1. Introduction

1.1 Prostate gland

In humans, prostate development occurs during the second and third trimester and is complete at the time of birth (Lowsley, 1912). The prostate sits under the bladder and is located at the base of the penis and in front of the anus. The structure of the prostate is that it has a narrow hole through the middle. It is fashioned this way as the urethra, the tube which empties urine from the bladder passes through this hole on its way to the end of the penis (The Cancer Council Victoria, 2007). The gland enlarges continuously in size to reach the adult weight of approximately 20 g by 25–30 years of age (Figure 1.1).

Urinary bladder
Seminal vesicle
Prostate
Seminal colliculus (verumontanum)
Prostatic utricle
Opening of ejaculatory duct
Urogenital diaphragm
Bulbourethral (Cowper's) gland
Opening of bulbourethral duct
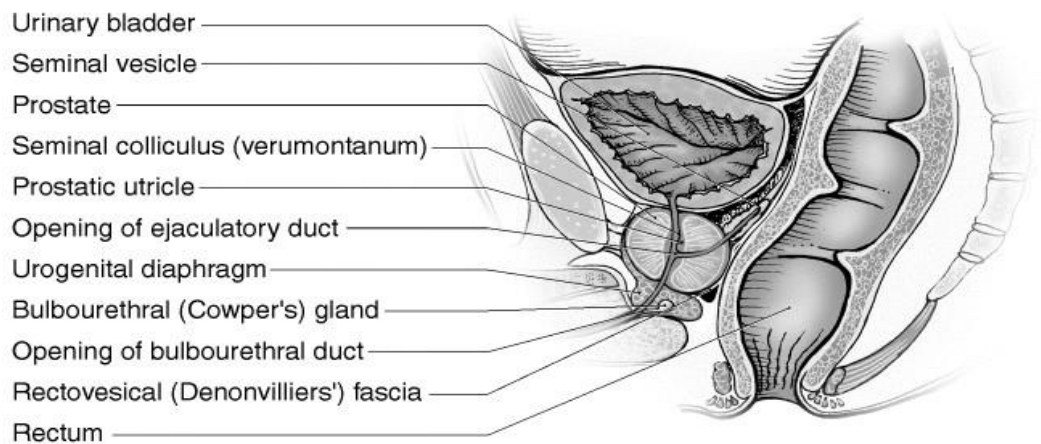Rectovesical (Denonvilliers') fascia
Rectum

Figure 1.1: Normal prostate anatomy (Robert et al., 2000)

Similarly to the breast, the prostate is a gland which produces and secretes fluids. The prostate also controls the flow of these fluids. Semen which was ejaculated from the penis during sexual climax (orgasm) was produced by the cells which line the prostate gland. The prostate also produces some of the fluid in the semen. The prostate also helps separate the urine from the semen. The way the semen is found to be in the liquid form was because of the prostate specific antigen (PSA). PSA is an enzyme, which is produced primarily by cells lining the ducts and the acini of the prostate gland. The PSA is found in the form of a glycoprotein (Strax, 2008).

The prostate overall structure however is the greatest setback. The wrap around structure around the urethra becomes problematic if the prostate swells or enlarges. Enlargement of the prostate could occur in man of all ages and could be cause by prostate infection, commonly known as prostatitis. It could also be caused by benign growth. In both cases, it will cause pain, discomfort and problems in urination (Strax, 2008).

1.2 Prostate Diseases

Generally the occurrence of prostate associated problems increase with ages. Symptoms or problems of the following are relatively common.

- Difficulty in getting the urinary flow started, especially when a person is in a hurry
- Difficulty stopping the urine flow
- Requires longer time, due to the urine stream is weak, or it stops and starts
- Dribbling of urine after a person have finished
- Frequency of going to the toilet throughout the day, even though there is not much urine to pass

- Getting up at night to go to the toilet, a person used to sleep through.

- Needing to go urgently at all times

- An unfinished feeling or that there is need to go again repeatedly, although nothing is produced

- Pain or a burning sensation when urinating

- Blood in the urine (The Cancer Council Victoria, 2007)

Due to the structure of the prostate (Strax, 2008) the prostate will be a source of three of the major health problems which affects men. The following are the major prostate disease:

- Benign prostatic hyperplasia (BPH); commonly known as the prostate enlargement. It is one of the most common benign tumors in men

- Prostatitis; the prostate inflammation and the most common cause of urinary tract infections in men. This is a highly painful condition

- Prostate cancer, the most common cancer in men (Walsh and Worthington, 2002).

BPH is commonly found in most men at the age of sixty or older. BPH is a non-malignant enlargement of the prostate effecting about 50% of men of that particular age category. This enlargement however is normal, and is caused by aging and is rarely life threatening but may produce up-setting symptoms (Rubenstein and McVary, 2008). BPH is not prostate cancer, as BPH and prostate cancer are two separate diseases which are formed in different parts of the prostate. Having developed BPH does not indicate that a man is more or less likely to acquire prostate cancer. The difference in prostate cancer and BPH is that prostate cancer initiates at the outer, marginal zone of the prostate and is growing outward and invades tissues surrounding it and that is primarily

the reason that it seldom produces symptoms until it is far advanced. Meanwhile BPH on the other hand begins in a small area of the inner prostate known as transition zone which is a ring of tissues that make a natural circle surrounding the urethra. While prostate cancer grows outwards, BPH grow inwards toward the prostate's core and constantly tightening around the urethra and interfering with urination, which provides many symptoms. BPH is a very common condition and it is not cancerous, but it can mimic cancer (Walsh and Worthington, 2002). BPH can be treated with medications or surgery. The medications will be able to alter hormone levels, or can relax or reduce the size of the prostate, hence reducing the pressure on the urethra. Medication may take time like months before the condition improves. Surgery procedures to remove part of the prostate could be taken to stop the squeezing of the urethra. Laser and microwave treatments could also be applied. (The Cancer Council Victoria, 2007).

Prostatitis in men is the most prevalent cause of urinary tract infection. It is anticipated that a quarter of all men who visits a doctor for urological problems have tell tale signs of prostatitis. In general, there are four conditions, which are categorized as "prostatitis". The first, two acute and chronic bacterial prostatitis, as the name suggest, are caused by bacteria and are not common but easily treated. These two prostatitis are accompanied by symptoms such as fever, chills, severe burning on urination, increased frequency of urination, and, in certain cases, a life-threatening infection in the bloodstream.

The third form of prostatitis is called chronic prostatitis or chronic pelvic pain syndrome. The actual causes these forms of prostatitis are not known and medications like antibiotics do not help at all. Medications are mainly targeted at relieving symptoms, with muscle relaxants such as alpha-blockers and other drugs, which ease muscle tension in the prostate and make urination easier. The other form of prostatitis is called the asymptomatic inflammatory prostatitis, which produces no symptoms and is

usually found by chance, when inflammatory cells are found in the prostatic fluid or inflammation is detected on a prostate biopsy. Again, in the case of prostatitis, it is not a cancer (Walsh and Worthington, 2002), treatment is with the help of antibiotics and consumption of medication may need to persist for several months. Some men may also need surgery to stop the swollen prostate from pressing on the urethra (The Cancer Council Victoria, 2007).

For the detection of those prostate diseases, the doctor normally collect a patient's urine and thoroughly exam his prostate gland. To check the prostate gland, the doctor undergoes digital rectal examination, which involves inserting a well-lubricated gloved finger into the rectum to check for any abnormalities of the gland. Meanwhile prostate specific membrane antigen (PSMA) test could also be carried out where a blood tests to look for this glycoprotein compound in the blood, which is produced by the prostate. Once there is a high level of detection it could be probable be prostate cancer but the doctor needs to carry out more tests to be certain. A biopsy could be carried out once PSMA test or digital rectal examination is abnormal. This involves an ultrasound probe being placed in the rectum to find areas of the prostate which are abnormal. Samples (usually six or more) of the prostate are subsequently taken and sent to be observed under the microscope. This procedure can be certain to diagnose if one has prostate cancer. The biopsy will also provide insights of how fast the cancer may develop and how much of a threat it may be (The Cancer Council Victoria, 2007).

1.3 Prostate Cancer

1.3.1 History of Prostate Cancer

The history of prostate cancer spans for about 200 years from the recognition of the disease to the development of the three primary types of treatment which are surgery, radiation and hormonal manipulation. Random cases of prostate cancer were noted by physician as way back as 1817. In the early days, histological examination was not yet in clinical use and hence, diagnoses were made at autopsies. Later on in 1853, the first case of prostate cancer established by histological examination was reported by Adams, a surgeon at The London Hospital, where a 59 year old man with a scirrhous (hard and firm) tumor at the prostate gland. The prostate cancer largely unrecognized until the turn of the last century, when prostatic adenectomy for urinary obstruction became widely performed resulting in more specimen available for histological examination.

In the year 1898, Fuller performed complete prostate removal together with a bladder neck resection and then patient bladder function recovered. After the discovery of X-ray and its applications, in 1909 Minet from Paris placed a radium tube in a catheter to irradiate prostate cancer and subsequently in 1913 Pasteau and Degrais reported a 3 year cure using this method. In the early 1900s experiments in animals established the relationship of the pituitary and testis as well as their effects on the prostate and this sparked the beginning of the research on the treatment through hormonal manipulation. All these studies subsequently lead to the major discovery of the dramatic effects of castration and estrogen administration on prostate cancer cells in 1941 by Huggins at the University of Chicago. During the period of 50 years, Huggins reported more than 230 scientific articles, mostly on the effects of hormones on cancer, with emphasis on the prostate and breast.

In 1966 he shared the Nobel Prize in physiology and medicine with Rous, who developed the first virally induced solid tumor in animals, called the Rous-chicken sarcoma (Lytton, 2001).

1.3.2 Definition of Prostate Cancer

Prostate cancer is a form of cancer that develops in the prostate. The cells are known to mutate and there is uncontrollable growth. Prostate cancer is the primary cause of cancer death and is the most common major cancer in men. A common fact is noted that when prostate cancer is small and curable, it is often also silent where it produces no tell tale signs. Hence, routine testing is very important where the cancer is detected as early as possible and once if it is detected too late, the prostate cancer can be often lethal and the symptoms are often painful if the disease is allowed to run its course. However if it is detected early prior to the cancer cells spreading beyond the wall of the prostate, prostate cancer can be cured with surgery or radiation (Walsh and Worthington, 2002).

In some cases, some men with small and slow-growing tumors, a process called expectant management which means following the disease closely may be a safer option. With the advances of technologies, treatment and successful rate of curing prostate cancer is better and with fewer side effects. However, although in some cases the cancers are incurable, the metastasis of the cancer cells can be contained with the advancement in the medical technology.

In prostate cancer the key to a better chance of recovery is as follows:

- Prevention—to ward off prostate cancer entirely, or at least delay its onset for decades.

- Earlier diagnosis—with the help of highly sensitive tests and sophisticated models for analyzing the results, detecting prostate cancer at the earliest and most curable stages yet.

- Better treatment for localized disease—expanding and refining effective treatments, and working to minimize side effects even further.

- Better control of advanced disease. (Walsh and Worthington, 2002).

1.3.3 Genetics of cancer

Similar to other cancers, prostate cancer also develops due to the consequences of genetic changes. There are quite a number of putative genes that have been isolated for the development of breast, ovarian (*BRCA1, BRCA2*) and colon cancer (*hMLH1, hMSH2*) but however, the etiology and pathogenesis of prostate cancer continues to be largely unknown. This is also due to the fact that prostate cancer is such a heterogeneous disease. The diversity of prostate cancer with the stepwise transition from benign cells through prostatic intraepithelial neoplasia (PIN), invasive carcinoma, the development of metastases to hormone refractory disease make this an interesting disease to study but also a difficult disease to cure. Many works have been published on clinical prognostic factors but with advanced development in molecular genetics, the genetic code analysis that will in the future give both prognostic information and targets for therapeutic intervention.

At present, the available prognostic factors have recently been ranked by a multidisciplinary group of clinicians, pathologists and statisticians and they are divided in three categories as follows:

- Category I: Factors of prognostic importance and are used frequently on a daily basis in patient management. These are inclusive of the preoperative prostate specific antigen (PSA), TNM stage, Gleason grade and surgical margin status.

- Category II: Factors that have been comprehensively studied but the importance remains to be validated statistically, e.g. tumor volume, histological type and DNA ploidy.

- Category III: All factors insufficiently studied to demonstrate their prognostic value: examples would be oncogenes, tumor suppressor genes and apoptosis genes, as well as perineural invasion, neuroendocrine differentiation microvessel density, nuclear roundness and chromatin texture (Bott et al., 2003).

1.3.4 Signs and symptoms

Prostate cancer could be identified with three different stages namely the early-stage disease, locally advanced disease and advanced disease. In the early-stage disease, patient with organ-confined prostate cancer most commonly without symptoms while patient with a large component of benign prostatic hyperplasia could be identified with bladder outlet obstruction unrelated to prostate cancer. In the case of locally advanced disease, bladder outlet obstruction is mainly the sign of locally advanced prostate cancer however there are few cases with locally advanced disease present with hematuria, urinary tract infections, and irritative voiding symptoms secondary to bladder outlet obstruction. Lastly, in the case of advanced disease there are rare instances where

patient with bulky lymph node metastasis may present with bilateral lower-extremity edema (Moul et al., 2008).

1.3.5 Screening and diagnosis

Prostate cancer could be screened using prostate specific membrane antigen (PSMA) and digital rectal examination (DRE). With such screening, this has increases the prostate cancer detection. With this, prostate cancer is detected at earlier stages, when they are potentially curable. Most prostate cancers were undetected until it produces local symptoms or distant metastases. The discovery at this stage is often too late and incurable.

The digital rectal examination could possible detect nodularity or induration, and this is followed by a biopsy. Even with the detection of such irregularities using DRE, from the subsequent biopsy procedure, it only leads to a diagnosis of prostate cancer in only 15% to 25% of cases. Although such DRE detection is neither accurate nor sensitive for prostate cancer detection, it is however that abnormal DRE is associated with a fivefold increased risk of cancer present at the time of screening. PSA is a serine protease produced by the prostatic epithelium and secreted in large quantities through the seminal fluid. PSA level in the serum could be increased through inflammation of the prostate, urinary retention, prostatic infection, benign prostatic hyperplasia, prostate cancer, and prostatic manipulation. The overall sensitivity for PSA levels ranges from 50% to 70% but this depends on the threshold used, and it is not as specific, and in addition, it does not allow for differentiation between indolent and aggressive disease. A more worthwhile approach for PSA screening may be to use the rate of rise in PSA (PSA velocity) in combination with the absolute PSA value. This approach has been

shown to be useful recently in the form of age-adjusted PSA velocity, but accepted guidelines are still controversial (Moul et al., 2008).

1.3.6 Prognosis

The most common and widely used staging system is the TNM system (Table 1.1). For this system, T1 and T2 are tumors, which are limited to the gland while T3 and T4 are the tumor having a local extension.

Table 1.1: TNM staging system of prostate cancer.

Localised disease	
T1a	Tumor incidental histologic finding in $\leq$ 5% of resected tissue: not palpable
T1b	Tumor incidental histologic finding in $\geq$ 5% of resected tissue
T1c	Tumor identified by needle biopsy (e.g. because of elevated PSA level)
T2a	Tumor involves one-half of one lobe or less
T2b	Tumor involves more than one-half of one lobe but not both lobes
T2c	Tumor involves both lobes
Local extension	
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Bladder invasion, fixed to pelvic side wall, or invasion of adjacent structures
Metastasic disease	
N1	Positive regional lymph nodes
M1	Distant metastasis

The management of patients with prostate cancer differs widely and is highly reliant upon the patient's age, overall health, and tumor risk assessment. The disease development process can be varied; it can be incidental that will not result to cancer-specific mortality, to a case of very aggressive, resulting in early widespread metastatic disease and death.

Patients with a low Gleason score (2– 4) that has clinically localized prostate cancer treated conservatively (observation or hormonal therapy alone), have a small risk of death from their cancer within 15 years (4%–7%). Meanwhile, those with poorly differentiated tumors (Gleason score 8–10), have a higher risk of death cause by prostate cancer than of any other causes, even when the cancer is diagnosed in the eighth decade of life. Besides that, if a man prior to sixty years old diagnosed with a clinically localized prostate cancer with a Gleason score of 8 to 10 and is left untreated, that patient has an 87% risk of dying of the disease within 15 years (Moul et al., 2008).

1.4 Prostate specific membrane antigen (PSMA)

1.4.1 Structure of PSMA

The PSMA gene consists of 19 exons that span ~60 kb of genomic DNA. This gene encodes a type II transmembrane protein with a short NH2-terminal cytoplasmic tail (19 amino acids), a single hydrophobic trans-membrane domain (24 amino acids), and a large extracellular domain (707 amino acids) at the COOH terminus (Figure.1.2), (Israeli et al., 1993; O'Keefe et al., 1998). The extracellular domain of PSMA is highly glycosylated, with linked oligosaccharides accounting for up to 25% of the molecular weight of the native protein (Holmes et al., 1996). Regions within this domain share modest degrees of homology with the transferrin receptor (TfR) (Israeli et al., 1993), and with members of the M28 family of cocatalytic aminopeptidases (Rawlings et al., 1997). Although the TfR has only a vestigial catalytic site, PSMA is known to possess both N-acetylated, α-linked acidic dipeptidase (NAALADase) and folate hydrolase (FOLH) activities (Pinto et al., 1996).
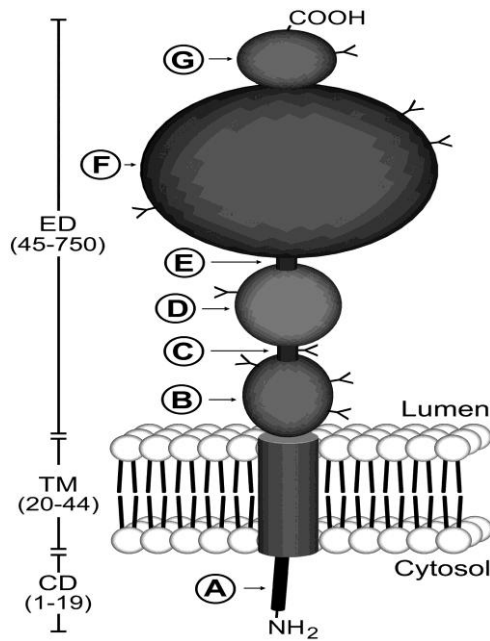
Figure 1.2: Schematic diagram of prostate-specific membrane antigen (PSMA) structure. PSMA is a type II transmembrane protein with a short NH_2-terminal cytoplasmic domain (CD), a hydrophobic transmembrane region (TM), and a large extracellular domain (ED). The CD contains an endocytic targeting motif and filamin A (FLNa) binding site (A). The large ED is highly glycosylated with nine predicted *N*-glycosylation sites (Y). The ED contains two domains of unknown function that span amino acid residues 44–150 (B) and 151–274 (D), proline- and glycine-rich regions that span amino acid residues 145–172 and 249–273, respectively (C and E), a catalytic domain that spans amino acid residues 274–587 (F), and a final domain of unknown function (amino acids 587–750) to which a helical dimerization domain (amino acids 601–750) is localized (G) (Israeli et al., 1993; O'Keefe et al., 1998).

These two related peptidase activities hydrolyze γ-peptide bonds between *N*-acetylaspartate and glutamate in the abundant neuropeptide *N*-acetylaspartylglutamate (NAAG) and the γ-glutamyl linkages in pteroylpolyglutamate, respectively. Thus this enzyme has been referred to alternatively as glutamate carboxypeptidase II (GCP-II) and folate hydrolase 1 (FOLH1). The enzymatic activity of PSMA is largely inhibited by phosphate, even at millimolar concentrations (Slusher et al., 1999), and is dependent on glycosylation and dimerization for proper function (Ghosh et al., 2003). In contrast to the large extracellular domain, the cytoplasmic tail of PSMA consists of just 19 amino acids. In spite of its diminutive stature, the cytoplasmic domain interacts with a number

of proteins and has a major impact on the localization and molecular properties of PSMA (Anilkumar et al., 2003).

1.4.2 Dimerization of PSMA

Homodimerization is a fundamental feature of many transmembrane receptors. Induction of homodimer formation is often induced by ligand binding, which is in turn necessary for mediating the cellular response of the receptor (Schlessinger et al., 2002). The TfR is an archetypal example of one such receptor. This type II transmembrane protein is involved in regulating cellular iron homeostasis through binding and internalization of iron-laden transferrin (Aisen et al., 2004). PSMA shares homology with the TfR at the levels of both amino acid identity and domain organization (Mahadevan et al., 1999).

Like the TfR, PSMA is expressed as a noncovalently linked homodimer on the cell surface (Lawrence et al., 1999; Schülke et al., 2003). This dimerization is apparently mediated by epitopes within the large extracellular domain, because truncated versions of PSMA lacking the cytoplasmic and transmembrane domains are still capable of interacting. PSMA dimerization is critical to maintaining the conformation and enzymatic activity of PSMA (Schülke et al., 2003). Although the possibility has yet to be addressed fully, the similarity between PSMA and TfR at the amino acid and structural levels, combined with the common dimerization requirement, may suggest that these proteins share similar receptor and ligand transport functions.

1.4.3 Potential role of PSMA enzyme activity

The prostate gland is composed mainly of stromal, epithelial, and neuroendocrine cells. The dynamic balance of cell proliferation, differentiation, and apoptosis in general maintains the cellular and tissue homeostasis. This balance is generated by the continuous cross talk among these cell populations (Sung et al., 2002). For this purpose, epithelial and stromal cells secrete various types of growth factors, chemokines, and neuropeptides (Wong & Wang, 2000). Deregulation in this paracrine communication can result in derangement of the prostate gland, such as benign prostate hyperplasia and prostate carcinoma (Dawson et al., 2004). For example, the peptidase NEP normally acts to inhibit the migratory properties of prostate epithelial cells. NEP achieves the inhibition of prostatic epithelial cell migration by cleaving critical neuropeptides such as bombesin and endothelin and thereby prevents the relay of signal transduction mediated by G protein-coupled receptors (Sumitomo et al., 2000). Like NEP, PSMA is also a type II transmembrane glycoprotein with cocatalytic metallopeptidase activity. The increased expression of PSMA in prostatic adenocarcinoma may indicate a role in the cleavage of signaling molecules involved in maintaining prostate gland architecture and function. The overexpression of PSMA could potentially disturb the growth balance of the prostate gland.

1.4.4 Genomic properties

PSMA expression in prostate cancer is significantly higher than in benign prostate hyperplasia or the normal prostate and is greater in prostate cancer with a higher Gleason score (Marchal and Ghosh et al., 2004), suggesting that the regulatory elements controlling PSMA expression become more active as prostate cancer progresses.

There are at least three PSMA variants produced by alternative splicing: PSM', PSM-C, and PSM-D. Interestingly, the ratio of full-length PSMA to variant PSM', which lacks nucleotides 114 to 380 of the PSMA cDNA and, as a result, is located in cytoplasm, increases as normal prostate progresses to the tumor (Su et al., 1995).

To date, two transcription regulatory elements have been characterized: the 1.2-kb promoter upstream of the PSMA-encoding gene (FOLH1) and the PSMA enhancer core (PSME) in the third intron of FOLH1. PSME exhibits high activity only in PSMA-positive LNCaP and C4-2 cells; very low activity in PC-3 cells; and no activity in other PSMA-negative cells tested (Watt et al., 2001, Lee et al., 2002). Consistent with PSMA expression, PSME is also negatively regulated by androgens, so it exhibits a much higher activity at low levels or in the absence of androgens (Wright et al., 1996, Watt et al., 2001), making PSME a strong candidate for mediating virus replication or expressing exogenous cytotoxic genes in hormone-refractory prostate cancer.

1.5 Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a selective amplification of a particular region or fragment of a DNA molecule. In order for a PCR to be carried out the border sequence of the region must be known. This is because of two short oligonucleotide flanking the fragment of interest is need for the amplification and this short oligonucleotide is known as primers. The primers delimit the region that would be amplified. Amplification is normally being conducted using DNA I polymerase enzyme from *Thermus aquaticus*. *Taq* polymerase the enzyme from *Thermus aquaticus* is resistant to heat denaturation.

The overall procedure begins with the addition of enzyme to the primed DNA template and incubated to produce new complementary strands. This is the heated to 94 °C so the newly synthesize strand will separate and this is subsequently cooled to let more primers to hybridize the respective position. This process of denaturation, hybridization and synthesis is repeated 25 to 30 times resulting hundreds of millions of copies amplified (Brown, 2002)

1.6 Overlap extension PCR

Overlap extension polymerase chain reaction is a type of PCR, which helps to produce polynucleotides from smaller fragments (Higuchi et al., 1988).

The following diagram is an overview of the overlap PCR:
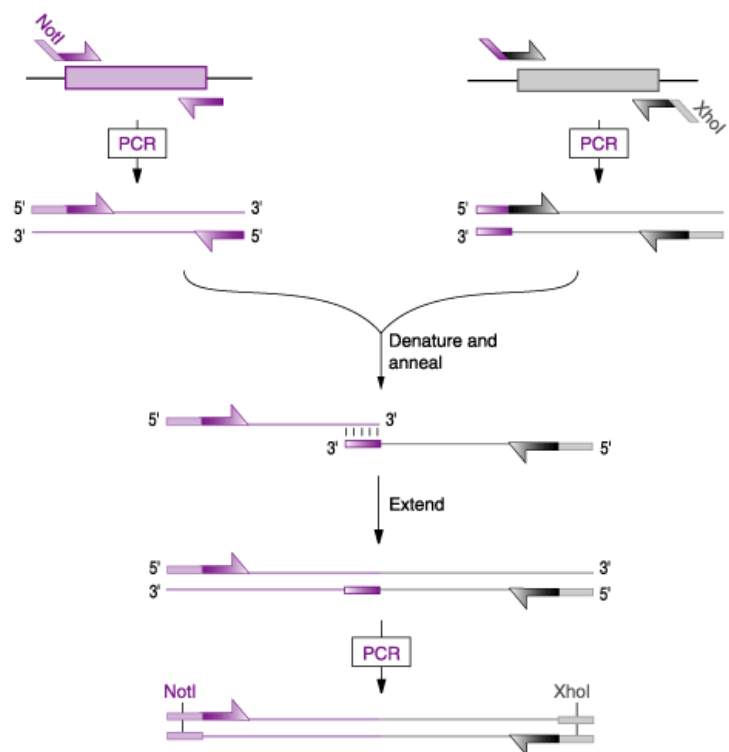
Figure 1.3: The process of overlap extension polymerase chain reaction.

PCR was used to amplify the smaller fragments of the polynucleotide that was to be formed. When PCR for the carried out, the primers used to amplify one of the fragment should by synthesis with part of the sequence of the other fragment, hence there would be part of an overlapping region. Subsequently, both of these products were used as a template for a PCR. The fragments are denatured and they anneal at the overlapping regions. This combination of fragment was subsequently extended. The complete polynucleotide was then used as a template for another PCR reaction to amplify this complete polynucleotide (Miesfeld, 2001).

1.7 TA Cloning

The procedure of cloning DNA fragment into a plasmid vector is a regular procedure in recombinant DNA technology. It is known that cloning methods can be divided into two main classes, depending on if ligase is use. Among the two methods, commonly used method for cloning is the method that requires the use of DNA ligase to link the compatible ends of the DNA fragment and the linearized plasmid, forming a single cyclic molecule that is capable of autonomous replication in host cells.

TA cloning is among the easiest and most effective way of the cloning of PCR products as this approach takes advantage of the terminal transferase activity of certain thermophilic DNA polymerases, like for example the *Thermus aquaticus* (*Taq*) polymerase. *Taq* polymerase is known to have non-template dependent activity, which adds a single adenosine to the 3'-ends of a double stranded DNA molecule. With this, the molecules PCR amplified by *Taq* polymerase contains single 3'-A overhangs. With this, the use of a linearized "T-vector" which has single 3'-T overhangs on both ends permits direct, high-efficiency cloning of PCR products which is facilitated by being complement between the PCR product 3'-A overhangs and vector 3'-T overhangs.

This strategy is commonly referred to as "TA cloning." This strategy is rather simple and much efficient than blunt-ended ligation for the cloning of PCR products (Zhou and Sanchez, 2000).

1.8 Expression system

The need to have functional studies like protein-protein interaction experiments, enzyme kinetics studies, functional studies of the protein, structural studies like protein crystallization and protein structure study and as well as production of antibodies for further experiments has lead to the expression recombinant protein. There are two main systems for the expression of recombinant protein. The two systems are the prokaryotic (bacterial) or eukaryotic (usually yeast or mammalian cell) system.

1.8.1. Bacterial expression system

The bacterial expression system is the use of prokaryotic cells as an expression system. *Escherichia coli,* a prokaryotic cell is among the most popular hosts in the production of recombinant proteins. The *E. coli* is in favour because of its simplicity, safety, and known genetic properties, which is a major asset. The capability of transformation of *E. coli* with foreign DNA is an easy with well-established genetic manipulation method. With the advantage of fast propagation, generations of stable cell lines are a quick process. The most important advantage of *E. coli,* however, is its capability to produce proteins in large amount and to grow very quickly in comparison to other cell lines like mammalian cells.

However, the application of *E. coli* for production of complex molecules like heterodimers, molecules containing complex disulfide bonds, or glycosylated proteins is still a problem. Besides that, over expression of recombinant genes often results in formation of inactive protein aggregates (inclusion bodies) from which biologically active proteins can only be obtained through complicated and costly denaturation–refolding processes. The other major setback of the usage of E. coli as an expression system is difficulty in recovering substantial yields of correctly folded proteins (Leonhartsberger, 2006).

1. 8.2 *Pichia Pastoris* expression system

The expression systems foreign gene by yeasts is known to be efficient and economical and becomes a source of a different of higher eukaryotic proteins which are important academically and commercially. Yeasts has both the microbial growth and genetic manipulation advantages of *E. coli* and an eukaryotic environment which allows many eukaryote-specific posttranslational protein modifications such as proteolytic processing, folding, disulfide bridge formation, and glycosylation. *E. coli* has the ability to produce eukaryotic foreign proteins at high levels; however with the absence of these eukaryotic post transcriptional modifications, this causes the proteins to be insoluble and inactive. This is why, for eukaryotic proteins that are needed in a biologically active and/or native form, in *vitro* refolding procedures have proven to be inefficient, hence a eukaryotic expression system is much desirable.

The *Pichia pastoris* which is methylotrophic yeast has two key advantages as a host for the production of foreign proteins. The first is the promoter used to transcribe foreign genes that is derived from the methanol-regulated *P. pastoris* alcohol oxidase I gene (*AOX 1*). This is where cells that are exposed to methanol as the sole carbon

source, transcription initiation at the *AOX 1* promoter *(AOX l p)* is highly efficient and comparable to that of promoters derived from highly expressed glycolytic pathway genes. The second key advantage of *P. pastoris* is that it is not a strong fermenter, which is beneficial as yeast fermentation, generates ethanol, where in high-density cultures; it can rapidly build to toxic levels. Besides that, in the case of secreted proteins, the concentration of a foreign protein in the medium is roughly proportional to the concentration of cells, hence a high-cell density fermenter culture is need and *P. pastoris* expression strains are relatively easy to culture at cell densities of -100 g/liter, dry cell weight, or more. (Cregg, 1999).

1.9 Objectives

This project is a continuation as a part of research project started by previous student. Due to the large size of this particular *PSMA* gene, it was divided into three fragments namely G1, G2 and G3. In the previous work, G2 and G3 have been constructed. The G1 fragment of *PSMA* gene failed to be joined, hence six sub-fragments namely fragment a, b, c, d, e, and f need to be overlapped in order to obtain G1 fragment. So in order to obtain the full length of this gene, these fragments need to be overlapped.

The project primarily aims would be:

1. Construction of the G1 fragment of the *PSMA* gene.

2. Amplification and overlapping of G1, G2 and G3 fragments of *PSMA* gene.

3. Construction the full length of *PSMA* gene and amplification.

4. Cloning of the *PSMA* gene in the *E. coli*.

5. Expression of *PSMA* gene in the *Pichia Pastoris* recombinant expression system.

2. Materials & Methods:

2.1 Overlapping PCR

For overlapping PCR, a master mix containing its reagent was carried out in a 1.5 ml microcentrifuge. Overlapping PCR was carried out in a 25 μl reaction containing $1\times$ MgSO$_4$ free *Pfu* buffer (Fermentas, Canada), 1.5 mM MgSO4, 200 μM per each dNTP (Fermentas, Lithuaya) (dATP, dTTP, dCTP, and dGTP), 1.0 to1.5 unit of *Pfu* polymerase (Fermentas, Canada), autoclaved distilled water, and ~100 ng of template DNA.

2.1.1 Optimization of overlapping PCR

The quantity of the *Pfu* polymerase enzyme used ranged from 1 unit to 1.5 units for one reaction. There were two PCR programs used in this project, each program was used based on the fragment size.

2.1.1.1 Fragment less than 1500bp

Fragment under the size of 1500bp was overlapped under the following conditions using the PCR machine (Peltier Thermal Cycler MJ Research): samples were subjected to 16 cycles of denaturation at 95ºC for 3 min, annealing at 60ºC for 1 minute, extension at 72ºC for 1 minute, and a final cycle of elongation for 5 minute at 72ºC.

2.1.1.2 Fragment more than 1500bp

Fragment over the size of 1500bp was overlapped under the following conditions using the PCR machine (Peltier Thermal Cycler MJ Research and PCR system 2400): samples were subjected to 16 cycles of denaturation at 95ºC for 3 minute, annealing at 60ºC for 1 minute, extension at 72ºC for 10 minute, and a final cycle of elongation for 10 minute at 72 ºC.

2.1.2 PCR amplification

For PCR amplification, a master mix containing its reagent was carried out in a 1.5 ml microcentrifuge. PCR amplification was carried out in a 25 µl reaction containing 1× MgCl free *Taq* buffer (EURx , Poland), 1.5 mM $MgCl_2$, 200 µM per each dNTP (Fermentas, Lithuaya) (dATP, dTTP, dCTP, and dGTP), 0.4 µM for each primer, 1-2 unit of *Taq* polymerase (EURx , Poland), autoclaved distilled water, and 2 µl of overlapping PCR product.

2.1.2.1 Optimization of PCR

The quantity of the *Taq* polymerase enzyme used ranged from 1 unit to 2 units for one reaction. The following are the primers combination and the fragments amplified.

Fragment	Primers	Primers Sequence
G1	SfiPSMA-F PSMA-961R	5'-cttcgggcccagccggccgatgtgcaatctccttcacgaaac-3' 5'-gaagattccaaccatctggataggacttcac-3'
G2	PSMA-961F PSMA-1741R	5'-tccagatggttggaatcttcctggaggtggt-3' 5'-gccactgaactctggggaaggactttttta-3'
G3	PSMA-800F PSMAfacXa-Not1R	5'-cttccccagagttcagtggcatgcccaggat-3' 5'-agctggcggccgcgcggccttcaatggctacttcactcaaa-3'
G1-G2	SfiPSMA-F PSMA-1741R	5'-cttcgggcccagccggccgatgtgcaatctccttcacgaaac-3' 5'-gccactgaactctggggaaggactttttta-3'
G2-G3	PSMA-961F PSMAfacXa-Not1R	5'-tccagatggttggaatcttcctggaggtggt-3' 5'-agctggcggccgcgcggccttcaatggctacttcactcaaa-3'
G1-G2-G3	SfiPSMA-F PSMAfacXa-Not1R	5'-cttcgggcccagccggccgatgtgcaatctccttcacgaaac-3' 5'-agctggcggccgcgcggccttcaatggctacttcactcaaa-3'

Table 2.1: Primer combination and the amplified products

There were two PCR programs used in this project, each program was used was based on the fragment size.

2.1.2.1.1 Fragments more than 1500bp

Fragments more than 1500bp was amplified using the following conditions in the PCR machine (PCR system 2400): the samples were subjected to 35 cycles of denaturation at 95 ℃ for 3 minutes, annealing at 50 ℃ for 1 minutes, extension at 72 ℃ for 2 minutes 30 seconds, and a final cycle of elongation for 5 minutes at 72 ℃.

Or amplification was performed under the following conditions in the PCR machine (PCR system 2400): the samples were subjected to 35 cycles of denaturation at 95 ℃ for 3 minutes, annealing at 55 ℃ for 1 minutes, extension at 72 ℃ for 2 minutes 30seconds , and a final cycle of elongation for 5 minutes at 72 ℃.

2.1.2.1.2 Fragment less than 1500bp

Fragments less than 1500bp was amplified using the following conditions in the PCR machine (Peltier Thermal Cycler MJ Research): the samples were subjected to 32 cycles of denaturation at 95 ℃ for 3 minutes, annealing at 55 ℃ for 45 seconds, extension at 72 ℃ for 1 minutes, and a final cycle of elongation for 5 minutes at 72 ℃.

Or amplification was performed under the following conditions in the PCR machine (Peltier Thermal Cycler MJ Research): the samples were subjected to 35 cycles of denaturation at 95 ℃ for 3 minutes, annealing at 60 ℃ for 45 seconds, extension at 72 ℃ for 1 minutes and 30 seconds, and a final cycle of elongation for 5 minutes at 72 ℃.

2.2 Gel electrophoresis

A 1% agarose gel was prepared mixing agarose powder (Vivantis, USA) and TBE Buffer (89 mM tris base, 89 mM boric acid, 2 mM EDTA) and was subsequently boiled using the microwave until all agarose powder was fully dissolved. The molten agarose was cooled down until it is only warm to touch and then Ethidium bromide was added in and mixed with the solution. The molten agarose was then poured into the mould and caster to be further cooled down and to let it solidify. Once solidify, the gel was immersed in TBE buffer in the gel electrophoresis tank. PCR sample was then mixed with 6× loading dye at the ratio of 5 units of samples to 1 unit of dye. Each sample was loaded in the wells separately. A DNA marker 100bp (seegene, Korea), 1kb (Promega, USA), was also loaded in the well. The gel was then electrophoresis at 120 volts for 20 minutes. The gel was then removed and visualised under ultraviolet light.

2.3 Gel Extraction

Gel extraction was carried out in this research to excise expected band of the PCR samples. All gel extractions that were carried out in this research were done using QIAquick Gel Extraction Kit from USA according to manufacturer's protocol.

2.4 Cloning

2.4.1 Competent Cell Preparation

2 μl of JM109 High Efficiency Competent Cells was added to 10 ml of sterile LB broth (Pronadisa, Spain) in a universal bottle under sterile condition using aseptic

techniques. The universal bottle was then capped loosely and was secured onto a 37 °C shaking bath at 220 rpm to be incubated overnight (16 hours). Then 1 ml of the overnight culture was added to 10 ml of sterile autoclaved LB in a universal bottle.

The universal bottle was then capped loosely and was secured onto a 37 °C shaking bath at 220 rpm to incubate. The density of the competent cells was monitored. This was done by randomly taking out 3 cultures and the cultures were diluted 100× by adding 495 μl of LB broth and 5 μl of culture and mixing them by pipetting in a disposable cuvette.

The culture was returned immediately to continue incubating. The spectrophotometer was set at a wavelength of 600 nm in ultraviolet spectrum. Before the sample was loaded, the spectrophotometer was "blanked" by dispensing clean and uncultured LB broth into the disposable cuvette. The cell density was determined by measuring the OD_{600}. This step was repeated at a 15 minute interval and more frequently (5 minutes) as the OD_{600} reading approaches close to 0.5. The incubation was stopped when the OD_{600} measures as 0.5. This was because, the cell culture at this stage was in the log phase and the cell at this phase was most optimal for competent cell preparation. The competent cell culture was then transferred into sterile 15 ml falcon tubes using aseptic technique. The tubes containing the cells were then left on ice for 15 to 30 minutes. The tubes of cells were then centrifuge at 3000 rpm at 4 °C for 5 minutes. The supernatant was discarded and 5000 μl of RF1 (every 100 ml contains 1.20 g $RbCl_2$, 0.99 g $MnCl_2.4H_2O$, 0.30 g KoAC, 0.15 g $CaCl_2.4H_2O$, 15 ml glycerol (AnalaR, England), sterile distilled water added and had the pH adjusted to 5.8, and the solution was filter sterilised) was added to the pellet and was resuspended (on ice).

The tubes were then left on ice for 20 minutes. The tubes were once again centrifuged at 3000 rpm at 4 °C for 15 minutes. The supernatant was discarded and was resuspended in RF2 (every 100 ml contains 0.21 g MOPS, 0.12 g $RbCl_2$, 1.10 g

CaCl$_2$.4H$_2$O, 15.0 ml glycerol and sterile distilled water and had the pH adjusted to 6.8, and the solution filter sterilised). The amount of RF2 added varies from 100 μl to 400 μl depending on the pellet size. The prepared competent cell was then aliquot into sterile 1.5 ml microcentrifuge. The microcentrifuge was then dipped into liquid nitrogen and then stored at -80°C.

2.4.2 LB plates with ampicillin / IPTG / X-Gal

LB agar (Pronadisa , Madrid-Spain) plates with (100 μg/ml) ampicillin (Sigma, USA), 0.5 mM IPTG, and 80 μg/ml X- gal were prepared prior to the transformation day as it requires time for autoclaving and for the agar to set. Prior to transformation conducted the LB plates were dried in 37ºC incubator. This was to ease the plating of the transformed cells and to avoid contamination.

2.4.3 LB Broth Preparation (Pronadisa, Madrid-Spain)

20 g of the dehydrated medium was dissolved in 900 ml of distilled water and then it was dissolved with frequent agitation until completely dissolved. Final volume was adjusted to 1000 ml. It was then sterilized at 121°C for 15 minutes.

2.4.4 Ligation reaction with pGEM®-T vectors

a pGEM ®-T vector (Promega, USA) was used in cloning. The pGEM ®-T vector DNA tube was briefly centrifuged to collect the contents in the bottom of the tube. The ligation reaction was set up in 0.5 ml microcentrifuge tubes as it is known to have low DNA binding capacity. The 2× rapid ligation buffer was thawed on ice and vortexed prior to each use. The 10 μl ligation reaction was set up containing   5.0 μl 2× Rapid ligation Buffer, 1.0 μl pGEM®-T vectors (50 ng), 3 μl purified PCR product and

1.0 µl T4 DNA Ligase (3 Weiss unit/ µl). The reactions were mixed by pipetting and incubated at 4ºC overnight for the maximum number of transformants.

2.4.5 Transformation into JM109 competent cells

The tubes containing ligation product were centrifuged to collect the content at the bottom of the tube. 2 µl of each ligation reaction was added to a sterile 1.5 ml microcentrifuge on ice. Tubes of frozen JM109 competent cells were removed from -80ºC storage and were placed on an ice bath until just thawed. The cells were mixed by flicking the tube gently. A 100 µl of cells were carefully transferred into each of the 1.5 ml microcentrifuge tubes containing the ligation product. The tubes were flicked gently to mix the cells and the ligation product.

The tubes were then place on an ice bath for 20 minutes. The cells were then heat-shocked for 45 seconds in a water bath at exactly 42ºC without shaking. The tubes were immediately returned to ice for 2 minutes. 900 µl room temperature LB broth was added to the tubes containing cells transformed with ligation reaction. It was then incubated for one and a half hour at 37ºC in rotating hybridization oven. The tubes were then centrifuged at $1000 \times g$ for 10 minutes to pellet the cells. 800 µl of the supernatant was discarded and the pellet was resuspended with the remaining supernatant. A 100 µl of the transformed cells were plated on the LB agar plates using a spreader. The plates were then incubated overnight (16 hours) at 37 ºC incubator.

2.4.6 Recombinant Colonies Screening

When the desired PSMA fragments was inserted into the *LacZ'* gene of M13 cloning vector, it inactivates the β-galactosidase synthesis causing the formation of white colonies on the LB/ Ampicillin/ IPTG/ X-gal plates after overnight incubation. Blue colonies were formed when there is no inserts in this gene.

Under a sterile condition using aseptic technique, white colonies which are represented by one cell each was picked to build library on a new LB/ Ampicillin/ IPTG/ X- gal plates. The library later was incubated at 37°C overnight (16 hours). While constructing the library, the loop that is used to pick up the colony is immersed in 50 μl of autoclaved distilled water in a 0.5 ml microcentrifuge tube before being flame sterilised. Tubes containing the transformed colonies were boiled at 99°C for 10 minutes to lyse the cells. This was then proceeded by colony PCR using M13 forward and reverse primers. The PCR product was then analysed by mixing 1 volume of 6× loading dye and 5 volumes of purified DNA and electrophoresis in a 1% stained agarose gel.

2.5 Isolation of Plasmids

A positive screened colony (with correct insert) was selected from the library plate and cultured in 10 ml sterile LB broth containing 50 μg/ml ampicillin (Sigma, USA) in a universal bottle. The universal bottle was capped loosely and was left incubating in a shaking (220 rpm) waterbath at 37 °C overnight (16 hours) to increase the transformed cells quantity for plasmid extraction. The following steps were carried out in sterile condition with aseptic techniques. 150 μl of 100% glycerol (AnalaR, England), was pipetted into each 1.5 ml microcentrifuge tube. Then, 850 μl of overnight

culture was added into the microcentrifuge tubes contain the culture and glycerol (AnalaR, England) were then mixed before being kept at -80 °C for future usage.

The remaining overnight culture was poured into 15 ml falcon tubes and was centrifuged at 6000 rpm for 15 minutes at room temperature. Then, the supernatant was discarded and the bacterial pellet was resuspended using 200 μl ice cold Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris Cl) by pulse vortexing. The suspended pellet was then transferred into a new 1.5 ml microcentrifuge tube and 200 μl of freshly prepared Solution II (0.2 N NaCl, 1% SDS) was added. The sample was then mixed by gently inverting and was left to stand in room temperature for 4 minutes. 200 μl of ice cold Solution III (3 M KoAc, 11.5% glacial acetic acid) was added. The sample was mixed by gentle inversion and was incubated on ice for 15 minutes. The sample was then centrifuged at 13000 rpm for 10 minutes. The supernatant was then transferred into sterile 1.5 ml microcentrifuge tubes, and 3 μl of RNase (50mg/ml) (Sigma-Aldrich, USA) was then added. This was subsequently incubated in a water bath for an hour at 37 °C.

After incubating, 600 μl of ice cold phenol (Pierce, USA) was added. It was then vortexed followed by centrifuging at 13000 rpm for 5 minutes. The upper layer of supernatant was transferred to a new 1.5 ml microcentrifuge tube. The supernatant was then added with 600 μl of chloroform (Merk, Germany). It was then vortexed and centrifuged at 13000 rpm for 5 minutes.

The upper layer of supernatant was transferred to a new 1.5 ml microcentrifuge tube. Then 0.1 volume of 5 M sodium chloride was then added followed by 2.5 volume of ice cold isopropanol (Merck, Germany). It was then left to incubate on ice for 20 minutes to collect precipitate. The sample was then centrifuged at 13000 rpm for 15 minutes and the supernatant was discarded.

1 ml of ice cold 70% ethanol was added. It was then centrifuged at 13000 rpm for 5 minutes. The supernatant was then discarded. The pellet formed was pure DNA and was dried in speed vacuum for 5 minutes. Lastly, 50 µl of autoclaved distilled water was added and the pellet was left in 4°C overnight for the pellet to dissolve. The dissolved pellet was subsequently quantified through spectrophotometric method to identify the purity and the quantity of the isolated plasmid.

2.5.1 Plasmid digestion with *Sfi*1 and *Not*1

To carry out the cloning of the desired PSMA fragments into plasmid vectors, both of them need to be modified to make integration possible. The plasmid and the PSAM fragments were subjected to RE digestion to generate identical cohesive or sticky ends. In this case, the inserted DNA and plasmids were cleaved with *Sfi*1 and *Not*1 restriction enzymes at the S*fi*1 /*Not*1 cleave sites.

Firstly, concentrations of DNA samples were determined using spectrophotometer (Eppendorf, USA). Later, based on OD reading a master mix was prepared. 1X Tango buffer (Fermentas, Canada), 1 U of *Sfi*1 restriction endonuclease (Promega, USA), 1 U of Not1 restriction endonuclease (Fermentas, Canada), 100 ng DNA samples, and adequate sterile distilled water, were mixed together and placed in 0.5 ml tubes and left in 37°C waterbath for 3 hours. Heat inactivated used for 10 minutes at 99°C. Small aliquot of digested plasmid was checked by agarose gel electrophoresis to check complete digestion. The digested plasmid Stored at -20°C.

2.5.2 Plasmid purification

After vector was completely digested, phenol/chloroform washing step was used, the solution was centrifuged to pellet DNA. 5M NaCl/ice cold isopropanol were added, and the pellet washed with 70% ethanol, air-dried and resuspended in 30 μl sterile distilled water. Incubated overnight at 4°C then stored at -20°C. Plasmids were checked by restriction and electrophoretic analysis, and then purified plasmids were sent for sequence analysis (Applied Biosystems, Japan).

2.6 Cloning with pPICZαA vector

2.6.1 Ligation reaction with pPICZαA vector

Ligation reaction involved a combination of 2X ligation buffer, DNA inserts and plasmid vectors, T4 DNA ligase enzyme (Promega, USA) and adequate volume of sterile distilled water. The mixture was prepared with consideration of the insert-vector ratio (3:1). The components were aliquoted in 0.5 ml tubes, then mixed by pipetting and incubated at 4°C overnight. After ligation, the vectors have to be transformed into host cells.

2.6.2 Preparation of low salt Luria Bertani (LSLB) medium

The LSLB mediums were prepared in two forms, agar plates and broth. For LSLB agar plate preparation, 2% of peptone (Pronadisa, Canada), 3% of agar (Amresco, Canada), 1% of NaCl (Promega, USA) and 1% of yeast extract (Pronadisa, Canada), were added before stirred with 190 ml of sterile distilled water using a stirrer (LMS, Japan).

The pH of the solution was adjusted to 7.5 using a pH meter (Sartorius, China) and the volume was brought up to 200 ml, the solution was then autocalved for 15 minutes at 121ºC. The autoclaved broth was cooled down with running tap water until temperature was around 55ºC. Next, 25 µg/ml of zeocin (Invitrogen, USA) was added to the broth before being poured into labeled clean petri-dishes. The plates were left at room temperature to solidify, and kept in 4ºC for maximum three months.

For LSLB broth preparation, 1% of peptone was mixed with 0.5% of yeast extract and NaCl in 90 ml of sterile distilled water. PH was adjusted to 7.5 and total volume was topped up to 100 ml. Broth were then aliquoted, 10 ml into each universal bottle and autocalved.

2.6.3 Transformations into Top10 cells

Prior to transformation, the host cells have to be prepared with the same procedure, which mentioned in (section 2.4.1). 100 µl of the frozen stocks of *E. coli* Top10 cells in each tube were thawed on ice for 5 minutes. Then, 5 µl of ligation reaction was transferred into each tube (s) containing competent cells, mixed gently by flicking and incubated on ice for 30 minutes. After that, the mixtures were heat shocked at 42°C for 90 seconds and immediately chilled on ice for 5 minutes. 900 µl room temperature LSLB broth (Appendix A) was added to the mixture and incubated for 1.5 hours at 37°C with shaking (~250 rpm). After transformation, 100 µl of each transformation mix were spread on the LSLB medium containing zeocin and incubated overnight (16-24 hours) at 37°C. Plates were placed at 4°C for short term storage.

2.6.4 Recombinant colonies screening

Cells that were successfully transformed will be conferred resistance to zeocin antibiotic present in the medium. Using sterilized looped wire, the colonies were picked randomly since all colonies were white. Tip touched onto library plate drawn with 6×6 grids while the rest were mixed in 30-50 μl of sterile distilled water. The tubes containing the transformed colonies were heated at 99°C for 10 minutes to lyse the cells before proceeding to colony PCR. Meanwhile, the library plates were incubated at 37°C overnight.

2.6.5 Colony PCR

This PCR method was employed for selection of colonies that were successfully transformed with vectors containing inserted DNA. Inserted sequences were amplified with a thermocycler (as previously mentioned). Clones providing an amplicon of correct size were identified by agarose gel electrophoresis. Right clones were picked up, and grown in 10 ml LSLB broth containing 2.5 μl zeocin, and then incubated overnight at 37°C. Cells were grown and recombinant product was isolated by using convential procedure (section 2.4.7) and linearized by restriction endonuclease *Sac*1 (Fermentas, Canada).

2.7 Transformation into *Pichia pastoris*

Recombinant plasmid DNA was first linearized by restriction endonuclease *Sac*1 (Fermentas, Canada) and then transformed into *P. pastoris* strain X33 (Prondisa, Canada) by using EasyComp Transformation kit (Invitrogen, USA), following manufacturer's instructions.

3. Results

In order to obtain a full length sequence of the PSMA gene for cloning and expression of the protein two approaches were used. One of the approaches is by overlapping the three fragments from the PCR product. The other approach was by cloning the gene fragments into a vector system (pGEM-T vector) and then overlapped them from the isolated plasmids.

3.1 Construction of *PSMA* gen*e* from PCR product

3.1.1. Construction of G1

The G1 fragment of *PSMA* gene is further divided into six sub fragments namely fragment a, b, c, d, e, and f. All the fragments were obtained from the previous students with the exception of fragment d. Fragment d was amplified by using two steps PCR with 3'oligo's (45bp), and primers PSMA4F as forward primer and PSMA4R in second step (Appendix b).
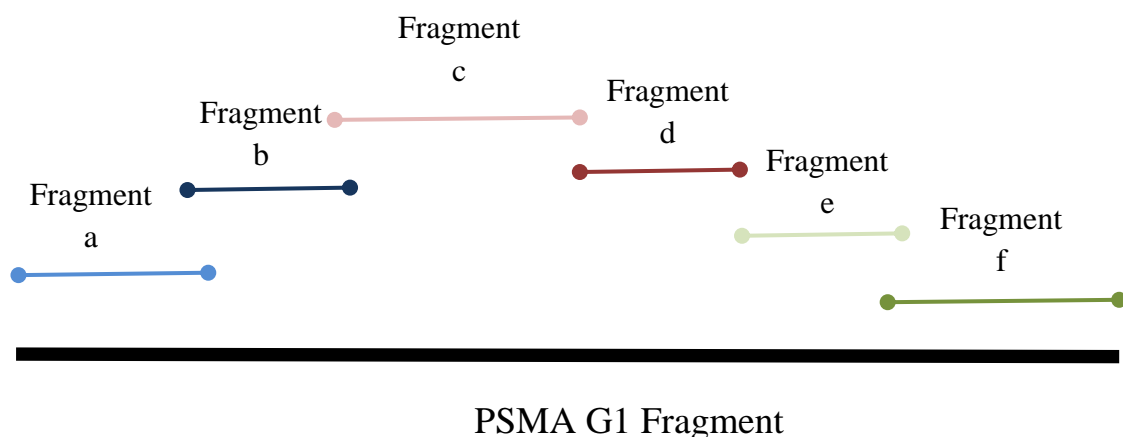
PSMA G1 Fragment

Figure 3.1 PSMA G1 fragment and its six subfragments

PCR amplification was carried out to amplify the desired fragments and sub fragments which was subsequently needed for the construction of the PSMA gene. In figure 3.1, the fragment G1-d was successfully amplified using two steps PCR and the band produced was sufficiently bright at the desired size of 101bp.
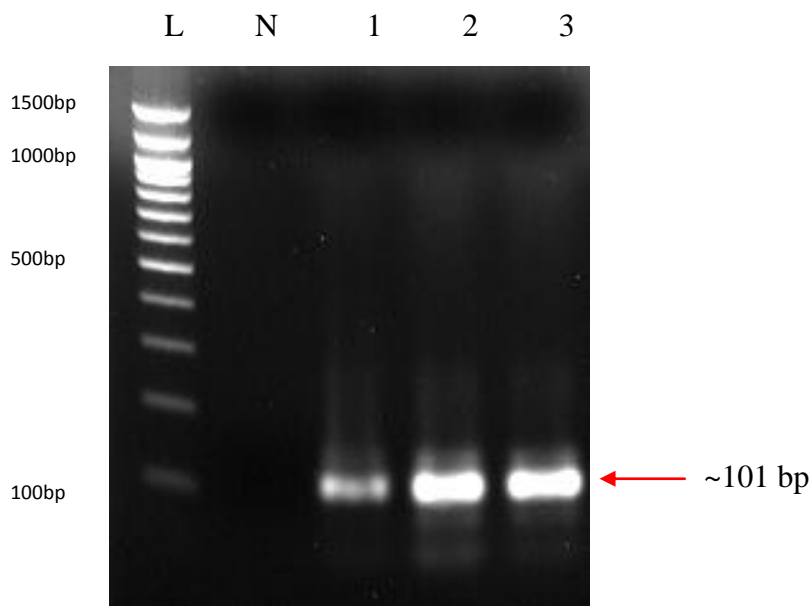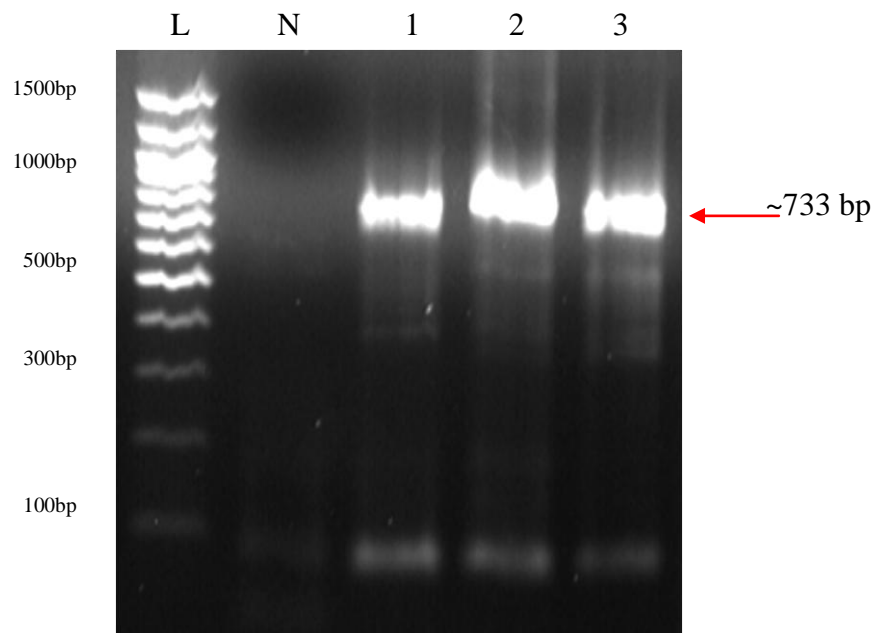

Figure 3.1: Two steps PCR for G1-d fragment of the *PSMA* gene. Lane L is the 100bp ladder (Seegene, Korea), lane N is the negative control, and lane 1, 2, and 3 is the amplification product of the two steps PCR for fragment G1-d of the *PSMA* gene.

As the gel picture shows there are traces of unspecific bands in this PCR product as well as primer dimers. In order to proceed with the construction of the PSMA gene only the specific fragment is needed. Hence the product here in figure 3.1 was subsequently purified using the gel extraction method to eliminate the unspecific band which are present in the gel.

As only the desired fragment was needed, purification of the PCR sample was required and to eliminate the unspecific bands and primer dimers, gel extraction was carried out.
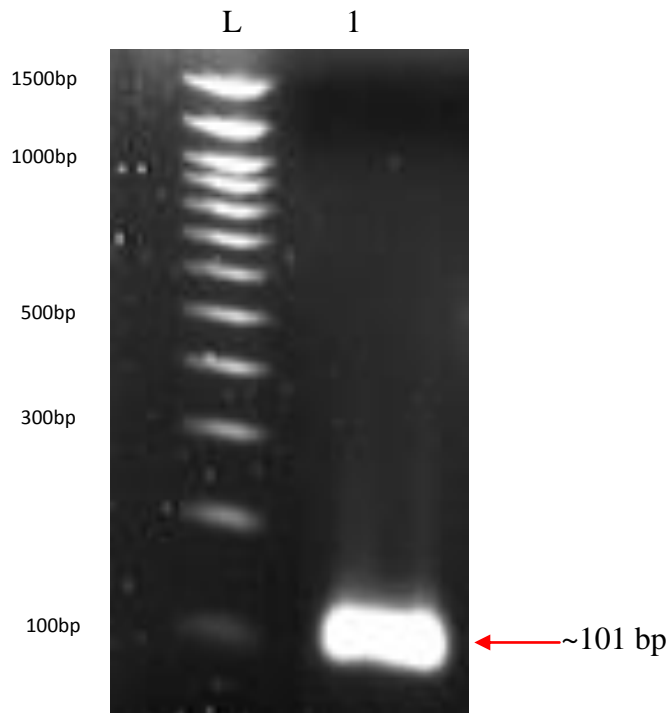
Figure 3.2: Gel extraction product for the G1-d fragment of the *PSMA* gene showed only a single band at the desired size 101bp with high intensity. L is the 100bp ladder (Seegene, Korea), and lane 1 is the purified product of the G1-d fragment of the *PSMA* gene.

Figure 3.2 illustrate the sample purified by means of gel extraction which shows only a single band with the desired size of 101bp in high intensity. Other undesired bands and dimers were eliminated from the PCR product with this procedure as only the desired fragment was excised to be purified.

After obtaining the six sub-fragments of G1, a two-steps PCR was carried out to construct G1. In the first step, all six fragments were overlapped. In the second step, G1 was amplified using G1 specific primer Sfi PSMA F as the forward primer & PSMA 961R as the reverse primer.

Figure 3.3: Two-steps PCR for the construction of G1 fragment of the *PSMA* gene. Lane L is the 100bp ladder (Seegene, Korea), lane N is the negative control, and Lane 1, 2 and 3 is the G1 fragment constructed.

All the six G1 sub fragments were successfully overlapped and the G1 fragment was successfully amplified at the desired size of 733bp as illustrated in figure 3.3. However, unspecific bands were also present in the PCR product. In order to construct the *PSMA* gene, G1, G2, and G3 fragment need to be overlapped and only the specific fragment of G1 is needed hence gel extraction need to be carried out.

3.1.2 Amplification of PSMA G2

The results shown on figure 3.4 is a gel picture for the PCR amplification of the G2 fragment of the *PSMA* gene using specific primers PSMA 961 F as forward primer and PSMA 1741 R as reverse primer. The G2 fragment was successfully amplified and this produces band with the expected size of 723 bp.
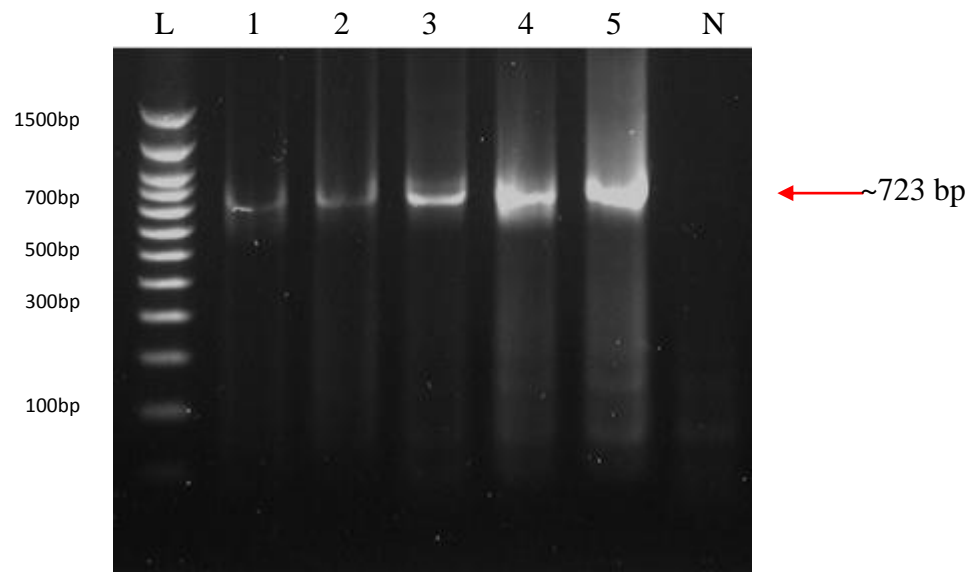
Figure 3.4: PCR amplification of the G2 fragment of the *PSMA* gene using *Pfu* polymerase. Lane L is the 100bp ladder (Seegene, Korea), Lane 1, 2, 3, 4 and 5 is the G2 fragment amplified which were amplified at the expected size, and lane N is the negative control.

Unspecific bands were also present in these PCR products and hence gel extraction was needed to be carried out to obtain the pure G2 fragment for subsequent procedure of overlapping the fragments *PSMA* gene.

3.1.3 Amplification of PSMA G3

The results illustrated on figure 3.5 is a gel picture for the PCR amplification of the G3 fragment of the *PSMA* gene using specific primers PSMA 800 F as forward primer and PSMA facXa-Not1R as reverse primer with the expected size 790 bp. The G3 fragment was successfully amplified and this produces band with the expected size of 790 bp.
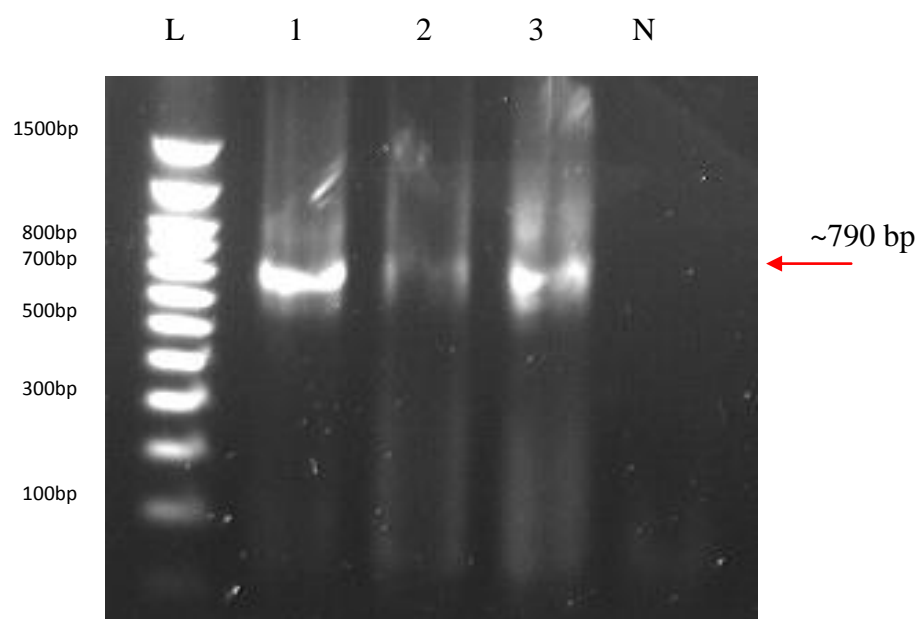
Figure 3.5: PCR amplification of the G3 fragment of the *PSMA* gene using *Pfu* polymerase. Lane L is the 100bp ladder (Seegene, Korea), lane 1, 2, and 3 is the G3 fragment amplified which were amplified at the expected size, and lane N is the negative control.

Unspecific bands were also present in these PCR products and hence gel extraction was needed to be carried out to obtain the pure G3 fragment for subsequent procedure of overlapping the fragments *PSMA* gene.

3.1.4 Purification of *PSMA* gene fragments

The result shown on figure 3.6 is a gel picture for PCR *PSMA* gene fragments (G1, G2 and G3) before purification (figure 3.6a) and product of the purified *PSMA* gene fragments (G1, G2 and G3) (figure 3.6b) by gel extraction, using QIAquick Gel Extraction kit (Qiagen, USA) according to the manufacturer's instructions.

(a)   (b)
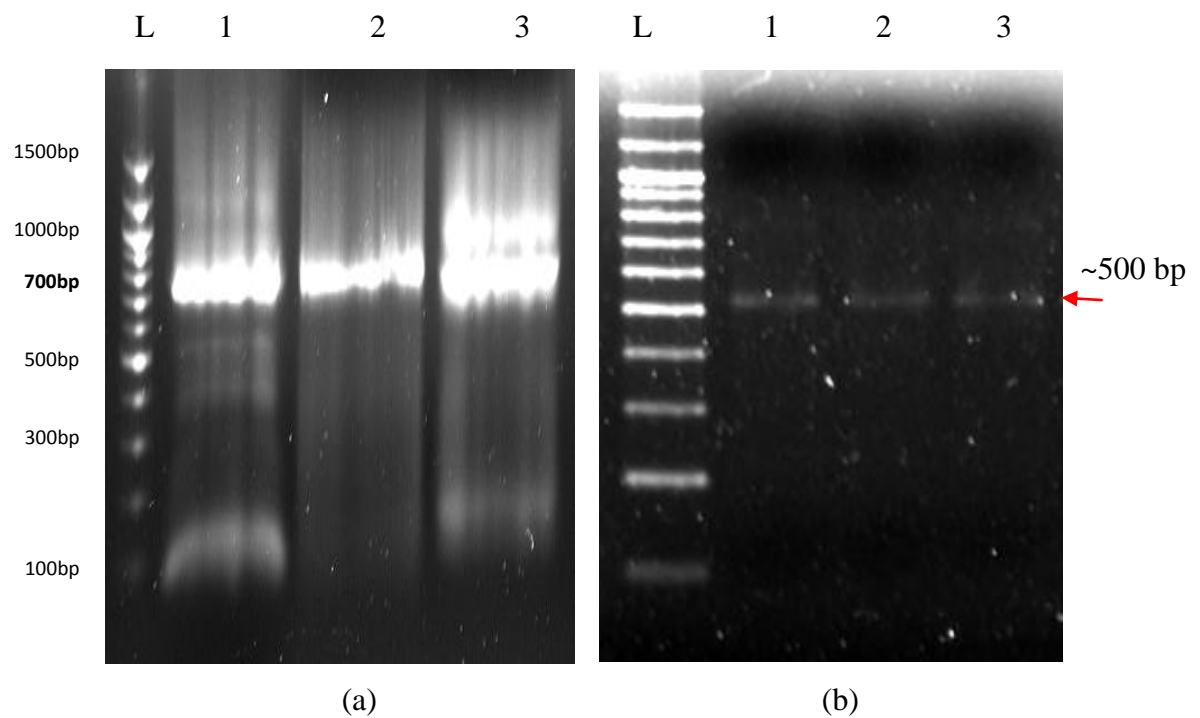
Figure 3.6: (a) *PSMA* gene fragments before gel extraction. (b) *PSMA* gene fragments after gel extraction. Lane L is the 100bp ladder (Seegene, Korea), lane 1 is G1 fragment, lane 2 is G2 fragment and lane 3 is G3 fragment.

After gel extraction, the bands obtained were significantly fainter and the band sizes obtained were smaller than the initial size of ~700bp. So, to overcome this problem the extracted fragments were heated up at 80° C for five minutes and then cooled down to room temperature.

3.1.5 Overlap extension from PCR product

The combination of the three fragments should be joined as following table:

Table 3.1: PSMA fragment combinations

Overlap extension PCR	Fragment 1	Fragment 2	Expected size
1st	PSMA G1	PSMA G2	1456 bp
2nd	PSMA G1-G2	PSMA G3	2246 bp

Figure 3.7 is the gel picture showing the overlapped extension from the fragment G1 and G2. The overlapped fragment was amplified using the primers Sfi PSMA –F as forward primer and Psma 1741 R as reverse primer with expected size 1456bp. The overlapped fragments shows smear after running in the gel and no clear band obtained.
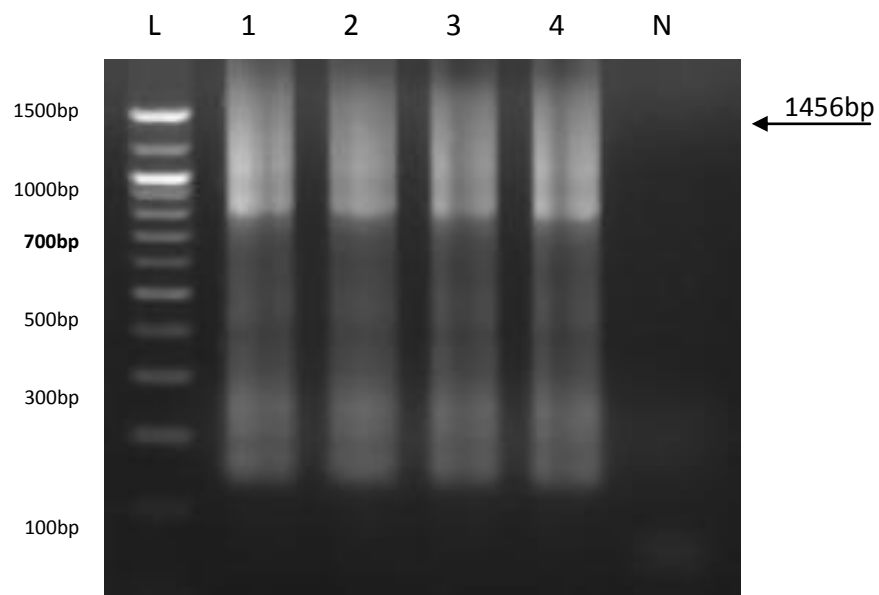
Figure 3.7: Overlap extension of G1 and G2 fragments. Lane L is the 100bp ladder (Seegene, Korea). Lane 1 to 4 is the result of the overlapped extension of G1-G2, and lane N is the negative control.

3.1.6 PCR optimization to amplify G1-G2 after overlapping

With no clear band obtained, a temperature gradient PCR was carried out to further optimize the amplification of G1-G2. Six different temperatures were chosen and the temperature ranged from 45ºC to 65ºC.

The best temperature for the amplification is 50.5 ºC in lane 3 as shown in figure 3.8; however, there is still no clear bands obtained at the expected size ~1456 bp.
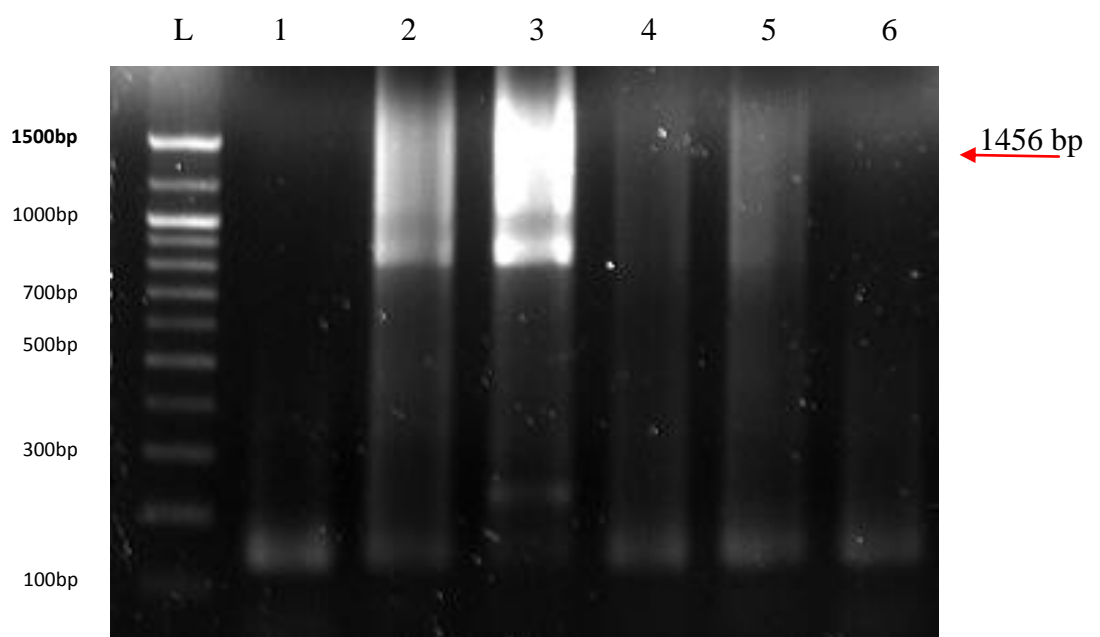
Figure 3.8: A temperature gradient PCR for the amplification of overlapped G1-G2. Lane L is the 100bp ladder (Seegene, Korea), Lane 1 is PCR at 45.0 ºC, lane 2 at 48.2 ºC, lane 3 at 50.5 ºC, lane 4 at 56.7ºC, lane 5 at 61.8 ºC and lane 6 at 65.0 ºC.

As no clear band was observed even at the optimum temperature of 50.5 ℃, a serial dilution was carried out to dilute the template for PCR. PCR product from lane 3 (50.5 ℃) figure 3.8 was used as the template.

Even with serial dilution of the template for PCR, no improvement was obtained as shown in figure 3.9. All amplification from different dilutions only produces smears and no specific band was produced.
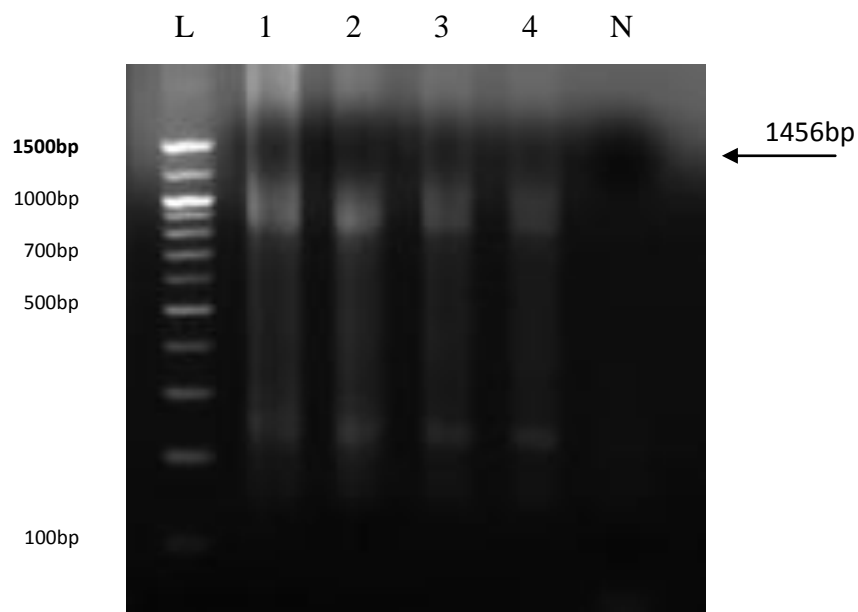
Figure 3.9: Serial dilution of template for G1-G2 PCR. Lane L is the 100bp ladder (Seegene, Korea). Lane 1 is G1-G2 PCR product as template, lane 2 is 10 × dilutions of the template, lane 3 is 100× dilutions of the template, lane 4 is 1000× dilutions of the template and lane N is negative control.

Since the approach of overlapping with PCR products not being successful, overlapping using plasmid from cloned fragments would be carried out as an alternative option to obtain the complete *PSMA* gene.

3.2. Cloning

3.2.1 Cloning of PSMA G1, G2and G3

Cloning method was used for each PSMA fragments to get high quantity of the fragments. Two approaches were applied in this project to clone the fragments with pGEM-T vector and transformed into JM109 competent cells. First approach was cloning of each fragment separately. Second approach was cloning of overlapped fragments that are PSMA G1-G2, PSMA G2-G3 & PSMA G1-G2-G3. After that, all these are to be used in construction *PSMA* gene.

G1, G2 and G3 fragments was amplified individually using *Taq* polymerase and the PCR product was subsequently purified and used for cloning.
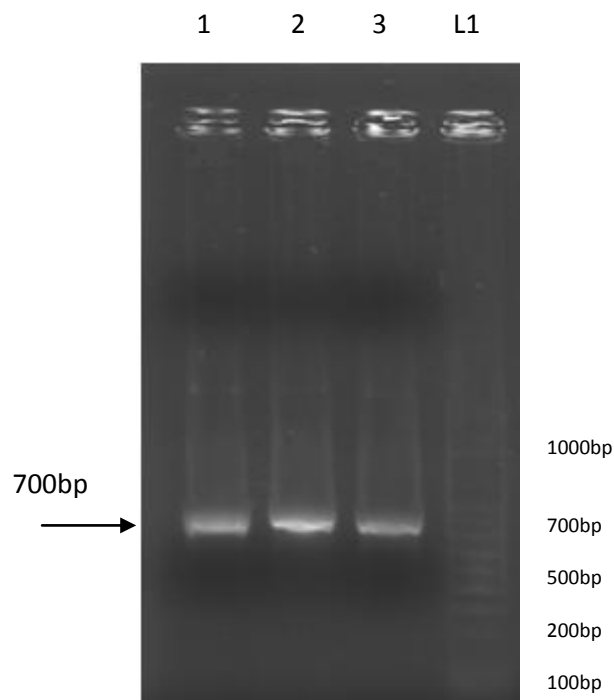
Figure 3.10: Gel extraction of amplified PCR product of G1, G2 and G3. Lane 1 is G1, lane 2 is G2 and lane 3 is G3. Lane L1 is 100bp ladder (Seegene, Korea).

Colony PCR was performed on colonies, which was transformed with pGEM-T vector ligated with G1, G2, and G3 fragment to select a colony containing the right insert. The expected size is approximately ~900bp for the colony PCR using M13F and M13R for all the fragments cloned.
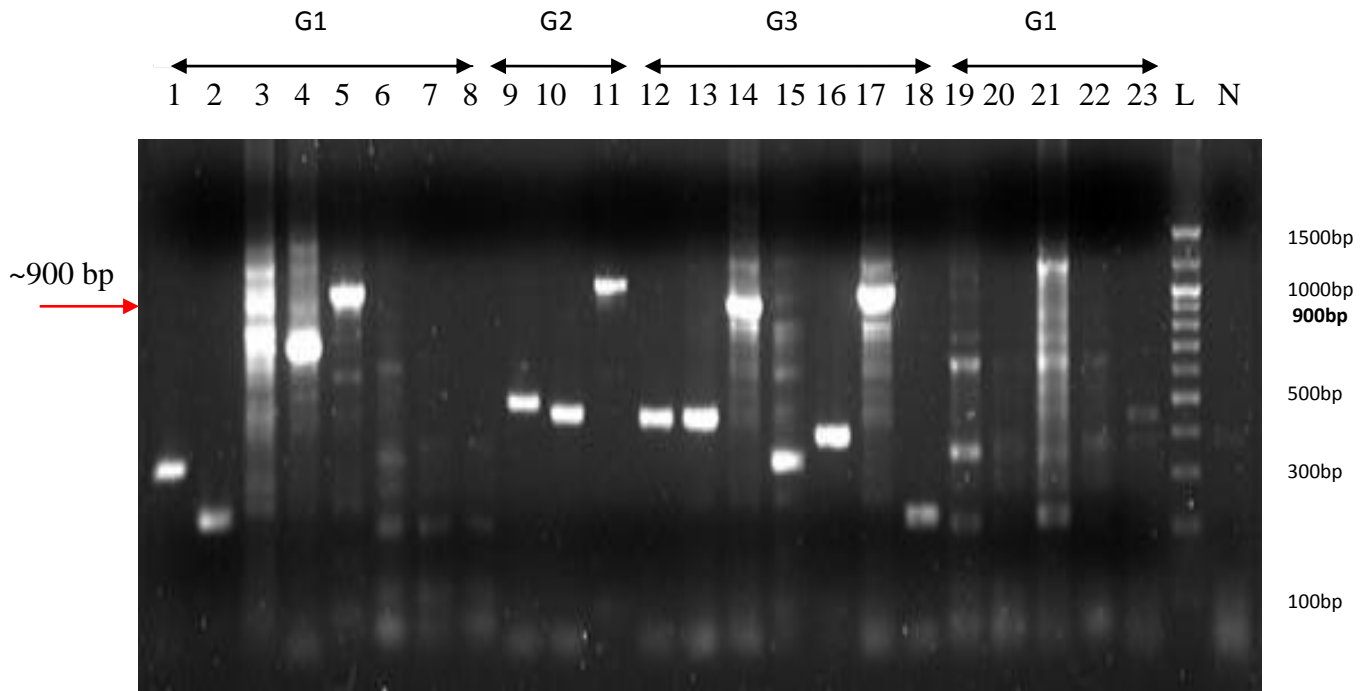
Figure 3.11: Colony PCR of PSMA G1, G2 and G3. Lane 1 to 8 and 19 to 23 is clones for PSMA G1 (~733+(265bp size of M13F/R primers). Lane 9 to 11 is clones for PSMA G2 (~723+265bp). Lane 12 to 18 is clones for PSMA G3 (~790+265bp). Lane L is 100bp ladder (Seegene, Korea), and lane N is the negative control.

The clones of PSMA G1, G2, and G3 with the right fragment size~900bp were then selected for plasmid isolation. Based on figure 3.11, colonies (G1) are 3, 5 and 21; (G2) is 11; and (G3) is 14 were selected and subsequently used for plasmid isolation.

Clones containing the right expected size fragments were cultured and the plasmids isolated, show in figure 3.12.
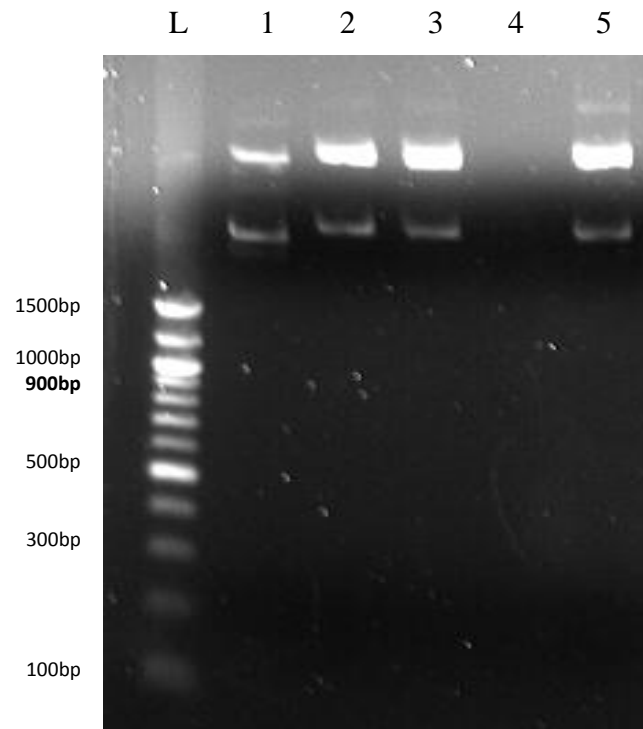
Figure 3.12: Plasmid Isolated for clones of PSMA G1, G2 & G3. Lane L is 100bp (Seegene, Korea), lane 1 is PSMA G1 fragment plasmid isolated from cloned 3, lane 2 is PSMA G1 fragment plasmid isolated from cloned 5, lane 3 is PSMA G2 fragment plasmid isolated from cloned 11, lane 4 is PSMA G3 fragment plasmid isolated from cloned 14 and lane 5 is PSMA G1 fragment plasmid isolated from cloned 21. Each one has two bands except G3 (14) no band appear due to failed in plasmid isolation or low concentration of the plasmid isolated.

The plasmid isolation step was successfully carried out as plasmids were obtained from most of the clones except one.

Subsequently, the PCR of the specific fragment was carried out to detect the presence of the desire insert. This is to verify that the desired fragment was cloned and not other DNA fragment of similar size.
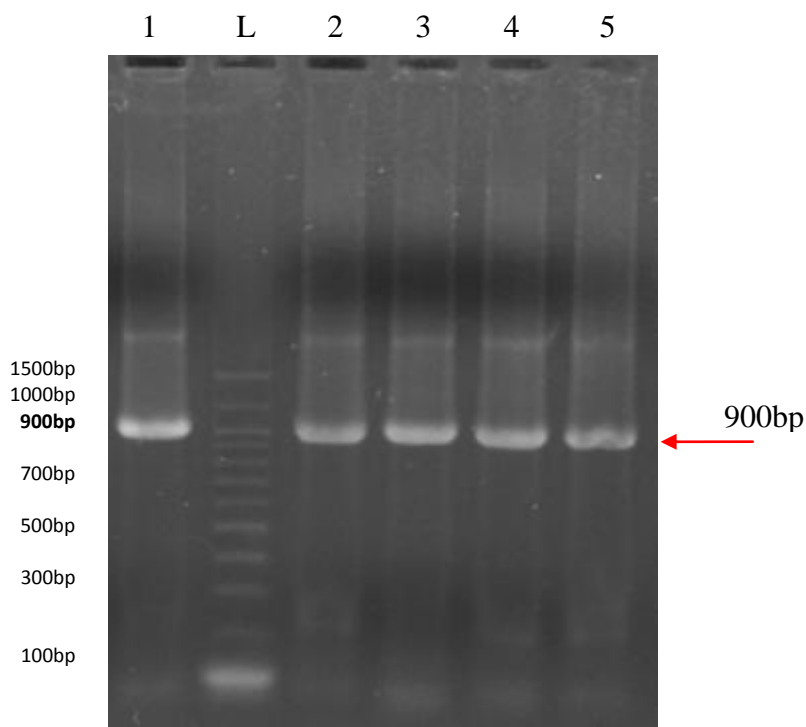
Figure 3.13: PCR product of amplifying PSMA G1, G2 & G3 from the diluted plasmid. Lane L is 100bp ladder (Seegene, Korea), Lanes 1, 2 and 5 is G1fragments amplified from cloned 3, 5 and 21 respectively G1 (~733+265bp expected size of specific primers). Lane 3 is fragment G2 amplified from cloned 11 (~723+265bp). Lane 4 is fragment G3 amplified from cloned 14 (~790+265bp).

.

Based on the fragment specific screening, plasmid from colonies 1 (lane1), 5 (lane2), 11 (lane 3) and 14 (lane 4) were used for sequencing.

3.2.2 Sequencing result of PSMA G1, PSMA G2 and PSMA G3

The sequencing results for the PSMA G1, result shows that there is a deletion in both sample of PSMA G1 that is clone a3 and a5 (figure 3.14a and 3.14b)
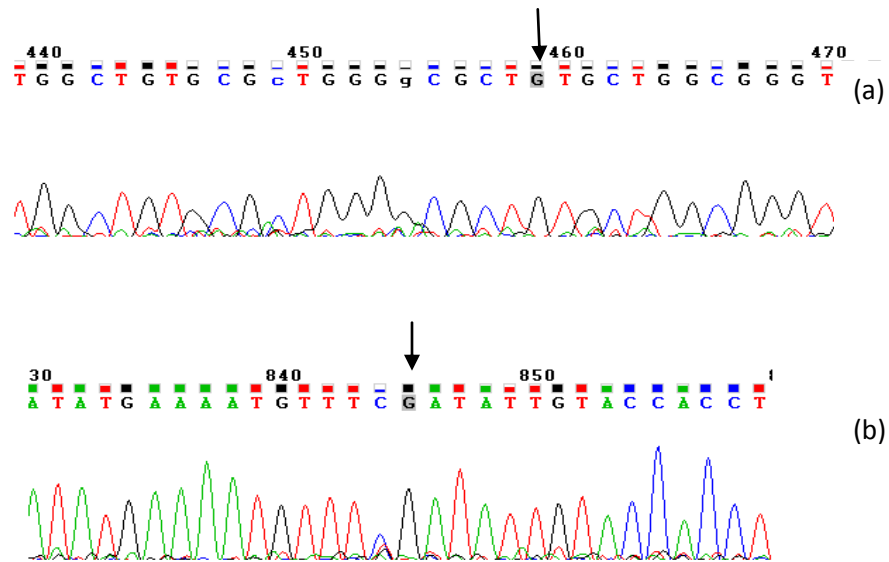
Figure 3.14: G1 sequencing results (a) showed the deletion of G nucleotide at 459 in G1 sequencing in sample number 3 (b) showed the deletion of G nucleotide at 845 in G1 sequence in sample number 5. Due to the deletion in G1 sequence, cloning of the PSMAG1 fragment was repeated.

The fragment for PSMA G2 (clone 11) and G3 (clone 14) were successfully cloned and the complete sequence of the fragment were obtained (Appendix C). Based on the sequencing result cloning of G1 have to be repeated.

3.2.3 Re-cloning of G1 Fragment

In figure 3.15, colonies that were selected were screened by carrying out colony PCR using M13F and M13R primers. Lanes 1, 2, 3, 4 and 5 shows faint bands of 900 bp, which was the expected size, while lane 7 and 9 are colonies with wrong inserts.
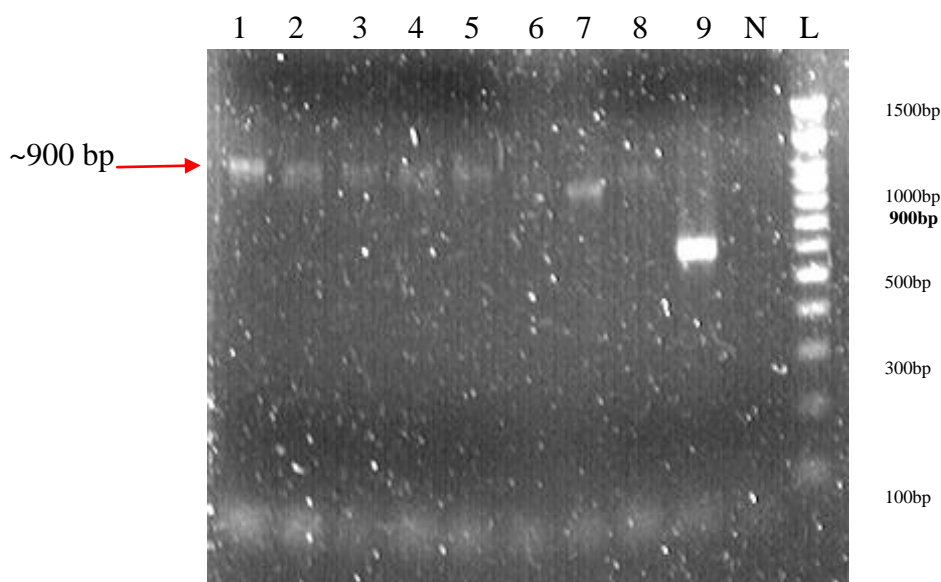
Figure 3.15: Colony PCR amplifications using M13F and M13R primers. Lane 1 to 9 (a1- a9), are all colonies picked from cloning of the fragment G1, lane N is negative control and lane L is 100bp ladder (Seegene, Korea).

Three colonies (a1, a2 and a4) were selected from the colony PCR and proceed for plasmid isolation process.
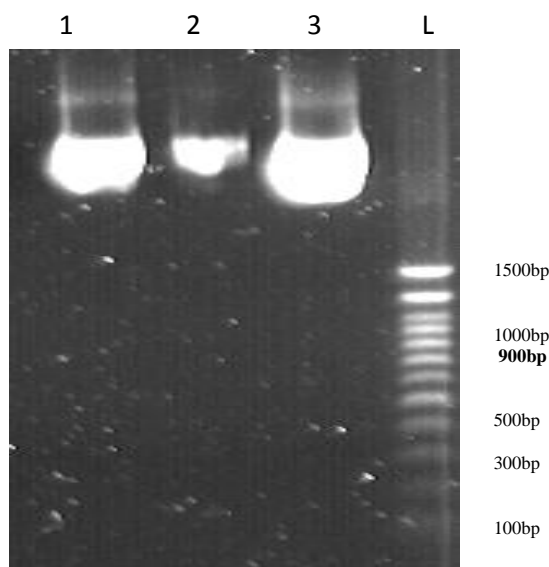
Figure 3.16: Plasmid isolated from colonies of G1. Lane 1 is plasmid from colony a4, lane 2 is plasmid from colony a2, lane 3 is plasmid from colony a1 and lane L is the 100 bp ladder (Seegene, Korea).

PCR confirmation was carried out on the plasmid isolated using G1 specific primers (Sfi PSMA F & PSMA 961 R) and the bands with expected size were obtained as illustrated in figure 3.17.   Correct amplification size of the G1 fragment indicates correct inserted fragment and hence sequencing of the plasmid could carried out.
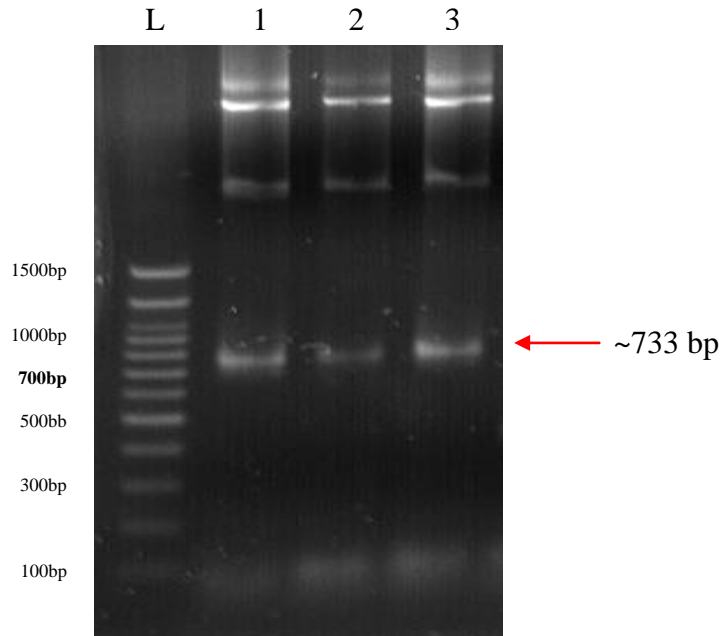
Figure 3.17: PCR verification of the plasmid isolated from colonies of G1: Lane L is the 100bp ladder (Seegene, Korea), lane 1 is a4 PCR of plasmid isolated colony, lane 2 is a2 PCR of plasmid isolated colony, and lane 3 is a1 PCR of plasmid isolated colony.

(a)

PSMA_G1_a2

```
The Sequence Manipulation Suite: Show Translation
Results for 2262 residue sequence starting "ATGTGGAATC".
    1 M   W   N   L   L   H   E   T   D   S   A   V   A   T   A   R   R   P   R   W
    1 ATGTGGAATCTCCTTCACGAAACCGACTCGGCTGTGGCCACCGCGCGCCGCCCGCGCTGG
   21 L   C   A   G   A   L   V   L   A   G   G   F   F   L   L   G   F   L   F   G
   61 CTGTGCGCTGGGGCGCTGGTGCTGGCGGGTGGCTTCTTTCTCCTCGGCTTCCTCTTCGGG
   41 W   F   I   K   S   S   N   E   A   T   N   I   T   P   K   H   N   M   K   A
  121 TGGTTTATAAAATCCTCCAATGAAGCTACTAACATTACTCCAAAGCATAATATGAAAGCA
   61 F   L   D   E   L   K   A   E   N   I   K   K   F   L   Y   N   F   T   Q   I
  181 TTTTTGGATGAATTGAAAGCTGAGAACATCAAGAAGTTCTTATATAATTTTACACAGATA
   81 P   H   L   A   G   T   E   Q   N   F   Q   L   A   K   Q   I   Q   S   Q   W
  241 CCACATTTAGCAGGAACAGAACAAAACTTTCAGCTTGCAAAGCAAATTCAATCCCAGTGG
  101 K   E   F   G   L   D   S   V   E   L   A   H   Y   D   V   L   L   S   Y   P
  301 AAAGAATTTGGCCTGGATTCTGTTGAGCTAGCACATTATGATGTCCTGTTGTCCTACCCA
  121 N   K   T   H   P   N   Y   I   S   I   I   N   E   D   G   N   E   I   F   N
  361 AATAAGACTCATCCCAACTACATCTCAATAATTAATGAAGATGGAAATGAGATTTTCAAC
  141 T   S   L   F   E   P   P   P   G   Y   E   N   V   S   D   I   V   P   P
  421 ACATCATTATTTGAACCACCTCCTCCAGGATATGAAAATGTTTCGGATATTGTACCACCT   ← Deletion
  161 F   S   A   F   S   P   Q   G   M   P   E   G   D   L   V   Y   V   N   Y   A
  481 TTCAGTGCTTTCTCTCCTCAAGGAATGCCAGAGGGCGATCTAGTGTATGTTAACTATGCA
  181 R   T   E   D   F   F   K   L   E   R   D   M   K   I   N   C   S   G   K   I
  541 CGAACTGAAGACTTCTTTAAATTGGAACGGGACATGAAAATCAATTGCTCTGGGAAAATT
  201 V   I   A   R   Y   G   K   V   F   R   G   N   K   V   K   N   A   Q   L   A
  601 GTAATTGCCAGATATGGGAAAGTTTTCAGAGGAAATAAGGTTAAAAATGCCCAGCTGGCA
  221 G   A   K   G   V   I   L   Y   S   D   P   A   D   Y   F   A   P   G   V   K
  661 GGGGCCAAAGGAGTCATTCTCTACTCCGACCCTGCTGACTACTTTGCTCCTGGGGTGAAG
  241 S   Y   P   D   G   W   N   L   P   G   G   G   V   Q   R   G   N   I   L   N
  721 TCCTATCCAGATGGTTGGAATCTTCCTGGAGGTGGTGTCCAGCGTGGAAATATCCTAAAT
  261 L   N   G   A   G   D   P   L   T   P   G   Y   P   A   N   E   Y   A   Y   R
  781 CTGAATGGTGCAGGAGACCCTCTCACACCAGGTTACCCAGCAAATGAATATGCTTATAGG
  281 R   G   I   A   E   A   V   G   L   P   S   I   P   V   H   P   I   G   Y   Y
  841 CGTGGAATTGCAGAGGCTGTTGGTCTTCCAAGTATTCCTGTTCATCCAATTGGATACTAT
```

[1] GCA→GTA
Ala → Val

[2] ACT→ACA
Thr → Thr

← Deletion

(b)

PSMA_G1_a4

```
The Sequence Manipulation Suite: Show Translation
Results for 2262 residue sequence starting "ATGTGGAATC".
    1 M   W   N   L   L   H   E   T   D   S   A   V   A   T   A   R   R   P   R   W
    1 ATGTGGAATCTCCTTCACGAAACCGACTCGGCTGTGGCCACCGCGCGCCGCCCGCGCTGG
   21 L   C   A   G   A   L   V   L   A   G   G   F   F   L   L   G   F   L   F   G
   61 CTGTGCGCTGGGGCGCTGGTGCTGGCGGGTGGCTTCTTTCTCCTCGGCTTCCTCTTCGGG
   41 W   F   I   K   S   S   N   E   A   T   N   I   T   P   K   H   N   M   K   A
  121 TGGTTTATAAAATCCTCCAATGAAGCTACTAACATTACTCCAAAGCATAATATGAAAGCA
   61 F   L   D   E   L   K   A   E   N   I   K   K   F   L   Y   N   F   T   Q   I
  181 TTTTTGGATGAATTGAAAGCTGAGAACATCAAGAAGTTCTTATATAATTTTACACAGATA
   81 P   H   L   A   G   T   E   Q   N   F   Q   L   A   K   Q   I   Q   S   Q   W
  241 CCACATTTAGCAGGAACAGAACAAAACTTTCAGCTTGCAAAGCAAATTCAATCCCAGTGG
  101 K   E   F   G   L   D   S   V   E   L   A   H   Y   D   V   L   L   S   Y   P
  301 AAAGAATTTGGCCTGGATTCTGTTGAGCTAGCACATTATGATGTCCTGTTGTCCTACCCA
  121 N   K   T   H   P   N   Y   I   S   I   I   N   E   D   G   N   E   I   F   N
  361 AATAAGACTCATCCCAACTACATCTCAATAATTAATGAAGATGGAAATGAGATTTTCAAC
  141 T   S   L   F   E   P   P   P   G   Y   E   N   V   S   D   I   V   P   P
  421 ACATCATTATTTGAACCACCTCCTCCAGGATATGAAAATGTTTCGGATATTGTACCACCT
  161 F   S   A   F   S   P   Q   G   M   P   E   G   D   L   V   Y   V   N   Y   A
  481 TTCAGTGCTTTCTCTCCTCAAGGAATGCCAGAGGGCGATCTAGTGTATGTTAACTATGCA
  181 R   T   E   D   F   F   K   L   E   R   D   M   K   I   N   C   S   G   K   I
  541 CGAACTGAAGACTTCTTTAAATTGGAACGGGACATGAAAATCAATTGCTCTGGGAAAATT
  201 V   I   A   R   Y   G   K   V   F   R   G   N   K   V   K   N   A   Q   L   A
  601 GTAATTGCCAGATATGGGAAAGTTTTCAGAGGAAATAAGGTTAAAAATGCCCAGCTGGCA
  221 G   A   K   G   V   I   L   Y   S   D   P   A   D   Y   F   A   P   G   V   K
  661 GGGGCCAAAGGAGTCATTCTCTACTCCGACCCTGCTGACTACTTTGCTCCTGGGGTGAAG
  241 S   Y   P   D   G   W   N   L   P   G   G   G   V   Q   R   G   N   I   L   N
  721 TCCTATCCAGATGGTTGGAATCTTCCTGGAGGTGGTGTCCAGCGTGGAAATATCCTAAAT
  261 L   N   G   A   G   D   P   L   T   P   G   Y   P   A   N   E   Y   A   Y   R
  781 CTGAATGGTGCAGGAGACCCTCTCACACCAGGTTACCCAGCAAATGAATATGCTTATAGG
  281 R   G   I   A   E   A   V   G   L   P   S   I   P   V   H   P   I   G   Y   Y
  841 CGTGGAATTGCAGAGGCTGTTGGTCTTCCAAGTATTCCTGTTCATCCAATTGGATACTAT
```

[1] TTC→TTT
Phe → Phe

[2] GTT→GCT
Val → Ala
Same group

[3] GCA→GCT
Ala →Ala

[4] TAT→TGT
Tyr →Cys
G1e

[5] TCC→TCA
Ser → Ser

(c)

Figure 3.18: Nucleotide sequences based on sequencing results of (a) G1-a1, (b) G1- a2, (c) G1-a4. The figures showed the replaced nucleotide and the repairing area according to the amino acid codon.

From the analyses of the sequencing results for all three sample of G1 (a1, a2 & a4), plasmid isolation of (G1_a4) showed the minimum mutation which can be repaired and does not affect the amino acid of the protein. Hence, sample (G1_a4) clone was used for subsequent work.

3.2.4 Overlapping of PSMA G1, G2 and G3 fragments from plasmid isolation product

Overlap extension PCR was conducted in three different combinations of template. All the overlapping combination and the expected size showed in the following table.

Table 3.2**: PSMA G1, G2 and G3 fragments combination for overlapping PCR**

combination	Fragment 1	Fragment2	Fragment 3	Expected size
1	G1	G2	-	1456bp
2	G2	G3	-	1513bp
3	G1	G2	G3	2246bp
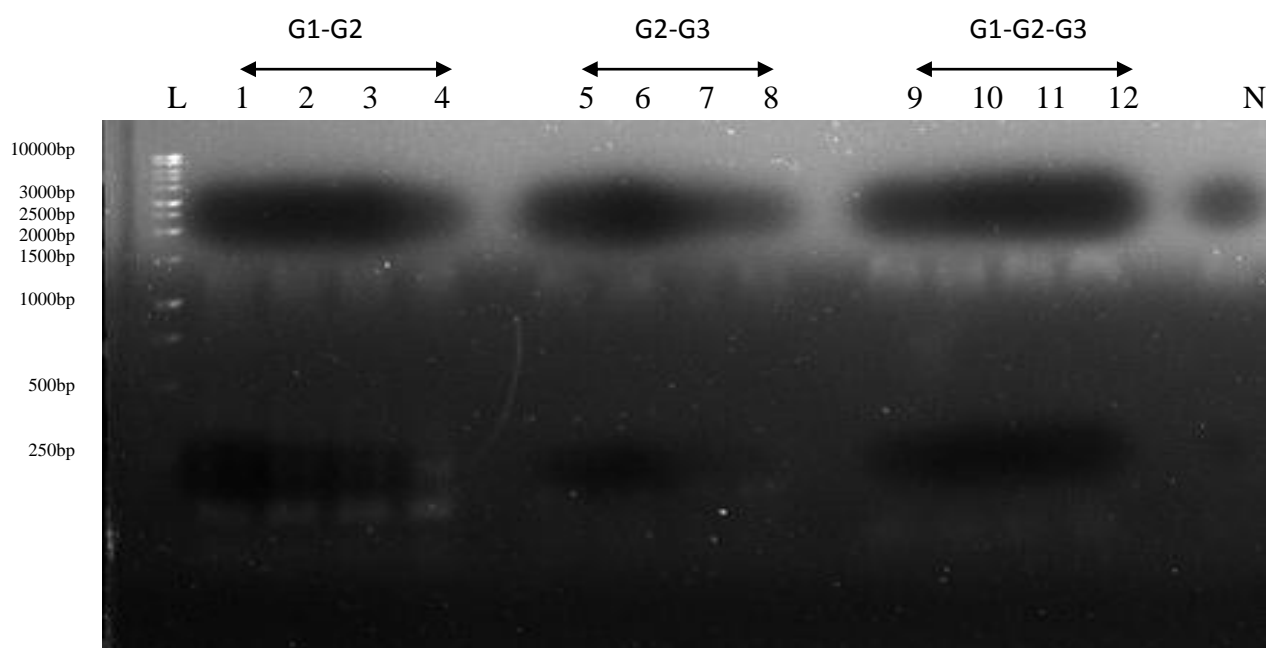
Figure 3.19: Two Steps PCR were done; both steps were carried out with *Pfu* polymerase enzyme. Lane L is 1kb ladder (Promega, USA), Lane 1 to 4 is G1-G2 fragment (1456bp), lane 5 to 8 is G2-G3 fragment (1513bp), lane 9 to 12 is G1-G2-G3 fragment (2232bp) and lane N is the negative control.
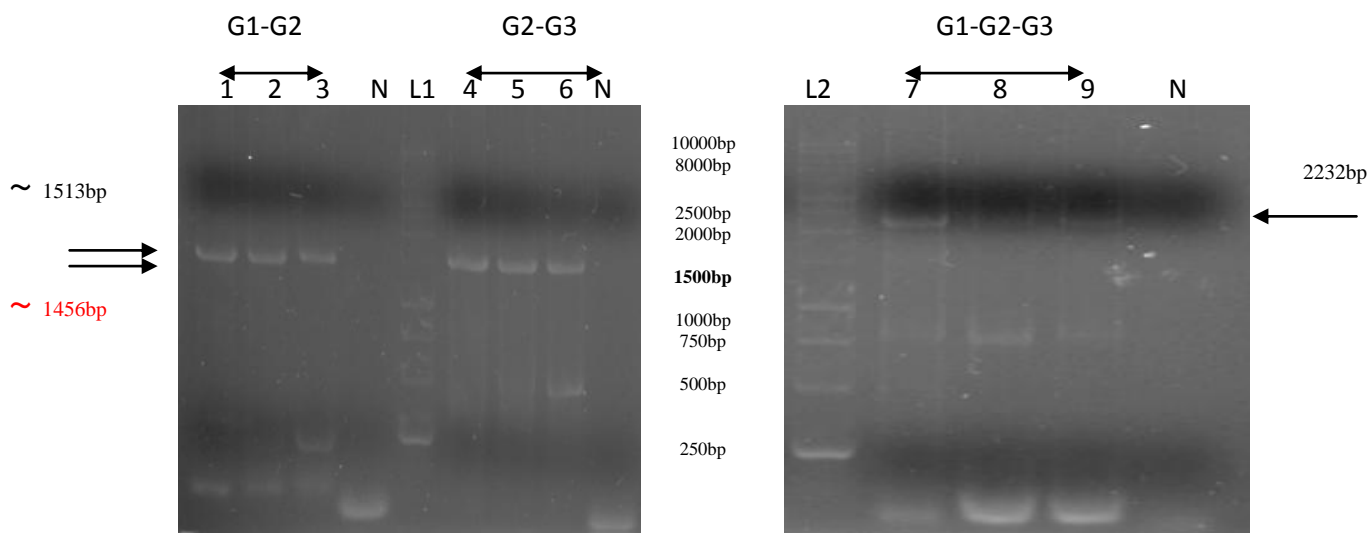
Figure 3.20: Two steps PCR were done, 1st step overlapping with *Pfu* polymerase enzyme and 2nd step amplifying with *Taq* polymerase enzyme. Lanes L1 and L2 is 1kb ladder (Promega, USA), Lane 1 to 3 is G1-G2 fragment (1456bp), lane 4 to 6 is G2-G3 fragment (1513bp), lane 7 to 9 is G1-G2-G3 fragment (2232bp), and lane N is the negative control.

In comparison of using different enzymes to amplify the fragments after overlapping it is shown that *Taq* polymerase (figure 3.20) is more efficient as compared to *Pfu* polymerase (figure 3.19). Amplification using *Pfu* polymerase shown no bands at the desired size whilst amplification using *Taq* polymerase successfully amplified the desired bands although the intensity could range from faint to intense bands.
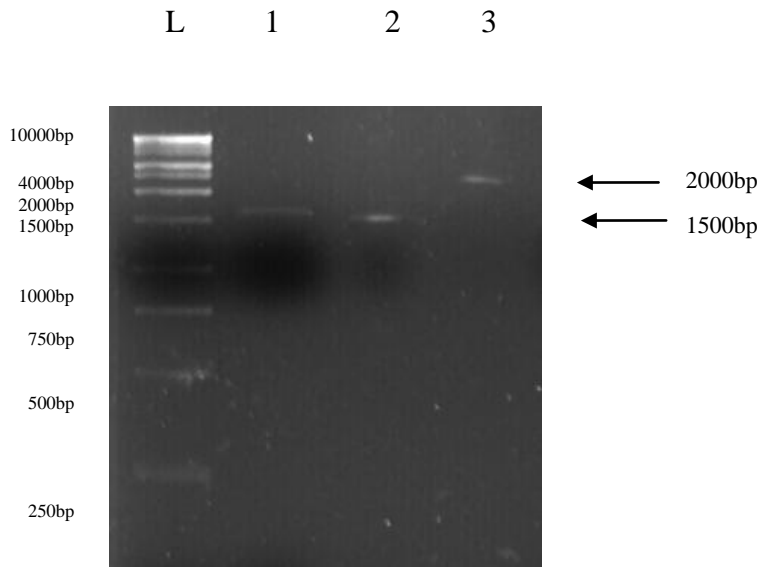
Figure 3.21: Gel extraction of PSMA G1-2, PSMA G2-3 & PSMA G1-2-3. Lane L is 1kb ladder (Promega, USA), lane 1 is PSMAG1-2, lane 2 is PSMA G2-3 and lane 3 is PSMA G 1-2-3.

3.2.5 Sequencing of the overlapped fragments

All fragments after purification only had shown faint bands due to much of it being lost in the purification process. The purified fragments PSMA G1-G2, PSMA G2-G3 & PSMA G1-G2-G3 were then cloned in JM109 cells but did not produce significant results as the sequencing results indicated the presence of partially inserted sequence and the sequence with mutation (data not shown).

Since this approach yielded no results, another attempt was done by amplifying the G1 fragment from the isolated plasmid and amplify G2-G3 overlapped fragment using the isolated plasmid and then overlapping both of these amplified products.

3.2.6 PCR optimization to amplify G1

In the PCR optimization of G1, the enzyme type and quantity were the parameters, which were manipulated at this stage. Two types of the enzymes were used, namely *Taq* polymerase and *Pfu* polymerase, to amplify PSMA G1 fragment. The amplified product using *Pfu* polymerase was used in overlapping while which amplified with *Taq* was used in cloning.
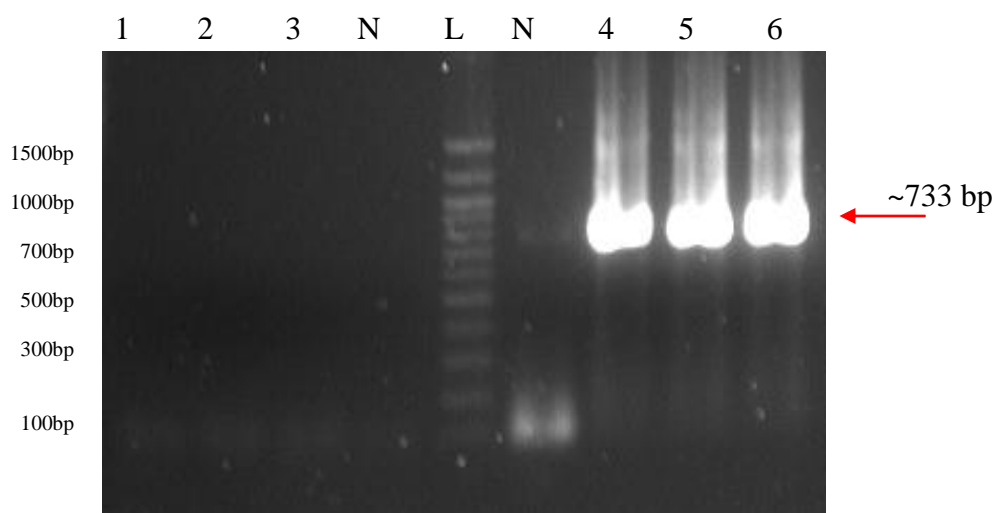

Figure 3.22: PCR amplification of G1using plasmid (colony a4) as template with one unit of *Pfu* polymerase enzyme and one unit of *Taq* polymerase enzyme. Lane 1 to 3 is the G1 fragment amplified with one unit of *Pfu* polymerase enzyme, lane 4 to 6 are G1 fragment amplified with one unit of *Taq* polymerase enzyme, lane N is the negative control and lane L is 100bp ladder (Seegene, Korea).
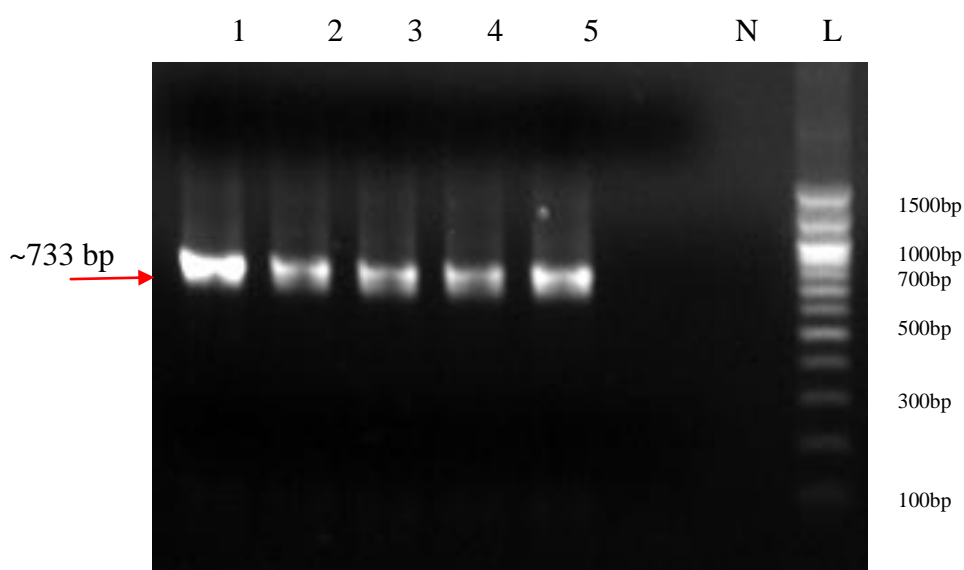

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Figure 3.23: PCR amplification of G1using plasmid (colony a4) as template with 1.5 unit of *Pfu* polymerase enzyme.

It is shown that *Pfu* polymerase could only amplify using a much higher concentration as shown in figure 3.23, using one unit of *Pfu* polymerase did not yield any bands (figure 3.22) while by using 1.5 units of *Pfu* polymerase the desired fragment could be amplified.

3.2.7 PCR optimization of overlapping G2-G3

PCR optimization was carried out to obtain optimal amplification of G2-G3 fragment after the overlapping PSMA G2 and G3, which carried out using plasmid of G2 and G3 clones. The parameter, which manipulated was the concentration of the enzyme for each PCR reaction.
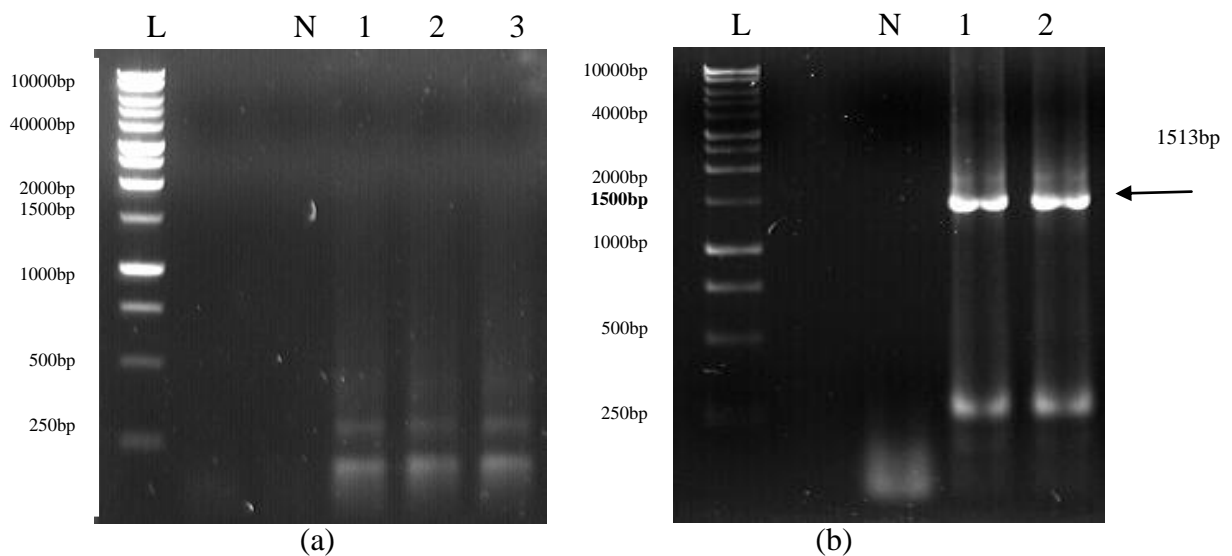

Figure 3.24: (a): Overlap PCR of PSMA G2 and G3 fragments using one unit of *Taq* polymerase enzyme. Lane L is 1kb ladder (Promega, USA), lane N is the negative control and lane 1 to 3 is the Overlap PCR amplification

(b): Overlap PCR of PSMA G2 and G3 fragments using two units of *Taq* polymerase enzyme. Lane L is 1kb ladder (Promega, USA), lane N is the negative control and lane 1 to 2 is the Overlap PCR amplification.
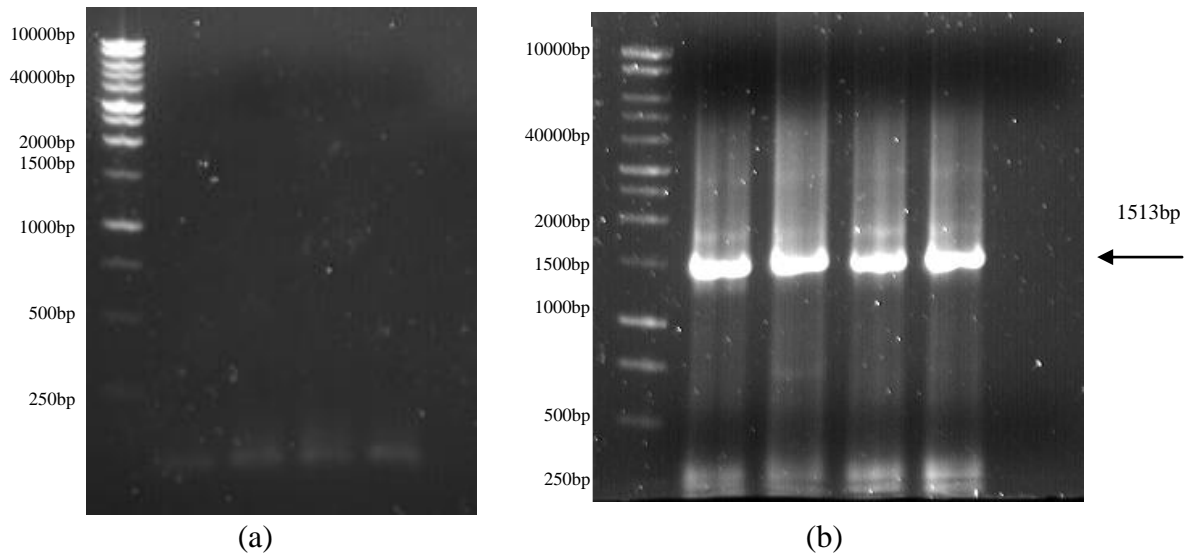
Figure 3.25: Overlap PCR of PSMA G2 and G3 fragments. Amplify PCR done by using 2 units of *Taq* for each PCR reaction .(a) The PCR run in PCR machine (Peltier Thermal Cycler 200 MJ Research.(b) The PCR run in PCR machine (PCR system 2400).

In figure 3.25, it is clearly shown that different PCR machine shows different efficiency. Under same parameters and same reagents and template, Peltier Thermal Cycler 200 MJ Research could not amplify the fragments whilst PCR system 2400 could.

Overlapping PSMA G2-G3 from different template source was one of the parameter, which manipulated in order to obtain the optimum amplification of the overlapped fragment of PSMA G2-G3. 2units of *Taq* polymerase enzyme was used in the PCR amplification using of two different templates, namely PCR product of G2 and G3, and the plasmids from clones G2 and G3.

As shown in figure 3.26, PCR product as the template for overlapping did not produce a clear band but instead a smear was produced while in comparison to the isolated plasmid as a template, clear band was produced at the desired size.
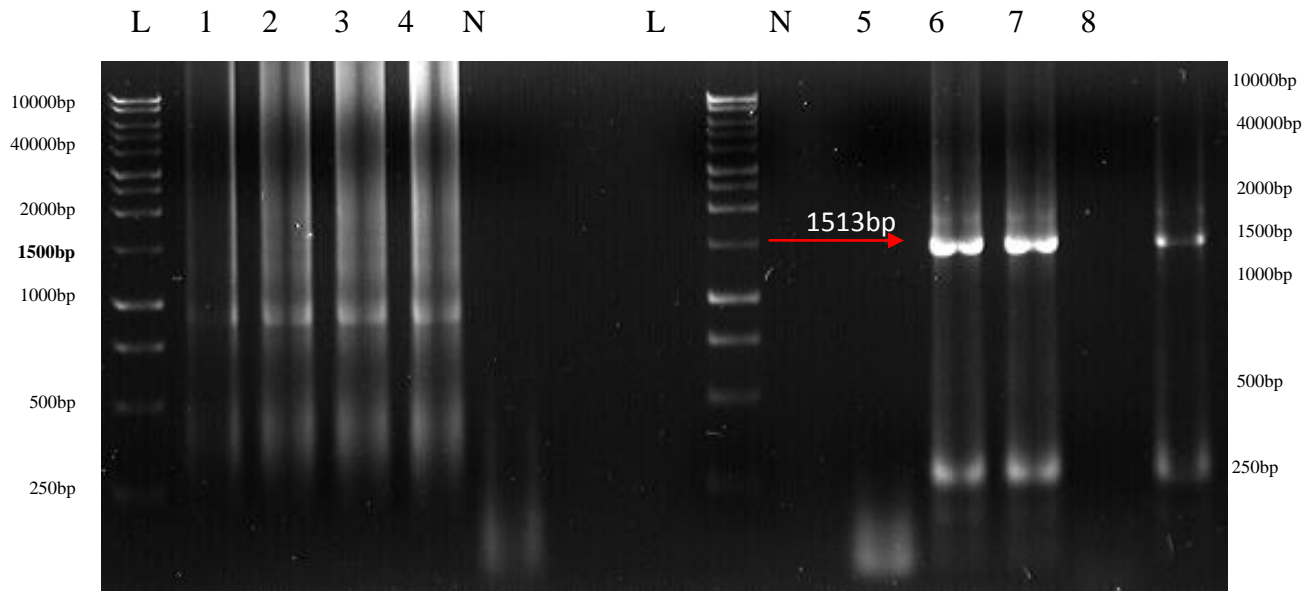

Figure 3.26: Overlap PCR of PSMA G2 and G3 fragments from different DNA templates. Lane 1 to 4 are overlapped PCR of G2 and G3 fragments using PCR product of G2 and G3, lane 5 to 8 are overlapped PCR of G2 and G3 fragments using isolated plasmid from cloning of G2 and G3 fragments.

Gel extraction for overlapped PCR of G1 and G2-3 fragments were done using QIAquick® Gel Extraction Kit (Qiagen) following protocol.
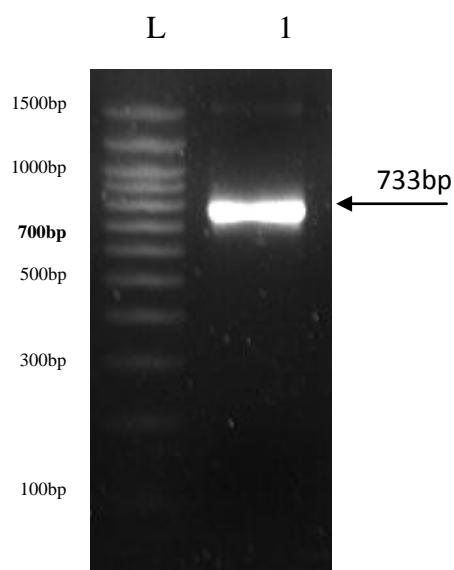

Figure 3.27: Gel extraction of the PCR of G1 fragment from a4 colony plasmids. Lane L is the 100bp ladder (Seegene, Korea) and Lane 1 is the purified G1 fragment.
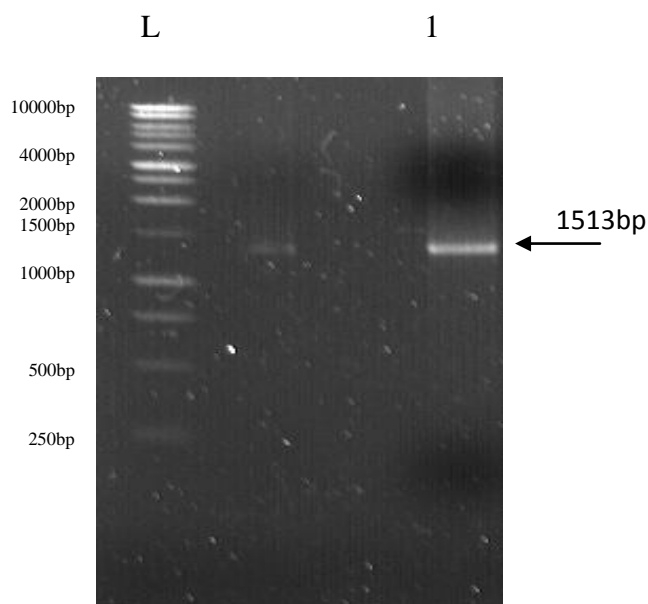

Figure 3.28: Gel extraction of the PCR of G2-G3 fragment. Lane L is the 1kb ladder (Promega, USA), and lane 1 is the purified G2-G3 fragment.
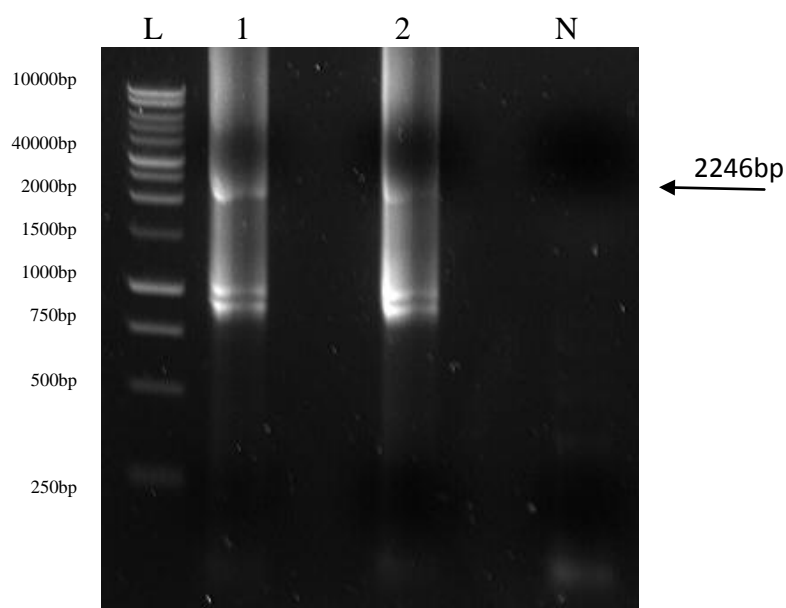
3.2.8 Overlapping PSMA G2-G3 with G1:

Figure 3.29: Overlap PCR of PSMA G2-G3 with PSMA G1. Lane L is 1kb ladder (Promega, USA), lane 1 and 2 is PSMA G1-G2-G3, and lane N is the negative control.
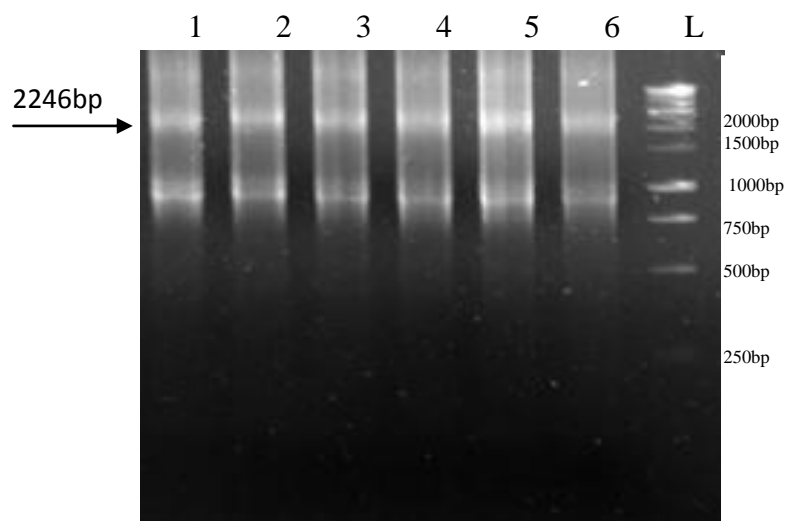

Figure 3.30: PCR optimization of PSMA G1-G2-G3 sample through annealing temperature ranging between 45ºC to 63.4ºC. Lane 1 at 45.0ºC, lane 2 at 48.2ºC, lane 3 at 50.5ºC, lane 4 at 56.7ºC, lane 5 at 59.6ºC and lane 6 at 63.4ºC and lane L is 1kb ladder (Promega, USA).

Although optimizing of the annealing temperature was carried out, it did not produce significant difference at different annealing temperature as all products formed from different annealing temperature was the same and have a smearing effect.

Hence, the product was then purified using gel extraction method and a faint band was produced as shown in figure 3.31.
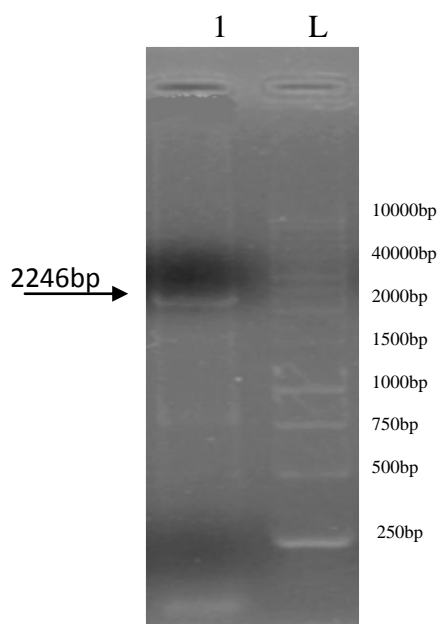

Figure 3.31: Gel extraction of the amplified PSMA G1-G2-G3. Lane L is 1kb ladder (Promega, USA), lane 1 is PSMA G1-G2-G3.

The purified product was subsequently cloned using pGEM®-T vectors to amplify the fragment.

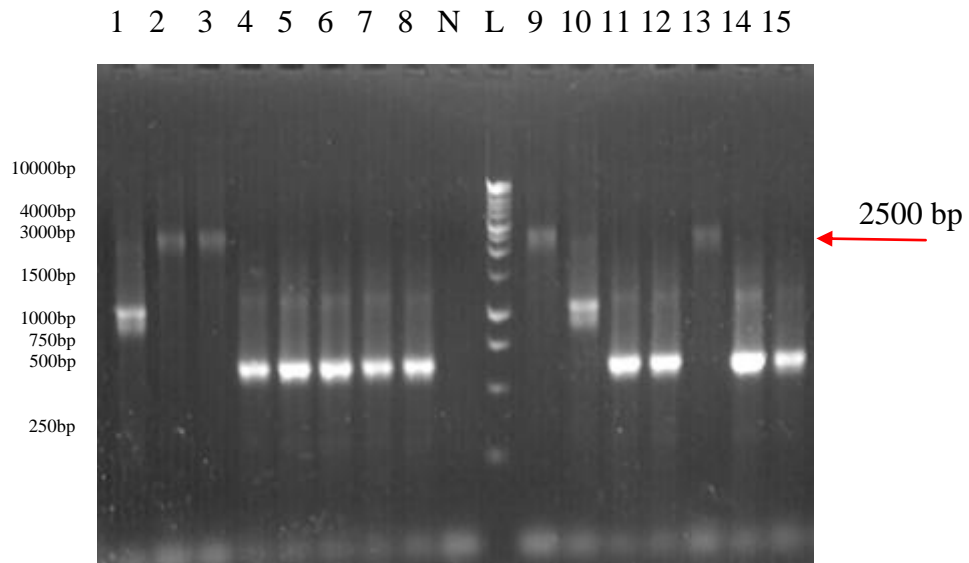1 2 3 4 5 6 7 8 N L 9 10 11 12 13 14 15

2500 bp

Figure 3.32: Colony PCR of PSMA G1-2-3 by using M13 forward and reverse primers. Colonies containing the insert were successfully identified through colony PCR. Lanes 2, 3, 9 and 13 showed bands at the expected size ~2500 bp (2246bp+ 256bp size of specific primers) due to colony having the correct insert. Lane L is 1kb ladder (Promega USA); lane N is the negative control.
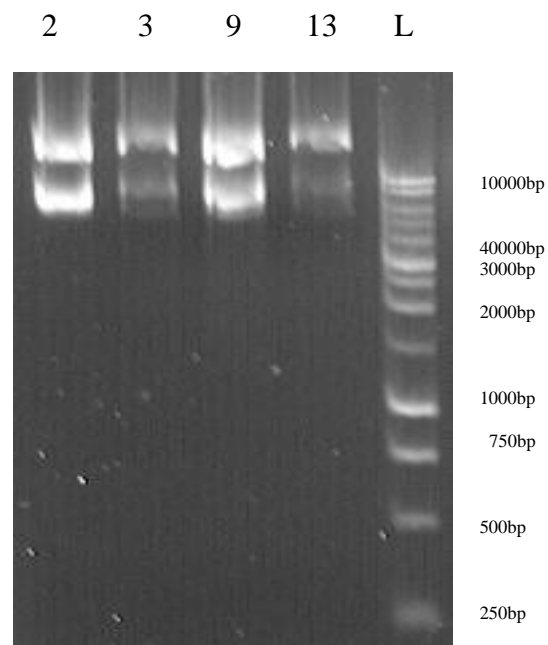
2   3   9   13   L

Figure 3.33: Plasmid isolation of PSMA G1-2-3 of the samples 2, 3, 9 and 13. Lane L is 1kb ladder (Promega USA).

The plasmid samples were sent for sequencing, and subsequently cloned using pPICZαA vector to amplify the fragment after digestion with *Sfi*I and *Not*I restriction enzymes.

3.3 Plasmid digestion with *Sfi*1 and *Not*1

To determine if the insert is in the correct size, the plasmid from both sides was digested with *Sfi*1 and *Not*1 in a total volume of 50 µl. The mixtures were incubated at 37ºC for 3 hours. Results of digestion revealing approximately 2246bp can be seen in figure 3.34. Digested and purified plasmids were analyzed by 1% agarose gel electrophoresis and sent for sequencing.
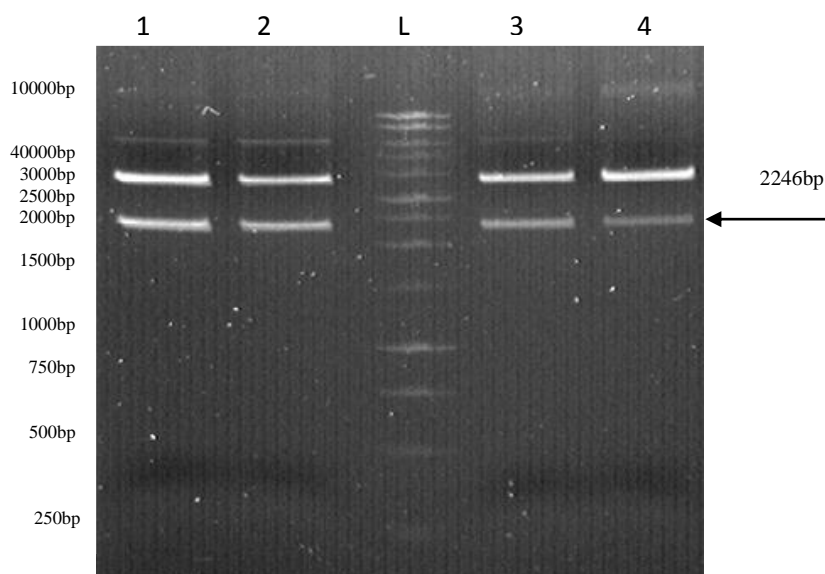

Figure 3.34: Analysis of *Sfi*I and *Not*1 restriction enzymes. Double digestion of plasmid isolated from PSMA G123 colonies. Lane L is 1kb ladder, lane 1- 4 showed a restricted plasmid, indicating that digestion has occurred.

3.4 Colony PCR of PSMA G123

Colony PCR was conducting after ligation of the restricted plasmid PSMA G123 into digested pPICZαA vector and transformed into *E. coli* strain (Top10). 23 colonies were selected and verified by PCR. The PCR was done by using vector primers (Forward primer: α-factor, Reverse primer: 3'AOX). Thus the expected PCR product was 2246bp + 300bp (vector primer size), approximately 2500bp. results of colony PCR are presented in figure 3.35.

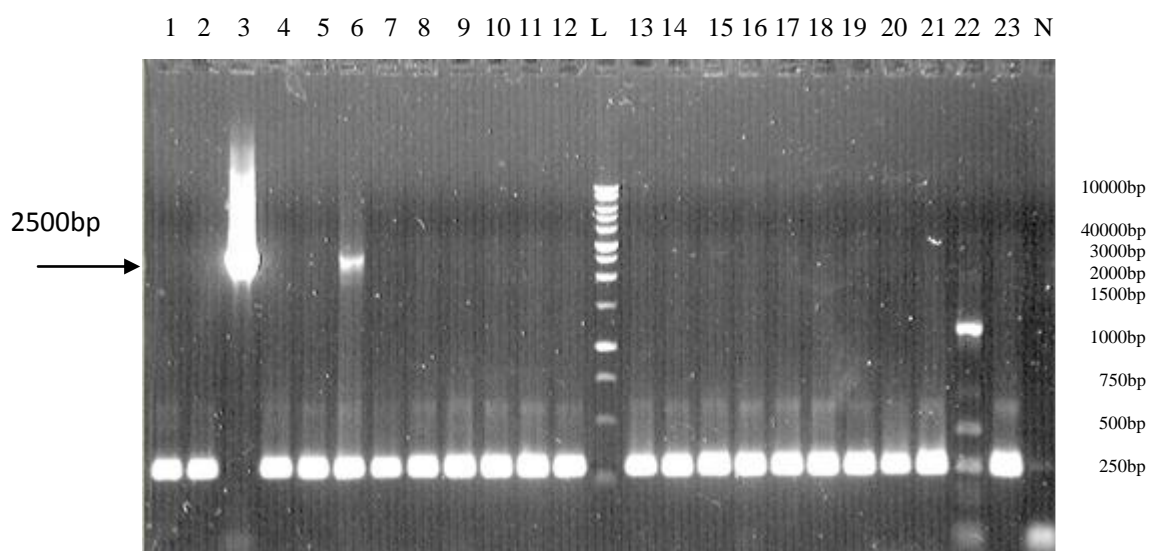Figure 3.35: Colony PCR: Colonies containing the insert were successfully identified through colony PCR. Lane L is 1kb ladder. Lane 1 to 23 showed inserted (3 and 6), and non-inserted colonies, which were determined according to exactly expected size. Lane N is a negative control (PCR reaction without DNA).

Selected colonies, which were determined according to expected size 2500bp, were proceeding for plasmid isolation.
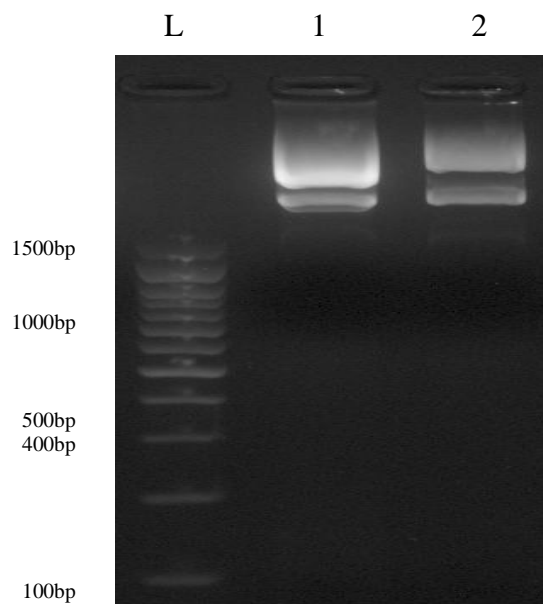
Figure 3.36: Plasmid isolated from colonies 3 and 6 of PSMA G123. Lane L is 100bp (Seegene, Korea), lane 1 is plasmid isolated from clone 3, lane 2 is plasmid isolated from clone 6.

3.5 Linearization of PSMA G123

The recombinant plasmid DNA was first linearized by restriction endonuclease *Sac1*, and then transformed into *P.pastoris* strain X33, by using EasyComp Transformation kit as described in manufacturer's manual (Invitrogen, USA).

Figure 3.37: Linearization of PSMA G123 after digestion with *Sac*1 Restriction enzyme. Lane L is 1kb ladder. Lane 1 is a result of digested plasmid, lane 2 is undigested plasmid.

4. Discussion

PSMA cDNA consists of 2.65 kilobase and a portion of the coding region from nucleotide 1250 to 1700 has 54% homology to the human transferrin receptor mRNA (Israeli RS et al., 1993). In contrast to PSA and prostatic acid phosphatase which are secreted proteins, the prostate specific membrane antigen is an integral membrane protein.
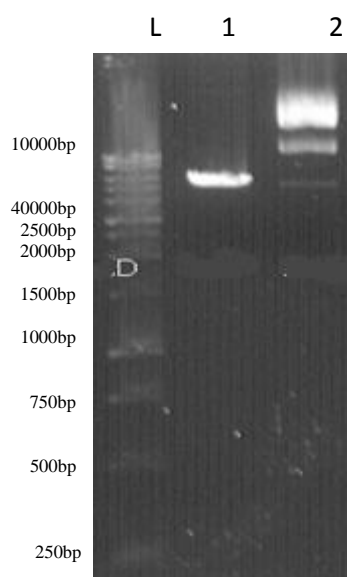
The PSMA (molecular weight 100,000) similarly has representation on both benign and neoplastic prostate cells with more intense staining seen with malignant cells. Moreover, PSMA is an integral membrane protein rather a secreted protein as is PSA and, therefore, may be an even more appropriate vaccine component. The foregoing list of known antigens which are over-represented on prostate: prostatic acid phosphatase (PAP); prostate specific antigen (PSA); and prostate specific membrane antigen (PSMA) is offered for the purpose of illustration. These well known antigens (or the epitope bearing fragments thereof) are proteins (or peptides) and are useful in the vaccines of the invention. However, the invention includes any other antigens substantially uniquely present on the prostate gland so that prostate derived tissue can be distinguished from other tissue by virtue of the presence of these antigens (Watt et al., 1986; Lundwall and Lija, 1987).

For antigens that are proteins or peptides, a number of options is available in addition to isolation and purification. In addition to genetic engineering techniques, peptides, and even proteins, can be prepared using standard chemical synthesis methods, preferably the commercially available solid-phase-based techniques. These techniques are well known and according to the manufacturer's instructions automated systems to conduct them can be purchased and employed.

In addition, protein or peptide antigens may be prepared using genetic engineering. Procedures for the production of pure antigens from the DNA encoding the desired antigen are well known to those skilled in the art. Briefly, the preferred DNA is expressed in a suitable recombinant expression vector such as those adapted for E. coli; yeast, such as Saccharomyces cerevisiae or Pichia pastoris; or filamentous fungi such as Aspergillus nidulans (Berzofsky J & Berkower I., 1989).

The preparation of recombinant forms of protein antigens in a variety of host cells results in a variety of posttranslational modifications which affect the immunogenicity and other pharmaceutical properties, such as pharmacokinetics, of the product. Accordingly, although human prostate-specific antigen (PSA) isolated from human tissues has been used to induce the production of antibodies for diagnostic use, the immunogen prepared in this way differs from the immunogen as prepared in nonhuman cells, such as insect cells. The post-translational modifications peculiar to the recombinant host result in alternations in glycosylation pattern, folding, and the like (Houghten R, 1985).

The technique of recombinant expression may also be used to produce portions of the desired antigen rather than the entire antigen. Whether the antigen or a suitable epitope is prepared synthetically or recombinantly, it may be prepared initially as a fusion protein containing amino acid sequence heterologous to the amino acid sequence of interest. Construction of such fusion proteins is common in recombinant production in order to stabilize the product produced in the cell. It may be unnecessary to stabilize the desired peptide or protein in this way, especially if it is to be secreted from the recombinant cell (Houghten R, 1985, Berzofsky J & Berkower I, 1989).

However, the fusion protein itself may be useful as an ingredient in the vaccine, especially if the additional heterologous amino acid sequence supplies an immunogenicity enhancing property on the relevant epitope. Thus, the fusion proteins which contain the relevant amino acid sequences may be used simply as precursors of the immunogen or may provide the end-product for use in the vaccine. If the fusion protein is intended as an intermediate, it is useful to provide a cleavage site between the heterologous portion and the desired epitope. Such cleavage sites include, for example, the target sequences for various proteolytic enzymes, or, if the epitope does not contain methionine, may constitute simply a methionine residue, which is cleaved by cyanogen bromide. Methods to provide suitable cleavage sites are well known in the art (Hruby D, 1988).

The aim of this study was to clone the complete fragment of *PSMA* gene, but since it is length was about 2.5 kb and it faced difficulties in cloning. So, in this study *PSMA* gene was divided into three parts named as G1 as the first part of the gene, G2 as the second part of the gene and G3 as the last part of the gene.

It is noted that in this study, two steps PCR were used to overlap and amplify the expected fragments to provide a template for amplification. To amplify G1, it was also divided into six sub fragments named a, b, c, d, e and f to facilitate cloning. All was cloned previously except d, so this sub-fragment was amplified by overlapping with other fragments and it later gave the complete G1. The length was 733 bp. To amplify G2 and G3, it was done successfully and the length was 723 bp and 790 bp respectively.

As it was shown in the results, when these three fragments were applied to gel extraction to excise accordingly, surprisingly the excised fragments appeared in

shorter length. The problems was solved by heating the extracted fragment at 80^0 C for five minutes and then cooled down to room temperature.

To find the whole *PSMA* gene, these three fragments were subjected to overlapping and amplification in two steps PCR. To overlap these three, G1-G2 were subjected to overlap and then were supposed to join to G3, but this step as was shown in the results did not give any results, so Gradient PCR was done to find the right annealing temperature. A concentrated and smear pattern was found at 50.5^0 C, whereas no clear band was shown in other temperatures, so this smear pattern was applied for dilution to overcome the problem. Unfortunately after three times dilution, no clear band was found. The expected length was around 1.5 kb based on the length of G1 and G2.

Since amplification of G1-G2 was failed, subsequently the amplification of the joint G1-G2-G3 would also failed, so it seemed that this approach is not correctly chosen. To find the whole *PSMA* gene containing G1-G2-G3, each fragment was cloned separately into pGEM-T vector to proceed for overlapping. On the other side, it is also useful to clone each fragments individually to prepare a good amount of DNA for overlapping step.

Therefore, amplified fragments individually applied for gel extraction and successfully obtained. All were cloned into pGEM-T vector individually and expected clones were picked for plasmid isolation. Although one of the samples showed a faint band (Refer to Fig3.12) in plasmid isolation step due to low resolution of agarose gel but the presence of inserted fragment was proved by PCR and showed a clear band at expected size (900bp), which means that a little amount of template would be enough for amplification in PCR whereas it would not be shown on agarose gel.

To find the nucleotide sequence, the plasmid isolation including the insertion was applied to sequencing. In G1, one deletion was found in each sequenced sample due to *Taq* polymerase enzyme errors as it was shown in the results (Figure 3.14). In G2 and G3 fragments, the successful complete sequence without any deletion was obtained. To check the presence of deletion in G1, this fragment was cloned again into pGEM-T vector and proceeds to sequencing. Some substitution in nucleotide sequences were found among three samples (Figure 3.18). Out of three sequenced G1 samples, one with less affected mutation in amino acid sequences was chosen to proceed to overlapping with G2 and G3. Hopefully the mutation in G1 sequence would not make overlapping failed.

To overlap these plasmid isolated fragments, two approaches were used. First was overlapping one fragment individually in one reaction and second was overlapping all three fragments in one reaction. To compare these two approaches theoretically, it is necessary to mention that first approach is time consuming, however, the second approach is complicated as there would be a competition among three fragments in one reaction to overlap which it affects the specificity and efficiency of the results. In this study both approaches were done.

Based on first approach, G1 and G2 as well as G2 and G3 were overlapped. Based on second approach all three fragments were overlapped and then proceed for amplification using *Pfu* enzyme in both steps of overlapping and amplification. Unfortunately, it was not shown any results. It was thought that changing the enzyme to *Taq* enzyme might solve the problem, so *Taq* enzyme was used in both steps in overlapping and amplification and successfully showed the expected band (Figure 3.20), as well as some unspecific bands may due to contamination of template,

usage of unsterilized pipette, repeated usage of pipette tips, mater mix and even PCR reagents.

Expected fragments were applied to gel extraction. Although they were faint after being extracted, but they all proceed to cloning. Unfortunately, all were failed in sequencing step due to the low ratio of ligation, transformation failure, and low numbers of clones containing overlapped fragments.

Probability, previous trial was failed due to the low concentration of fragments, so the amplification of G1 as well as G2-G3 was optimized to get high concentration and purity. To optimize G1 amplification, one unit of *Taq* and one unit of *Pfu* were used to amplify the fragment. As it was shown in the results (Figure 3.22), by using of *Taq* compared to *Pfu*, fragment was amplified expectedly. It might be due to high efficiency of *Taq* enzyme in amplification. It was also shown that changing the amount of *Pfu* from one unit to one unit and a half changed the result and gave the expected amplicon, which means that increasing the amount of enzyme will help amplification accordingly.

Three optimization steps were done for G2-G3 as increasing the amount of enzyme, testing PCR machine and changing the template. Using of *Taq* enzyme from one unit to two units was shown that expected fragment was being amplified when two units of *Taq* enzyme was used (Figure 3.24). This can explain that increasing the amount of enzyme again can lead to more product amplification.

Testing two PCR machine surprisingly gave different results as follows (Figure 3.25). When PTC 200 was used for amplification, no band was amplified whereas with PCR system 2400 expected band was amplified. It seems that PCR machine itself also affects the results in such way that when they should adjust to exact

temperature and time, they fail. Also it was thought that PCR 200 machine fails to amplify the longer fragment more than 1500 bp.

For template optimization, using of amplified fragments by PCR as a template failed to provide a good template for overlapping compared to cloned fragments in plasmid which shows that when fragments clone into vector give better template for overlapping and amplification. This is might be due to the amount and concentration of DNA as a template.

To summarize all optimization work which was done, it is worth to mention that two units of *Taq* enzyme, PCR machine 2400 and Plasmid isolation as a template were ended to result. To continue G1 overlapping with G2-G3, gel extraction was done successfully which was applied to overlapping. The expected band was obtained as well as some unspecific bands due to contamination mentioned before. To remove the unspecific bands, gradient PCR was done but this step apparently did not change the previous result, as different temperature in gradient PCR did not produce different result accordingly due to errors in PCR machine in adjusting the exact temperature. However, the expected fragments were excised and proceed for cloning. The length of plasmid together with the inserted fragment was about 2500bp (figure 3.32), which was found and proceed for sequencing.

To carry out the cloning into pPICZαA vector, plasmid samples were digested with *Sfi*1 and *Not*1 restriction enzyme from the both sides. Digested and purified plasmids were sent for sequencing. However, the digested plasmids were cloned into digested pPICZαA vector, clones were amplified using vector specific primer; the result shown some of the colonies at the expected size 2500bp (Figure 3.35); therefore the clones with the corrected fragment size was proceed for sequencing.

PSMA G123 fragment was then linearized by using *sac*1 restriction enzyme. Additionally, linearized DNA can be inserted in high efficiency via homologous recombination procedures to generate stable cell lines whilst expression vectors can be readily prepared that allow multiple copies of the target protein.

6. Conclusion

In this project, partial PSMA was successfully sequenced but due to limit of time, it was not troubleshoot to get the complete fragment. By looking into the importance of PSMA, it is worth to find the complete fragment of PSMA to be able to be expressed in Pichia pastoris as an expressing system with more effort and time.

To overcome this problem, it is suggested to use special *Taq* enzyme with a special technique such as LA technique which can help to amplify PSMA gene. In LA technique, conventional Taq polymerase mixes with a thermostable proof reading enzyme with a 3' to 5' exonuclease activity. The proof reading enzyme removes mismatched nucleotides as they are incorporated and replace them with the correct nucleotides, thus allowing Taq polymerase to continue amplifying the target DNA. The presence of both enzymes significantly improves fidelity and processivity which results in high yields of highly accurate.

Moreover, in order to study the substitution of nucleotides, new primers could be designed to fix the differences in these nucleotide and subsequent amino acid sequences after overlapping the three fragments of the gene.

In conclusion, the purpose of this research was to providing PSMA to be used at the expression point in *Pichia pastoris*.

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Appendix
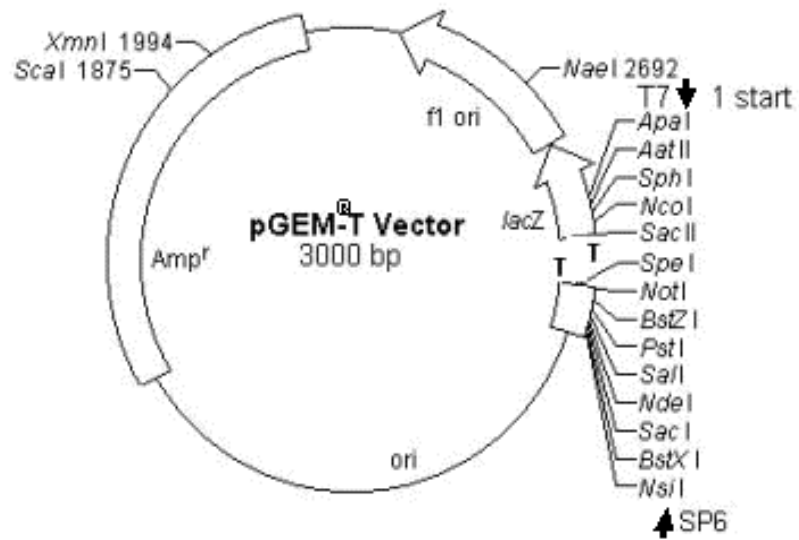
Source: www.promega.com
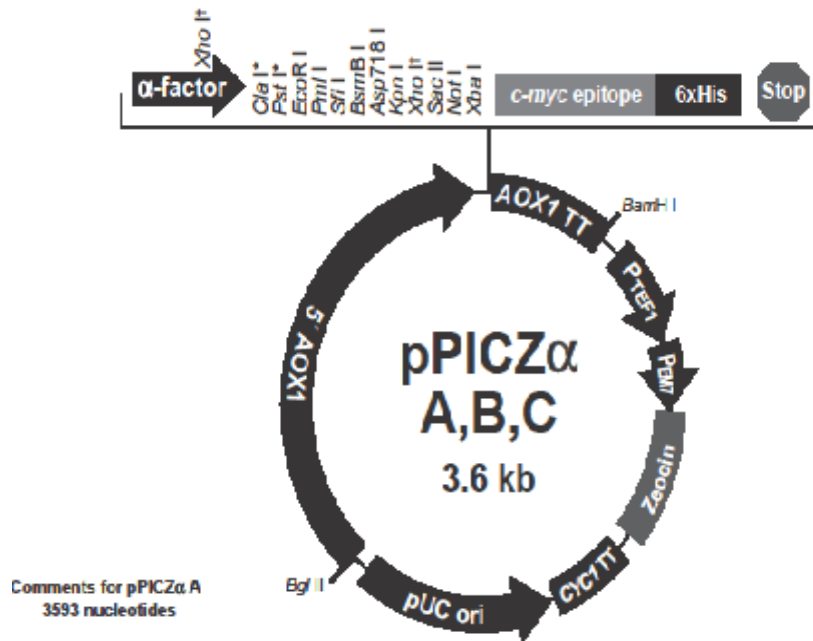
Source: www.invitrogen.com

(a)   The summary of features available on the pGEM-T and pPICZαA vectors used to clone the PSMA fragments into JM109 and Top10 competent cells respectively.

(b)   Primers used for amplification of G1, G2 and G3 fragments of PSMA (overlapping extension).

Fragment	Primers	Primers Sequence
G1		
a	SfiPSMA-F	5'-cttcgggcccagccggccgatgtgcaatctccttcacgaaac-3'
	PSMA-961R	5'-gaagattccaaccatctggataggacttcac-3'
b	PSMA2Fo	5'- tcttcgggtggtttataaaatcctcca-3'
	PSMA2Ro	5'- aaattatataagaacttcttgatgttc-3'
c	PSMA3Fo	5'- cttatataattttacacagataccaca-3'
	PSMA3Ro	5'- gaaaatctcatttccatcttcattaat-3'
d	PSMA4Fo	5'- aatgagattttcaacacatcattattt-3'
	PSMA4Ro	5'- atcgccctctggcattccttgaggaga-3'
	G1d_oligo1	5'- AAT GAG ATT TTC AAC ACA TCA TTA TTT GAA CCA CCT CCT CCA GGA-3'
	G1d_oligo2	5'- CAC TGA AAG GTG GTA CAA TAT CCG AAA CAT TTT CAT ATC CTG GAG-3'
	G1d_oligo3	5'- CCA CCT TTC AGT GCT TTC TCT CCT CAA GGA ATG CCA GAG GGC GAT-3'
e	PSMA5Fo	5'- ccagagggcgatctagtgtatgttaac-3'
	PSMA5Ro	5'- tttaaccttatttcctctgaaaacttt-3'
f	PSMA6Fo	5'- aataaggttaaaaatgcccagctggca-3'
	PSMA-961Ro	5'-attccaaccatctggataggacttcac-3'
G2	PSMA-961F	5'-tccagatggttggaatcttcctggaggtggt-3'
	PSMA-1741R	5'-gccactgaactctggggaaggacttttttta-3'
G3	PSMA-800F	5'-cttccccagagttcagtggcatgcccaggat-3'
	PSMAfacXa-Not1R	5'-agctggcggccgcgcggccttcaatggctacttcactcaaa-3'

(c)   Full sequence of PSMA G1, G2 and G3

G1 sub-fragment sequences

FRAGMENT A

ATGTGGAATCTCCTTCACGAAACCGACTCGGCTGTGGCCACCGCGCGCCGCCCGCGCTGGCT
GTGCGCTGGGGCGCT**G**GTGCTGGCGGGTGGCTT**C**TTTCTCCTCGGCTTCCTCTTC

FRAGMENT B

GGGTGGTTTATAAAATCCTCCAATGAAGCTACTAACATTACTCCAAAGCATAATATGAAAGC
ATTTTTGGATGAATTGAAAGCTGAGAACATCAAGAAGTTCTTATA

FRAGMENT C

TAATTTTACACAGATACCACATTTAG**C**AGGAACAGAACAAAACTTTCAGCTTGCAAAGCAA
ATTCAATCCCAGTGGAAAGAATTTGGCCTGGATTCTG**T**TGAGCTAGCACATTATGATGTCCT
GTTGTCCTACCCAAATAAGAC**T**CATCCCAACTACATCTCAATAATTAATGAAGATGGAAATG
AG

FRAGMENT D

ATTTTCAACACATCATTATTTGAACCACCTCCTCCAGGATATGAAAATGTTTC**G**GATATTGTA
CCACCTTTCAGTGCTTTCTCTCCTCAAGGAATGCCAGAG

FRAGMENT E

GGCGATCTAGTGTATGTTAACT**A**TGCACGAACTGAAGACTTCTTTAAATTGGAACGGGACAT
GAAAATCAATTGCTCTGGGAAAATTGTAATTGCCAGATATGGGAAAGTTTTCAGAGGAAAT
AAG

FRAGMENT F

GTTAAAAATGCCCAGCTGGCAGGGGCCAAAGGAGTCATTCTCTACTC**C**GACCCTGCTGACTA
CTTTGCTCCTGGGGTGAAGTCCTATCCAGATGGTTGGAATCTTCCTGGAGGTGGTGTCCA
GATATCCTAAATCTGAATGGTGCAGGAGACCCTCTCACACCGCAAATG

G2 fragment sequence

GCAGGAgAcCCTCTCACACCAGGTTACCCAGCAAATGAATATGCTTATAGGCGTGGAATT
GCAGAGGCTGTTGGTCTTCCAAGTATTCCTGTTCATCCAATTGGATACTATGATGCACAG
AAGCTCCTAGAAAAAATGGGTGGCTCAGCACCACCAGATAGCAGCTGGAGAGGAAGTCTC
AAAGTGCCCTACAATGTTGGACCTGGCTTTACTGGAAACTTTTCTACACAAAAAGTCAAG
ATGCACATCCACTCTACCAATGAAGTGACAAGAATTTACAATGTGATAGGTACTCTCAGA
GGAGCAGTGGAACCAGACAGATATGTCATTCTGGGAGGTCACCGGGACTCATGGGTGTTT
GGTGGTATTGACCCTCAGAGTGGAGCAGCTGTTGTTCATGAAATTGTGAGGAGCTTTGGA
ACACTGAAAAAGGAAGGGTGGAGACCTAGAAGAACAATTTTGTTTGCAAGCTGGGATGCA
GAAGAATTTGGTCTTCTTGGTTCTACTGAGTGGGCAGAGGAGAATTCAAGACTCCTTCAA
GAGCGTGGCGTGGCTTATATTAATGCTGACTCATCTATAGAAGGAAACTACACTCTGAGA
GTTGATTGTACACCGCTGATGTACAGCTTGGTACACAACCTAACAAAAGAGCTGAaAAGC
CCTGATGAAGGCTTTGAAGGCAAATCTCTTTATGAAAGTTGGACTAAAAAAAGTCCTTCC
CCAGA

G3 fragment sequence

GTTCAGTGGCATGCCCAGGATAAGCAAATTGGGATCTGGAAATGATTTTGAGGTgtCTTC
CAACGACTTGGAATTGCTTCAGGCAGAGCACGGTATACTAAAAATTGGGAAACAAACAAA
TTCAGCGGCTATCCACTGTATCACAGTGTCTATGAAACATATGAGTTGGTGGAAAAGTTT
TATGATCCAATGTTTAAATATCACCTCACTGTGGCCCAGGTTCGAGGAGGGATGGTGTTT
GAGCTAGCCAATTCCATAGTGCTCCCTTTTGATTGTCGAGATTATGCTGTAGTTTTAAGA
AAGTATGCTGACAAAATCTACAGTATTTCTATGAAACATCCACAGGAAATGAAGACATAC
AGTGTATCATTTGATTCACTTTTTTCTGCAGTAAAGAATTTTACAGAAATTGCTTCCAAG
TTCAGTGAGAGACTCCAGGACTTTGACAAAAGCAACCCAATAGTATTAAGAATGATGAAT
GATCAACTCATGTTTCTGGAAAGAGCATTTATTGATCCATTAGGGTTACCAGACAGGCCT
TTTTATAGGCATGTCATCTATGCTCCAAGCAGCCACAACAAGTATGCAGGGGAGTCATTC
CCAGGAATTTATGATGCTCTGTTTGATATTGAAAGCAAAGTGGACCCTTCCAAGGCCTGG
GGAGAAGTGAAGAGACAGATTTATGTTGCAGCCTTCACAGTGCAGGCAGcTGCaGAGACT
TTGAGTGAAGTAGCCATTGAAGGCCGC