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Kun Xu

DETECTION OF INSULIN RECEPTOR, EPIDERMAL GROWTH FACTOR RECEPTOR, AND INTERLEUKIN-6 ON INDIVIDUAL MOUSE EMBRYOS BY IMMUNO-POLYMERASE CHAIN REACTION

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By Kun Xu

Thesis Advisor: Dr. James Weber

An Abstract of the Thesis Presented
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Three series of experiments were conducted to: 1) optimize the conditions for the production of pUC19 plasmid and of biotinylated pUC19 fragments; 2) optimize the conditions for the production of protein A-streptavidin chimera (chimeric protein); and 3) detect soluble antigens [bovine serum albumin (BSA) and interleukin-6 (IL-6)] and membrane-bound antigens [insulin receptor and epidermal growth factor receptor (EGFr)] of mouse embryos by immuno-polymerase chain reaction (I-PCR).

The first experimental series, which included bacterial culture, chimeric protein purification, and chimeric protein functional experiments, was performed to investigate the effects of IPTG (isopropyl-β-D-thiogalactopyranoside) induction time and temperature, bacterial culture medium, and protein purification procedure on chimeric protein yield. The yield of chimeric protein was affected by IPTG induction time, but not by induction temperature. The yield of chimeric protein from TB broth culture was five to ten times higher than from either M9 or LB medium culture. Eliminating a centrifugation step just before the biotin affinity chromatography increased the yield of

chimeric protein three times more than when the centrifugation step was included. Data demonstrated that our chimeric protein was fully functional.

The second series, including culturing and biotinylation experiments, was conducted to investigate the effects of culture conditions [Lennox L broth (LB broth) versus Terrific broth (TB broth) and 15 versus 28 hours] on pUC19 plasmid yield from pUC19 plasmid-transformed *Escherichia coli* (*E. coli*), and the effect of incubation conditions on the efficiency of pUC19 biotinylation. Culture medium but not culture time affected pUC19 plasmid yield. pUC19 biotinylation at 37 °C for 30 min seemed to be superior compared to 15 min either on ice or at room temperature. Data demonstrated that biotinylated pUC19 fragments were fully functional.

The third series, including BSA, IL-6, insulin receptor, and EGFr assays, was performed using I-PCR and enzyme-linked immunosorbent assay (ELISA) assays to determine the absolute sensitivity of I-PCR technique and to compare the relative sensitivity between I-PCR and ELISA for detecting these antigens. The sensitivity limits of I-PCR were 5.8 BSA and 5.8 x 10^3 IL-6 molecules immobilized on the surface of a microtiter plate, which were six and seven orders of magnitude more sensitive than ELISA, respectively. By using I-PCR technique, we were able to detect insulin receptors on two mouse embryos and EGFr on only one mouse embryo at the morula stage. Data from these studies should be valuable for the future development of a non-invasive, I-PCR-based assay of individual embryo viability.

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Ultra-Sensitive Detection of Membrane-Bound and Soluble Antigens by Immuno-Polymerase Chain Reaction

The Application of Immuno-Polymerase Chain Reaction

A new antigen detection technique named Immuno-Polymerase Chain Reaction (Immuno-PCR) was developed within the past decade. The term, Immuno-PCR, can be dated back to 1991 (Widjojoatmodjo et al., 1991). In that year, a research group in the Netherlands used monoclonal antibodies to trap Salmonella onto magnetic particles and used the PCR technique to amplify a particular sequence of bacterial DNA with primers from the *Salmonella typhimurium* origin of DNA replication. The PCR products were visualized either by ethidium bromide-stained agarose gel or by Southern blotting. This new technique was called the Magnetic Immuno-PCR Assay (MIPA). A year later, another research group (Sano et al., 1992) detected bovine serum albumin (BSA) with a similar system. With this system, BSA molecules were immobilized onto the surfaces of wells of a microtiter plate. Anti-BSA immunoglobulin G (IgG) was subsequently allowed to interact with the plate-bound BSA molecule. Finally a linker molecule (a fusion protein), which contained both protein A and streptavidin moieties, was introduced and allowed to attach to the Fc portion of the IgG molecule with the protein A side and the biotin portion of a biotinylated DNA molecule with the streptavidin side. The marker DNA was used to generate PCR products and the products were visualized

by ethidium bromide-stained agarose gel. Sano's group called this antigen detection system Immuno-Polymerase Chain Reaction or Immuno-PCR, which is the most commonly used term today.

The three basic components of an Immuno-Polymerase Chain Reaction (referred to I-PCR thereafter) assay are the antigen recognition system, the marker DNA amplification system, and the signal manifestation system. The antigen recognition system contains the target antigen, an antibody which is specific to the antigen, and a DNA molecule (marker DNA) that is linked to the antibody indirectly through a linker molecule, or directly through antibody-DNA conjugation. The antigen recognition system in an I-PCR is based on the same format as ELISA and RIA (radioimmuno-assays). The only difference among them is that an enzyme in ELISA or an isotope in RIA is replaced by a piece of DNA in I-PCR. I-PCR is the most sensitive antibody-based antigen detection system presently available since it combines the high specificity of antibody and antigen interaction with the high sensitivity of polymerase chain reaction (PCR). This combination makes it the most powerful tool to date for detecting minute amount of antigens. In principle, this technique could be used to detect a single antigen molecule.

Since I-PCR was first described, it has been extensively used to detect a variety of antigens, including both membrane-bound and soluble antigens, with tremendous improvements in sensitivity and efficiency compared to other commonly used assay methods. Like all other techniques, I-PCR has been subjected to modifications and refinement during its development due to the cooperative efforts of scientists worldwide.

Detection of Membrane-Bound Antigens. Although I-PCR has been used to detect membrane-bound antigens on different cell types, including both eukaryote and prokaryotic cells, the general body of information on cellular I-PCR is relatively small compared to I-PCR of soluble antigens. The ability of I-PCR to detect membrane-bound antigens on samples of small number of cells has a great significance, because these antigens are usually undetectable, or are very difficult to detect with traditional assay methods, such as ELISA. All the data generated so far demonstrate that I-PCR is very useful for detecting these membrane-bound antigens.

Detection of Membrane-Bound Antigens on Eukaryote Cells. Little data are available pertaining to the detection of membrane-bound antigens on eukaryote cells. Even fewer data are available on the detection of membrane-bound antigens on embryos. Only a few papers have been published dealing with I-PCR for detecting antigens on embryos and all the data were generated from the same research group at Northeastern University. McElhinny and Warner (1997) were the first to use I-PCR on mammalian embryos when they reported the use of I-PCR to detect major histocompatibility complex class I (H-2 D^b) molecules on murine blastocysts. They were able to detect signal on one mouse blastocyst. In contrast, more than 15 mouse blastocysts were needed in order to obtain a positive H-2 D^b signal by ELISA. Using the same technique, McElhinny et al. (1998) successfully detected Qa-2 antigens not only on whole mouse embryos but also on isolated inner cell mass (ICM) cells. Wu et al.(1999) from the same lab microinjected *Q7* and *Q9* genes (which encode for the Qa-2 antigen) into *Ped* (preimplantation embryonic development)-*slow* mouse zygotes (Qa-2 negative), and could detect Qa-2 protein expression on a single microinjected embryo at the blastocyst stage by I-PCR.

Ke and Warner (2000) also introduced *Q7* and *Q9* genes into the male pronucleus of 1 cell F1 *Ped slow* embryos through microinjection, and the protein expression of the injected gene was detected on 15 pooled one-cell mouse embryos by I-PCR. McElhinny et al. (2000) attempted to incorporate Qa-2 antigens onto the surface of *Ped slow* preimplantation mouse embryos through a process called protein "painting", and subsequently detected the painted Qa-2 molecules on a single *Ped slow* preimplantation embryo. In addition to mouse embryos, I-PCR has also been used to detect membrane-bound antigens on other cell types including the gastric cancer-associated antigens on the cell line KATO III (Ren et al., 1994), and carbohydrate tumor-antigens on cancer cells (Zhang et al., 1998) with a detection limit of less than10 cells.

Detection of Membrane-Bound Antigens on Prokaryote Cells. Widjojoatmodjo et al. (1991) pioneered the detection of antigens on prokaryote cells by a technique called the Magnetic Immuno-PCR Assay (MIPA). With MIPA, one hundred colony forming units (CFU) of *Salmonella typhimurium* were detectable by visualization of the amplified products by ethidium bromide-stained agarose gel electrophoresis. The same technique was also used to detect Salmonellae at 10^5 CFU/mL in human fecal samples (Widjojoatmodjo et al., 1992). The results indicated that using MIPA not only improved detection sensitivity, but also significantly reduced the work needed to dilute fecal samples for detection. With primers specific for the iroB gene common to all strains of *Salmonella enteridica*, *Salmonella enteridica* and other bacterial species could be distinguished by using MIPA (Baumler et al., 1997).

Wolfhagen et al.(1994) also used MIPA to detect toxigenic *Clostridium difficile* in fecal samples. Using a monoclonal antibody (MAb), specific for *Clostridium difficile,*

Clostridium sordellii, and *Clostridium bifermentans*, these researchers achieved a sensitivity of 96.7%, and specificity of 100% without elaborate extraction of DNA from fecal samples. They concluded that this method was rapid, easy, and sensitive for detecting the presence of toxigenic *C. difficile* in stool samples. Kakizaki et al. (1996) used I-PCR to detect the pathogenic bacteria, *Pasteurella piscicida.* Immuno-polymerase chain reaction could detect as little as 3.4 CFU/mL of bacteria, whereas ELISA's limit of detection was much higher (3.4×10^4 CFU/mL).

Leptospires in urine samples of 10 experimentally infected cattle were consistently detected by MIPA (Taylor et al., 1997), and the results indicated that the immunomagnetic separation of leptospires from inhibitors in frozen formalin-fixed bovine urine prior to PCR detection significantly improved both sensitivity and speed. Giardia in environmental water were also detected by I-PCR (Mahbubani et al., 1998). It was claimed that the reliability for detection of this pathogen with the required sensitivity was improved by using I-PCR.

The pathogenic bacteria *Escherichia coli* (*E. coli*) 0157:H7 in both contaminated dairy products and ground beef samples was detected using I-PCR (Ogunjimi and Choudary, 1999). Results were obtained within eight h with detection sensitivities approaching one CFU/g or mL. The authors concluded that this method was suitable for rapid screening of *E. coli* 0157:H7 in fruit juices and plant produce.

Cao et al. (2000) used *in situ* I-PCR to detect hepatitis B viral antigens in hepatic tissues. In this research, amplified DNA sequences were detected *in situ* by hybridization with digoxigenin-labelled DNA probe followed by immuno-staining. Paraffin sections of liver samples from patients with hepatitis B virus (HBV) infection were examined for

HBsAg expression by *in situ* I-PCR. It was concluded that *in situ* I-PCR might be the only technique available to detect minute quantities of biological macromolecules such as proteins, carbohydrates, and lipids in intact cells or tissue sections.

I-PCR was compared with other assays in detecting nonstructural protein (NS 1), a common antigen among influenza A viruses (Ozaki et al., 2001). The sensitivity was 10 to 100 times higher with I-PCR than that with RT-PCR for the detection of the viral genome. Using anti-hemagglutinin (HA) monoclonal antibodies to detect HA subtype-specific antigen, the sensitivity was up to 10^{7-8} times higher than by virus isolation or by RT-PCR.

Detection of Soluble Antigens. Soluble antigens must be immobilized first in order to be detected. The most common method for immobilization is to bind the antigens onto the surface of a solid support, such as the surface of the wells of a 96-well plate. Immobilization may be achieved directly through electro-magnetic interactions, or indirectly through the action of a capturing antibody coated onto the solid support.

Detection of Antigens Immobilized Directly onto a Solid Support. The first attempt to detect the soluble antigens immobilized directly onto the surface of solid support by I-PCR was reported by Sano et al. (1992). The antigen to be detected was BSA which was immobilized on the surface of wells of a 96-well plate. A piece of biotinylated-pUC19 molecule was indirectly linked to an anti-BSA IgG by a protein A-streptavidin chimera (linker molecule). The signal was generated by amplifying a segment of pUC19 molecule by PCR. The PCR products were detected by agarose gel electrophoresis with ethidium bromide staining. The results revealed that I-PCR improved detection sensitivity about 10^5 times over ELISA. Ruzicka et al. (1993), who

simplified Sano's chimeric protein-based method by using all reagents from commercial sources, were still able to detect as little as 10 fg of anti-apolipoprotein E per milliliter of coating solution.

A full-length recombinant human proto-oncogene (ETS1) was detected by using I-PCR (Zhou, 1993). In Zhou's I-PCR assay, the antigen, ETS1, was coated onto ultra thin-wall PCR tubes overnight at 4 °C to immobilize the antigen. Then purified ETS1-specific monoclonal antibody, biotinylated sheep anti-mouse secondary antibody, free streptavidin, and biotinylated *Eco*RI-digested pαTEL14 (marker DNA) were added sequentially. The immobilized linear DNA was subjected to PCR amplification. Signal from ETS1 could be detected with as few as ~5.8×10^3 molecules.

Bovine herpesvirus 1 (BHV-1) antigens were detected by I-PCR after immobilization on microtiter plates (Mweene et al., 1996). BHV-1-specific monoclonal antibody, biotin-labeled goat anti-mouse antibody, streptavidin, and biotin-labeled marker DNA were added sequentially, with washes after each step. The plates were subjected to PCR and an agarose gel was used to detect the amplified PCR products. A sensitivity of up to 10^7 higher than that of ELISA for antigen detection was obtained. I-PCR was also demonstrated to be very useful for detecting BHV-1 in the nasal secretions and various tissues of experimentally infected calves (Mweene et al., 1996).

The application of a single I-PCR protocol for the detection of multiple antigens was studied by Case and colleagues (1997, 1999). These antigens were diverse both in chemical structure and size: O^6-methylguanosine conjugated to BSA (O^6-MeG-BSA, Mr 6.76×10^4), pyruvate dehydrogenase complex (PDC) oligomer (Mr 8.5×10^6), O^6-methylguanine-DNA methyltransferase (MGMT) monomer (Mr 2.17×10^4), and an

N-terminal oligopeptide of MGMT (Mr 2.31 x 10^3). The detection limits were between 180 and 6.9 x 10^7 molecules, with O^6-MeG and PDC to be the most sensitive and MGMT to be the least sensitive.

Human serum gastric carcinoma-associated antigen MG7-Ag was detected after immobilization on the surface of microtiter plate wells (Ren et al., 2000). It was concluded that this technique could be applied in the clinical setting readily and could be a potential screening tool in mass surveys of high-risk populations with gastric carcinoma.

Another variant of I-PCR, called immuno-detection amplified by T7 RNA polymerase (IDAT), has recently been described (Zhang et al., 2001). With this technique, a double-stranded oligonucleotide containing the T7 promoter was conjugated to an antibody, and then the T7 RNA polymerase was used to amplify RNA from the double-stranded oligonucleotides coupled to the antibody in the antibody-antigen complex. This technique was 10^9- fold more sensitive than the conventional ELISA method for detecting the p185 (her2/neu) receptor on T6-17 cells. The researchers claimed that this technique was capable of monitoring proteins, lipids, metabolites, and their modifications at the single-cell level. They also claimed that IDAT represented a significant improvement over conventional I-PCR in terms of sensitivity.

Clostridium botulinum neurotoxin type A (BTx-A) was assayed recently with I-PCR technique (Wu et al., 2001). A murine monoclonal antibody against BTx-A was covalently coupled to double-stranded marker DNA using a heterobifunctional cross-linker reagent. A dose-dependent relationship was demonstrated. Detection sensitivity

of I-PCR for BTx-A (3.33×10^{-17} moles) exceeded that of the conventional ELISA (3.33×10^{-14} moles) by 1000-fold. The DNA-antibody conjugates can be used to detect BTx-A antigen directly, without the addition of biotinylated reagents, binding proteins, and numerous washing steps. Such a simplified I-PCR technique may prove valuable for antigen detection in clinical diagnosis.

Detection of Antigens Indirectly Immobilized onto the Surface of a Solid Support by a Capture Antibody. A sandwich is generally used to perform this type of assay. In a sandwich format, an antibody against a target antigen is bound to the surface of a solid support. Antigen is allowed to bind to coated antibody, then a second antibody against a different epitope on the antigen binds to the immobilized antigen. The second antibody is linked to a reporter molecule, such as an isotope, enzyme, or a marker DNA. Because the Hepatitis B virus (HBV) antigen in serum is undetectable by currently available immunoassays, Maia et al. (1995) tried to use an I-PCR sandwich assay to detect this antigen in serum. The two antibodies chosen were high affinity anti-HBV monoclonal antibodies directed against distinct and separate determinants on the HBV antigen. After the antigen was captured by the first (coated) antibody, the second antibody (biotinylated) was allowed to bind to captured antigen. This step was followed by the addition of free streptavidin. Finally, a biotinylated linear DNA molecule derived from a bluetongue virus (BTV) gene was allowed to bind to the free binding site on the capture streptavidin. PCR was conducted on the bound marker DNA with BTV-specific primers. As little as 0.5 pg of hepatitis B surface antigen (HbsAg) in a serum sample could be detected.

The I-PCR assay has been used to detect two clinical analytes, human thyroid-stimulating hormone (hTSH), and human chorionic gonadotropin (hCG) (Goerger et al., 1995). DNA-antibody conjugates were generated by using heterobifunctional cross-linker chemistries. These approaches yielded molecular chimeras both for analyte binding and PCR amplification. Dose-response relationships were observed for I-PCR assays of both analytes. Detection limits for hTSH and hCG were 1×10^{-19} and 5×10^{-17} M, respectively. These sensitivities exceeded those of conventional ELISA by three to four orders of magnitude.

I-PCR has also been used to detect tumor necrosis factor alpha (TNF-alpha). Sanna et al. (1995) investigated the time course of TNF-alpha induction in rat cerebrospinal fluid after intracerebroventricular administration of bacterial lipopolysaccharide (LPS). I-PCR sensitivity was 0.625 pg/well and the sensitivity of the ELISA version of the same assay was 100 pg/mL (159 -fold difference in sensitivity). Due to the high sensitivity of I-PCR, TNF-alpha in the cerebrospinal fluid could be detected as early as 15 min after LPS administration.

TNF-alpha cannot be measured in human serum by existing methods other than I-PCR due to its low concentration. However, it has been detected by I-PCR (Saito et al., 1999). The detection limit of this assay was 0.001 ng/mL, an approximately 5×10^4 fold improvement compared to a conventional ELISA.

The sensitivity of I-PCR was compared to that of ELISA for detecting soluble murine T cell receptors (sTCR) (Sper et al., 1995). With the direct ELISA employing hamster anti-TCR beta monoclonal antibody (which detects all types of alpha and beta TCR) the detection limit was 6000 pg/mL; with the indirect sandwith ELISA using

anti-V beta 8 as capture antibody, the detection limit was 600 pg/mL; with ELISA using anti-V beta 8, the detection limit was 100 pg/mL; and with I-PCR assay, the detection limit was 0.8 pg/mL.

Simultaneous detection of multiple antigens in solution by a single PCR reaction was also possible with I-PCR. Three analytes (hTSH, hCG and β-Gal) were detected in a single reaction (Hendrickson et al., 1995). Assays were performed using a two-antibody sandwich assay. With this system, three marker DNAs, different in length (55, 85, and 99 bp), were covalently attached to three different second antibodies. These DNA sequences were identical at 5' end and at 3' end, so that a single primer pair could be used to amplify the three marker DNAs to generate 55, 85, and 99 bp products. Analytes were detected at sensitivities exceeding those of ELISA assays by approximately three orders of magnitude. Detection limits for hTSH, hCG, and β-Gal were 1 $x10^{-19}$, 1 x 10^{-17}, and 1 x 10^{-17} moles, respectively.

Suzuki et al. (1995) detected soluble intercellular adhesion molecule-1 (sICAM-1) from Panc-1 cells using I-PCR, with a detection limit 10^3 times higher than that achieved by ELISA. I-PCR was also used to detect this antigen in sera at a level below the detection limit of traditional ELISA.

An I-PCR sandwich assay was used to detect α-atrial natriuretic peptide (ANP) in human plasma (Numata and Matsumoto, 1997). ANP is a 28-amino acid polypeptide with an intermolecular disulfide bridge that is synthesized mainly in the heart atria. Plasma ANP levels are increased in patients with heart failure, therefore, they serve as good indicators of atrial distension in these patients. It usually takes 2 or 3 days after heart damage to quantify plasma ANP by immunoradiometric assay (Yamauchi et al.,

1991) or ELISA (Hashida et al., 1988) because of its very low concentration in plasma, but using I-PCR sandwich assay, one can quantify plasma ANP within five h of initial heart damage. The lowest level of ANP detected by I-PCR was 2.0 ng/L (50 fg per tube).

Chang and Huang (1997) used I-PCR to detect *E. coli* beta-glucuronidase (GUD). The surface of 96 V-bottomed polycarbonate microtiter plate wells were coated with the first anti-GUD antibody (rabbit anti-GUD antibody). Then GUD molecules were added to the wells and were captured by the first antibody. This was followed by adding biotinylated second anti-GUD antibody bound to a different epitope on the captured GUD molecule. The biotin group was allowed to interact with the avidin molecule on avidin-biotinylated λ phage DNA. Amplification of this DNA fragment generated 500 bp PCR products. The detection limit was 1×10^{-17} g/mL (or 5×10^{-19} g/well), which was equivalent to two GUD molecules in 50 microliters of a sample solution. This technique was so sensitive that it could detect the enzymes released from a single E. coli cell in a solution of 10 L.

Furuya et al. (Furuya et al., 2000) used an I-PCR sandwich assay to detect human interleukin-18 (IL-18) in serum, where the concentration in patients with acquired immunodeficiency syndrome (AIDS) was too low to be measured accurately with conventional ELISA. The plate was coated with mouse anti-human IL-18 monoclonal antibody, followed by sequential addition of IL-18 antigen, rabbit polyclonal anti-human IL-18 antibody, and biotinylated goat $F(ab')_2$ anti-rabbit IgG to each well. After washings between steps, free streptavidin was added to each well. Finally, 50 μl of biotinylated DNA was added and this marker DNA was replicated by PCR. With I-PCR assay, the detection limit was 2.5 pg/liter, 1.6×10^4 times more sensitive than that of

ELISA. The authors claimed that this I-PCR for human IL-18 was the most sensitive method for detecting human IL-18 reported to date.

Sugawara et al. (2000) used I-PCR assay to detect human recombinant angiotensinogen using identical first and second polyclonal antibodies. A rabbit polyclonal antibody (first PAb) immobilized on an ELISA microtiter plate was used to capture antigen sandwiched with biotinylated rabbit polyclonal antibody (second PAb), after which streptavidin was added. Biotinylated DNA (which was initially generated by PCR amplification using a biotinylated primer) was then allowed to bind to plate-bound streptavidin. Finally, the marker DNA was amplified through PCR. The detection limit was 0.1 ng/L, an approximately 2.5 x 10^5 fold improvement compared to a conventional ELISA assay.

I-PCR Components and I-PCR Assay Sensitivity

The First Antibody. The first antibodies are the molecules that make direct contact with target antigens. Their physical properties have a great impact on the sensitivity and selectivity of I-PCR assays. Both monoclonal (Wolfhagen et al.1994, Numata and Matsumoto, 1997) and polyclonal (Case et al., 1997) antibodies can be used in I-PCR assays. The concentration used depends the antibody chosen and the user's preference. McElhinny and Warner (1997) used a high concentration (500 µg/mL) of anti-H-$2D^b$ antibody to detect major histocompatibility complex Class I antigens on the surface of murine embryos. In contrast, Zhang et al. (1998) found that less than one ng/mL of DNA-HumAb complex was required to detect carbohydrate tumor-antigens on tumor cells. Case et al. (1999) demonstrated that, as the concentration of polyclonal antibodies was

increased from one μg/mL to ten μg/mL, assay sensitivity increased 100-fold. One of the major factors affecting antigen detection limits is primary antibody titers. Case et al. (1999) showed that antigen detection limits were positively correlated with primary antibody titers and antigen molecular weight did not affect the sensitivity of I-PCR.

Linker Molecule. Several techniques are available for linking antibodies to DNA molecules. Sano et al. (1992) used a protein A-streptavidin chimera to link protein A-compatible IgG molecules to biotinylated DNA molecules. Streptavidin binds biotin with an extremely high affinity (Kd ~ 10^{-15} M) (Sano, et al., 1990). Protein A binds specifically to the Fc domain of most immunoglobulin G (IgG) types of molecules without disturbing their antigen-binding ability (Sjoholm, 1975). The gene of Sano's linker molecule was cloned into an expression vector (Uhlen et al., 1984; Sano et al., 1995), and is efficiently expressed in *E. coli* (Studier and Moffatt, 1986). Ruzicka et al. (1993) used avidin tetramer to link biotinylated antibodies to biotinylated DNA molecules.

Avidin-biotinylated DNA complex can be prepared by mixing avidin and biotinylated DNA (Law et al., 1993). A primary antibody can be also linked to a DNA molecule through a biotinylated second antibody-streptavidin-biotinylated DNA complex (Zhou, 1993). Although avidin and streptavidin are commercially available, the following potential drawbacks reduce the attractiveness of these molecules as antibody-DNA linkers. Firstly, every primary antibody has to be biotinylated using a tedious procedure that decreases antibody-antigen affinity (Ruzicka et al., 1993). Secondly, the avidin-DNA conjugate lacks homogeneity (i.e., one avidin molecule may bind zero, one, two, three or four biotinylated DNA molecules), which reduces the reproducibility of the

I-PCR system. Thirdly, binding avidin tetramer without marker DNA to antigen-biotinylated antibody complex will reduce the overall sensitivity of this system because no DNA tag will be present for amplification during PCR. Finally, binding four molecules of marker DNA to avidin tetramer (saturated) will prevent avidin from binding to biotinylated antibody, again reducing the number of DNA tags available for PCR. A primary antibody can also be linked to a marker DNA directly through cross linking technology that creates covalent bonds between the antibody and the DNA without using a linker molecule (Joerger et al., 1995). Although direct linking can be applied to any class or sub-class of immunoglobulin, it is not generally recommended due to the complexity of the manipulation involved (Sano et al., 1995).

The advantages of using a protein A-streptavidin chimera as a bispecific linker molecule include: 1) the protein A moiety is a "universal linker" because it can bind specifically with high affinity to most immunoglobulin G from many species; 2) streptavidin moiety binds biotin with high affinity, and biotin can be easily incorporated into a marker DNA molecule; 3) because streptavidin can form a subunit tretramer, four protein A-streptavidin chimera molecules form an aggregate, which can bind four IgG and four biotin molecules independently by using an appropriate relative molar ratio of biotinylated DNA to protein A-streptavidin chimera (Niemeyer et al., 1999). One can link up to four DNA molecules to each bound antibody in situations when antigens are scarce, thus, amplifying the target signal (Sano et al., 1995). One potential problem associated with using protein A-streptavidin chimera as a linker molecule in a sandwich assay format is that the protein A portion of the chimera might bind to Fc portion of the antibody used to coat the surface of a microplate well (Ruzicka et al., 1993). However,

this problem can be minimized by using $F(ab')_2$ or Fab fragments, which lack Fc portion, as capturing antibodies (Sano et al., 1993).

Marker DNA. Any DNA molecule (referred to as marker DNA thereafter) can be used in I-PCR assay, as long as this DNA sequence is not present in the sample to be analyzed. Case et al. (1997) used a fragment of gene from a plant as the marker DNA. Maia et al. (1995) produced a marker DNA from a virus gene. McElhinny and Warner (1997) made their marker DNA out of a plasmid, while Kakizaki et al. (1996) derived their marker DNA from a phagemid sequence.

Two general methods are available for the preparation of biotinylated marker DNA. The first method uses DNA polymerase to incorporate a biotinylated deoxynucleotide into a DNA molecule with sticky ends. For example, McElhinny and Warner (1997) used *Acc* I and *Hin*d III to digest pUC19 plasmids to generate a 1.67 kb linear molecule. Then they used biotinylated deoxyadenine and the three other deoxynucleotides to produce a biotinylated pUC19 fragment under the action of Klenow large fragment. Another method is the enzymatic amplification (PCR) of a template DNA with a set of primers, one of which contains one biotin per molecule. The resulting PCR product contains one biotin molecule at one of its termini (Kakizaki et al., 1996; Saito et al., 1999).

Factors that affect the efficiency of marker DNA amplification include primer size, strand numbers, and the length of PCR products. Adequate size of primers increases PCR amplification efficiency. Joerger et al. (1995) used two primer sets, P10 and P20 (16 bp each) vs (G3 (20 bp) and G4 (22 bp) in their I-PCR assay. They found that the amplification potential of the G3 and G4 primer set (longer in length and higher thermal

stability) was superior to that of the P10 and P20 primer set. Joerger et al. (1995) also studied the effects of double stranded (ds) versus single stranded (ss) DNA as a template. The results indicated that 25 PCR cycles of ds DNA and 40 PCR cycles of ss DNA generated similar amounts of PCR products. The higher efficiency of double stranded DNA might be attributable to improved stability and increased number of templates with ds DNA compared to ss DNA. The lengths of PCR product also affect the efficiency of marker DNA amplification. In general, the molar yield was higher for shorter PCR products than for longer ones under identical PCR conditions (Joerger et al., 1995).

Factors that affect the detection of PCR products include the lengths of PCR products and detection methods. The length of PCR products influences the amount of DNA-specific dye bound and therefore affects the band intensity on agrose or acrylamide gel. On the same molar base, the longer the PCR product is, the more intense the band on the gel will be. Hendrickson et al. (1995) generated three different lengths of PCR products in a single I-PCR reaction. They found that the band of the shorter PCR product (55 bp) generated from shorter marker DNA (55 bp) was less intense than that of the longer PCR products (85 and 99 bp) from the longer marker DNA (85 and 99 bp) on agarose gel stained by ethidium bromide.

Methods of detecting PCR products also affect assay sensitivity. Niemeyer et al. (1997) compared three methods for detecting PCR products: 1) direct staining with a fluorescent intercalating dye, 2) an enzymatic assay utilizing doubly hapten-labeled products, and 3) gel electrophoresis. They concluded that the most sensitive method among the three was the fluorometric dye.

In conclusion, I-PCR has been extensively used to detect a variety of membrane-bound and soluble antigens, and abundant evidence indicates that I-PCR is the most sensitive of currently available techniques to detect minute amounts of antigen. In addition, I-PCR technique is an exquisitely sensitive and non-invasive technique for detecting antigens on living cells. Therefore, we hypothesized that an I-PCR assay could be used to validate a model system for the detection of protein expression in single mammalian embryos and to work toward the goal of a non-invasive assay that could determine viability of a single mammalian embryo.

Proteins Expressed by Preimplantation Embryos and Potential Candidates for Embryo Viability Assay

Many proteins are expressed by pre-implantation embryos. These proteins can be classified into two general categories, e.g., membrane-bound proteins and soluble proteins. Each category can be further divided into groups. The membrane-bound proteins that embryos produced include major histocompatibility complex (MHC) molecules, such as H-2D^b and Qa-2 antigens in mice (McElhinny and Warner, 1997; McElhinny et al., 1998a, 1998b, 2000; Warner et al., 1993; Wu et al., 1999; Xu et al., 1994); cell adhesion molecules, such as integrins in mice (Cai et al., 2000), N-cadherin in chickens (Garcia-Castro, 2000), connexins in mice (Lo and Gilula, 1979; McLachlin et al., 1983; Goodall and Johnson, 1984); and growth factor receptors (Lighten et al., 1997; Wiley et al., 1992). Soluble proteins produced by embryos include growth factors (Harvey and Kaye, 1990; Paria et al., 1991), cytokines (Orvieto and Bar-Hava, 1999; Orvieto and Schwartz, 1997), and prostaglandins (Niimura and Ishida, 1987). These

membrane-bound and soluble proteins have diverse functions in embryo growth and development. However, the research reported herein was designed to concentrate on insulin receptor, epidermal growth factor receptor, and interleukin-6 based on the reasons discussed in the following sections.

Expression and Functions of Insulin and Its Receptors

Insulin is a small protein with a molecular weight of 5,700. It contains two polypeptide chains, A and B, joined by two disulfide bonds (Nelson and Cox, 2000). The first step in insulin action consists of binding of the hormone to specific cell surface receptors. The insulin receptor is a hormone-dependent protein tyrosine kinase that belongs to the family of tyrosine kinases associated with growth factor receptors and oncogene products. This receptor has two functional domains: an extracellular alpha-subunit containing the majority or the totality of the hormone binding site and an intracellular beta-subunit possessing insulin-stimulated tyrosine kinase activity. This receptor's enzymatic function is essential for generation of the metabolic and growth-promoting effects of insulin. The activity of the insulin receptor kinase is regulated by the phosphorylation state of specific domains of the protein (Herrera and Rosen, 1987). Interaction of insulin with the receptor alpha-subunit triggers a conformational change, which is propagated to the beta-subunit and activates it. The active receptor kinase then leads to the phosphorylation of cellular protein substrates, which are likely to belong to two broad categories, those generating metabolic effects and those resulting in growth-promoting effects. The phosphorylated and active substrates then generate the final effects (Ballotti et al., 1989).

Evidence suggests that insulin receptor (IR) or insulin like growth factor receptors (IGFR) are expressed on pre-implantation embryos. Insulin like growth factor II transcripts were detected in mouse pre-implantation embryos (Lighten et al., 1997). Messenger RNA and protein for IGF-II, but not for insulin, were expressed in pre-implantation mouse embryos (Harvey and Kaye, 1992). Messenger RNAs of insulin receptor and IGF I and II receptors (IGF1R and IGF2R) are present in rat embryos during the pre-implantation period (Lighten et al., 1997). Insulin-like growth factor II mRNA was present in embryos, oviducts, and uteri during the rat pre-implantation period (Zhang et al., 1994). In addition, transcripts for IR, IGF1R, and IGF2R were present in human pre-implantation embryos (Lighten et al., 1997). Insulin receptor expression was seen in the ureteric bud branches and early nephron precursors in mouse metanephroi harvested at day 13 of gestation (Liu et al., 1997). Addition of insulin into the culture medium at low concentrations, ranging from 40 to 400 ng/mL, increased [3H] thymidine incorporation in mouse embryonic renal explants, and inclusion of IR beta-subunit-specific anti-sense oligodeoxynucleotide caused marked dysmorphogenesis and growth retardation of the metanephroi (Liu et al., 1997). Functional studies showed that incubating mouse embryos with physiological levels of insulin results in increased synthesis of RNA and DNA (Heyner et al., 1989). Indirect evidence also exists that insulin receptors are present on the ICM of mouse embryos (Harvey and Kaye, 1990) and proteins for IGF-II were expressed in pre-implantation mouse embryos (Harvey and Kaye, 1992). These findings suggest that insulin and related factors may play a role in early mammalian development (Heyner et al., 1989; Zhang et al., 1994).

High resolution electron microscopy in conjunction with colloidal gold-labeled insulin has been used to provide direct evidence that insulin is internalized by pre-implantation mouse embryos by means of receptor-mediated endocytosis (Heyner et al., 1989). In addition, immunocytochemical analyses at the blastocyst stage, using gold-labeled anti-insulin receptor immunoglobulin (IgG), have confirmed the expression of insulin receptors on all cells of the embryo, including the inner cell mass. This evidence also suggests that insulin receptor is expressed on pre-implantation mouse embryos, but its concentration is too low to be detected by conventional techniques (Lighten et al., 1997).

The functions of insulin and insulin like growth factor receptors were determined directly from culture studies. Insulin or IGF-1 at physiological concentrations stimulated pre-implantation mouse embryo growth (Harvey and Kaye, 1992). Insulin-like growth factor II (IGF-II) mediated growth in early mouse embryos (Harvey and Kaye, 1992). Exogenous IGF-I or IGF-II increased the cell number in cultured blastocysts, but a mutant form of IGF-II that strongly bound only the IGF-II receptor did not have an effect. Reduction of IGF-II expression by antisense IGF-II oligonucleotides decreased the rate of progression to the blastocyst stage and decreased the cell number in blastocysts. Pig blastocysts responded to physiological levels of insulin by increasing in diameter and in the rate of protein synthesis. Insulin at concentrations as low as 50 pM increased the number of mouse blastocysts and decreased the number of morulae by 10% after 54 h culture from 2-cell embryos. These results support the hypothesis that insulin has an important role in the regulation of growth during pre-implantation development (Harvey

and Kaye, 1990), and support the hypothesis that insulin may act as a general embryonic growth factor. (Lewis et al., 1992).

Expressions and Functions of Epidermal Growth Factor and Its Receptors

Epidermal Growth Factor (EGF) is a small polypeptide (Fine et al., 1981) that exerts its functions by binding to specific receptors embedded in the cellular plasma membrane. After binding to its receptors, EGF moves to the cytoplasm in coated vesicles (CVs), and finally appears in lysosomes, where it is degraded and released from the cells (Fine et al., 1981). EGF has been isolated from rodent and human tissue, and probably exists in nearly all animal species. In humans, EGF has been detected in many body fluids (Carpenter, 1985). The expression of EGF in 22 to 30-day goat pregnancies was studied and the results indicated that EGF was expressed in the luminal and glandular endometrial epithelium on all days studied, but it was not detectable in trophoblastic cells or other embryonic structures (Flores et al., 1998). Epidermal growth factor, which is not synthesized by the mouse pre-implantation embryo, is present in both the oviduct and the uterus. Epidermal growth factor of maternal origin is present in both inner cell mass and trophectoderm cells in freshly isolated blastocysts but is present only in greatly reduced amounts following overnight culture of blastocysts *in vitro* (Dardik et al., 1992), indicating that blastocysts incorporate maternal EGF *in vivo*.

Epidermal Growth Factor receptor (EGFr) in mice is a 170-kDa protein (Paria et al., 1991; Wiley et al., 1992) which is encoded by a single gene that produces two main transcripts that are translated and processed into a single polypeptide chain (Adamson, 1990). In the mid 1980s, it was found that the EGFr has no subunit structure, and

functions not only in ligand recognition, but also may produce an intracellular 'second messenger'. The receptor contains a protein kinase activity that is activated by the binding of EGF and this enzymatic function may, in turn, yield the critical 'second messenger', by phosphorylation of an intracellular protein (Carpenter, 1985). This intracellular protein is phosphorylated when exposed to both EGF and ATP. Epidermal growth factor induced approximately a two-fold increase in protein tyrosine kinase (PTK) activity in Day 4 mouse blastocysts when they were incubated in the presence of a peptide substrate with a tyrosine moiety and ATP (Paria et al., 1991), indicating that EGF has a metabolic effect on pre-implantation embryos.

Through antisense DNA inhibition and immunoprecipitation studies, it was shown that EGFr protein in mice was first synthesized at the blastocyst stage (Paria et al., 1991; Wiley et al., 1992). In humans, receptors for EGF are ubiquitous (Carpenter, 1985). It was observed that EGFr existed in the oocytes of primordial, primary, preantral, and antral follicles. Strong immuno-staining for EGFr was present in human thecal cells and EGFr was also expressed in some granulosa cells of primary to antral follicles (Qu et al., 2000). Wiley et al. (1992) used immunoprecipitation and RT-PCR techniques to study the EGFr expression pattern of mouse embryos. They observed that unfertilized eggs and 2-cell embryos had a very low reactivity to antimouse EGFr antibodies, but by the 4-cell stage and later the reactivity increased. Messenger RNA concentrations increased starting after 4-cell stage and EGFr protein was synthesized by 8-cell and older embryos. The widespread expression of EGFr throughout development from the beginning of gestation in mice suggests that it has important roles, which could include autocrine and paracrine stimulation (Wiley et al., 1992). Also in mice, EGFr is preferentially distributed

on the basolateral surface of the trophectoderm and is present on the inner cell mass (Dardik et al., 1992). Evidence also exists that transforming growth factor-alpha (TGF-alpha) may act as a regulating factor for follicular development through binding to the EGF receptor (Qu et al., 2000).

Epidermal Growth Factor acts as a mitogen for many cell types (Fine et al., 1981). Epidermal growth factor stimulated oocyte maturation by increasing prostaglandins (PG) in granulosa cells (Tsutsumi, 1993). Nuclear and cytoplasmic maturation of in vitro-grown mouse oocytes was also promoted by EGF treatment during meiotic maturation (De La Fuente, 1999). The presence of EGF during in vitro maturation, irrespective of concentration, stimulated bovine cumulus expansion and significantly increased the proportion of oocytes attaining metaphase II, the rate of embryo cleavage, and the proportion of embryos reaching the 5 to 8 cell stage at 72 h post insemination. The presence of EGF also altered the pattern of protein neosynthesized during oocyte maturation in cattle (Lonergan, 1996). Evidence indicated that if placental EGFrs malfunction, the fetus is at a disadvantage, which may be attributable to poor placental growth and development, as well as a reduced ability of the placenta to perform a barrier function (Adamson, 1990). Evidence also exists that the response on the binding of EGF to EGFr has a greater effect on the regulation of cellular differentiation than on cellular proliferation of two-cell mouse pre-implantation embryos cultured for 48 h (Brice et al., 1993).

Interleukin-6 (IL-6)

Molecular Structure and Biological Functions. Interleukin-6 (IL-6) is a multifunctional glycoprotein of 185 amino acids that belongs to a class of hormone-like molecules termed cytokines. Interleukin 6 shares a structure of an antiparallel four α-helical bundle core with leukemia inhibitory factor (LIF), oncostatin M (OM), ciliary neurotrophic factor (CNTF), and IL-11 (Brakenhoff et al., 1995). IL-6 exerts its activity through binding to a high affinity receptor (Yamasaki *et al.*, 1988).

The IL-6 receptor (IL-6R) complex consists of two membrane proteins: an 80 kDa IL-6-binding receptor protein (Yamasaki *et al.*, 1988) and a 130 kDa signal-transducing protein (gp 130; Hibi *et al.*, 1990). The IL-6R complex consists of 468 amino acids, including a signal peptide of approximately 19 amino acids and a domain of approximately 90 amino acids that is similar to a domain in the immunoglobulin(Ig) superfamily (Yamasaki et al., 1988). A cDNA encoding human gp130 has been cloned, which consists of 918 amino acids with a single transmembrane domain. The extracellular region comprises six units of a fibronectin type III module, and part of this region of approximately 200 amino acids has features typical of the cytokine receptor family. A cDNA-expressed gp130 showed no binding property to IL-6 or several other cytokines. Although a transfectant with an IL-6R cDNA expressed mainly low affinity IL-6 binding sites, an increase in high affinity binding sites was observed after cotransfection with a gp130 cDNA. This confirmed that a gp130 is involved in the formation of high affinity IL-6 binding sites.

Production and Distribution. The production, secretion, and distribution of IL-6 in reproductive tissues have been extensively researched. Interleukin-6 activity was detectable in porcine uterine luminal fluid throughout the estrous cycle and pre-implantation period of pregnancy, and IL-6 mRNA was only detectable in day 11 endometrium (Anegon et al., 1994).

A research group in the United Kingdom used RT-PCR analysis to investigate expression of cytokine and receptor mRNA and found that IL-6R was expressed in a stage-specific manner throughout the preimplantation development of cryopreserved human embryos (Sharkey et al., 1995). Interleukin-6 was detectable in all seven-day old mouse embryos tested, which suggested that this cytokine may play an important role in early embryonic development (Kita et al., 1994). The results of an *in vitro* study revealed that IL-6 was differentially expressed at several stages of mouse preimplantation development and IL-6R was also detectable at these stages of development (Gerwin et al., 1995).

A group of researchers in Switzerland used RT-PCR to investigate the expression of mRNA for IL-6 and IL-6R, and found that both mRNAs were expressed in normal rat brain in a region-specific manner and were developmentally regulated. The most pronounced expression of both genes was observed in the adult hippocampus. Lower levels were seen in the hypothalamus and the striatum. It was found that IL-6 mRNA is predominantly located in neurons, indicating that IL-6 may have a specific role in neuronal functions such as differentiation or survival (Gadient and Otten, 1995). It was also found that IL-6 was produced in the whole placental tissue, isolated trophoblast (TC), and the villous core (VC) compartments of the placenta *in vitro*. Dot blot analysis

and ELISA revealed that all three tissues linearly produced IL-6 over a 28-h culture period. It was found that IL-6 was detectable in all samples of coelomic and amniotic fluids and in most extracts of placental and decidual tissues of normal human pregnancies between 7 and 12 weeks of gestation. The concentration of IL-6 varied among tissues and with gestational age, was significantly higher in decidual than in placental tissues, was significantly higher in coelomic fluid than in amniotic fluid, and was positively correlated with gestational age (Jauniaux et al., 1996). IL-6 mRNA levels decreased markedly during trophoblast differentiation *in vitro*. After 6 days in culture, when almost all the cytotrophoblast cells had fused and differentiated into syncytiotrophoblast cells, the amount of IL-6 mRNA was decreased by 60.9%. Interleukin-6 plays important roles in host defense, acute phase reactions, immune responses, and haematopoiesis. Exogenous IL-6 had differential effects on cytokine mRNA expression. When added to placental cultures during the first 6 days of culture, IL-6 markedly inhibited its own expression, and the expression of IL-1 alpha and IL-1 beta mRNA. However, when added to the cells during days 6 to 9 of culture, when most of the cells were syncytiotrophoblast cells, IL-6 stimulated IL-1 alpha and IL-1 beta mRNA expression (Stephanou et al., 1995).

Evidence also exists that when continuously exposed to IL-6 (500 U/mL) for up to 6 days, an enriched fraction of human trophoblast cells (prepared by the isopycnic centrifugation of enzymatically dispersed term placenta) released significantly higher amounts of placental lactogen and placental lactogen mRNA in a dose-dependent fashion (Stephanou and Handwerger, 1994). A cloned gp130 could associate with a complex of

IL-6 and soluble IL-6R and transduce the growth signal when expressed in a murine IL-3-dependent cell line (Hibi et al., 1990).

Research Objectives

From the above discussion, we can see that insulin, epidermal growth factor, and interleukin 6 have many important functions in animal reproduction, especially in the regulation of preimplantation embryo growth and development. Any abnormality of these growth factors and their receptors could result in undesirable reproductive performance. Therefore, a simple, accurate, and speedy assay for these factors and/or their receptors may be of paramount importance to improving animal reproductive performance by accurate prediction of the viability of individual embryos.

Many assay methods have been used to study insulin, epidermal growth factor, and interleukin 6, such as RT-PCR for detecting EGFr expression in human (Chia et al., 1995) and bovine embryos (Watson et al., 1992), for detecting IL-6 expression on mouse embryos (Kita et al., 1994); commercial ELISA kits for measuring IL-6 secreted from human embryos (Seifer et al., 1993); immunocytochemical assays for studying the production of IL-6 by normal human trophoblast (Kameda et al., 1990). However, these techniques suffered either low sensitivity, were invasive in nature, or too were complicated to be commercially useful in single embryos. So, a quick and sensitive technique to detect these cell surface proteins needed to be developed.

Immuno-polymerase chain reaction, as mentioned above, is the most sensitive technique currently for the detection of either membrane-bound or soluble proteins. In addition, I-PCR is non-invasive and easy to perform. A number of molecules produced

by preimplantation embryos are viability-related. These molecules, such as insulin receptor, epidermal growth factor receptor, and interleukin-6, could be used as an indicator of an embryo's health status. The purpose of this series of experiments was to determine the feasibility of using I-PCR to detect insulin receptor, and epidermal growth factor receptors on individual mouse embryo. In addition, recombinant IL-6 molecules instead of embryo-secreted IL-6 molecules were measured by I-PCR in this preliminary study because the former is free from other cellular protein contamination. Based on the recombinant IL-6 data, the I-PCR assay could subsequently be tuned to measure embryo-secreted IL-6 molecules. The data generated by these studies should be valuable for the future development of a single-embryo viability assay based on I-PCR.

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Chapter 2

THE PRODUCTION AND ASSAY OF

PROTEIN A-STREPTAVIDIN CHIMERA FOR I-PCR

Abstract

Protein A-streptavidin chimera produced in *E.coli* is a component of I-PCR assay that was used to link antigen-antibody complex to biotinylated pUC19 fragment. In order to increase the production of this critical protein, a series of studies was conducted to determine factors that affected its yield. Isopropyl-β-D-thiogalactopyranoside induction time had a positive effect on the yield of chimeric protein. This protein increased from zero through four h of IPTG induction. After five h, the yield remained about the same or was reduced slightly. The effect of induction temperature was not clear-cut. However, it appeared that a slight increase in temperature from 37.0 °C to 37.5 °C increased protein A-streptavidin chimera production. The method of bacterial cell lysis also affected chimeric protein yields, with higher yields by sonication than by chemically induced lysis. Culture medium played a major role in the induction of chimeric protein. Terrific Broth was preferable to either M9 medium or Lennox L broth for inducing chimeric protein. Timing of centrifugation of bacterial lysate had a major effect on chimeric protein yields. Although centrifugation just before IgG-affinity chromatography did not seem to affect overall chimeric protein yields, centrifugation just before biotin-affinity chromatography column significantly reduced chimeric protein yield because substantial amounts of chimeric protein were discarded in the pellet. After purification,

the physical and functional integrity of this protein was also analyzed. It appeared that this protein was highly purified and fully functional.

Key words: chimeric protein, culture medium, protein purification.

Introduction

The bacteria transformed by pTSAPA-2 vector was produced by Sano et al. (1992). This vector contains a truncated protein A gene fused to a truncated streptavidin gene. Under induction by IPTG, a protein A-streptavidin chimera was produced. This chimeric protein binds many of the isotypes of IgG molecules at one end (protein A end) and binds to biotin at the other end (streptavidin end). This protein is one of the important components in I-PCR assay, because it provides a connection between antibody-antigen complexes and the biotinylated pUC19 fragment.

Although the use of a protein A-streptavidin chimera was first described almost a decade ago by Sano et al. (1992), only one other research team has reported the use of this protein in an I-PCR assay (McElhinny and Warner, 1997). Very limited information was available from either of these groups concerning the factors affecting the chimeric protein yields. Increasing the chimeric protein yield is not only economically sound, but also simplifies subsequent testing of this protein. Preliminary data in our lab suggested that induction time and temperature appeared to affect the chimeric protein yield. So, controlled studies were conducted for critically testing the effects of these two factors on chimeric protein yield. The chemical lysis of bacterial cells in the original method described by Sano and Cantor (1990) was very tedious and time-consuming, so we chose to use a simpler sonication method (the improved method) instead. A simple comparison

between these two methods was conducted to determine which method was superior in terms of chimeric protein yield. Culture medium plays an important role in both plasmid and protein production of bacteria. A very low yield of chimeric protein was produced by using M9 medium, which was suggested by Sano et al. (1992). So, two other culture media, TB and LB broth, were also explored for producing chimeric protein. According to the original protocol (Sano et al., 1992), after the crude extract of chimeric protein was obtained, it was first dialyzed in respective column buffer before going through an IgG column which bound to the protein A moiety and a biotin column which bound to the streptavidin moiety of the protein A-streptavidin chimera. It was observed that after passing through the first affinity column (IgG column), the chimeric protein was almost pure. However, a large proportion of protein was lost during the centrifugation step that occurred just before the biotin affinity column. In order to confirm the effect of centrifugation on the chimeric protein loss, a controlled experiment was also conducted to compare the effects of centrifugation versus non-centrifugation on the chimeric protein yields.

In addition to the chimeric protein yield, the functional integrity of this protein is also important for conducting I-PCR assay. Two separate studies were conducted in this regard. The first study was immuno-blotting which was able to determine the molecular weight and identity of the chimeric protein. The second study was an ELISA assay which was able to determine whether the chimeric protein was bifunctional (could bind to both IgG and biotin).

Materials and Methods

Reagents and Apparatus

The following ingredients were from Sigma Chemical Co. (St. Louis, MO): PMSF (Phenylmethanesulfonyl fluoride), deoxyribonuclease I and ribonuclease A, anti-streptavidin (rabbit, antisera), biotinamidocaproyl-alkaline phosphatase, LB Broth Base, and LB Agar Tablets, 2-iminobiotin agarose beads, and fish gelatin; the following ingredients were from Spectrum Laboratories, Inc. (Rancho Dominguez, CA): IPTG (Isopropyl-β-D-thiogalactopyranoside), Spectra/Por* 7 dialysis membrane (MWCO: 25,000) and Spectra/Por® RC Irradiated DispoDialyzer (MWCO: 25,000); the following ingredients were from Bio-Rad Laboratories (Hercules, CA): Immuno-Blot™ PVDF membrane, EIA Grade Reagent Gelatin, GAR-HRP (goat-anti-rabbit IgG-horseradish peroxidase conjugate), and Trans-Blot SD Semi-Dry Transfer Cell; IgG Sepharose 6 Fast Flow gel and PD-10 column were from Amersham Pharmacia Biotech Inc. (Piscataway, NJ); Aprotinin and Leupeptin were purchased from United States Biochemical Corp. (USB) (Cleveland, OH); Whatman No. 1 filter paper was from Fisher Scientific (Hanover Park, IL); Pepstatin A was from Roche Molecular Biochemicals (Indianapolis, IN); Branson Sonifier 450 (model 450) was from Branson Ultrasonics Corporation (Danbury, CT); Transformed *E. Coli* strain, BL21(DE3)(pLysS)(pTSAPA-2), was a gift from Dr. Takeshi Sano (Harvard Medical School, Cambridge, MA).

Medium Preparation and Bacterial Culturing

Preparation of LB Agar Plate. LB Agar Tablets (Sigma) were added to distilled water (one tablet/50 ml) in a beaker on a stirring hot plate while stirring. After the tablets melted, the solution was autoclaved for 15 min at 121 °C. Ten ml of LB agar solution was added to 100-mm petri dishes (plates without antibiotics). After the liquid agar in the beaker had cooled, ampicillin was added to a concentration of 150 μg/mL. Ten mL of LB agar/ampicillin solution was added to other 100-mm petri dishes (plates with ampicillin). After that, twenty-five μg/mL of chloramphenicol was added to the remaining melted agar and 10 mL of the agar was put into other petri dishes (plates with both ampicillin and chloramphenicol). The plates stayed at room temperature for four h. Then they were wrapped in parafilm and stored at 4 °C for up to 24 h before use. Before plating, the plates were inverted and incubated face down at 37 °C for 20 min to dry the plates.

Preparation of LB and TB Broth. LB broth base powder (two g/100 mL, 1% tryptone, 0.5% yeast extract, and 0.5% NaCl) or TB broth powder (4.76 g/100 mL plus 0.4 mL of glyceral, 1.2% tryptone, 2.4% yeast extract, 0.94% K_2HPO_4, 0.22% KH_2PO_4, and 0.4% glycerol) was dissolved in distilled water at room temperature without stirring. Once dissolved, the LB broth was autoclaved for 15 min at 121 °C and then cooled down to room temperature before use.

Original Bacterial Culture. Ten mL of autoclaved LB broth were added into each of two 50-mL flasks. Then 150 μg/mL ampicillin and 25 μg/mL chloramphenicol were added to each flask. A stab culture of BL21(DE3)(pLysS)(pTSAPA-2) was put into each

flask and cultured at 37 °C with shaking at 300 rpm for 11 h. One loopful of bacteria culture from one of the flasks was streaked on LB agar plate with selective agents.

Plating Bacteria on LB Agar Plates. The original culture with LB broth was first diluted to achieve an absorbance of 1.000 at 600 nm, then was further diluted 500 times in LB broth and 200 times in SOC medium. Ten µl of final bacteria dilution was added to 90 µl of SOC medium on agar plates with or without selective agents and spread with a sterilized spatula. Plates were incubated at 37 °C with maximium humidity for 24 h.

M9-Supplemented Medium and Starter Culture. M9 medium was prepared by sequentially adding one mM MgSO4, 0.2% glucose, 1.5 µM thiamine, 0.5% casamino acids, two µg/mL d-biotin, 150 µg/mL ampicillin, and 25 µg/mL chloramphenicol to M9 salt solution (42.3 µM Na2HPO4, 22 µM KH2PO4, 8.6 µM NaCl, 18.7 µM NH4Cl, pH 7.4). Ten mL of sterilized M9 medium was poured into a 50 mL flask, and one isolated bacterial colony was added into the beaker and cultured for 15 h at 37 °C with shaking at 300 rpm. Four mL of the cultured cell suspension was added to 100 mL of M9 medium and cultured under the same condition for an additional four hours.

Preparing Large Batch Culture. Four mL of starter culture was added to 220 mL of M9 medium in each of 1000-ml flasks and incubated at 37 °C with vigorous shaking (300 rpm) for about four h. When the A_{600} reached 0.8 to 1.0, either zero (control) or 0.5 mM IPTG (treatments) was added and cultures were continued to be incubated for 0, 1, 2, 3, 4, 5, or 6 h with shaking.

Harvesting Bacteria. Fifty-mL of bacterial culture was centrifuged at 2300 x g at 4°C for 15 min. The supernatant was discarded. The centrifuge tube was reloaded and centrifuged under the same conditions. So each tube contained a bacterial pellet from

100 ml original bacterial culture. After the supernatant was discarded, the centrifuge tube with bacterial pellet was frozen at - 70 °C (for improved method), or 10 ml of wash buffer (100 mM NaCl, 10 mM tris-HCl, and 1mM EDTA of pH 8.0, at 4 °C) was added to the bacterial pellet (for original method). Cells were resuspended by vigorously shaking and were then re-centrifuged under the same conditions. The supernatant was discarded and ten mL of lysis buffer (2 mM EDTA, 30 mM Tris HCl, 0.1% Triton X-100, 0.5 mM PMSF) was added. The bacteria were resuspended by shaking and vortexing. The bacterial suspensions were stored at –70 °C.

Purification of Sano's Chimeric Protein

Original Method. Ten mL of frozen bacterial suspension in lysis buffer was thawed at room temperature for 45 min, at 4 °C for 1 h, and on ice for 4 h. After vortexing to homogenize the pellet, 86.05μg/mL PMSF, 0.4756 μg/mL leupeptin, and 0.6859 μg/mL pepstatin A were added and stirred occasionally for 20 min. When the lysate became very viscous, 10 μg/mL deoxyribonuclease I and 10 μg/mL ribonuclease A were added, and the lysate was incubated in a room-temperature water bath for 20 min with occasional mixing. After the lysate lost its viscosity, the tube was centrifuged at 39,000 x G for 15 min at 4 °C.

The pellet was dissolved in 3 mL of 7 M guanidine hydrochloride by vortexing at high speed. Then another 7 mL of guanidine hydrochloride was added followed by more vortexing and mixing. After the pellet was completely dissolved, the volume of the solution was increased to 100 mL by adding 7 M Guanidine hydrochloride and mixed thoroughly. The mixture was then put into a dialysis tube (Spectrum Laboratories, Inc.)

and dialyzed against one liter of TST dialysis buffer (150 mM NaCl, 50 mM Tris-HCl, 0.05% Tween 20, 0.02% sodium azide, pH 7.5) supplemented with 86.05μg/mL PMSF, 0.4756 μg/mL leupeptin, 0.6859 μg/mL pepstatin A in a one liter graduated cylinder overnight at 4 °C without stirring. The dialysis buffer was discarded and one liter of fresh dialysis buffer was added and then dialyzed for 6 h with stirring. The dialysis was repeated for three additional times.

IgG Sepharose 6 Fast Flow gel (Pharmacia) packed in a Pharmacia PD-10 column was equilibrated sequentially with 1) three mL of 0.5 M acetic acid at pH 3.4, 2) three mL of TST buffer (TST dialysis buffer without sodium azide), 3) three mL of 0.5 M acetic acid at pH 3.4, and 4) ten mL of TST buffer. The fluid was removed from dialysis tube and was centrifuged at 39,000 x G for 15 min at 4 °C. The supernatant was adjusted to a pH between 7.0 and 7.5. The supernatant was slowly applied to the IgG Sepharose 6 Fast Flow column twice at 8 °C. After all the supernatant had passed through the column, the column was rinsed with 40 mL of TST buffer and the unbound proteins were washed off with 20 mL TST buffer. The column was washed with 2.5 mL of 5 mM ammonium acetate at pH 5.0. The bound protein was eluted with ten mL of 0.5 M acetic acid at pH 3.4 (eluting buffer I). Aliquots of 0.5 mL fractions were collected. The binding and eluting procedures were repeated twice with the flowthrough to make sure all the chimeric protein in the dialysate was harvested. All fractions with absorbances at 280 nm higher than the eluting buffer I were pooled. The pooled fractions were dialyzed in a Spectra/Por® RC Irradiated DispoDialyzer overnight with stirring at 4 °C in 500 mL of pH 11.0 dialysis buffer (1 M NaCl, 50 mM sodium carbonate). The dialysate and washings of the DispoDialyzer were pooled and centrifuged at 39,000 x g for 15 min.

The supernatant was applied to a biotin column (previously equilibrated with the pH 11.0 dialysis buffer) at 8 °C. The column was rinsed with 40 mL of pH11 dialysis buffer and unbound proteins were washed off with 20 mL of pH 11 dialysis buffer. The bound proteins were eluted with ten mL of six M urea and 50 mM ammonium acetate buffer at pH 4.0 (eluting buffer II). A volume of 0.5 mL fractions were collected. All fractions with absorbances at 280 nm higher than eluting buffer II were pooled. These binding and eluting procedures were repeated for three times with the flowthrough to make sure all the chimeric protein in the dialysate was harvested. Pooled fractions were dialyzed in a Spectra/Por® RC Irradiated DispoDialyzer against 500 mL of Tris-buffered saline (150 mM NaCl, 20 mM Tris-Cl, pH = 7.5) overnight with stirring. The volume was recorded and protein concentration was measured spectrophotometrically at absorbance of A_{280}. The chimeric protein was sterilized by passing through a 0.22 micron filter, aliquoted in 100 to 500 microliter volumes and stored at –70 °C.

Improved Method. Ten mL of TST buffer supplemented with the protein inhibitors (2 μg/mL aprotinin, 2 μg/mL leupeptin, and 1 μg/mL pepstatin A) was put into each tube containing a frozen bacterial pellet and the bacteria were resuspended by hand-shaking. The contents of each tube (ten mL of the bacteria suspension) were transfered into a 30 mL Pyrex beaker. The bacteria cells were lysed by sonifying for one min with a Branson Sonifier (Branson Ultrasonics Corporation) at Output Control #5 and Duty Cycle of 50%, and then stopped for three min. The sonifying and stopping cycle was repeated three times. The lysate was centrifuged (first centrifugation) at 37,000 x g for 20 min at 4 °C. The supernatant (supernatant of first centrifugation) was poured into a 15-mL centrifuge tube, and two mL of six M urea in TST buffer was added to the pellet.

A ten-µl pipet tip was used to break the pellet. After that the content was pipetted a few times every 10 min for one h with a three-mL plastic pipette to dissolve the pellet. The suspension (pellet of first centrifugation) was centrifuged (second centrifugation) at 37,000 x g for 20 min at 4 °C. The supernatant (supernatant of second centrifugation, or crude extract of chimeric protein) was poured into a 15-mL centrifuge tube and frozen at - 20 °C until protein purification. A volume of 0.8 mL of 6 M urea in TST buffer was added to dissolve the pellet (pellet of second centrifugation).

The crude extract of chimeric protein in the 15 mL centrifuge tube was mixed with eight mL of 6 M urea in TST dialysis buffer at room temperature. The solution was loaded into Spectra/Por* 7 dialysis membrane (MCWC = 25,000) and dialyzed against 100 mL of 6 M urea in TST dialysis buffer without protein inhibitor for 7 h at 6 – 8 °C with gentle stirring twice. The sample was again dialyzed against decreasing concentrations of urea in 100 mL TST dialysis buffer, 3 M urea with neither inhibitor nor stirring for 2 h and with stirring for 2 h, 1.5 M urea with proteinase inhibitors (2 µg/mL of aprotinin, 2 µg/mL of leupeptin and 1 µg/mL of pepstatin A) for 4 h with stirring, and 0.75 M urea in TST dialysis buffer with proteinase inhibitors for 4 h with stirring. Finally the sample was dialyzed in 100 mL TST dialysis buffer without urea with proteinase inhibitors for 4 h with stirring and this last dialysis step was repeated twice.

The dialysate and the washings of the dialysis tube were transferred into a centrifuge tube and centrifuged (or not centrifuged) at 37,000 x g for 15 min at 4 °C. The supernatant or the dialysate were diluted with TST buffer (with proteinase inhibitors) to a final volume of 20 mL. Then the content was mixed with a 3 mL plastic pipette for one h. The pH of supernatant was measured and adjusted to 7.0 to 7.5. The supernatant was

slowly applied to an IgG Sepharose 6 Fast Flow column (prepared as described in the original method) twice at 8 °C. The column was rinsed with 40 mL of TST buffer, washed with 20 mL of TST buffer, and washed with 2.5 mL of 5 mM ammonium acetate at pH 5.0. Bound protein was eluted with ten mL of eluting buffer I. A volume of 0.5 mL fractions were collected. The flowthrough was loaded and eluted twice to ensure that all chimeric protein was harvested. All fractions with absorbances at 280 nm higher than eluting buffer I were pooled. The pooled fractions were dialyzed in a Spectra/Por® RC Irradiated DispoDialyzer overnight with stirring at 4 °C in 500 mL of pH 11.0 dialysis buffer. The dialysis tube was gently shaken for 30 min at room temperature to dissolve any precipitated protein. The dialysate and washings of the dialysis membrance were pooled and centrifuged (or not centrifuged) at 39,000 x g for 15 min. PH 11.0 buffer was added to the supernatant or dialysate to make a total volume of 20 mL and the diluted supernatant or dialysate were mixed with a three-mL plastic pipette for one h at room temperature. The 2-imino-biotin column was equilibrated with 40 mL of pH 11.0 dialysis buffer. The chimeric protein solution was applied to the 2-iminobiotin agarose column three times at room temperature. The column was rinsed with 40 mL of pH 11 dialysis buffer. Unbound proteins were washed off with 20 mL of pH 11.0 buffer. Bound proteins were eluted with ten mL of eluting buffer II. A volume of 0.5 ml fractions were collected. All fractions with absorbances at 280 nm higher than eluting buffer II were pooled. The pooled fractions were dialyzed against 100 mL of 4.5 M urea in Tris-buffered saline for two h without stirring and two h with stirring, then dialyzed against 100 mL of 3 M, of 1.5 M, and of 0.75 M urea in Tris-buffered saline for two h each with stirring. Finally the pooled fractions were dialyzed against 500 mL of Tris-buffered

saline overnight with stirring. The volume of the dialysate was recorded and chimeric protein concentration was measured at A_{280}. The dialysate was sterilized by passing through a 0.22 micron filter. Samples were stored in 50 µl volumes at –70 °C before use.

Immunoblotting and Chimeric Protein Detection

Separating Proteins. Protein samples were mixed with sample buffer and heated at 85 °C for six min. Ten microliter of the mixture was loaded in each well of a 3.0 % (upper) and 12.0 % (lower) discontinuous SDS-PAGE gel. The sample was electrophoresed at 5 mA for 30 min and then at 8 mA for another 90 min.

Transfering Proteins from a SDS-PAGE Gel to PVDF Membrane. The construction of one TRANS-UNIT of Trans-Blot SD Semi-Dry Transfer Cell (BIO-RAD) as follows (from top to bottom).

a. Graphite electrode (-) fixed to the plastic frame;

b. Six layers of filter paper (Whatman No. 1, same size as the gel) wetted by slow immersion in cathode solution (0.04 M 6-amino-n-hexanoic acid/0.025 M Tris/20% (v/v) methanol of pH 9.4);

c. Three layers of filter paper (Whatman No. 1) wetted by slow immersion in Cathode solution;

d. Polyacrylamide gel;

e. PVDF membrane (pre-rinsed in distilled water, pore size 0.45 um)

f. Three layers of filter paper (Whatman No. 1, same size as the gel slab) wetted by slow immersion in Anode #2 solution (0.025 M Tris/20% (v/v) methanol, pH 10.4).

g. Six layers of filter paper (Whatman No. 1, same size as the gel slab) wetted by slow immersion in Anode #1 solution (0.3 M tris/20% (v/v) methanol, pH 10.4).

h. Graphite electrode (+) fixed to the plastic frame.

Electrophoretic transfer was carried out at a constant current of 42 mA (0.8 mA/cm^2) for 1 h at room temperature.

Colorimetric Detection. The PVDF membrane with proteins transferred from the SDS-PAGE gel was dried completely in the air by placing the membrane between filter papers for six days at 4 °C. The membrane was cut with a razor blade into 3 portions, I, II and III. The cuts were made just to the left of the standard lane at the left most of each portion. The membrane was wetted three times in PBST (138.6 mM NaCl, 2.716 mM KCl, 2.147 mM KH_2PO_4, 9.879 mM Na_2HPO_4, and 0.1% tween 20) at pH 7.37 and was washed twice in ten mL of PBST, each for five min at room temperature. The membrane was then immersed at a 45° angle into ten mL of blocking solution (three % gelatin in PBS) and the solution was gently agitated on a rocking platform at room temperature for 60 min. The blocking solution was decanted and the membrane was washed twice with ten mL PBST for five min each. Ten mL of primary antibody solution [rabbit anti-streptavidin, Sigma) was diluted to 1:500, 1:2,500, or 1:12,500 with antibody dilution buffer (1% gelatin in 1x PBST), then the diluted antibody solution was added to the membrane and incubated for two h with gentle agitation. The primary antibody solution was decanted. The membrane was washed twice, each for five min with gentle agitation, first with ten mL PBST and then with seven mL PBST. The PBST was decanted. Then ten mL of secondary antibody solution (GAR-HRP, BIO-RAD; 1:6667 dilution in antibody dilution buffer) was added to the membrane and incubated with gentle agitation

for two h. The secondary antibody solution was decanted. The membrane was washed with ten mL PBST twice, each for five min with gentle agitation. The PBST was poured off. Ten mL of substrate mixture [one mL of Opti-4CN (4-chloro-1-naphthol) diluent concentrate mixed with 9 mL of distilled water and 0.2 mL of Opti-4CN substrate] was added to the membrane and incubated for 5 min without agitation. The substrate was poured off and the membrane was washed with 20 ml of distilled water for 15 min. Photographs of the membrane were taken using a digital camera with reflective light. The digital image was then manipulated with a densitometer.

Immuno-Sandwich Assay for Linker Molecule

In order to detect functional integrity of the chimeric protein obtained previously, an Immuno-sandwich assay was performed as follows. A 96-well plate (EIA plate) was coated (from top to bottom) overnight at 4 °C with 100 µl per well of one of the following: IgG (100, 50, 25, 12.5, 6.25, 3.125, 1.563 and 0.781 µg in 0.2 M carbonate-bicarbonate buffer at pH 9.6, from columns 1 through 4; IgM (20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 µg in 0.2 M carbonate-bicarbonate buffer at pH 9.6, from columns 5 through 8; or 0.2 M carbonate-bicarbonate buffer at pH 9.6, from columns 9 through 12. The plate was washed three times with distilled water. The last distilled water wash was discarded and the wells were blotted dry. Non-specific sites were blocked by adding 200 µl per well of 1% fish gelatin with 0.01% sodium azide in PBS. The well was covered with an adhesive plastic sheet to prevent evaporation and was incubated at 37 °C for 30 min. The blocking medium was discarded and the plate was washed twice with distilled water. Chimeric protein was diluted with 1% fish gelatin and 0.01% sodium azide in PBS to concentrations of 2.616×10^{-7}, 2.616×10^{-9}, 2.616×10^{-11}, and 2.616×10^{-13} M. Diluted

chimeric protein (100 µl) was added into each well: 2.616 x 10^{-13} M for column 1, 5, and 9; 2.616 x 10^{-11} M for columns 2, 6, and 10; 2.616 x 10^{-9} M for column 3, 7, 11; and 2.616 x 10^{-7} M for column 4, 8, and 12. The plate was then incubated at room temperature for 45 min. After the incubation, the wells were washed twice with distilled water. Biotinamidocaproyl-alkaline phosphatase (Sigma) was diluted one thousand fold with 1% fish gelatin and 0.01% sodium azide in PBS and mixed on a tumbling mixer for about 3 min. One hundred µl of diluted alkaline phosphatase was added to each well and incubated for 45 min at room temperature. The plate was washed five times with distilled water. Para-nitrophenol phosphate solution was prepared by mixing 22 mg of p-nitrophenol phosphate into 22 mL (one mg/ml) of Sorensen's glycine buffer (0.1 M, pH 10.4) supplemented with 1 mM $MgCl_2$. Para-nitrophenol phosphate solution (200 µl) was added to each well. The absorbance was read at 405 nm immediately and then every five min for two h.

Statistical Analysis

The data in Chapter 2 were analyzed either by Student's t-test or by Analysis of Variance.

Results and Discussion

Effects of Induction Conditions on Chimeric Protein Expression

Many attempts were made to increase chimeric protein yield. The early results appeared to indicated that IPTG induction time and temperature had effects on chimeric protein

yield. However, the data did vary somewhat in terms of optimal induction temperature and induction time.

Length of Induction Time. The effect of induction time by IPTG on the yield of chimeric protein is shown in Figure 2.1. Chimeric protein yield increased from zero to five h (lane 1 through 6) of induction, with a sharp increase at five h. The protein yield subsequently decreased at six h of induction.

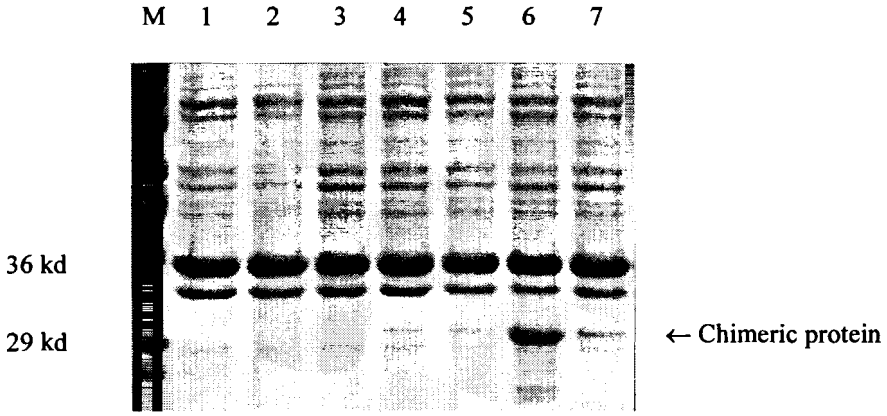


Figure 2.1. The effects of induction time by IPTG on chimeric protein yields. IPTG (0.5 mM) was added to six out of seven bacterial cultures when the absorbance of bacterial culture at 600 nm reached between 0.8 and 1. The flasks then cultured from zero (without IPTG) to 6 h. The chimeric protein crude extract was obtained as described in Materials and Methods (improved method). Eight microliters of crude extract and two microliters of sample buffer were loaded onto each well of the discontinuous SDS-PAGE gel. Lane M, protein marker; Lanes 1 through 7, 0, 1, 2, 3, 4, 5, and 6 h IPTG induction, respectively.

The effect of IPTG induction time on chimeric protein yield was clearly visible. The longer induction time appeared to be beneficial for chimeric protein production, for example, both five and six h induction were better than zero to five h induction. However, we have no clear explanation for the sudden increase in chimeric protein

production at five h (lane 6) of induction time. It seemed that factors other than the IPTG induction time might also have contributed to the sudden increase in the yield of the protein A-streptavidin chimera. The sudden decrease in chimeric protein yield at six h (lane 7) induction time might have resulted from the death of bacteria.

Induction Temperature. The variations in chimeric protein induction in different flasks are shown in Figure 2.2. Bacteria grown in Flasks 3 and 4 (lane 9 and 10) appeared to produce more chimeric protein than in Flasks 1 and 2 (lane 7 and 8). Because the same culture medium and the same type of culture flask were used in this study, theoretically, the chimeric protein yield should have been the same among the 4 flasks. However, it certainly was not the case, because more chimeric protein was produced in flasks 3 and 4 compared to flasks 1 and 2. The most likely reason for the variation among the flasks might be culture temperature. In the type of shaker used during incubations, hot air blows in from one side. Quite possibly, the temperature closer to the hot air vent might have been higher than the temperature further from the vent. The variations seen in this study led us to suspect that suboptimal temperature during incubation may have inhibited the protein A-streptavidin chimera expression.

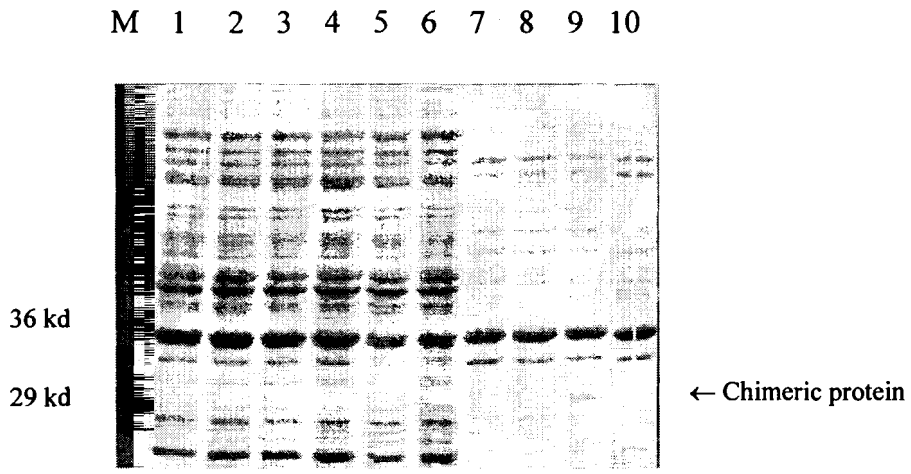


Figure 2.2. Chimeric protein yields from different portions of bacteria cultures and different flasks. M9 medium was put into four 1000-ml flasks (220 mL each). After adding bacterial culture, the culture was incubated at 37 °C for about four h. The flasks were incubated for two h after IPTG induction. Crude chimeric protein extraction and gel electrophoresis were performed as described in Materials and Methods. Lane M, protein marker; Lanes 1 through 4, proteins in cell lysate of flask 1, 2, 3, and 4; Lane 5 and 6, protein in supernatant of flask 1 and 2; Lane 7 through 10, proteins in crude extract of flask 1, 2, 3, and 4.

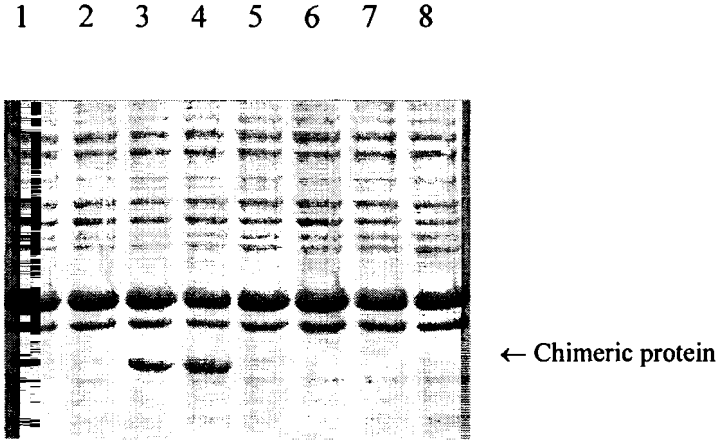


Figure 2.3. The effect of induction temperature on the yield of chimeric protein. Bacterial culture, chimeric protein induction, and crude chimeric protein extraction, and gel electrophoresis were done as described in Materials and Methods except that 4 different induction temperatures 37.5 °C (lanes 1 and 2), 38.0 °C (lanes 3 and 4), 38.5 °C (lanes 5 and 6), and 39.0 °C (7 and 8) were used.

The effect of the induction temperature on chimeric protein yield is shown in Figure 2.3. Three different temperatures were compared during induction with IPTG to determine whether incubation temperature influenced the yield of chimeric protein. More chimeric protein was produced if the induction temperature was adjusted to 38 °C (Lane 3 and 4), but not to 37, 37.5, 38.5, or 39 °C. It seemed the induction temperature was very crucial for chimeric protein induction and the optimal temperature was confined within a very narrow temperature zone.

In order to see clearly the effect of temperature and induction time on the chimeric protein yield, two factors (induction temperature and induction time) were combined in the following study. The effects of induction temperature and induction time on chimeric protein yield are shown in Figure 2.4.

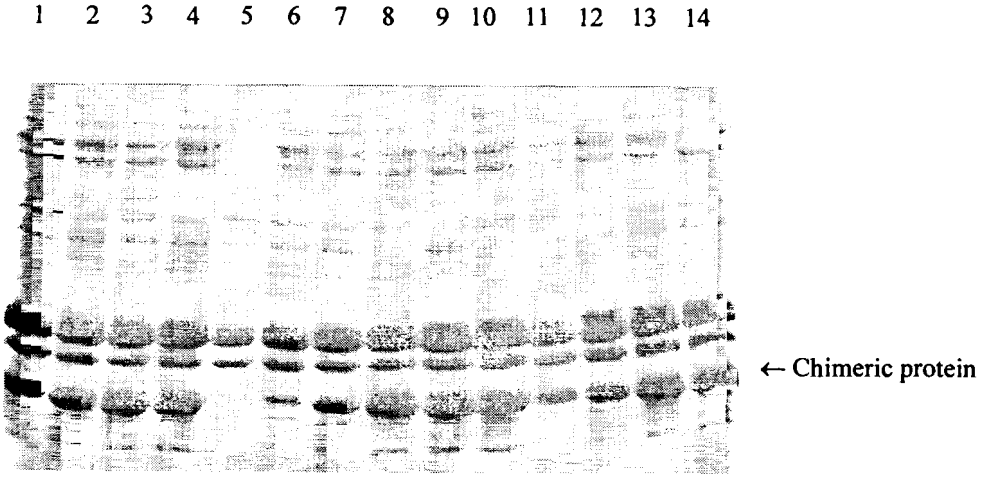


Figure 2.4. The effects of induction temperature and induction time on chimeric protein yield. After addition of IPTG, three different temperatures (37, 37.5, and 38.0 °C) and 6 different induction times (0, 1, 2, 3, 4, 5 h) were used to induce chimeric protein expression. Crude extract production and gel electrophoresis were performed as stated in Materials and Methods. Lanes 1 through 4, 2 to 5 h induction at 37 °C; Lanes 5 through 10, 0 to 5 h induction at 37.5 °C; Lanes 11 through 14, 2 to 4 h induction at 38.0.°C.

As induction time increased, the yield of chimeric protein increased up to four h (Lanes 1 to 3, 5 to 9, and 11 to 13), then decreased at five h (Lanes 4, 10, and 14). These findings were somewhat different from the timing of the previous study (Figure 2.2) where the optimal induction time seemed to be five hours rather than four hours. The reason(s) for this difference was not understood. Induction at 37.5 °C appeared to favor chimeric protein production compared to either 37 °C or 38 °C, but the differences seemed to be minor. This was somewhat different from what was found in the earlier study (Figure 2.3). In Figure 2.3, the optimal induction temperature seemed to be 38.0 °C rather than 37.5 °C. This difference in results might be due to the fact that the shaker used in this study was not the same as used in the previous studies, and its thermostat might have been slightly off scale. It is not difficult to see that a slight change in induction temperature would cause a measurable effect on chimeric protein induction.

The Effect of Bacterial Lysis and Centrifugation on Chimeric Protein Yields

The relative amount of chimeric protein in different fractions during chimeric protein extraction from *E. coli* is displayed in Figure 2.5. The highest proportion of chimeric protein to total cellular protein was obtained in the supernatant of the second centrifugation step (Lane 4).

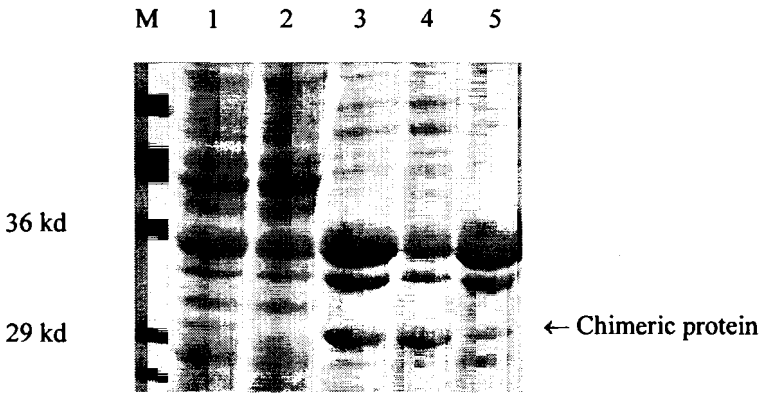


Figure 2.5. Relative amount of chimeric protein in different fractions during chimeric protein extraction from *E. coli*. Chimeric protein was extracted from bacteria as stated in Materials and Methods (improved method). Lane M, protein marker; Lane 1, proteins in lysate; Lane 2, proteins in supernatant of the first centrifugation; Lane 3, proteins in pellet of the first centrifugation; Lane 4, proteins in supernatant of the second centrifugation; Lane 5, proteins in pellet of the second centrifugation.

The chimeric protein purification protocol was adjusted so that the cellular proteins were gradually eliminated. Because of this selective elimination, the proportion of the chimeric protein to the total proteins was gradually increased as the purification went on. This is exactly what is shown in Figure 2.5. A portion of chimeric protein in the supernatant of the second centrifugation (Lane 4) was higher than in the pellet of the first centrifugation (Lane 3), which was still higher than in the lysate (Lane 1). Unexpectedly, a portion of chimeric protein was lost late in the purification process, in the pellet of the second centrifugation.

The effect of bacterial lysis method on the yield of chimeric protein is shown in Figure 2.6. The yield of chimeric protein was higher when the improved method was

used (Lane 1 and 2) than when the original method was used (Lane 3 and 4) to extract the chimeric protein.

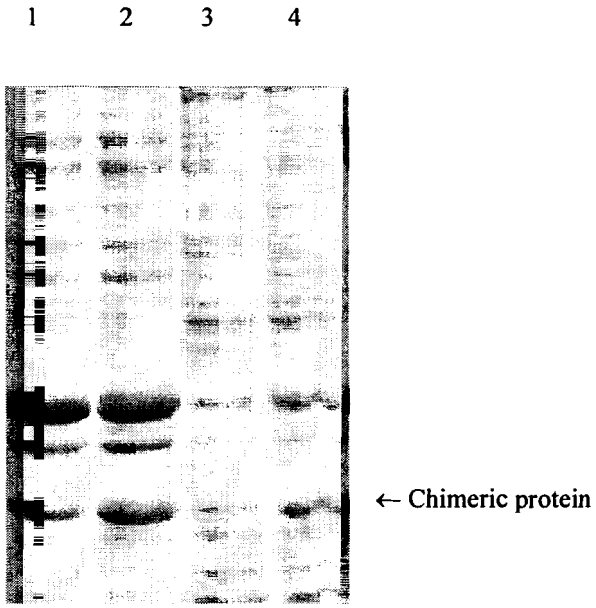


Figure 2.6. The effect of bacterial lysis methods on the yield of chimeric protein. Bacteria were either lysed by lysis buffer (original method) or by sonication (improved method). The crude extracts were obtained as stated in Materials and Methods. Lanes 1 and 2, bacteria lysed by improved method; Lanes 3 and 4, bacteria lysed by original method.

The original method for lysing bacterial cells was tedious and time consuming. There might be several reasons for the differences in chimeric protein yield between these two methods. For example, it was very difficult to completely dissolve the pellet of the first centrifugation in the original method and this might have contributed to the loss of a portion of the chimeric protein.

The effect of centrifugation on chimeric protein yield is shown in Figure 2.7. Centrifugation of the dialysate before the IgG affinity chromatography column did not reduce the yield of chimeric protein significantly. However, centrifugation before the

biotin affinity chromatography column significantly reduced the yield of chimeric protein, indicating that the solubility of chimeric protein in the pre-biotin dialysis was sub-optimal. The highest yield of chimeric protein after IgG and biotin chromatography columns was obtained when centrifugation steps prior to both chromatography columns were eliminated.

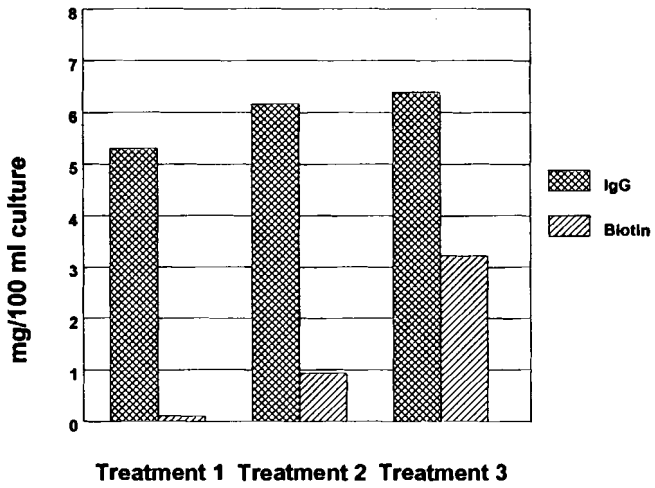


Figure 2.7. The effect of centrifugation on chimeric protein yield. The crude extract of chimeric protein from 900 mL of bacteria culture was pooled and randomly divided into 9 portions. Three portions were assigned to each of three treatments. In Treatment 1, the dialysates before both IgG and biotincolumns were centrifuged. In Treatment 2, the dialysate before IgG affinity chromatography was not centrifuged, but it was centrifuged before biotin affinity chromatography. In Treatment 3, the dialysate was not centrifuged before either chromatography step.

Without centrifugation priors to IgG and biotin chromatography columns (Treatment 3), the chimeric protein yield from 100 mL of bacteria culture was 6.392 mg after IgG chromatography and 3.217 mg after biotin chromatography. With centrifugations prior to IgG and biotin chromatography columns (Treatment 1), the

chimeric protein yield from 100 mL of bacteria culture was 5.2 mg after IgG chromatography which did not differ from Treatment 3, and 0.11 mg after biotin chromatography which was significantly lower ($p < 0.01$) than from Treatment 3. The chimeric protein yield after biotin column in Treatment 2 was 0.921 mg and the yield was intermediate to Treatment 1 and 3.

In the original protocol, dialysis of the crude extract of chimeric protein was followed by centrifugation in order to clarify the dialysate before applying it to the affinity columns. However, we found that protein loss was associated with centrifugation. As a result, less than one mg of protein A-streptavidin chimera was usually obtained from 100 mL bacterial culture. In order to confirm this finding, a controlled study was conducted with a direct comparison between centrifugation versus noncentrifugation before the affinity columns. The experimental results demonstrated that the centrifugation just before the imino-biotin affinity chromatography step was detrimental to the chimeric protein yield, and this centrifugation step was deleted from our purification protocol in later experiments.

The Effect of Media on Bacterial Growth and Chimeric Protein Yields

The effects of media on bacterial growth and chimeric protein yield are shown in Figure 2.8. In general, concentration (estimated by absorbance at 600 nm) after culture in TB broth was higher than in either M9 or LB media. Bacterial growth in TB broth was significantly higher than in M9 medium starting at one h of culture ($p < 0.0001$), and remained significantly higher throughout the rest of the 7 h incubation period. The difference was not significant until five h of culture ($p<0.0061$) when the absorbance of

TB culture was compared to that of LB culture. The absorbance was significantly higher in LB culture compared to M9 culture starting at two h of culture ($p=0.0076$), and this trend continued throughout the rest of the culture period. At the end of the culture, the absorbance of TB culture was about twice as high as that of either M9 or LB culture.

M9 medium [as recommended by Sano et al. (1992)] appeared to be inferior to TB broth for supporting bacterial growth to high density. Therefore, TB broth should be superior for M9 medium to maximizing chimeric protein production. To test this hypothesis, three different media were used in this study, namely M9 medium, LB broth, and TB broth.

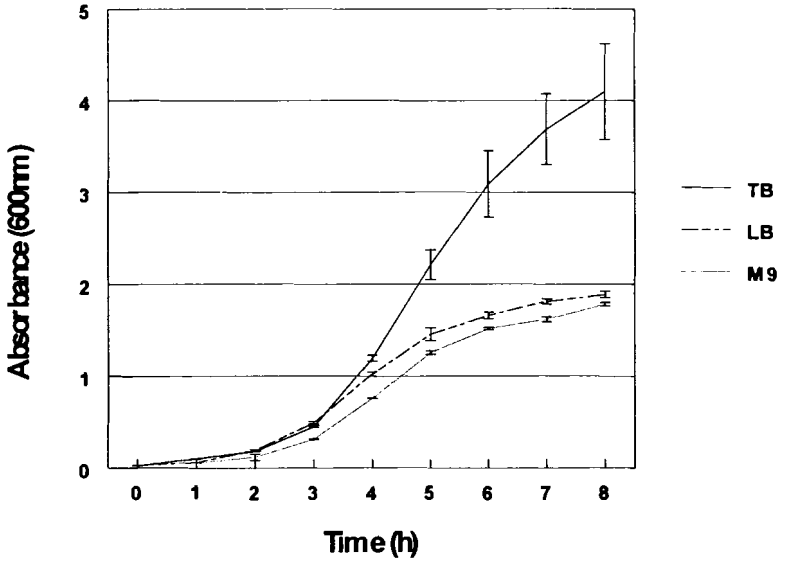


Figure 2.8. The effects of media on bacterial growth. Bacteria were cultured in three different media, TB broth, LB broth, and M9 medium for 8 h. The absorbances were measured hourly with a spectrophotometer at 600 nm. Each data point in the graph was represented by the mean of three readings.

The reason to include LB broth was because it belongs to the category of rich medium (contains about half as much tryptone and yeast extract as TB broth and about twice the concentration of nutrients as M9 medium), and more importantly it contains no buffering system. By including this medium, the effect of buffering system on the chimeric protein induction might be separated from other factors. The results mentioned above supported the hypothesis that TB broth best supported high density of bacteria growth.

The effect of media on chimeric protein yield is shown in Figure 2.9. The yields did not appear to be substantially different between LB and M9 cultures. However, bacteria cultured in TB broth produced about five to ten times more chimeric protein than bacteria cultured in LB broth or M9 medium.

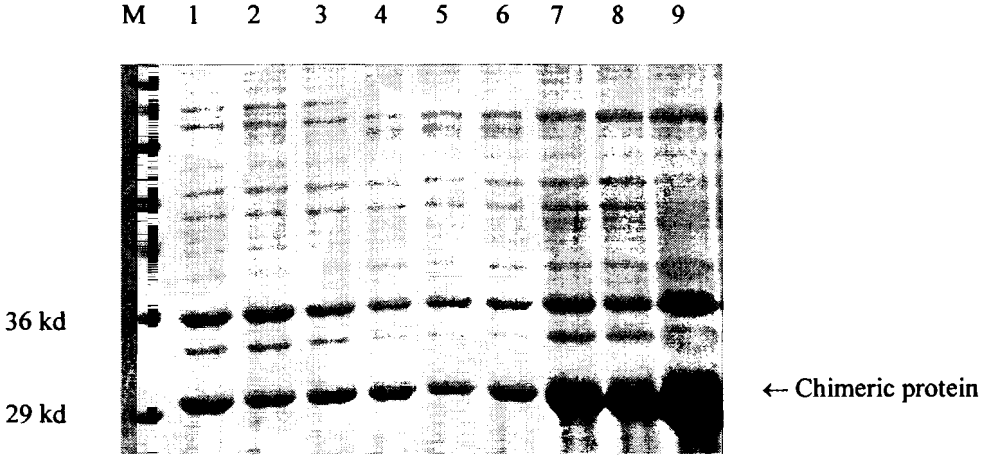


Figure 2.9. The effect of media on chimeric protein yields. Bacteria were cultured in three media, M9 medium, LB broth, or TB broth. The crude extracts from the bacteria were obtained as stated in Materials and Methods. Lane M, protein marker; Lanes 1, 2, and 3, crude extracts from M9 cultured bacteria; Lanes 4, 5, and 6, crude extracts from LB cultured bacteria; Lanes 7, 8, and 9, crude extracts from TB cultured bacteria.

This result was not exactly consistent with the effect of these media on bacterial growth shown in Figure 2.8. About twice as much chimeric protein should be produced in TB broth in comparison to either LB broth and M9 medium if the concentration of bacteria in the media was the sole contributor to the chimeric protein yield. However, the yield was about 5 to 10 times more in TB culture than in either LB or M9 culture. The additional positive effect of TB broth on protein yield might be due to a positive effect of TB broth on plasmid amplification (personal communication; Sigma) at the time of IPTG induction. Both the bacterial growth and chimeric protein production suggested that TB broth was the best of the three media for maximum chimeric protein production.

The Functional Assay of Chimeric Protein

The results of immuno-blotting and chimeric protein detection are shown in Figure 2.10. More than 90% of proteins were transported from the SDS-PAGE gel to the PVDF membrane after blotting (Figure 2.10 a and b). As the primary antibody was diluted from 500 to 12,500 times, the band intensity was decreased substantially. However, even at the1:12,500 dilution, the signal on the lane with the lowest amount of proteins (4.8 ng) from crude extract was still detectable.

Both the yield and functional integrity of chimeric protein are important for I-PCR assay. The objective of the first study was to test whether the chimeric protein was intact by immuno-blotting. If the band detected corresponded to the known size of this protein (31 kd), we can assume that the chimeric protein was intact. The results demonstrated that the protein appeared to be intact, based on the presence of a single band at 31 kd (arrow).

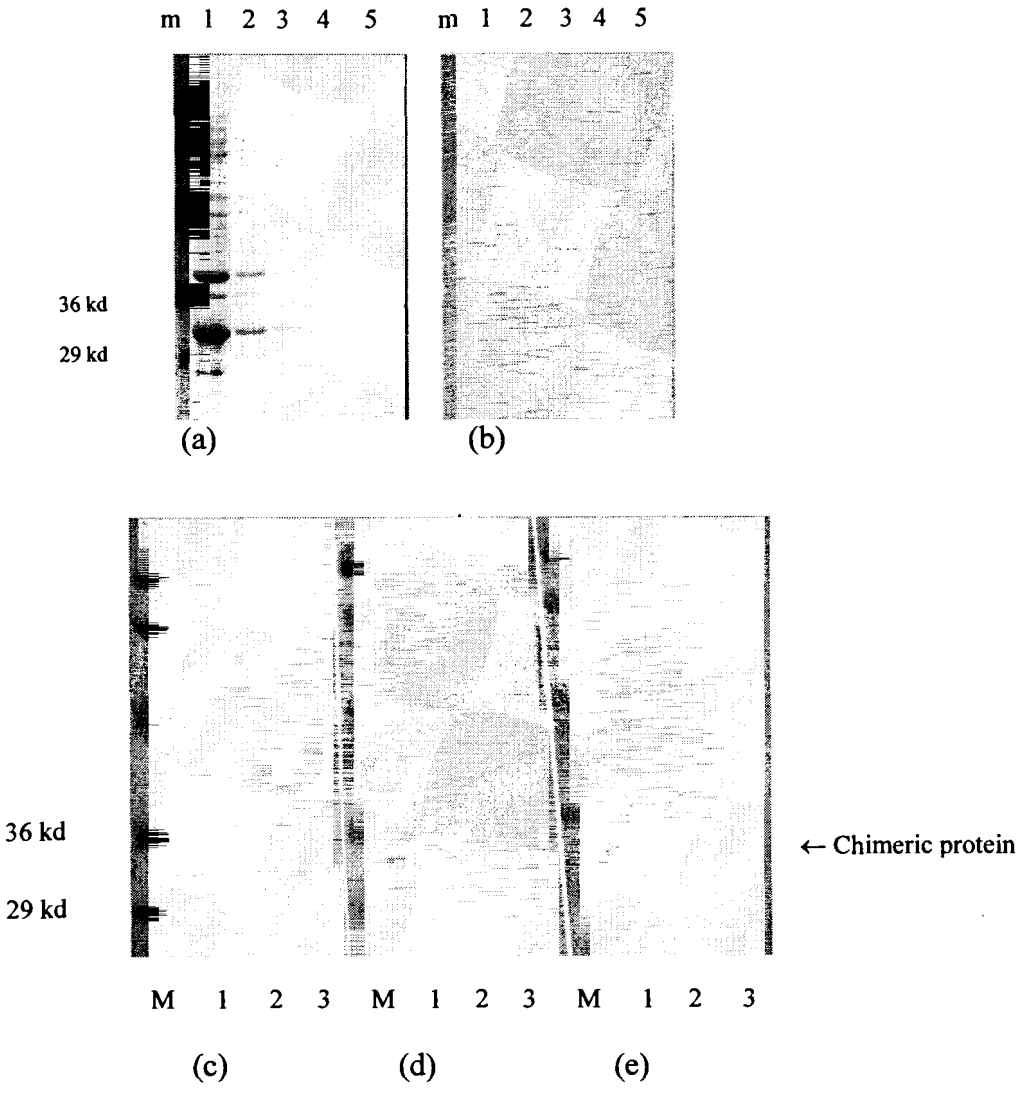
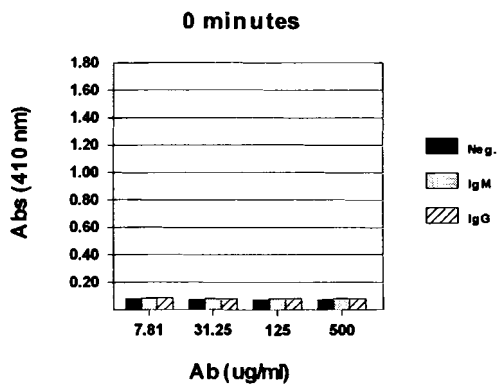


Figure 2.10. The results of Immuno-blotting and chimeric protein detection. (a) Lane M, protein marker; Lanes 1 through 5, 3000, 600, 120, 24, and 4.8 ng of chimeric protein per lane of agarose gel before blotting. (b) the same gel as in (a) after blotting. In (c), (d), and (e), the primary antibody was diluted 500, 2,500, and 12,500 times, respectively. Lanes M, protein marker; Lanes 1, 2, and 3 contained 120, 24, and 4.8 ng chimeric protein per lane, respectively.

The results of ELISA assay for determining the functional integrity of chimeric protein are shown in Figure 2.11. For Negative Control and IgM coated wells, the absorbances at 410 nm did not change substantially over the course of 1 h incubation. However, the absorbances for the

IgG coated wells increased during the entire incubation period and were observable even after only 10 minutes. The result demonstrated that the chimeric protein was fully functional, because the enzymatic reaction that resulted in increased absorbance was dependent on the binding of enzyme to the immobilized IgG molecules using both moieties of the bi-functional chimeric protein. The demonstration of functional integrity of chimeric protein used in our lab lays a firm foundation for the successful detection of target antigens by subsequent I-PCR assays.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Abs (410 nm)
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1.20
1.00
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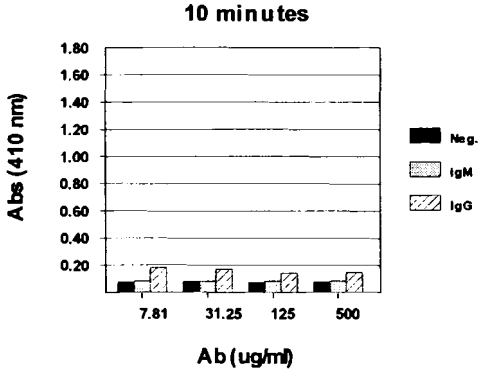
31.25
125
500
Ab (ug/ml)
Neg.
IgM
IgG


10 minutes
Abs (410 nm)
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1.60
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1.20
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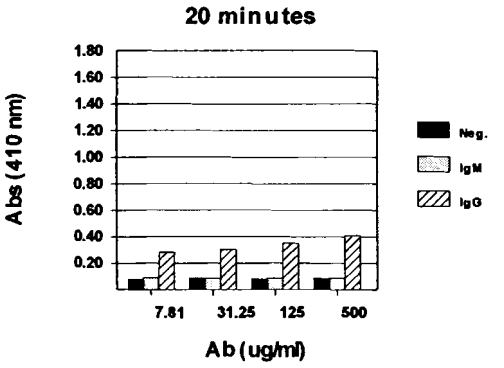
20 minutes
Abs (410 nm)
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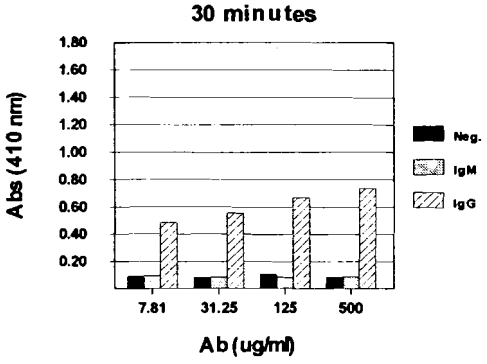
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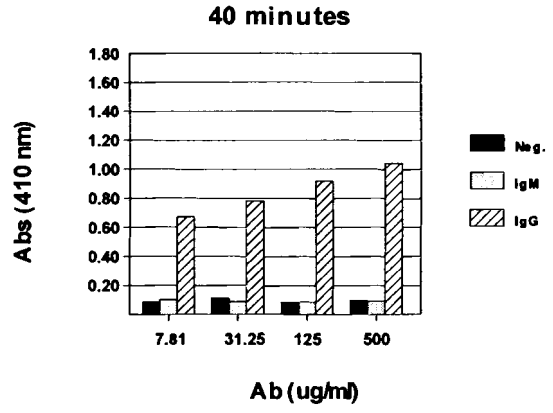
Ab (ug/ml)
Neg.
IgM
IgG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Abs (410 nm)
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Ab (ug/ml)
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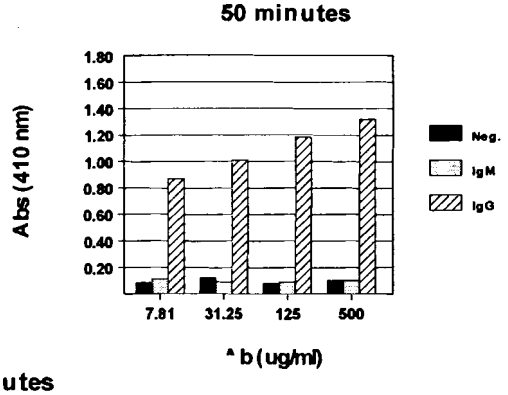
IgM
IgG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Ab (ug/ml)
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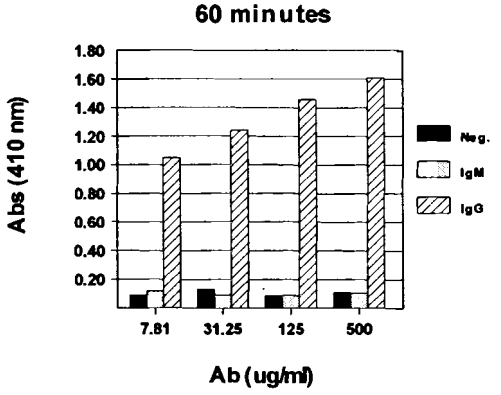
60 minutes
Abs (410 nm)
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500
Ab (ug/ml)
Neg.
IgM
IgG

Summary

Chimeric protein is one of the important components used in I-PCR. A series of experiments was conducted to increase the yield of this protein through modifications to the original protocols. A number of factors were studied and several of them were demonstrated to play a role in increasing the production of the chimeric protein. By using the newly optimized protocol, the yield of chimeric protein was increased substantially. Experiments were also completed to test the function of this protein, and its physical and functional integrity was confirmed by these studies.

The length of the bacterial culture period was shown to have a major influence on chimeric protein production by the transformed bacteria. Although some variation did exist in our data, the general trend was that the production of chimeric protein increased from zero to four h after IPTG induction [compared to two h after induction as suggested by Sano et al. (1992)]. Chimeric protein yield either remained the same or decreased slightly after four h IPTG induction.

The effect of induction temperature tested on chimeric protein induction seemed to be ambiguous. In one study, 38.0 °C appeared to be optimal temperature and in the other, 37.5 °C appeared to be the best. This narrow shift in optimal temperature might be due to variation between the different shakers used in these two studies. The position of a flask in the shaker might also play a role due to differing proximities to the hot air vent.

The method used to lyse bacterial cells also affected the chimeric protein yield. Lysing by sonication appeared to be superior in term of chimeric protein yield compared to chemically induced lysis. The increased yield with the sonication method might have resulted from the rapid and complete breakdown of the bacterial cells. Although

chemically induced lysis immediately eliminated much of the other cellular proteins at an early step in the purification, much of the chimeric protein was also lost. In addition, the sonication method was simpler and shorter than the chemical lysis method. These advantages would be even more valuable when multiple samples are processed simultaneously. Culture medium played a major role in chimeric protein production. Terrific broth was the best medium among the three media used and supported high density bacterial cell growth. A five to ten-fold increase in chimeric protein yield was obtained with TB culture compared to either M9 or LB culture. With M9 medium, the optimal yield of pure chimeric protein was greater than 3 mg per 100 mL of bacterial culture when the improved method was used. LB broth did not seem to confer any advantage over M9 medium for chimeric protein induction, although LB broth contained twice the level of essential nutrients as M9 medium. This non-parallel result might be due to the lack of a buffering system in LB broth. Therefore, LB broth might not support a suitable pH range for bacterial growth at high density, which was confirmed by the similar rates of bacterial growth between M9 medium and LB broth.

The centrifugation of dialysate just before immino-biotin affinity chromatography significantly decreased chimeric protein yield. After IgG affinity chromatography, the chimeric protein was almost pure. During the following dialysis, a visible precipitate formed. This was probably due to the high pH of the dialysis buffer (pH 11), which made the protein relatively insoluble. After centrifugation, most of the chimeric protein was lost in the pellet, leaving only a limited amount in the clarified dialysate. Chimeric protein loses were prevented by eliminating the centrifugation step, which certainly contributed to the high yield of chimeric protein using our improved method.

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Chapter 3

THE PRODUCTION AND ASSAY OF BIOTINYLATED PUC19 FRAGMENTS FOR I-PCR ASSAY

Abstract

pUC19 is a commonly used plasmid in molecular biology and its yield and quality are crucial for many analytical processes. Biotinylated pUC19 molecules were used as marker DNA to target antigens and these molecules were amplified by I-PCR assay to identify the presence of target antigens. Commercially available bacteria were transformed by pUC19 plasmid from a commercial source. The transformed bacteria were cultured under various conditions and pUC19 plasmid was purified and processed. Bacterial cells grew to significantly higher densities ($p<0.01$) when bacteria were cultured in TB broth [A_{600} 3.235±0.199 (15 h) and A_{600} 3.596±0.394 (28 h)] compared to Lennox L broth (LB) cultures [(A_{600} 1.516±0.04 (15 h) and A_{600} 1.676±0.055 (28 h)]. Similarly, significantly higher plasmid yields ($p<0.01$) were obtained in TB cultures [483.2±15.5 μg (15 h) and 439.4±14.9 μg (28 h) per 100 ml bacterial culture] compared to LB culture [15 and 28 h were 184.6±7.4 μg (15 h) and 207.1±9.3 μg (28 h). No difference ($p > 0.05$) was observed in bacterial cell density or plasmid yield between the two time periods within medium. In addition, the quality of pUC19 plasmid was independent of medium type and culture time. A novel procedure was developed for testing pUC19 biotinylation efficiency, which was simpler, less expensive, and more efficient than existing procedures. For pUC19 biotinylation, it appeared that incubation

for 30 min at 37 °C led to higher biotinylation efficiency of the pUC19 fragment than for 15 min either on ice or at room temperature. By using the optimized procedure, our biotinylated pUC19 fragments could be detected by I-PCR with as few as 58 PCR-generated or 6 Klenow fragment-generated biotinylated pUC19 molecules in 50 µl of PCR reaction mix. We concluded that: (1) using TB broth, but not increasing the culture time, was beneficial for pUC19 yield; (2) pUC19 quality was not affected by the conditions under study; (3) our new method for testing the efficiency of pUC19 biotinylation was very effective and could be used for testing the biotinylation of other molecules; and (4) our biotinylated pUC19 fragments were fully functional.

Key words: Culture medium, Plasmid, PCR, pUC19

Introduction

One of the important components in our I-PCR assay is a biotinylated pUC19 fragment, which was linked by a recombinant chimeric protein to a target-specific antibody. The generation of biotinylated pUC19 fragments requires very pure and high quality pUC19 plasmid. Although pUC19 plasmid is commercially available, it becomes expensive to purchase in a large quantity. A large amount of pUC19 was needed in our research due to the complicated testing procedures involved, thus it was necessary to produce pUC19 plasmid in a large quantity. Initially, we tried to use pUC19-transformed bacteria from another lab. However, our results were not very encouraging in terms of either pUC19 yield or quality. The reason(s) for our poor results were not completely understood since only very limited information was available on the factors that affect

pUC19 yield and quality. Although Tartof and Hobbs (1987) found that using rich media (i.e., TB broth) resulted in increased yield of pUC18 plasmid DNA, no information was available on the quality of their plasmid produced. Frenkel and Bremer (1986) found that culture time affected the yields of pBR322 and pBR327 plasmids. However, pBR322 and pBR327 are low copy number plasmids, and it is not known whether this information is applicable to high copy number plasmids such as pUC19. To solve the ambiguity, we transformed commercially available bacteria with commercial source pUC19 plasmids. After transformation, the effects of culture media and culture time on the yield and quality of plasmid from *E. coli* were determined. In addition, a novel procedure was developed for testing the efficiency of pUC19 biotinylation, which is less expensive and more efficient than the procedures presently available.

Materials and Methods

Reagents

The following ingredients were from Sigma Chemical Co. (St. Louis, MO): streptavidin, rabbit anti-streptavidin (antisera), Lennox L broth, Terrific broth, LB agar tablets. The following ingredients were from New England BioLabs, Inc. (Beverly, MA): *Pvu* II, *Acc* I, *Hin*d III, DNA polymerase I (Klenow large fragment), and pUC19 plasmid. The following ingredients were from Gibco BRL (Grand Island, NY): isopropyl β-D-thiogalactopyranoside, 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), biotin-14-dATP, deoxynucleotides, dNTP mix, and Platinum® Taq DNA polymerase. The following ingredients were from Bio-Rad Laboratories (Hercules,CA): goat anti-

rabbit IgG-horseradish proxidase conjugate (GAR-HRP), Zeta-Probe® Blotting membrane, and Immun-Blot™ PVDF membrane. The EndoFree Plasmid Maxi Kit and QIAquick PCR Purification Kit were from Qiagen Inc. (Valencia, CA). Clonables™ Kit was from Novagen (Madison, WI). Whatman paper was from Fisher Scientific (Hanover Park, IL). The pUC19 transformed bacteria was a gift from Dr. Carol Warner (Northeastern University, Boston, MA).

Bacteria Transformation and Culture

Transformation of Bacteria with Plasmids. The transformation reaction was carried out according to the manufacturer's instructions (Novagen) with a few modifications. Clonables Positive Control (5 µl with insert to vector molar ratio of 2.5:1) was put into a 1.5-mL microcentrifuge tube and this was followed by adding 5 µl of 2X Ligation Premix. After thorough mixing, the tube was incubated at 22 °C for 15 min for the ligation reaction to take place and then the reaction product was stored on ice until transformation. Four tubes of NovaBlue Singles™ competent bacteria cells (Novagen) were thawed by immediately placing them on ice (all but the cap) for 3 min. The tubes were gently flicked 3 times, and 1 µl of the positive control plasmid (Novagen), Test plasmid (0.2 ng) (Novagen), pUC19 plasmid dilution (2.5 pmole/mL, New England Biolabs, Inc.), and TE buffer (negative control) were added into tubes one to four. The tubes were immediately but gently flicked after each addition. After the addition of all reagents, the tubes were incubated on ice for 5 min, heated for exactly 30 seconds in a 42 °C water bath, and placed on ice for 2 min. After this, 250 µl of room temperature SOC medium was added to each tube and then tubes were stored on ice before use.

Preparation of LB Agar Plates. LB Agar Tablets (Sigma) were put into 50 °C distilled water (one tablet/50 mL). The tablets were stirred on a stirring hot plate until completely dissolved. After autoclaving for 15 min and then cooling down to 45 °C, IPTG, X-gal, tetracycline, and ampicillin were added to final concentrations of 80 μM, 70 μg/mL, 15 μg/mL, and 50 μg/mL, respectively. For plates without antibiotics, the antibiotics were excluded. After mixing the content and putting the beaker on a pipette tip box containing hot water (to prevent gel from forming inside of the beaker), ten mL of gel solution was introduced into each 100-mm petri dish. The petri dishes were shaken to let the liquid gel uniformly cover the bottoms. The plates were stacked to prevent condensation and were cooled to room temperature. The plates were wrapped in parafilm, and were stored at 4 °C for up to one day before use.

Plating and Culturing Transformed Bacteria on LB Agar Plates. These procedures were carried out according to manufacturer's instructions (Novagen) with a few modifications. Prior to transformation, the LB agar plates were placed (cover-side down) in a 37 °C incubator for about 20 min and removed just before plating. The transformation and negative control tubes were flicked 7 times, samples were removed from the middle of the transformation reaction and put on the LB agar plate (5 and 40 μl for Positive Control, 3 and 1.5 μl for Test plasmid, 0.2, and 0.04 μl for pUC19 plasmid, which was put in 50 μl SOC on LB agar plates). After adding the bacterial suspension, a sterilized and cooled glass plating spreader was placed on the plate and the sample was evenly spread on the plate. Plates were incubated with cover-side down at 37 °C with maximum humidity for 24 h. Photograph of the plates were taken with transmitted light and the colonies were counted.

Preparation of Starter #1 and #2. A volume of 4 mL of autoclaved LB broth was put into a 25-ml flask. Ampicillin and tetracycline were added at concentrations of 120 μg/mL and 50 μg/mL, respectively. One blue colony was placed into the flask and cultured in a Controlled Environment Incubator Shaker (New Brunswick Scientific Co. Inc.) for 15 h with vigorous shaking at 250 rpm (Starter #1). The Starter #1 was diluted, plated, and cultured as stated below. To make Starter #2, a colony isolated from Starter #1 plate was cultured as stated above.

Plating Bacterial Culture on Agar Plates. Bacteria culture was diluted with either LB or TB broth to an absorbance of 1:000 at 600 nm. Two μl of diluted sample was mixed with 998 μl of LB broth. Then 5 μl of this dilution was further diluted by mixing with 995 μl of SOC. Ten microliters of the end dilution plus 90 μl of SOC were evenly spread over each plate. Plates were cultured at 37 °C with maximum humidity for 24 h.

Preparation of Large Culture. A volume of 220 mL of either LB or TB broth was poured into each 1000-mL flask. Ampicillin and tetracycline were added to the flask to reach concentrations of 120 μg/mL and 50 μg/mL, respectively. After adding 0.22 mL Starter #2 to each flask, the flasks were cultured at 37 °C for 15 to 28 h with shaking at 300 rpm. Bacterial growth (absorbance at 600 nm) was measured hourly in triplicates up to 15 h. Single readings were sequentially taken at 17, 23, and 2 8 h of culture.

Harvesting Bacteria. After cultured for 12, 17, 23, and 28 h, the bacteria were harvested by loading 50 mL of bacteria culture into a 50-mL centrifuge tube. The bacterial cells were centrifuged at 6,000 x g for 15 min. The supernatant was discarded. The tube was reloaded once with additional 50 mL of bacterial culture and centrifuged under the same

conditions. The resulting pellet (from 100 mL of bacterial culture) was frozen dry at -70 °C until use.

pUC19 Plasmid Purification

pUC19 plasmid was purified using an EndoFree Plasmid Maxi Kit from Qiagen according to the manufacturer's instructions, with a few modifications. Briefly, one frozen bacterial pellet was suspended in 10 mL of Resuspension Buffer (Buffer P1). Bacterial cells were lysed by adding 10 mL of Lysis Buffer (Buffer P2) and incubating for 5 min at room temperature after mixing. The reaction was stopped by adding 10 mL of Neutralization Buffer (Buffer P3) and mixing well. The lysate was poured into the QIAfilter Cartridge and incubated at room temperature for 10 min, then was filtered into a 50-mL centrifuge tube by gently pushing a plunger into the cartridge. After Buffer ER (2.5 ml) was added to the filtered lysate and mixed, the filtered lysate was incubated on ice for 30 min. The solution was then applied to the Buffer QBT- Equilibrated QIAGEN-tip and allowed to enter the resin by gravity flow. After washing the QIAGEN-tip with Buffer QC, pUC19 plasmid was eluted with 15 mL of Buffer QN. The eluted DNA was precipitated with isopropanol, washed with ethanol, and resuspended with endotoxin-free Buffer TE. The purified pUC19 plasmid was frozen and stored at - 20 °C until use.

Biotinylated pUC19 Fragment Production and Assay

***Pvu* II Digestion.** pUC19 plasmid was digested by *Pvu* II restriction enzyme (cuts at base number 306 and 628) according to the manufacturer's instructions (New England BioLabs, Inc.), with only a few modifications. Briefly, 5 µg of circular pUC19 plasmid

were digested in a total volume of 50 µl of 1X NEBuffer 2 supplemented with 10 units of *Pvu* II for 2 h at 37 °C.

***Acc*I and *Hind* III Digestion.** pUC19 plasmid was digested by *Acc* I and/*or Hin*d III restriction enzymes according to the manufacturer's instructions (New England BioLabs, Inc.), with only a few modifications. Briefly, between 10 to 200 µg pUC19 plasmid was diluted in NeBuffer4 (100 µg/mL final concentration) in microcentrifuge tubes. *Acc* I (1 unit/µg pUC19) and *Hin*d III (4 units/µg pUC19) were added. The tubes were incubated at 37 °C for up to 4 h. Because *Acc* I and *Hin*d III cut pUC19 at base number 429 and 447, respectively, a 2.67 kb fragment with sticky ends was produced.
The digested pUC19 was either run on an agarose gel directly to check digestion efficiency or was purified by QIAquick PCR Purification Kit for producing biotinylated pUC19 fragments.

pUC19 Fragment Purification. Biotinylated and non-biotinylated pUC19 molecules were purified by QIAquick PCR Purification Kit (Qiagen Inc.) according to manufacturer's instructions. In brief, five volumes of Buffer PB were added to 1 volume of DNA solution. After mixing, the mixture was transferred into a QIAquick spin column and was centrifuged at 10,000 x g for 1 min. To wash, 0.75 mL of Buffer PE was added to the column and the column was centrifuged under the same conditions. To elute DNA, 30 µl of Buffer EB was added to the column, incubated at room-temperature for 1 min and centrifuged at 10,000 x g for 1 min. The eluate was collected.

Biotinylation of pUC19.

By Klenow Fragment. The pUC19 biotinylation was done according to the recommended protocol (New England BioLabs) with slight modifications. In brief,

2.67 kb pUC19 fragments (digested by *Acc* I and *Hind* III) were diluted to a concentration of 33.33 μg/mL in 1X DNA polymerase I buffer containing 1 unit DNA polymerase I (Klenow large fragment) per ug of DNA and 16.67 μM of each dNTP (biotinylated dATP, dCTP, dGTP, and dTTP) in volumes of 30 or 300 μl. After mixing in microcentrifuge tubes, the reaction mixtures were incubated for 15 min either on ice or at room temperature, or at 37 °C for 30 min. After the reaction was completed, pUC19 fragments were purified by QIAquick PCR Purification Kit.

By PCR. Non-biotinylated 2.6 kb pUC19 fragments were diluted with Buffer EB (10 mM Tris.HCl, pH 8.5) to a concentration of 2.0×10^{-9} M. Thin-walled PCR tubes were each loaded with 48 μl of master mix (see below) and 2 μl of the prepared pUC19 dilution. The primers were 5'CTACTTACTCTAGCTTCCCGG3' (upper primer) and 5'Biotin-CGCAGGAAAGAACATGTGAGC3' (lower primer). The upper primer binding site on pUC19 molecule starts (5' to 3') at 1901 bp and the lower primer starts at 795 bp. This generated a biotinylated pUC19 fragment that was 1107 bp in length. The PCR cycle was described below. After PCR reaction, biotinylated pUC19 fragments were purified by a QIAquick PCR Purification Kit.

Assay of Biotinylated pUC19 Fragments

Blotting pUC19 DNA onto a Zeta-Probe Membrane. Biotinylated pUC19 fragments were subjected to electrophoresis through an agarose gel. The agarose gel was then shaken in 70 mL 0.5 M NaOH and 1 M NaCl for 30 min at room temperature on a rotating platform. The solution was changed once and the gel was shaken for an additional 30 min at room temperature. A piece of sponge (5 mm thick and larger than

the gel) was placed on the bottom of a buffer tray. A volume of 1500 ml of 10X SSC buffer (1.5 M NaCl and 0.15 M trisodium citrate at pH 7.0) was poured into the tray to immerse the sponge, and bubbles were squeezed out of the sponge. The SSC buffer was sucked out of the tray with a large syringe to a level that barely covered the sponge. Three sheets of prewetted (with 10 SSC) Whatman paper (a little larger than the sponge) were placed on the sponge. The surface of the paper was flooded with 10X SSC buffer, and the agarose gel was placed on top of the stack. A plastic wrap with a little window to allow only the gel and the Whatman paper through was rested on the sponge. A pre-wetted (with distilled water) Zeta-Probe membrane was placed on top of the Whatman paper. The surface of the Zeta-Probe membrane was flooded with 10X SSC. Two sheets of pre-wetted Whatman paper were placed on top of the Zeta-Probe membrane, and bubbles trapped between papers were removed. A 15-cm thick layer of paper towels was stacked on the Whatman paper. The DNA was transferred for 24 h at room-temperature. The level of 10X SSC was kept 4 cm deep during the entire transfer process. After transfer, the Zeta-membrane was separated from the gel and rinsed briefly in 50 ml of 2X SSC, then was blotted briefly with 3 layers of filter paper.

DNA Alkaline Fixation. The Zeta-Probe membrane was placed (with DNA surface upper-most) on a pad of 3M paper saturated with 0.4 M NaOH for 10 min and rinsed in 50 mL 2x SSC. The membrane was air-dried, then was stored between two pieces of filter paper in a plastic bag at room temperature until use.

Colorimetric Detection of Biotinylated DNA. The dried membrane was wetted in 50 mL PBST (phosphate buffered saline/0.1% Tween-20), and then washed twice (5 min each) in 50 mL PBST in a plastic tray. The wetted membrane was put into 50 mL of

room-temperature blocking solution (3% gelatin in PBS) and was agitated on a rocking platform for 60 min at room temperature. The blocking solution was discarded and replaced by 50 mL PBST. The membrane was washed in PBST twice, each for 5 min. After decanting the PBST, the membrane was incubated sequentially with 50 mL of streptavidin (1 μg/mL), 50 mL of primary antibody solution (rabbit anti-streptavidin, 1:500 dilution), and 50 mL of secondary antibody solution [goat anti-rabbit horseradish proxidase (GAR-HRP), 1:5000 dilution), each for 2 h with gentle agitation. Between the incubations, the membrane was washed twice with 50 ml of PBST, each for 5 min. After the final wash with PBST, the membrane was incubated with 51 ml of Opti-4CN diluent solution (substrate) for 2.5 min, then rinsed with 50 mL of distilled water for 15 min. Finally, the membrane was photographed.

Polymerase Chain Reaction (PCR)

The PCR reaction was performed in a thin-walled PCR tube containing a 48 μl mixture of template (2 μl of pUC19 molecules) and master mix (48 μl). The 50 μl of master mix contained 1.5 mM MgCl, 0.2 mM of each dNTP, 2 units of Platinum Taq DNA Polymerase, and 0.2 mM of each primer in 1X PCR buffer. The upper primer was 5'CAGGCAACTATGGATGAACGA'3, and the lower primer was 5'GGCGAAACCCG-ACAGGACTAT3'. The PCR reaction was carried out using the following settings: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min with a final extension at 72°C for 5 min. With this set of primers, 769 base pair (bp) fragments from pUC19 templates were generated after PCR reaction.

Gel Electrophoresis

For gel electrophoresis, a horizontal mini-gel system (model MGU-202T, C.B.S. Scientific Company, Inc.) was used with an EC 105 electrophoresis power supply (E-C Apparatus Corporation). A 0.5% Agarose gel was made with molecular biology-certified agarose (Shelton Scientific, Inc.) in 1X TBE buffer containing 0.11 μg/mL of ethidium bromide. The gel running buffer was also 1X TBE with 0.11 μg/mL of ethidium bromide. After loading the wells (8 μl of pUC19 sample and 2 μl of gel loading solution for the small wells, or 12.8 μl of pUC19 sample and 3.2 μl of gel loading solution for the large wells), the gel was run for 1 h at a constant voltage of 95 V and then visualized and photographed under ultraviolet light to identify DNA bands.

Statistical Analyses.

The data in this section were analyzed either by Student's *t*-test or by Analysis of Variance.

Results and Discussion

The Yield and Quality of pUC19 Plasmid Purified from Bacteria Transformed at Northeastern University

The yield of purified pUC19 plasmid from 100 mL of transformed bacteria culture is presented in Table 3.1. The average yield of purified pUC19 plasmid was 76.5 μg/100 mL of culture, which was lower than what would be expected from a high copy number plasmid (300 to 500μg/100 mL culture; Qiagen, 1998).

Table 3.1. The yield of purified pUC19 plasmid from 100-mL culture of transformed bacteria.

Flask #	1	2	3	4	5
Yield (μg)	65.45	77.19	78.09	79.2	82.45
Mean (μg)	76.48				

The quality of pUC19 purified from the bacteria was shown in Figure 3.1. The purity of this plasmid was greater than 95% as shown on agarose gel. There were two bands (a lighter upper band and a heavier lower band) for every lane. The lighter band represented dimeric and the heavier band represented monomeric circular pUC19 plasmid. No genomic DNA contamination was detected.

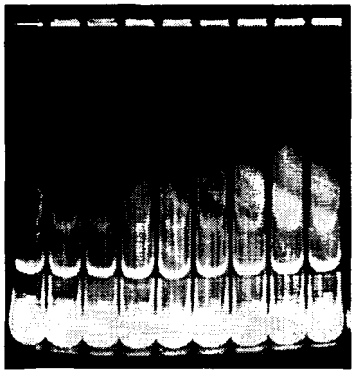


Figure 3.1. The quality of purified pUC19 plasmid by EndoFree Plasmid Maxi Kit from the transformed bacteria. About 10 μg of pUC19 plasmid was loaded in each well of an agarose gel. DNA bands were visualized by ethidium bromide staining.

The minimum amount of biotinylated pUC19 fragment detectable after PCR amplification was 2 μl of 7.4×10^{-12} M in 50 μl of PCR reaction (Figure 3.2).

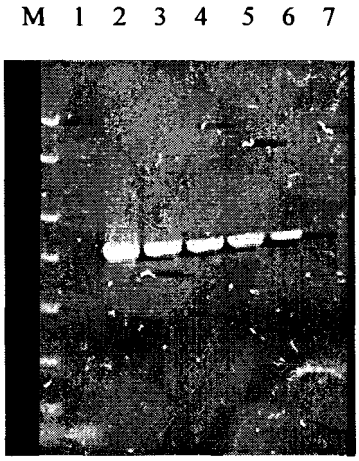


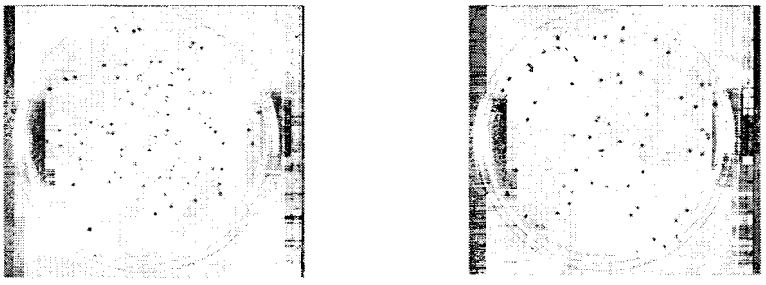
Figure 3.2. Efficiency of biotinylation of pUC19 fragments. pUC19 plasmid was purified from transformed bacteria (from Warner lab), then was digested with *Acc*I and *Hin*dIII restriction enzymes. The linearized pUC19 molecule was purified with QIAquick PCR Purification Kit (Qiagen) and biotinylated with biotinylated-14-dATP through the action of Klenow large fragment of DNA polymerase I. The quality of biotinylated pUC19 fragment was tested by PCR. Lane M, PCR marker; Lane 1, negative control; Lane 2 through 7, two μl of 7.4×10^{-8} to 10^{-13} M biotinylated pUC19 fragment (8.9×10^{10} to 8.9×10^{5} molecules) as template. Eight μl of PCR reaction mixture and 2 μl of gel loading solution were loaded in each well.

The amplification power was too low to be useful in I-PCR assay because 4×10^{-13} M of biotinylated pUC19 is normally used (McElhinny and Warner, 1997). The reason (s) for such a low amplification power is unknown. It might be due to damage to the plasmid during purification process or due to intrinsic problem(s) associated with the plasmid, such as mutation of the primer-binding site. Due to the low amplification of this plasmid, we explored the possibility of transforming bacteria in our lab with commercially available pUC19 plasmid.

The Yield and Quality of pUC19 Plasmid Purified from Bacteria Transformed in Our Lab

The Growth Status of Bacteria Under Culture.

Newly Transformed Bacteria. Two types of colonies were expected from these transformed bacteria. In the case of transformed bacteria with test and pUC19 plasmids, a blue colony was produced when the bacteria contained the respective plasmid and a white colony was produced when the bacteria did not contain the respective plasmid. In the case of transformed bacteria with positive control plasmid, a blue colony was produced when the bacteria contained the plasmid, but lacked the insert in the plasmid so the integrity of the gene was not interrupted. Alternatively, a white colony was produced when the bacteria either had the plasmid with the insert or did not have the plasmid. After the competent bacteria were transformed in our lab, they were cultured on LB agar plates. After 12 h incubation, the change of colony color was visible. After 24 h incubation, blue-white colonies were readily distinguished (Figure 3.3), and individual bacterial colonies were large enough for colony isolation.



(a) (b)

Figure 3.3. Blue and white colonies formed by bacteria transformed in our lab on LB agar plates. (a) Blue and white colonies formed by bacteria transformed by test plasmids (1.5 μl of transformed bacteria solution. (b) Blue colonies were formed by bacteria transformed by pUC19 plasmids (0.04 μl of transformed bacterial solution).

The numbers of colonies formed by the transformed bacteria on LB agar plates were shown in Table 3.2. The positive plasmid produced 93.1% white colonies which was very close to the 95% predicted value. When 0.04 μl of pUC19 transformed bacteria solution was incubated on agar plate with antibiotics, the density of colonies on the plate was optimal for colony isolation.

Table 3.2. Number of colonies formed on LB plates by transformed bacteria.

Treatment	+/- antibodies (plating volume in microliters)	Number of colonies	
		White (%)	Blue
Positive control	+ (40)	366 (93.1)	27
	- (5)	~ 20,000	0 (too small to see color)
Test plasmid	+ (1.5)	95	103
	- (1.5)	~ 20,000	0 (too small to see color)
pUC19 plasmid	+ (0.2)	78	430
	+ (0.04)	0	72
	- (0.2)	~ 40,000	0 (too small to see color)
Negative control (LB broth only)	+ (90)	0	0
	- (10)	0	0

Starter Cultures. After 12 h of culture, a portion of colonies on the plates turned blue in color. White and blue colonies could be distinguished very easily after 24 h of culture. Colony numbers of Starter culture 1 (SC1) and Starter culture 2 (SC2) were shown in Table 3.3. There was no significant difference in colony numbers between

antibiotic and non-antibiotic plates. This might be due to the fact that after first round selection, the bacteria without antibiotic resistance genes were drastically reduced.

Table 3.3. Number of colonies formed by Starter Cultures at 24 h after culturing.

SC#	AB*		Colonies		
		Replicate	W	B	Blue-White
SCI	AB	1	0	87	4
		2	0	76	4
		3	0	66	0
	No	1	0	69	3
		2	0	119	7
	AB	3	0	73	3
SC2	AB	1	0	79	2
		2	0	90	2
		3	0	87	3
	No	1	0	55	1
		2	0	41	1
	AB	3	2	98	3

* Antibiotics

Cultures 1 and 2. After 20 h of culture, the absorbance at 600 nm of culture 1 (C1) was 1.948. After 19 h of culture, the absorbance at 600 nm of culture 2 (C2) was 1.875. After the plates were cultured for 24 h, white and blue colonies of both cultures could be seen very easily, and colonies were almost large enough for colony isolation. Colony numbers at 24 h of plate culture were shown in Table 3.4. Again, the number of colonies was similar between antibiotics and non-antibiotic plates.

Table 3.4. Number of colonies formed by Cultures 1 and 2 at 24 h of culturing.

C#	Med	AB	Replicate	Colony number		
				White	Blue	Blue-White
C1	LB	AB	1	0	71	2
			2	0	9	0
			3	0	30	0
		No AB	1	0	43	0
			2	0	46	0
			3	0	74	2
	TB	AB	1	0	27	2
			2	0	18	2
			3	0	28	1
		No AB	1	0	32	1
			2	0	37	0
			3	0	40	2
C2	LB	AB	1	0	62	2
			2	0	64	2
			3	0	41	1
		No AB	1	0	28	0
			2	0	31	1
			3	0	44	1
	TB	AB	1	0	2	0
			2	0	3	0
			3	0	4	0
		No AB	1	0	5	3
			2	0	6	2
			3	0	16	5

Large Culture. The relationship between the number of colonies formed on the LB agar plates and the absorbances at 600 nm was represented in Figure 3.4. The number of colonies formed on the plates increased in a linear fashion as the absorbances increased. By using the equation of $y = 22.68x + 4.18$, the number of colonies (y) that will form on a plate under the same conditions can be predicted if the absorbance (x) is given.

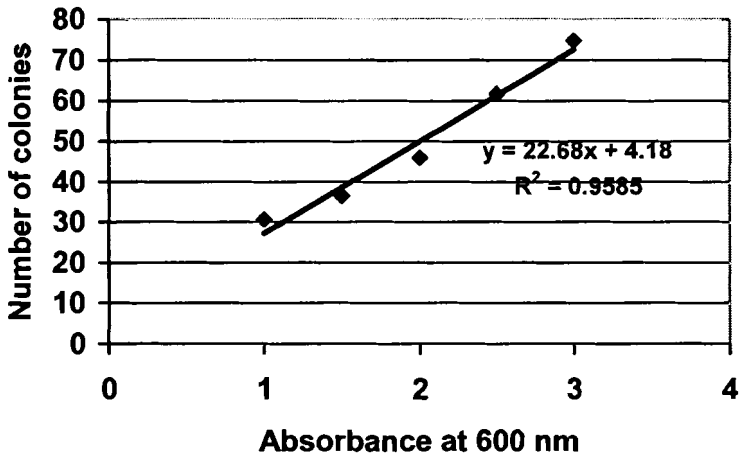


Figure 3.4. The relationship between the absorbance of a culture and the number of colonies formed on a plate. Transformed bacteria were cultured in TB broth with ampicillin and tetracycline as selective agents at 37 °C and 300 rpm rotation for 12 h. The absorbances at 3.0, 2.5, 2.0, 1.5, and 1.0 at 600 nm were obtained by diluting the original culture with TB broth. Then every tube, except the last one (1.0 at 600 nm), was further diluted to achieve the absorbance of 1.0. In this way, every tube has the same concentration of cells before plating. Each tube was then plated as stated in the Material and Methods. Each point was represented by the mean number of colonies from 3 plates.

The absorbances of bacteria cultured on LB and TB broth is presented in Figure 3.5. From 1 to 9 h, the absorbances of LB and TB cultures did not differ ($p > 0.05$). After 10 h of culture, the absorbances of TB cultures were significantly higher than LB cultures at the same time point. The highest absorbance for LB culture (1.916) was obtained at 17 h (not shown). The highest reading for TB (4.296) was obtained at 28 h (not shown). The logarithmic growth phase for both LB and TB culture was between 11 h to 13 h of culture. At the end of culture, the absorbance of TB broth was more than double that of LB broth. This may be because TB broth is richer than

LB broth (Sambrook et al., 1989), so TB broth could support higher density of bacterial growth.

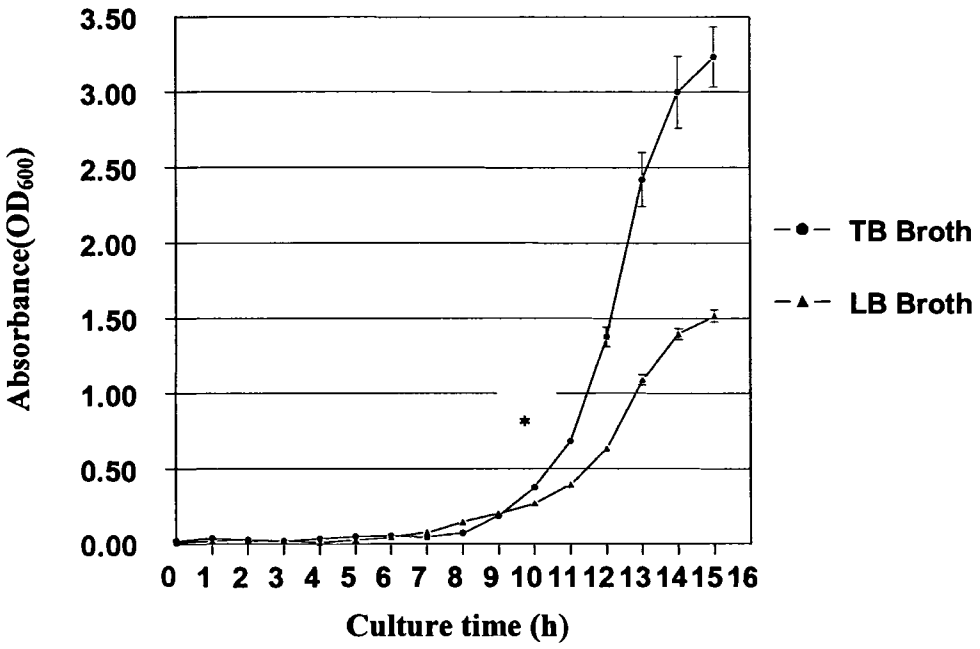


Figure 3.5. The absorbances of bacteria cultured in either TB or LB broth. Samples were taken hourly from zero to 15 h and absorbances were measured with a spectrophotomer at 600 nm. The average absorbance from three flasks of either TB culture (•) or LB culture (▲) was calculated and the mean (with standard error) was plotted against time in h.
* Absorbances of TB and LB cultures starting from 10 h to the end of culture (15 h) differ significantly ($p<0.01$).

Figure 3. 6 represents the mean number of colonies formed on LB agar plates by bacteria cultured in TB or LB broth for different periods of time. Overall, as culture time increased, the number of colonies formed on the plates decreased. The colonies from TB culture decreased at a faster rate over time when compared to LB culture. The most likely reason for the sharp reduction in colony number was that the survival rate of bacteria were decreased as the culture time extended.

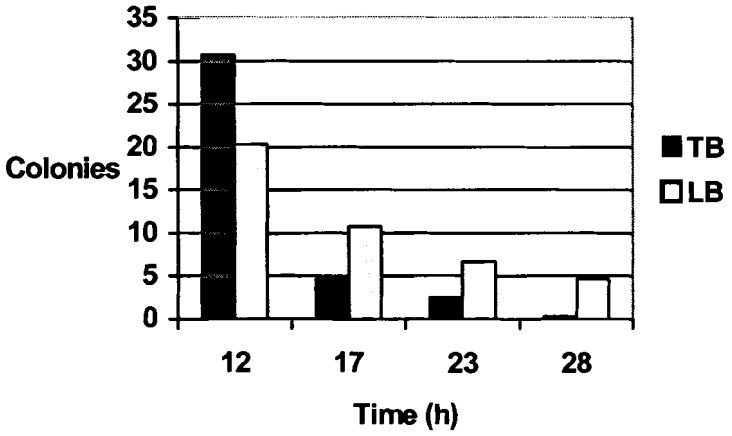


Figure 3.6. The mean number of colonies formed on LB agar plates by bacteria cultured in TB and LB broth for different periods of time. Samples were taken at 12, 17, 23, and 28 h of cultures. The sample was diluted and plated on LB agar plates. The plates were cultured at 37 °C for 24 h. The numbers of colonies were counted. The mean colony number from three plates was presented at each time point.

The Yield and Quality of pUC19 Plasmid. Table 3.5 shows the yields of purified pUC19 plasmid from 100 mL cultures of bacteria grown either in LB or TB medium and for either 15 or 28 h. There was no difference ($p > 0.05$) in the mean yield between the two culture periods within the same medium. However, a significantly higher yield was produced in bacteria cultured in TB broth than in LB broth. The mean yield of pUC19 plasmid from 100-ml TB culture was 483.2 μg and 439.4 μg for 15 h and 28 h of culture, respectively, which was within the predicted range of 300 to 500 μg per 100 ml of culture (Qiagen, 1998), but was about half of what was obtained using another high copy number plasmid, pUC18 (Tartof and Hobbs, 1987). The reason for the difference in yield is not clear because pUC18 and pUC19 are high copy number plasmids and should not differ in

plasmid yield. However, Tartof and Hobbs (1987) used the alkaline lysis procedure to extract DNA. This might have contributed to the increased DNA yield in their study.

Table 3.5. The pUC19 yields purified from bacteria grown in either LB or TB broth and for either 15 or 28 h.

Media	LB		TB	
Culture time (h)	15	28	15	28
Yield (μg)	170.0 189.8 194.1	210.2 189.6 221.5	452.6 502.4 494.5	409.7 456.5 452.0
X±SE	184.6±7.4	207.1±9.3	483.2±15.5	439.4±14.9

Table 3.6 compares the yield of pUC19 plasmid purified from bacteria transformed either in our lab or in Warner lab. The yield of pUC19 plasmid purified from the bacteria transformed in our lab was significantly higher ($p>0.05$) than the yield from bacteria transformed in Warner's lab. We do not know exactly why the yield of pUC19 plasmid from bacteria from Warner's lab was lower, but speculate that mutations might have occurred to the origin of replication on pUC19 plasmid and this might have reduced its replication efficiency.

Table 3.6. The yield (ug per 100 ml) of pUC19 plasmid purified from bacteria transformed either in our lab or in Warner lab and grown in LB broth.

Our Lab	Warner Lab
178.237	72.36
169.496	78.435
193.510	75.225
X = 180.414	X = 75.34

The quality of purified pUC19 plasmid is shown in Figure 3.7. Only one band is shown in most of the lanes. A few lanes have one or two faint bands above the major band. The fainter bands on the top of lane 9, 12, and 13 appeared to be genomic DNA contamination based on this gel.

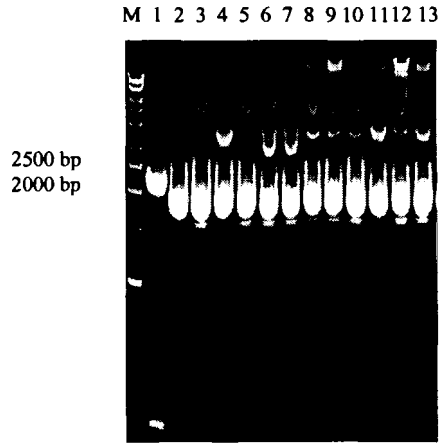


Figure 3.7. The quality of purified pUC19 plasmid shown in agarose gel. Bacteria transformed with pUC19 plasmid in our laboratory were cultured in either LB or TB for either 15 h or 28 h. One microgram of purified pUC19 plasmid from each 100-ml culture was loaded per lane. Lane M, 1 kb ladder; Lane 1, *Pvu* II-digested pUC19; Lanes 2 through 4, bacteria cultured in LB broth for 15 hours; Lanes 5 through 7, bacteria cultured in LB broth for 28 h; Lanes 8 through 10, bacteria cultured in TB broth for 15 h; Lanes 11 through 13, bacteria cultured in TB broth for 28 h.

Figure 3.8 shows the *Pvu* II-digested pUC19 fragment on an agarose gel. The DNA was 100% digestable and a single band was shown in every lane loaded with digested DNA. The results appear to disprove the possibility of genomic DNA

contamination. Consequently, the accessary bands likely represented the multimeric forms of pUC19 plasmid (Qiagen, 1998).

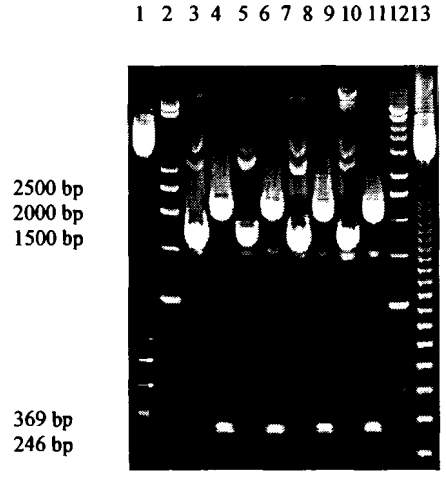


Figure 3.8. Non-digested and *Pvu* II-digested pUC19 plasmid on agarose gel. pUC19 plasmid from the same sources as Lanes 3, 6, 9, and 12 in Figure 3.7 were digested with *Pvu* II. This produced two fragments. The large fragment was 2363 bp and the small fragment was 323 bp. One microgram of digested pUC19 plasmid was loaded in each lane. Lanes 1 and 13, 123 bp ladder; Lanes 2 and 12, 1 kb ladder; Lanes 3, 5, 7, and 9, non-digested pUC19 from the same sources as in lanes 3, 6, 9, and 12 in Figure 3.7. Lanes 4, 6, 8, and 10, one microgram of *Pvu* II-digested pUC19 from lanes 3, 6, 9, and 12 in Figure 3.7.

Figure 3.9 shows the amplification efficiency of *Pvu* II-digested pUC19 plasmid purified from bacteria incubated for different times and in different media. Incubation time and media had no effect on the amplification efficiency of linear pUC19 molecule by PCR. The significance of this information is that using TB broth is superior to LB broth because the yield was significantly increased on one hand, and the quality of pUC19 was not lowered on the other hand.

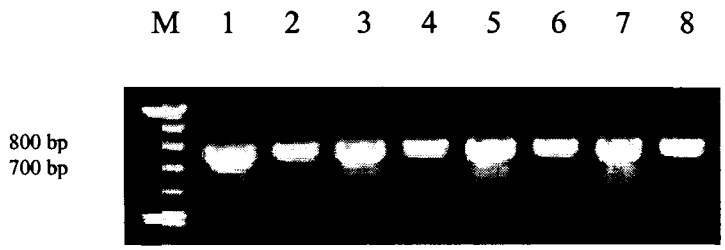


Figure 3.9. PCR amplification efficiency of pUC19 molecules from bacteria incubated in different media and for different time periods. Purified pUC19 plasmid was digested by *Pvu* II and the digested molecules were purified. Two microliter of digested pUC19 at 2×10^{-13} (heavy band) and at 2×10^{-14} M (light band) were amplified by PCR and eight microliter of PCR reaction was loaded in each lane of an agarose gel. Lanes 1 and 2, *Pvu* II-digested pUC19 from the same source as in lane 3 of Figure 4.7; Lane 3 and 4, *Pvu* II-digested pUC19 from the same source as in lane 6 of Figure 4.7; Lane 5 and 6, *Pvu* II-digested pUC19 from the same source as in lane 9 of Figure 4.7; Lane 7 and 8, *Pvu* II-digested pUC19 from the same source as in lane 12 of Figure 4.7.

PUC19 Plasmid Biotinylation and Detection of pUC19 Biotinylation Efficiency.

Figure 3.10 shows the time required for the complete digestion of one ug of pUC19 plasmid with one unit of *Acc* I. One hundred and sixty min were needed for 100% digestion (lane 9) under the conditions used.

Figure 3.11 shows the time required for the complete digestion of pUC19 plasmid by *Hind* III (4 units/mg of pUC19). One hundred and forty (Lane 8) min were needed for 100% digestion under the conditions used.

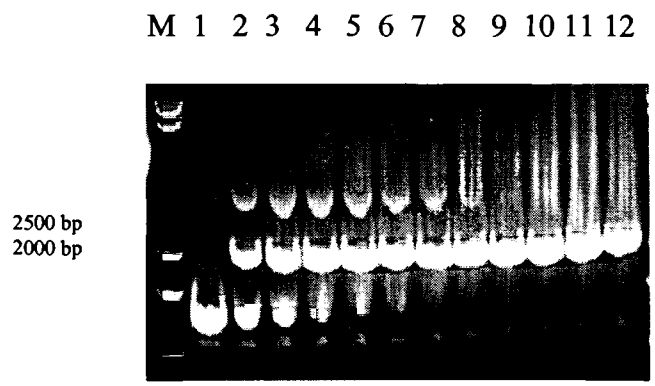


Figure 3.10. The time course of pUC19 digestion by *Acc* I. The purified pUC19 plasmid was digested with 1 unit of *Acc*I per microgram of pUC19 plasmid. The digestion lasted up to 220 min and samples were taken every 20 min. One microgram of pUC19 DNA was loaded in each lane of an agroase gel. Lane M, 1 kb ladder; Lane 1, undigested pUC19; Lane 2 through 12, 20 to 220-minute *Acc* I-digested pUC19.

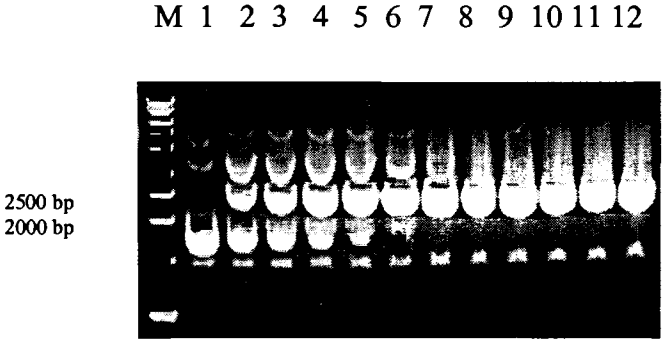


Figure 3.11. The time course of pUC19 digestion by *Hin*d III. The purified pUC19 plasmid was digested with 4 units of *Hin*d III per microgram of pUC19 plasmid. The digestion lasted up to 220 min and a sample was taken every 20 min. One microgram of pUC19 DNA was loaded in each lane of an agroase gel. Lane M, 1 kb ladder; Lane 1, undigested pUC19; Lane 2 through 12, 20 to 220-min *Hin*d III digested pUC19.

Knowledge of the minimum time necessary for complete digestion of the pUC19 plasmid is very important because incomplete restriction digestion would lead either to low biotinylation rate or to a pUC19 fragment with both ends biotinylated. In either case, the sensitivity of I-PCR would be reduced. In addition, incubation after complete

restriction digestion of pUC19 may lead to nonspecific plasmid breakdown, which would also have a negative effect on the efficiency of I-PCR. With this information in mind, we used double enzyme digestions of pUC19 plasmid for 4 h because the two enzymes in the same system might influence each other and reduce the digestion efficiency. The advantage of using double digestion is that the incubation time was reduced compared to two single digestions. This minimized non-specific destruction of plasmid DNA.

Figure 3.12 is the schematic representation of a system for detecting pUC19 biotinylation.

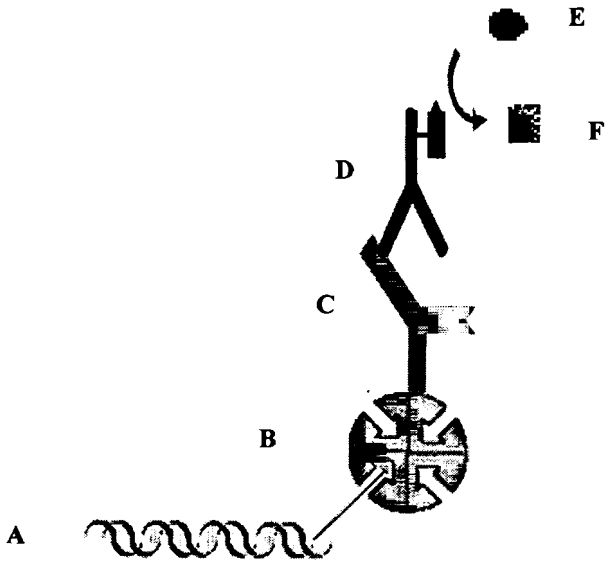


Figure 3.12. Schematic representation of pUC19 biotinylation detection system. Biotinylated pUC19 molecules (A) were fixed onto the Zeta-probe membrane. Streptavidin (B) was introduced to interact with the biotin group (arrow) on the DNA. Rabbit anti-streptavidin antibody (1^{st} Ab, IgG) (C) was then added to bind to streptavidin. This was followed by goat-anti rabbit IgG (2^{nd} Ab) (D) conjugated to horseradish peroxidase (pointed box), which interacted with 1^{st} Ab. Opti-4 CN (4-chloro-1-naphthol) (E) was added and purple precipitate (F) was generated under the action of horseradish peroxidase. Biotinylation efficiency was directly related to the intensity of the purple precipitate.

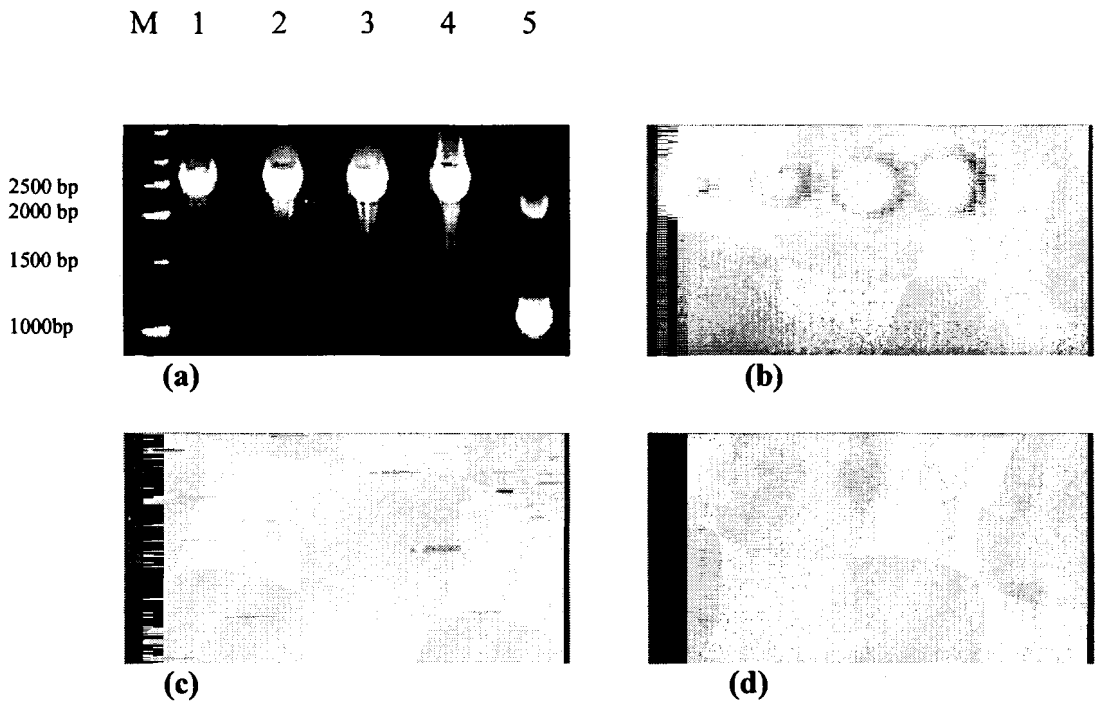


Figure 3.13. A new method for testing biotinylation efficiency of pUC19 fragments. PUC19 plasmid was digested by *Acc* I and *Hin*d III. The linear DNA molecules were purified by QIAquick PCR Purification Kit. The pUC19 biotinylation was achieved either by Klenow large fragment with biotin-dATP or by PCR reaction with biotinylated primer using linear pUC19 molecules as templates. One microgram of pUC19 was loaded in each well of an agarose gel. After electrophoresis, the linear DNA molecules were transferred from the agarose gel onto a Zeta-probe membrane and were detected with Opti-4 CN system. (a). Biotinylated pUC19 bands on an agarose gel detected by ethidium bromide staining. Lane M, 1 kb ladder, Lane 1, non-biotinylated linear pUC19 molecule; Lanes 2, 3, and 4, Klenow large fragment-generated biotinylated pUC19 fragment under the conditions of 15 min on ice, 15 min at room temperature, and 30 min at 37 °C, respectively; Lane 5, PCR-generated biotinylated pUC19 fragment. (b) pUC19 molecules transferred onto Zeta-probe membrane; (c) agarose gel after transferring; and (d) biotinylated pUC19 on the Zeta-probe membrane as detected by the Opti-4 CN system.

Figure 3.13 shows a novel and quantitative method for detecting pUC19 biotinylation efficiency. The biotinylation efficiency of PCR-generated pUC19 fragment with biotinylated primer did not differ from that of Klenow large fragment-generated biotinylated pUC19 fragment. When pUC19 fragments were biotinylated by incubation

with Klenow Fragment at 37 °C for 30 min, more pUC19 fragments were biotinylated compared with 15 min incubation either on ice or at room temperature.

Up to now, it has been very difficult, if not impossible, to do side by side comparisons of DNA biotinylation efficiency. The most widely used method for detecting DNA biotinylation is streptavidin-agarose affinity chromatography. This involves a tedious handling procedure, including boiling for a long time period to dissociate the binding between biotin and streptavidin. In addition, a relatively large quantity of biotinylated DNA is required to perform affinity chromatography. Furthermore, it is very expensive to compare multiple samples. However, using our detection system with all the commercially available ingredients, one microgram of biotinylated pUC19 per sample was sufficient for making side by side comparisons of biotinylation efficiency. This system proved to be effective, efficient, and ecomonical.

Figure 3.14 shows PCR amplification efficiency of biotinylated pUC19 fragments. For PCR-generated biotinylated pUC19 fragments, at least 50 molecules in 50 μl were required for detecion after PCR amplification (Lane 6). For Klenow fragment-generated biotinylated pUC19 fragments, as few as 5 molecules in 50 μl were readily detectable after PCR amplification (Lane 7).

The above results demonstrate that the biotinylated pUC19 fragments generated in our lab were very high in quality, because: 1) no DNA contamination was observed, 2) biotinylation efficiency was very high, and 3) signals were generated by PCR amplification even with a small number of pUC19 molecules.

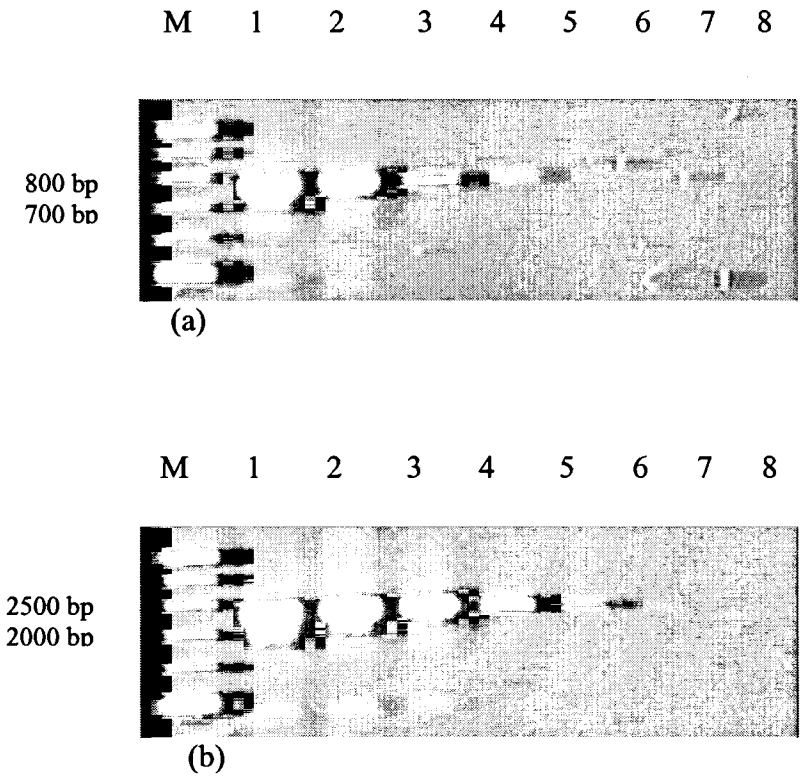


Figure 3.14. The amplification efficiency of biotinylated pUC19 fragments. Various amounts of biotinylated pUC19 fragments (both PCR-generated and Klenow large fragment-generated) were added to 50 µl of PCR master mix in thin-wall PCR tubes. After PCR reaction, 8 µl of PCR reaction and 2 µl of gel loading solution were loaded in each well of agarose gel containing ethidium bromide. Bands on agarose gels were visualized under UV-light. In (a), Lane M, PCR ladder; Lanes 1 through 8, 4.8×10^6, 4.8×10^5, 4.8×10^4, 4.8×10^3, 4.8×10^2, 4.8×10^1, 4.8×10^0, and 0 molecules of PCR-generated pUC19 fragments used as templates. In (b). The same molar concentrations of pUC19 as in Figure 3.14 (a) were used as templates, but Klenow large fragment-generated biotinylated pUC19 molecules were used as templates.

Summary

High quality biotinylated pUC19 was prepared as one of the components of our I-PCR assay. Commercially acquired bacteria were transformed with commercially-acquired pUC19 plasmids. The transformed bacteria were cultured under various conditions to increase the production of this plasmid. After harvesting the bacteria, the plasmids were purified with Qiagen EndoFree Plasmid Maxi Kit, then were cut by *Acc* I and *Hind* III restriction enzymes. Biotinylated pUC19 fragments were produced either with biotin-dATP through the enzymatic action of DNA polymerase I (Klenow large fragment) or by PCR amplification of a pUC19 fragment using a biotinylated primer.

The effect of a number of factors were studied in order to increase the yield and quality of biotinylated pUC19 fragments. Several of them were correlated with either yield or quality, while other factors appeared to be ineffective. First of all, culture medium played a key role in increasing plasmid yield. Yield of pUC19 plasmid was almost doubled when TB broth rather than LB broth was used to culture transformed bacteria. The contribution of TB broth was reflected by increased cell density at the end of culture, since the absorbance of TB culture averaged twice that of LB culture. Culture time did not seem to affect plasmid yield. Comparing 15 and 28 h of culture time within culture medium, pUC19 yield did not differ significantly ($p > 0.05$) in either TB broth cultures or LB broth cultures. The quality (estimated by the amount of end-product after PCR amplification) of pUC19 plasmid was not influenced by either culture media or culture time used. However, biotinylation conditions appeared to affect pUC19 fragment biotinylation efficiency. Incubation at 37 °C for 30 min seemed to increase biotinylation

efficiency compared to 15 min either on ice or at room temperature. Additional research is needed to confirm this observation.

Until recently, comparing DNA biotinylation efficiency under different conditions has been a painstaking process. Our novel membrane-based colorimetric procedure for testing pUC19 biotinylation efficiency was very effective, especially for side by side comparison, and was inexpensive and easy to perform. This procedure is useful not only for testing biotinylated DNA, but could potentially be used to test biotinylation efficiency in proteins.

After optimizing the procedure of biotinylated pUC19 production, we could detect as few as 48 PCR-generated or 5 Klenow fragment-generated biotinylated pUC19 molecules in 50 µl of immuno-PCR reaction. Only one-sixth of the total reaction volume (8 µl) was needed to detect a band on agarose gel. Based on these results, biotinylated pUC19 produced and tested using the optimized procedure may be used to enhance the sensitivity of our I-PCR assay.

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Chapter 4

Detection of Soluble and Membrane-Bound Antigens by I-PCR

Abstract

Both soluble antigens and cell membrane-bound antigens were studied using ELISA and I-PCR assays. For the detection of bovine serum albumin molecules immobilized on microtiter plates, the detection limit of ELISA was 5.8×10^7 molecules and the detection limit of I-PCR was 5.8×10^1 molecules, six orders of magnitude more sensitive than ELISA. For detecting recombinant interleukin-6 molecules immobilized on microtiter plates, the detection limit of ELISA was 5.8×10^{10} molecules and the detection limit of I-PCR was 5.8×10^3 molecules, seven orders of magnitude more sensitive than ELISA. I-PCR was also performed on individual mouse embryos using insulin receptors and EGF receptors on embryo plasma membranes as targets. In both cases, the intensity of the signal was positively correlated to the number of the embryos. The detection limit for insulin receptor was two embryos at the morula stage and the detection limit for EGF receptor was one embryo at the morula stage. Immuno-polymerase chain reaction was also used to compare differences in H-$2D^b$ expression on embryos from two strains of mice, Balb/c (H-$2D^b$ negative) and C57BL/6 (H-$2D^b$ positive). Strong signals were detected from C57BL/6 mouse embryos, while signals were not detected from Balb/c embryos.

Introduction

Bovine serum albumin immobilized on microtiter plates was detected by I-PCR in a classical experiment by Sano et al. (1992). The detection limit of BSA by I-PCR was 5.8×10^2 molecules, which was five orders of magnitude more sensitive than ELISA (5.8×10^7 molecules). This experiment was repeated in our lab in order to: 1) test ingredients produced in our lab for I-PCR, and 2) determine the relative sensitivity of I-PCR versus ELISA under our experimental conditions.

Detection of IL-6 by I-PCR has never been reported in the literature. IL-6 belongs to a group of low-molecular-weight secretory proteins, collectively designated as cytokines, which have important functions in coordinating immune and inflammatory responses (Arai et al., 1990), in neuronal functions such as differentiation or survival (Gadient and Otten, 1995), and in growth signal transduction (Hibi et al., 1990). Messenger RNA coding for IL-6 was detectable in human (Sharkey et al., 1995) and mouse embryos (Kita et al., 1994), which indirectly suggested that IL-6 played an important role in early embryonic development. In a preliminary study, we used recombinant IL-6 (mouse) immobilized on microtiter plates as target antigens. If I-PCR assay could detect IL-6 molecules with high sensitivity, our next step would be to determine whether IL-6 was secreted by preimplantation embryos and whether an I-PCR assay would be sensitive enough to detect IL-6 secretion by individual mouse embryos. Future research would be focused on determining embryo viability by measuring IL-6 and other viability-related embryo products using I-PCR.

There are only very limited data available that describe the detection of cell membrane-bound antigens on individual embryos by I-PCR. The first reported

experiment was completed by McElhinny and Warner (1997). By using H-2D^b histocompatibility antigens as targets, they detected signal on individual mouse embryos at the blastocyst stage. Following this initial report, the usefulness of I-PCR was again demonstrated by this group for detecting other embryo surface antigens, including Qa-2 protein, which increases the cleavage rate of mouse embryos (McElhinny et al., 1998a, 1998b; Waner et al., 1993; Wu et al. 1999). Our objective was to use I-PCR to detect receptors for insulin and EGF on individual mouse embryos because these two proteins: 1) have very important functions in cell signal transduction; 2) may be expressed by mouse embryos based on the reported expression of insulin receptor and EGF receptor mRNA in the inner cell mass (Harvey and Kaye, 1990; and 3) may have an expression pattern that is predictive of embryo viability (Heyner et al., 1989; Zhang et al., 1994). The objectives of the present studies were to: 1) validate the I-PCR technique in our lab by comparing I-PCR vs ELISA sensitivity in a 96-well plate-bound assay format using BSA as the target antigen; 2) detect a potential viability-related soluble protein by I-PCR and ELISA assay in a 96-well plate-bound assay format using recombinant IL-6 molecules as the target antigens to determine absolute and relative sensitivities; and 3) detect the expression of viability-related cell-surface proteins on individual mouse embryos using an embryo I-PCR assay.

Materials and Methods

Reagents and Experimental Animals

The following ingredients were from Sigma Chemical Co. (St. Louis, MO): recombinant Interleukin-6, bovine serum albumin, fatty acid free BSA (FAF-BSA), rabbit anti-mouse interleukin-6 (IgG fraction of antiserum), monoclonal anti- BSA (mouse IgG2a), biotinylated alkaline phosphatase, alkaline phosphatase yellow (pNPP) liquid substrate, sonicated salmon sperm DNA, equine chorionic gonadotropin (ECG), monoclonal anti-red blood cell (RBC) Wrb antigen (mouse, IgG2b), mineral oil, sodium penicillin G, streptomycin sulfate, gentamycin sulfate, Dulbecco's phosphate buffered saline (DPBS). The following ingredients were from Gibco BRL (Grand Island, NY): dNTP mix, and Platinum® Taq DNA polymerase. The following ingredients were from Biodesign International (Saco, Maine): monoclonal antibody (mouse IgG_{2b}) to epidermal growth factor receptor, monoclonal antibody to mouse transferrin receptor (CD71, rat IgG2a). Anti-mouse H-2 D^b Ab (IgG_{2b}) was from BD PharMingen (San Diego, CA). Gelatin was from Bio-Rad Laboratories (Hercules,CA); Ninety-six well plates (Maxisorp, flat bottom) were from Fisher Scientific and Fisherbrand Siliconized/Low Retention Microcentrifuge tubes with Snap Caps were from Fisher Scientific (Hanover Park, IL). C57BL/6 mice were from Jackson Lab (Bar Harbor, ME). Balb/c mice were from Charles River Laboratories (Wilmington, MA). The horizontal mini-gel system (model: MGU-202T) was from C.B.S. Scientific Company, Inc. (Del Mar, CA).

ELISA Assay to Detect BSA and IL-6 Immobilized onto the Surface of 96-well Microtiter Plates

The BSA and IL-6 molecules on the surface of microtiter plates were detected using the assay method described by Sano et al. (1992). Our assay procedure, which included minor changes from the original procedure, was described briefly as follows:

Preconjugation of Chimeric Protein with Biotinylated Alkaline Phosphatase. Fifty µl of 8.5×10^{-6} M chimeric protein solution in tris-buffered saline (TBS: 150 mM NaCl, 20 mM tris-HCl at pH 7.5) with 0.02% sodium azide was mixed with 50 µl of 8.5×10^{-6} M biotinylated alkaline phosphatase (in TBS with 0.02% sodium azide). The mixture was incubated at room temperature for one h. Just before use, 100 µl of preconjugate was mixed with 4900 µl of ETBS (TBS with 0.1 mM EDTA) at pH 7.5, containing 0.45% gelatin, to produce an 8.5×10^{-8} M solution of conjugate.

ELISA Assay. The surface of microtiter plate wells was coated with different amounts of FAF-BSA (from 5.8×10^{15} to zero molecules per well) or IL-6 (from 5.8×10^{12} to 5.8×10^{7} molecules per well) in 50 µl of coating buffer at pH 9.5 [150 mM NaCl, 20 mM tris-HCl, and 0.02% sodium azide] at 0 °C for 15 h to immobilize BSA or IL-6 molecules on the surface of the wells. The treatments of wells are shown in Table 4.1. Coated wells were washed four times for five min each with 150 µl of TBS containing 0.02% sodium azide. After emptying the wells, 200 µl of ETBS containing 2% gelatin was added to each well and the plates were incubated at room temperature for one h to block reactable sites on the surface of the wells. After washing wells three times (five min each) with 200 µl of TETBS (ETBS with 0.04% Tween 20), 75 µl of ETBS containing 0.45%

gelatin and 500-fold diluted mouse monoclonal anti-BSA antibody or rabbit anti-mouse interleukin-6 antibody were added to wells.

Table 4.1. Concentration of BSA or IL-6 (molecules/well) for ELISA assay.

Well #	Concentration	
	BSA	IL-6
1	5.8×10^{15}	5.8×10^{12}
2	5.8×10^{14}	5.8×10^{11}
3	5.8×10^{13}	5.8×10^{10}
4	5.8×10^{12}	5.8×10^{9}
5	5.8×10^{11}	5.8×10^{8}
6	5.8×10^{10}	5.8×10^{7}
7	5.8×10^{9}	
8	5.8×10^{8}	
9	5.8×10^{7}	
10	5.8×10^{6}	
11	5.8×10^{5}	
12	0	

Wells were incubated at room temperature for one h to allow the antibody to bind to immobilized BSA or IL-6 molecules. The wells were washed six times with 200 µl of TETBS. Six pmol of preconjugate of biotinylated alkaline phosphatase and streptavidin-protein A chimera in 70 µl of ETBS containing 0.45% gelatin were added to each well and were incubated at room temperature for one h to allow the conjugate to bind to the antigen-antibody complexes. The wells were washed 10 times with 200 µl of TETBS and washed once with 200 µl of TBS without sodium azide. Two hundred µl of p-nitrophenyl phosphate (pNPP) was added to each well and was incubated at 37 °C for up to one h.

The absorbance (at 405 nm) of each well was measured at 0, 15, 30, 45, and 60 min after incubation. Fifty µl of 3N sodium hydroxide was added to the wells after 60 min of incubation to stop the reaction.

Immuno-PCR Assay to Detect BSA and Recombinant IL-6 Molecules Immobilized onto the Surface of 96-Well Microtiter Plates

BSA and IL-6 molecules on the surface of microtiter plates were detected using the assay method described by Sano et al. (1992). Only minor changes from the original protocol were included in the assay procedure, which is described briefly as follows:

Preconjugation of Chimeric Protein with Biotinylated pUC19 Molecules. Protein A-streptavidin chimera and biotinylated pUC19 stock solutions were diluted and mixed in TBS solution (with sodium azide) at a concentration of 1.26×10^{-12} M each. The mixture was incubated at room temperature for one h. Just before use, the mixture was further diluted with TETBS (containing 0.45% gelatin and 0.1 mg/ml sonicated salmon sperm DNA, pH 7.5) to a final concentration of 2.8×10^{-14} M each.

Immuno-PCR Assay. Wells of 96 well plates were coated with different amounts of BSA or recombinant IL-6 molecules (Table 4.2) in 50 µl of coating buffer at 0 °C for 15 h to immobilize BSA or IL-6 molecules on the surface of the wells. The wells were washed three times with 125 µl and three times with 250 µl of TBS with 0.02% sodium azide for five min each. Two hundred µl of ETBS containing 2% gelatin and 1 mg/mL of sonicated salmon sperm DNA were added to each well, and were incubated at 37 °C for 80 min to block reactable sites on the surface of the wells.

Table 4.2. Concentration of BSA or IL-6 (molecules/well) for I-PCR assay.

Well #	Molecules/well	
	BSA	IL-6
1	5.8×10^4	5.8×10^7
2	5.8×10^3	5.8×10^6
3	5.8×10^2	5.8×10^5
4	5.8×10^1	5.8×10^4
5	5.8×10^0	5.8×10^3
6	0	5.8×10^2

The wells were washed three times with 200 µl and three times with 400 µl of TETBS (TBS with 0.1 mM EDTA & 0.1% Tween 20) by a plate washer. A volume of 50 µl of TETBS [containing 0.1 mg/mL of sonicated salmon sperm DNA, and 800,000-fold diluted mouse monoclonal anti-BSA or rabbit anti-mouse IL-6 antibody] was added to each well and incubated at room temperature for one h to allow the antibody to bind to immobilized BSA or IL-6 molecules. The wells were washed 30 times with 400 µl of TETBS. Fifty µl of TETBS (containing 0.45% gelatin, 0.1 mg/mL of sonicated salmon sperm DNA, and 2.8×10^{-14} M of biotinylated pUC19 conjugated to the streptavidin-protein A chimera) were added to each well, which was then incubated at room temperature for 50 min to allow the conjugate to bind to the antigen-antibody complexes. The wells were washed 40 times with 400 µl TETBS and 6 times with 250 µl of TBS without sodium azide. Fifty µl of Master mix was added to each well and the plates were heated in a water bath at 85 °C for 10 min to disrupt antibody-antigen and antibody-chimeric protein bonds, thud releasing plate-bound biotinylated pUC19 molecules into the Master Mix solution. The solution in each well was transferred into a thin-walled

PCR tube. Polymerase chain reaction was performed with an automated thermal cycler (*GeneAmp* PCR System, Perkin Elmer, NorWalk, CT), with the use of the following temperature profile: initial denaturation at 94 °C for five min; 35 cycles of denaturation at 94 °C for one min, annealing at 58 °C for one min, and extension at 72 °C for one min; and final extension at 72 °C for 5 min. The 21-mer primers (upper primer: 5'CAGGCAACTATGGATGAACGA'3 and lower primer: 5'GGCGAAACCCGACAG-GACTAT3') hybridize to a segment of the biotinylated pUC19 fragment and generate a 769-bp fragment upon PCR amplification. Eight µl of PCR product and 2 µl of gel loading solution were electrophoresed for 60 min at 95 Volts in a 1.0% agarose gel. The intensity of 769 bp DNA bands on the agarose gel was viewed under ultraviolet light to determine the presence of PCR products.

Immuno-PCR Assay to Detect Insulin Receptor and Epidermal Growth Factor Receptor on Mouse Embryos

Insulin receptors and EGF receptors on the plasma membranes of individual mouse morulae were detected with a modification of the method described by McElhinny and Warner (1997). Assay procedures were described briefly as follows:

Mouse Superovulation. C57BL/6 mice and Balb/c mice (at 10 weeks of age) on a 12 h light/12 h dark light cycle (lights on at 0600 h) were superovulated by ip injection at 1300 h of 7.5 to 10 IU of eCG in normal saline. Forty-eight h after the eCG injection, 7.5 to 10 IU of hCG was injected ip. Female mice were introduced to male mice immediately after the second injection, and were separated from males early the next morning.

Mouse Embryo Recovery and Handling. Sixty-eight h after hCG treatment, superovulated female mice were killed by cervical dislocation. The ovaries and uteri were removed and were placed into drops of HEPES-buffered KSOM medium (pH 7.4) containing one mg/mL of BSA (Fraction V; HEPES-buffered KSOM) in Petri dishes at room temperature. The oviducts and uterine horns were flushed with the same medium to harvest embryos. High-quality embryos at the 8-cell stage or older were passed through eight drops of HEPES-buffered KSOM and one additional drop of bicarbonate-buffered KSOM medium (pH 7.35) containing one mg/mL of BSA (Fraction V; bicarbonate-buffered KSOM). Embryos were cultured overnight in 25 μl drops of bicarbonate-buffered KSOM under mineral oil in 35 x 10 mm petri dishes at 37 °C with 5% CO_2 and maximum humidity. For some embryos, the zona pellucida was digested after three h of culture by incubating embryos in acidic Tyrodes solution (pH 2.5) for one min. Zona-free embryos were washed twice in Hepes-buffered KSOM and were transferred into bicarbonate-buffered KSOM drops, then were cultured overnight under the same conditions as stated above.

Preconjugation of Biotinylated pUC19 with Protein A-Streptavidin Chimera. A volume of 1.39 μl of 1.44 x 10^{-8} M pUC19 in modified Dulbecco's phosphate buffered saline (mdPBS) (0.49 mM MgCl, 2.68 mM KCl, 1.47 mM KH2PO4, 136.89 mM NaCl, 8.10 mM Na2HPO4, 0.68 mM CaCl, one mg/mL glucose, 36 μg/mL pyruvic acid, 60 μg/mL sodium penicillin, and 50 μg/mL streptomycin sulfate) at pH 7.5 was mixed with 0.76 μl of 2.62 x 10^{-8} M protein A-streptavidin chimera in mdPBS. The mixture was incubated at room temperature for one h. Just before use, the preconjugate was diluted with 500 μl of dilution buffer (mdPBS at pH 7.5 containing 0.5% FAF-BSA, 0.1%

sodium azide, 25 μg/mL of gentamycin and 0.1 mg/mL of sonicated salmon sperm DNA) to obtain a final pUC19/chimera concentration of 4.0×10^{-11} M.

Immuno-PCR of Mouse Embryos. After incubation overnight, zona-free embryos at the compact morula to blastcyst stage were rinsed in four drops of mdPBS containing 0.5% of FAF-BSA, 0.1% of sodium azide, and 25 μg/mL of gentamycin (Wash solution II). Embryos were incubated in 30 μl of diluted antibody solution [mixing 15 μl of original antibody solution (anti-H-2 D^b at 250 μg/mL, anti-insulin receptor at 250 μg/mL) and 15 μl of the Wash Solution II] or 30 μl of undiluted antibody solution (anti EGF-R at 100 μg/ml) for four hours on ice. The embryos were washed six times in mdPBS containing 0.5% FAF-BSA, 0.1% sodium azide, 0.002% tween 20, and 25 μg/mL of gentamycin (Wash Solution I) and then six times in Wash Solution II. Embryos were transferred into 50 μl of 4.0×10^{-11} M chimeric protein/ biotinylated pUC19 conjugate in mdPBS containing 0.5% of FAF-BSA, 0.1% salmon sperm DNA, 0.1% sodium azide and 25 μg/mL gentamycin, and were incubated for one h on ice. Embryos were washed in six drops of wash solution I and then six drops of wash solution II, then were rinsed briefly in two drops of sodium azide-free mdPBS containing 0.5% FAF-BSA, then were transferred into thin-wall PCR tubes containing 3 μl of distilled water. After adding 48 μl of Master Mix, PCR was performed under the same conditions as used for the amplification of BSA and recombinant IL-6. Bands of amplified pUC19 were visualized by ultraviolet light.

Stasistical Analysis

The data generated in these experiments were analyzed by Student's *t*-test or by Analysis of Variance.

Results and Discussion

ELISA and I-PCR Detection of BSA Immobilized on Microtiter Plates

The results of BSA detection by ELISA are presented in Figure 4.1. When 5.8×10^7 or more BSA molecules were used, the absorbances at 405 nm were all significantly higher ($p < 0.05$) than when the wells received 5.8×10^7 or fewer molecules of BSA. The absorbances were not significantly different among the wells that received 5.8×10^6 or fewer BSA molecules. The absorbances fell into a linear range when the amount of BSA was between 5.8×10^{10} and 5.8×10^8 molecules. The detection limit of the ELISA assay for BSA was 5.8×10^7 molecules per well.

The results of BSA detection by I-PCR are presented in Figure 4.2. As the concentration of coating antigens was reduced from 5.8×10^4 molecules (Lane 2) to 5.8×10^1 molecules (Lane 5), the pUC19 band was clearly detectable by I-PCR, and its intensity decreased in a dose-dependent manner. When the concentration of BSA molecules was reduced to 5.8×10^0 molecules (Lane 6) per well, the signal was no longer detectable by I-PCR. The signal lessened sharply when the number of BSA molecules was reduced from 5.8×10^2 (Lane 4) molecules to 5.8×10^1 (Lane 5) molecules.

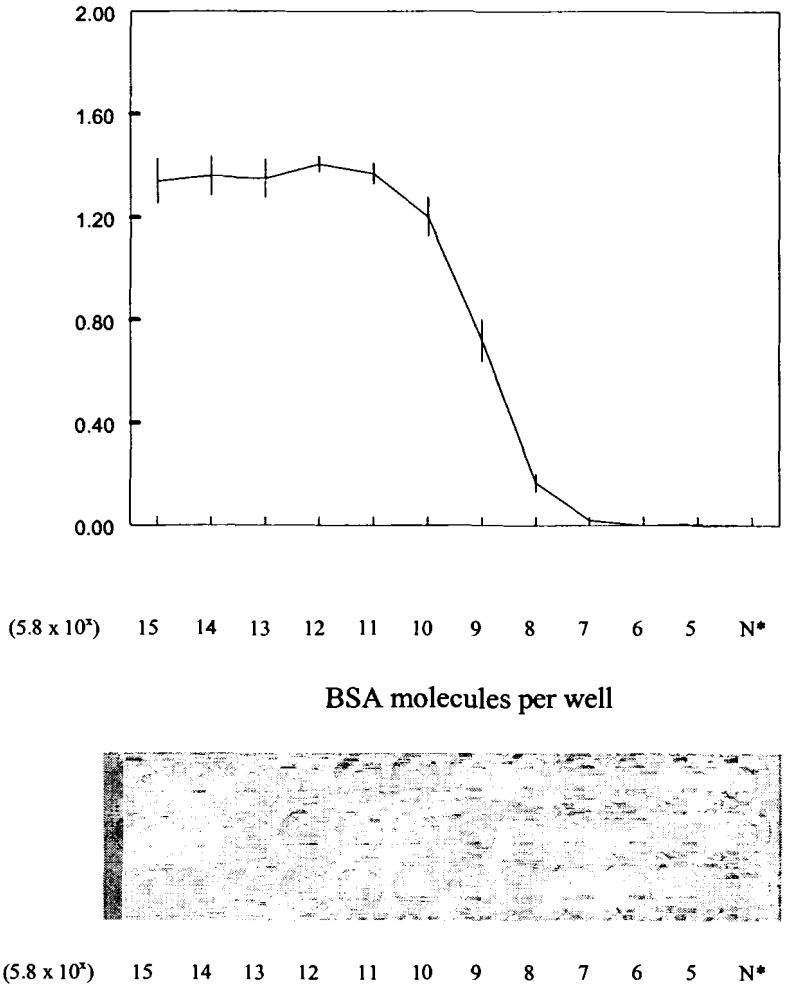

Figure 4.1. BSA detection by ELISA. The surface of microtiter plate wells was coated with different amounts of BSA at 0 °C for 15 h. After the wells were washed with TBS, reactable sites on the surface of the wells were blocked with 2% gelatin in ETBS at room temperature for 1 h. After washing wells with TETBS, mouse monoclonal anti-BSA antibody in ETBS containing 0.45% gelatin was added to the wells and was incubated at room temperature for 1 h. After washing wells, a conjugate of 6 pmol of biotinylated alkaline phosphatase and the streptavidin-protein A chimera in ETBS containing 0.45% gelatin was added to each well and was incubated at room temperature for 1 h. The wells were washed with TETBS first and then with TBS without sodium azide. P-nitrophenyl phosphate (pNPP) substrate was added to each well and was incubated at 37 °C for up to 1 h. Absorbances at 405 nm was measured at 0, 15, 30, 45, and 60 min of incubation.

N*-Negative control, no BSA molecules coated on the well.

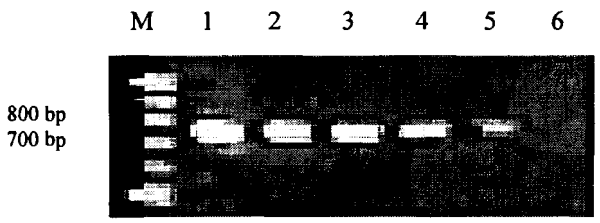

Figure 4.2. BSA detection by immuno-PCR. Microtiter plate wells were coated with different amounts of BSA at 0 °C for 15 h. Reactable sites on the surface of the wells were blocked with ETBS containing 2% gelatin and one mg/mL of sonicated salmon sperm DNA. Mouse monoclonal anti-BSA in TETBS containing 0.1 mg/mL of sonicated salmon sperm DNA was added to each well and was incubated at room temperature for one h. This was followed by adding biotinylated pUC19 conjugated to streptavidin-protein A chimera in TETBS (containing 0.45% gelatin and 0.1 mg/mL of sonicated salmon sperm DNA). Wells were incubated at room temperature for 50 min. Fifty uL of Master Mix was added to each well and the plates were heated at 85 °C for 10 min. The solution in each well was transferred to a thin-walled PCR tube. PCR products were loaded into wells of an agarose gel. The DNA band intensity on the agarose gel was viewed under UV-light and photographed. Lane M, DNA ladder; Lane 1, positive control (2 µl of 1.4×10^{-13} M of biotinylated pUC19; Lanes 2 through 6, 5.8×10^{4} to 5.8×10^{0} molecules of BSA per well.

Immuno-PCR was 1.0×10^{6} times more sensitive than the comparable ELISA assay for detecting BSA molecules on the surface of microtiter plates (5.8×10^{1} versus 5.8×10^{7} molecules, respectively). The ELISA results were similar to those obtained by Sano et al. (1992), who also reported a sensitivity of 5.8×10^{7} molecules of BSA with an ELISA. However, our I-PCR detection limit of 5.8×10^{1} BSA molecules, was 10 times more sensitive for the detection of plate-bound BSA than Sano's (5.8×10^{2} molecules). The reason for the increased sensitivity in our assay is not completely understood. Our more stringent washings and 100-fold lower antibody concentration may have contributed to the increased sensitivity by reduction of the background signal.

Detection of Recombinant IL-6 on Microtiter Plates by ELISA and I-PCR

The results of IL-6 detection by ELISA are presented in Figure 4.3. When 5.8×10^{10} or more IL-6 molecules were used, the absorbances at 405 nm were all significantly higher ($p < 0.05$) than that obtained from the wells that received 5.8×10^{9} or fewer IL-6 molecules.

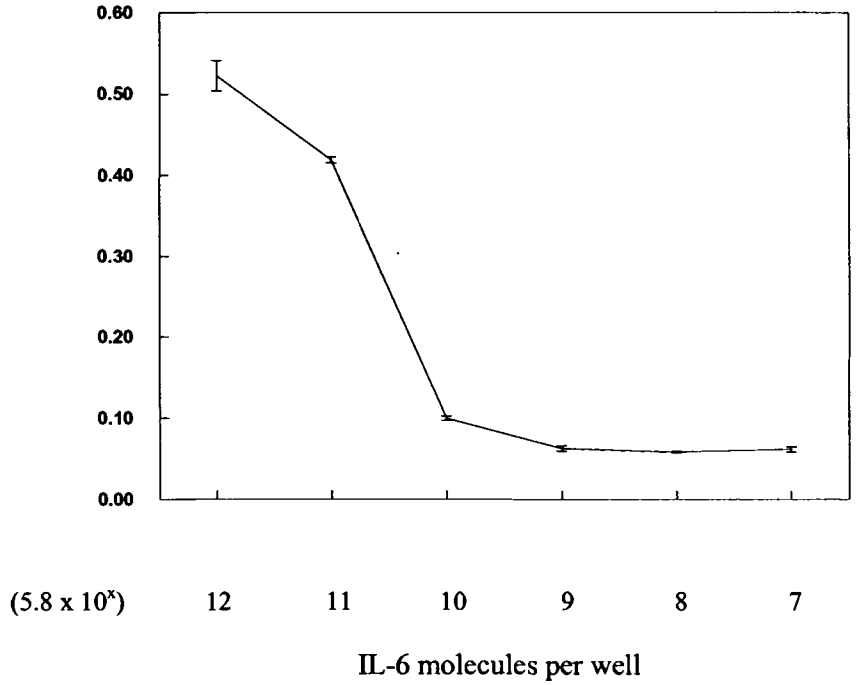

Figure 4.3. IL-6 detection by ELISA. The surface of microtiter plate wells was coated with different amounts of IL-6 at 0 °C for 15 h. After the wells were washed with TBS, the reactable sites on the surface of the wells were blocked with 2% gelatin in ETBS at room temperature for 1 h. After washing wells with TETBS, mouse monoclonal anti-BSA antibody in ETBS containing 0.45% gelatin was added to the wells and incubated at room temperature for 1 h. After a second series of washes, a conjugate of 6 pmol of biotinylated alkaline phosphatase and streptavidin-protein A chimera in ETBS containing 0.45% gelatin was added to each well and was incubated at room temperature for 1 h. The wells were washed with TETBS and then with TBS without sodium azide. P-nitrophenyl phosphate (pNPP) substrate was added to each well and was incubated at 37 °C for up to 1 h. The absorbance at 405 nm was measured at 0, 15, 30, 45, and 60 min after incubation.

The absorbances were not significantly different among the wells that received 5.8×10^9 or fewer BSA molecules. The detection limit of the ELISA assay for IL-6 was 5.8×10^{10} molecules.

The results (one of the triplicate gels) of IL-6 detection by I-PCR are presented in Figure 4.4. When the coating antigens were reduced from 5.8×10^7 (Lane 2) to 5.8×10^3 (Lane 6) molecules/well, the PCR bands were visible, and decreased in intensity in a linear manner. Repeatable band patterns (in triplicates) were obtained.

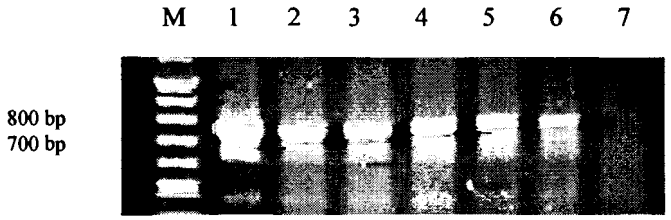

Figure 4.4. IL-6 detection by immuno-PCR. The microtiter plate wells were coated with different amounts of IL-6 at 0 °C for 15 h. The reactable sites on the surface of the wells were blocked with ETBS containing 2% gelatin and one mg/mL of sonicated salmon sperm DNA. The mouse monoclonal anti-IL-6 in TETBS containing 0.1 mg/mL of sonicated salmon sperm DNA was added to each well and was incubated at room temperature for one h. This was followed by adding biotinylated pUC19 conjugated to the streptavidin-protein A chimera in TETBS (containing 0.45% gelatin, 0.1 mg/mL of sonicated salmon sperm DNA) to each well and incubated at room temperature for 50 min. After washing, fifty microliters of Master Mix was added to each well and the plates were heated at 85 °C for 10 min. The solution in each well was transferred into a thin-walled PCR tube. After PCR amplification, the PCR products were loaded into each well of an agarose gel. The DNA band intensity on the agarose gel was viewed under ultraviolet light and photographed. Lane M, DNA ladder; Lane 1, positive control (2 µl of 1.4×10^{-13} M of biotinylated pUC19; Lanes 2 through 7, 5.8×10^7 to 5.8×10^3 molecules of IL-6 per well.

When IL-6 was reduced to 5.8 x 10^2 molecules/well (Lane 7), the I-PCR signal was no longer detectable. Although the absolute sensitivity of I-PCR for detecting IL-6 was not as high as for BSA, its relative sensitivity (in comparison to ELISA) was higher compared to the BSA. The I-PCR achieved seven orders of magnitude more sensitivity than ELISA for detecting immobilized IL-6 molecules compared to six orders of magnitude more sensitivity obtained in the BSA assays. The reason for the lower absolute sensitivity of the IL-6 I-PCR may be attributable to the presence of a 50-fold excess of BSA in the IL-6 sample. BSA molecules may have competed with IL-6 molecules for the limited binding sites on the surface of the microtiter plate well and therefore may have reduced the absolute number of IL-6 molecules that bound to the wells.

Detection of Insulin Receptors and EGF Receptors on Mouse Embryos by I-PCR

Figure 4.5 presents results that were typical of our early efforts to use I-PCR assay to detect cell surface markers on C57BL/6 mouse blastocysts. After zona-free embryos from C57BL/6 mice were cultured overnight and rinsed in three drops of mdPBS containing 1% BSA and 0.1% sodium azide, they were incubated on ice for one h in a mixture of diluted antibodies [two ul of each of eight antibodies plus eight-four µl of mdPBS (containing 0.1% sodium azide), so, each antibody was diluted 50 fold]. The antibodies were: 1) monoclonal antibody to epidermal growth factor receptor, IgG_{2b} (mouse), 0.25 µg/mL, Biodesign International™; 2) monoclonal antibody to transferrin receptor, IgG_{2a} (rat), 1.25 µg/mL, Biodesign International™; 3) monoclonal antibody to integrin α5 chain , IgG_{2a}, κ (rat), 1.25 µg/mL, BD PharMingen; 4) monoclonal antibody to integrin β1 chain , IgG_{2a}, κ (rat), 0.25 µg/mL, BD PharMingen; 5) monoclonal

antibody to hyaluronate receptor, IgG_{2b}, κ (rat), 1.25 μg/mL, BD PharMingen; 6) monoclonal antibody to H-$2D^b$, IgG_{2b}, κ (mouse), 1.25 μg/mL, BD PharMingen; 7) monoclonal antibody to Qa-2, IgG_{2a}, κ (mouse), 0.25 μg/mL, BD PharMingen; and 8) monoclonal antibody to E-Cadherin, IgG_{2a}, (mouse), 0.625 μg/mL, Sigma Chemical Co. Embryos were washed in five drops of 100 μl of mdPBS (1% BSA, 0.1% sodium azide, and 0.02% tween 20) (Wash I) and five drops of mdPBS (1% BSA and 0.1% sodium azide) (Wash II), then were incubated in protein A-streptavidin chimera that was diluted to 1×10^{-11} M in Wash II. After washing with five drops each of Wash I and Wash II, embryos were incubated in 4×10^{-11} M biotinylated pUC19 fragments. After washing as above to remove unbound biotinylated pUC19 molecules, mouse embryos were transferred into thin-walled PCR tubes containing 3 ul of distilled water. Fifty microliters of Master Mix was added to each tube. After PCR amplification, PCR products were resolved on agrose gels and were visualized over ultraviolet light. No band was observed in the negative control lane (Lane 2). When one embryo (Lane 7) was incubated with a mixture of antibodies, only a very weak band was seen on the gel. The band became brighter when six embryos (Lane 6) were used. The brightest band (except positive control, Lane 1) was obtained when antibodies were excluded from the assay (Lane 5). The second brightest band was obtained when both antibody mixture and protein A-streptavidin chimera were excluded from the assay (Lane 4). Based on these inconsistent results we hypothesized that the background signals (without antibodies or without either antibodies and protein A-streptavidin chimera) were too high and this masked the specific signals.

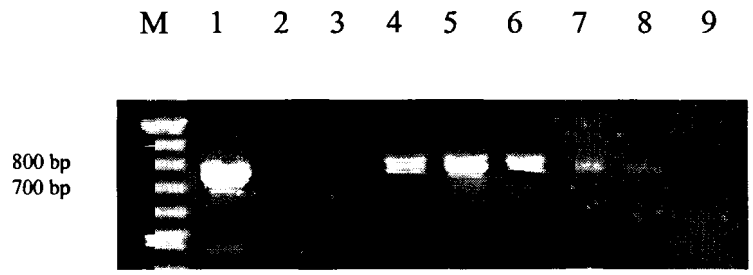

Figure 4.5. Surface antigen detection on C57/BL6 mouse blastocysts by I-PCR assay. After zona-free embryos were cultured overnight and rinsed in mdPBS once, they were incubated in a mixture of antibodies for one h on ice. After washing, embryos were incubated in chimeric protein and biotinylated pUC19 for one h each under the same conditions. After washing, mouse embryos were transferred into thin-walled PCR tubes and fifty microliters of Master Mix was added to each tube. The PCR product was resolved on agrose gel and visualized over ultraviolet light. Lane M, DNA ladder; Lane 1, 2 ul of 4 x 10^{-11} M biotinylated pUC19 fragments put directly in PCR tube (positive control); Lane 2, 2 µl of PBS put directly in PCR tube (negative control); Lane 3, without antibodies, without protein A-streptavidin chimera, without biotinylated pUC19 (with 3 blastocysts); Lane 4, without antibodies, without protein A-streptavidin chimera, with biotinylated pUC19 (4 x 10^{-11} M) (with 2 blastocysts); Lane 5, without antibodies, with protein A-streptavidin chimeria, with biotinylated pUC19 (4 x 10^{-11} M) (2 blastocysts); Lane 6, with antibody mixture, with protein A-streptavidin chimera, with biotinylated pUC19 (4 x 10^{-11} M) (6 blastocysts); Lane 7, with antibody mixture, with protein A-streptavidin chimera, with biotinylated pUC19 (4 x 10^{-11} M) (1 blastocyst); Lane 8, 3 µl of last embryo rinse put directly in PCR tube; and Lane 9, Master Mix only without PCR amplification.

McElhinny and Warner (1997) used anti-H2-D^b antibody and 10^{-13} M pUC19 in their embryo I-PCR assay and reported a visible signal with single embryo sensitivity. However, when we followed their protocol exactly, we were unable to duplicate their results in our lab. We also performed embryo I-PCR assays using other antibodies for mouse embryo surface antigens, again following the published procedure, but no signal was obtained. Two possible reasons might have accounted for our failure to produce a signal: 1) biotinylated pUC19 concentration in our assay was too low, and/or 2) the concentrations of target antigens on embryos were too low to be detectable. In order to test the first possibility, we increased the concentration of biotinylated pUC19 in our

assay from 4×10^{-13} M to 4×10^{-11} M. To test the second possibility, we incubated embryos with a mixture of eight antibodies (to increase the number of targets) instead of one antibody in our current I-PCR assay. However, this gave us very high background signals. In order to reduce or eliminate this high background signal, sonicated salmon sperm DNA was added to both chimeric protein and biotinylated pUC19 incubation step, so that the non-specific binding of DNA to embryos was blocked.

The results of detecting insulin and EGF receptors on BALB/c mouse morulae by I-PCR assay were presented in Figure 4.6. The pivotal discovery in this study is that the nonspecific signal of " no antibody negative control " (Lane 4) was decreased to an undetectable level by the addition of 0.1 mg/mL of sonicated salmon sperm DNA to the incubation steps of antibody, chimeric protein, and biotinylated pPC19. Because the background signal no longer obscured specific binding of pUC19 to embryo-bound antibody, the target signals were revealed (Lanes 6 and 7). While insulin and EGF receptors both appeared to be expressed by Balb/c mouse morulae, CD71 molecules did not seem to be expressed at a detectable level on these embryos at the morula stage (Lane 8). An unexpected finding was that the zonae pellucida of mouse embryos did not trap significant amounts of the conjugate of protein A-streptavidin chimera and biotinylated pPC19 (Lane 9). This finding was not consistent with experimental results obtained by another research group (Warner, 1998), and may be due to the blocking action of sonicated salmon sperm DNA.

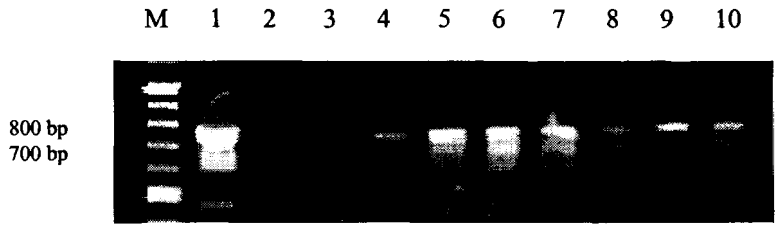

Figure 4.6. Detection of insulin receptor and EGF receptor on Balb/c mouse morulae by I-PCR. Zona-free embryos were cultured overnight, then were washed in mdPBS, then were incubated with or without antibodies on ice for three h. After washing to remove unbound antibodies, embryos were incubated with or without 4 x 10^{-11} M of chimeric protein/ biotinylated pUC19 conjugate for one h on ice. After washing to remove the unbound conjugate, embryos were transferred into thin-walled PCR tubes containing 3 µl of distilled water and 50 µl of Master Mix was added to each tube. The PCR product was resolved on an agrose gel and was visualized over ultraviolet light. Lane M, DNA ladder; Lane 1, 2 µl of 1.4 x 10^{13} M biotinylated pUC19 fragments put directly in PCR tube (positive control); Lane 2, 2 µl of PBS put directly in PCR tube (negative control); Lane 3, without antibody, without protein A-streptavidin chimera, without Marker DNA, with 5 morulae (embryo negative control); Lane 4, without antibody, with protein A-streptavidin chimera, with biotinylated pUC19, with 5 morulae (antibody-free negative control); Lane 5, with anti-Red Cell Wrb antibody, with protein A-streptavidin chimera, with biotinylated pUC19, with 4 morulae (negative antibody control); Lane 6, with anti-insulin receptor antibody, with protein A-streptavidin chimera, with biotinylated pUC19, with 6 morulae; Lane 7, with anti-EGF receptor antibody, with protein A-streptavidin chimera, with biotinylated pUC19, with 5 morulae; Lane 8, with anti-CD71 antibody, with protein A-streptavidin chimera, with biotinylated pUC19, with 5 morulae; Lane 9, without antibody, with protein A-streptavidin chimera, with biotinylated pUC19, with 4 zona-enclosed expanded blastocysts; and Lane 10, 3 µl of fluid from the last embryo rinse of the negative antibody control treatment.

Another unexpected finding was obtained when anti-Red Cell Wrb antibody was used in the assay. Because Red Cell Wrb antigen was supposed to be expressed only on red blood cells (Telen and Bolk, 1982; Telen et al., 1987), we used it as a negative antibody control in the present I-PCR assay. However, anti-RBC Wrb antibody produced a positive signal in the I-PCR (Lane 5). The reason for this unexpected result is beyond our comprehension, but may be due to cross-reactivity of the anti-RBC Wrb antibody

with embryo surface proteins. Based on this result, we discontinued our use of anti-RBC Wrb antibody as a negative antibody control in our later experiments.

In later studies, we included a strain-specific histocompatibility antibody, anti-H-2 D^b antibody, as both a negative antibody control (when incubated with embryos from a H-2 D^b negative mouse strain (Balb/c) and a positive antibody control (when incubated with embryos from a H-2 D^b positive mouse strain (C57BL/6). Since very strong signals were obtained with anti-insulin receptor (Lane 6) and anti-EGF receptor (Lane 7) antibodies in this study, we also explored the possibility of using fewer embryos per incubation in later studies.

Our final I-PCR experiment utilized antibodies against H-2D^b antigen, insulin receptor, and EGF receptor and each antibody was incubated with one to six BALB/c or C57BL/6 mouse morulae. Results are presented in Figure 4.7.

When anti-insulin receptor antibody was used, the PCR band was fairly intense for 5 embryos (Lane 10), clearly observable for two embryos (Lane 11), and not detectable for one embryo (Lane 12). When anti-EGF receptor antibody was used, the PCR band was very strong for six embryos (Lane 13), fairly strong for three embryos (Lane 14), and clearly above background for one embryo (Lane 15). When Anti H_2-D^b antibody was used for detecting target antigen on BALB/c embryos which are negative for this antigen (BD PharMingen, 2000), the signal was very weak even using six embryos (Lane 4), barely observable for three embryos (Lane 5), and completely absent for one embryo (Lane 6). When Anti H-2 D^b antibody was used for detecting target antigen on C57/BL6 mouse embryos [positive for H-2 D^b molecules (McElhinny and Warner, 1997)], a different picture appeared: the signal was extremely strong for six

embryos (Lane 7), strong for three embryos (Lane 8), and clearly above background for one embryo (Lane 9). Consequently, the anti- H_2-D^b antibody was successfully used as both a positive and a negative control.

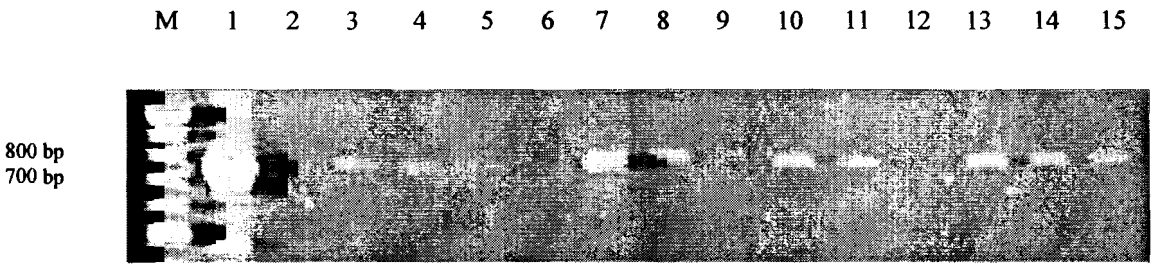

Figure 4.7. Detection of H-2 D^b molecules, insulin receptor, and EGF receptors on C57BL/6 and Balb/c mouse morulae by I-PCR. Zona-free embryos were cultured in KSOM medium overnight, then were washed in mdPBS, and then were incubated in a solution with or without respective antibody for three h on ice. After removing unbound antibodies by washing, embryos were incubated in a solution with or without the conjugate of protein A-streptavidin chimera and biotinylated pUC19 for 1 h on ice. After washing to remove the unbound conjugate, mouse embryos were transferred into thin-walled PCR tubes that contained three µl distilled water and forty-eight microliters of Master Mix was added to each tube. The PCR products were resolved on agarose gel and were visualized under ultraviolet light. Lane M, DNA ladder; Lane 1, 2 µl of 1.4×10^{-13} M biotinylated pUC19 fragments put directly in PCR tube (positive control); Lane 2, 3 µl of mdPBS put directly in PCR tube (negative control); Lane 3, without antibody, with protein A-streptavidin chimera, with 4×10^{-11} M biotinylated pUC19 fragments (Marker DNA), with 6 Balb/c morulae (antibody-free negative control); Lanes 4 through 6, anti-H-2 D^b antibody, with protein A-streptavidin chimera, with Marker DNA, with 6, 3, or 1 morulae from Balb/c mice, respectively; Lanes 7 through 9, with anti-H-2 D^b antibody, with protein A-streptavidin chimera, with Marker DNA, with 6, 3, or 1 morulae from C57/BL6 mice, respectively; Lanes 10 through 12, with anti-insulin receptor antibody, with protein A-streptavidin chimera, with Marker DNA, with 5, 2, or 1 morulae from Balb/c mice, respectively; Lanes 13 through 15, with anti-EGF receptor antibody, with protein A-streptavidin chimera, with Marker DNA, with 6, 3, or 1 morulae from Balb/c mice, respectively.

To our surprise, cell surface antigens on a single embryo at the morula stage could be detected by I-PCR assay. This is very significant in terms of the sensitivity of our modified I-PCR technique for detecting cell surface antigen. Previously, the highest

sensitivity with I-PCR for detection of mouse embryo surface antigens was reported by McElhinny and Warner (1997) on a single mouse embryo at the blastocyst stage, which contains more cells than a morula-stage embryo.

Summary

Both soluble and membrane-bound antigens were successfully detected by I-PCR technique. The soluble antigens were BSA and IL-6 molecules and the membrane-bound antigens were insulin receptor, EGF receptor, and H-2 D^b histocompatibility antigens on mouse embryos. Plate-based I-PCR of soluble BSA and IL-6 demonstrated much higher sensitivity in comparison to assays of these compounds using ELISA.

The detection of BSA fixed on microtiter plates has been reported previously (Sano et al., 1992), but we chose to repeat this classic experiment in order to demonstrate that our protein A-streptavidin chimera and biotinylated pUC19 were fully functional. For purposes of comparing relative sensitivity with a known assay, ELISA and I-PCR for plate-bound BSA molecules were performed concurrently. The detection limit of ELISA on plate-bound BSA was 5.8×10^7 molecules. This result is exactly the same as obtained by Sano et al (1992). The detection limit of I-PCR was 5.8×10^1 molecules. Our sensitivity was 10 times higher than the sensitivity reported by Sano et al (1992), which was 5.8×10^2 molecules. This high detection sensitivity (six orders of magnitude higher than ELISA) of BSA by I-PCR has further proved that our protein A-streptavidin chimera and biotinylated pUC19 were fully functional. In addition, these results also demonstrated that Sano's I-PCR technique worked under the conditions in our laboratory.

Plate-bound IL-6 was also detected by I-PCR and ELISA. The detection limit of I-PCR for IL-6 was 5.8×10^3 molecules. The absolute sensitivity of I-PCR for IL-6 was 2 orders of magnitude lower than for BSA. The detection limit of ELISA for IL-6 was 5.8×10^{10} molecules, which was three orders of magnitude lower than for BSA. The reduced sensitivity for IL-6 molecules might be due to the "carrier" protein (BSA, at 50 times the concentration of IL-6) in the commercial preparation of IL-6. The overwhelming number of BSA to IL-6 molecules may have reduced the number of IL-6 molecules bound to the surface of microtiter plate wells because of competition between BSA and IL-6 for limited number of binding sites. Although the absolute detection limit of I-PCR for IL-6 was lower than I-PCR for BSA, the relative sensitivity of I-PCR for IL-6 (compared to IL-6 ELISA assay) was seven orders of magnitude higher than that of IL-6 ELISA assay and was one order of magnitude more sensitive than the relative sensitivity of I-PCR for BSA. These results have demonstrated that I-PCR is preferable method to ELISA for the detection of a soluble antigen in a impure sample.

Detecting cell surface proteins on embryos by I-PCR was the most challenging application of I-PCR technology. By using an existing published protocol from the other lab, we were unable to obtain PCR signals after incubation of mouse embryos with antibodies to putative embryo antigens. However, by increasing the concentration of protein A-streptavidin and biotinylated pUC19 conjugate from 4×10^{13} M to 4×10^{11} M, we produced strong signals, but simultaneously increased non-specific binding enough to mask the specific signal. The increased background made the I-PCR useless for the identification of the target antigens. Based on numerous unsuccessful embryo-I-PCR assays using the original published I-PCR protocol, we hypothesized that an unacceptably

high level of non-specific binding was overshadowing any signal generated by specific antibody-antigen binding. In an attempt to solve this problem, we kept the conjugate concentration at the same level, but added sonicated denatured salmon sperm DNA (to reduce non-specific binding of pUC19 to embryos) to incubation drops that contained biotinylated pUC19. This change allowed us to increase the concentration of protein A-streptavidin/biotinylated pUC19 conjugate by a factor of 100, while reducing non-specific binding of pUC19 to undetectable levels.

The embryo surface proteins of interest were insulin receptor, EGF receptor, and H-2 D^b molecules. We chose to use anti- H-2 D^b antibody as both a positive and a negative control since the existing data demonstrated that H-2 D^b molecules were expressed only on C57/BL6 mouse embryos, but not on Balb/c embryos (BD PharMingen, 2000). Our I-PCR results confirmed these earlier findings: H-2 D^b molecules existed only on C57BL/6 mouse embryos, but not on Balb/c embryos. By using I-PCR technique, we were able to detect positive signals on two mouse embryos at the morula stage by using antibody to insulin receptor, and on only one mouse embryo at the morula stage by using antibody to EGF receptor. Such a high sensitivity of detecting cell surface antigens expressed on mammalian embryos has not previously been reported. This result demonstrated that I-PCR is a very powerful and a very useful technique for detecting cell surface antigens on embryos.

Our experimental results demonstrated that I-PCR is the most sensitive technique for detecting both soluble and membrane-bound antigens. Comparing to ELISA, I-PCR was six orders of magnitude more sensitive for detecting BSA molecules and seven orders of magnitude more sensitive for detecting IL-6 molecules. The detection limit for

insulin receptor was two embryos at the morula stage and the detection limit for EGF receptor was one embryo at the morula stage. Because IL-6 molecules, insulin receptors, and EGF receptors are viability-related embryo proteins, I-PCR assay may become a potential technique for the testing of embryo viability.

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Chapter 5

SUMMARY

Protein A-streptavidin chimera was one of the important components used in I-PCR. Experiments were conducted to increase the yield of this protein through modifications to the original protocol. A number of factors were studied and several of them were demonstrated to play a role in the yield of this protein. The protein yield was increased substantially by using the newly optimized protocol. Experiments were also conducted to test the binding of this protein to both antibodies and biotinylated DNA, and its physical and functional integrity were confirmed by these studies.

The yield of protein A-streptavidin chimera increased from zero to four h after IPTG induction. Under our experimental conditions, the optimal induction time was four hours. IPTG induction temperature did not appear to affect the yield of Protein A-streptavidin chimera, while culture medium had a significant effect on the yield of Protein A-streptavidin chimera. The yield was five to ten times higher when *E. coli* cells were cultured in TB broth than in either LB broth or M9 medium. This increased bacterial yield did not seem to result from increase bacterial density alone because the yield of plasmid was increased by TB broth even when the effect of bacterial concentration was equalized among treatment groups. The strong buffer system in TB broth may have played a role in its superior growth-promoting effect.

The methods used for bacterial cell lysis and protein purification also affected the chimeric protein yield. Lysing by sonication appeared to be superior in terms of chimeric protein yield compared to chemically induced lysis. More than three times as

much protein A-streptavidin chimera was obtained when a centrifugation step just before biotin affinity chromatography was eliminated. Both immuno-blotting and ELISA assays were used to test the functional status of the protein A-streptavidin chimera. By immuno-blotting, a single band of approximately 31 kd was obtained. In an ELISA assay, the intensity of color depended on the antigen concentration in a dose-dependent manner. These data suggested that our protein A-streptavidin chimera was fully functional.

The factors affecting pUC19 plasmid yield from pUC19-transformed *E. coli* cells were studied in a series of experiments. The yield of pUC19 plasmid was almost doubled ($p < 0.05$) when TB broth rather than LB broth was used to culture transformed bacteria. Culture time did not affect plasmid yield. The quality of pUC19 plasmid was not influenced by either culture media or culture time used. However, biotinylation conditions appeared to affect pUC19 fragment biotinylation efficiency. Incubation at 37 °C for 30 min seemed to increase biotinylation efficiency compared to incubation for 15 min either on ice or at room temperature. A novel membrane-based colorimetric technique for testing the efficiency of pUC19 biotinylation was described. It was very effective, especially for side by side comparison, and inexpensive and easy to perform compared to presently available procedures. Our results indicated that the efficiency of pUC19 fragment biotinylation by biotinylated primer did not appear to be superior to Klenow fragemen-catalyzed insertion of biotin-dATP. Our membrane-based colorimetric technique is useful not only for testing biotinylated DNA, but could potentially be used to test biotinylation efficiency in proteins. After developing an optimized PCR procedure, we could detect as few as 48 PCR-generated or 5 Klenow fragment-generated biotinylated pUC19 molecules in 50 µl of I-PCR reaction. Only one-sixth of the total

reaction (8 μl) was needed to detect a band on agarose gel. Based on these results, biotinylated pUC19 produced and tested using the optimized procedure may be used to enhance the sensitivity of our I-PCR assay.

I-PCR technique was used to detect BSA and IL-6 soluble antigens. I-PCR demonstrated much higher sensitivity than ELISA for the detection of these antigens. The detection limit of I-PCR for BSA was 5.8×10^1 molecules which was six orders of magnitude more sensitive than ELISA. The detection limit of immuno-PCR for IL-6 was 5.8×10^3 molecules, which was seven orders of magnitude more sensitive than that of ELISA for IL-6. These results have demonstrated that; 1) I-PCR is a more sensitive method than ELISA for the detection of a soluble antigen; and 2) our protein A-streptavidin chimera and biotinylated pUC19 were fully functional

I-PCR was also used to detect insulin receptor, EGF receptor, and H-2 D^b molecules that were expressed on the plasma membranes of murine embryos. By using I-PCR technique, we were able to detect positive signals on as few as two mouse embryos at the morula stage by using antibody to insulin receptor, and on only one mouse embryo at the morula stage by using antibody to EGF receptor. Such a high sensitivity of detecting embryo natural surface antigens has not previously been reported.

Two breakthroughs were made in studying membrane-bound antigens by I-PCR technique. The first was our decision to include denatured salmon sperm DNA to reduce non-specific binding of pUC19 to embryos. The second was to use anti-H-2 D^b antibody as both a positive and a negative, strain-specific experimental controls. These modifications increased our ability to generate meaningful experimental data with the I-PCR assay. Originally, 4×10^{-13} M of biotinylated pUC19 molecules were used in the

I-PCR for detecting membrane-bound antigens on mouse embryos, and a positive signal was never obtained. By increasing the concentration of biotinylated pUC19 from 4×10^{-13} M to 4×10^{-11} M, strong signals were generated, but meanwhile the background signal was so high that it masked any specific binding to the target antigen. In an attempt to solve this problem, we used a biotinylated pUC19 concentration of 4×10^{-11} M, but added 0.1 mg/mL sonicated salmon sperm DNA as a blocking agent in the antibody and pUC19 incubations. This change allowed us to increase biotinylated pUC19 concentration by a factor of 100 (to 4×10^{-11} M), while reducing non-specific binding of pUC19 to undetectable levels. We also used embryos from both H-2 D^b positive (C57BL/6) and H-2 D^b negative (Balb/c) strains of mice. This combination allowed us to use anti-H-2 D^b antibody as both a positive and a negative control. A positive signal was detected on the embryos of C57/BL6 mice and no signal was detected on the embryos of Balb/c mice by I-PCR assay.

I-PCR is the most sensitive technique for detecting both soluble and membrane-bound antigens. By using this technique, we obtained six orders of magnitude more sensitivity for detecting BSA molecules and seven orders of magnitude more sensitivity for detecting IL-6 molecules than using ELISA. In addition, we were able to detect signals on two morula-stage mouse embryos by using antibody against insulin receptors and on one morula-stage mouse embryo by using antibody against EGF receptors.

Preimplantation embryos secret a number of factors and molecules which may be related to their viability. Ultra-sensitive detection of these factors (such as insulin growth factor and EGF factor) and molecules (such as IL-6) may potentially contribute to animal

husbandry by increasing the success rate of embryo transfer and contribute to basic science by leaning more about gene expression.

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APPENDIX I

PROTOCOL NUMBER: A98-02-03

Course/Project Title: Production of monoclonal antibodies to H-Y antigen for use in an embryo sexing assay

PI: Jim Weber

As principal investigator/instructor on this protocol, please note that the Policies and Procedures for the Humane Care and Use of Animals require that you report at once to the IACUC any unanticipated harm to animals.

As principal investigator/instructor, I certify that I have provided an accurate description of the animal care and use protocol to be followed in the proposed project/course, and that the activities proposed do not unnecessarily duplicate previous experiments. I understand that federal and institutional regulations require that significant changes to this protocol cannot be implemented without prior IACUC approval. I assume responsibility for compliance with such regulations by all personnel involved with this protocol. If using laboratory animals, I verify that all personnel handling the animals have had a tetanus shot within the past ten years.

James A. Weber ______________________ ______________________
Signatures of Principal Investigator(s)/Instructor(s)

2/20/98 ______________________ ______________________
Date **Date**

I have reviewed and approved this protocol for animal care and use.

* Charles R Willen ______________________ 2/20/98
Signature of Department Chair or Unit Director **Date**

For Committee Use Only: Date of IACUC Receipt: 2/23/98 Date of IACUC Review: 2/23/98

Committee Action:

✓ 1. Approved until 2/23/01
Species and # of animals approved: 24 mice
___ 2. Modifications required. See attached statement.
___ 3. Disapproved. See attached statement.

Full IACUC Review: ____________ Subcommittee Review: ✓

IACUC Signatures:

Elaine E. Bean Susan Hunter ______________
[signature] [signature] ______________
[signature] ______________ ______________

PROTOCOL.DOC October 1997

APPENDIX II

PROTOCOL NUMBER: A2001-05-01[7]

Course/Project Title: Production and testing of monoclonal antibodies to cell surface antigens of bovine and murine embryos

PI: James A. Weber

As principal investigator/instructor on this protocol, please note that the Policies and Procedures for the Humane Care and Use of Animals require that you report at once to the IACUC any unanticipated harm to animals.

As principal investigator/instructor, I certify that I have provided an accurate description of the animal care and use protocol to be followed in the proposed project/course, and that the activities proposed do not unnecessarily duplicate previous experiments. I understand that federal and institutional regulations require that significant changes to this protocol cannot be implemented without prior IACUC approval. I assume responsibility for compliance with such regulations by all personnel involved with this protocol. If using laboratory animals, I verify that all personnel handling the animals have had a tetanus shot within the past ten years.

James A Weber — April 18, 2001
Signatures of Principal Investigator(s)/Instructor(s)

Date — Date

I have reviewed and approved this protocol for animal care and use.

Charles R Wellens — 4/18/01
Signature of Department Chair or Unit Director — Date

For Committee Use Only: Date of IACUC Receipt: 4/20/01 Date of IACUC Review: 5/21/01

Committee Action:

✓ 1. Approved until 5/21/04
Species and # of animals approved: 1200 mice
____ 2. Modifications required. See attached statement.
____ 3. Disapproved. See attached statement.

Full IACUC Review: _____ Subcommittee Review: ✓

IACUC Signatures:

Elaine E Bean — S J Hunter

Jayne E Longacre

PROTOCOL.DOC October 1997

APPENDIX IIII

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Dear Dr. Weber:

Per your request, we would like to make available to you the biological material described below.

UCB Registry Number: #127
Laboratory Designation: pTSAPA-2
Description: Plasmid pTSAPA-2 carries the gene fusion of streptavidin and two IgG-binding domains of protein A under the bacteriophage T7 *Φ10* promoter. The encoded streptavidin-protein A chimeric protein can be expressed in *Escherichia coli* by using T7 expression system, which has been patented by Associated Universities, Inc. (Patent Number; 4,952,496).
Sano, T. and Cantor, C. R. (1991) *BioTechnology* 9, 1378-1381.

The Biological Material is provided for non-clinical, non-commercial research purposes. Please do not distribute the Biological Material, including any progeny or any genetically engineered modification, which is substantially based on and incorporates an essential element of the Biological Material, to any other individual or entity without our prior consent.

Because the Biological Material is experimental in nature, please note that it is provided without any warranties, such as merchantability or fitness for a particular purpose, and the University of California or its employees have no liability in connection with its use.

If you agree with the above, please sign and return a copy of this letter to us for our laboratory records and we will promptly ship the material to you.

Sincerely yours,

Takeshi Sano/ Charles R. Cantor

AGREED AND ACCEPTED:

James A Weber Charles R Wallace May 20, 1998
Signature Date

James A. Weber Charles R Wallace - Chair, Department of Biosystems Science and Engineering, U Maine.
Print name

This Letter Agreement is recommended by the Office of Technology Licensing, University of California at Berkeley, for distribution of Biological Materials to scientific colleagues at non-profit scientific organizations.

BIOGRAPHY OF THE AUTHOR

Kun Xu was born in July 13, 1958 in Nehe County, Heilongjiang Province, P. R. China. He spent his early years in the remote countryside where he received his elementary to high school education in public schools. After high school, he worked as a farmer in a local Commune Production Team for more than two and a half years.

He entered Heilongjiang August-First Agricultural University in 1978, and obtained his B.S. degree in animal science in 1982. Upon completion of his undergraduate education, he worked in the Animal Husbandry Bureau on Len Bei state farm and technically supervised overall animal production. In November of 1985, he was accepted into the graduate program in The Western College of Veterinary Medicine, University of Saskatchewan, and completed his M. Sc. Degree in January, 1988. After the completion of his graduate study, he went back to the Peoples' Republic of China. He did research on bovine embryo fast freezing technology in the Animal Breeding and Technical Improvement Division of Heilongjiang Animal Breeding and Guiding Station, which is the largest cattle breeding station in Asia. Due to his outstanding performance, he was promoted to be senior scientist in 1989, a member of the Board of Science and Technology Committee in 1990, a member of the Board of Heilongjiang Animal Husbandry and Veterinary Committee in 1991, vice-division chief of the Animal Breeding and Technical Improvement Division in 1992 and division chief in 1993. Although he was extremely busy with management, he still remained active in science and research. In 1994, he became the youngest recipient of the Heilongjiang Academy of Science Award.

In October 1995, he enrolled for graduate study in the Department of Biosystems Science and Engineering at the University of Maine and worked as a Research Assistant in this Department. He is a candidate for the Doctor of Philosophy degree in Biochemistry and Molecular Biology from the University of Maine in December, 2001.