (2) λ DNA (amplified from 500 pg/50 µl).

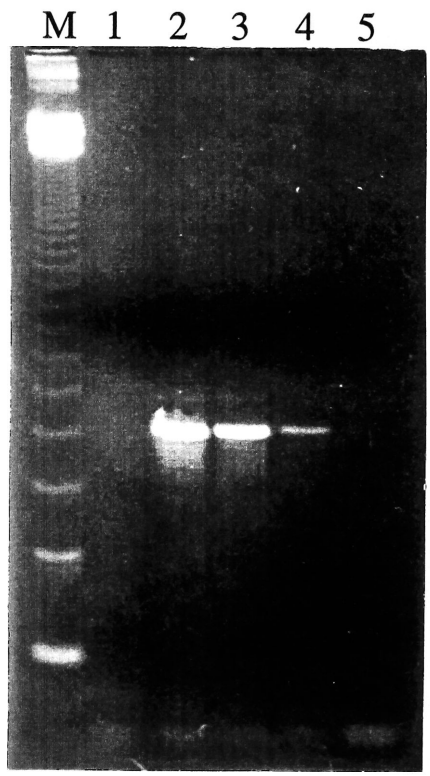


Figure 3.2 Amplified products after 25 cycles from different concentrations of λ DNA using specific primers. (M) 123 bp marker ladder; (1) negative control (no DNA); (2-5) amplification from 500 pg/50µl, 50 pg/50µl, 5 pg/50µl, and 0.5 pg/µl λ DNA respectively.

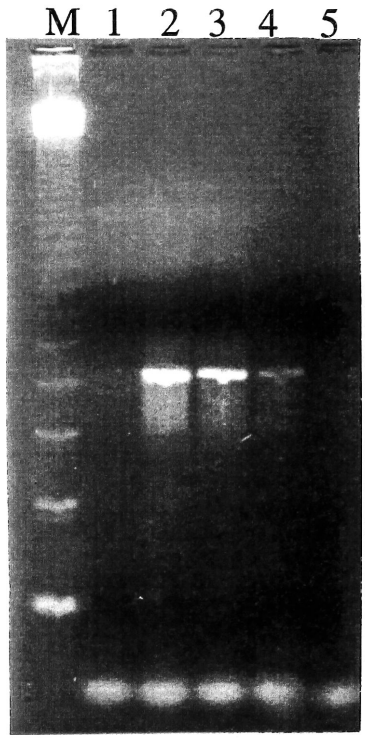


Figure 3.3 Amplified products after 35 cycles from different concentrations of λ DNA using specific primers. (M) 123 bp marker ladder; (1) negative control (no DNA); (2-5) amplification from 500 pg/50µl, 50 pg/50µl, 5 pg/50µl, and 0.5 pg/µl λ DNA. No amplification was detected with less than 5 pg/50µl λ DNA. The background smear was more than that seen in Fig. 3.2.

3.2 Degenerate oligonucleotide primed PCR (DOP-PCR) with initial low temperature cycles ramped and not ramped

Thermal cycling conditions that have worked with a wide variety of DOP, are based on the use of a low annealing temperature of 37°C during the initial two to eight cycles of amplifications. The other parameter that has been reported to be critical during the early cycles of the amplification is a slow ramp between the annealing temperature and extension temperature for the Taq polymerase (Compton, 1990).

Starting with 20 pg λ DNA, we could amplify λ DNA with a universal primer of partially degenerate sequence with six fixed bases at its 3' end (5'CCGACTCGAGNNNNNNATCTGG 3') (Table 3.3 and 3.4). At least 7 discrete fragments were amplified and readily detected over a slight background smear (Fig. 3.4). Adding Taq DNA polymerase after the first denaturation step to avoid non-target amplification ("hot start") did not give any apparent advantage (Fig.3.4, lane 2).

Because there was not any significant difference between the amplified products obtained by initial low temperature ramped or not ramped early cycles (data not shown), we decided to employ PCR cycles that did not involve a ramped increasing temperature.

To find the optimal concentration of DOP, varying concentrations of DOP were tested. The components of DOP-PCR for this experiment are listed in Table 3.5. The cycling conditions are summarized in Table 3.6. It is clear from the data in Fig.3.5 that the optimal concentration was 5 μM since maximum amounts of amplified fragments were obtained while putative primer-dimer products (indicated bands by the arrow) were less than those produced with 10 μM primer.

To examine the sensitivity of this protocol, different amounts of the template were tested. Although some products were just detected when using 0.2 pg DNA, far more significant amounts of PCR product were obtained with 2 pg and 20 pg DNA (Fig. 3.6).

The appearance of 2 new low molecular weight bands may represent the formation of a direct primer-related product, since after decontaminating all the reagents by exposure to UV and in the absence of added template, these two bands were still formed during the reaction (Fig.3.6 bands indicated by arrows).

Table 3.3. The reaction mixture for the amplification with DOP.

Components	**Volume**	**Final Concentration**
Sterile distiled water	14.5 µl	
[10x] Reaction Buffer	5.0 µl	1x
dATP (10 mM)	1.0 µl	200 µM
dTTP (10 mM)	1.0 µl	200 µM
dCTP (10 mM)	1.0 µl	200 µM
dGTP (10 mM)	1.0 µl	200 µM
$MgCl_2$ (25 mM)	1.0 µl	0.5 µM
Primer (20 µM)	15 µl	6 µM
Taq DNA polymerase (5 U/µl)	0.4 µl	2 U/50 µl
Experimental Template (2 pg/µl)	10 µl	20 pg/50 µl
Total Mix	50 µl	

Table 3.4. Amplification cycle for DOP-PCR with initial low temperature cycles ramped.

Step	Temperature	Time	No.of Cycles
1.1	94° C	1.00 Min	
1.2	30° C	1.30 Min	
1.3	37° C	0.30 Min	
1.4	42° C	0.30 Min	
1.5	49° C	0.30 Min	
1.6	56° C	0.30 Min	
1.7	63° C	0.30 Min	
1.8	71° C	0.30 Min	
1.9	72° C	3.00 Min	X5 cycles
2.1	94° C	1.00 Min	
2.2	62° C	1.00 Min	
2.3	72° C	3.00 Min	X20 cycles
3.1	94° C	1.00 Min	
3.2	62° C	1.00 Min	
3.3	72° C	3.30 Min	X19 cycles
4.1	94° C	1.00 Min	
4.2	62° C	1.00 Min	
4.3	72° C	10.00 Min	X1 cycle

Table 3.5. The reaction mixture for different concentrations of DOP.

Components	Neg.Control	Positive 1	Positive 2	Positive 3
Sterile distiled water	34.6 µl	24.6 µl	17.1 µl	4.6 µl
[10x] Reaction Buffer	5.0 µl (1x)	5.0 µl (1x)	5.0 µl (1x)	5.0 µl (1x)
dATP (10 mM)	1.0 µl (200 µM)	1.0 µl (200 µM)	1.0 µl (200 µM)	1.0 µl (200 µM)
dTTP (10 mM)	1.0 µl (200 µM)	1.0 µl (200 µM)	1.0 µl (200 µM)	1.0 µl (200 µM)
dCTP (10 mM)	1.0 µl (200 µM)	1.0 µl (200 µM)	1.0 µl (200 µM)	1.0 µl (200 µM)
dGTP (10 mM)	1.0 µl (200 µM)	1.0 µl (200 µM)	1.0 µl (200 µM)	1.0 µl (200 µM)
$MgCl_2$ (25 mM)	1.0 µl (0.5 mM)	1.0 µl (0.5 mM)	1.0 µl (0.5 mM)	1.0 µl (o.5 mM)
Primer (20 µM)	5.0 µl (2.5 µM)	5.0 µl (2.5 µM)	12.5 µ l (5 µM)	25 µl (10 µM)
λ DNA(2 pg/µl)	0.0	10.0 µl (20 pg/µl)	10.0 µl (20 pg/µl)	10.0 µl (20 pg/µl)
Taq DNA polymerase	0.4 µl (2U)	0.4 µl (2U)	0.4 µl (2U)	0.4 µl (2U)
Total Mix	50.0 µl	50.0 µl	50.0 µl	50.0 µl

Note: The final concentration of each reagent is shown in a parentheses.

Table 3.6. The Taq- PCR program for varying amounts of DOP

Step	Temperature	Time	No.of Cycles
1.1	93° C	4.00 Min	X1cycle
2.1	94° C	1.00 Min	
2.2	30° C	1.00 Min	
2.3	72° C	3.00 Min	X8 cycles
3.1	94° C	1.00 Min	
3.2	56° C	1.00 Min	
3.3	72° C	2.00 Min	X27 cycles
4.1	94° C	1.00 Min	
4.2	56° C	1.00 Min	
4.3	72° C	10.00 Min	X1 cycle

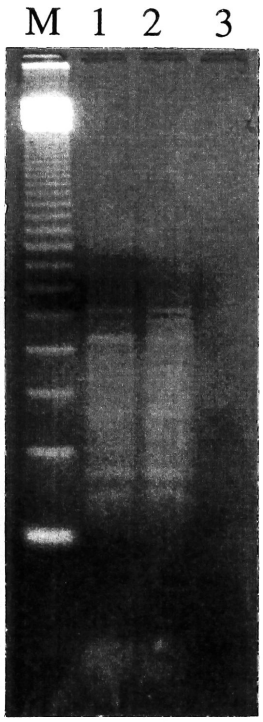


Figure 3.4 DOP-PCR with Taq DNA polymerase. (M) 123 bp marker ladder; (1) products resulting from 20 pg λ DNA; (2) amplified fragments from 20 pg λ DNA but Taq enzyme was added after the first denaturation step; (3) negative control (no DNA).

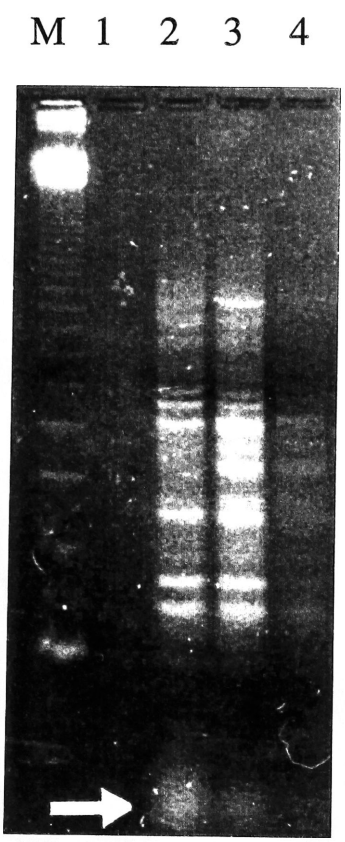


Figure 3.5 λ DNA amplified using different concentrations of DOP primer. (M) 123 bp marker ladder; (1) negative control (no DNA); (2-4) 10 μM, 5 μM, and 2.5 μM primer respectively.The arrow shows the primer-related products. While with 5 μM primer the maximum amounts of amplified fragments were obtained, the primer-dimer products were less than those detected with 10 μM primer.

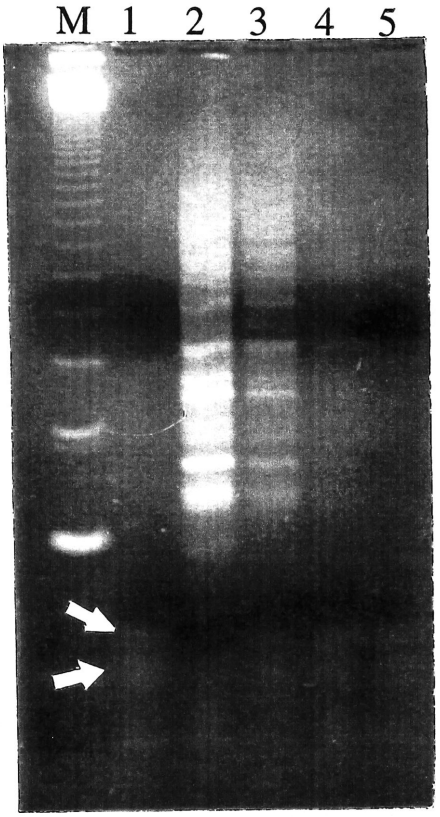


Figure 3.6 DOP-PCR using different amounts of λ DNA. (M) 123 bp marker ladder; (1) negative control (no DNA); (2-5) 20 pg/50µl, 2 pg/50µl, 0.2 pg/50µl, and 0.02 pg/50µl respectively. The bands indicated by arrows represent the formation of primer-dimer products.

3.3 DOP-PCR utilizing a small initial volume

To avoid the formation of primer-dimer and also to reduce the amount of template required for the amplification, we investigated amplification of different amounts of λ DNA placed in 2 µl Taq PCR reaction mixture (Table 3.7) under 25 µl paraffin oil and then used the amplified products of this initial reaction for subsequent amplification in 50 µl of the Taq PCR mixture. After an initial 13 PCR cycles carried out in a 2 µl reaction mixture, 50 µl of the Taq PCR mixture was added to the each tube and the remaining cycles were performed in the larger volume (Table 3.6).

It was anticipated that since, in this protocol, the ratio of the primer to the template was far less than in a normal PCR, the formation of primer-dimer would be reduced and as a result the product yield would be increased. However, while the primer-dimer products were much less than those produced in the previous experiments, the sensitivity of this protocol was less than that obtained with the DOP-PCR. With this procedure no amplified fragment was detected with less than 2 pg DNA and even the product yield from 2 pg λ DNA was much less than that achieved in a Taq PCR carried out in 50 µl volume from the outset (Fig. 3.7).

Table 3.7. The reaction mixture for DOP-PCR

Components	Volume	Final Concentration
Sterile distiled water	27.5 µl	
[10x] Reaction Buffer	5.0 µl	1x
Taq DNA polymerase	0.4 µl	2 Units/50 µl
dATP (10 mM)	1.0 µl	200 µM
dTTP (10 mM)	1.0 µl	200 µM
dCTP (10 mM)	1.0 µl	200 µM
dGTP (10 mM)	1.0 µl	200 µM
$MgCl_2$ (25 mM)	1.0 µl	0.5 µM
Primer (20 µM)	12.5 µl	5 µM
Total Mix	50 µl	

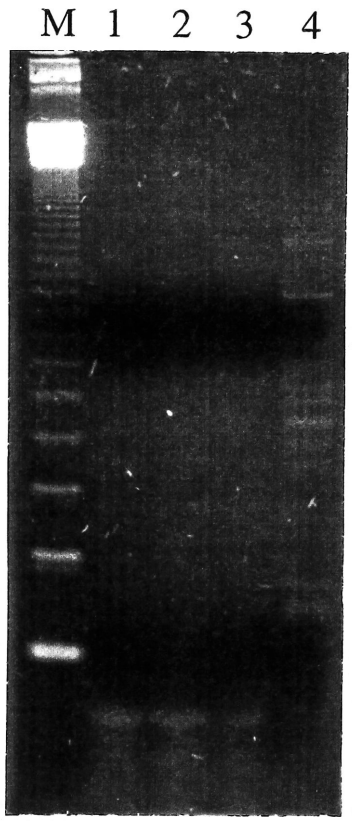


Figure 3.7 DOP-PCR using different amounts of λ DNA placed in small drop to elevate the initial concentration. (M) 123 bp marker ladder; (1) negative control (no DNA); (2-4) 0.02 pg/50µl, 0.2 pg/50µl, and 2 pg/50µl respectively.

3.4 DOP-PCR with modified T7 DNA polymerase (Sequenase) catalysed pre-amplification cycles.

T7 DNA polymerase was chosen for this procedure because it has been reported that pre-amplification with modified T7 DNA polymerase (Sequenase) enhances the efficiency of DOP-PCR by increasing priming at the lower extension temperatures, at which T7 DNA polymerase is active (Bohlander *et al*, 1992).

20 pg of λ DNA was added into 5 µl of the reaction buffer for T7 DNA polymerase (Table.3.8) and overlaid with mineral oil. An initial 6 cycles of PCR were conducted by adding 0.17 µl (0.2 units) of T7 DNA polymerase (Sequenase version 2.0, USB) at each annealing step [Sequenase (13U/ml) was diluted 1 to 8 in enzyme dilution buffer (USB)]. The program for the PCR cycles is recorded in Table 3.9.

Following this pre-amplification step, 50 µl PCR reaction mixture (Table 3.8) containing 2 units of Taq DNA polymerase was added directly to the reaction tube and further amplification carried out as shown in Table 3.10.

In our hands, the amplified products obtained by this procedure were less than those given by the PCR in which Taq DNA polymerase was used throughout (Fig. 3.8). This is probably due to an inhibitory effect of the buffer in which Sequenase is diluted; when we added 2 units of the diluted enzyme to an ordinary Taq-PCR reaction mixture containing 20 pg λ DNA, the product yields were about 10 fold lower than that obtained with the Taq-PCR mixture alone (data not shown). However, when we investigated the sensitivity of this protocol, detectable products were obtained down to 0.2 pg λ DNA template (Fig. 3.9).

Table 3.8. The reaction buffer for Sequenase

Components	Volume	Final Concentration
Sterile distiled water	14.50 µl	
$MgCl_2$ (1.M)	2.24 µl	20 mM
Tris-HCl (1 M, pH:7.5)	4.48 µl	40 mM
NaCl (5 M)	1.12 µl	50 mM
dATP (10 mM)	2.25 µl	200 µM
dTTP (10 mM)	2.25 µl	200 µM
dCTP (10 mM)	2.25 µl	200 µM
dGTP (10 mM)	2.25 µl	200 µM
Primer (20 µM)	5.60 µl	1 µM
Total Mix	100 µl	

Table 3.9. The PCR program for pre-amplification with Sequenase

Step	Temperature	Time	No.of Cycles
1.1	94° C	5.00 Min	X1cycle
Stop at 30°C to add 0.17 µl enzyme.			
2.1	94° C	1.00 Min	
2.2	30° C	1.00 Min	
2.3	37° C	3.00 Min	X6 cycles
Stop at 30°C to add 0.17 µl enzyme.			

Table 3.10. The PCR program cycle with Taq DNA polymerase

Step	Temperature	Time	No.of Cycles
1.1	94° C	1.00 Min	
1.2	356 °C	1.00 Min	
1.3	72° C	2.00 Min	X35 cycles
2.1	94° C	1.00 Min	
2.2	56° C	1.00 Min	
2.3	72° C	5.00 Min	X1 cycle

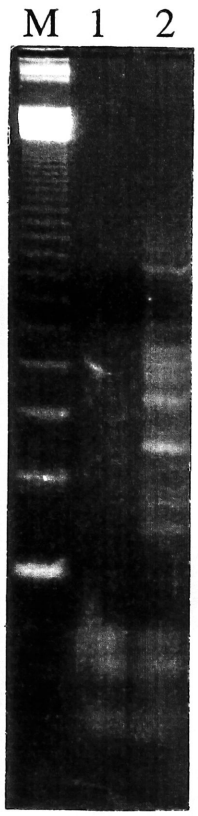


Figure 3.8 Pre-amplification with T7 DNA polymerase (Sequenase). (M) 123 bp marker ladder; (1) negative control (no DNA); (2) 20 pg/50µl λ DNA.

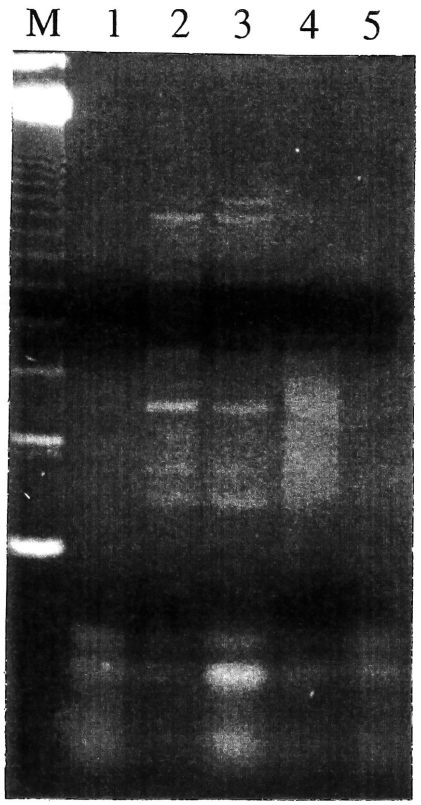


Figure 3.9 Pre-amplification with Sequenase using varying amounts of the template. (M) 123 bp marker ladder; (1) negative control (no DNA); (2-5) 20 pg/50µl, 2 pg/50µl, 0.2 pg/50µl, and 0.02 pg/50µl respectively.

3.5 DOP-PCR with boosting of the Taq polymerase concentration at cycle 20.

An important factor limiting the yield of DOP-PCR was found to be the concentration of Taq DNA polymerase. In the case of DOP-PCR there are many sites to be amplified. However, the number of available polymerase molecules limits the amplification and thus a linear rather than exponential amplification is observed by DOP PCR (Telenius *et al*, 1992). For this reason, increasing the original concentration of polymerase should increase the product yield, but the formation of direct primer-related products places an upper limit on the amount of enzyme that can be used. Thus we tried to introduce Taq DNA polymerase in two stages, initially and then after 20 cycles.

Varying amounts of template were placed into 50 µl of the Taq PCR mixture (Table 3.7). After the first 20 cycles, Taq DNA polymerase (2 units) was added to the each tube. Then the remaining 23 cycles were performed as indicated in Table 3.11.

With this procedure, amplified fragments were obtained even from 0.02 pg λ DNA per 50 µl reaction mixture (Fig. 3.10). Moreover, a comparison between Fig. 3.10 and Fig. 3.6 shows that the product yields obtained by this protocol are greater than those achieved by the procedures described in the earlier experiments (see above). The appearance of some new bands with high molecular weight (Fig. 3.10, indicated bands by arrows) suggests that this protocol is capable of amplifying longer DNA fragments.

Table 3.11. The PCR program cycle using extra Taq DNA polymerase and more cycles

Step	Temperature	Time	No.of Cycles
1.1	93° C	4.00 Min	X1 cycle
2.1	94° C	1.00 Min	
2.2	30° C	1.00 Min	
2.3	72° C	3.00 Min	X8 cycles
3.1	94° C	1.00 Min	
3.2	56° C	1.00 Min	
3.3	72° C	2.00 Min	X11 cycles
Stop at the 20th cycle to add 0.4 µl (2 units) Taq DNA polymerase.			
3.1	94° C	1.00 Min	
3.2	56° C	1.00 Min	
3.3	72° C	2.00 Min	X22 cycles
4.1	94° C	1.00 Min	
4.2	56° C	1.00 Min	
4.3	72° C	10.00 Min	X1 cycle

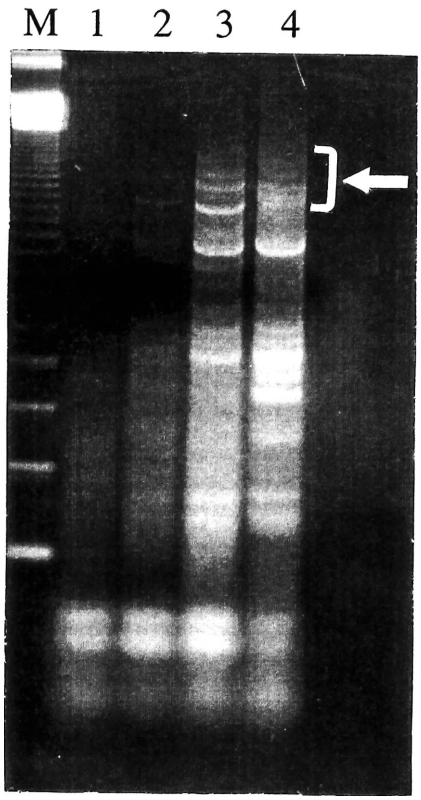


Figure 3.10 DOP-PCR using extra Taq DNA polymerase in the middle of the cycles. (M) 123 bp marker ladder; (1) negative control (no DNA); (2-4) 0.02 pg/50µl, 0.2 pg/50µl, and 2 pg/50µl respectively. The arrow shows new bands with high molecular weight.

4. Discussion and Conclusion

4.1 General discussion

Since its introduction the polymerase chain reaction (PCR) has transformed many aspects of DNA analysis in both research and clinical laboratories. The PCR involves the *in vitro* enzymatic synthesis of millions of copies of a specific target DNA segment. However, although the PCR was initially introduced to amplify a single locus in target DNA, it is increasingly being used to amplify multiple loci simultaneously allowing, for example, amplification of whole segments of mitotic chromosome isolated by microdissection. Widely used primers for this universal amplification are those based on repetitive sequences within the genome, which allow amplification of segments between suitably positioned repeats (Ledbetter *et al*, 1990).

The linker adaptor technique is an alternative procedure which is based on the digestion of microdissected chromosome bands with a restriction enzyme and then ligating them to a short adapter, one strand of which serves as a primer for a general PCR. However, since repetitive sequences are not uniformly distributed and because linker adapter PCR is technically difficult, we investigated the optimization of a PCR technique called degenerate oligonucleotide primed PCR (DOP-PCR) which involves multiple locus priming and allows a general amplification. This technique can be used for amplification of DNA in microdissected chromosome fragments. In DOP-PCR, a mixture of oligonucleotides of fixed length and varying in base sequences is used. DOP-PCR rests on the principle of priming from short sequences specified by the 3' end of the oligonucleotides used , during the initial low annealing temperature cycles of the PCR protocol. Since these short sequences occur frequently, amplification of target DNA proceeds at multiple loci simultaneously.

Annealing of the specified 3'-most primer sequence is stabilized by the adjacent six bases of degenerate sequence. At the 5' end of the primer is a further specified sequence including a restriction site for cloning, if required. This 5' sequence also stabilizes annealing of the primers to the initially amplified DNA, enabling a higher annealing temperature to be used in later PCR cycles.

When λ DNA was used as a target for DOP-PCR, at least 7 discrete fragments were amplified, although with a background smear. Also under most reaction conditions tested, although the product yield varied, the fragment pattern obtained did not vary qualitatively. This suggests preferential priming from specific sites within the λ DNA, rather than "random" priming, presumably governed by the specified sequence at the 3' end of the degenerate primer.

While changes to monovalent and divalent cation concentrations have a profound effect on the specificity and yield of an amplification of a specific locus and parameters such as magnesium concentration for each procedure have to be optimized, DOP-PCR is less dependent on these factors. Amplification was shown to take place under varying concentrations of $MgCl_2$ (ranging from 2 mM to 5 mM) (Telenius *et al*, 1992). However, the efficiency of DOP-PCR is mainly dependent on polymerase and primer concentrations. These effects are to be expected, since DOP-PCR has the potential to amplify at many sites. Indeed, theoretically, all degenerate primers would be depleted after a few cycles because of the huge number of priming sites, whereas single-locus primers would last longer. However, the number of available polymerase molecules limits the reaction before primers are exhausted. In our experiments, we found that the optimal concentration for DOP to be 5 mM. However, we could not avoid formation of direct primer-related

products; even when primers were added in two different stages, the formation of putative primer-dimer could not be avoided completely.

We also investigated the effect of varying the amount of Taq DNA polymerase and found that 2 units of the enzyme are sufficient to amplify the target DNA. However, in contrast to the single target PCR, in which all polymerase molecules will be engaged after at least 25 cycles, in DOP-PCR the enzyme may be limiting during the earlier cycles and thus a linear amplification can be observed if all priming sites are available (Telenius *et al* 1992). For this reason, we introduced an additional 2 units of Taq polymerase after 20 cycles and found that we could achieve higher product yields. However, after doubling the concentration of the polymerase, a faint background smear appeared in the negative control. Since we had decontaminated all the reagents, this background could be related to the formation of primer-dimer or minute amounts of contaminants introduced from air, gloves and other equipment during addition of the necessary reagents.

Efficient amplification of DNA by DOP-PCR relies on initial low temperature annealing cycles. The low temperature annealing conditions are required for hybridization of the short region of the primer to the template. After the early cycles of amplification, the unit-length product that includes the 5' extensions will serve as the template for subsequent rounds of amplification. When the low temperature cycles were omitted, no amplification was detected, demonstrating the requirement for initial low temperature annealing (data not shown). However, we found that, for our working conditions, a slow ramp time between the annealing temperature and extension temperature as employed by some investigators did not offer any advantage.

Perhaps one of the most difficult problems in working with degenerate primers is contamination. Since DOP-PCR is very efficient and sequence-independent, and because with six degenerate positions actually 4^6 different unique primers are used, these circumstances allow hybridization to almost any DNA which can be amplified. Thus, stringent procedures to minimize contamination should be adopted. These include: wearing gloves in all steps of operation, autoclaving water, all buffers and reagents, preparing all buffers and reagents in a sterile hood and storing them in small aliquots for one use only, and employing either displacement pipettes or aerosol barrier pipette tips. Sterile water, tubes, and tips should be stored in the hood under UV light.

To improve the efficiency and sensitivity of DOP-PCR, we have investigated different protocols suggested by other researchers with our own modifications. It is proposed by Guan *et al* (1993) that pre-amplification with T7 DNA polymerase during the low-temperature cycles, results in increased efficiency of amplification. In our hands, it has not been possible to obtain detectable amplification even with 20 pg of DNA and with 1 mM primer concentration as proposed by those researchers (data not shown). While increasing the concentration of the primer to 5 mM resulted in amplified fragments being detected, no fragment patterns were seen with less than 0.2 pg λ DNA as a template. Furthermore, because the T7 DNA polymerase is denatured each cycle, the reaction tube must be repeatedly opened to add fresh enzyme thus increasing the risk of contamination.

We have devised a protocol using 5 mM primer concentration and 2 units Taq DNA polymerase which can amplify 0.2 pg DNA. The yield

obtained by this protocol was greater than that produced by pre-amplification with T7 DNA polymerase.

Finally, with optimization of this procedure and addition of more Taq polymerase half way through the PCR, we could reduce the amount of template required to generate amplified fragments to 0.02 pg with insignificant background. Since a human diploid cell contains 6 pg DNA (Rees *et al*, 1972), this optimized DOP-PCR should be able to usefully amplify an amount of DNA corresponding to 0.3% [(0.02/6)x100=0.3%] of that present in a human diploid cell. Since in a cell undergoing mitosis all chromosomes have replicated (and contain two chromatids), a complete spread of forty six human mitotic chromosomes will contain 12 pg of DNA. Consequently, an "average chromosome" should contain 12/46 or 0.26 pg DNA. Thus, this optimized procedure can amplify an amount of DNA corresponding to approximately one tenth or less ($0.02/0.2 \sim 0.1$) of an average human mitotic chromosome. The reduction of the number of chromosome segments required for the amplification can significantly decrease the time-consuming and labour-intensive aspect of microdissection, and at the same time reduce the risk of contamination.

4.2 Conclusion

The ability to amplify specific segments of DNA, with the polymerase chain reaction (PCR), provides the capacity to approach both fundamental and applied biological problems. The versatility and power provided by the PCR to amplify large amounts of target sequence has resulted in its involvement in almost every aspect of the Human Genome Project. This method is also being employed in the study of molecular evolution, and gene expression. The technique provides an efficient tool for diagnosis of inherited diseases and cancer.

While the template for PCR may simply be provided by adding DNA or RNA molecules released from cells or even adding whole cells to the PCR reagent mixtures, for access to defined chromosomal regions chromosome microdissection is a powerful technique. This method, coupled with PCR is now contributing to a variety of genome and human molecular genetics studies, particularly in the cloning of disease-related genes with known map positions.

As the performance of PCR has improved, several strategies have been developed to overcome one of the apparent limitations of this technique, namely the requirement for specific sequence information for design of the amplification primers. This limitation has been overcome by a variety of specific strategies such as using linker-adaptors or primers based on interspersed repetitive sequences.

A new technique which employs a mixture of oligonucleotides of a fixed length but differing in sequences, termed degenerate oligonucleotide primer (DOP) has been applied for general amplification of target DNA. The degeneracy, together with a PCR protocol utilizing a low initial annealing temperature, ensures priming from multiple dispersed sites

within a given genome. The reaction begins by using a low annealing temperature of 37°C during the initial two to eight cycles of amplification. After the second cycle of amplification, the unit-length products that include the 5' extensions of the primers will serve as the template for subsequent rounds of amplification.

Unquestionably, no single protocol will be appropriate for all situations. Consequently, each DOP-PCR application is likely to require optimization. For this reason, in this investigation different conditions giving the maximum efficiency and sensitivity have been investigated. Thus different concentrations of DOP and Taq DNA polymerase (which are critical in DOP-PCR) were examined and the optimal concentration of both variables has been determined. Different strategies have been investigated to find the most efficient and sensitive protocol which can amplify as little starting DNA as possible. Finally, a modified strategy was developed for general amplification by DOP-PCR. This protocol has the ability to generate amplified products from as little as 0.02 pg DNA. This modified method offers several advantages. It is fast: the actual time for setting up the reactions is less than an hour. If it is applied to microdissected chromosomes, the specificity and quality of the microdissection and the amplified products can be verified by other fast techniques such as FISH. The danger of contamination is minimized because the number of steps and manipulations is reduced.

This technique has the potential to contribute to a variety of applications such as gene mapping and the study and diagnosis of genetic or infectious diseases and cancers. In fact, since the completion of this work, the method has been successfully applied to the analysis of a human chromosome translocation in collaboration with the Department of Cytogenetics, John Hunter Hospital, Newcastle.

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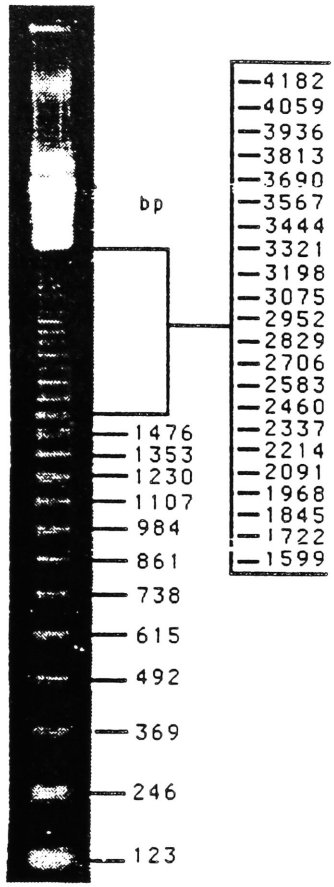
Appendix I



123 DNA ladder, 3 μg/lane, 1.5% agarose gel, stained with ethidium bromide.