Novel approaches in quantitative polymerase chain reaction.

Monique Elise. Verhaegen

University of Windsor

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NOVEL APPROACHES IN QUANTITATIVE POLYMERASE CHAIN REACTION

by

Monique Elise Verhaegen

A Thesis
submitted to the Faculty of Graduate Studies and Research
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
1998
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ABSTRACT

NOVEL APPROACHES IN QUANTITATIVE POLYMERASE CHAIN REACTION

by

MONIQUE ELISE VERHAEGEN

Perhaps the most challenging current analytical problem associated with PCR is the determination of the starting quantity of target DNA. The objective of this research was to devise novel quantitative PCR assays for DNA and RNA which are automatable, highly sensitive and easily adaptable to the routine clinical chemistry laboratory. One project focused on the development of the first quantitative assay for prostate-specific antigen (PSA) mRNA based on co-amplification with a recombinant RNA internal standard (IS) and hybridization performed in microtiter wells. The PSA mRNA and the IS, differing only in a 24 bp centrally located sequence, contained the same primer recognition sites and generated amplification products of identical size. After reverse transcriptase-polymerase chain reaction, the products were captured through biotin/streptavidin, hybridized separately to specific digoxigenin-labeled probes and detected with alkaline phosphatase labeled anti-digoxigenin antibody and time-resolved fluorometry. Samples containing total RNA from prostate cancer cells amidst 1 μg of RNA from healthy cells were analyzed. The ratio of the fluorescence values obtained for PSA mRNA and IS was linearly related to the number of cells in the range of 4 to 3000 cells.

The goal of the second project was to develop a dual-analyte chemiluminescence hybridization assay for simultaneous determination of both amplification products in the
same well, thereby facilitating automation of quantitative PCR. Aequorin and alkaline phosphatase were used as reporter molecules. The proposed quantitative PCR assay could detect as low as 430 target DNA molecules and the linear range extended up to 315000 molecules.
ACKNOWLEDGEMENTS

I wish to begin by thanking my supervisor Dr. Christopoulos (TKC), for his remarkable and infectious enthusiasm, passion, and excitement regarding research. His guidance, teaching and not to mention, insatiable quest for results, have truly challenged me and provided me with a valuable learning experience.

My regards to all who have come and gone in the TKC lab: Susan, Barbara, Norman, Stephanie, James, Elita, Dr. Ioannou, Bakhos and Pierre. Thanks for the help, the advice, the sanity, and the craziness. Special thanks to “PCR Queen” Sue for being a fabulous mentor, and to Elita for being a fabulous girlie and fellow Monita member.

Thanks to all other Essex Hall students (big bro lab Joe), staff and faculty for their guidance, assistance and kindness; Dr. Adeli and Dr. Cotter, for serving as committee members; the math boys who once allowed me to be an accessory member; my friends, parents and sisters (Genevieve and Claudine) for their support and tolerance of me during times of frustration; and NSERC and the University of Windsor for financial assistance.
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<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>anti-DIG-ALP</td>
<td>alkaline phosphatase conjugate of anti-digoxigenin antibody</td>
</tr>
<tr>
<td>amol</td>
<td>attomole</td>
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<tr>
<td>B</td>
<td>biotin</td>
</tr>
<tr>
<td>BL</td>
<td>bioluminescent</td>
</tr>
<tr>
<td>bp</td>
<td>basepair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CL</td>
<td>chemiluminescent</td>
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<tr>
<td>CSPD</td>
<td>1,2-dioxetane chemiluminescent enzyme substrate:</td>
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<td></td>
<td>Disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-5-chloro) tricyclo[3.3.1.1&lt;sup&gt;3,7&lt;/sup&gt;]decan)-4-yl)phenyl phosphate</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine 5' triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylypyrocarbonate</td>
</tr>
<tr>
<td>DFP</td>
<td>diflunisal phosphate</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DIG-dUTP</td>
<td>digoxigenin-11,2'-deoxyuridine triphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>DRE</td>
<td>digital rectal exam</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid disodium salt</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis-(β-amino ethyl ether) N,N,N',N'-tetra acetic acid</td>
</tr>
<tr>
<td>Eu&lt;sup&gt;3+&lt;/sup&gt;</td>
<td>europium ion</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>G</td>
<td>guanine</td>
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<tr>
<td>IS</td>
<td>internal standard</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LC</td>
<td>long chain</td>
</tr>
<tr>
<td>LH-RH</td>
<td>leutening hormone-releasing hormone</td>
</tr>
<tr>
<td>M</td>
<td>molar = mol/L</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>M-MLV RT</td>
<td>Moloney murine leukemia virus reverse transcriptase</td>
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<tr>
<td>mmol</td>
<td>millimole</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide ester</td>
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<tr>
<td>nt</td>
<td>nucleotide</td>
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<tr>
<td>NTP</td>
<td>ribonucleoside triphosphate</td>
</tr>
<tr>
<td>oligo</td>
<td>oligonucleotide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate specific antigen</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
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<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription PCR</td>
</tr>
<tr>
<td>SA</td>
<td>streptavidin</td>
</tr>
<tr>
<td>S/B</td>
<td>signal-to-background</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
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<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus Aquaticus</em></td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>Tb⁺⁺</td>
<td>terbium ion</td>
</tr>
<tr>
<td>Tₘ</td>
<td>melting temperature</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>TRUS</td>
<td>transurethral ultrasound</td>
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<td>UV</td>
<td>ultraviolet</td>
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CHAPTER 1
GENERAL INTRODUCTION

1.1 The Polymerase Chain Reaction

1.1.1 The Principle of the Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a chemical means of increasing the concentration of a specific nucleic acid in vitro (1). This technique was originally the brainchild of Dr. Kary Mullis who initially tried to devise a method which would utilize dideoxy sequencing and DNA polymerases to identify nucleotides at particular positions in a DNA molecule (2). Eventually this idea led to the advent of the polymerase chain reaction. It was first introduced as a powerful technique to amplify specific nucleic acid sequences in 1985 (3). Mullis received the Nobel Prize in Chemistry in 1993 for his contribution.

The principle of PCR is based on the enzymatic amplification of nucleic acid sequences by repeated cycling at different temperatures (1). Three temperature steps favouring denaturation (~95°C), oligonucleotide (oligo) annealing (37°C-65°C), and DNA synthesis (~72°C) make up one reaction cycle (4). In the first heating step, the double stranded (ds) DNA is denatured to form two single strands. Subsequent cooling allows the binding of complementary oligo primers to opposite strands of the target fragment. The final temperature change promotes the 5' to 3' extension of the oligo primer, catalyzed by a DNA polymerase. The primers are positioned so that the newly synthesized strands will overlap the binding site of the opposite oligo primer (5).

Following one complete cycle, two copies containing the sequence of interest are generated. As the cycling continues, the primers bind the original target as well as the newly synthesized strands. After repeated denaturation, oligo annealing and DNA extension, the end result is an exponential increase in the total number of DNA fragments, reaching a theoretical abundance of $2^n$ copies where $n$ is the number of cycles.
performed. These resulting amplification products are a discrete length, defined by the 5' termini of the primers. The principle of PCR is illustrated in Figure 1.1.

1.1.2 The Components of PCR

The essential components for PCR amplification are relatively simple. A source containing the desired sequence to be amplified is required. In the presence of a molar excess of the four deoxyribonucleoside triphosphates (dNTPs), the two oligo primers, the DNA polymerase and an appropriate MgCl₂ containing buffer, the specific template can be amplified in a single reaction tube in an automated thermal cycler (4,5).

1.1.2.1 The Target

The sequence to be amplified can be either ss-DNA, ds-DNA or RNA. Reverse transcriptase PCR (RT-PCR) allows RNA to be used as a starting template, provided that an initial reverse transcription reaction be carried out (4). Using a reverse transcriptase enzyme, acting as an RNA-directed DNA polymerase, a complementary DNA (cDNA) copy of the RNA is created that can be amplified by PCR.

It is not necessary that the desired nucleic acid sequence be present in a pure form. It can be a minor fraction of a complex mixture, a discrete molecule or even part of a larger molecule (1). The optimal target sequence to be amplified should be in the 100-1000 nucleotide (nt) size range, but sequences ranging several kilobases in length have now been successfully amplified (6). Regardless of the starting material, the product will be a discrete molecule defined at both termini by the position of the primers (1).

1.1.2.2 The Primers

The oligo primers define the amplification products and are chosen so that they are complementary to the end regions of opposing strands. For this reason some knowledge of the target base sequence is desirable. The primers are oriented with their 3' OH groups facing one another so as to allow the polymerase to extend in the 5' to 3' direction (4, 5).
FIGURE 1.1
The Principle of the Polymerase Chain Reaction

Legend

A simplified schematic of the amplification of DNA by the polymerase chain reaction. Exponential amplification occurs as the number of ds DNA copies doubles, following each cycle of denaturation, primer annealing and polymerase extension. After repeated cycling, the major product is defined by the distance spanning the 5' ends of the two primers. Following n cycles, the theoretical yield of PCR is $2^n$ copies.

[Adapted and modified from Mullis (1).]
Primers are usually about 20-30 bases in length since shorter primers will yield non-specific products and much longer sequences will produce a low yield. The melting temperature (T_m) of the primers, dependent on the G-C content, should be identical or similar for both primers to allow equal hybridization to the target during the annealing step.

PCR was initially constrained by the fact that the starting template had to be known in order to create primers that were complementary to the end regions of the desired sequence to be amplified. Specific strategies have now been employed to directly overcome this limitation. The use of generic primers complementary to repetitive DNA families or the addition of flanking sequences through ligation (7) or terminal transferase (8) to introduce known priming regions, can be used to initiate annealing and extension of unknown sequences (4).

An alteration or addition to the primer sequences can be used to introduce new nucleotide information into a PCR product. This allows for the creation of restriction sites (9), specific mutations, new base sequences, insertions or regulatory elements such as promoters (10). Sequence modification through \( \text{Pci} \_1 \) has greatly improved upon older, more time-consuming techniques such as chemical mutagenesis or construction of oligos to be cloned into copies of the normal sequence (5).

Primers can also be used to incorporate binding moieties such as biotin or fluorescent and radioisotope tags into the amplification products (11). Because the DNA polymerase adds to the 3' end of the primer, the 5' end can be successfully altered to create modified PCR products differing from the starting template.

Amplification of the same sequence by two different PCR reactions, allows for site-directed mutagenesis whereby a mutation at any position in the fragment may be introduced, rather than only at the 5' ends. Two overlapping DNA fragments, both bearing the same mutation introduced via primer mismatch, are produced such that the complete fragment can then be reconstructed, simply by 3'-terminal overlap annealing.
and polymerase extension of each 3' end templated by the complementary fragment, provided the primers contain the 5'-terminal extensions into one another’s sequence (12).

1.1.2.3 The DNA Polymerase

DNA polymerase catalyzes the addition of dNTPs to the 3'-OH group of a DNA template allowing the chain to grow in a 5' to 3' direction. Initial PCR studies utilizing the Klenow fragment of Escherichia coli (E. coli) DNA polymerase I required the addition of enzyme after each denaturation step (3). A major improvement contributing to PCR’s success was the introduction of the heat-resistant DNA polymerase from the thermophilic bacterium Thermus Aquaticus (Taq) which could withstand the high temperature of the denaturation step, and was therefore added only once to the amplification reaction with the other necessary reagents in a single tube (13).

The purification and commercial availability of this thermostable DNA polymerase enabled the automation of PCR as well as the increased specificity and yield (13). PCR products as long as 10 kilobases (kb) could now be amplified, compared to the limited 400 base size obtained with the Klenow fragment (4). While Taq has no 3' to 5' exonuclease (proofreading) activity, it has a 5' to 3' exonuclease activity during polymerization (13). Depending on the goal of PCR, the degree of the misincorporation rate of bases can be somewhat controlled by altering conditions that affect the specificity of PCR.

1.1.3 Specificity of PCR

The specificity of PCR refers to the ability to amplify the desired sequence with correct sequence and size. Since PCR is dependent on the binding of complementary primers, the specificity of PCR may therefore be increased by increasing the stringency with which the primers bind. This can be achieved by decreasing the concentration of MgCl₂ and increasing the annealing temperature to promote only exact complementary hybridization. Concentrations of primers, dNTPs, and enzyme as well as annealing and extension times and number of cycles also affect the specificity (4).
Specific strategies such as the manual addition of one of the essential PCR reagents in a "hot start" protocol after the ds DNA target is denatured, minimize the non-target amplification (4), hence increasing the specificity. This protocol also minimizes the formation of "primer-dimers" which may result as ds-PCR products of the two primers and their complementary sequences (4).

1.1.4 Efficiency of PCR

A discrepancy remains between the theoretical and observed efficiency of PCR. This is directly dependent on the initial lag phase, the rate of doubling and the eventual saturation of the reaction. A low efficiency during the first few cycles will greatly influence the overall yield of the reaction due to the exponential nature of the reaction (5).

The initial binding of the primers may be hindered due to structural constraints, strand breaks or reannealing of the original parent strands. Although the PCR ingredients are added in excess so as not to limit the reaction, a plateau phase is reached due to substrate saturation of the enzyme and competition between primer/template annealing and template/template annealing.

Until the plateau phase is reached, the accumulation of product is an exponential function defined by the equation \( P = T(1+E)^n \), where \( P \) is the amplification product, \( T \) is the initial target, \( n \) is the number of cycles and \( E \) is the average efficiency of the reaction. Theoretically, \( E \) is 1 and the product doubles after each cycle. However, the typical gain per cycle during exponential amplification is 1.80-1.95, slightly less than the theoretical maximum of 2.0 (6). PCR product accumulates exponentially up to about 10⁻⁸ M, rapidly declining to linear accumulation between 10⁻⁴ M and 10⁻⁷ M (6).

1.1.5 PCR Contamination

The extreme sensitivity of PCR renders it vulnerable to carryover DNA contamination from previous amplification reactions. This carryover contamination is difficult to control and even more difficult to eliminate. Careful attention is required to
laboratory procedure, such as pre-aliquoting reagents, use of dedicated pipettes, physical separation of the reaction setup and reaction product analysis and ultraviolet (UV) irradiation of lab equipment to minimize the carryover contamination (14). Negative controls lacking starting template are also employed to detect contamination which may occur during preparation of the PCR.

1.1.6 Quantitative-PCR

Qualitative analysis of PCR products has traditionally allowed detection and identification of amplified products by size or sequence dependent means. Ethidium bromide stained gel electrophoresis (6) or annealing of a labeled complementary probe to a specific sequence, were the accepted methods of detection (13,15). Quantitative methods using Northern and Southern blot hybridizations, along with densitometry to measure band intensity, proved to be only semi-quantitative at best, and unsuitable for clinical laboratory analysis (16). The continuous development of PCR technology has demanded a method for the quantification of PCR products.

The principle of quantitative PCR (Q-PCR) is to relate the magnitude of amplification to the initial starting concentration of the specified target. The main concern in the development of a Q-PCR technique has been to account for the reaction-to-reaction variability inherent in PCR. Slight variations in the reaction set-up or in the early cycles of PCR will significantly alter the kinetics of the reaction (16). This variability results from thermocycler-dependent temperature deviations, the presence of DNA polymerase inhibitors, pipetting variations and the abundance of the target sequence in the specimen of interest (17).

To date, Q-PCR techniques have relied on the inclusion of some type of known control to correct the yield for any variations in amplification. The three strategies adopted thus far are based on external standards, non-competitive internal standards (IS) or competitive internal standards (17).
External standards are based on serial dilutions of a known amount of standard amplified in parallel with the sample of interest. Provided a linear relationship between the target and the product is observed for the standard dilutions, the relative amount of sample target can be deduced (17). The flaw of this procedure is that any variation in the setup may drastically affect the precision and reproducibility.

Quantification with non-competitive internal standards is achieved via a co-amplification with a second endogenous target or housekeeping gene, such as β-actin (18), with the target of interest. This second target shares neither the primer binding sites nor the region spanning the target of interest and is expected to be quantitatively invariant in the experimental system but functionally related to the target (19,20).

Competitive PCR involves co-amplification of the primary target with an added synthetic IS, using a single set of primers for both sequences. Various strategies have been employed including co-amplification with the same primers of an added IS; i) differing in length from the target and distinguishable by gel electrophoresis (21), ii) identical to the target with a base substitution introducing a novel restriction site (22), or iii) identical to the target except for a centrally located sequence distinguishable by hybridization (23).

1.1.7 Applications of PCR

Since PCR has the ability to selectively and exponentially amplify nucleic acid sequences to levels that are orders of magnitude higher than the starting material, it has potential in any situation, research or medical, that requires the examination or analysis of DNA or RNA. Applications may be relevant in the following areas: archeology and evolutionary studies, forensics, diagnosis and characterization of genetic disease, diagnosis and characterization of infectious bacterial or viral diseases, and diagnosis and characterization of neoplasia.

The superior sensitivity of PCR has, over the past 10 years, made it one of the most important analytical techniques in all aspects of biological research and molecular
diagnosis. The multi-faceted nature of PCR has enabled its specific application to
different testing needs in the clinical laboratory. Current trends favour the routine
implementation of PCR as a potential clinical diagnostic and quantitative tool.

1.2 Prostate Cancer

The prostate is a small-walnut sized gland situated just below the bladder where it
encircles the urethra in the male. It consists of three lobes: two lateral lobes and a middle
lobe encased in a firm fibrous capsule. During ejaculation the gland secretes a mixture of
prostaglandins, proteolytic enzymes, growth factors, sugars, acids and trace metals into
the urethra. These substances are thought to nourish and aid in the transport of sperm.

Adenocarcinoma of the prostate is the most commonly diagnosed solid tumor
with approximately 334,500 cases diagnosed in the United States in 1997 (24). It is
responsible for more than 40,000 deaths annually (24) and is the second leading cause of
cancer related deaths (behind lung cancer) (25).

1.2.1 Prostate Cancer Metastasis

Cancer metastasis, or the spread of cells from the primary site to a distant site, is
the most dreaded aspect of cancer, as most cancer deaths are due to metastases that are
resistant to therapies (41). Tumor metastasis requires the exodus of cancer cells from the
primary site, survival in the blood stream, adhesion to an extracellular matrix, invasion
and penetration at a distant site, and establishment of metastatic tissue at the secondary
site (42). Immune clearance makes it highly unlikely for normal, benign hyperplastic
prostate cells, or cancer cells that have broken from the primary site, to survive in the
peripheral circulation (41).

The presence of these cancer cells in the peripheral circulation does not
necessarily indicate metastases has occurred. However, the detection of circulating
prostate cancer cells may indicate the tendency for metastases and may serve as an
independent prognostic indicator for patients with prostate cancer (29). The ability to
detect malignant spread at its earliest stage is desirable to allow for efficient therapeutic intervention before the tumor burden becomes too great.

1.2.2 Prostate Specific Antigen

Many cancer cells synthesize products that are inappropriate, abnormal or produced at much higher concentrations than normal. These tumour markers may serve as biochemical indicators of a specific malignancy. The ideal tumour marker would have the following characteristics: specificity and sensitivity to the tumour of interest, serum levels correlating with the tumour mass, uses in monitoring response to treatment and uses in prediction of recurrence and prognosis.

Prostate specific antigen (PSA) is a 33 kD single chain glycoprotein with kallikrein-like serine protease activity, believed to be involved in the liquefaction of the seminal coagulum formed at ejaculation (27). Produced by the epithelial cells lining the acini and ducts of the prostate gland, it is secreted in seminal fluid at concentrations of 0.5-5.0 g/L (26). PSA is thought to be produced exclusively by prostatic tissue, making it significant as a tumour marker (28).

Although PSA is tissue specific it is not cancer specific. Prostatic cells from normal, benign hyperplastic, malignant and metastatic tissue all secrete PSA independent of the degree of dysplasia (29). Therefore, while PSA has become widespread as a serum marker for prostatic disease, it is not useful alone as a marker of prostate cancer or predictor of tumour behaviour (30).

While the clinical applications of PSA measurement remain somewhat controversial to date, and further research and clinical trials are required, it is clearly the best marker currently available for prostate cancer. Because PSA should fall to undetectable levels following radical prostatectomy and the removal of the tissue source (31,32), one of the most common uses of PSA has been in monitoring prostatic cancer therapy (28). In conjunction with other testing, PSA measurement has proven beneficial
in staging, prognosis, tumor volume evaluation, detection of recurrent disease, differential diagnosis, and in some cases, screening and early diagnosis (28,33).

In its early analytical evaluation, PSA was measured by isoelectric focusing, immunodiffusion, immunoelectrophoresis, and rocket immunoelectrophoresis (34). These techniques were commonly replaced with immunoradiometric assays (IRMA) (35,36), until the extensive development of non-radioisotopic procedures became prevalent. These latest assay developments currently provide serum based PSA assays adapted to automated analyzers using a variety of labeled antibodies and a variety of fluorescent and chemiluminescent detection systems (37,38-40).

Knowledge of PSA extends to the genetic level. The 6 kb gene, containing 4 introns and 5 exons has been sequenced and localized on chromosome 19 (30). While examination at the DNA level can detect changes in the gene, it cannot relate information on gene expression. PSA gene expression is believed to be regulated at the transcriptional level and recent studies indicate that the sequence of PSA is conserved in normal and malignant tissue (43). Data to date strongly suggest that detection of PSA mRNA in circulating prostate cells should correlate with the presence of circulating prostate cancer cells or a “micrometastasis” (29).

1.2.3 Screening and Diagnosis of Prostate Cancer

At present, the recommended strategy for screening asymptomatic men for prostate cancer as suggested by the American Cancer society (44) and American Urological Association (45) includes a serum PSA level along with the digital rectal exam (DRE), yearly after the age of 50. If any irregularities in size, contour or consistency are found in the DRE, the patient should undergo a transurethral ultrasound (TRUS)-guided biopsy of the abnormal area. If the serum PSA level is between 4.0 and 10.0 ng/mL, a TRUS should be performed and sextant biopsies of the prostate should be performed if abnormal regions are seen with the ultrasound. Serum PSA levels >10.0 ng/mL dictate sextant biopsy regardless of TRUS findings (46,47). Biopsy of the
suspicious tissue allows for microscopic analysis which confirms the presence of malignancy.

While it should be noted that PSA testing became widely available in 1986, PSA screening remains a controversial issue among urologists and oncologists with respect to its usefulness and cost effectiveness. Because of the overlap in PSA concentrations between healthy, non-cancerous and diseased individuals, it is difficult to define a critical PSA value to distinguish between the two (28). Adjustment of this cutoff value ultimately alters the specificity and sensitivity of the test. The apparent observation to date is that PSA is at least as good as DRE and TRUS for evaluating prostate cancer, since none of these three screening methods are 100% effective (28,46).

1.2.4 Staging of Prostate Cancer

Once malignancy has been confirmed by microscopic analysis, the stage of prostate cancer advancement is determined through information gathered from the rectal exam, ultrasound, biopsy and other non-invasive tests such as computed tomography (CT)-scans. PSA concentrations are generally proportional to the clinical stage of disease (48, 49), but cannot solely be relied upon for staging. Staging is necessary since present treatment guidelines are based on the extent of progression.

As listed in Table 1.1, four stages (A-D) of prostate cancer have been recognized with the first three stages distinguished from one another by the size of the tumor and the fourth stage indicating tumor metastasis (28). Unfortunately, the surgical-staging techniques cannot detect stray cancerous cells that have escaped into the blood and have lodged at different locations.

A Gleason score is also given based on the examination of shape, size, and arrangement of the cancerous cells in the prostate. Well differentiated cells are small, uniform in shape and tightly packed, whereas poorly differentiated cells are fused in irregular masses. This grade is indicative of tumour virulence, since well differentiated
<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
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| A     | Incidental or unsuspected cancer, small or microscopic tumors confined to one small area of the prostate; subdivided into two stages  
A1 (focused): well differentiated tissue, closely packed, large nuclei  
A2 (diffuse): moderately to poorly differentiated tissue |
| B     | Unilobular, palpable nodules confined to the prostatic capsule; subdivided into two stages  
B1: single nodule  
B2: multiple nodules (indicates possibility of aggressive cancer behaviour) |
| C     | Capsular penetration and extension of tumor into adjacent tissue, cancer occurs throughout gland making it rock hard; subdivided into two stages  
C1: periprostatic tissue involvement  
C2: extension into one or both seminal vesicles |
| D     | Metastatic spread of the cancer; subdivided into two stages  
D1: lymph node involvement  
D2: metastases to distant sites such as bone or viscera |

Adapted from Armbruster (28).
tumours are thought to behave less aggressively than less organized or poorly differentiated cells (25).

1.2.5 Therapy and Treatment Options

Generally it is recommended that patients with Stage A or B disease be treated promptly with a potentially curative treatment such as radical prostatectomy or radiation. However, the treatment of stage A and B is becoming increasingly controversial as conflicting data, supporting either aggressive therapy or no treatment, have both been reported as options for an improved quality of life and higher survival rate (50,51). Because surgery cannot fully eradicate tumors spilling from the prostate, radiation is generally the therapy of choice for stage C. Stage D patients are most suited to systemic or hormonal therapy to slow or inhibit progression of the metastatic disease (25).

1.2.5.1 Radical Prostatectomy

This procedure involves the removal of the prostate gland and some surrounding tissue, along with a thorough examination of the pelvic lymph glands and their removal pending local metastasis. For confined tumors, this procedure can be curative, although it runs the risk of impotence and incontinence (26).

1.2.5.2 Radiation Therapy

The gland is irradiated with an external beam to destroy the cancerous cells of the prostate. There is a small risk of damage to the bowel, bladder and other organs and impotence is not uncommon.

1.2.5.3 Hormone Ablation

This form of treatment is often used when the tumor extends beyond the confines of the prostate gland. Actual castration (orchiectomy) or chemical castration using anti-androgens, LH-RH analogs or drugs blocking the endogenous steroid biosynthetic pathway may be used. Impotence is a universal feature of effective treatment (26).
1.2.5.4 Cytotoxic Drugs

This type of treatment is not common for prostate cancer, since these drugs attack fast growing cells and prostate cancer is generally a slow-growing cancer.

1.2.5.5 Watchful Waiting

This waiting approach involves no treatment. While PSA levels and TRUS are regularly checked to identify aggressive tumor behaviour, this approach often spares the patient inconvenience and adverse side effects of treatment. It is an especially suitable choice for elderly patients or patients with limited life-span due to concurrent disease.

1.3 The Trend in Assay Detection Systems

Sensitive bioanalytical assays including immunoassays and nucleic acid hybridization assays have become widespread tools in diagnostic medicine, forensics, genetics, and drug and agricultural testing. These assays depend on the ultrasensitive detection methods available to allow measurement of minute quantities of analyte. Over the years, a vast array of techniques have been used as convenient means of detection.

Historically, absorbance spectrophotometry with chromogenic labels, such as fluorescein or rhodamine, was practical and acceptable for automation. However, sensitivity for direct detection was limited to the 0.1-1.0 μM range (52). The introduction of fluorescence methods increased the sensitivity by 10-1000 fold (53). Radiolabel techniques using $^{32}$P and $^{125}$I provided far higher sensitivity with a minimum label detectability of ~12 attomoles (atmol) for $^{125}$I (52). These radioisotopic immunoassays (RIAs) have the capability of detecting analyte concentrations of picograms per mL (54).

The health hazards, short shelf life, and disposal problems plaguing radioisotopic detection systems, forced the search for alternative ultrasensitive non-isotopic methods. The use of time-resolved fluorescence improved fluorescence techniques, previously limited by high backgrounds, to extend anywhere from the nM to pM range (55). Other methods included the use of multiple labeling (56), and enzyme labels which provided
amplification through rapid turnover of substrate to detectable product (57,58). Highly sensitive chemiluminescent (CL) and bioluminescent (BL) labels have been demonstrated to equal or exceed sensitivities reached with RIAs and detection limits often reach attomole and sub-attomole ranges (55,59,60).

1.4 Luminescence

1.4.1 The Fate of Electronically Excited States

Luminescence procedures are based on molecules that are excited, generating a species whose emission spectrum provides information for qualitative and quantitative analysis. Absorption of energy by a molecule leads to an electronically excited state which may be lost in a variety of ways including radiative decay or non-radiative decay.

In radiative decay, excitation energy is lost as a photon in fluorescence, phosphorescence or chemiluminescence. Non-radiative decay involves the excess energy being transferred to the surrounding molecules, creating thermal motion of the environment. The excitation required for fluorescence and phosphorescence is brought about by the absorption of photons, while chemiluminescence is based on an excited species formed as a result of a chemical reaction.

The energy diagram shown in Figure 1.2 [A] indicates the various types of radiative and non-radiative decay that may occur as a result of an electronically excited state. The lowest heavy line indicates the ground state energy of the molecule \( S_0 \) in a singlet state. The upper heavy lines represent the ground vibrational states of the three excited electronic states; the first \( S_1 \) and second \( S_2 \) singlet states and the first \( T_1 \) triplet state. Excitation of the molecule occurs through absorption of energy which excites the molecule to any of the vibrational states of the higher electronic singlet states. Direct excitation to the triplet state is forbidden.

The excited molecule can return to the ground state in a combination of steps, favouring a route that minimizes the lifetime of the excited state. Internal conversion
FIGURE 1.2

Energy Diagram of Luminescent Systems

Legend

Energy diagram illustrating the possible fates of electronically excited states following absorption of energy by a molecule. The molecule returns to the ground state by either radiative or non-radiative decay.

[A]: Fluorescence and phosphorescence occur following the absorption of photons, while chemiluminescence is a result of a chemical reaction.

[B]: An intramolecular energy transfer may occur between molecules, whereby energy is absorbed characteristic of one molecule and the emission spectrum is characteristic of the molecule to which the energy was transferred.

[Adapted from (67) and Diamandis and Christopoulos (64).]
from $S_2$ to $S_1$ may occur, as well as vibrational relaxation within each singlet state. Intersystem crossing may occur if electron spins become unpaired (parallel) and the excited molecule crosses to the triplet state. After losing sufficient energy to occupy the lowest vibrational level of the first excited singlet state or corresponding triplet state, the molecule returns to the ground state with either radiative or non-radiative decay.

Energy dissipated as light emission may take several forms depending on the excitation energy source and the excited level from which decay occurs. Fluorescence or chemiluminescence occurs with decay from singlet states in the nanosecond time scale. Decay from triplet states occurs in a slower $\mu$sec-msec time scale and is referred to as phosphorescence.

1.4.2 Time Resolved Fluorescence

1.4.2.1 Conventional Fluorometry vs. Time-Resolved Fluorometry

The sensitivity of conventional fluorescence measurements are limited to nM concentrations due to the high backgrounds present in any routine analysis (53). Traditional fluorophores generally exhibit a broad absorption and emission spectrum with a short fluorescence emission lifetime. The use of conventional fluorometry is additionally hindered by Rayleigh and Raman scattering, fluorescence quenching, background fluorescence from cuvettes, optics, the sample matrix and non-specific binding of the reagents.

The advent of time-resolved fluorescence is based on the design of new fluorophores possessing large Stokes shifts and longer emission lifetimes (10-1000 $\mu$sec) (53). The large separation between absorption and emission spectra allow the use of wavelength filtering to eliminate scattered excitation light. The long lifetime allows the use of pulsed-excitation time-gated detection methods to measure only the desired signal after the short lived background signals from scattered light have decayed (57). This eliminates the background signal from serum, solvents, cuvettes, and reagents, thereby improving signal to noise ratio. Any observed background signal is the result of
non-specific binding of the labeled reagents. The principle of time-resolved fluorescence is illustrated in Figure 1.3.

Time-resolved fluorometers have all the usual components of a conventional fluorometer plus a system for time-gated measurements. The photomultiplier tube (PMT) is inactive during the unwanted short-lived fluorescence. The CyberFluor 615™ Immunoanalyzer used in this work is designed for time-gated measurements and also employs a modified optical system to allow solid-phase measurements to be taken at the bottom of a white microtitration well (61).

1.4.2.2 Lanthanide Chelates

The large Stokes shift, long lifetime and narrow band emission of lanthanide chelates enable them to be suitable compounds for various applications of time-resolved fluorometry (63). These lanthanide chelates consist of two parts; i) an organic chelating agent with several nucleophilic coordinating groups and an aromatic chromophore and ii) a trivalent lanthanide ion such as Eu³⁺ or Tb³⁺ that is bound to the chelator.

The unique emission pattern of these lanthanide chelates results from an intramolecular energy transfer as shown in Figure 1.2 [B]. The radiation absorbed by the molecules is characteristic of the chromophore. If the energy level of the chromophore and lanthanide ion are suitably matched, the ion is located close to the chromophore in the chelate and intersystem crossing to the triplet excited state of the ligand has occurred, then excitation energy of the chromophore may be transferred to the appropriate energy level of the central metal ion (57,64). This transferred energy can in turn move up to its own excited singlet state and be emitted as a line spectrum characteristic of the metal ion (57,64).

1.4.2.3 The Ternary Tb³⁺-EDTA-Diflunisal Complex

Various enzyme substrates have recently been synthesized which upon enzymatic conversion are able to form highly luminescent chelates with lanthanide ions (57). It has been shown that dephosphorylation of the phosphate ester of salicylic acid and a number
FIGURE 1.3

The Principle of Time-Resolved Fluorescence

Legend

The measurement principle for time-resolved fluorescence with the CyberFluor 615™ Immunoanalyzer is shown for one cycle of 50 msec. Pulsed excitation (337 nm) occurs at the beginning of each cycle followed by a delay time. This allows short lived (background) fluorescence to decay prior to the 400 μsec counting time. After a recovery period, the cycle is repeated.

[Adapted from Diamandis et al. (61).]
FIGURE 1.3

Fluorescence Intensity

Flash Excitation

Short Lived Fluorescence

Long Lived Fluorescence

Time (μsec)

Delay Time  Counting Time  Recovery Time

1 Cycle = 50 msec
of its derivatives form luminescent terbium chelates in the presence of EDTA at alkaline pH (65). These products can then be measured using conventional or time-resolved fluorometry. This has allowed the wide use of alkaline phosphatase (ALP) as a label in DNA hybridization assays.

The following work utilizes diflunisal phosphate (DFP) which upon ALP dephosphorylation, forms a highly fluorescent ternary complex with Tb$^{3+}$-EDTA in alkaline solution (Figure 1.4). The excitation light is absorbed by the diflunisal anion and the energy transferred to Tb$^{3+}$ via an intramolecular energy transfer (Figure 1.2 [B]). The resultant fluorescence is characteristic of the Tb$^{3+}$ lanthanide ion. The fluorescence yield is increased by the presence of the co-chelator EDTA which excludes water molecules from the coordination sphere, maintaining water solubility of the complex, without the danger of hydroxide precipitation (57, 58).

1.4.3 Chemiluminescence and Bioluminescence

Chemiluminescence, as previously discussed and illustrated in Figure 1.2, refers to the emission of light by a substance as a result of a chemical reaction. This process encountered in nature, and exhibited by species such as the firefly, jellyfish, and certain bacteria, protozoa and crustaceans is often termed bioluminescence.

The excited molecules that eventually emit light are supplied with energy from an enthalpy change during the chemical conversion of a reactant A, to a product B. The product B is reached via a light-emitting intermediate species B* that is an electronically excited state relative to the product B. It is the decay of the metastable B* intermediate to the product B that is responsible for the emission of light (52).

Light emission from chemical or biological reactions have a wide variety of analytical applications including nucleic acid hybridization assays, protein blotting, immunoassays and sequencing (60). This luminescence allows superior sensitivity in the attomole and sub-attomole range (60), because low light can be measured in the absence of noise (66) and radiation attenuation by a filter or monochromator is avoided. CL
FIGURE 1.4
Formation of Tb\textsuperscript{3+}-EDTA-Diflunisal Fluorescent Complex

Legend

Alkaline phosphatase dephosphorylation of the diflunisal phosphate substrate forms a highly fluorescent ternary complex with Tb\textsuperscript{3+}-EDTA in alkaline solution. The excitation light is absorbed by the diflunisal anion and the energy transferred to Tb\textsuperscript{3+} via an intramolecular energy transfer. Subsequent fluorescence is characteristic of the Tb\textsuperscript{3+} lanthanide.

[Adapted from Evangelista et al. (57).]
FIGURE 1.4

F
F
F
F

COO'  H^+ + HPO_4^{2-}

ALP

COO'

OH

diflunisal phosphate

diflunisal anion

+ Tb^{3+}-EDTA

pH 12-13

Tb^{3+}-EDTA-Diflunisal Fluorescent Complex
methods require no hazardous reagents and are generally rapid and simple procedures, often employing single-step methods.

Detection limits are generally dependent on reagent purity rather than simply detector sensitivity (67). Although increasing the detectable signal for a chemiluminescent label can be achieved by increasing the sensitivity of the detecting instrument, it can also be achieved by improving the quantum efficiency of the light-emitting reaction by modifying the label or its microenvironment (52).

1.4.3.1 Kinetics of Light Emission

Analytical applications of chemiluminescence involve two distinct types of response. The first type of response employs the CL molecule as a detection label which is present in limiting concentrations. This produces a "pseudo" first order reaction since the slowest process in the sequence of events leading up to the light emission is the reaction itself (52). These labels produce flash type reactions that proceed through a short initial lag phase followed by a rapid increase. Once the reactants are used up and can no longer be converted to an excited state, light emission decays. Flash reactions generally last from one second to tens of seconds (52).

The second form of CL detection utilizes an enzyme label capable of catalyzing a chemiluminescent reaction upon addition of appropriate substrate. In this case, the limiting amount of enzyme is detected in the presence of a large excess of substrate. The reaction rate or substrate turnover rate ultimately determines the light intensity. The kinetic profile reflects the enzyme kinetics; initially there is a lag phase due to the formation of the excited state, followed by an increase and then a plateau as the enzyme turns over a constant amount of substrate. This steady state of light output, termed a glow reaction, may remain constant for minutes or even hours (52).

1.4.3.2 Instrumentation

The light output from a CL or BL reaction is measured using a luminometer. The instrumentation may be fairly simple and consist of only a suitable reaction vessel and a
PMT. Innovations have made possible semi-automated, or high-throughput automated systems that conduct complete assays followed by readout (52). Generally, no wavelength-restricting device is necessary since the only source of radiation is the chemical reaction between the reagent and analyte. Light emission signals are usually integrated over a fixed period of time.

Since the light emission from a flash reaction is short-lived, the measurement of these reactions generally requires a microplate or tube luminometer that is capable of simultaneously injecting a reagent that triggers the reaction prior to measurement. The MLX™ Microtiter® Plate Luminometer used in the subsequent CL and BL detection systems is a general purpose instrument intended for automated initiation of the reaction, measurement of emitted light and calculation of assay results.

1.4.3.3 The Bioluminescence of Aequorin

Aequorin is a bioluminescent protein isolated from the umbrella of the jellyfish _Aequorea victoria_ (68). The aequorin complex consists of a 22 000 Da molecular weight apoaequorin protein, some form of bound molecular oxygen and the prosthetic luminophore coelenterazine. Once the aequorin complex binds calcium, a conformational change occurs and in the presence of the bound oxygen, coelenterazine is oxidized to coelenteramide with a simultaneous emission of blue light (λ<sub>max</sub> = 469 nm) and release of CO₂ (69,70). The principle of aequorin bioluminescence is depicted in Figure 1.5. Emission of the light, lasting ~3 sec, occurs as coelenteramide falls from its excited state to the ground state (71).

The primary structure of the protein has been determined by sequencing the apoaequorin, and is now known to consist of 189 amino acid residues with three characteristic Ca<sup>2+</sup> binding sites (72). Recombinant aequorin has recently been produced by purifying apoaequorin from _E. coli_ bacteria transformed with a plasmid containing the apoaequorin gene and regenerating the complex _in vitro_ with pure coelenterazine (73, 74). The recombinant protein can readily be conjugated to other small molecules or
FIGURE 1.5

The Bioluminescence of Aequorin

Legend

The protein aequorin from the jellyfish *Aequorea victoria* emits a flash of blue light (\(\lambda_{\text{max}} = 469\) nm) following the binding of Ca\(^{2+}\) in the three sites (I, II, and III). A conformational change causes the intramolecular oxidation of the non-covalently bound coelenterazine in the aequorin complex to coelenteramide that yields the characteristic flash of blue light and CO\(_2\).

[Adapted from Tsuji *et al.* (75)]
Figure 1.5

- **NH₂**
- **COO⁻**
- **Ca⁺⁺**
- **bound O₂**

**sequorin complex**


coeleterazine

sposequorin

coeleteramide

+ CO₂
+ light

(λₑₒᵣ = 469 nm)
happens to be used as a detection molecule in bioluminescence based assays. The amount of label present can then be determined by adding a calcium salt solution and measuring the flash of blue light in a luminometer.

The high quantum yield of aequorin bioluminescence allows detection limits to reach the attomole ($10^{-18}$) level (59). The low background associated with the elimination of any excitation energy source and the broad dynamic range, spanning six orders of magnitude, makes aequorin conjugate labels more sensitive than fluorescent, chemiluminescent, colourimetric or enzyme-mediated techniques (76).

1.4.3.4 Dioxetane Substrates for Alkaline Phosphatase

Many compounds that exhibit chemiluminescence undergo reactions in which a cyclic peroxy intermediate or dioxetane is formed, followed by its spontaneous breakdown to give an excited-state product (52). A breakthrough was made when the highly stable phosphate analogue of the meta-substituted phenyl dioxetane was synthesized as an alkaline phosphatase (ALP) substrate (77,78). The advent of these new dioxetane substrates, specifically adamantyl-substituted 1,2-dioxetanes, allowed CL methodologies to be applied to enzyme-amplified assays (55).

The slight possibility of a chemiluminescent signal in the absence of enzymatic triggering, has generated several derivatives of the adamantyl methoxy phenyl dioxetane phosphate. Substitutions on the adamantane ring to produce hydroxy or halogeno-derivatives of the parent dioxetane, claim to reduce the background signal and facilitate the emission kinetics by reducing the half-life of the enzymatically produced phenoxy anion (79).

The following work uses a modified adamantyl 1,2-dioxetane phosphate ALP substrate termed CSPD®, which has a chloro group substituted on the adamantane ring. As depicted in Figure 1.6, the hydrolytic phosphate cleavage of CSPD by ALP releases the electron rich dioxetane phenolate (CSPD). Charge transfer from the phenolate to the dioxetane ring promotes concerted breakdown of the cyclic peroxide. This peroxide
FIGURE 1.6

Chemiluminescence of 1,2-Dioxetane ALP Substrate

Legend

Enzymatically triggered decomposition mechanism of a 1,2-dioxetane substrate (CSPD®). Upon dephosphorylation of the highly stable substrate by ALP, a metastable phenolate anion intermediate is formed. Charge transfer from the phenolate to the dioxetane ring promotes concerted breakdown of the cyclic peroxide, releasing energy that excites the methyl m-oxybenzoate product to a higher electronic state. Conversion to the ground state occurs with light emission at $\lambda_{\text{max}} = 477$ nm.

[Adapted from Pringle (52) and CSPD® Substrates Product Insert (80).]
FIGURE 1.6

1,2-Dioxetane Substrate
(X=Cl in CSPD*)

Alkaline Phosphatase \[ \text{pH 9.0} \]

Metastable Intermediate (CSP'D)

excited state methyl \( m \)-oxybenzoate

Emission of Light
\( \lambda_{\text{max}} = 477 \text{ nm} \)
breakdown releases energy that excites the methyl m-oxybenzoate product to a higher electronic state which chemiluminesces ($\lambda_{\text{max}}=477 \text{ nm}$) upon conversion to the ground state (55). The ALP enzyme label present in limiting concentrations produces a glow reaction in the presence of excess chemiluminescent substrate.

1.5 Project Overview

The polymerase chain reaction is a powerful analytical technique for the in vitro exponential amplification of specific nucleic acid sequences. Because of its superior sensitivity, PCR has rapidly replaced the conventional methods of nucleic acid analysis such as Southern and Northern blots. In recent years, several methods have been developed for analysis of PCR products. These include electrophoretic separation of amplified DNA, chromatography, capillary electrophoresis and hybridization followed by radioactive or nonradioactive detection of the hybrids.

Perhaps the most challenging current analytical problem associated with PCR is the determination of the starting quantity of target DNA. Quantification requires the establishment of a reproducible relation between the analytical signal obtained from the amplification product and the number of target DNA molecules in the sample prior to amplification.

As a consequence of the exponential amplification, small sample-to-sample variations in efficiency lead to dramatic changes in the amount of product and large errors in estimating the initial quantity of the target. For these reasons, the most reliable approach to quantitative PCR is to coamplify, in the same reaction tube, the target DNA and a known amount of an internal standard. The majority of reports on quantitative PCR, are based on an IS containing a deletion or insertion large enough to allow electrophoretic separation of the products. The fragments are then quantified by scanning densitometry.
The objective of my research was to devise novel quantitative PCR assays for DNA and RNA which are automatable, highly sensitive and easily adaptable to the routine clinical chemistry laboratory.

Recently there has been a great interest in PSA mRNA as a specific marker of circulating prostate cancer cells and its use for molecular staging of prostate cancer. One project, described in Chapter 3, focused on the development of the first quantitative assay for PSA mRNA based on co-amplification with a recombinant RNA IS and hybridization performed in microtiter wells. In the proposed Q-PCR methodology, PSA mRNA and the IS contained the same primer recognition sites and generated amplification products of identical size, differing only in a 24 bp centrally located sequence. After RT-PCR, the products were immobilized in wells through biotin/streptavidin and hybridized (in separate wells) to specific probes labeled with digoxigenin (DIG). The hybrids were then detected by ALP labeled anti-DIG antibody conjugates and time-resolved fluorometry.

Studies were first performed to prove that the IS was distinguishable from the target. Then, the sensitivity, linearity and reproducibility of the hybridization assays for the amplification products were assessed. Finally, quantitative PCR assays were performed on mixtures containing RNA from prostate cancer cells in the presence of a large excess of healthy RNA and a constant amount of RNA IS. The ratio of the fluorescence obtained from the target RNA and IS was plotted as a function of the number of cancer cells in the sample.

The goal of the second project was to facilitate the automation of quantitative PCR. In Chapter 4, the development of a dual-analyte chemiluminescence hybridization assay for simultaneous determination of both amplification products in the same reaction vessel is described. The products were labeled with biotin and captured on streptavidin (SA) coated microtiter wells. Probes specific for target DNA and IS were labeled with DIG and fluorescein haptens, respectively. Aequorin-anti-DIG and ALP-antifluorescein conjugates were used as reporter molecules.
First it was proven that the dual-analyte approach was achievable by analyzing mixtures of target DNA and IS. Then, the sensitivity, linear range and reproducibility of the dual protocol assays were assessed. Finally, a quantitative PCR methodology based on the proposed dual-analyte chemiluminescence assay was developed and studied.
CHAPTER 2
MATERIALS

The following materials are used throughout the course of my research. The methods for particular procedures are described in the subsequent chapters.

2.1 Chemicals, Biochemicals and Supplies

The human prostate adenocarcinoma cell line, LNCaP, which expresses the prostate specific antigen mRNA was obtained from the American Type Culture Collection (ATCC CRL 1740, Rockville, MD).

RPMI 1640 with L-glutamine, fetal bovine serum (FBS), fungizone, penicillin and streptomycin used to make complete media were all from Gibco Laboratories Life Technologies Inc. (Gaithersburg, MD). Polymorphprep™ used to isolate normal lymphocytes from whole blood, Moloney murine leukemia virus reverse transcriptase (M-MLV RT), dithiothreitol (DTT), trypsin and Trizol® LS Reagent were also from Gibco.

Cells were grown in 25 cm² polystyrene culture flasks obtained from Corning (Corning, NY).

Disposable 3 and 10 mL cc syringes and disposable 10mL graduated, plugged, polystyrene serological pipettes by Falcon® were from Becton Dickinson Labware (Lincoln Park, NJ).

Nalgene™ disposable 25 mm syringe filters (0.2 μm pore size) and soda lime glass Pasteur pipettes were from Baxter Diagnostics Corp. (Toronto, ON).

Sterile, conical 15 mL and 50 mL graduated polypropylene centrifuge tubes as well as disposable universal fit pipette tips were supplied by VWR (West Chester, PA).

The VacuCap™ disposable bottle-top filter used for sterilization of cell media was from Gelman Sciences (Ann Arbor, MI).
T7 RNA polymerase, Sephadex® G-25 gel filtration columns (Nap-5), RNase inhibitor, and Ultrapure 2'-deoxyribonucleoside 5'-triphosphates (dNTPs) were from Pharmacia LKB (Montreal, PQ, Canada).

The Wizard™ PCR preps DNA purification kit, and the RQ1 RNase-free DNase were from Promega Corp. (Madison, WI).

The ribonucleoside triphosphates (NTPs), digoxigenin-11-2'-deoxyuridine triphosphate (DIG-dUTP). Yeast tRNA, Fluorescein-5(6)-carboxamidocaproyl-[5-(3-aminoallyl)-2'-deoxy-uridine-5'-triphosphate] (fluorescein-dUTP), blocking reagent (Cat. No. 1096 176), the sheep polyclonal anti-DIG alkaline phosphatase conjugate (anti-DIG-ALP), alkaline phosphatase-labeled anti-fluorescein antibody (antifluorescein-ALP) and terminal deoxynucleotidyl transferase (TdT) were from Boehringer Mannheim Biochemica (Laval, PQ, Canada).

Sulfo-succinimidyl 6-(biotinamido) hexanoate, the long chain derivative of the N-hydroxysuccinimide ester of biotin (NHS-LC-Biotin), was from Pierce (Rockford, IL).

Agarose was from the Eastman Kodak Company (Rochester, NY).

Ultratherm Taq DNA Polymerase was from Bio/Can Scientific (Mississauga, ON).

pUC 18 Hae III digest, polyoxyethylene sorbitan monolaurate (Tween-20), mineral oil, dimethylsulfoxide (DMSO), diethylpyrocarbonate (DEPC), ethidium bromide, ethyleneglycol-bis-(β-amino ethyl ether) N,N,N',N' tetra acetic acid (EGTA), spermidine, calcium chloride, and streptavidin (SA) were obtained from Sigma (St. Louis, MO). SA from Boehringer Mannheim was also used.

Opaque, flat-bottom polystyrene microtiter wells (Microlite™ 2) were from Dynex Technologies, Inc. (Chantilly, VA).

A covalent conjugate of aequorin with anti-digoxigenin antibody (Fab fragments) was purchased from Sealite Sciences (Atlanta, GA).

Diflunisal phosphate (DFP) was from CyberFluor Inc. (Toronto, ON).
Terbium chloride hexahydrate was from Aldrich Chemical Co. (Milwaukee, WI).
Disodium 3-(4-methoxyspiro\{1,2-dioxetane-3,2'-\(5'\) chloro\}tricyclo [3.3.1.1\(^3\)7] decan)-4-yl)phenyl phosphate (CSPD\(^\circ\)) and the chemiluminescence enhancer Sapphire II were purchased from Perkin-Elmer (Mississauga, ON, Canada). CSPD\(^\circ\) from Tropix (Bedford, MASS) was also used.

All general chemicals including sodium chloride, potassium chloride, magnesium chloride, sodium hydroxide, maleic acid, ethylenediaminetetra-acetic acid disodium salt (EDTA), diethanolamine, disodium hydrogen phosphate, potassium dihydrogen orthophosphate, sodium acetate, chloroform, ethanol, sodium hydrogen carbonate, trishydroxymethylaminomethane (Tris), 2-propanol, glacial acetic acid and hydrochloric acid were purchased from BDH Inc. (Toronto, Canada).

2.2 Apparatus and Instrumentation

PCR experiments were performed using the 48-well Perkin Elmer Cetus DNA thermal cycler (Perkin Elmer, Norwalk, CT).

Absorbance measurements for RNA were carried out using the Shimadzu UV-160 spectrophotometer.

Agarose gel electrophoresis of DNA was performed using the Miniature Horizontal Gel System MLB-06 from Tyler Research Instruments (Edmonton, AB) along with the Fotoforce 500\(^\text{TM}\) Power supply by Fotodyne Inc. from Bio/Can Scientific (Mississauga, ON). Gels were viewed using the hand held Model UVG-II ShortWave UV Mineralight\(^\circ\) Lamp from Ultra-Violet Products Inc. (San Gabriel, CA).

Positive and negative photographs of the gels were taken with Polaroid 665 film using the Model MP4 Polaroid Camera System (Polaroid Corp., Cambridge, MA). The Bio-Rad Model GS-670 Imaging Densitometer from Bio-Rad Laboratories, Ltd. (Mississauga, ON), was used for scanning negative photographs of the gels.
The CyberFlour 615™ Immunoanalyzer from CyberFlour Division, Nordion International (Toronto, ON), was used for time-resolved fluorescence measurements of Tb³⁺ solutions. The gated characteristics of the CyberFlour 615™ [Fig. 1.3] are as follows: nitrogen laser emission wavelength 337.1 nm; laser pulse duration 3 to 4 nsec; repetition rate 20 pulses/sec; delay time 200 µsec; measurement time 400 µsec; recovery time after completion of measurement to next flash 49.4 msec; cycle time 50 msec; measurement time/well 1 sec (20 flashes); with 16 usable fluorescence measurements/well (62). Excitation and emission wavelengths are set at 337 and 615 nm, respectively.

Luminescence measurements for both the ALP and aequorin reactions, were carried out using the MLX™ Microtiter® plate luminometer from Dynex Technologies (Chantilly, VA). The luminometer contained two positive displacement syringe dispensers for automated injection of triggering reagents, and was capable of performing glow endpoint, glow kinetic or flash reactions with readings taken as frequently as 10 msec (66). The Revelation™ Software controlled the MLX® and collected all data.

Cell counting was carried out using the Nikon TMS Microscope and the Neubauer Brightline hemocytometer by Hausser Scientific (American Scientific Products, McGraw Park, IL).

Centrifugations were performed using either a swinging bucket centrifuge from Beckman Instruments, Inc. (Mississauga, ON), a desktop Safety-Head Centrifuge from Canlab (Mississauga, ON) or an Eppendorf Model 5415C microcentrifuge from Baxter/Canlab, Inc. (Mississauga, ON).

The Amerlite Shaker/Incubator from Amersham Canada Ltd. (Oakville, ON) was used for shaking and incubating microtiter wells during hybridization assays. Microtiter wells were washed using the Model EAW II plate washer from SLT-Lab Instruments (Groedig/Salzburg, Austria).

Other general laboratory equipment used were as follows:
2.3 Reagents and Buffers

- **Phosphate-Buffered Saline** (PBS). Consisted of 0.14 mol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄ and 1.76 mmol/L KH₂PO₄, pH 7.4.
- **LNCaP Complete Culture Media.** Consisted of 90% (v/v) RPMI 1640, 10% (v/v) FBS, 100 kU/L penicillin, 100 mg/L streptomycin and 0.25 mg/L Fungizone.
- **Cell Freezing Media.** Consisted of 20% (v/v) FBS, 10% (v/v) DMSO in complete RPMI 1640 culture media.
- **Sodium Phosphate Buffer.** Consisted of 10 mmol/L sodium dihydrogen phosphate (NaH₂PO₄), pH 6.8.
- **NAP™-5 and NAP™-10 Columns.** Disposable columns containing DNA grade Sephadex® G-25 medium in distilled water with 0.15% Kanthon® CG as a preservative.

For Hybridization Assays:

- **Wash Buffer.** Consisted of 50 mmol/L Tris, pH 7.4, 0.15 mol/L NaCl and 1 mL/L Tween-20. For dual-analyte assay, wash buffer also consisted of 2 mmol/L EGTA.
- **Blocking Solution.** Consisted of 1% (w/v) blocking reagent (10 g/L) in 0.1 mol/L maleic acid and 0.15 mol/L NaCl, pH 7.5. For dual-analyte assay, blocking solution also consisted of 2 mmol/L EGTA.
- **DFP Substrate Buffer.** Stock solution consisted of 10 mmol/L diflunisal phosphate (DFP) in 0.1 mol/L NaOH, stored at 4 °C. Final substrate buffer made fresh prior to
use, consisted of a 10 fold dilution of the stock (1 mmol/L DFP) in 1 mmol/L MgCl₂, 0.1 mol/L NaCl and 0.1 mol/L Tris, pH 9.1.

- **Developing Solution.** Consisted of 0.4 mol/L NaOH, 2 mmol/L Tb³⁺, 3 mmol/L EDTA and 1 mol/L Tris.
- **CSPD Substrate (1x) Buffer.** Used for single assay protocols only. Consisted of 0.1 mol/L diethanolamine, 1.0 mmol/L MgCl₂, 1 mg/mL Sapphire-II™ Enhancer, 0.4 mmol/L CSPD, pH 9.5.
- **CSPD Substrate (2x) Buffer.** Used for dual-analyte assay only. Consisted of 0.2 mol/L diethanolamine, 2.0 mmol/L MgCl₂, 2 mg/mL Sapphire-II™ Enhancer, 0.8 mmol/L CSPD, pH 9.5.
- **Ca²⁺ Triggering Solution.** Consisted of 25 mmol/L CaCl₂, 20 mmol/L Tris, pH 7.5.

2.4 Oligonucleotides

The following oligonucleotide sequences used in the course of this work were all synthesized by Bio-Synthesis (Lewisville, TX):

i) 5'-(NH₂)-CTC TCG TGG CAG GGC AGT CT-3', a 20mer used as the upstream primer (u) in quantitative PCR homologous to a sequence in exon 2 of the PSA gene;

ii) 5'-GGT CGT GGC TGG AGT CAT CA-3', a 20mer used as the downstream primer (d) in quantitative PCR complementary to a sequence in exon 3 of the PSA gene;

iii) 5'-CTT GCT GAA CTT CTG ACT ACG ACT TGG GCA GCT GTG AGG-3', a 39mer (a) used as the downstream primer for synthesis of short product A;

iv) 5'-AGT CGT AGT CAG AAG TTC AGC AAG CTT GCT GGG TCG GCA-3', a 39mer (b) used as the upstream primer for synthesis of short product B;

The underlined segments in primers (a) and (b) represent the new sequence to be introduced in the internal standard and are complementary to one another.
v) 5'-CTA ATA CGA CTC ACT ATA GGG CTC TCG TGG CAG GGC A-3', the 37mer upstream primer (T7-u) homologous to PSA exon 2 and bearing the T7-promoter sequence (in italics);

vi) 5'-ATC ACG CTT TTG TTC CTG ATG CAG-3', the 24mer probe (p₁) used in the hybridization assays of amplified target RNA sequences. The probe spans the exon 2/exon 3 junction in the PSA mRNA with a complementary sequence to the last 12 bases of exon 2 and the first 12 bases of exon 3 of the PSA gene.

vii) 5'-CTT GCT GAA CTT CTG ACT ACG ACT-3', the 24mer probe (p₂) used in the hybridization assay of amplified RNA internal standard.

The relative positions of primers and probes are shown in Figure 2.1.
FIGURE 2.1

Position of Primers and Probes for PSA mRNA

Legend

A schematic presentation of the PSA mRNA and the relative positions of primers and probes used in this work. The Latin numbers correspond to the exons. Oligonucleotides (a) and (b) are used exclusively for the synthesis of RNA IS. Oligonucleotides (u) and (d) are the upstream and downstream primers for RT-PCR of PSA mRNA and RNA IS. Primer (T7-u) is used in the synthesis of the T7 promoter-bearing DNA template. Oligonucleotides (p₁) and (p₂) are the probes specific for the PSA mRNA and RNA IS, respectively.
CHAPTER 3
QUANTIFICATION OF PROSTATE SPECIFIC ANTIGEN mRNA BY
COAMPLIFICATION WITH A RECOMBINANT RNA INTERNAL STANDARD
AND MICROTITER WELL-BASED HYBRIDIZATION

3.1 Introduction

In clinical management of patients with prostate cancer it is important to
distinguish between organ-confined and metastatic disease because of the significant
therapeutic and prognostic implications. Organ-confined disease is potentially curable by
radical prostatectomy but once the disease has spread to distant sites, the patients are
offered systemic treatment aimed at retarding progression of the malignancy. However,
the ability of current staging techniques to detect metastases is poor and as many as 25-
30% of patients who undergo prostatectomy are found to possess metastatic disease
subsequent to surgery (81-84). Detection of prostatic cells in peripheral blood may be an
earlier indication of metastasis.

RT-PCR provides highly sensitive detection of mRNA sequences and has been
used widely for the study of gene expression in malignant and physiological states
(85,86). In 1992, Moreno et al. first applied RT-PCR methodology to the detection of
PSA mRNA as a specific marker of circulating prostatic cells in patients with metastatic
prostate cancer (87). This methodology was further used for preoperative staging of
prostate cancer patients (88). In recent years, the analytical sensitivity of the technique
has been improved substantially by increasing the number of PCR cycles, using nested
PCR, using hot-start PCR or enhancing the detectability of the amplification products
(29,81,89-92). Several clinical studies have since been performed using peripheral blood

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(29,81,89-92), lymph nodes (93) and bone marrow (94,95) from patients with prostate cancer.

Most of the PSA mRNA assays reported previously have been qualitative, that is, they detect the presence or absence of the particular mRNA without referring to its quantity. This has produced considerable controversy regarding the usefulness of the RT-PCR test in staging prostate cancer. For example, highly sensitive RT-PCR protocols have led to the detection of PSA mRNA in the blood of healthy individuals and patients with hyperplasia (96-99). Furthermore, several studies have detected PSA gene expression in male and female breast tumors (100,101), and in milk from lactating women (102).

To date, RT-PCR has been able to detect small numbers of circulating PSA expressing cells currently undetectable by other means in patients with localized or metastatic cancers (29). The ability of circulating tumour cells to cause metastasis is still controversial and a relation between PSA mRNA concentration and metastatic potential has not been reported. Several reports have addressed the need for a quantitative assay for PSA mRNA (29,96-98).

The limited number of reported quantitative protocols for PSA mRNA have been based on the parallel amplification of PSA mRNA with the mRNA of β-actin, a housekeeping gene (103-105). A crucial assumption of this procedure is that the housekeeping gene has an even level of transcription and is independent of different degrees of cellular activation. However, it has been reported that β-actin mRNA levels increase with the malignant transformation of cells (106). Also, the efficiency of reverse transcription and amplification for PSA and actin mRNA may vary, thus limiting the usefulness of the technique in a clinical setting.

In this work, a quantitative analytical methodology for PSA mRNA was developed, based on the co-amplification of the target PSA RNA with a recombinant RNA internal standard. The IS has the same primer binding sequences as the PSA mRNA.
and both amplification products are the same size, differing only by a 24 bp centrally located sequence. The RT-PCR products are captured on microtiter wells and analyzed by two separate nonradioactive and highly sensitive hybridization assays using specific probes.

3.2 Methods

3.2.1 Procedure Precautions

All glassware used for cell culture was autoclaved to avoid bacterial contamination. All supplies used in cell culture (media bottles, sterile tubes, cell culture flasks) were consistently sprayed with 70% ethanol and opened only in the laminar flow hood. The LNCaP culture media was sterilized with the VacuCap™ vacuum filter unit prior to use.

Several precautions were taken to prevent carryover DNA contamination during PCR and to prevent RNA degradation by RNases prior to reverse transcription. Disposable gloves were worn continually and changed frequently during both instances. All pipette tips, glassware, microcentrifuge tubes and PCR tubes were autoclaved prior to use. A dedicated set of pipettes and pipette tips for RNA and PCR work were UV irradiated for several hours prior to use. Set up of PCR and product analysis were carried out in separate rooms to avoid the possibility of contamination.

DEPC-treated water, autoclaved twice, was used in all solutions for the preparation of the RNA IS and all subsequent RNA work. RNA was immediately stored at -80°C following isolation and RNA dilutions were held on ice during RT-PCR set up procedures, to minimize degradation by RNases.

3.2.2 Cell Culture

3.2.2.1 Culturing Cells

Frozen LNCaP cells (positive for PSA mRNA) were thawed by rapid agitation and immediately washed with PBS in a 15 mL conical tube. Cells were pelleted by
centrifugation at 300 g for 5 min, resuspended in 5 mL of complete media and pipetted into a single 25 cm² culture flask. The cells were grown as a monolayer at 37°C with 5% CO₂. The culture medium was replaced with fresh medium every 2-3 days depending on the cell density.

3.2.2.2 Subculturing and Freezing of Cells

Once the cells became confluent, the expired medium was aspirated and the cells were detached from the flask by incubating with 3 mL of a 0.25% trypsin-0.03% EDTA solution for 10 min at 37°C. Complete media were then added and the suspension aspirated up and down several times. Cells were removed to a 15 mL conical tube, centrifuged as usual, and washed 3 times with complete media. Fresh media (6 mL) were added, the cells resuspended and split into two 25 cm² culture flasks.

To store cells for later use, cell pellets were resuspended in cell freezing media at a minimum density of 1 x 10⁶ cells/mL and aliquoted into 2.0 mL cryogenic vials. Vials were immediately placed at -20°C for 1 hr and then stored at -80°C until further use.

3.2.2.3 Counting Cells

Following trypsinization, an aliquot of the suspended cells was diluted 2 fold with PBS and placed on a hemocytometer grid. The cells in the outer 4 corner squares were counted and a average cell number/square was obtained. This value was divided by the volume of the total square (1mm x 1mm x 0.1 mm = 1 x 10⁻⁴ mL) and multiplied by the dilution factor of 2, the end result producing the number of cells/mL.

3.2.3 Total RNA Isolation

3.2.3.1 Procedure for LNCaP Cells

Ten million LNCaP cells were sedimented by centrifugation for 2 min at 12000 g. One mL of Trizol™ LS reagent was added and total RNA was isolated (107) according to the manufacturer's instructions. RNA, precipitated with 2-propanol, was washed with 75% ethanol and redissolved in 20 μL of DEPC-treated water containing 37 units of RNase inhibitor.

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3.2.3.2 Procedure for Whole Blood

Whole blood (5 mL), used for isolation of total RNA from healthy cells, was separated with Polymorphprep™ according to the manufacturer's instructions. Polymorphonuclear cells were washed once in PBS, pelleted and resuspended in 1 mL of PBS. The cells were pelleted at 12000 g for 2 min followed by the addition of Trizol® LS reagent and total RNA isolation was carried out as described above for the LNCaP cells. The RNA concentration was determined by measuring absorbance at 260 nm using the Shimadzu UV-160 spectrophotometer.

3.2.4 Polymerase Chain Reaction

PCR reactions were carried out in a total volume 100 μL, consisting of 20 mmol/L ammonium sulfate, 75 mmol/L Tris-HCl (pH 8.8), 0.1% Tween-20, 2.5 mmol/L MgCl₂, 20 μmol/L of each dNTP, 50 pmol of each primer and 2.5 units of Taq polymerase. The appropriate starting target DNA and primers are discussed in their corresponding sections. The mixtures were layered with mineral oil and placed in the Perkin-Elmer DNA thermal cycler. Primers were added to the mixture during a "hot start" protocol (123) once the block temperature had reached 95 °C. Upon completion of the cycling (95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min), samples were incubated at 72 °C for 10 min and then cooled to 4 °C until further analysis.

3.2.5 Synthesis of RNA Internal Standard

3.2.5.1 Synthesis of Recombinant DNA Template

3.2.5.1.1 Creation of Short Products

A DNA template was first generated from the recombinant plasmid pA75 (108), containing a 1.4 kb PSA cDNA insert. Two separate PCRs were set up to create two short products, A and B, each containing a newly introduced sequence of 24 bp. PCR-A used oligonucleotides (u) and (a) as upstream and downstream primers, respectively (Figure 2.1). The downstream primer (a) contained a 15 bp sequence at the 3' end, necessary for binding to the cDNA, and a 24 bp extension at the 5' end. Thus, the
amplification product A consisted of a 66 bp segment identical to the starting DNA with a 24 bp addition. For PCR-B, oligonucleotides (b) and (d) were used as upstream and downstream primers (Figure 2.1). The upstream primer (b) contained a 15 bp sequence, at the 3' end, complementary to the cDNA and a 24 bp extension at the 5' end. Product B consisted of a 143 bp segment identical to the starting DNA plus the 24 bp extension.

Sixteen million molecules of plasmid DNA were used as the starting template for the creation of both A and B short fragment. The PCR was carried out for 30 cycles with the following cycling parameters: 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min. The products of PCR-A and PCR-B were 90 bp and 167 bp, respectively.

3.2.5.1.2 Joining of Short Products

Twenty μL aliquots each of short product A and B were electrophoresed on a 2% agarose gel and the DNA was stained with ethidium bromide. The 90 bp and 167 bp bands were excised from the gel and a small slice from each band was used as starting template in a PCR mixture containing no primers. The mixture was subjected to 40 cycles of denaturation (95 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 2 min).

Subsequently, 5 μL of this reaction mixture were amplified for 30 cycles using oligos (u) and (d) as upstream and downstream primers, respectively. The PCR conditions were as described above for the short fragments. This amplification produced a 233 bp recombinant DNA fragment.

3.2.5.2 Synthesis of RNA Template

3.2.5.2.1 Fusion of T7 Promoter

The new DNA fragment was fused to a T7 promoter through PCR in order to create a complete transcription unit. The PCR was carried out with oligos (T7-u) and (d) as upstream and downstream primers respectively and 1 μL of a 100 fold dilution of the previous recombinant amplification product. The cycling protocol and PCR setup were
carried out as described above for the short fragments with an annealing temperature of 60 °C instead of 55 °C.

3.2.5.2.2 Wizard™ Purification

The products of three amplifications were pooled and purified with the Wizard™ PCR preps DNA purification system according to the manufacturer’s instructions. Through this process, excess primers were removed and the desired DNA template containing the T7 promoter was concentrated in 50 μL of water.

3.2.5.2.3 Transcription

The RNA internal standard (IS) was synthesized by in vitro transcription of the T7 promoter-bearing DNA template. The transcription reaction was carried out in a total volume of 75 μL containing 40 mmol/L Tris-HCl (pH 8.0), 6 mmol/L MgCl₂, 2 mmol/L spermidine, 10 mmol/L NaCl, 10 mmol/L dithiothreitol (DTT), 55 units RNase inhibitor, 2.5 mmol/L NTPs, 200 units T7 RNA polymerase and 10 μL of the DNA template. The reaction was allowed to proceed at 37 °C for 90 min.

3.2.5.2.4 RNA Purification and DNase Treatment

The RNA was purified with Trizol® LS reagent, dissolved in 20 μL of DEPC-treated water and then diluted with 180 μL of 1 g/L yeast tRNA containing 37 units of RNase inhibitor. The resulting stock RNA IS solution was treated with RQ1 RNase-free DNase to destroy the DNA. To 20 μL of stock RNA IS was added 1 μL (1 unit) of DNase and 2 μL of a 10 mmol/L MgCl₂, 500 mmol/L Tris-HCl (pH 7.5) solution. After a 15-min incubation at 37 °C, the DNase was inactivated by heating at 75 °C for 5 min.

3.2.5.2.5 RNA IS Working Solution

Various dilutions of the purified RNA IS were prepared by using a solution containing 0.1 g/L yeast tRNA and 200 units/mL RNase inhibitor as a diluent. A 3x10⁶ fold dilution of the stock RNA IS was used as the RNA IS working solution.
3.2.6 Labeling Primers and Probes

3.2.6.1 Biotinylation of Upstream Primer

The upstream primer (u) was synthesized with an amino group at the 5' end and was labeled with NHS-LC-biotin. To 0.1 mL of primer (10 nmol) diluted in water, 20 µL of carbonate buffer and 56 µL (5 µmol) of NHS-LC-biotin, dissolved in DMSO, were added. After a 2 hr incubation at room temperature the biotinylated primer was purified three times by size exclusion chromatography using NapTM-5 columns with sodium phosphate buffer. The purified primer solution was concentrated 4 fold by lyophilization.

3.2.6.2 Tailing Probes with Digoxigenin

The probes (p1) and (p2) were tailed enzymatically with multiple DIG moieties (109). The tailing reactions were performed in a total volume of 20 µL which consisted of 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl (pH 6.6), 0.25 g/L bovine serum albumin (BSA), 5 mmol/L CoCl2, 50 µmol/L DIG-dUTP, 0.5 mmol/L dATP, 25 units of terminal deoxynucleotidyl transferase (TdT) and 100 pmol of probe. The (p1) and (p2) tailing reactions were carried out at 37 °C for 1 hr and were then terminated by adding 2 µL of 0.2 mol/L EDTA. The labeled probes containing multiple DIGs at the 3’ end were used without purification.

3.2.7 Quantification of PSA mRNA

3.2.7.1 RT-PCR

For reverse transcription, a solution of total volume 12 µL, containing 10 pmol downstream primer (d), 1 µg total RNA (containing PSA mRNA) and 2 µL of RNA IS working solution (Refer to Section 3.2.5.2.5) was heated for 5 min at 70 °C and placed on ice. Then an 8-µL aliquot of a buffer containing 125 mmol/L Tris (pH 8.3), 187.5 mmol/L KCl, 25 mmol/L DTT, 7.5 mmol/L MgCl2, 1.25 mmol/L of each dNTP and 200 units of reverse transcriptase (M-MLV RT) was added to the sample. The reaction was allowed to proceed for 1 hour at 37 °C at which time the reverse transcriptase was inactivated by heating at 95 °C for 5 min and then placing on ice.
PCR was then performed in a total volume of 100 μL containing (final concentrations) 20 mmol/L ammonium sulfate, 75 mmol/L Tris-HCl (pH 8.8), 0.1% Tween-20, 2 mmol/L MgCl₂, 50 μmol/L dNTPs, 50 pmol of each of the biotinylated upstream primer (u) and the downstream primer (d), 2.5 units of Taq polymerase, and 8 μL of the reverse transcription mixture. The “hot start” protocol was followed in which the mixture was heated to 95 °C for 5 min and then the primers were added. PCR was carried out for 33 cycles of denaturation at 95 °C for 30 sec, annealing at 65 °C for 30 sec and extension at 72 °C for 1 min. Finally, the mixtures were incubated at 72 °C for 10 min and then cooled to 4 °C until analysis by hybridization.

3.2.7.2 Hybridization Assay

3.2.7.2.1 Preparation of Streptavidin Coated Wells

Opaque, polystyrene microtiter wells were coated, by physical adsorption, with 50 μL of 1.4 mg/L streptavidin diluted in PBS. The wells were covered with transparent tape and incubated at room temperature overnight. Wells to be used at a later date were stored at 4°C.

3.2.7.2.2 Hybridization Assay Procedure

Prior to use, the SA coated microtiter wells were washed three times with wash buffer, using the automated plate washer. Then, 50 μL of PCR product diluted 10 fold in blocking solution, were pipetted into each of four wells and incubated at room temperature with shaking for 30 min. The wells were then washed as above and 50 μL of a 0.2 mol/L NaOH solution was added. After a 20 min incubation, the unbound DNA strand was removed by washing as above. Each DIG-labeled probe, (p₁) and (p₂), was diluted in blocking solution to 7 nmol/L and heated to 42°C. Subsequently, 50 μL of each probe was pipetted in duplicate to the wells containing the immobilized single-stranded PCR products. Hybridization was carried out for 30 min at 42°C with shaking in the Amerlite shaker/incubator. The wells were washed and 50 μL of 750 units/L anti-DIG-ALP conjugate, diluted in blocking solution, were added to each well and incubated for
30 min. The wells were then washed and 50 µL of DFP substrate solution was added to each well. The enzymatic dephosphorylation of DFP to produce diflunisal, was allowed to proceed with shaking, for 30 min at room temperature. At the end of this incubation period, 50 µL of developing solution was added to each well, incubated for 1 min and the fluorescence was measured with the CyberFluor 615 time-resolved fluorometer.

3.3 Results and Discussion

The recombinant RNA IS was constructed by replacing a 24-bp sequence spanning the exon 2/exon 3 junction of the PSA mRNA (shown by the two vertical arrows in Fig. 2.1) with a new segment of equal size. This was accomplished by using PCR as a synthetic tool (13) as shown in Fig. 3.1. The PSA cDNA was amplified with primers (u) and (a) to create the short 90 bp product A. PCR B used primers (b) and (d) to create the short 167 bp product. Subsequently, products A and B were mixed and subjected to cycles of denaturation, annealing and extension in the presence of Taq polymerase, but without added primers.

Because the 5' extensions of the fragments are complementary to each other, during the cycling process, the two fragments act as primers for one another leading to the joining of A and B and production of the 233 bp fragment. This fragment was then fused to the T7 promoter through PCR, and served as the template for the synthesis of the RNA IS (Figure 3.1). Following transcription and purification, the RNA IS was treated with DNase to degrade any traces of DNA template that may have been present. Amplification of RNA IS aliquots by PCR and analysis by hybridization gave no signal, thus confirming the absence of contamination from DNA template.

In order to verify the presence of the new 24 bp sequence in the RNA IS and to confirm that the amplification products from PSA mRNA and RNA IS were distinguishable by hybridization, RT-PCR was performed on two samples containing either PSA mRNA or RNA IS. Each PCR product was then analyzed in triplicate by
FIGURE 3.1

Synthesis of Recombinant RNA Internal Standard

Legend

Schematic presentation of the RNA internal standard synthesis. Two short products, A (90 bp) and B (167 bp), were first created from a plasmid containing the PSA cDNA insert. Product A used oligos (u) and (a) as upstream and downstream primers and product B used oligos (b) and (d) as upstream and downstream primers, respectively. Products A and B were joined and extended to produce a 233 bp recombinant DNA template, with a centrally located different 24 bp sequence. The new DNA fragment was fused to the T7 promoter through use of the T7-(u) primer in PCR. In vitro transcription, followed by purification and DNase treatment, were carried out to produce the recombinant 233 base RNA IS.
FIGURE 3.1

PSA cDNA

PCR A
primers (u) and (a)

PCR B
primers (b) and (d)

90 bp
5' product A

167 bp
5' product B

joining
extension

(A) 5' 3'

no extension

PCR
primers (u) and (d)

233 bp
5' recombinant DNA

T7-u primer

T7 promoter bearing
5' 254 bp DNA fragment

transcription
purification
DNase treatment

233 base RNA IS

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hybridization to both probes, \((p_1)\) and \((p_2)\). The average signals obtained from amplified PSA mRNA assayed with DIG-labeled probes \((p_1)\) and \((p_2)\) were \(114990\pm4646\) and \(1232\pm86\), respectively. The amplified RNA IS tested with probes \((p_1)\) and \((p_2)\) gave signals of \(2567\pm275\) and \(139830\pm4494\), respectively. The fluorescence signals obtained from both amplification products with both labeled probes are presented as a diagram in Figure 3.2.

It was observed that the amplified PSA mRNA binds exclusively to probe \((p_1)\) whereas the RNA IS binds only to probe \((p_2)\). The fluorescence signals obtained after testing each PCR product with the non-complementary probe reflect the non-specific binding of the probe and the ALP-labeled anti-DIG antibody to the solid phase (typical readings for the assay blank). This experiment also confirmed that cross hybridization between targets and probes did not occur and that the RNA IS solution was free of contamination from PSA mRNA and/or its amplification product, and vice versa.

The detection of the amplification products following RT-PCR was carried out by two separate hybridization assays as shown in Figure 3.3. Biotinylated products from PSA mRNA and RNA IS were captured on SA coated wells. Following removal of the one DNA strand by NaOH, either DIG-labeled probe \((p_1)\) or \((p_2)\) was allowed to hybridize. Anti-DIG-ALP conjugates were reacted with the DIG-labeled hybrids and a diflunisal phosphate substrate solution added. Dephosphorylation of the DFP by ALP, allowed the formation of the fluorescent ternary complex upon addition of a Tb\(^{3+}\)-EDTA Developing solution.

The sensitivity and linear range of the two hybridization assays were established as follows. A stock solution of biotinylated amplification product was prepared by pooling several RT-PCRs of the PSA mRNA. The DNA concentration of the stock was determined by scanning densitometry of negative photographs prepared from ethidium bromide stained agarose gels. The pUC 18 DNA fragments were used as standards in the quantification of the DNA bands. The Molecular Analyst v. 1.0 software was employed
FIGURE 3.2
Confirmation of Distinguishable IS

Legend

Fluorescence signals for hybridization of both amplification products with both DIG-labeled probes. Signals for hybridization with PSA specific probe ($p_1$) and IS specific probe ($p_2$) are shown by striped lines or solid colour, respectively. Detection of the targets with only one specific probe indicates that the two targets are distinguishable and there is no cross hybridization between targets and probes. The PCR Neg product (containing no starting DNA) gives fluorescence readings typical of the assay blank.
FIGURE 3.2

[Graph showing fluorescence levels for PSA probe and IS probe across different target RNAs: NEG, PSA mRNA, IS RNA. The x-axis represents target RNA categories, and the y-axis represents fluorescence in arbitrary units (arb. units).]
FIGURE 3.3
Hybridization Assay for PCR Product Detection

Legend

Hybridization assays for detection of both the amplified PSA mRNA and RNA IS RT-PCR products. SA-coated wells were washed and biotinylated PCR product was added. Following washing and NaOH incubation, one strand of the DNA was washed out. This allowed DIG-tailed probe to bind the immobilized strand in a 42°C incubation step. For PSA and IS detection, DIG-labeled (p₁) and (p₂) were used respectively. The wells were washed and an anti-DIG-ALP conjugate added. After washing, a DFP substrate solution was added, followed by the addition of the Tb³⁺-EDTA developing solution. Fluorescence was measured in the CyberFluor time-resolved fluorometer.
**FIGURE 3.3**

Wash SA-coated wells 3x.

Add 50 μL biotinylated PCR product (10x diluted 1% Blocking).
Incubate 30 min RT.

Add 50 μL 0.2 M NaOH.
Incubate 20 min RT. Wash.

Add 50 μL pre-heated DIG-probe (p₁ or p₂).
Incubate 30 min at 42°C. Wash.

Add 50 μL anti-DIG ALP.
Incubate 30 min RT. Wash.

Add 50 μL DFP substrate.
Incubate 30 min RT.

Add Tb³⁺-EDTA Developing
Read fluorescence in CyberFluor 615

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and the dependence of ethidium bromide incorporation on the fragment size was taken into account. A stock solution of amplification product for the RNA IS was also prepared as above.

Following quantification, various dilutions of each stock solution were analyzed by hybridization to probe \( p_1 \) or \( p_2 \). In Figure 3.4, the fluorescence (corrected for the background) was plotted versus the concentration of the amplified DNA. The background is defined as the fluorescence obtained when a sample containing no amplification product was assayed. Each data point represents the average of two assays. Concentrations as low as 4 pmol/L (200 amol/well) of amplification products from PSA mRNA and RNA IS were detected with signal-to-background ratios (S/B) of 1.5 and 3.0, respectively. The linear range extends up to 1000 pmol/L and 500 pmol/L, respectively.

It was observed that the hybridization assay for the IS was more sensitive than that of the target. The probes were designed with the same GC content and therefore the hybrids are expected to have the same melting temperature. The difference in the amplification graphs of Figure 3.4, was probably due to folding of the captured single stranded target DNA (i.e., after the NaOH step) which might have interfered with the hybridization.

The reproducibility (within-run) of the hybridization assay was tested by analyzing samples containing 31, 125 and 500 pmol/L of amplified DNA. The %CVs were 4.2, 5.2 and 7.9, respectively (n=5).

The ability of the proposed analytical system to quantify PSA mRNA from a few PSA expressing cells in the presence of total RNA from normal cells was estimated by preparing mixtures containing total RNA representative of 4 to 3000 LNCaP cells in the presence of 1 μg total RNA from healthy cells (isolated from whole blood as described in Section 3.2.3.2). A fixed amount of RNA IS was added into each sample. The reverse transcription and amplification procedure for the Q-PCR method is shown in Figure 3.5.
FIGURE 3.4
Sensitivity and Linearity of Hybridization Assays

Legend

Calibration curves for the hybridization assays of the amplification products from PSA mRNA (solid line) and RNA IS (dashed line). The amplified DNA was labeled with biotin (through PCR), captured on SA-coated wells and hybridized to DIG-labeled probes \( p_1 \) and \( p_2 \) respectively. The linear ranges of the assays for PSA mRNA and RNA IS were, 4-1000 pmol/L and 4-500 pmol/L, respectively.
FIGURE 3.4

Fluorescence (arb. units) vs. Amplified DNA, pmol/L.
Legend

Procedure for the quantification of PSA mRNA by co-amplification of the PSA mRNA with RNA IS in RT-PCR. Reverse transcription was first carried out with the downstream primer (d) to create both PSA and IS RNA-cDNA hybrids. This was followed by PCR, using biotinylated (u) and (d) as the upstream and downstream primers, respectively. The two 233 bp DNA products, distinguishable by the altered 24 bp sequence characteristic of the IS, were detected by separate hybridization assays.
FIGURE 3.5

PSA mRNA 5' __________________________ 5' __________________________ RNA IS 233 bases

 Addition of primer (d)

 5' _______ d 5' _______ d

 Reverse Transcription

 PSA mRNA-cDNA hybrid

 5' __________________________ 5' __________________________ IS RNA-cDNA hybrid

 PCR

 Primers BIO-(u) and (d)

 PSA DNA 5'-BIO 233 bp 5'-BIO IS DNA 233 bp

 Detection of co-amplified PCR products by hybridization assays
Following RT-PCR, the products were analyzed by the two hybridization assays. The fluorescence signals obtained for both the PSA mRNA and the RNA IS were plotted as a function of the number of LNCaP cells in Figure 3.6. The S/B ratios for PSA mRNA corresponding to 4 and 12 LNCaP cells were 1.6 and 2.5, respectively.

The fluorescence corresponding to the RNA IS remained constant for small numbers of LNCaP cells, but then decreased as the amount of PSA mRNA in the sample increased. This was due to the plateau phenomenon of PCR. When the amplification was still in the exponential phase, the efficiency was constant. Because the same amount of RNA IS was used in every sample, the amount of amplified RNA IS, and therefore the signal, was relatively constant and independent of the starting amount of the PSA mRNA. However, as the PSA mRNA increased, the signal for IS decreased due to competition of target RNA for amplification as well as the PCR plateau effect.

The amount of RNA IS added to each sample may affect the sensitivity and the analytical range of the technique. If a large amount of IS is added, small quantities of PSA mRNA will not be significantly amplified to generate a signal. On the other hand, a small amount of RNA IS in the presence of relatively high PSA mRNA levels would be undetectable following amplification. The amount of IS was optimized empirically by testing serial dilutions of the RNA IS stock such that the amplification product gave a fluorescence signal that was in the middle of the linear range of the calibration curve for the hybridization assay.

In Figure 3.7, the ratio $F/F_{IS}$, of the fluorescence values obtained for the PSA mRNA and RNA IS was plotted against the number of LNCaP cells. The data suggest that the quantitative assay was linear in the range of 4 to 3000 cells. Since only 40% of the reverse transcription mixture was amplified and only 1/20th of the PCR mixture was used for analysis, the fluorescence signal represents amplification product from PSA mRNA corresponding to less than 0.1 LNCaP cell.
FIGURE 3.6
Sensitivity of Quantitative RT-PCR for PSA mRNA

Legend

Study of the variation in fluorescence as the PSA mRNA corresponding to 4-3000 LNCaP cells was co-amplified with a constant amount of RNA IS in the presence of 1 µg of healthy RNA. The solid and dashed lines represent signals obtained from the PSA mRNA and RNA IS respectively.
FIGURE 3.7
Calibration Curve for the Quantification of PSA mRNA

Legend

Calibration curve for the quantification of PSA mRNA by co-amplification with the RNA IS. Mixtures of both targets, with varying amounts of PSA mRNA and a constant amount of RNA IS were subject to RT-PCR. The ratio of fluorescence signals (F/F<sub>IS</sub>) obtained for the PSA mRNA and RNA IS was plotted against the initial number of LNCaP cells present in the sample prior to amplification.
FIGURE 3.7

![Graph showing the relationship between F/Fis and the number of LNCaP cells. The graph is a log-log plot with F/Fis on the y-axis and the number of LNCaP cells on the x-axis. The data points are connected by a straight line, indicating a linear relationship.]
To assess the overall reproducibility (between-run) of the quantitative RT-PCR, CV studies were carried out at three PSA mRNA levels in the presence of 1 µg of healthy RNA. RT-PCRs for the three pools, containing different concentrations of PSA mRNA, were performed four times on different days. The %CVs of the F/F<sub>ts</sub> ratios obtained from the three pools were 16.1, 16.2 and 14.2. In addition, from the F/F<sub>ts</sub> ratios and the graph of Figure 3.7 it was estimated that the pools contained 31, 381 and 1626 cells with %CVs of 19.1, 19.1 and 17.0, respectively.

The proposed analytical methodology for quantitative RT-PCR of PSA mRNA may have potential in, a) distinguishing between clinically relevant and non-relevant levels of PSA mRNA, the latter possibly occurring due to illegitimate transcription (110); b) providing information about the relation of PSA mRNA levels and recurrent disease following surgery; c) research as a powerful tool in the study of the biology of prostate cancer and the metastatic process; d) applications to non-prostatic diseases since PSA may be a new favorable prognostic indicator in female breast cancer as has been found in recent years (111-113).

A drawback of the proposed quantitative assay for PSA mRNA is that it cannot distinguish if the increased mRNA concentration is due to a large number of PSA expressing cells (containing small amounts of the particular mRNA) or due to a small number of cells containing high amounts of PSA mRNA. This point should be taken into account in the clinical evaluation of the quantitative PCR assay.

3.4 Conclusions

The proposed analytical methodology for quantification of PSA mRNA has several advantages. First, the method is based on the use of a recombinant RNA internal standard which contains the same primer binding sites as the target RNA. Thus, in the absence of mispriming events of the RT primer to the considerably larger native RNA, any variation in the efficiency of the reverse transcription and/or the PCR step affects
both RNAs equally. The ratio of the analytical responses therefore reflects the initial ratio of the two RNAs in the starting mixture. This is proven by the linearity and the reproducibility of the assays. Secondly, the PCR products are confirmed by hybridization assays performed in microtiter wells, thus avoiding time-consuming electrophoresis and densitometry, as well as facilitating automation for use in the routine laboratory. Finally, time-resolved fluorometry offers the high sensitivity required for this type of analyses while eliminating the need for radioisotopic labeling of PCR products or probes.
CHAPTER 4
QUANTITATIVE POLYMERASE CHAIN REACTION BASED ON A DUAL-
ANALYTE CHEMILUMINESCENCE HYBRIDIZATION ASSAY FOR TARGET
DNA AND INTERNAL STANDARD

4.1 Introduction

The polymerase chain reaction amplifies nucleic acid sequences, selectively and
exponentially, to levels that are several orders of magnitude higher than those in the
starting material (1). Conventional methods of nucleic acid analysis such as Southern and
Northern blots are not sensitive enough to detect DNA or RNA in limited amounts from
biological samples, or in samples containing low copy numbers of the sequences of
interest. Because of its superior sensitivity, PCR has rapidly become one of the most
important analytical techniques in all aspects of biological research and molecular
diagnosis.

In recent years, several methods have been developed for analysis of PCR
products. These include electrophoretic separation of the amplified DNA, high-
performance liquid chromatography (114), capillary electrophoresis (115) and
hybridization followed by radioactive or nonradioactive detection of the hybrids.
Nonradioactive hybridization assays based on fluorescent, chemiluminescent or enzyme
labels have been developed. Sensitivity has been further improved by the replacement of
conventional chromogenic substrates with alternatives, allowing the monitoring of
enzyme activity by chemiluminescence or time-resolved fluorescence (116-117).

Despite the advances described above, the exponential nature of PCR poses
serious difficulties in its utilization for the determination of the starting quantity of target
DNA. Quantification requires the establishment of a reproducible relation between the
analytical signal obtained from the amplification product and the number of target DNA
molecules in the sample prior to amplification. The amount of product (P) accumulated
after n cycles is given by the equation \( P = T(1+E)^n \), where \( T \) is the initial amount of target DNA and \( E \) is the average efficiency. As a consequence, small sample-to-sample variations of \( E \) lead to dramatic changes in the amount of product and large errors in estimating the initial quantity of the target (especially when \( n \) is large).

The variability of the efficiency can be circumvented by coamplifying, in the same reaction tube, the target with an internal standard. The ratio of the two amplification products can then be related to the initial amount of target in the sample (21-22,118-119). The IS should have the same primer binding sites as the target DNA. Moreover, it must be possible to analyze the amplification products from target DNA and IS in the PCR mixture without interference from each other. In the majority of reports on quantitative PCR, the IS is designed to contain a deletion or insertion large enough to allow electrophoretic separation of the products (21-22,118-119). The fragments are then quantified by scanning densitometry.

Alternatively, the IS has the same size as the target but contains a new restriction site and the products are subjected to digestion prior to electrophoresis. The use of HPLC may facilitate the separation and quantification of the two products (120). Furthermore, the target DNA and the IS may be analyzed by hybridization to specific probes (23,121-122). The hybridization-based methods offer significant advantages because they allow confirmation of the amplified sequences and provide much higher sensitivity.

Until now, quantitative PCR methodologies required that the hybridization assays for amplified target DNA and IS be performed separately, by splitting each sample in different reaction vessels (e.g. microtiter wells). To facilitate automation of Q-PCR, a dual-analyte chemiluminescence hybridization assay for simultaneous determination of both amplification products in the same reaction vessel has been developed. Aequorin and alkaline phosphatase are used as reporter molecules. It was first proven that the dual-analyte approach was valid, by using mixtures of target DNA and IS. The sensitivity,
linear range and reproducibility of the dual protocol assay were then assessed. Finally, a quantitative PCR methodology based on the proposed dual-analyte assay was developed.

4.2 Methods

4.2.1 Preparation of Target DNA and DNA IS Stock Solutions

A 233 bp DNA fragment was used as a target. The target DNA stock solution was synthesized by PCR as described in Section 3.2.4. Starting from the recombinant plasmid pA75 (108) containing a 1.4 kb PSA cDNA insert, the 233 bp fragment was created using biotinylated primer (u) and (d) as the upstream and downstream primers, respectively. The amplification products of three PCR reactions were pooled and purified with the Wizard™ PCR Preps DNA purification system according to the manufacturer’s instructions.

The concentration of the target DNA was determined by scanning densitometry of negatives prepared from pictures of ethidium bromide-stained agarose gels, using the pUC 18 DNA fragments as standards. Solutions with various target DNA concentrations were prepared by diluting the stock in 20 mmol/L ammonium sulfate, 75 mmol/L Tris-HCl (pH 8.8), 0.1% Tween-20, 2 mmol/L MgCl₂.

Using PCR as a synthetic tool, the recombinant DNA internal standard was constructed as described previously in Section 3.2.5.1. A 24 bp segment spanning region 67-90 of the 233 bp target DNA, was replaced with a different sequence of the same size (5'-CTT GCT GAA CTT CTG ACT ACG ACT-3'). The final amplification to produce the 233 bp recombinant IS DNA also used the biotinylated primer (u) and (d) as upstream and downstream primers, annealing at positions 1-20 and 214-233, respectively. Wizard™ purification and quantification with scanning densitometry were carried out for the DNA IS as for the target DNA.
4.2.2 Labeling of Primers and Probes

4.2.2.1 Biotinylation of Upstream Primer

The upstream primer (u) was synthesized with an -NH$_2$ group at the 5' terminus. The primer was biotinylated using NHS-LC-biotin according to the procedure described in Section 3.2.6.1.

4.2.2.2 Tailing Probe (p$_1$) with Digoxigenin

Probe (p$_1$), specific for the target DNA, was tailed with multiple digoxigenin molecules. The tailing reaction was performed in a total volume of 20 μL which consisted of 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl (pH 6.6), 0.25 g/L BSA, 5 mmol/L CoCl$_2$, 50 μmol/L Dig-dUTP, 0.5 mmol/L dATP, 25 units of TdT and 100 pmol of probe. The reaction was carried out at 37 °C for 60 min. The tailed probe was used without purification.

4.2.2.3 Tailing Probe (p$_2$) with Fluorescein

The IS probe (p$_2$) was tailed with multiple fluorescein molecules, exactly as above, using 50 μmol/L of fluorescein-dUTP instead of the DIG-dUTP.

4.2.3 Dual-Analyte Chemiluminescence Hybridization Assay

Opaque polystyrene wells were coated with streptavidin as described in Section 3.2.7.2.1. Prior to use the wells were washed three times with wash buffer. PCR products containing amplified target DNA and DNA IS, both biotinylated at their 5' end, were diluted ten times in blocking solution and 50 μL pipetted into each well in duplicate. The DNA fragments were allowed to bind to the streptavidin for 30 min with shaking at room temperature. The wells were washed as above. The non-biotinylated strand was dissociated by incubating for 20 min with 50 μL of 0.2 mol/L NaOH, followed by three washings. A 50 μL solution containing both Fluorescein-labeled probe (p$_2$) and DIG-labeled probe (p$_1$), each diluted in blocking solution to a concentration of 7 nmol/L and preheated at 42 °C, was pipetted into each well. The immobilized single stranded amplification products for target DNA and IS were allowed to hybridize simultaneously.
with their specific probes for 30 min, shaking at 42 °C in the Amerlite shaker/incubator. The wells were washed three times to remove the unbound probes. A 50 μL solution containing 75 mU/L ALP-labeled anti-fluorescein antibody and 10 μg/L aequorin-labeled anti-DIG antibody, diluted in blocking solution, was added to each well. The labeled antibodies were allowed to bind simultaneously to their corresponding haptens for a period of 30 min at room temperature and then the excess reagent was removed by washing. The wells were then placed in the luminometer and 50 μL of the aequorin luminescence Ca²⁺ triggering solution was injected into each well. The light emitted was integrated for 3 sec. Subsequently, 50 μL of the ALP CSPD substrate solution was injected into each well. The enzymatic reaction was allowed to proceed for 20 min, at which point the light emitted was integrated for 10 sec. The dual-analyte chemiluminescence hybridization assay protocol is illustrated in Figure 4.1.

4.2.4 Quantitative Polymerase Chain Reaction

PCRs were performed in a total volume of 100 μL containing (final concentrations) 20 mmol/L ammonium sulfate, 75 mmol/L Tris-HCl (pH 8.8), 0.1% Tween-20, 2 mmol/L MgCl₂, 50 μmol/L dNTPs, 50 pmol of each of the biotinylated upstream primer (u) and the downstream primer (d), 2.5 units of Taq polymerase, a constant amount of IS (in the range of 10 000-40 000 molecules) and target DNA varying from 400 to 300 000 molecules. The hot-start protocol (123) was employed in which the reaction mixture was heated to 95 °C for 5 min, followed by the addition of primers. PCR was carried out for 25 cycles of denaturation at 95 °C for 30 sec, annealing (65 °C, 30 sec) and extension (72 °C, 1 min). Finally, the mixtures were incubated at 72 °C for 10 min and then cooled to 4 °C until their analysis by the dual-analyte chemiluminescence hybridization assay described previously.
4.3 Results and Discussion

The proposed dual-analyte chemiluminescent hybridization assay, illustrated in Figure 4.1, allowed the detection of both target DNA and DNA IS, in the same reaction vessel. SA coated microtiter wells were washed prior to use and biotinylated PCR products containing amplified target DNA and DNA IS were captured on the wells. After washing, the non-biotinylated strands were dissociated with 0.2 M NaOH and removed by washing. Both immobilized single stranded target DNA and IS amplification products were allowed to simultaneously hybridize with their specific probe, DIG-tailed (p1) or fluorescein-tailed (p2), respectively. The excess of unbound probes was removed by washing. ALP-labeled anti-fluorescein antibody and aequorin-labeled anti-DIG antibody conjugates, in the same mixture, were then allowed to simultaneously bind their corresponding haptens.

The injection of Ca^{2+} triggering solution by the luminometer initiated the first chemiluminescent reaction. Subsequent light emission, from the aequorin-labeled anti-DIG antibody bound to the target DNA hybrids, was integrated for 3 sec. This was immediately followed by the addition of the CSPD substrate solution without prior washing of the wells. The dephosphorylation of the CSPD by the ALP-labeled antifluorescein antibody bound to the IS hybrids, was allowed to proceed for 20 min, upon which light emission was integrated for 10 sec.

In Figure 4.2, the kinetics of light emission, for both the aequorin [A] and the ALP [B] reactions, are presented. The flash-type reaction of aequorin reaches an emission peak at about 1 sec and is completely over in 10 sec. Dephosphorylation by ALP triggered a glow-type chemiluminescent reaction, stable for several hours.

In order to assess the ability of the dual-analyte hybridization assay to provide accurate determination of the two DNA fragments in a mixture, two pools were prepared containing a low concentration of target DNA in the presence of a high concentration of IS and vice versa. Pool #1 contained 15 pmol/L of target DNA and 500 pmol/L of IS,
FIGURE 4.1
Dual-Analyte Chemiluminescent Hybridization Assay Protocol

Legend

Dual-analyte chemiluminescent hybridization assay for simultaneous detection of target DNA and DNA IS in a single well. Biotinylated PCR amplification products were captured on SA coated wells. Following removal of one DNA strand by NaOH, the labeled probes, DIG-(p₁) and fluorescein-(p₂), were allowed to hybridize. The immobilized hybrids were reacted with anti-DIG-aequorin (Aq) and antifluorescein-ALP conjugates. Injection of Ca²⁺ triggering solution, initiated the flash-type reaction of aequorin (Aq) bioluminescence. Light emission was measured for 3 sec in the MLX Luminometer. An ALP substrate solution (CSPD) was immediately added, and after a 20 min incubation, chemiluminescence was measured for 10 sec.
Capture of biotinylated PCR products on SA-coated wells.

Removal of one strand by 0.2 M NaOH. Addition of DIG-\(p_1\) and Fluorescein-\(p_2\).

Addition of anti-DIG-Aq and anti-fluorescein ALP.

Injection of Ca\(^{2+}\). Flash Reaction. Measurement of light emission.

Injection of CSPD substrate. Glow reaction. Measurement of light emission after 20 min.
FIGURE 4.2

Kinetics of Light Emission

Legend

Kinetics of light emission, for both the aequorin and ALP reactions.

A: Following the addition of Ca²⁺, aequorin emits a flash of light (λ_{max}=469 nm) that peaks at about 1 sec. The chemiluminescent reaction was integrated for the first 3 sec.

B: ALP dephosphorylation of CSPD produces a glow-type chemiluminescent reaction, stable for several hours. After 20 min, luminescence was integrated for 10 sec.
while Pool #2 contained 500 pmol/L of target DNA and 15 pmol/L of IS. The pools were first analyzed by single assay protocols. In the single assay protocol, the hybridization solution contained only one labeled probe and the hybrids were reacted with the corresponding labeled antibody. The wells were washed and Ca\textsuperscript{2+} containing solution and CSPD substrate solution were injected.

The dual protocol was carried out with mixtures of probes, antibodies and detection reagents as described under Methods Section 4.2.3. The results are presented in Table 4.1. It was observed that the dual-analyte protocol did not interfere with the determination of either DNA fragment. Apparently, the fluorescein moieties immobilized in the wells did not interfere with the aequorin emission. Also, the alkaline phosphatase reaction was not inhibited by the presence of the Ca\textsuperscript{2+} light triggering solution for aequorin, thus allowing the two reporter molecules to be determined in the same well.

The sensitivities and the linear ranges of the hybridization assays were established as follows. Various dilutions of each target DNA and DNA IS stock solution were analyzed by hybridization to labeled probes (p\textsubscript{1}) or (p\textsubscript{2}), respectively. The hybridization assay for the IS was performed in the presence of the aequorin triggering solution. In Figure 4.3, the luminescence (corrected for the background) is plotted as a function of the amplified DNA in the well. The background is defined as the luminescence obtained when a sample containing no amplification product was analyzed.

Each data point represents the average of two assays. Concentrations as low as 0.97 pmol/L (48.5 amol/well) of amplification product for the target DNA were determined with a S/B ratio of 3.7. The linearity extends up to 500 pmol/L. Also, 5.5 pmol/L (267 amol/well) of the amplification product for DNA IS was determined with a S/B ratio of 2.3 and linearity extending up to 700 pmol/L.

The results presented in Figure 4.3 indicate that the dual-analyte assay provides higher sensitivity for the amplified target DNA compared to the IS. The reasons for this difference were investigated as follows. To directly compare the detectabilities of the two
Table 4.1. Comparison Between Single and Dual-Analyte Hybridization Assays

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Assay Type</th>
<th>Luminescence</th>
<th></th>
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</tr>
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<tr>
<td></td>
<td></td>
<td>Pool #1</td>
<td>Pool #2</td>
<td></td>
</tr>
<tr>
<td>Target DNA</td>
<td>Single</td>
<td>30.0±1.4</td>
<td>904±20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dual</td>
<td>36.3±4.7</td>
<td>832±96</td>
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<tr>
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<td>Single</td>
<td>37003±4167</td>
<td>2674±182</td>
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</tr>
<tr>
<td></td>
<td>Dual</td>
<td>40986±1706</td>
<td>2714±251</td>
<td></td>
</tr>
</tbody>
</table>

a. The mean values and standard deviations are given (n=4).
FIGURE 4.3

Sensitivity and Linearity of Chemiluminescent Hybridization Assays

Legend

Calibration curves for the chemiluminescent hybridization assays of the target DNA (solid line) and DNA IS (dashed line). The amplified biotinylated target DNA and IS were captured on SA coated wells and hybridized to DIG-labeled probe (p1) and fluorescein-labeled probe (p2), respectively. The linear ranges of the assays for the target DNA and IS were, 0.97-500 pmol/L and 5.5-700 pmol/L, respectively.
FIGURE 4.3

![Graph showing the relationship between log(target) and amplified DNA concentration.](image-url)
chemiluminescence detection systems, solutions containing 0, 20, 60 and 180 pmol/L of biotinylated amplification product from target DNA were analyzed using DIG-labeled probe (p.). After completion of hybridization, the hybrids were reacted either with anti-DIG-aequorin or with anti-DIG-ALP conjugates. The results, expressed as S/B ratios, are presented in Table 4.2. It was observed that the aequorin based detection system provides a 3.5 fold improvement of the S/B ratio compared to the ALP based detection system.

In the proposed hybridization assay, the hybrids were linked to the corresponding reporter molecules through a hapten/antibody interaction. The DIG/anti-DIG and fluorescein/antifluorescein linking systems were compared as follows. Solutions containing 0, 20, 60 and 180 pmol/L of biotinylated amplified target DNA were analyzed by hybridization to probe (p.) labeled with either DIG or fluorescein. The hybrids were reacted with either anti-DIG-ALP or antifluorescein-ALP antibody conjugates. A 2 fold improvement in S/B ratios was obtained with the DIG/anti-DIG system (Table 4.2).

In order to study the effect of the altered 24 bp centrally located sequence on the hybridization, solutions containing 0, 20, 60, 180 pmol/L of either target DNA or IS were analyzed using probes (p,) or (p,) labeled with fluorescein. The hybrids were then reacted with antifluorescein-ALP. The results are presented in Table 4.2. It was observed that the S/B ratios are slightly higher for the IS compared to the target DNA. The 24 bp centrally located sequence of the IS was designed with the same GC content as the native sequence of the target DNA and therefore, the hybrids were expected to have the same melting temperature. The small difference in hybridization may be attributed to the folding of the captured single-stranded DNA fragments after the alkaline denaturation step.

To summarize, the higher sensitivity observed with the target DNA is due to the combined use of aequorin, as a reporter molecule, and the DIG/anti-DIG interaction as a linker.

The reproducibility of the hybridization assays was tested by analyzing three pools, each containing the same concentration of both target DNA and IS. Pool 1
Table 4.2. Study of the Two Chemiluminescent Detection Systems Used in the Dual-Analyte Hybridization Assay

<table>
<thead>
<tr>
<th>Analyte</th>
<th>pmol/L</th>
<th>Anti-Dig/Aequorin (S/B)ᵃ</th>
<th>Anti-Dig/ALP (S/B)ᵇ</th>
<th>Anti-F/ALP (S/B)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target DNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>66</td>
<td>19</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>203</td>
<td>53</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>679</td>
<td>168</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>DNA IS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td></td>
<td></td>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ. The values represent the average of two measurements.
contained 15 pmol/L of each, pool 2 contained 100 pmol/L of each and pool 3 contained 300 pmol/L of each type of DNA. The pools were analyzed five times with the dual hybridization assay. The %CVs obtained for target DNA and IS were 6.7 and 7.7 (pool 1), 4.9 and 2.9 (pool 2) and 6.1 and 5.9 (pool 3), respectively.

The quantitative PCR assays were carried out by co-amplifying samples containing target DNA concentrations varying from 430 to 315000 molecules with a constant amount of DNA IS (in the range of 10000 to 40000 molecules). Following amplification, the products were determined by the dual-analyte hybridization assay using probes (p1) and (p2). For each standard curve, a negative was prepared (PCR mixture containing IS but no target DNA), amplified and measured by the same protocol. The luminescence values obtained, \( L_{\text{target}} \) and \( L_{\text{IS}} \), reflect the concentrations of amplification products from target DNA and DNA IS, respectively. In Figure 4.4, the luminescence (corrected for the background) was plotted as a function of the number of target DNA molecules in the sample prior to amplification. The background is defined as the luminescence obtained from the PCR negative.

The luminescence corresponding to the DNA IS remains constant for a small number of target DNA molecules. However, as the target DNA increases, the PCR enters its plateau phase (124) where amplification efficiency drops for both DNA fragments and the total amount of product remains practically constant regardless of the starting amount of target DNA. As a consequence, a further increase of target DNA results in suppression of the amplification of IS.

In Figure 4.5, the ratio \( L_{\text{target}}/L_{\text{IS}} \) of the luminescence values obtained for the target DNA and DNA IS amplification products, was plotted against the number of initial target DNA molecules. A linear relationship was observed which extends over almost three orders of magnitude (from 430 to 315000 molecules). Moreover, the figure demonstrates the effect of the amount of IS on the calibration graphs. The three lines correspond to quantitative PCR assays performed in the presence of 10000, 20000 and
FIGURE 4.4
Sensitivity of Quantitative PCR for Target DNA

Legend

Study of the variation in luminescence as the target DNA corresponding to 430-315000 molecules was co-amplified with a constant amount of IS (40000 molecules). The luminescence signals obtained from the dual-analyte chemiluminescent assay for the target DNA (solid line) and IS (dashed line), are plotted versus the number of target DNA molecules initially present.
FIGURE 4.5

Calibration Curve for the Quantification of Target DNA

Legend

Study of the linearity and sensitivity of the quantitative PCR assay. The ratios of luminescence signals ($L_{\text{target}}/L_{\text{IS}}$) obtained from the target DNA and IS were plotted against the initial number of target DNA molecules present prior to amplification. The three lines correspond to quantitative PCR assays performed in the presence of 10000 (circles), 20000 (squares) and 40000 (diamonds) molecules of DNA IS.
40000 molecules of DNA IS. In theory, the ratios of the luminescence signals are inversely related to the number of DNA IS molecules used. This is reflected by the parallel shift of the double logarithmic plot to higher L_{target}/L_{IS} values as the amount of IS decreases. The signal-to-background ratios observed for 430 molecules of target DNA were 4.4, 2.4 and 2.7, respectively. Because only 5% of the initial PCR mixture was used in the hybridization assay, the luminescence signal is essentially obtained from amplification product corresponding to 22 target DNA molecules.

The overall reproducibility of the proposed quantitative PCR assays (including the PCR step and the dual-analyte hybridization assay) was tested by analyzing samples containing 1300, 13000 and 130000 target DNA molecules. To each sample were added 40000 molecules of DNA IS and the PCRs were performed on different days. The %CVs obtained for the L_{target}/L_{IS} ratios were 7.3, 8.9 and 17.6, respectively (n=4).

The most widely used internal standards for quantitative PCR share the same primers with the target, but contain a deletion or insertion to enable electrophoretic separation of the target DNA and IS amplification products. Sequence length is however, also a major determinant of the amplification efficiency. It has been shown that the efficiency is inversely related to the size of the DNA (125). In the present work, the target DNA and the DNA IS not only share a common set of primers but also have identical sizes. Moreover, the IS closely resembles the target DNA, differing only in a 24 bp segment (10% of its size).

It has been observed that coamplification of DNA fragments sharing considerable sequence homology leads to the formation of heteroduplexes during PCR, even if their sizes are different (125). Indeed, in the annealing phase of the PCR cycle, a fraction of the single stranded fragments of target DNA will hybridize with the complementary strand from the IS and vice versa. Upon electrophoresis, the heteroduplexes migrate between the target DNA and the IS and may cause errors in the determination of the products, especially if they cannot be resolved from the homoduplexes. In the case of internal
standards having the same size but differing only in a restriction site, heteroduplexes interfere because they are resistant to digestion. In the proposed hybridization assay, all the amplified fragments are captured in the well followed by alkaline denaturation and washing of the strands. The subsequent hybridization essentially measures immobilized single stranded DNA. Therefore, heteroduplex formation is not a concern for this assay.

Recently, methods have been developed for continuous determination of the generated products during PCR, i.e., for real-time monitoring of DNA amplification (126-128). One such approach uses the compound SYBR Green I whose fluorescence increases upon binding to ds DNA. The co-determination of undesired products represents a serious limitation of this method. An alternative strategy employs an oligonucleotide probe labeled with a donor fluorescent molecule (6-carboxy- fluorescein) at the 5' end and an acceptor molecule (6-carboxy-tetramethylrhodamine) at the 3' end. The close proximity between donor and acceptor allows for energy transfer to occur and therefore no fluorescence from the donor is observed. During annealing, the probe hybridizes to the template DNA at a position flanked by the primers. However, because of the 5' exonuclease activity of Taq DNA polymerase, the hybridized probe is degraded during the extension phase of each cycle and the fluorescence increases. Specific probes labeled with different donor molecules and the same acceptor molecule may be used for determination of amplified target DNA and DNA IS.

Real-time quantitative PCR methods are advantageous in that they do not require post-PCR sample processing. However, the detectability of the fluorescent label is in the nmol/L range and the florescence is an indirect measure of the amplification product. It has also been reported that hybridization is a necessary but not sufficient condition for probe hydrolysis, i.e. all probes are not cleaved efficiently. In contrast, the proposed chemiluminescence assay allows confirmation of the final amplification product after PCR and offers at least 1000-fold higher sensitivity, since it can detect 1 pmol/L of amplified product (see Figure 4.2). Also, the labeling of probes in the proposed system
was accomplished by enzymatic tailing which is much easier than the conjugation techniques required for double labeling of the probes in real-time PCR methods.

In conclusion, a quantitative PCR methodology was developed, based on a dual-analyte chemiluminescence hybridization assay for the detection of amplified target DNA and the DNA IS in a single reaction vessel. The assay is highly sensitive and, because it is performed in microtiter wells, it is suitable for automation and high-throughput analysis.
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