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Methods for antigen and nucleic acid analysis.

Bakhos Antonios. Tannous

University of Windsor

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Methods for Antigen and Nucleic Acid Analysis

by

Bakhos Antonios Tannous

A Dissertation
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 Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada
2002
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Abstract

One aspect of this dissertation involves the investigation of T7 RNA polymerase (T7RP) as a label for antigen quantification. In an in vitro, coupled (one-step) transcription/translation reaction, T7RP catalyzes the expression of an enzyme-coding DNA template to produce free enzyme (luciferase) in solution. We demonstrate that the generated luciferase is linearly related to the input T7RP in a range covering over four orders of magnitude. It is also shown that T7RP exhibits a significant level of self-replication (100-fold) in vitro by acting on a DNA template comprising the T7RP cDNA downstream of a T7 promoter. By combining the self-replication reaction with the expression of luciferase DNA, as low as 1400 T7RP molecules are detectable. Furthermore, the T7RP is biotinylated, complexed with streptavidin and used for antigen quantification in a microtiter well-based assay with high detectability and reproducibility.

Another aspect of the dissertation deals with the design of universal heterobifunctional linkers between antibodies and reporter genes for immunoassay development. The linkers consist of avidin or streptavidin conjugated to the oligonucleotide (dA)$_{40}$. (Strept)avidin interacts with a biotinylated detection antibody whereas the oligonucleotide hybridizes with a complementary poly(dT) tail added enzymically to the 3' end of the reporter gene. The linker is evaluated in a model two-site (sandwich-type) immunoassay performed in microtiter wells. A 4.3 kb plasmid containing the firefly luciferase cDNA is used as a reporter. Upon in vitro expression of the reporter gene, multiple enzyme molecules are generated and detected in solution.

In another study, we investigated five strategies for immobilization of a DNA probe to polystyrene microtiter wells for hybridization assays. Probes were immobilized: (a) through digoxigenin-antidigoxigenin interaction, (b) through biotin-streptavidin interaction, (c) by preparation and immobilization of oligonucleotide-albumin conjugates, (d) by activation of the polystyrene surface with poly (Lys, Phe) and subsequent conjugation with the probe, (e) by direct coating via physical adsorption of the probe to the wells. The hybrids were detected by indirect labeling through biotin-streptavidin or digoxigenin-antidigoxigenin and using alkaline phosphatase as a reporter molecule.
We also developed a dual-analyte microtiter well-based chemiluminometric assay for genotyping single point mutations. The IVS-1-110 mutation of the human β-globin gene (responsible for β-thalassaemia) was studied as a model. Genomic DNA was extracted from whole blood followed by PCR amplification. The oligonucleotide ligation assay was performed on the amplified DNA using a common probe and two allele-specific probes. Ligation products were analyzed by a microtiter well-based assay in which aequorin and alkaline phosphatase are used as labels. The assay format enables simultaneous detection of normal and mutated allele in a single microtiter well.
Dedication

To My mom for all her hard work and pain
To my family for their love and support
To the one I live in his remembrance!
Acknowledgements

First, I would like to thank my supervisor Dr. T. K. Chrsitopoulos (aka TKC) for his guidance throughout the course of my Ph.D. Sir, your continuous encouragement and support is appreciated. Check it out sir!

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Special Acknowledgement goes to TKC’s lab members. Not everyday you get to meet people that you enjoy working with. NC, thanks for keeping up with me in my first years, I learned a lot from you. MV and EL, Thanks for being BAT’s Girlies, I already miss “Coffee Time” and “Hareeeeeeee”. PO, was nice having you around in Greece. SW, thank you.

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List of Abbreviations

A    adenosine
Å    angstrom
AEQ  aequorin
Ag   antigen
ALP  alkaline phosphatase
AMP  adenosine monophosphate
Anti-Dig anti-digoxigenin antibody
ATP  adenosine triphosphate
B    biotin
bp   base pair
BS₃  bis-(sulfosuccinimidyl) suberate
BSA  bovine serum albumin
C    cytidine
cDNA complementary DNA of the mRNA
CV   coefficient of variation
Da   Dalton
dA   deoxyriboadenine
Dig  digoxigenin
DNA  deoxyribonucleic acid
DNase deoxyribonuclease
dNTP deoxyribonucleoside triphosphate
dT   deoxyribothymine
DTT  dithiothreitol
E. coli Escherichia coli
EDTA ethylenediamine tetraacetic acid
EGTA ethyleneglycol bis (2-aminoethyl ether) N,N',N tetraacetic acid
G    guanosine
H₂O₂ hydrogen peroxide
HRP  horseradish peroxidase
Ig   immunoglobulin
Ka   affinity constant
Kb   kilo base
Kd   dissociation constant
kDa  kilodalton
LB   Luria-Bertani
Luc  firefly luciferase
M    molar
min  minute
mRNA messenger RNA
RNA  ribonucleic acids
RNase ribonuclease
MW   molecular weight
NHS  N-hydroxysuccinimide ester
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<tr>
<td>NTP</td>
<td>ribonucleoside triphosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>Ppi</td>
<td>inorganic pyrophosphate</td>
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<td>PSA</td>
<td>prostate specific antigen</td>
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<td>pT7</td>
<td>T7 RNA polymerase promoter</td>
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<tr>
<td>R</td>
<td>correlation coefficient</td>
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<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>s</td>
<td>second</td>
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<td>SA</td>
<td>streptavidin</td>
</tr>
<tr>
<td>SATA</td>
<td>N-succinimidyl S-acetylthioacetate</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SMCC</td>
<td>succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate</td>
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<td>snRNA</td>
<td>small nuclear RNA</td>
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<tr>
<td>T</td>
<td>deoxythymidine</td>
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<td>T7RP</td>
<td>T7 RNA polymerase</td>
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<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
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<td>Tris</td>
<td>tris (hydroxymethyl)aminomethane</td>
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<td>transfer RNA</td>
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Chapter 1

General Introduction
1.1. Immunoassays

The high specificity and affinity of antibody-antigen interaction forms the basis of immunoassay, a technique that has had a profound impact in laboratory medicine. Immunoassay is the technique of choice for determination of hormones, drugs, allergens, infectious agents etc, to aid the diagnosis and monitoring of the disease.

Immunoassays were first introduced in the early 1960s for detection of plasma insulin (Yalow RS et al., 1959) and thyroxine (Ekins RP, 1960). These assays were based on the addition of a fixed known concentration of radioisotopically labeled analyte (tracer), and monitoring the distribution of the analyte between the free and the bound forms (radioimmunoassay) by separation and measurement of radioactivity.

Over the past forty years, progress in improving assay configuration, reagents, as well as detection systems have emerged. The first immunoassay using labeled antibodies was introduced in 1968 (Miles LEM et al., 1968), followed by the first “two site”, sandwich-type, immunoassay in 1971 (Wide L., 1971). Furthermore, the development of monoclonal antibodies (Kohler G et al., 1975), and the transition from radioisotope labels to chromogenic, fluorogenic and more recently chemiluminogenic labels has led to an increase in immunoassay sensitivity. and facilitated the development of fully automated immunoassay systems with random access capability (Chan DW, 1996).

In this section, the theory of immunoassays, the structure of antibodies, the antigen-antibody interaction and the basic immunoassay configurations will be discussed.

1.1.1. Antibody structure

Immunoglobulins share a common structural unit, yet offer enormous diversity needed for their central function in recognizing and binding a specific foreign molecule (antigen), activating cellular immune system, and leading to phagocytosis. The basic structure of immunoglobulins consists of four subunits: two identical light chains (25 kDa each) and two identical heavy chains (50 kDa each), which form the characteristic Y-shape (Figure 1.1). The light chains are connected to the heavy chains by one or more disulfide bonds, along with other non-covalent interactions. Similarly, the constant heavy chains as well as the light chains are connected to each other by intrachain disulfide
Figure 1.1. Schematic diagram of an immunoglobulin molecule. Immunoglobulin consists of two light chains (\(\lambda\)) and two heavy chains (\(\mu\)). Each light chain is composed of a variable (V) and a constant (C) region. Each heavy chain is composed of four subunits, three constant and one variable region. The heavy and light chains are connected to each other by a number of disulfide bonds. The hinge region gives the flexibility of the immunoglobulin. The variable region of the heavy and light chain forms the antigen-binding site. Digestion of the immunoglobulin with the proteolytic enzyme papain results in cleavage in the hinge region yielding two Fab fragments and one Fc fragment.
linkage, which contribute to the secondary structure of the antibody. Immunoglobulins are glycoproteins and each heavy chain has an N-linked oligosaccharide (Voet D et al., 1990).

There are five classes of secreted immunoglobulins (Ig) in human, IgA, IgG, IgM, IgD, and IgE, each with different physiological function. They have two similar types of light chains (κ or λ), but differ in the heavy chain constant region, designated α, γ, μ, δ, and ε respectively, which are coded by different genes. Both light and heavy chains contain regions with similar amino acid sequence (constant region) as well as regions that vary among different antibodies (variable region). The variable regions for both the light and heavy chain are the amino terminal ends.

The three dimensional structure of immunoglobulins shows that the protein is made up of six globular domains, two that form the stem of the Y (Fc fragment), and another two domains form each of its two arms (Fab region). Each domain is a folded β-sheet structure formed by alternating antiparallel sequence connected by loop regions. Immunoglobulin fold exhibits intersegmental flexibility due to the hinge region joining each Fc and Fab fragment (Schumaker V et al., 1991). This flexibility facilitates the antigen binding, by permitting an optimal fit between the antigen and its combining sites.

Digestion of immunoglobulin with certain proteolytic enzymes results in predictable fragments. For example, papain digestion will result in three 50 kDa fragments: two identical Fab fragments each containing an antigen binding site and one Fc fragment. Pepsin digestion however, will result in a bivalent fragment containing both antigen binding sites F(ab')2 and non-recovered small pieces of Fc fragment.

1.1.1. Antibody Production

Antibodies are produced by the immune system of animals in response to the presence of foreign substances. Originally, all antibodies used in immunoassay were derived from animal serum after immunization with the antigen of interest. The antiserum contains a heterogeneous population of antibodies that recognize several epitopes on the antigen (polyclonal antibodies).
Polyclonal antibodies are particularly useful for simultaneous detection of various antigens that share similar epitopes (e.g. immunoassay screening for drugs of abuse). However, in the case of determination of a specific antigen, the lack of specificity poses serious limitations because it leads to cross-reactivity problems with antigen of related structure (Neoh SH et al., 1974). To overcome this problem, the hybridoma technique was developed by which monoclonal antibodies are produced (Kohler G et al., 1975). In this technique, the animal is immunized with the antigen of interest, and subsequently killed. Then, B-lymphocytes are obtained from the spleen and are fused with a cancerous multiple myeloma cells. Unfused cells will die, fused cells grow to give a hybrid that undergoes cell division and produces a clone, called hybridoma. Each clone secretes large amounts of identical monoclonal antibodies. Among thousands of hybridomas, the one that produces the desired antibody is cultured while the rest are discarded. The antibodies made in this way are known as monoclonal since they are produced from cell lines derived from single recombinant parent cells. The advantages of monoclonal antibodies include specificity since they are directed to a single epitope of antigen, sensitivity (high affinity constants), reproducibility and continuous production from one cell clone.

The most convenient laboratory animal for producing large quantities of polyclonal antibodies is the rabbit, since it yields high volume of serum and can be easily bled from the marginal ear veins. For monoclonal antibody production, the animal of choice is the Balb-C mouse, the strain from which most of the myeloma cell fusion partners were derived.

1.1.2. Antigen-antibody interaction

The crystal structure of an antibody-antigen complex reveals that the binding site for the antigen is located at the N-terminus of each Fab fragment in a cleft between the variable heavy and light chains. The size and shape of the cleft depends on the amino acid sequences of these regions. It was shown that a maximum of seventeen charged amino acid residues (not necessarily in the same peptide) are directly involved in the antigen-antibody binding (Price CP, 1991). The number of amino acid residues of the
antigen that are bound to the antibody is restricted so that the antigen molecule will have on its surface many structurally dissimilar antigenic determinants (epitopes).

Similar to enzyme-substrate interaction, the antigen-antibody interaction involves a number of non-covalent bonds including electrostatic, hydrogen, van der Waals and ionic interactions. Long-range interactions such as ionic and hydrogen bonds are important in the formation rate of the antigen-antibody complexes, however, short-range forces are important for bond strength, therefore dictating their dissociation constant (Tedford M et al., 1991). The value of the dissociation constant for antigen-antibody complex is around $10^9$ M.

1.1.2.1. Antigen-antibody binding theory

The binding of an antigen to an antibody can be presented mathematically by the Scatchard model, which focuses on the individual binding sites of the antibody for the antigen (ligand, L) and the law of mass action applies for each site (s) to define the association constant (K).

$$s + L \overset{\text{\(\rightleftharpoons\)}}{\text{\(\rightarrow\)}} sL \quad (1)$$

therefore,

$$K = \frac{(sL)}{(s) (L)}$$

The mass conservation of the ligand requires that the total concentration of the antigen equals the sum of the bound plus the free antigen, therefore,

$$K = \frac{B}{F(N - B)} \quad (2)$$

Where B and F represent the concentration (molarity) of the bound and free antigen respectively, and N is the total concentration of the antibody binding sites (total concentration of antibody times 2 (antigen binding sites)), $N - B$ represents the concentration of the free antigen binding sites on the antibody.

Rearranging equation (2) gives

$$B/F = K(N - B)$$

$$B/F = KN - KB$$
Figure 1.2. Scatchard plots for the binding of the antigen (ligand) to different concentrations of antibody (binder) binding sites (N). a. N = 10 nM, b. N = 20 nM, and C. N = 30 nM. With increasing concentration of antibody, the line shifts to the right, however, the slope (-K) remains constant. The x-axis intercept gives the total concentration of the antibody binding sites.
The plot of B/F vs B for various antigen concentrations and a constant antibody concentration is a straight line with a slope of \(-K\) (Figure 1.2). The x-axis intercept corresponds to an infinitely high antigen concentration (saturation of antibody) and gives the total concentration of antibody binding sites (N).

1.1.3. Immunoassay configurations

Immunoassays can be divided into two groups, competitive immunoassays (limited reagent), and noncompetitive (reagent excess) methods.

1.1.3.1. Competitive immunoassays

This type of immunoassay can be divided into two groups, the immobilized antibody or the immobilized antigen approach.

1.1.3.1.1. Immobilized antibody approach

The anti-analyte antibody is immobilized on solid support (such as polystyrene microtiter wells or beads) by physical adsorption through the hydrophobic side chains of the protein. After washing the unbound antibody, the excess binding sites on the solid phase are blocked with a protein such as bovine serum albumin, followed by the addition of the sample along with the tracer (labeled analyte). The analyte in the sample competes with the tracer for limited antibody binding sites. After a certain incubation time, the excess unbound tracer is removed by washing the solid support and the signal from the bound tracer is measured. The signal is inversely related to the analyte concentration (Figure 1.3A).

The antibody used for coating should be affinity-purified, otherwise, other proteins that are present in the antibody solution compete for adsorption to the solid phase. If the antibody is labeled with a hapten such as biotin, then the solid support can be coated with streptavidin (biotin binding protein), followed by the capture of the biotinylated antibody through the biotin-streptavidin interaction. The advantage of this system is that a universal solid support can be used for different assays. Furthermore, the antibody does not have to be affinity-purified due to the specificity of the biotin streptavidin interaction (refer to biotin-streptavidin system, refer to section 1.4.2).
Figure 1.3. Schematic presentation of different immunoassay configurations. A. Competitive immunoassay with the immobilized antibody approach. B. Competitive immunoassay with the immobilized antigen approach. C. Two-site (sandwich-type), non-competitive immunoassay. D. Non-competitive immunoassay for specific immunoglobulin determination.
1.3.1.1.2 Immobilized antigen approach

Affinity-purified antigens are immobilized on solid support by adsorption. After removal of excess unbound antigens and blocking, the sample is added followed by the addition of the labeled detection antibody. The analyte from the sample competes with the immobilized analytes for the limited detection antibody binding sites. After removal of the excess unbound reagents, the signal is measured (Figure 1.3B). As the analyte concentration in the sample increases, the concentration of the labeled antibody captured by the immobilized analyte decreases (for discussion on antibody labeling, refer to section 1.3).

1.1.3.2. Non-competitive immunoassays

This type of immunoassay offers higher detectability over competitive ones since their ultimate detectability is determined by the non-specific binding of the labeled antibody as well as the detectability of the label. These assays have the potential for single molecule detection, by using highly detectable label along with low non-specific binding. In contrast, the sensitivity of competitive immunoassays is limited by the affinity constant of the antibody used (Jackson TM and Ekins RP, 1986). Non-competitive immunoassays can be divided into three different approaches that will be discussed separately.

1.1.3.2.1. Two-site (sandwich-type) immunoassays

In these assays two antibodies are used, one for capturing, and the other for detection. In order to avoid steric hindrance, the antibodies should bind to non-overlapping epitopes of the antigen. First, the solid phase is coated with an affinity-purified anti-analyte antibody. After washing and blocking, the sample is added and the antigen is allowed to bind to the antibody. The solid support is then washed and a detection antibody carrying a label is added (two-step assay). After the completion of the immunoreaction, the excess unbound detection antibody is washed followed by the detection of the label (Figure 1.3C).

The sandwich-type immunoassay can be performed in one-step by adding both the sample and the detection antibody simultaneously. One disadvantage of this approach is
that if the amount of analyte exceeds the amount of capture and detection probe, excess analyte remains in solution and the detection antibody is distributed between the two analyte fractions. As the analyte concentration becomes higher, less fraction of the detection antibody will be bound to the solid phase, leading to a decrease in signal. This is referred to as the high-dose hook effect, which can lead to underestimation of the analyte concentration. To solve this problem, the sample can be diluted, or the immunoassay can be repeated with the two-step protocol.

1.1.3.2.2. Immunoassay for immunoglobulins

This assay is performed in order to detect specific immunoglobulins in the sample. The solid phase is coated with the purified antigen. After washing and blocking, the sample is added and the immunoglobulin is allowed to bind to the immobilized antigen. After washing the solid support, a secondary labeled anti-immunoglobulin antibody is added. After certain incubation time, the excess unbound antibody is washed and the bound label is detected (Figure 1.3D). This approach is used when screening hybridomas for monoclonal antibody production.

1.1.3.2.3. Epitope mapping immunoassays

This approach is used when several monoclonal antibodies are available for a single antigen in order to determine if the antibodies bind to the same or different epitopes. A labeled monoclonal antibody is added with various concentrations of another unlabeled antibody to an antigen-coated solid support. If both antibodies bind to the same epitope, a decrease in signal is observed as the concentration of the unlabeled monoclonal antibody increases. However, if each monoclonal antibody binds to a different epitope, a constant signal is observed.
1.2. Nucleic Acid Analysis

The determination of the DNA double helical structure by James Watson and Francis Crick in 1953 marked the birth of modern molecular biology. However, at that point, none would have predicted the impact this discovery would later have on our understanding of normal human biological processes and disease. Over the last decade, the mapping, sequencing, and analysis of the 3.2 billion base pairs of the human genome through the Human Genome Project have opened the door to great advances in basic science and medicine. Although it is likely to be a number of years before many of the expected benefits of the genomic revolution are realized, the impact of these scientific breakthroughs on disease diagnostics and treatments is likely to become apparent relatively quickly.

The fruits of the human genome project include the understanding of the function of various genes and the mechanisms of their regulation as well as linkage of gene function to various diseases. This in turn will stimulate the development of new diagnostic tests and therapeutic approaches.

Nucleic acid based tests have found an increasing number of applications in diverse areas such as molecular diagnosis of disease, environmental monitoring, forensic science, etc. Because of their importance it is worthwhile to describe some of these techniques.

1.2.1. DNA hybridization assays

DNA is composed of four deoxyribonucleotides consisting of the bases, adenine and guanine (purines), cytosine, and thymine (pyrimidines) attached through phosphodiester bonds (Figure 1.4). DNA hybridization is a fundamental tool in molecular cloning, characterization and analysis of genes. The basis of DNA identification by hybridization is hydrogen bonding between two strands of DNA with Watson-Crick pairing restrictions. Adenine (A) pairs with thymine (T) and guanine (G) pairs with cytosine (C) (Figure 1.5). DNA is coiled to form a double helix (double-stranded DNA, dsDNA) composed of two strands held together by hydrogen bonds that can be broken by heat or high pH (denaturation).
Figure 1.4. DNA sequence composed of deoxyribonucleotides linked together by phosphodiester bonds in the 5’ to 3’ direction. The deoxyribose atom numbers are primed to distinguish them from the bases atom positions. Deoxyribonucleotides are always added to the DNA growing chain, by DNA polymerases, in the 5’ to 3’ direction.
Figure 1.5. Watson and Crick base pairing. Deoxyriboadenine pairs with deoxyribothymine by two hydrogen bonds, whereas, deoxyriboguanine joins to deoxyriboctosine by three hydrogen bonds. The distance between the deoxyribose (dR) C1' of both base pairs is the same.
The denatured, single-stranded DNA (ssDNA) is relatively stable, however, upon condition changes (temperature decrease or removal of extreme pH), the DNA molecule will reanneal into the double-stranded configuration by re-forming the hydrogen bonds with specific complementary bases. When the two ssDNAs are from different sources, the reannealing process is called hybridization. If an unknown sequence of single-stranded DNA hybridizes perfectly with a known sequence, the unknown DNA sequence is just the base complement of the known sequence.

1.2.1.1. Stability of DNA hybrids

The melting temperature (Tm) of a nucleic acid is defined as the temperature at which 50% of the bases are paired. The stability of the DNA double helix, and hence its melting temperature depends on several factors including the base composition, length, as well as the nature of the solvent, the presence of denaturants such as formamide, the identities and concentrations of the ions in solution, and the pH (Voet D et al., 1990). Furthermore, the extent of precise base pairing between the two strands of DNA influences Tm. A perfect match in the sequence of nucleotides produces very stable dsDNA, however, one or more base mismatches decrease the stability that can lead to weak hybridization of strands.

The melting temperature of a nucleic acid can be estimated by using the following empirical equation (Sambrook J et al., 1989):

\[
Tm \ (^\circ \text{C}) = 81.5^\circ \text{C} - 16.6 \ (\log_{10} [M^+]) + 0.41 \ (% \ G + C) - \ %\text{mismatch} - 0.63 \ (%\text{formamide}) - (600/L)
\]

Where L is the length of the hybrid in base pairs, and M⁺ is equivalent monovalent cation (a divalent cation is approximately equivalent to 100 times higher concentration of monovalent cation). This equation is valid only if the monovalent ion concentration is in the range of 0.01 M to 0.4 M and G + C content is 30% - 70%. Dissimilar conditions will result in a less accurate prediction of Tm. The effect of mismatches on the stability of DNA hybrids is as follows: For every 1 % of base-mismatching in a dsDNA, there is a reduction of Tm by 1-1.5° C. However, the precise effect of mismatches depends on the G + C content of the oligonucleotide and on the distribution of mismatched bases in the dsDNA.
It is clear that hydrogen bonding is essential for the specificity of base pairing required for the fidelity of DNA replication. However, hydrogen bonding does not contribute much to the stability of the double helical structure of DNA. In fact, hydrophobic interactions between the heterocyclic rings of the bases are largely responsible for the stability of DNA (Tijssen P, 1993). Furthermore, purines and pyrimidines in the dsDNA structure are usually partially overlapped or "stacked" by van der Waal interactions, thus stabilizing the DNA.

As mentioned above, the hydrogen bonds between the two DNA strands can be destroyed by increased temperature or by alkaline conditions. For example, 0.2 M NaOH will denature the DNA due to the deprotonation of the bases, preventing the formation of hydrogen bonds. Similarly, acidic conditions will protonate the bases and prevent hydrogen bonding. However, low pH catalyzes the spontaneous cleavage of the glycosidic bond of purine residues, converting them to apurinic acid (Sambrook et al., 1989).

The most convenient way of monitoring the native state of DNA is by ultraviolet absorbance spectrum. When DNA denatures, its UV absorbance, which is entirely due to its aromatic bases, increases by 40% at all wavelengths (usually it is measured at 260 nm) as a result of disruption of the electronic interactions among nearby bases. This phenomenon is known as the hyperchromic effect (Voet D et al., 1990). This hyperchromic shift occurs over a narrow temperature range, which indicates that DNA denaturation is a cooperative phenomenon in which the collapse of one part of the structure destabilizes the remainder.

A denatured solution of DNA can be easily renatured by maintaining the temperature 25° C below its Tm so just enough thermal energy is available for short base paired regions to rearrange by melting and reforming the correct long complementary stretches. However, if the denatured DNA solution is cooled quickly below its Tm, the resulting DNA will only be partially paired because the complementary strands will not have had enough time to find each other before the partially base paired structures becomes rigid.
1.2.1.2. DNA probe characteristics

A DNA probe is normally a short sequence of nucleotides that binds to a specific region of a target DNA. In developing a probe, the sequence must be identified, isolated, reproduced in sufficient quantities, as well as tagged with a label that can be detected if necessary. Many methods have been developed for probe production. A typical approach is to clone the DNA sequence into a vector that can be replicated in bacterial cells to produce more copies of this DNA. After purification of the vector DNA, the DNA of interest (the probe) is cut out by restriction endonucleases. This difficult and time-consuming process has been replaced by much faster methodology that permits oligonucleotide synthesis. Presently, the most widely used method for probe synthesis is based on the phosphoramidite chemistry, a solid phase based method that adds a suitably protected nucleotide to a growing end of an oligonucleotide chain. After removal of the protecting group, the process is repeated until the desired oligonucleotide has been synthesized (Caruthers MH et al., 1987).

Oligonucleotide probes can be composed of DNA or RNA, with DNA probes being more common since RNA can be easily hydrolyzed by ubiquitous ribonucleases due to the hydroxyl group at the 2' position (Hilborne LH et al., 1992). DNA probes can also be hydrolyzed by deoxyribonucleases, but, this problem can be efficiently solved by using EDTA or EGTA to chelate the divalent cations (Mg^{2+} or Ca^{2+}) that are required for DNase activity.

In general, the entire sequence of the probe represents the binding site to the DNA target of interest and affects the specificity and sensitivity of the assay. Longer probes are specific, but, they may require longer reaction time (hours) to achieve a stable hybrid. In contrast, shorter probes are more favorable due to their advantages of rapid hybridization (minutes), ease of preparation, and their ability to detect minor changes in target DNA, despite the fact that they are subjected to more non-specific binding and are more difficult to label.
The specificity of the probe is determined by its base sequence as well as the hybridization conditions. The only requirement for an ideal DNA probe is to hybridize to its target. However, special considerations should be taken before the preparation of a certain probe:

a. Interstrand and intrastrand complementary regions of the probes as well as runs of three or more G’s and C’s should be avoided to prevent the formation of probe dimers or hairpin structure.

b. The probe must only bind to the sequence of interest, and it should not bind to non-target nucleic acids in the hybridization reaction or in the clinical specimen.

c. Length of the oligonucleotide should be 15-40 nucleotides, however, for statistical uniqueness, a minimum of 20 nucleotide bases are usually needed (Berry AJ et al., 1984).

d. The base composition should be 40-60% G + C.

Many computer programs are available which allow accurate selection of DNA probes, taking into account the points discussed above (Keller GH et al., 1989).

1.2.2. Polymerase Chain Reaction

In 1983, a technique for the in vitro enzymatic exponential amplification of specific DNA sequences was developed by Kary Mullis. In 1989, this technique was declared to be the “major scientific development” of the year (Guyer RL et al., 1989). In 1993, Kary Mullis won the Nobel prize in chemistry for his invention. This process was termed polymerase chain reaction (PCR). Today, PCR is considered to be one of the most essential techniques in molecular biology laboratories for amplification of nucleic acids. Furthermore, PCR is the technique of choice for detection of inherited mutations associated with genetic diseases as well as infectious agents in a variety of clinical and environmental samples (refer to section 5). PCR is based on the repetition of a three-step process (Figure 1.6):

a. Denaturation at 94-97°C of the dsDNA to form two single strands. The time of this step depends on the sequence and the length of target DNA. A higher G + C content and a longer DNA requires longer incubation time.
Figure 1.6. The principle of the polymerase chain reaction (PCR). 3 cycles are illustrated. In the first step, the target DNA is denatured followed by the annealing of both upstream and downstream primers to their complementary sequence on the target DNA in a way that their 3' ends point towards one another along the intervening sequence of interest. DNA polymerase will catalyze the extension of the hybridized primers resulting in an exponential accumulation of target DNA. The arrows represent the direction of primer extension and darker lines represent the newly generated target DNA.
b. Annealing of the two, downstream and upstream, oligonucleotide primers to the complementary sequence on opposite strands of the DNA. This step is done at a temperature ranging from 40-72°C depending on the melting temperature of the primers (refer to DNA probes characteristics, section 1.2.1.2). The primers are oriented so that their 3’ ends point toward one another along the intervening sequence. The DNA sequence amplified by PCR is determined by the position of the two primers.

c. 5’ to 3’ extension of template-primer heteroduplexes at 72°C catalyzed by a thermostable DNA polymerase.

Following one complete cycle, two copies containing the sequence of interest are generated. As the cycling continues, the primers bind to the original DNA as well as to the newly synthesized strands and the number of DNA copies approximately doubles at each cycle resulting in an exponential DNA amplification. A typical amplification is 20 to 40 cycles that results in a one million fold amplification of the original DNA.

1.2.2.1. Efficiency of PCR

The accumulation of PCR product during the exponential phase can be described by the following equation

\[ P = T(1 + E)^n \]

Where \( P \) is the amount of amplification product, \( T \) is the initial amount of DNA target, \( E \) is the efficiency of PCR, and \( n \) is the number of amplification cycles. The theoretical value of \( E \) is 1, which means 100% efficiency. However, in practice, this efficiency is not obtained during PCR, mainly due to the initial lag phase attributed to many factors such as unavailability of the DNA target due to strand breaks, lack of DNA dissociation from other macromolecules, structural constraints, or a tendency of the parental strands to reanneal (Christopoulos TK, 2000). This low efficiency in the first few cycles greatly affects the overall yield of PCR. PCR enters the exponential phase once the lag phase is overcome. After a certain number of cycles, exponential amplification of the target DNA stops gradually and enters a linear and eventually a stationary phase, a phenomenon known as the plateau phase of PCR. This decrease is mainly due to competition between primer-template and template-template annealing, incomplete denaturation of DNA.
strands and competition for reactants by any accumulated non-specific products. Other factors that contribute to the plateau phase of PCR are depletion or destruction of PCR reactants such as primers, dNTPs and polymerase, as well as inhibition of reaction by end products such as pyrophosphate. The point at which a PCR reaction reaches its plateau phase depends primarily on the amount of DNA target present originally in the reaction and the numbers of cycles.

1.2.2.2. PCR conditions

Due to the wide variety of PCR applications, it is probably impossible to outline a single set of conditions that can be used for all PCR reactions with guaranteed success. However, a general guideline can be described to provide adequate information that can be used as a common starting point from which optimization studies should be performed for each application.

The standard PCR is typically done in a 50 or 100 µL volume containing 20-200 µM of each deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, dTTP). Higher concentrations tend to promote misincorporation by DNA polymerase at non-target sequences, leading to a decrease in PCR specificity. Like the PCR primers, labeled dNTPs (e.g. with a fluorophore or a hapten) can be used in the reaction to incorporate a specific label to the PCR product that can later be used for capture or detection of the DNA target. Since Mg$^{2+}$ appears to bind the dNTPs quantitatively, and is required for DNA polymerase activity, the concentration of Mg$^{2+}$ required for the reaction will be determined by the concentration of dNTPs and DNA polymerase present. Furthermore, the concentration of Mg$^{2+}$ should be optimized for each DNA target since it may affect the primer annealing as well as target denaturation.

1.2.2.3. Specificity of PCR

There are a number of factors that could affect the specificity of PCR. In order for PCR to be efficient, leading to exponential amplification, the primer must bind only to the specific complementary sequence on the target. The stringency of the annealing and extension step should be optimized, by adjusting the annealing temperature as well as extension time, thus avoiding mispriming and extension of non-specific products.
However, the extension time should be long enough to allow complete extension of the DNA target (usually 1 min/Kb). Furthermore, reducing the concentrations of primer, enzyme, dNTPs, and Mg$^{2+}$ can further improve PCR specificity.

A practical approach used to avoid mispriming and non-specific product extension is the "hot start" protocol. In this protocol, an important PCR reactant, such as DNA polymerase, dNTPs or primers, is added to the reaction, only after the temperature of the mixture has reached 90$^\circ$C. An alternative approach is to include in the reaction a DNA polymerase inactivator such as a monoclonal antibody that binds and inactivates the enzyme at ambient temperature. When the temperature is raised to 90$^\circ$C, the antibody is dissociated from the polymerase, thus allowing the enzyme to function normally during PCR.

1.2.2.4. Reverse transcriptase-PCR (RT-PCR)

RT-PCR entails the exponential amplification of specific RNA sequences. First the RNA is converted to the complementary DNA (cDNA) by using reverse transcriptase (from moloney murine leukemia virus or amian myeloblastosis virus) along with a suitable primer, e.g., a poly (dT) primer for reverse transcription of eukaryotic mRNA sequences (Christopoulos TK, 2000). The downstream PCR primer may also be used as a primer for reverse transcription. Subsequently, reverse transcriptase is heat inactivated at 95$^\circ$C and the mixture is subjected to PCR. During PCR, the upstream primer will act on the synthesized cDNA to produce a double-stranded DNA that will serve as a template for exponential amplification. Care should be taken in the RNA isolation step to avoid contamination from genomic DNA.

1.2.2.5. Pitfalls of PCR

As with every other amplification approach, PCR does have some disadvantages. Due to the extreme sensitivity of PCR, it is highly susceptible to contamination with extraneous DNA fragments carried over from previous amplifications (amplicon contamination) or introduced from other sources that could be amplified with the sample leading to false-positive results and therefore faulty diagnosis (Victor T et al., 1993). Many precautions should be taken to avoid contaminations such as physical isolation of
sample preparation from amplification or detection areas, ultraviolet irradiation of all materials used for PCR preparation such as dedicated pipettes, pipette tips, solutions, and glassware. Also, prealiquoting of all reagents used for the reaction such as buffers, enzyme, and dNTPs could decrease the chances of contamination. In addition to false positive results, false negative results could also be an issue due to carry-over of compounds that inhibit DNA polymerase activity or due to a mutation in the area of primer annealing on the target DNA. Finally, a reaction that serves as a negative control (no target), as well as one for a positive control, should be included in every series of samples for accurate interpretation of results.

1.2.2.6. Analysis of PCR products

Several methods have been developed for the analysis of PCR product. The most commonly used one is based on separation by agarose gel electrophoresis followed by staining with planar aromatic cations such as ethidium bromide (showing below).

![Ethidium Bromide](image)

Ethidium bromide binds to duplex DNA by a process called intercalation (slipping in between stacked base pairs) and exhibits fluorescence under ultraviolet light (Freifelder D, 1982). As low as 50 ng of dsDNA can be detected with this technique.

Other common methods used for PCR analysis are based on hybridization assays. The classical approach to DNA hybridization is the Southern blotting technique. First DNA fragments are separated by electrophoresis followed by soaking the gel with NaOH solution to denature the dsDNA. Afterward, single-stranded DNA is transferred onto a nylon or nitrocellulose paper followed by drying at 80° C, which permanently fixes the DNA. Finally, a DNA probe, with a complementary sequence to the DNA target, containing a label such as biotin is allowed to hybridize with the immobilized target. The biotin is detected by adding a streptavidin-enzyme conjugate such as horseradish
peroxidase or alkaline phosphatase followed by the addition of the corresponding substrate (refer to section 1.3).

PCR products can also be detected using a solid phase-based hybridization assay. In this case, the target DNA is labeled through PCR by incorporating a deoxyribonucleotide or a primer that is labeled with a fluorophore or a hapten. The PCR target is first denatured, and then captured on a solid-phase that is coated with a DNA probe containing a complementary sequence to the target DNA. Subsequently, the fluorophore or the hapten is detected.

More recent, homogeneous methods for real-time monitoring of DNA amplification have been developed (Heid CA et al., 1993; Gibson UE et al., 1996; Wittwer CT et al. 1997). Some of these methods are based on the 5’ nuclease assay, molecular beacons or fluorescence resonance energy transfer (FRET) technologies, which are performed in a similar manner to SNPs detection (refer to section 1.5.2).
1.3. Labels (Reporters) used in Immunoassays and Hybridization Assays

DNA hybridization techniques have many similarities to immunoassays. In hybridization assays, the analyte is a DNA sequence that reacts with a specific oligonucleotide probe. Similarly, in immunoassays, the analyte (antigen) reacts with a specific antibody. Studies showed that hybridization between a 20 bp DNA probe and its complementary target sequence is as strong as an antigen-antibody interaction (Okahata Y et al., 1998).

Both hybridization assays and immunoassays require a detection system (label) that enables quantification of hybrids and immunocomplexes respectively. The principal factors determining the suitability of a substance as a label includes high specific activity, ease of labeling without interfering with the activity of the reactants, simplicity of detection with high sensitivity and reproducibility, associated bio-hazards, stability, and possibilities for convenient assay development.

Traditionally, radioisotope labels are used for detection of proteins and nucleic acids. However, due to the safety issues related to the use of radioactive labels, newer nonradioactive methods have been developed that allow sensitive determination of these analytes.

1.3.1. Radioisotope labels

Until recently, radioisotope labels, such as $^{125}$I and $^{32}$P, have been used for detection of antigen and nucleic acids. Radioactivity is the result of an unstable combination of protons and neutrons in the nucleus, and the transition to a more stable combination by emission of an electron (β particle), or a photon (γ radiation). This process is known as radioactive decay (Chapman JM et al., 1981). Detection of radioactivity is usually performed in the following way: The sample is mixed with a scintillation cocktail that contains a fluorophore (such as 2,5-diphenyloxazole) and a solvent such as toluene. The energy of the radioactive decay is absorbed by the solvent molecules and then it is transferred to the fluorophore molecules which emit fluorescence.
As mentioned above, due to the health hazards associated with the use and disposal of radioisotopes, as well as their relatively short half life, the current trend is towards nonradioactive labels.

1.3.2. Fluorescent labels

Fluorescence is defined as the radiative decay of an electronically excited molecule to its ground state by emitting a photon. Fluorescence is short lived and requires only $10^{-5}$-$10^{-8}$ s to occur. Fluorescence generally has a longer wavelength (low energy) than the excitation light (Skoog D et al., 1992). Because the emission takes place from the lowest excited state ($S_1$), the shape of the emission spectrum is independent of the excitation wavelength. When measuring fluorescence, it is important that the excitation and the emission wavelength monochromators are at a 90° angle with respect to one another to avoid measuring the excitation light. The kinetics of fluorescence are depicted by a constant increase of light over time due to the repeating cycles of excitation and emission (Figure 1.7A).

Many fluorescent compounds are known, however, and in order to be suitable as a label for immunoassays and hybridization assays, a fluorophore should have high quantum yield. Fluorescence quantum yield is defined as the ratio of the number of molecules that fluoresce to the total number of excited molecules (Christopoulos TK et al, 1996). The most widely used fluorescent label is fluorescein (Figure 1.8A) due to its high absorptivity and quantum yield (0.85). Derivatives of fluorescein (rhodamines, Figure 1.8B) that emit light at a longer wavelength have been developed. However, these compounds have lower quantum yield than fluorescein (Diamandas EP and Christopoulos TK, 1996).

Although fluorometry offers 10-1000 fold higher detectability in comparison to spectrophotometry, it has several limitations which include:

a. Excitation light scattering from solvent molecules leads to a decrease in detectability of fluorometry (nM range) due to the increase of the background.
Figure 1.7. Kinetics of the light production for fluorescent and chemiluminescent reactions. **A.** The production of light in a fluorescence reaction, showing the constant increase of light over time due to the repeating cycles of excitation and emission. **B.** Flash-type light emission of chemiluminescent reaction depicted by a short burst of light due to depletion of reactants. **C.** Glow-type light emission of enzyme-catalyzed chemiluminescent reaction illustrated by the steady-state production of light due to the substrate turnover.
Figure 1.8. Fluorescent and bioluminescent molecules.  

A. Structure of the fluorescent molecule fluorescein isothiocyanate.  
B. Structure of the fluorescent molecule rhodamine-B-isothiocyanate. The thiocyanate group allows conjugation of these fluorophores to amino groups.  
C. Structure of the chemiluminescent molecule luminol.
b. Biological samples may contain a variety of fluorescent components, leading to higher background.

c. Light-absorbing molecules such as hemoglobin or bilirubin can absorb the excitation or the emission energy causing quenching. Furthermore, when the difference between excitation and emission wavelengths (Stokes shift) is somewhat small, fluorescence emitted by one fluorophore is absorbed by adjacent fluorophores in the same labeled reagent or solution, causing inner filter quenching.

d. Upon continuous excitation, fluorescence can be decreased by a photochemical reaction. This photodestruction is a serious problem when high-intensity excitation and several repeated measurements are performed during a short time interval.

Due to all these limitations, the trend now is towards chemiluminescence or bioluminescence labels, which do not require excitation.

1.3.3. Chemiluminescent and bioluminescent labels

Chemiluminescence is defined as the light emission that is produced in certain chemical oxidation reactions. This light emission is due to the decomposition of chemically excited-state intermediates or product molecules that decay to the electronic ground state (Kricka LJ, 1991). Bioluminescence is a natural phenomenon found in many lower forms of life (DeLuca M, 1978 and 1986). In this special type of chemiluminescence, the light emission is facilitated by an enzyme such as luciferase, or a photoprotein such as aequorin.

Chemiluminescent labels were initially tested as possible alternatives to radioisotopes in the early 1970s (Kricka LJ, 1985). This type of label has become very popular for immunoassay and hybridization assay detection. Chemiluminescent substrates have also been developed for quantification of enzyme labels (e.g. horseradish peroxidase and alkaline phosphatase).
There are two types of chemiluminescence emission:

a. Flash-type light emission in which a short burst of light is produced (Figure 1.7B). In this case, the chemiluminescent molecule is used as the detection label and therefore is present in limited amounts. The kinetics of this type of chemiluminescence is depicted by an initial lag phase followed by a rapid increase in the rate of product formation to a certain maximum, after which a sharp decrease in light is observed due to depletion of reactants.

b. Glow-type light emission in which a continuous measurement of light intensity is observed over a predetermined period of time (Figure 1.7C). In this case, the enzyme is used as the label, and is detected by using excess of substrate. The kinetics for the glow type chemiluminescence is illustrated by an initial lag phase until initial excited state molecules are formed followed by a constant production of light (steady state) due to the substrate turnover.

The most studied chemiluminescent reaction is the oxidation of luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) (Figure 1.8C) by hydrogen peroxide in the presence of a catalyst such as horseradish peroxidase to produce an intermediate which decomposes to produce 3-aminophtalate molecules in an excited state, which emit light upon their return to the ground state. Without enzyme, this reaction is very inefficient, with a quantum yield of 0.01. Chemiluminescence quantum yield is the product of three components: the fraction of molecules entering the reaction pathway, the fraction of these molecules that become chemically excited, and the fluorescence quantum yield of the excited state emitter (Diamandas EP and Christopoulos TK, 1996).

Bioluminescence of certain naturally occurring reactions such as firefly luciferase and aequorin photoprotein have higher quantum yield.

1.3.3.1. Firefly luciferase

The bioluminescent firefly luciferase reaction is one of the most studied systems due to its high quantum yield (>0.88, the highest ever known for any bioluminescent reaction). Therefore, for every one excited molecule, approximately one photon is emitted (Seliger HH et al., 1960).
The firefly luciferase from the American firefly *Photinus pyralis* is found in the light-emitting organ known as the lantern within the abdomen of the insect. The gene encoding the firefly luciferase was cloned and expressed actively in bacteria (De Wet JR et al., 1985). Firefly luciferase is a 62 kDa protein that consists of a single polypeptide chain (550 amino acids) which is folded into two compact domains (Elena C et al., 1996). The major N-terminal domain of the molecule (residues 4-436) consists of a distorted antiparallel β-barrel (β-sheets forming cylindrical structure) and two β-sheets that are flanked by α-helices to form an αβαβα layered structure. The C-terminal portion of the enzyme comprising residues 440-544, contains two short antiparallel β-strands and a three-stranded mixed β-sheet, with three helices packed against the sides (α + β structure). The small portion of the molecule is separated from the large N-terminal domain by a wide cleft. The highly conserved residues located on the surface of the two domains across the cleft, is identified to be the location of the active site in the firefly luciferase structure. Recently, it was shown that stepwise removal of the last 12 amino acids of the C-terminus resulted in a stepwise loss of luciferase activity from 50–99% between −8 and −12 aa (Sala-Newby GB et al., 1994), yet, deletion of the last 7 amino acids had no detectable effect on its specific activity, therefore, the C-terminus plays a crucial role in the bioluminescence activity of firefly luciferase.

Firefly luciferase bioluminescent reaction requires ATP, Mg$^{2+}$, pyrophosphate (product of the reaction), coenzyme-A, luciferin, and molecular O$_2$. The specific mechanism of this reaction is not known, however a general understanding of how the luciferase reaction occurs had been suggested.

Firefly luciferase has a catalytic active site for luciferin and two sites for ATP. One ATP site has low-affinity (Km = 10$^{-4}$ M) is responsible for the initial flash of light and is rapidly inhibited by the reaction product and/or AMP and pyrophosphate after a single catalytic event. A second high-affinity ATP binding site (Km = 2 x 10$^{-5}$ M) becomes apparent after the initial flash of light and is responsible for the activity of the steady state (Deluca M et al., 1984; Lemert N et al., 1995). During the process of luciferase reaction, magnesium is combined with ATP to form the actual substrate (MgATP complex). Once Mg-ATP and luciferin are bound to luciferase, the reaction is initiated as outlined in the reaction scheme shown in Figure 1.9A.
Figure 1.9.  

A. Reaction scheme for firefly luciferase. The carboxylate group of d-luciferin reacts with the α-phosphate of ATP to produce an enzyme-bound luciferyl adenylate. The luciferase adenylate is oxidized, by molecular oxygen, to produce a dioxetanone intermediate that is spontaneously cleaved to produce an excited state of oxyluciferin that emits light upon returning to the ground state. 

B. Scheme for the aequorin bioluminescence reaction. Oxygen and coelenterazine are bound tightly to the apoaequorin to produce an active aequorin molecule. Upon calcium binding, a conformational change is triggered and coelenterazine is oxidized to coelenteramide by the bound oxygen releasing energy as a flash of light.
In the presence of ATP and Mg\(^{2+}\), firefly luciferase catalyzes the oxidative decarboxylation of \(\Delta\)-luciferin to produce pyrophosphate and luciferyl adenylate. The latter is then converted by molecular oxygen to dioxetanone intermediate, which, upon spontaneous cleavage of the dioxetane ring, produces an excited molecule of oxyluciferin that emits light upon returning to the ground state (White EH et al., 1975; McElroy WD et al., 1969).

The emission of luciferase at neutral or alkaline pH and at room temperature is a single band in the yellow-green spectrum at 562 nm. However, it was shown that at acidic pH, in the presence of phosphate, SO\(_4\)\(^{2-}\) anions, divalent metal ions (such as Cd\(^{2+}\), Zn\(^{2+}\)), or with increased temperature (37° C), the emission spectrum shifts to 620 nm (red) leading to dramatic decrease in quantum yield (0.45 at pH 6.5) (Seliger HH et al., 1960; Kricka LJ et al., 1982). Therefore, it is essential to perform the luciferase reaction under optimum conditions at 25-30° C. The addition of EDTA in the reaction mixture prevents the inhibition by metal ions.

In the original assay procedure, a flash of light in less than 1 second is produced that decays rapidly (Bronstein I et al., 1994) and the total light emission is proportional to the luciferase activity. Nevertheless, this procedure has many drawbacks due to the product inhibition effect of oxyluciferin on the enzyme, which is presumably due to the slow release of oxyluciferin from the enzyme, inhibiting its turnover. Furthermore, measurement of a flash of light requires an automatic injector and the reaction must be performed in front of the light detector.

Several procedures have been developed that will convert the flash of light emission into a glow type. Coenzyme A was found to enhance the reaction of luciferin by stimulating light production through binding to luciferase, producing a conformational change in the enzyme, and allowing the release of oxyluciferin (Ford SR et al., 1995), thus, preventing the rapid inhibition of light production and eliciting a nearly constant production of light. It was also shown that the sulfhydryl group of coenzyme A is required for this activity, therefore, a reducing agent such as dithiothreitol should be included in the reaction of luciferase. Furthermore, luciferase contains two free SH groups that are essential for its activity, hence, dithiothreitol can also stabilize luciferase by preventing their oxidation.
In addition to coenzyme A, cytidine nucleotides, inorganic pyrophosphate as well as detergents such as Triton X-100 and polymers such as polyvinylpyrrolidone were found to have similar effect on luciferase reaction, leading to a constant production of light (Ford SR et al., 1992; Kricka LJ et al., 1982).

A drawback of luciferase as a label is that upon conjugation to antibodies or DNA probes, it loses its activity. Therefore, this limits the use of luciferase as a direct label. The in vitro synthesis of firefly luciferase (Wood KV et al., 1984), and the fact that it does not require post-translational modification for enzyme activity, led to the development of expression immunoassays and hybridization assays in which the gene encoding luciferase under the control of T7 RNA polymerase promoter is used as a label (Christopoulos TK et al., 1995; Laios E et al., 1998). After formation of the immunoreaction or the hybrid, the gene encoding luciferase is expressed and luciferase is produced, by using an in vitro coupled cell free transcription and translation system.

1.3.3.2. Aequorin Photoprotein

Aequorin is a calcium-activated photoprotein isolated originally from the jelly fish Aequorea Victoria (Shimomura O et al., 1962). Aequorin is widely used as a probe to monitor intracellular levels of free calcium, due to its high sensitivity to calcium ions and because it is biologically harmless. Aequorin is a complex of 22 kDa composed of apoaequorin protein, molecular oxygen, and the luminophore coelenterazine (2-(p-hydroxy-benzyl)-6-(p-hydroxyphenyl)-3,7-dihydroimidazo-[1,2a]-pyrazin-3-one). The apoaequorin gene has been cloned (Inouye S et al., 1985). Recently the crystal structure of apoaequorin was resolved (Head JF et al., 2000). Aequorin is composed of a single polypeptide chain of 189 amino acids containing four helix-loop-helix domains arranged in pairs in a way that each pair is back-to-back forming short stretches of β-sheets. Three of these domains can bind calcium (Inouye S et al., 1985), however, recent studies showed that the binding of only two calcium ions to aequorin triggers its bioluminescence (Shimomura O, 1995). The hydrophobic binding cavity of coelenterazine is situated in the center of the protein. The bound coelenterazine exists in a peroxidized form, with the hydroperoxide group attached to the C2 position of the ligand.
The mechanism of aequorin chemiluminescent reaction is depicted in Figure 1.9B. In the presence of calcium ions, aequorin undergoes a conformational change that triggers an intramolecular reaction resulting in the oxidation of coelenterazine to an excited state coelenteramide, which decays by emitting a flash of blue light (469 nm) that lasts for 2-3 seconds (Actor JK et al., 1999). This reaction has a quantum yield of 0.15 (Shimomura O et al., 1970). Apoaequorin can be regenerated into active aequorin, in the absence of calcium, by incubation with coelenterazine, oxygen and a thiol agent such as mercaptoethanol (Shimomura O et al., 1975).

The in vitro expression and purification of apoaequorin in bacteria followed by reconstitution of the complex with pure coelenterazine led to the possibility of exploring aequorin as a label (Prasher D et al., 1985; Patel A et al., 1984). Recently, conjugates of aequorin with biotin and streptavidin were employed for development of highly sensitive hybridization assays (Galvan B and Christopoulos TK, 1996). Furthermore, aequorin cDNA proved to be a highly sensitive label for expression hybridization assays, which were developed recently (White SR and Christopoulos TK, 1999).

1.3.4. Enzymes as labels

Although, many labels have been tested for use in immunoassays and hybridization assays, enzymes remain the most widely used labels. This popularity is due to the favorable properties of an enzyme label such as amplification through the rapid turnover of the substrate to the product and the diverse range of sensitive and convenient assay methods. Nevertheless. enzymes are proteins and therefore have limited stability. The most widely used enzymes as labels are horseradish peroxidase (HRP) and alkaline phosphatase (ALP). Initially, chromogenic substrates were used for detection of these enzymes. More recently, fluorogenic and chemiluminogenic substrates have been developed for higher detectability of analytes (as described above).

1.3.4.1. Horseradish peroxidase.

Horseradish peroxidase (HRP) is a plant glycohemoprotein with a molecular weight of 44 kDa that consists of a hemin prosthetic group, 2 Ca\(^{2+}\) and 308 amino acid residues, including four disulfide bridges that carries eight carbohydrate side chains
(Welinder KG, 1979). The enzymatic activity arises from the cyclic reduction and oxidation of the iron atom in the hematin group. HRP combines with hydrogen peroxide and the resultant (HRP-H₂O₂) complex can oxidize a wide variety of chromogenic hydrogen donors. In the presence of HRP, the substrate such as o-phenylenediamine forms a 1:1 complex with hydrogen peroxide to produce an orange colored complex that can be monitored spectrophotometrically. Several other chromogenic substrates are known that can be used for the detection of HRP.

Fluorogenic substrates such as the amplex red reagent (10-acetyl-3,7-dihydroxyphenoxazine) are also used for HRP detection. In the presence of HRP, this reagent reacts with hydrogen peroxide to produce resorufin, a highly fluorescent compound (Figure 1.10A).

Chemiluminogenic substrates are also known for HRP. For instance, Luminol (Figure 1.10B) has been used as a hydrogen donor in the peroxidase catalyzed reaction. Some of these substrates are based on acridinium esters. At alkaline pH, HRP catalyzes the oxidation of acridinium ester, by hydrogen peroxide to produce a flash of light (Figure 1.9E) (Diamandas EP, 1990).

1.3.4.2 Alkaline phosphatase

Alkaline phosphatase (ALP, 140 kDa), from E. coli., catalyzes the hydrolysis of a phosphate group from substrates such as p-nitrophenyl phosphate to produce p-nitrophenol, a substance that can be monitored spectrophotometrically at 405 nm.

Fluorogenic substrates have also been developed for ALP detection, including coumarin derivatives such as 4-methylumbelliferyl phosphate. Upon enzymatic hydrolysis of the phosphate ester bond by ALP, the fluorescent compound 4-methylumbelliferone is produced (Figure 1.10C).

More recently, chemiluminogenic substrates have been developed for ALP detection. These substrates are based on the 1,2-dioxetane phosphate compound. Upon cleavage of the phosphate, a phenoxide intermediate is produced which decomposes to give an excited molecule that upon relaxation to the ground state emits light (Figure 1.10D) (Olesen CE et al., 2000).
Figure 1.10. Detection of horseradish peroxidase (HRP) and alkaline phosphatase (ALP). **A.** Fluorescent detection of HRP. HRP catalyzes the oxidation of amplex red reagent, in the presence of peroxide, to produce fluorescent resorufin. **B.** Chemiluminescent detection of HRP. acridan substrate reacts with HRP to produce acridinium ester which in turn is converted to a weak-bond intermediate that reacts with peroxide at alkaline pH, to generate a chemiluminescent substance. **C.** Fluorescent detection of ALP which catalyzes the hydrolysis of 4-methyl-umbelliferone phosphate ester to produce a fluorescent molecule. **D.** Chemiluminescent detection of ALP. ALP catalyzes the phosphate cleavage of adamantyl 1,2-dioxetane phosphate to produce an intermediate that is decomposed spontaneously to generate a chemiluminescent molecule.
1.4. Antibody and DNA Labeling Strategies

Labeling of antibodies and nucleic acids can be achieved in two ways. One approach involves direct conjugation of the label to the antibody or the DNA probe. An alternative strategy employs a mediator system that uses an affinity tag and a specific interaction to link one of the primary reactants, such as the antibody or the DNA probe, with another component of the assay system (e.g., the solid support or the label). The most commonly used mediator in immunoassays and hybridization assays is the streptavidin or avidin ((strept)avidin)-biotin system. Another mediator system that has also been used especially in nucleic acid detection is based on the digoxigenin-antidigoxigenin interaction. Each one of these systems will be discussed separately. But before that, it is important to note the essential factors that are required to achieve a successful labeling reaction.

a. The label should remain fully active upon conjugation to the reactants.

b. The binding ability of reactants such as antibodies, DNA probes, biotin, and (strept)avidin should not change after conjugation. The active group used for conjugation (in the case of protein) should not be essential for full activity of the reactant, and the protein should remain fully active after conjugation. Also, polymerization of reactants should be avoided, since their reactivity is impaired by steric hindrance.

c. The non-specific binding of the labeled reactant to the solid phase should be as low as possible. Polymerization is also a cause of the non-specific binding.

d. The conjugates should be stable if stored under appropriate conditions.

1.4.1. Direct conjugation

Direct conjugation of proteins to the label is usually achieved through their active groups (NH₂, SH, carboxyl, and carbohydrate moiety). However, for DNA probes, functional groups such NH₂ and SH should be introduced, which is accomplished by two methods: either by incorporating them at the 5'-end or 3'-end of the DNA probe during synthesis, or by the action of DNA polymerases such as Klenow, Taq, or terminal transferase along with a modified deoxyribonucleoside triphosphate. It has been shown
that direct incorporation of the label during DNA synthesis or with the action of DNA polymerase using a labeled dNTP is efficient, without interfering with the activity of the label or the binding of the probe. Labeling of DNA probes with both methods will be discussed below in a separate section.

Cross-linker reagents are used for conjugation of the label to the reactant. There are two major categories of cross-linkers, homobifunctional and heterobifunctional. Homobifunctional cross-linkers were the first cross-linking reagents used for modification and conjugation of biomolecules. These reagents consist of bireactive compounds bearing two identical functional groups at both ends separated by a carbon chain as a spacer to reduce steric hindrance (Hermanson GT, 1996). Like molecular ropes, these reagents can tie one molecule to another by reacting with the same common groups on both molecules. The ability to link so easily two molecules with different binding specificity or catalytic activity opened the door for inventing new, unique, and powerful reagent systems for use in immunoassay and hybridization assays. The production of these cross-linker reagents increased rapidly through the 70s and 80s and today dozens of commercially available cross-linkers possessing almost every length and reactivity desired are available (Figure 1.11).

A drawback of using a homobifunctional cross-linker for connecting two molecules bearing the same active group arises from the possibility of creating a wide range of poorly defined conjugates. For example, when conjugating two proteins together through their amino groups using BS3 (Figure 1.10), various types of conjugates are expected. One conjugate is composed of the two different proteins, or another composed of two molecules of the same protein (homodimers). Other possibilities include the formation of a wide range of homopolymers or heteropolymers. Despite these disadvantages, homobifunctional reagents are still used widely for cross-linking of biomolecules.

In order to eliminate the shortcomings of homobifunctional cross-linkers for conjugation reactions, heterobifunctional cross-linkers carrying two different functional groups that can couple two different active groups were developed. The advantages of these reagents include the ability to direct the conjugation reaction to selected parts of the molecule, thus gaining a better control over the conjugation process.
Figure 1.11. Reagents for labeling antibodies and DNA probes. All of these reagents have a spacer arm between the reactive groups or the label to decrease steric hindrance. Maleimide reacts with sulfhydryl groups, succinimide and isothiocyanate react with primary amino groups. SATA reacts with amino groups to produce an acetylated sulfhydryl group that can be deprotected using hydroxylamine. NHS-LC-biotin is used to conjugate biotin to proteins through their ε-amino group of lysine residues, and to DNA probes through their reactive amino groups.
A typical conjugation reaction using heterobifunctional cross-linkers is to link an SH group of one molecule to an NH₂ group of another using SMCC (Figure 1.10). In the first step of the reaction, the protein is treated with the cross-linker to allow the succinimide active group to react with the amino group. Then, the derivatized protein is purified from excess linker followed by the addition of the second protein bearing the SH group, which in turn reacts with the maleimide part of the cross-linker to form an active conjugate (Figure 1.12). This multistep protocol offers good control of the resultant conjugate as well as the molar ratio of components within the cross-linked products.

Other reagents were further developed which assist in the conjugation process of two molecules bearing the same active group. These reagents have the ability to introduce a protected sulfhydryl group to any molecule. For example, when conjugating two molecules through their NH₂ group, one molecule is first treated separately with a reagent such as SATA (Figure 1.11) to allow the amino group to react with the succinimide functional group, resulting in a protected sulfhydryl group. Simultaneously, the second molecule bearing an NH₂ group is treated with SMCC as above. After purification of both molecules from excess cross-linker, the sulfhydryl group is deacetylated using hydroxylamine and the SATA derivatized molecule is conjugated to the SMCC treated molecule as previously discussed.

Possible reactions of active reactant (protein, DNA, label) groups with cross-linker functional groups are shown in Figure 1.12. In general, N-hydroxysuccinimide ester and isothiocyanate derivatives react with primary amines at alkaline pH to produce a covalent amide and an isothiourea bond respectively. Similarly, maleimide and α-haloacetate derivatives (such as iodoacetate) react with free sulfhydryl groups of the reactants to yield a permanent thioether bond, at neutral and alkaline pH, respectively. Carboxyl groups of the reactants can also be derivatized by cross-linkers bearing carbodiimide derivatives at neutral pH to produce an active ester intermediate, which in turn reacts with a nucleophile such as primary amine to form an amide bond, in a similar manner as the N-hydroxysuccinimide ester reaction. Carbodiimide derivatives are zero-length cross-linkers, which mediate the conjugation reaction of two molecules by forming a covalent bond without any additional atoms, thus, one atom of a molecule is covalently attached to an atom of a second molecule with no intervening linker or spacer. Finally,
Figure 1.12. Reaction scheme for the conjugation of some common cross-linker (R₄) functional groups, with reactant (protein, DNA, or label (R')) active groups: A. primary amines, B. free sulphhydryl groups, C. carboxyl groups. D. carbohydrates moiety. NHS represents N-hydroxy succinimide and NaIO₄ denotes sodium periodate.
the carbohydrate moiety of the reactants can be conjugated by first oxidizing them with sodium periodate (cleaves adjacent carbon-carbon bonds that possess hydroxyl groups) to produce an aldehyde residue, which in turn is reacted with hydrazide derivatives, at slightly alkaline pH, to produce a covalent hydrazone linkage.

Many factors can influence the course as well as the yield of the conjugation reaction. Some of these factors are:

a. The concentration of the molecules to be conjugated (mass action law), and the molar ratio of both molecules with respect to one another.
b. The relative reaction rates of the cross-linker with the two molecules. Homobifunctional cross-linkers have the tendency to create polymers of one molecule.
c. The activity of the cross-linker reactive group and the efficiency of the conjugation reaction, which depend on the pH and ionic strength of the buffer solution.
d. The purity of the solutions used and possible reactivity of the buffers components with the cross-linker. For example, when conjugating through primary amine groups, all solutions should be amine free.
e. The solubility of the cross-linkers. Most cross-linkers are usually not water-soluble and they need an organic solvent such as dimethyl sulfoxide or dimethyl formamide. When conjugating proteins, the concentration of the organic solvent should be <10% in the conjugation reaction, since these solvents are known to denature protein. Analogues of these cross-linkers have been developed in which a negatively charged sulfonate group is added to the cross-linker giving just enough polarity to the molecule to provide solubility in water (Hermanson GT, 1996).

1.4.2. The (strept)avidin-biotin system

The most popular non-covalent conjugation method is based on the naturally occurring binding of the small molecule named biotin to the protein (strept)avidin. This strong binding has led to the use of this system in a variety of different applications especially, immunoassays and hybridization assays development.
Biotin, also known as vitamin H, is a small molecule of 244.31 Da found in all living cells. Biotin serves as a co-factor of carboxylating enzymes such as pyruvate carboxylase by carrying CO₂ to the substrate (Wood HG et al., 1977).

Avidin, a glycoprotein found in eggwhite, is composed of four identical subunits of 16.4 kDa (128 amino acids) giving a total intact protein of approximately 66 kDa (Delange RJ et al., 1971). Each subunit contains one binding site for biotin. This glycoprotein is basic, having an isoelectric point (pI) of 10. The major disadvantage of using avidin in assays is the high non-specific binding due to the presence of carbohydrate and the high pI. Deglycosylation of avidin by enzymatic cleavage or concanavalin-A affinity columns that adsorb only the glycosylated fraction proved to decrease the pI of the protein. Furthermore, reducing the charge of avidin by formylation, acetylation, or succinylation of the amino groups of lysine, or arginine leads to a decrease in the isoelectric point to approximately 6.3, making it suitable for many applications.

Streptavidin, from *Streptomyces avidinii*, is another biotin binding protein that overcomes the disadvantages of avidin. This protein (60 kDa) is also composed of four identical subunits, each containing one biotin-binding site, however, it does not contain carbohydrates and it has an isoelectric point of 5-6 (Chait L et al., 1964). Even though streptavidin binds biotin less efficiently (2-4 times lower than avidin), it is the biotin-binding protein of choice for assay development due to its low non-specific binding.

The interaction of (strept)avidin with biotin is among the strongest non-covalent interactions known, with a dissociation constant of approximately 10⁻¹⁵ M, which is approximately 10²-10⁶ higher than antigen-antibody interaction (Diamandis EP et al., 1991). This high affinity ensures that once the complex is formed, it will not be disturbed by pH changes, presence of chaotropic agents, or multiple washing when the complex is immobilized on a solid support. When biotin is bound to streptavidin, it induces a conformational change to the protein so that biotin is buried in the interior of the particular subunit. Furthermore, this interaction is strengthened by tryptophan 120 of the adjacent subunit, which makes hydrophobic contact with the biotin (Sano T et al., 1995).

The specificity of biotin binding to (strept)avidin provides the basis for development of assay systems to detect antigens and nucleic acids. In immunoassays, the specificity of antibodies to their antigen provides the basis of protein analysis. If the
antibody carries a biotin, (strept)avidin conjugated to the label can be used to detect the formation of the immunocomplex. The same concept is applied for hybridization assays if the detection oligonucleotide probe carries a biotin. Therefore, the (strept)avidin-biotin system does not confer a measure of specificity to the immunoassays and hybridization assays, however, it serves as a mediator between the reactant (antibody or DNA probe) and the label or the solid phase.

Biotinylation of antibodies can be carried out by conjugating N-hydroxysuccinimide ester derivatives of biotin (NHS-biotin) to the amino group of the antibody. The reaction is carried out in a similar manner as shown in Figure 1.12. Biotinylation of nucleic acids however is done by one of the techniques discussed below for DNA probe labeling using chemical or enzymatic methods, in which deoxyribonucleoside derivatives such as biotin-11-dUTP (Figure 1.13) are incorporated in the DNA target. Also, if the DNA probe is modified with an amino group, conjugation through NHS-biotin can be performed as above.

1.4.3. Digoxigenin-antidigoxigenin system

Similar to (strept)avidin-biotin interaction, the digoxigenin-antidigoxigenin is the basis of a mediator system that links the reactant to the label or the solid phase. Digoxigenin is a cardenolide-steroid which occurs exclusively in Digitalis plants as a secondary metabolite (O'Sullivan MJ 1981). It is a cyclopentano-perhydrophenanthrene with the chemical formula C_{23}H_{34}O_{3} (Figure 1.13). The anti-digoxigenin antibody isolated from sheep is a polyclonal immunoglobulin obtained by immunization with digoxigenin. The specificity of the anti-digoxigenin antibody is high and it is the basis of many protein as well as nucleic acid detection systems (Laios et al., 2001; Dooley S et al., 1988).

In order to avoid non-specific binding reactions of the Fc portion of the antibody, usually the Fab fragment is used as the anti-digoxigenin antibody as the indicator group in the digoxigenin detection. The Fab fragment is obtained by papain digestion and purification from the Fc segment. However, when this system is employed as a means for immobilization of reactants to the solid phase, the whole antibody is used.
Figure 1.13. dUTP derivatives used for chemical or enzymatic labeling of DNA. dUTP is derivatized at the C-5 position. The number (11) denotes the number of atoms in the spacer between the label and the nucleoside. dATP analogues (biotin-14-dATP) derivatized at the N-6 position can also be synthesized. Nucleoside analogues can carry an antigen such as digoxigenin, a fluorophore such as fluorescein, or even an enzyme such as horseradish peroxidase in a similar manner.
Labeling of assay reagents with digoxigenin is achieved in a similar manner as biotin. N-hydroxyssuccinimide esters of digoxigenin derivatives are available as well as digoxigenin-11-dUTP (Figure 1.13).

1.4.4. DNA probe labeling

In contrast to proteins, DNA does not bear a functional group that is available for conjugation. However, there are two main mechanisms by which nucleic acids can be modified: enzymatic or chemical. Both of these procedures produce active conjugates for sensitive hybridization assays.

1.4.4.1. Chemical labeling of nucleic acids

Since DNA probes are now synthesized in vitro by many companies, one of the ways to label a DNA probe is by incorporating an active group such as an amine or sulfhydryl at precisely defined sites of the oligonucleotide during synthesis, linked to the DNA base by a carbon chain (6-14 atoms) spacer (Connolly BA, 1985 and 1987). These groups can then be conjugated to the label as previously discussed. Moreover, direct incorporation of the label during synthesis has proven to be efficient, without any loss of the label activity or the DNA specificity. Therefore, biotin, digoxigenin, or a fluorophore can be simply incorporated in the same way during synthesis of the DNA probe.

Another way to chemically modify the DNA is by adding an active group or a label at specific sites within individual nucleosides which in turn is incorporated in the DNA growing chain during synthesis. Many nucleoside analogues have been developed, one of which is biotin-11-dUTP (Figure 1.13), in which dUTP is modified with a biotin group at the C-5 position of uridine. The number (11) denotes the size of the atom spacer between the nucleoside and the active group. Other nucleoside modifications such as biotin-14-dATP have been developed in which the biotin is linked to the N-6 position of dATP by a 14 atom spacer. Similarly, the biotin can be replaced with an active group such as an amino or sulfhydryl group, a fluorophore, or even an enzyme such as horseradish peroxidase (Urdea M et al., 1988).
1.4.4.2. Enzymatic labeling of nucleic acids

DNA polymerases such as DNA polymerase I or terminal deoxynucleotidyltransferase can be used to add controlled amounts of modified nucleosides to an existing DNA strand. There are four main procedures for DNA labeling by enzymatic activity which will be discussed below.

1.4.4.2.1. Polymerase chain reaction

Polymerase chain reaction is a DNA amplification technique based on three steps: DNA denaturation, annealing of a specific primer, and extension of the hybridized primer catalyzed by heat stable DNA polymerase (refer to section 1.2.2). dNTP derivatives, such as biotin-11-dUTP, can be included in the PCR reaction mixture. During the extension step, DNA polymerase will incorporate the modified dUTP leading to multiple copies of DNA target containing biotin at different sites. Another way to incorporate the label during PCR is by using a 5' end labeled oligonucleotide primer in the PCR mixture thereby introducing a single label per amplicon.

1.4.4.2.2. Nick translation

This procedure involves two steps with the use of two enzymes. In the first step, dsDNA is subjected to limited DNase I digestion leading to multiple nicks in the DNA sequence. In the second step, the 3' → 5' exonuclease activity of DNA polymerase removes a number of nucleotides beginning at each nick site and its 5' → 3' activity will fill in the gap by incorporation of dNTPs including the labeled (modified) dNTP. This technique is suitable for labeling circular or linear double-stranded DNA greater then 1 kb but not single-stranded or small DNA probes.

1.4.4.2.3. Random-primed labeling

This procedure uses the Klenow fragment of DNA polymerase I (large fragment with 5' → 3' polymerase activity without the exonuclease activity) and random specific oligonucleotide primers. First, the double stranded DNA is denatured followed by the annealing of primers at multiple sites of the DNA target. The Klenow fragment of DNA polymerase I catalyzes the extension of the hybridized primers in the 5' → 3' direction,
incorporating labeled dNTP. The length of the DNA fragment does not influence the reaction, therefore small DNA fragments (< 100 bp) can be labeled as efficiently, and both double and single-stranded DNA can be used as template for random priming.

1.4.4.2.4. Tailing using terminal deoxynucleotidyl transferase

Terminal deoxynucleotidyl transferase (TdT, 60 kDa) is an unusual DNA polymerase that recognizes and binds both ribo- and deoxyribonucleoside triphosphates. In the presence of a divalent cation, the enzyme catalyzes the addition of dNTPs to the 3' hydroxyl termini of the DNA (minimum 3 dNTPs). This process is referred to as "tailing reaction". The incorporation of labeled dNTPs to DNA using TdT is also efficient. When the nucleotide to be added is a purine, Mg$^{2+}$ is the preferred cation, however, when the nucleotide is pyrimidine, Co$^{2+}$ is the cation of choice (Bolum FJ, 1974). Single nucleotide can be added to the 3'-end of the termini by using a dideoxynucleoside triphosphate as the substrate. The enzyme strongly prefers the use of single-stranded DNA or double-stranded DNA with protruding end. However, blunt termini are used with lower incorporation efficiency. Ribonucleoside triphosphates, and particularly ATP, are known to be potent competitive inhibitors for DNA tailing, since they bind and block the dNTPs binding sites on the enzyme (Pandey VN et al., 1989).
1.5. Single Nucleotide Polymorphisms Genotyping Methods

The Human Genome Project has stimulated the investigation of numerous DNA sequence variants in the human genome, including microsatellites (tandem repeats of multiple copies of the same base sequence motif on the chromosome) and single nucleotide polymorphisms (SNPs, insertion/deletion, or substitution). These DNA variants are potential markers for molecular diagnosis of genetic disorders and large-scale population studies for genetic analysis of complex traits such as linkage analysis, i.e., studies of the tendency of certain genes to be inherited together in families.

Microsatellites have been used in linkage analysis in the past. However, it is becoming a trend to use SNP markers since they are the most frequent DNA sequence variations found in the human genome. SNPs are single-base variations at a unique physical location. As of the end of 2000, over 1.5 million SNPs have been found in the human genome and have been deposited to public databases (Int. SNP Map Work. Group, 2001). A single nucleotide polymorphism may occur in one of every 300 to 500 bp of human DNA (Collins FS. 1999). The availability of an ultra-high density SNP map will shed a new light in the understanding and studying of linkage analysis.

Technologies to identify genetic polymorphisms have been rapidly increasing over recent years, especially since the advent of the PCR technique. An ideal genotyping technique should be based on a robust and flexible reaction format, have low cost, be easily automatable, and allow for user friendly data analysis. In the following section, the current techniques for mutation detection and genotyping are reviewed.

1.5.1. Conformation-based polymorphism analysis
1.5.1.1. Single-strand conformation polymorphism

This technique is one of the most widely used for mutation detection. In single-strand conformation polymorphism, the DNA of interest is first amplified by PCR, followed by denaturation of the products and separation on a non-denaturing polyacrylamide gel. The DNA fragment with a single base modification forms a
conformer that migrates differently than the wild-type DNA when run on a polyacrylamide gel (Makino R et al., 1992).

1.5.1.2. Denaturing gradient gel electrophoresis

In this method, the DNA of interest is first amplified by PCR, and the products are subjected to denaturation followed by a renaturation step to form heteroduplex molecules. Then, these DNA heteroduplexes are resolved on a polyacrylamide gel with an increasing denaturing gradient of urea and formamide under careful temperature control. The melting characteristics of double-stranded DNA are defined by its sequence. Therefore, a single-base mismatch produces conformation changes in the double DNA helix that cause the differential migration of homoduplexes and heteroduplexes containing base mismatches during electrophoresis (Korkko J et al., 1998).

Another technique that uses the same principle is the mismatch cleavage detection technique which takes advantage of the fact that mismatched bases are sensitive to cleavage by enzymes or chemicals. In this technique, samples are treated with resolvases or chemicals to cleave the DNA heteroduplexes formed as above. The wild-type and the variant products are resolved electrophoretically side by side to check for the presence of mismatch-cleaved molecules (Mashal RD et al., 1995).

1.5.2. Sequence-specific polymorphism analysis

Although sequence-nonspecific detection of polymorphisms, such as the techniques described, is the basis in mutation discovery, it is not an acceptable approach to genotyping. Genotyping of SNPs plays an essential role in disease diagnostics and treatment, and will probably be a major part of every linkage analysis study. Thus, the appropriate genotyping method is critical to the success of the study.

Sequence-specific detection techniques have been developed for detection and genotyping of SNPs. These methods rely on four general mechanisms for allelic discrimination: allele-specific hybridization, allele-specific primer extension, allele-specific oligonucleotide ligation, and allele-specific invasive cleavage (Kwok PY, 2000).
1.5.2.1. Allele-specific hybridization assays

In this approach, two allele-specific probes (one for the wild type and another for the variant) are designed to hybridize to the target sequence only when they are perfectly matched. Under optimized assay conditions (controlled stringency), the one-base mismatch sufficiently destabilizes the hybridization and prevents the allelic probe to anneal to the DNA target. When the allele-specific probes are immobilized on a solid support, the labeled target DNA samples are captured followed by the detection of the label. Knowing the location of the probe sequences on the solid support, allows one to deduce the genotype of the sample.

Allele-specific hybridization is the basis of several elegant homogeneous genotyping assays that differ in the way they report the hybridization event.

1.5.2.1.1. 5’ nuclease assay

In this technique, an oligonucleotide is included in the PCR amplification reaction that hybridizes to the target polymorphic site along with the forward and reverse primers. If the target sequence of the probe is amplified in the reaction, then the probe will hybridize to this target sequence during the annealing/extension step of PCR. When Taq DNA polymerase encounters the hybridized probe, the probe is cleaved by the 5’ nuclease activity of Taq polymerase. This cleavage occurs only if the probe is specifically hybridized to the target sequence (Figure 1.14A). Monitoring the cleavage event will determine whether or not hybridization occurred. Using fluorogenic probes in the 5’ nuclease assay combines PCR amplification and detection into a single step. The fluorogenic probe consists of an oligonucleotide labeled with a fluorescent reporter dye at the 5’ end and a quencher dye at the 3’ end. In the intact probe, proximity of the quencher reduces the fluorescence signal observed from the reporter dye (fluorescence resonance energy transfer). Cleavage of the fluorogenic probe during the 5’ nuclease assay liberates the reporter dye, causing an increase in its fluorescence intensity (Livak KJ, 1999). Specific genotyping can be determined with this assay by using two different fluorogenic probes with different reporter dyes, one probe specific to the wild-type and another specific to the variant DNA target.
Figure 1.14. Principle of allele-specific hybridization assays. **A.** 5' nuclease assay. Two probes targeted at the polymorphic site are labeled with different fluorophores (F1 and F2) and a common quencher dye. Only a perfectly hybridized probe will be cleaved by Taq polymerase during the extension phase of PCR. If there is a mismatch, the probe will be displaced without being cleaved. Genotyping is determined by the ratio of the intensities of the two fluorophores. **B.** Principle of molecular beacons. Free molecular beacons are nonfluorescent because the stem hybrid keeps the fluorophore close to the quencher. When the probe sequence in the loop hybridizes to its target, forming a rigid double helix, a conformational rearrangement occurs that removes the quencher from the vicinity of the fluorophore, thus restoring fluorescence.
1.5.2.1.2. Molecular beacons detection

Molecular beacons are oligonucleotide probes that have a stem and loop structure. A fluorophore is covalently linked to one end of the molecule and a quencher linked to the other. The hairpin stem keeps the fluorophore so close to the quencher that fluorescence is quenched. The energy absorbed by the fluorophore is transferred to the quencher and released as heat (Marras SAE et al., 1999). However, when the probe sequence in the loop is annealed to its target sequence, the rigidity of the probe-target hybrid forces the hairpin stem to unwind, separating the fluorophore from the quencher, and restoring fluorescence (Figure 1.14B). Mismatched probe-target hybrids dissociate at substantially lower temperature than perfectly complementary hybrids. This thermal instability of mismatched hybrids increases the specificity of molecular beacons. Because the presence or absence of fluorescence reflects the open or close status of the stem-loop structure, no purification or separation steps are involved. Since molecular beacons can possess a wide variety of differently colored fluorophores, multiple targets as well as genotyping discrimination can be accomplished in the same solution using several different molecular beacons each designed to detect different target, and each labeled with a different fluorophore. This “closed-tube” system has real advantages because cross-contamination is minimized and automation is easily achievable.

1.5.2.1.3. Homogeneous solution hybridization using fluorescence resonance energy transfer (FRET) detection.

FRET between an excited fluorescent donor and an acceptor molecule is observed when two fluorophores are in close proximity and when the donor emission spectrum overlaps the acceptor excitation spectrum (Lopez-Crapez E et al., 2001). As a result of this energy transfer, the donor’s emission is diminished (quenched) while the acceptor’s emission is increased. The changes in fluorescence intensities of donor and acceptor dyes can therefore be monitored to determine whether a donor dye and an acceptor dye have been joined together and are part of a new molecule or whether the donor and acceptor dyes found on the same probe have been separated form each other. The latter case has been applied to the 5’ nuclease assay using an oligonucleotide specific to the target
polymorphic site that is labeled at the 5' end with the acceptor molecule and at the 3' end with the donor molecule.

1.5.2.1.4. DNA microarray genotyping

The DNA microarray offers many advantages that include the use of planar solid support such as glass or silicon, miniaturization and high-density analysis as well as powerful high-resolution detection offered by fluorescence. DNA chip dimensions offer hybridization sites in the 50-200 micron range, producing arrays ranging from 100 to over 10,000 or even 400,000 different probes on a 1 cm² area (Cuzin M, 2001) therefore leading to simultaneous analysis of thousands of polymorphisms.

DNA chip manufacturing is achieved by two methods. Either by “on chip” technologies where oligonucleotides are chemically synthesized in situ, by using parallel standard photolithographic (light-directed chemistry) techniques combined with specially developed, photochemically sensitive phosphoramidites, or by “off-chip” technologies where DNA fragments are previously synthesized, purified and controlled before being grafted onto the substrate using a number of different technologies such as mechanical, ink-jet, or electrochemical deposition (Fodor SPA et al., 1991).

The first large-scale genotyping method was developed by the Whitehead Institute and Affymetrix, Inc. (Wang DG et al., 1998). A total of 3241 candidate SNPs were identified and the locations of 2227 of these SNPs were identified from the constructed genetic map. Prototype genotyping chips were developed that allow simultaneous genotyping of 500 SNPs.

Polymorphism analysis is usually achieved by first amplifying the DNA of interest by PCR to incorporate fluorescently labeled nucleotides followed by hybridization of the products to the array. Each oligonucleotide in the high-density array acts as an allele specific probe. This technology is based on the fact that perfectly matched sequences hybridize more efficiently to their corresponding probe on the array and, therefore, give stronger fluorescent signals over mismatched probe-target combinations. Hybridization signals are usually quantified by high-resolution fluorescent scanning and analyzed by computer software (Chee M et al., 1996; Lipshutz RJ et al., 1999).
Recently, a DNA chip was designed that offers genotyping information of 1494 SNPs in a single experiment (Mei R et al., 2000). The major breakthrough of this approach is the degree of multiplexing achieved by designing PCR assays that amplify very small segments, followed by a second round of PCR using a common set of primers. In this assay, the entire set of 1494 SNPs is amplified in just 24 multiplex reactions. Advantages of this technology include the requirement of only small amounts of starting genomic DNA (120 ng total for all 1494 markers), the large number of SNPs that are typed in one experiment, and the minimal manual steps. However, this approach has a major drawback due to the expensive design and manufacturing of the microarray, the set of markers selected cannot be changed quickly or arbitrarily. Furthermore, it has been shown that 20% of the SNPs tested in this approach do not yield reliable results (Kwok PY, 2001). This rate of failure is too high for many applications.

Another assay for single nucleotide polymorphisms detection that is based on the microarray technology is the dynamic allele-specific hybridization assay (Howell et al., 1999). This assay is based on the fact that the mismatched probe-target hybrids dissociate at substantially lower temperature than exactly complementary hybrids. Therefore, this approach looks for the melting temperature differences between the allele specific probe when it is annealed to the matched and mismatched targets by monitoring the hybridization over a range of different temperatures. Using a DNA intercalating dye (such as Sybr Green I) that fluoresces in the presence of double-stranded DNA, fluorescence is observed only when hybridization occurs and double-stranded DNA species are formed. A disadvantage of this technique is that the double-stranded PCR target must be rendered single stranded before being used in the assay because the double-stranded PCR product will cause the dye to fluoresce.

1.5.2.2. Allele-specific primer extension

This method is a robust allelic discrimination mechanism, highly flexible, and requires the least number of primers/probes. There are numerous variations of the primer extension approach that are based on the ability of DNA polymerase to incorporate specific deoxyribonucleosides complementary to the sequence of the template DNA.
Allele-specific primer extension methods can be grouped into two categories, allele-specific nucleotide incorporation and allele-specific PCR approach.

1.5.2.2.1. Allele-specific nucleotide incorporation

This technique tests for the specific nucleotide that is incorporated onto the primer at the polymorphic site. Oligonucleotides specific to target sequence immediately upstream of the polymorphic site are immobilized via their 5′ end on solid support (glass surfaces). PCR amplified target DNA is first denatured, and allowed to hybridize to the immobilized oligonucleotides. In the presence of dideoxyribonucleoside triphosphates, DNA polymerase extends the immobilized oligonucleotides by one base containing the SNP sequences. With each of the four dideoxy terminators labeled with different fluorescent dyes, the identity of the incorporated base, and therefore the SNP, can be easily identified (Shumaker JM et al., 1996; Tonisson N et al., 2000).

FRET-based technologies have been applied to the allele-specific nucleotide incorporation in which the oligonucleotide used is labeled at the 5′ end with a donor dye and is extended by one base using acceptor dye-labeled dideoxy terminator. This assay is referred to as template-directed dye-terminator incorporation (Chen X et al., 1999).

Detection of this type of primer extension reaction can also be performed based on pyrosequencing where the identity of the polymorphic base in the target DNA is determined (Alderborn A et al., 2000). Pyrosequencing is a new DNA sequencing method based on detecting the formation of pyrophosphate, the by-product of DNA polymerization (Ronaghi M, 2001). The produced pyrophosphate is converted to ATP in the presence of adenosine 5′-phosphosulfate, which in turn fuels luciferase (for discussion on luciferase reaction, refer to section 1.3.3.1). Therefore light is observed when a nucleotide is added to the growing DNA chain. ATP and unincorporated dNTPs are continuously degraded by apyrase. The light is switched off and the next dNTP is added. The SNP is uncovered by monitoring the incorporated dNTP.

1.5.2.2.2. Allele-specific PCR approach

In this approach, DNA polymerase extends a primer only when its 3′ end is perfectly complementary to the target. The 3′-end of the primer extension probe consists
of the allelic base. The allele found on the target DNA can be deduced by determining whether or not PCR product is produced. Several innovative approaches have been utilized to detect the formation of PCR products in homogeneous assays. Some are based on melting curve analysis in which a GC-rich sequence was added to one of the allele-specific PCR primers to increase the melting temperature of one of the PCR products. Others are based on the usage of DNA intercalating dye to detect the presence of double stranded DNA. Moreover, hybridization of target specific probes with the application of FRET assay or molecular beacons have been applied for PCR product detection (Germer S et al., 1999; Lopez-Crapez E et al., 2001; Marras SAE et al., 1999).

1.5.2.3. Allele-specific oligonucleotide ligation

The enzyme DNA ligase is highly specific in repairing nicks in the DNA molecule. When two adjacent oligonucleotides are annealed to a DNA template, they are ligated together only if the two oligonucleotides perfectly match the template. However, the ligation fails to occur if a mismatch is present at the junction. Therefore, the alleles present in the target DNA can be inferred by determining whether or not ligation has occurred. Moreover, the development of new thermostable DNA ligases allows increase in sensitivity and specificity of ligation assays (Barany F et al., 1991; Tong J et al., 1999). Many methods have been developed for polymorphisms detection using the allele specific oligonucleotide ligation approach. Some of them have the potential of genotyping without prior target amplification by PCR such as the padlock probe and the rolling circle amplification protocols, and will be discussed below.

1.5.2.3.1. Oligonucleotide ligation assay (OLA)

OLA is based on the ability of DNA ligase to covalently join two adjacent oligonucleotide probes when they are perfectly hybridized to the DNA template. If a mismatch, however, is present at the junction, then ligation fails. The OLA reaction contains an allele-specific oligonucleotide and a common oligonucleotide along with PCR amplified product. After heat denaturation of the target DNA, the 3’ end of the allele-specific probe hybridizes immediately adjacent to the 5’ end of the common probe on the DNA target. DNA ligase (such as T4 DNA ligase) joins the adjacent ends of the allele
specific probe and the common probe, only when there is a perfect match. These ligation products can be viewed by electrophoresis. The genotype of the DNA sample can be deduced by running two separate reactions, one containing an oligonucleotide specific to the wild-type and another reaction with an oligonucleotide specific for the variant allele.

Solid phase detection is another way to measure the OLA products in which the allele-specific probes are labeled with a fluorophore at the 3' end and the common probe is labeled with biotin at the 5' end. After ligation, the biotin is captured on streptavidin-coated solid phase. The target DNA is removed by a sodium hydroxide step, and the fluorophore is detected (Figure 1.15A).

FRET approach has also been used for the detection of OLA. In this case, the allele specific oligonucleotide is labeled with an acceptor molecule and the common probe is labeled with the donor dye. Once both oligonucleotide are ligated together, intramolecular FRET is observed. This method is referred to as dye-labeled oligonucleotide ligation assay (Chen X et al., 1999).

1.5.2.3.2. Padlock probes and rolling circle amplification

Padlock probes are circularized oligonucleotide probes in which the 5' and 3' end regions are designed to base pair next to each other on a target DNA strand, where the polymorphism occurs. If properly hybridized, then the ends can be joined by DNA ligase, converting the probes to covalently closed molecules (80-90 bases) that are padlocked to the target sequence (Baner J et al, 1998). Ligation, and therefore polymorphism can be detected by the rolling circle amplification (RCA), a mechanism that is used by many viruses to rapidly produce multiple copies of their genome (Kornberg A et al., 1992). During the RCA reaction, an oligonucleotide complementary to the circularizable probe is added after ligation to serve as a primer for RCA. In the presence of a strand-displacing DNA polymerase (such as phage 29 or T7), the primer is extended, eventually displacing itself at its 5' end once one complete revolution of the circularized probe is made (Lizardi PM et al., 1998). Continuous polymerization and displacement generates single-stranded, concatameric DNA copies of the original circularized probe (Figure 1.15B).
Figure 1.15. Principle of two allele-specific oligonucleotide ligation assays. A. Oligonucleotide ligation assay with solid phase detection. DNA target is first denatured. Then, common and allele specific probes are annealed to the target DNA immediately adjacent to each other at the junction of polymorphism. After ligation with DNA ligase, the biotin on the common probe is captured on streptavidin coated solid support, followed by a sodium hydoxide wash to remove the target DNA. Finally the fluorophore is detected. B. Padlock probe and rolling circle amplification (RCA). The open circle probe is first annealed to the denatured target DNA followed by ligation. An oligonucleotide that is complementary to the circle probe is allowed to hybridize and the RCA is initiated by the action of DNA polymerase to produce multiple copies of the circle.
Several methods have been developed for detection of the RCA products. Some of them are based on electrophoresis, others use fluorophore-labeled dNTPs during the RCA reaction followed by the detection of the fluorophore. A major advantage of rolling circle amplification is the ability to obtain the genotype of SNPs directly from genomic DNA without previous amplification by PCR.

1.5.2.3.3. Ligase chain reaction

Ligase chain reaction (LCR) is an exponential in vitro amplification technique that allows discrimination of SNPs, using the activity of thermostable DNA ligase. In this technique, two sets of DNA probes are used. One set (one allele-specific oligonucleotide and one common DNA probe) is complementary to the sense strand of target DNA and are hybridized adjacent to each other at the junction of the SNP (similar manner to OLA). A second set of DNA probes specific to the antisense strand of DNA target sequence is also used. After target denaturation, the two sets of DNA probes are allowed to hybridize to their corresponding DNA strand followed by ligation using thermostable DNA ligase. The ligated products can then serve as a template in the following cycle of LCR leading to an exponential amplification process. However, if there is a mismatch at the primer junction, ligation will not occur, thus amplification product will not be formed.

Many methods have been developed for detection of LCR products. Solid phase-based assays can be employed in which one probe in each set is labeled with a fluorophore, and the second is labeled with biotin. The biotin is captured on streptavidin coated solid support and the fluorophore is detected in a similar manner to OLA solid phase detection (Figure 1.15A). Homogeneous assays based on molecular beacons or FRET technologies can also be used for detection of LCR products.

1.5.2.4. Allele-specific invasive cleavage

The invader assay is based on structure-specific enzymes that cleave a complex formed by hybridization of overlapping oligonucleotide probes. Three probes are used in each reaction, one probe specific for the wild-type allele, a second probe specific for the variant allele, and a third upstream invader probe. These probes are designed such that the polymorphic site is the point of overlap. When the 3’ end of the upstream invader
probe overlaps the hybridization site of the 5' end of the downstream allele-specific probe by at least one base pair, the structure will be recognized and cleaved by cleavases, a class of natural and engineered enzymes (Lyamichev V et al., 1999). Therefore, the correct overlapping structure and the cleavage occurs only with the allele-specific probe. However, if one base mismatch is present, no overlapping structure and therefore no cleavage occurs. The allele-specific probe contains a flap sequence (an arbitrary sequence unrelated to the SNP) downstream of the polymorphic site. Upon cleavage, the flap released from the allele-specific probe is used in a second reaction where it serves as an invader oligonucleotide, directing the cleavage of a combined labeled FRET probe-template construct. This secondary probe is 5' end labeled with the donor fluorophor, which is quenched by an internal acceptor dye. Upon cleavage, in the presence of the flap probe, the donor and acceptor dyes are no longer in close proximity, quenching is abolished, and fluorescence is generated (Figure 1.16).

The advantage of the invader assay is that it is conducted isothermally, with a linear increase in signal over time. This signal amplification step increases the amount of the produced labeled cleavage-product by $10^6$-$10^7$ fold per target sequence per hour, an amount sufficient for detection without the need for target amplification (Hall JG et al., 2000).
Figure 1.16. Principle of the invader assay for detection of polymorphisms. Two sequence-specific oligonucleotide probes, allele-specific probe and invader probe, are hybridized to the DNA target to generate an overlapping structure that is recognized and cleaved by cleavase. The flap released from the allele-specific probe serves as an invader in a second invasion reaction, directing the cleavage of a universal FRET probe-template construct. If there is a mismatch between the invader probe and the DNA target, the invasion is lost and no fluorescent from the donor fluorophore is generated. $F_a$ denotes acceptor fluorophore and $F_d$ indicates the donor fluorophore.
1.6. Gene Expression

In 1902, Archibald Garrod hypothesized that a defective gene inherited at birth results in lack of enzyme function. This hypothesis was confirmed forty years later by George Wells Beadle and Edward Tatum proposing that genes mediate chemical reactions in cells through the synthesis of specific enzymes and that there is a one-to-one correspondence between a mutation and the lack of specific enzyme. They referred to this finding as the "one gene/one enzyme" hypothesis. In 1958, Francis Crick proposed the central dogma of molecular biology. This theory, which has become the basis of molecular biology, states that DNA directs its own replication as well as its transcription to RNA, which in turn, directs its translation to protein. In 1960, Charles Yanofsky stated that the gene sequence is colinear with the specific polypeptide sequence. One year later, Crick and Sydney Brenner discovered the triplet characteristics of the genetic code, called the codon.

The expression of genetic information is a two-step process: transcription and translation. Each of these processes will be discussed separately.

1.6.1. Transcription

In transcription, a DNA strand serves as a template for the synthesis of a complementary strand of RNA in the nucleus. RNA is generally single-stranded and similar to DNA with the exception that thymine is replaced by uracil, and a ribose sugar instead of deoxyribose. There are four types of RNA: messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), and small nuclear RNA (snRNA). Each RNA will be discussed in the corresponding section.

RNA synthesis is catalyzed by RNA polymerases, multisubunit nuclear enzymes, which are among the largest known soluble enzymes. There are three different types of eukaryotic RNA polymerases. RNA polymerase I which transcribes rRNA genes, RNA polymerase II that transcribes mRNA genes, and RNA polymerase III which transcribes tRNA and snRNA genes.

Transcription can be divided into 3 steps:

(i) Initiation. In this step, the RNA polymerase recognizes the promoter sequence on the DNA, unwinding (melting) the double-helical structure of the DNA template to
produce a single stranded DNA region referred to as the transcription bubble or initiation complex (Figure 1.17B). The 3' → 5' DNA strand of the bubble acts as a template for RNA synthesis. The first ribonucleoside triphosphate (NTP) that is incorporated is usually ATP or GTP. Recognition of the promoter sequence is essential for the correct initiation of transcription. The initiation phase is characterized by abortive cycling, a process of repeated synthesis and release of short RNA products (2-8 bases). This process continues until some transition or conformational change occurs to form the more stable elongation complex that is able of transcribing the complete DNA template (Gralla JD et al., 1980). In eukaryotes, DNA transcription is activated by the binding of transcription factors (TFIIC, TFIID, TFIIE) to specific regions in the promoter such as the CAAT (CAAT binding protein, CBP). TATA box (TATA binding protein, TBP), or GC rich region (SP1). The TBP is a component of TFIID transcription factor. Upon binding of TBP to the TATA box, a second transcription factor known as ATF interacts with its specific binding site in the promoter, causing TFIID to change its conformation so it interacts with both the TATA box and the transcriptional start site instead of the TATA box alone (Figure 1.17A). This altered binding, facilitates the binding of other transcription factors such as TFIIC and TFIIE along with RNA polymerase to form a stable initiation complex (Latchman DS, 1993). Transcription factors can also bind to regulatory sequences (enhancers), which can be found upstream, downstream, or within the gene. The exact function of enhancers is unknown, however, it is hypothesized that these sequences enhance the access of RNA polymerase to the transcription start site, stimulating transcription.

(ii) Elongation. Once the first NTP is incorporated, the RNA chain is elongated in the 5' → 3' direction according to the DNA nucleotide sequence. The formation of phosphodiester bond between the incorporated NTPs is accompanied by the release and subsequent hydrolysis of inorganic pyrophosphate. As soon as the ribonucleosides are incorporated in the transcription bubble (approximately 8), T7 RNA polymerase moves downstream along the DNA template (translocation), forming another transcription bubble, and elongation continues. Simultaneously, previously unwound DNA regains its double helical structure allowing the formation of another transcription bubble around the promoter region. Studies showed that elongation is activated by the binding of
Figure 1.17. Transcriptional mechanism of initiation and elongation. A. Diagram showing the interactions of transcription binding protein (TBP) of the transcription factor (TFIID) with the TATA box of the gene promoter to form transcription initiation complex. Transcriptional activator (ATF) binds to the promoter and triggers a conformational change in TFIID so that it contacts both the TATA and start site, where RNA polymerase initiates transcription. B. Schematic diagram showing the transcriptional elongation complex with the DNA binding site (DBS), RNA binding site (RBS) and the RNA-DNA heteroduplex binding site on RNA polymerase. The RNA is extended in the 5' → 3' direction.
transcription elongation factors, such as TFIIS (Reines D et al., 1989), as well as transactivators (Yankulov K et al., 1994). Furthermore, it was observed that in E. coli, elongation occurs with a speed of 60-80 nucleotides per second (Gotta SL et al., 1991).

Recently, it was shown that RNA polymerases contain three binding sites in the elongation complex that hold the nucleic acid residues (Nudler E, 1999). The dsDNA binding site with 9 bp immediately downstream of the transcription bubble, the RNA-DNA heteroduplex binding site with 6 bp at the 3' end of the nascent RNA, and the ssRNA binding site with 9 bases just upstream to the RNA-DNA heteroduplex site (Figure 1.17B).

(iii) Termination. The DNA template contains specific sequences at the 3' end that triggers transcription termination. It has been observed that a 3' end G + C rich region, which forms a hairpin in the RNA, followed by a series of A's on the template strand are common eukaryotic and prokaryotic genes. The stability of the hairpin structure in the RNA causes the RNA polymerase to pause for few seconds at the termination site. This mechanism induces a conformational change in the RNA polymerase which permits the non-coding DNA strand to displace the weakly bound poly (U) tail from the template strand, thereby triggering transcription termination. Furthermore, transcription termination factors, such as rho protein, stimulate the release of RNA. Rho protein, with ATPase activity, binds to a specific sequence and moves along the nascent RNA in the 5' → 3' direction. Once this protein encounters the paused RNA polymerase at the termination site, 70-80 bases segment of the RNA wraps around it, resulting in the activation of the ATPase, which leads to its efficient movement along the RNA, unwinding the RNA-DNA duplex, and releasing the RNA transcript (Shigesada K et al., 1982).

1.6.1.1. Posttranscriptional modification of mRNA

mRNA transmits the genetic code from the genes in the nucleus to the cytoplasm where it is used as the template for protein synthesis. The pre-mRNA produced in the nucleus is usually different than the mature mRNA that is translated in the cytoplasm. Pre-mRNA undergoes several modifications in the nucleus before the initiation of translation.
The first posttranscriptional modification is 5' capping of mRNA, which is achieved by adding a guanine residue to the 5' end of mRNA in reversed orientation (5' end of guanine is attached to the 5' end of mRNA). This cap structure, added to the RNA chain by a specific guanylyltransferase before it is 20 nucleotides long, defines the eukaryotic translation start site (Song H-J, et al., 1995).

Polyadenylation in which a poly (A) sequence is added at the 3' end of the mRNA by the action of poly (A) polymerase is another type of posttranscriptional modification. This enzyme recognizes a highly conserved AAUAAA sequence at the 3' end of mRNA, which acts as a signal for polyadenylation (100-200 adenosine). The Poly (A) tail is known to have a protective role for mRNA (Zhao J et al., 1999), as well as translation stimulation (Sachs AB et al., 1997).

RNA splicing, in which the non-coding intervening (introns) sequences are cut or spliced from mRNA, followed by linking the coding (exons) sequences is an essential modification of mRNA before it is translated. Splicing reactions are performed by spliceosomes, which consist of several protein subunits and snRNA molecules. SnRNAs bind proteins to form small nuclear ribonucleoproteins (snRNPs). It was observed that the 5' end of a U-rich snRNA (U1-snRNA) is partially complementary to the 5' mRNA splice junctions. Similarly, U2-snRNA recognizes the intron regions and U5-snRNA has a complementary sequence to the 3' mRNA splice junctions. Spliceosomes bring together the pre-mRNA, U1, U2 and U5-snRNA, along with other snRNA (U1-U6) and Ser-Arg rich splicing factors (recognizing U1-snRNA) and trigger splicing reactions (Wise JA, 1993; Staknis D et al., 1994). The exact splicing site is important for the production of a mature mRNA. Incorrect splicing results in a defective mRNA which is the basis of certain molecular diseases such as beta-thalassemia (refer to section 6.2).

1.6.2. Translation

The transcribed RNA is first translocated from the nucleus to the cytoplasm where protein synthesis occurs. Since prokaryotes do not have a nucleus, transcription and translation occurs simultaneously as soon as a segment of mRNA is synthesized. Translation can be divided into four steps:
(i) Activation of amino acids. In the first step of translation, all amino acids are activated by tRNA before they can be incorporated in the polypeptide chain. There is at least one tRNA per amino acid. tRNAs have similar structure with approximately 80 bases. They consist of a 5' end phosphate group, four short double helices stems three of which contain a loop (one of them carries the anticodon), many unusual bases, and a 3' end CCA sequence with a hydroxyl group (site of attachment of amino acid). Each tRNA molecule is recognized by its corresponding aminoacyl-tRNA synthetase, which catalyzes the addition of the specific amino acid to the 3' end of tRNA. The anticodon loop of the tRNA carrying the correct amino acid recognizes the codon on mRNA so that the amino acid can be added to the growing polypeptide chain (Ochoa S, 1977).

(ii) Initiation. In this step, a number of initiation factors (IF1, IF3, IF4) bind to the small ribosomal subunit, promoting the dissociation of the large subunit. The 5' cap of mRNA interacts with IF4, which in turn unwinds the secondary structure of the mRNA, allowing the met-tRNA anticodon to hybridize to the AUG start codon. The met-tRNA usually binds IF2 carrying a molecule of GTP to fuel the formation of the initiation complex (Figure 1.18A). Therefore mRNA interacts with the small ribosomal subunit via protein-protein, protein-RNA, as well as mRNA-rRNA interaction (Sachs AB et al., 1997). The entry of the large subunit to form the initiation complex requires the hydrolysis of GTP along with the release of IF1 and IF2. Recently, it was shown that translation can also be stimulated through the binding of a poly (A) binding protein (PAB1P) to the poly (A) tail of mRNA (Tarum SZ et al., 1996). Upon this binding, the IF4 interacts with PAB1P, triggering a conformational change of mRNA which allows the 5' cap to interact with the IF4, stimulating the binding of the small ribosomal subunit as previously described. Furthermore, an eight base sequence flanking the AUG start codon in mRNA was identified to modulates translation by eukaryotic ribosomes. This sequence is ACCATGG and is referred to as “Kozak sequence” (Kozak M, 1986).

(iii) Elongation. In this step, an elongation factor (eEF1) carrying GTP interacts with aminoacyl-tRNA, which in turn hybridizes to the mRNA by anticodon-codon as well as protein-RNA interaction. This aminoacyl-tRNA is located at the A site of the large ribosomal subunit, however, the initial met-tRNA is located at the P site. The peptidyl transferase activity of the large subunit catalyzes the formation of a peptide bond between
Figure 1.18. Translational mechanism initiation and termination. A. Schematic diagram showing the interaction of the small ribosomal subunit with mRNA, tRNA, and initiation factors (IF1, IF2-GTP, IF3, and IF4) to form translational pre-initiation complex. Upon the binding of the large ribosomal subunit, GTP is hydrolyzed and initiation factors IF1 and IF2 are released. B. Mechanism of translation termination. Release factor-complex (eRF1-eRF2-GTP) binds to the Stop codon upon its arrival to the A-site of the large ribosomal subunit, mimics the binding of eEF1, and therefore induces the ribosomal peptidyl transferase to transfer the peptidyl group to water resulting in the release of the polypeptide chain.
the two amino acids (COOH group of methionine and the NH₂ group of the second amino acid). Subsequently, the mRNA moves resulting in the translocation of the second aminoacyl-tRNA codon from the A site to the P site of the large ribosomal subunit, driving out the met-tRNA, and making the A site available for the binding of another aminoacyl-tRNA. It has been shown that elongation factor (eEF2) binds to the ribosome and activates translocation in an energy dependent fashion (Proud CG, 1994). However, upon translocation and GTP hydrolysis, this elongation factor is released from the ribosome, allowing the next elongation cycle.

(iv) Termination. Once the ribosome encounters one of the stop codons (UAA, UGA, UAG), the elongation of the polypeptide chain ceases, resulting in the disassembly of the translational complex. It has been shown that a complex of eukaryotic release factors (eRF1-eRF3) carrying a GTP molecule, binds to the stop codon, following its arrival to the A site (Stansfield I et al., 1995). This interaction inhibits the binding of eEF1, and therefore induces the ribosomal peptidyl transferase to transfer the peptidyl group to water and not to aminoacyl-tRNA resulting in the release of the polypeptide chain (Figure 1.18B). At this point, the resulting inactive ribosome releases the mRNA as well as the release factors, and start preparing for a new round of protein synthesis.

1.6.2.1. Posttranslational processing of proteins

Many proteins are translated on free ribosomes with a signal peptide at their N-terminal. As soon as this sequence is synthesized, it is recognized by a signal recognition particle (SRP), which in turn, binds a specific receptor on rough endoplasmic reticulum (ER) membrane in eukaryotes or plasma membrane in bacteria (Fesler LI et al., 1978). As the polypeptide synthesis continuous, it is translocated across the membrane through a protein-rich channel. As soon as the polypeptide chain enters the luminal side of the membrane, signal peptidase catalyzes the cleavage of the signal peptide.

Most of the synthesized proteins undergo a series of co-translational as well as post-translational modifications (Wold F, 1981). The most important one is N-linked glycosylation in which carbohydrate moieties are attached to asparagine residues in the sequence Asn-X-Ser or Asn-X-Thr, where X is any amino acid residue except proline, by forming a β-N-glycosidic bond. This type of glycosylation is mediated by dolichol, a
lipid with attached carbohydrate moieties, in which a specific glycosyl transferase transfers the carbohydrate from dolichol to the protein in ER (Kornfield R et al., 1985). Glycoproteins are then processed starting in ER where some of their sugar residues are trimmed, followed by their transport to the Golgi apparatus via membrane vesicles where other types of sugars are covalently linked to them. Furthermore, O-linked glycosylation occurs in the Golgi where carbohydrate moieties are attached to serine or threonine residues by the corresponding glycosyl transferases. Glycosylation plays an essential role in sorting and distribution of the nascent proteins to their proper cellular destination. Moreover, it has been suggested that carbohydrate plays a role in stabilization of protein conformation, protection from proteolysis as well as non-specific interactions with other carbohydrates (West CM, 1986).

Other types of posttranslational modifications include methylation of lysine as well as arginine residues by the action of specific methyltransferases (Aletta JM et al., 1998), phosphorylation of tyrosine, serine, and threonine residues by specific kinases, hydroxylation of proline, lysine, and aspartic acid residues by hydroxylases, acetylation of lysine residues by acetylase, carboxylation of glutamate by carboxylase as well as ADP-ribosylation of arginine residue by a specific ADP-ribosyltransferase (Saxty BA et al., 1998).

1.6.2.2. Protein folding

The 3-D structure of a protein, and therefore its most stable conformation, is determined by its amino acid sequence. The synthesized protein rapidly folds by removing the hydrophobic side chains away from the aqueous solution to form the hydrophobic core. This folding appears to proceed cotranslationally as well as posttranslationally via one or more specific pathways, facilitated by ATP-dependent molecular chaperones. Chaperones are proteins that bind and stabilize the nascent as well as the translocating polypeptide chains, preventing their aggregation. The most studied chaperone system is the ATP-dependent Hsp70 (heat shock protein) chaperone. This type of chaperone contains two functionally coupled domains, the N-terminal domain (44 kDa) that mediates the binding of ATP and the C-terminal domain (19 kDa) which binds the substrate polypeptide (Flaherty KM et al., 1994; Zhu XT et al., 1996). The binding and
release of the substrate is performed by cycles of ATP binding and hydrolysis. ATP-bound chaperone interacts with its substrate, followed by interaction with a binding protein, which triggers ATP hydrolysis. The ADP-bound chaperone form results in a stable conformation of the substrate protein. Subsequently, a second binding protein acts on the ADP-bound chaperone and triggers the release of ADP. Rebinding of ATP induces the dissociation of the substrate-chaperone complex and another cycle is initiated (Harrison CJ et al., 1997). It has been observed that the substrate binding site of this chaperone contains a cleft that recognizes linear polypeptide sequences rich in hydrophobic amino acids. Due to the hydrophobic nature of the polypeptide substrate, the binding motif of chaperones is probably located in the interior of a correctly folded protein. These hydrophobic regions are found on an average of every 40 amino acid residues (Rudiger S et al., 1997).

This type of chaperone-mediated pathway was proven to be sufficient to promote some in vitro model protein folding such as luciferase (Szabo A et al., 1994). It has been shown that when luciferase is expressed in rabbit reticulocyte lysate or wheat germ, it folds within one minute. The N-terminal domain of luciferase (residues 1-190) folds cotranslationally on ribosome-bound nascent chain to produce a folded polypeptide intermediate that does not aggregates, followed by rapid formation of native protein upon the release of the full polypeptide from the ribosome (Frydman J et al., 1994 and 1999). More complex protein, however, requires a combination of chaperone systems such as the chaperonin complexes for sufficient folding (Kusmierczyk A et al., 2001). Lastly, if ribosome-bound polypeptides are unable to fold properly, cells possess a machinery that recognizes the non-correctly folded polypeptide and targets them for degradation (Frydman J et al., 1996). However, little is understood about when the newly synthesized polypeptides become accessible for degradation.
Chapter 2

Materials and Methods
2.1. DNA Plasmids Preparation

The plasmid (circular double stranded DNA with self-replication property) containing the luciferase coding gene under the control of T7 RNA polymerase promoter (Luc-DNA, 4.3 kb, Appendix 1.1) was propagated in E. coli JM109 strain. The plasmid containing the T7 RNA polymerase coding DNA under its cognate promoter (T7RP-DNA, plasmid pT7G1, 6.1 kb, a kind gift from J.A. Wolff, Depts. of Pediatrics and Med. Genetics, Waisman Center, Madison, WI, (Deng H et al., 1994), Appendix 1.2) was propagated in E. coli XL1-blue strain. Both plasmids contain an ampicillin-resisting gene (β-lactamase gene), and therefore the procedure for the preparation of both DNA plasmids was similar (Micklos DA et al., 1990).

In the first step, the E. coli strain was plated on an LB agar plate followed by incubation at 37° C over night, with plates up-side down. A negative control, (agar plate with no bacteria) was included during the incubation period. In order to obtain a homogenous cell population, a single colony was picked and inoculated into 5 mL of LB broth followed by over night incubation at 37° C with vigorous shaking. Liquid bacterial culture enters a lag phase for about 30 minutes after inoculation, followed by a log phase with rapid division until it reaches a stationary phase in which cells stop division due to nutrients depletion. If cells continue to be incubated, they enter a death phase due to the accumulation of waste products. Cells that reached the stationary phase (A_{600} = 1.2) were stored at −70° C with 15% glycerol until needed.

In order to make the bacterial cells competent for DNA transformation, cells from the above preparation were inoculated in 10 mL LB broth and incubated at 37° C until they reach their midlog phase (A_{600} = 0.5). A negative control in which LB broth that has not been inoculated with bacteria was included during every incubation step. Subsequently, the cells were pelleted by centrifugation at 1,000 g at 4° C followed by resuspension with 5 mL of 100 mM CaCl₂ and incubated for 20 minutes on ice. The cells were pelleted again by centrifugation and resuspended in 2 mL of CaCl₂. The cells that were not used are treated with 14% DMSO for 20 min on ice, and stored at −70° C until needed.

DNA plasmid transformation was performed as follows. 10-50 ng of plasmid DNA was mixed with 0.2 mL of competent cells and incubated on ice for 30 min, during
which the DNA adhered to the cell wall assisted by the ionic interaction of the negatively charged phosphate groups of the DNA and the calcium cations. Subsequently, the mixture was incubated at 42° C for 1.5 minutes in order to drag the DNA plasmid inside the cell by an influx of water, followed by 1 min on ice to recover the transformed cells. Furthermore, the volume of transformed cells was increased to 1.5 mL with LB broth and incubated at 37° C for one hour to allow expression of the β-lactamase gene. After that, 200 μL of the transformed cells were plated on agar plates containing ampicillin and were incubated over night at 37° C. Ampicillin, a penicillin derivative (Figure 2.1), inhibits transpepсидation enzymes involved in the cross-linking of the polysaccharide chains of the bacterial cell wall peptidoglycan layer that lies between the inner and outer cell membrane of *E. coli*. Ampicillin does not affect existing cells with intact cell envelope, but it kills dividing cells. The β-lactamase cleaves a specific bond in the β-lactam ring (4 membered ring, Figure 2.1) in ampicillin, which is required for its activity. Thus, only cells with ampicillin resistance, and therefore with plasmid DNA, are replicated (Cohen SH et al., 1972). A single colony is inoculated into a 20 mL of LB broth containing ampicillin, and is allowed to grow at 37° C overnight. Subsequently, the transfected cells are transferred into 2 mL LB broth with ampicillin and allowed to grow at 37° C until they have passed their midlog phase.

![β-Lactamase attacks here on the β-lactam ring](image)

**Figure 2.1.** Structure of ampicillin and the action of β-lactamase.
In order to purify the plasmid DNA from the lysate, the transformed cells were collected by centrifugation at 6,000 g for 10 min at room temperature. After removal of the supernatant, the cells were resuspended in 30 mL of cell resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 100 μg/mL ribonuclease (RNase) A). Once the mixture becomes homogeneous or clear, 30 mL of cell lysis solution (0.2 N NaOH with 1 % sodium dodecyl sulfate (SDS)) is added. The hydrophobic tail of SDS interacts with the lipid components of the cell envelope, solubilizes them, and along with the strong alkaline solution, causes the cells to burst. The hydrophobic tail of SDS also associates with the non-polar side chains of proteins producing protein-detergent complexes with an anionic surface (Voet D et al., 1990). NaOH denatures the bacterial chromosomal and plasmid dsDNA. While chromosomal DNA is denatured and separated, the closed circular plasmid DNA remains close to each other due to its supercoiled structure (Birnboim HC, 1983). RNase A is an endoribonuclease that specifically attacks ssRNA, 3’ to pyrimidine residues, cleaving the phosphodiester bond between the adjacent nucleotide (Moussaoui M et al., 1996), leading to the killing of bacterial endogenous RNA. Next, the alkaline solution is neutralized by adding 30 mL of the neutralization solution (1.3 M potassium acetate, pH 4.8) and stirred until a coarse white precipitate forms. The rapid decrease in pH causes denatured chromosomal DNA to re-hybridize randomly at multiple sites resulting in formation of insoluble aggregates. In contrast, plasmid DNA is properly renatured and remains soluble. Furthermore, the high concentration of cations from K⁺ interacts with the SDS-protein complexes, causing them to precipitate. The precipitate was removed by centrifugation at 12,000 g for 10 minutes, and the supernatant was filtered, using sterilized cheesecloth, into another tube. The plasmid DNA was precipitated by the addition of isopropanol (final concentration of 50 % v/v) and centrifugation at 12,000 g for 20 min. After removal of the supernatant, the DNA was dissolved in 2 mL of 1 x TE buffer.

The DNA was further purified by adding equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) solution equilibrated to a pH of 7.8. At this pH, the DNA remains in the aqueous phase. The phenol contains 0.1% of the antioxidant hydroxyquinoline, a weak chelator of metal ions, prevents phenol oxidation, catalyzed by metals, which can damage the DNA. In addition, the yellow color of the oxidized form of
hydroxyquinoline provides a convenient way to identify the organic phase. Phenol
denatures the proteins and precipitates them, the chloroform facilitates the separation of
the aqueous and organic phase, and isoamyl alcohol reduces foaming that occurs after
mixing (Marmur J, 1961). After mixing, the solution was centrifuged at 12,000 g for 15
seconds in a microcentrifuge. The organic solvent forms the bottom phase, the proteins
precipitate forms the middle phase, and the aqueous solution forms the upper phase. The
aqueous phase was collected and the extraction was repeated until no precipitate is
observed. Subsequently, a single chloroform extraction is performed to remove traces of
phenol, which inhibits subsequent enzymatic reactions.

In order to remove salts and traces of phenol and chloroform, ethanol precipitation
of the plasmid DNA was performed. Sodium acetate, pH 5.2 was added to the DNA
solution to a final concentration of 0.3M, followed by the addition of double the volume
of ice-cold ethanol (98%). After mixing and incubating at −20° C for 30-60 min, the
DNA was pelleted by centrifugation at 12,000 g for 20 min. Subsequently, the
supernatant was removed and the DNA pellet was washed with 200 μL of 80% ethanol.
Centrifugation is repeated for 2 minutes and the supernatant was removed. After air-
drying of the pellet, the DNA was reconstituted in 0.5 mL of 1 x TE buffer.

The purified DNA was quantitated spectrophotometrically by using the molecular
absorptivity of purines and pyrimidines at 260 nm (Blout ER et al., 1954). An
absorbance of 1 is equivalent to 50 mg/L of dsDNA (40 mg/L of ssDNA and RNA and
20mg/L of single stranded oligonucleotides). Since proteins absorbs at 280 nm, the purity
of the DNA plasmid was detected by measuring the absorbance ratio at 260 to 280 nm. A
ratio >1.8 depicts a pure DNA plasmid.

The plasmid DNA was characterized by agarose (0.7 %) gel electrophoresis,
followed by ethidium bromide staining (refer to section 1.2.2.6), and visualizing under
UV-light. Since the DNA has a negative charge, it travels from the cathode towards the
anode, separating according to their size and shape. Two forms of the plasmid DNA were
observed, the supercoiled and the relaxed form with the supercoiled form moving faster.
For determining the DNA size, a control supercoiled DNA marker in the range from 2-16
Kbp was included during gel electrophoresis.
2.2. In vitro Transcription and Translation

*In vitro* transcription requires the following components (Gurevich V *et al.*, 1991):

a. DNA template (circular or linear) under the control of RNA polymerase promoter (such as bacteriophage T7).

b. High Mg$^{2+}$, low K$^+$, and ribonucleoside triphosphates (NTPs). The substrate for T7 RNA polymerase is Mg$^{2+}$-NTPs complex. Therefore, Mg$^{2+}$ is added at a concentration 10% greater than NTPs.

c. Polyamines, such as spermidine, have distribution of positive charges, which are used as counter ions for DNA. They play a major role in stabilizing the transcription initiation as well as the elongation complex. Furthermore, they act as cofactors for activation of T7 RNA polymerase (Frugier M *et al.*, 1994), modulation transcriptional efficiency.

d. RNase inhibitor such as protein inhibitors (50 kDa) from human placenta, which form strong equimolar noncovalent complexes with RNases ($K_d = 3 \times 10^{-10}$ M) that are enzymatically inactive (Blackburn P *et al.*, 1977).

e. Dithiothreitol, used as a reducing agent for the free sulphydryl group required for RNA polymerase activity as well as RNase inhibitors (Scheele G *et al.*, 1979).

f. Diethylpyrocarbonate (DEPC) treated water. DEPC carboxy-ethylates histidine residues of RNAse (which are required for their activity) and inactivates them (Jones GH, 1976).

g. RNA polymerase (600-5000 U/mL).

*In vitro* translation requires the following components besides mRNA (Pelham HRB *et al.*, 1976):

a. Translation system such as rabbit reticulocyte lysate or wheat-germ extract (usually 50 % of the reaction). The rabbit reticulocyte lysate contains all cellular components required for protein synthesis such as tRNAs, rRNAs, amino acids and initiation, elongation, and termination factors, as well as a variety of posttranslational processing activities including acetylation, proteolysis, and phosphorylation activity (Jackson RJ *et al.*, 1983). The lysate is prepared by injecting the rabbit with a drug (acetylphenylhydrazine, 1.2 % w/v), which increases the production of reticulocytes,
making the rabbit anemic (red blood cell destruction) and leading to an increase in erythrocytes (immature red blood cells), which are active in translation. After bleedin the rabbit, the blood is collected, and cells are lysed. Then, the lysate is treated with Hemin (0.02 mM), which inhibits a protein called heme control inhibitor in lysate, which phosphorylate a specific serine residue of the translation initiation factor (eIF2), inhibiting translational initiation. The lysate is further treated with micrococal nuclease (75 U/mL) to denature any endogenous mRNA. This enzyme, which does not damage tRNA or rRNA, has an absolute requirement for CaCl$_2$. After treatment, Ca$^{2+}$ is chelated with EGTA to inactivate the nuclease.

b. Low Mg$^{2+}$ and high K$^+$ concentrations.

c. tRNA from liver or yeast (70 µg/mL). Some species of tRNAs are present in low amounts in the rabbit reticulocyte lysate, therefore lysate is supplemented with tRNA from a less specialized cell to expand the range of mRNA which can be translated. For most mRNA, the tRNA source does not make a difference.

d. ATP-regenerating system such as creatine phosphate (10 mmol/L) derived from muscle contraction. This system, catalyzed by creatine kinase (50 µg/mL), produces ATP constantly as soon as the ATP concentration is decreased due to the amino acid activation by aminoacyl-tRNA synthetase.

\[
\text{Creatine phosphate} + \text{ADP} \overset{\text{Creatine kinase}}{\rightleftharpoons} \text{Creatine} + \text{ATP}
\]

e. Amino acids mix (20 µM of each). Lysate contains amino acids, however, excess is added in order to increase translational efficiency.

Initially, \textit{in vitro} transcription and translation were performed in two separate steps since, in contrast to translation (low Mg$^{2+}$, high K$^+$), transcription requires different conditions (high Mg$^{2+}$, low K$^+$). Applying the transcription conditions to translation could lead to very low or even no protein synthesis. Recently, these two processes were combined in a single step \textit{in vitro} (Craig D \textit{et al.}, 1992). The optimum conditions in this combined transcription-translation (TNT) system are much closer to those of translation than transcription. Therefore, the transcription is relatively inefficient. However, the stringency of promoter selection does not seem to be compromised. Using high enough concentration of DNA, sufficient RNA is transcribed to saturate the capacity of the
system and to translate the correct protein. Comparison of separate versus one-step transcription and translation conditions is presented in the table 2.1.

**Table 2.1.** Comparison of the reaction conditions for the two-step versus one-step (coupled) transcription and translation.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Transcription</th>
<th>Translation</th>
<th>Coupled TNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$ (mM)</td>
<td>15-20</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>K$^+$ (mM)</td>
<td>100-120</td>
<td>5-10</td>
<td>60-80</td>
</tr>
<tr>
<td>Spermidine (mM)</td>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>NTPs (mM)</td>
<td>3</td>
<td>-</td>
<td>0.4</td>
</tr>
<tr>
<td>DTT (mM)</td>
<td>10-40</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>Amino acids (μM)</td>
<td>-</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Temperature °C</td>
<td>37</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

Through the first and second project (chapter 3 and 4), one-step in vitro transcription-translation was performed. The conditions applied for coupled TNT were as indicated in table 2.1 with 25 μL reaction volume for the solid-phase detection, or 12.5 μL volume for liquid-phase detection. 50% of the rabbit reticulocyte lysate was used in each reaction.

2.3. Detection of Luciferase

After in vitro transcription-translation, Luciferase was detected as follows: 2 μL of the TNT was mixed with 50 μL of luciferin substrate solution containing 20 mM tricine, pH 7.8, 1.07 mM magnesium carbonate pentahydrate, 2.67 mM magnesium sulfate, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme-A, 530 μM ATP, and 470 μM luciferin (4,5 - dihydro - 2 - [6-hydroxy-2-benzothiazolyl] - 4 - thiazole - carboxylic acid). Luminescence was monitored using a liquid scintillation counter in the single-photon monitoring mode.
2.4. Instrumentation

Immunoassays were performed in microtiter wells using the Amerlite shaker/incubator (Amersham, Oakville, ON, Canada). The microtiter plate washer, Model EAW II, was from SLT-Lab Instruments, (Salzburg, Austria). Luciferase luminescence measurements were carried out using a liquid scintillation counter (Model LS-6500, Beckman Instruments Inc, Fullerton, CA) in the single-photon monitoring mode. Alkaline phosphatase detection with paranitrophenyl phosphate was carried out using a microplate reader (Model EL-307C, Bio-Tek instruments, Winooski, VT). Hybridization assays were performed in microlite 2 polystyrene microtiter wells (Dynatech, Chantilly, VA) using a shaker/incubator available from Heidolph-instruments GmbH & Co. KG (Schwabach, Germany). Luminescence measurements for aequorin and alkaline phosphatase were carried out using a luminometer/photometer available from Mediators diagnostics systems (Vienna, Austria). Polymerase chain reactions and oligonucleotide ligation assays were performed in the PTC-0150 Minicycler available from MJ Research, Inc. (Watertown, MA, USA).

2.5. Materials

Bgl I, streptavidin-alkaline phosphatase conjugate (SA-ALP), Anti-digoxigenin alkaline phosphatase conjugate (antidig-ALP), and anti-digoxigenin polyclonal antibody from sheep (antidig antibody) were obtained from New England Biolabs (Beverly, MA). Terminal deoxynucleotidyl transferase, streptavidin (SA), bovine serum albumin (BSA), Digoxigenin-11-dUTP (Dig-dUTP), deoxyribonucleotides (dNTPs, 100 mmol/L solutions), and blocking reagent (cat.# 1096176) were purchased from Boehringer Mannheim Corporation (Indianapolis, IN). N-succinimidyl S-acetylthioacetate (SATA), sulfo-N-hydroxysuccinimide ester of biotin (sulfo-NHS-LC-biotin), sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), Bis (sulfosuccinimidyl) suberate (BS³), and maleimide-activated neutravidin were from Pierce (Rockford, IL). Spin-Pure sephadex G-25 columns were obtained from CPG (New Jersey, USA). ethyleneglycol bis (2-aminoethyl ether) N,N,N',N tetraacetic acid (EGTA) was obtained
from Serva (Heidelberg, Germany). T7 RNA polymerase (used in the heterobifunctional linker project), deoxyribonucleotides (dNTPs, 100 mmol/L solutions), NAP columns were from Amersham Pharmacia Biotech (Piscataway, NJ). Centricon-30, and microcon-30 concentrators were from Amicon Inc. (Beverly, MA). Monoclonal capture (cat. #8301) and detection (cat #8311) anti-PSA antibodies were obtained from Diagnostic Systems Laboratories (Webster Tx). Prostate specific antigen (PSA) was obtained from Scripps Laboratories (San Diego, CA). Linear DNA markers (lambda-DNA digested with Eco R I and Hind III and containing fragments from 1.2 to 21.2 Kbp), ethylene diamine tetraacetic acid (EDTA), ethidium bromide, coenzyme A, dithiothreitol, tricine, salmon testes DNA, streptavidin (used in self-replication project), Poly (Lys, Phe) 1:1 hydrobromide (MW 20000-50000), and hydroxylamine hydrochloride were from Sigma (St. Louis, MO). U-bottom, transparent (Nunc, Maxisorp) microwell wells were obtained from Life Technologies (Burlington, ON, Canada). White flat-bottom microlite 2 polystyrene microwell wells were obtained from Dynatech (Chantilly, VA). The T7 RNA polymerase (used in the self-replication project), Taq DNA polymerase, E. coli XL1-blue and JM109 strains were obtained from Stratagene (La Jolla, CA). The TNT T7 rabbit reticulocyte lysate, used for the in vitro transcription-translation reactions, and beetle luciferin are available from Promega Corp. (Madison, WI). Alkaline phosphatase chemiluminescent substrate Lumigen PPD (4-methoxy-4-(3-phosphatephenyl)spiro [1,2-dioxetane-3,2'-adamantane], disodium salt) is available from Aureon Biosystems Gmbh (Vienna, Austria). Ampligase thermostable DNA ligase was purchased from Epicentre (Madison, WI). Biotin-14-dATP (B-dATP) was obtained from GibcoBRL Life technologies (Vienna, Austria). QIAmp DNA blood mini kit available from Qiagen (Valencia, CA).

2.6. Buffers and Solutions

Luria-Bertani (LB) broth: 10g/L tryptone, 5 g/L yeast, 10 g/L NaCl, 2 mmol/L NaOH.
LB agar plates: LB broth, 15 g/L agar.
LB broth/agar with ampicillin: LB broth/agar, 0.1 g/L ampicillin (added after solution temperature reached 60° C. Higher temperature inactivates ampicillin).
Wash solution: 50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, and 10 mL/L Tween-20.
Conjugation buffer: 0.1 mol/L sodium phosphate, pH 7.0, and 5 mmol/L EDTA.
Maleic acid buffer: 0.1 mol/L maleic acid, 0.15 mol/L NaCl, pH 7.5.
Blocking solution: 10 g/L blocking reagent, 100 mmol/L maleic acid, and 150 mmol/L NaCl, pH 7.5.
1x TE: 10 mmol/L Tris, 1 mmol/L EDTA, pH 7.5.
T7 RNA polymerase buffer: 20 mmol/L magnesium phosphate, pH 7.7, 0.1 mol/L NaCl, 1 mmol/L dithiothreitol, and 1 mmol/L EDTA.
Coating buffer: 50 mmol/L Tris, pH 7.8, 0.5 g/L Na$_3$N$_3$
PSA diluent: 50 mmol/L Tris, pH 7.8, 60 g/L BSA.
Assay buffer: 50 mmol/L Tris, pH 7.8, 60g/L BSA, 0.5 mmol/L KCl, 0.5 g/L Na$_3$N$_3$, and 0.5 g/L Triton X-100.
Hybridization buffer: 60 mmol/L sodium citrate pH 7.5, 0.6 mol/L NaCl and 10 g/L blocking reagent.
PBS: 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L sodium phosphate, and 1.76 mmol/L potassium phosphate, pH 7.4.

Note: When Aequorin is used as the label, all solutions contained 2 mmol/L EGTA.

When performing cell culturing, all solutions were autoclaved prior to use.
Chapter 3

T7 RNA Polymerase as a Self-Replicating Label for Antigen Quantification
3.1. Introduction

Whole-genome sequencing projects have led to the identification of thousands of new genes. The challenge ahead is to unravel gene function and regulation on a genome-wide scale. Most studies of gene function are based on the comparison of expression profiles between control and perturbed states, which allows for the identification of genes whose expression is induced or suppressed. DNA microarrays provide valuable information on gene expression at the mRNA level (Wodicka L et al., 1997; Schena M et al., 1996). Gene function, however, is manifested through the activity of the encoded protein. mRNA abundances do not always correlate with protein concentrations due to significant post-translational regulation (Gygi SP et al., 1999). Consequently, the direct quantitative analysis of proteins provides more accurate information about biological systems. Moreover, the comparison of protein expression profiles in patients and normal samples (differential profiling) reveals potential biomarkers for diagnosis, prognosis and monitoring of disease progression, as well as, new therapeutic targets. The challenge, however, lies in the fact that proteins present at low concentrations are usually the ones that mediate the cellular response to various stimuli and are involved in the early stages of pathological processes. A recent study has showed that half of the yeast proteome was undetectable using two-dimensional electrophoresis followed by mass spectrometry (Gygi SP et al., 2000). Thus, high sensitivity, along with specificity, are essential requirements for any new technique in the field of proteomics, because they permit quantification of minute amounts of antigen and/or the use of smaller number of cells. Furthermore these qualities must be combined with the ability for automation and high-throughput protein analysis, in order to exploit the information provided by large-scale sequencing projects.

Target amplification techniques analogous to polymerase chain reaction (PCR) that offer exquisite sensitivity to nucleic acid analysis, are not available for protein analytes. The most sensitive protein assays are based on the interaction of the analyte with a specific binder (antibody, receptor or peptide) that is linked to a signal-generating molecule (label). The assay sensitivity is determined, mainly, by the detectability of the label and the affinity of the binder. DNA fragments have been used as labels that provide signal amplification through replication by PCR (Sano T et al., 1992) or rolling circle DNA replication (Schweitzer B et al., 2000) or by expression (Chiu NHL et al., 1999).
However, the most widely used labels are enzymes (alkaline phosphatase, horseradish peroxidase etc.) because they provide signal amplification through the turnover of many substrate molecules to detectable product. For almost thirty years, research efforts have been focused on the synthesis of novel substrates to allow more sensitive detection of enzyme labels. Thus, chromogenic substrates were gradually replaced by fluorogenic (Christopoulos TK et al., 1996) and, more recently, chemiluminogenic ones (Kricka LJ, 1996). On the contrary, this work introduces an enzyme label, T7 RNA polymerase (T7RP), which (i) has the unique ability for self-replication (or self-amplification) in vitro and (ii) catalyzes the in vitro synthesis of the mRNA encoding a second enzyme (firefly luciferase). The resulting signal amplification is due to the generation of higher mRNA template for higher number of enzyme molecules in solution. The assay allows for antigen quantification with high sensitivity, wide dynamic range and very good reproducibility.

3.1.1. T7 RNA polymerase

The RNA polymerase from bacteriophage T7 is a single polypeptide chain of 883 amino acids with a molecular weight of 99 kDa (Moffatt BA et al., 1984). The crystal structure of T7 RNA polymerase resembles a cupped right hand, with fingers, palm, and thumb subdomains that form a cleft that can accommodate the double-stranded DNA template, features that are common to all DNA polymerases described up to now (Sousa R et al., 1993). T7 RNA polymerase recognizes a highly conserved promoter sequence of composed of the following sequence: TAATACGACTCACTATAG (Chen Y-C et al., 2000). The initiation kinetic of T7 RNA polymerase is consistent with the two-step DNA binding mechanism:

\[
R + P \overset{K_1}{\rightleftharpoons} K_{-1} \rightarrow RPC \overset{K_2}{\rightarrow} K_{-2} \rightarrow RPo \overset{NTPs}{\rightarrow} \overset{Mg^2+}{\rightarrow} RNA
\]

T7 RNA polymerase binds to the dsDNA promoter (P) to form a closed complex (RPC), which rapidly triggers the formation of an open complex (RPo) in which the promoter is partially melted. The open complex formation is thermodynamically unfavorable, and the initiation of transcription to form RNA competes with RPo renaturation to RPC. The
addition of GTP (the initiating NTP for all T7 RNA polymerase promoter) followed by other rNTPs enlarges the T7 RNA polymerase protected area, stabilizes the complex, and facilitates RNA synthesis.

RNA polymerase consists of two distinct domains: A C-terminal domain from residues 180-883 (80 kDa) which contains the active site and the amino acids involved in the promoter binding (palm domain), and N-terminal domain (20 kDa) which binds the single-stranded RNA (Gardner LP et al., 1997). The promoter of T7 RNA polymerase in turn consists of a binding region that extends from -17 to -6 and an initiation region that extends from -5 to +6, with +1 being the initiation site (Li T et al., 1996). It was shown that the binding region is recognized as a double-stranded duplex and that the initiation region melts very rapidly upon polymerase binding (Maslak M et al., 1993). T7 RNA polymerase interacts with promoter asymmetrically, contacting the sequences -17 to -13, -7 to -1, and -14 to -9 of the coding strand, and -3 to +2 of the non-coding strand. These contacts seem to be located mainly in the DNA major groove (Kochetkov SN et al., 1998). The interaction between the active domain of T7 RNA polymerase and the promoter is made at position -11 and -10 with Asn748, and at position -8 with Gln758 (Rong M et al., 1998). Moreover, Asp537 and Asp812 were observed to be involved with the binding of metal ions, such as magnesium, required for activity (Woody A et al., 1996), as well as Lys631 which interacts with the triphosphate moiety of the incoming ribonucleotide substrate essential for the formation of phosphodiester bond (Osumi-Davis PA et al., 1992). His811 and His784 are also involved in phosphodiester bond formation as well as recognition of the ribonucleotide to be added. These amino acid residues are positioned in a way that enables hydrogen bonding with the 2'-OH group of the priming nucleotide (GTP is the initiating NTP for all T7 RNA polymerase promoters), assuring initiation of ribonucleotide and not deoxyribonucleotide (Cheetham GMT et al., 1999a). Moreover, the presence of an amino acid with no side chain (Gly542) rather than a bulky one (Glu480 in the case of DNA polymerase I), allows the accommodation of the C3'-endo ribose conformation of the incoming ribonucleotide (Cheetham GMT et al., 1999b).

Recent crystallographic studies indicated that amino acids 230-245 form a β-hairpin structure containing Val237 that intercalates in place of base-pair at position -4 to stack on base-pair -5, distorting the DNA during the open complex formation, by
providing a binding site for the melted template strand (Stano NM et al., 2002). The packing of this β-hairpin motif is essential for the maintenace of the upstream part of the transcriptional bubble during elongation. It was observed that Asp240 and Glu242 anchor the hairpin to the DNA in a way that the intercalation process occurs. Furthermore, four side-chains of residues 739-770 lying on one side of an extended antiparallel β-hairpin motif interacts with specific nucleotides of the promoter which form the basis for discrimination between T7 promoter and other DNA sequence (Cheetham GMT et al., 1999). It was shown that Arg756, Gln758, Arg746, and Asn748 interact with G-9 (guanine at position -9), A-8, G-7, and G -11 respectively.

Initially, before the formation of the first phosphodiester bond, the template strand at position −1 binds to a hydrophobic pocket (Trp422, His300, and Tyr73) adjacent to the catalytic active site. The aromatic side chain of Trp422 stacks the −1 base, inducing a sharp bend in the template strand, which puts the base at position +1 for initiating ribonucleotide. After the synthesis of a trinucleotide, the base at −1 flips out of this pocket and assumes a new position without changing the structure of the enzyme.

Similar to eukaryotic RNA polymerases, the initiation phase of transcription with T7 RNA polymerase is characterized by abortive cycles with the synthesis of short RNA products before the polymerase moves from the promoter to form a stable elongation complex (Diaz GA et al., 1996). During abortive cycling, the downstream contacts between the polymerase and the template expand and contract while the upstream contacts are constant (Cheetham GMT et al., 1999b). Synthesis of long enough RNA (8 to 12 nucleotides) that allows strong binding to the 144-168 amino acid residues of the NH2-terminal domain (Sastry S et al., 1999), causes a conformational change in the enzyme, releasing the upstream promoter contacts, and leading in transition of the reaction from abortive to processive mode (Kocketkov SN et al., 1998).

During the elongation phase of transcription, T7 RNA polymerase rarely releases the nascent RNA and readily makes chains of 15,000 bases long at an elongation rate of 200 bases per second (Lyakhov DL et al., 1998). The elongation bubble of T7 RNA polymerase is approximately 9 bases long with RNA:DNA hybrid of 7-8 bp in size (Huang J et al., 2000). Furthermore, 4-6 bases immediately upstream of the hybrid interact with T7 RNA polymerase. It was shown that Thr636-Met666, and Ala724-
Met750 regions of T7 RNA polymerase C-terminal are involved in the binding of the ssRNA (Shen H et al., 2001). Upon binding of NTPs, the polymerase is translocated downstream on the template either by a series of conformational changes (Chamberlin MJ, 1995) or, alternatively by passive sliding along the DNA template (Nudler E, 1999).

Transcription termination by T7 RNA polymerase is achieved by two mechanisms (Lyakhov DL et al., 1998; ). Either by recognizing a stable stem-loop structure in the nascent RNA followed by a run of U residues (class I site), homologous to eukaryotic RNA polymerase transcription termination refer to section 6.1.1), or by recognizing a specific 7 bp sequence (5'-ATCTGTT-3') at the 3' end of the non-template strand which causes T7 RNA polymerase to pause (class II site). In the latter case, termination 6 to 8 bp past this sequence is favored by the presence of runs of U, perhaps to destabilize the RNA:DNA hybrid. In both mechanisms, the less progressive conformation seems to be similar to the initiation conformation of the enzyme.

Since T7 RNA polymerase is one of the simplest DNA-dependent enzymes, capable of transcribing a complete gene without the need of additional proteins, and is cloned and overexpressed in E. coli (Davanloo P et al., 1984), it is widely used as a tool for synthesis of specific transcripts, as well as transcription mechanism studies.

3.2. Experimental Protocols

3.2.1. Biotinylation of T7 RNA polymerase

1 mg (1.8 μmol) of sulfo-N-hydroxysuccinimide ester of biotin (NHS-LC-biotin, Pierce, Rockford, IL) was dissolved in 3 mL of dimethyl sulfoxide and then diluted to 15 μmol/L in T7RP buffer. 2 μL of the NHS-LC-biotin solution was mixed with 1 μL (6 pmol) of T7RP and incubated for 1 hour at 4 °C. The volume was increased to 50 μL with T7RP buffer containing 0.2 g/L BSA. The biotinylated T7RP was purified from free biotin with size exclusion chromatography using NAP columns. The enzyme was eluted with 1 mL sodium phosphate buffer pH 6.8. 100 μL of 10x concentrated T7RP buffer containing 1.4 g/L BSA was added to the purified biotinylated T7RP solution and the mixture was concentrated by ultrafiltration using microcon-30 filters (MW cutoff = 30000).
3.2.2. Streptavidin-biotinylated T7 RNA polymerase complexation (SA-BT7RP)

Purified biotinylated T7RP (3 pmol) was mixed with 4.8 pmol of streptavidin, diluted in T7RP buffer (final volume of 150 μL). The complexation reaction was allowed to proceed for 10 min at room temperature and the SA-BT7RP complex was used without purification.

3.2.3. Biotinylation of monoclonal anti-PSA antibody

The detection monoclonal anti-PSA antibody solution was dialyzed overnight against 3.5 L of 0.1 mol/L sodium bicarbonate at 4 °C. 0.2 mg of the antibody was diluted with 0.5 mol/L carbonate buffer, pH 9.1, to a final concentration of 0.5 g/L. For biotinylation, 1 mg of NHS-LC-biotin was dissolved in 50 μL dimethyl sulfoxide and a 12.5 μL (0.25 mg) aliquot was added to the antibody solution. The mixture was incubated for 2 hours at room temperature. The biotinylated antibody was stored at 4 °C and used without purification.

3.2.4. T7 RNA Polymerase as a label for antigen quantification

U-bottom, polystyrene microtiter wells were coated overnight at room temperature with 25 μL of 5 mg/L capture anti-PSA antibody, diluted in coating buffer. Before use, the wells were washed six times with wash. A 10 μL aliquot of prostate specific antigen standard diluted in PSA buffer along with 15 μL of 0.5 mg/L biotinylated anti-PSA antibody, diluted in assay, were added into each well. The immunoreaction was allowed to proceed for 1 hour with continuous shaking. At the end of the incubation, any unbound biotinylated anti-PSA antibody was removed by washing the wells six times as above. Afterwards, 25 μL of 2.4 nmol/L SA-BT7RP complex (diluted in T7RP buffer containing 1% fat free dry skim milk) was added into each well and incubated for 10 min. The wells were then washed six times followed by two times with 50 mmol/L potassium acetate. Subsequently, 25 μL of transcription/translation mixture containing 52.5 fmol of Luc-DNA was added into each well. The coupled in vitro transcription/translation reaction was allowed to proceed for 90 min at 30 °C and the activity of synthesized firefly luciferase was measured as described in section 2.3.
3.2.5. Antigen quantification using a self-replicating T7 RNA polymerase label

The formation of the immunocomplex on microtiter wells and the binding of the SA-T7RP complex were carried out as described above. Subsequently, 23.5 μL of transcription/translation mixture containing 37.5 fmol of T7RP-DNA and 150 ng of salmon testes DNA was added into each well and incubated for 60 min (self-replication phase). Afterwards, 1.5 μL of Luc-DNA (26 fmol) was added into the wells and incubated for another 60 min (detection phase). The activity of synthesized firefly luciferase was measured.

3.3. Results and Discussion

The principle of antigen quantification using T7RP as a label is illustrated in Figure 3.1. Two approaches for measuring T7RP, based on in vitro transcription/translation (with and without self-replication), are also shown diagrammatically in Figure 3.1. Each of these mechanisms will be discussed separately.

The first attempt in this study was to verify the ability to quantify T7 RNA polymerase by coupled in vitro transcription and translation. The goal of these experiments was to establish a relationship between the input T7 RNA polymerase (T7RP) and the synthesized protein in an in vitro transcription/translation system. The expression of firefly luciferase was chosen because this enzyme can be detected with high sensitivity by using its characteristic bioluminogenic reaction (Wilson T et al., 1998). Various amounts of T7RP were added to a coupled transcription/translation reaction (final volume 12.5 μL) that contained the firefly luciferase cDNA (Luc-DNA) under the control of the T7 promoter. The reaction was allowed to proceed for 90 min at 30° C and then the activity of synthesized luciferase was measured by adding 2 μL of the expression mixture to 50 μL of luciferin substrate solution. It was observed (Figure 3.2) that the luminescence was linearly related to the number of T7RP molecules in a range extending over four orders of magnitude (5.2 x 10⁴ - 8 x 10⁸ molecules of T7RP). The signal-to-background (S/B) ratio for the 5.2 x 10⁴ molecules was 2.8.
Figure 3.1. Assay configuration for quantification of antigens by using T7 RNA polymerase (T7RP) as a label. The antigen (Ag) is bound simultaneously to an immobilized capture antibody and a biotinylated detection antibody. Biotinylated T7RP complexed with streptavidin (SA) is then added to the immunocomplex. The bound T7RP is determined by in vitro coupled transcription/translation. Two approaches were explored: (a) T7RP acts on firefly luciferase cDNA (Luc-DNA), located downstream of the T7 promoter, to produce several molecules of active luciferase which is measured by its characteristic bioluminogenic reaction. (b) T7RP acts on T7RP cDNA (T7RP-DNA), positioned downstream of the T7 promoter, to generate several T7RP molecules (Self-replication phase) which, in turn, act on Luc-DNA to produce luciferase (Detection phase). B = Biotin; T7 promoter is represented by a hatched square.
Figure 3.2. Establishing a quantitative relationship between the input T7 RNA polymerase (T7RP) and the synthesized firefly luciferase in a coupled, *in vitro* transcription/translation system with and without self-replication of T7RP. (■) The transcription/translation mixture contains only Luc-DNA as a template (no self-replication). (▲) Two expression reactions (12.5 µL each) are carried out. T7RP–DNA, placed downstream of the T7 promoter, serves as the template for the first reaction (self-replication). Then, 2 µL are transferred to the second expression reaction in which the Luc-DNA serves as a template (detection). (●) A single expression reaction is carried out with a delayed addition of Luc-DNA. Transcription/translation is allowed to proceed for 60 min with T7RP-DNA as a template (self-replication) and then, Luc-DNA is added and the reaction proceeds for another 60 min prior to luciferase measurement. cpm represents counts per minute.
The coupled transcription/translation process consists of a series of complex reactions that require the concerted action of numerous factors, such as RNA polymerase, ribosomal subunits, translation initiation, elongation and termination factors, aminoacyl-tRNA synthetases etc. Nevertheless, our data demonstrate that the final outcome is a simple linear relationship between input T7RP and the in vitro synthesized protein in a wide range of T7RP concentrations. This forms the basis for the development of a T7RP-based signal amplification system exploiting T7RP as a label.

The second step was to design a system that confirms the detectability of T7 RNA polymerase through self-replication. Two consecutive, 90-min, in vitro expression reactions were carried out (12.5 µL each); in the first reaction the T7RP catalyzed the transcription of its cognate gene positioned downstream of the T7 promoter (T7RP-DNA, Dubendorff JW et al., 1991; Deng H et al., 1994) and the generated RNA was translated simultaneously into active T7RP molecules. The newly synthesized T7RP also acted on the T7RP-DNA template to produce more of the enzyme (self-replication). The T7RP was then measured by transferring 2 µL into another expression reaction, containing 35 fmole of Luc-DNA, and monitoring the synthesized luciferase. The extent of self-replication is a function of the T7RP-DNA level, as indicated by the increase of the luminescence as the T7RP-DNA concentration becomes higher (Figure 3.3). At the optimum level of T7RP-DNA, the self-replication process caused a 110-fold increase of the signal compared to a reaction that contained no T7RP-DNA.

Similar experiments with decreasing amounts of T7RP (aimed at estimating the detectability of the polymerase) revealed a low level of "illegitimate" transcription of T7RP-DNA in the absence of T7RP. This was attributed to a eukaryotic RNA polymerase activity that is present in the rabbit reticulocyte extract and initiates a low level of transcription of T7RP-DNA, generating a few T7RP molecules which, in turn, are amplified by entering the self-replication cycle. This activity was not detectable in the absence of T7RP-DNA. Because illegitimate transcription compromises the detectability of T7RP, we carried out experiments to minimize it by adding various amounts of salmon DNA in the rabbit reticulocyte extract. Addition of 100 ng salmon DNA suppressed illegitimate transcription by 98%, whereas it caused only a 15% decrease in the T7RP-catalyzed transcription of T7RP-DNA (Figure 3.4).
Figure 3.3. Effect of the amount of T7RP-DNA on the extent of self-replication of T7RP. The luminescence is plotted against the amount of the T7RP-DNA in the transcription/translation reaction. 35 fmol of Luc-DNA was used. The arrow indicates the signal obtained without self-replication (absence of T7RP-DNA).
Figure 3.4. Effect of the concentration of salmon DNA on the extent of “illegitimate” expression of T7RP-DNA in the absence of T7RP (solid line) and on the extent of T7RP-catalyzed expression (dashed line). 25 fmol of T7RP-DNA and 35 fmol of Luc-DNA were used in the self-replication protocol. The percent (%) luminescence is plotted as a function of the amount of salmon DNA in the transcription/translation reaction mixture. The value of 100% is defined as the luminescence obtained with no salmon DNA present.
In order to estimate the detectability of T7RP in a self-replication system, various amounts of the enzyme were added into the first expression reaction containing 25 fmoles of T7RP-DNA followed by a separate expression reaction containing 35 fmoles of Luc-DNA. The linearity extends from $1.4 \times 10^3$ - $10^7$ molecules (Figure 3.2). The S/B ratio at 1400 T7RP molecules, was 2.5.

We investigated the possibility of combining the self-replication with the detection of T7RP in a single reaction mixture containing both T7RP-DNA and Luc-DNA templates. However, it was observed that self-replication was suppressed dramatically due to competition between the two templates for binding to a limited number of T7RP molecules. Therefore, a delayed addition protocol was designed in which the T7RP was first allowed to act on its cognate gene (self-replication) followed by the addition of Luc-DNA, in the same reaction mixture. The T7RP-DNA/Luc-DNA ratio, as well as the incubation times required before and after the addition of Luc-DNA were optimized to ensure efficient self-replication and detection with the delayed addition protocol. The T7RP-DNA/Luc-DNA molar ratio was studied in the range of 0.05 - 3 at three levels of Luc-DNA (Figure 3.5). The luminescence increases as the ratio becomes higher due to increased self-replication, and reaches a plateau when the molar ratio of the two templates becomes 1.1.5. For the same molar ratio, the signal increases by increasing the concentration of Luc-DNA.

Various combinations of reaction times before and after Luc-DNA addition were studied (Figure 3.6). The maximum signal was achieved with 60 min - 60 min, respectively, giving a 50-fold enhancement over the assay that contains no T7RP-DNA. Other combinations, such as 30 - 60, 30 - 90 and 60 - 30 compromised either the yield of the self-replication reaction or the detection reaction, thus giving a lower signal. In particular, the 0-90 combination (both templates added simultaneously at the beginning of the expression) gave the lowest yield of self-replication (Figure 3.6).

The detectability of T7RP using the optimized single expression reaction protocol (delayed addition protocol) was $8.5 \times 10^3$ molecules, with a S/B ratio of 4.3. The luminescence was a linear function of the amount of T7RP, up to $10^7$ molecules (Figure 3.2).
Figure 3.5. Study of the effect of T7RP-DNA to Luc-DNA molar ratio on the yield of an expression reaction that combines self-replication of T7RP and luciferase synthesis. A delayed addition protocol was performed (60 min-60 min). Increasing amounts of T7RP-DNA were used with 8.75 fmol (■), 17.5 fmol (○), and 35 fmol (▲) of Luc-DNA.
Figure 3.6. Effect of the transcription/translation incubation time on the yield of luciferase expression (delayed addition protocol). The reaction starts with the addition of T7RP and T7RP-DNA. The first and second numbers of each pair on the x-axis correspond to the incubation time before and after the addition of Luc-DNA, respectively. The first column (90 min) represents the signal obtained in the absence of self-replication (no T7RP-DNA).
After optimization of T7RP detection conditions in the liquid-phase, the subsequent step was to establish optimum conditions for its detection when bound to the solid-phase. The first experiment was to construct a mediator system that allows the binding of T7RP to the immunocomplex on the solid phase. T7RP was first biotinylated followed by complexation to streptavidin and then applied to the antigen quantification.

The effect of biotinylation on the activity of T7RP was studied by reacting with increasing concentrations of the sulfo-N-hydroxysuccinimide ester of biotin (NHS-LC-biotin) at pH 7.7 and 9.0. All reactions were incubated for one hour at 4°C and the activity of T7RP was measured by *in vitro* coupled transcription/translation. Inactivation of T7RP becomes significant at biotin:T7RP molar ratios greater than 5 (Figure 3.7), due to modification of free amino groups that are necessary for full activity. The inactivation was more extensive at pH 9.0 than pH 7.7 because the biotinylation reaction is more efficient when the -NH₂ groups are deprotonated.

A microtiter well-based 'two-site' immunoassay was developed for prostate-specific antigen (PSA), as a model (Figure 3.1). The antigen was bound both by an immobilized capture antibody and a biotinylated detection antibody. Biotinylated T7RP was complexed to streptavidin and added to the immunocomplex. The solid phase-bound T7RP was measured by *in vitro* expression.

The complexation of streptavidin (SA) with biotinylated T7RP (BT7RP) was studied at SA:BT7RP molar ratios ranging from 0.5 to 12 (Figure 3.8) and the complexes were applied directly to the assay of 20 fmol of antigen. The luminescence reached a maximum at a SA:BT7RP molar ratio of 1.5. The signal dropped sharply at lower or higher ratios. When biotinylated T7RP is in excess, all four biotin-binding sites of streptavidin are occupied and the complexes cannot bind to the biotinylated antibody on the solid phase. On the other hand, when streptavidin is in excess, free streptavidin competes with SA-BT7RP complex for binding to the well (Diamandis EP *et al*., 1991).

Furthermore, the time-course of the *in vitro* transcription/translation process with immobilized T7RP was studied up to 180 min (Figure 3.9). The luminescence increased with time but the signal-to-background ratio (S/B) reached a plateau at 120 min. The background was defined as the luminescence obtained when no antigen was present in the well.
Figure 3.7. Study of the effect of biotinylation on the activity of T7 RNA polymerase. T7RP (220 fmol) was determined by in vitro expression using Luc-DNA (35 fmol) as a template. The percent (%) of luminescence is plotted versus the molar ratio NHS-LC-Biotin:T7RP at pH 7.7 (solid line) and pH 9.0 (dashed line). The value 100% is defined as the signal obtained from non-biotinylated T7RP.
Figure 3.8. Optimization of streptavidin (SA):biotinylated T7RP (BT7RP) molar ratio for the preparation of the SA-BT7RP complex. The complexes were used for antigen quantification without prior purification as described under Experimental Protocols with 20 fmol of PSA, 60 fmol of complex and 47.5 fmol of Luc-DNA without self-replication. The solid and dashed lines correspond to the signal and signal-to-background ratio (S/B), respectively.
Figure 3.9. Time dependence of the transcription/translation reaction with T7 RNA polymerase immobilized on the solid phase. Luc-DNA was used as a template. The solid and dashed lines correspond to the signal and signal-to-background ratio (S/B), respectively.
The concentration of SA-BT7RP added to the well also affected both the signal and the background of the assay and therefore its detectability (Figure 3.10). The signal increases with the SA-BT7RP concentration and a plateau is reached at 7 nmol/L. The S/B ratio, however, is highest at 3 nmol/L and then drops because of the increasing nonspecific binding of the complex to the solid phase.

The sensitivity and dynamic range of the optimized immunoassay were assessed by analyzing serial dilutions of the antigen, in the appropriate buffer. The signal to background ratio was plotted as a function of the amount of prostate-specific antigen in the assay mixture. In the absence of T7RP-DNA (no self-replication), 195 attomoles of antigen were detected with a S/B ratio of 2.3. When the self-replication system was used (single expression reaction with a delayed addition of Luc-DNA), as low as 12 amoles of PSA could be detected with S/B ratio of 1.9. The dynamic range of the assay extended up to 50000 amoles (Figure 3.11).

Direct comparison of the proposed immunoassay using T7RP as a label and applying the self-replication protocol showed a 600-fold improvement over a traditional ELISA technique (S/B of 2.1 for 7.4 fmoles), using streptavidin-alkaline phosphatase as a label and paranitrophenyl phosphate colorimetric substrate (figure 3.11).

To assess the reproducibility of the immunoassay (including all the steps, i.e., coating of the wells, immunocomplex formation, binding of SA-BT7RP, in vitro coupled transcription/translation and luciferase measurement), we analyzed samples containing 0.1, 1 and 10 fmoles of PSA. The %CVs were 7.8, 7.5 and 8.1, respectively (n=7).

Optimizing the structure of T7RP-DNA and Luc-DNA templates may further enhance the sensitivity of the system. For instance, suitable enhancer and transcription termination sequences may be incorporated to increase the yield of both self-replication of T7RP and luciferase synthesis. Insertion of both T7RP-DNA and Luc-DNA templates, under the control of T7 promoter, in a single vector may also be investigated for higher yields. Besides firefly luciferase DNA, cDNAs for other highly detectable proteins may be employed, e.g., green fluorescent protein (Tsien Y, 1998), and alkaline phosphatase, aequorin (White SR et al., 1999).
Figure 3.10. Effect of the concentration of streptavidin-biotinylated T7 RNA polymerase complex (SA-BT7RP) on the luminescence (solid line) and the signal-to-background ratio (dashed line) obtained from the assay. The sandwich immunoassay was performed as described under Experimental Protocols with 10 fmol of antigen and the Luc-DNA (47.5 fmol) as the template.
Figure 3.11. Assessing the sensitivity and dynamic range for antigen quantification using T7 RNA polymerase as a label, (●) without self-replication (absence of T7RP-DNA) and (■) with self-replication of T7RP. The signal over background ratio was plotted against the amount of PSA present in the well. For self-replication, T7RP-DNA is included in the expression reaction mixture and the delayed addition protocol is employed. (▲) Traditional ELISA with streptavidin-alkaline phosphatase (SA-AP) conjugate as a label and paranitrophenyl phosphate substrate. After formation of the immunocomplex (as described under experimental section for T7RP as a label), SA-AP is added (1000 U) and incubated for 15 minutes. After washing excess unbound conjugate, the substrate was added and incubated for 30 min in dark, followed by absorbance measurement at 405 nm.
In recent years, research efforts have focused increasingly on the identification of binders, with the requisite affinity and specificity, for a large number of protein analytes. Various approaches include recombinant antibodies selected by phage- (deWildt RMT et al., 2000) or ribosome-display (Hanes J et al., 2000), RNA or DNA aptamers (Hesselberth J et al., 2000) and small organic compounds selected through combinatorial library methods (Han Y et al., 2000; Shuker SB et al., 1996). The extent and/or the position of biotinylation of proteins, nucleic acids and small molecules, can be controlled to achieve minimal interference with the interaction between the binder and the protein analyte. Consequently, SA-BT7RP is a universal detection reagent that can bind with high affinity ($K_D = 10^{14}$ M) to any biotinylated binder-analyte complex.
Chapter 4

Heterobifunctional Linker between Antibodies and Reporter Genes for Immunoassay Development
4.1. Introduction

A reporter gene is a DNA fragment coding for a protein that can be readily detected at low concentrations. Most widely used reporter genes encode enzymes (e.g., chloramphenicol acetyl transferase, alkaline phosphatase, luciferase etc), fluorescent proteins (GFP), and photoproteins (aequorin). Analytical applications of reporter genes include (Lewis JC et al., 1998): (i) the spatial and temporal monitoring of gene expression \textit{in vivo} (Bronstein I et al., 1994), (ii) the investigation of the strength and developmental regulation of promoters/enhancers fused to the reporter gene (Srikantha, T et al., 1996), (iii) the construction of light-emitting biosensors based on whole cells transfected with a reporter gene that is fused to an inducible promoter (Lewis JC et al., 1998; Ramanathan S et al., 1997), and (iv) the development of \textit{in vitro} immunoassays and hybridization assays for determination of antigens and nucleic acids (Christopoulos TK et al., 1995; White SR et al., 1998; Chiu NHL et al., 1999; White SR et al., 1999).

Expression immunoassay uses a reporter gene as a label (Christopoulos TK et al., 1995; White SR et al., 1998). Following completion of the immunoreaction, the reporter gene undergoes an \textit{in vitro} (cell-free), coupled transcription and translation to generate multiple active enzyme molecules per immunocomplex. Therefore, gene expression provides another significant amplification step in addition to substrate turnover, thereby improving greatly the detectability of the assay. All reagents required for gene expression are added as a mixture in a single step. It has been shown that, although the transcription and translation process consists of a series of reactions that require the concerted action of several factors, the final outcome is a reproducible, linear relationship between the amount of input DNA and the activity of the synthesized enzyme.

Currently the most challenging task in the development of an expression immunoassay is to link the antibody to the reporter gene (or the entire vector), which is a several kilobase long double-stranded DNA fragment that also contains control elements for transcription and translation. A functional macromolecular complex between antibody and reporter gene should retain both the binding affinity for the antigen and the expressibility of the DNA fragment. In previous reports (Christopoulos TK et al., 1995; Chiu NHL et al., 1999), a streptavidin-reporter gene complex was prepared by using a series of tedious and time-consuming steps. A plasmid was first linearized followed by a
fill-in reaction using Klenow DNA polymerase and biotin-dNTP to produce fragments that were biotinylated at both 3' termini. There are four binding sites on each (strept)avidin molecule (Diamandis EP et al., 1991). In order to avoid cyclization and polymerization of biotinylated DNA fragments during complexation, one of the DNA termini was first removed by digestion. The digested fragments were separated by gel electrophoresis, and the fragment containing the reporter gene was purified directly from the gel. The complexation was carried out with an excess of streptavidin and the complexes were purified by size-exclusion HPLC. The overall yield was 10-20% due to the multiple purification steps involved. The difficulty in preparing the complex constitutes the main obstacle for the wider use of gene expression as a highly sensitive amplification system in immunoassays.

The objective of the present work was to prepare a heterobifunctional linker between antibody and reporter gene. It consists of (strept)avidin covalently attached to an oligonucleotide (dA)$_{40}$. The linker is universal because (strept)avidin interacts with any biotinylated antibody or any biotinylated binder in general, such as peptides or receptors. The oligonucleotide (dA)$_{40}$ hybridizes with a complementary poly(dT) tail added enzymically to any reporter gene. Both biotinylation of binders and tailing of DNA are reactions performed readily without interfering with the respective biological activity. The linker is applied to a model sandwich-type immunoassay for prostate specific antigen using a 4.3 kilobase plasmid containing the firefly luciferase cDNA as a reporter.

4.1.1. Prostate-specific antigen

Prostate-specific antigen (PSA) is a 33 kDa single-chain glycoprotein comprising 237 amino acid residues with five intrachain disulfide bonds and approximately 8% carbohydrates in the form of N-linked oligosaccharide sidechains (Wang MC et al., 1981; Belanger A et al., 1995). This antigen has five major epitope domains, four of which have been localized to amino-acid residues 1-13, 53-64, 80-91, and 151-164 (Piironen T et al., 1998). PSA is produced by the prostate tissues and is released in the seminal fluid in high concentrations (0.5-5 g/L). This serine protease serves to increase sperm mobility by liquefying the sperm-entrapping seminal coagulum after ejaculation (Lilja H, 1985). Furthermore, PSA may have a bioactive role in fertilization (Fitchner J et al., 1994).
The release of PSA into the blood stream is a rare event in young, healthy man. It occurs at concentrations <4 μg/L, which is equivalent to less than one PSA molecule per million secreted PSA molecules (Lilja H, 1993). Elevated serum PSA is a sensitive marker for prostate cancer (Armbruster DA, 1993). However, noncancerous alteration of the prostate, such as prostatitis (inflammation of prostate gland) and benign prostatic diseases (noncancerous enlargement of prostate gland), have challenged the usefulness of PSA as a tumour marker for early detection of prostate cancer.

Prostate cancer is the most common malignancy in men, and is the third leading cause of cancer-related death (behind lung and large bowel cancer). In 2001, it was estimated that there were 200,000 new cases of prostate cancer with 32,000 deaths (Greenlee RT et al., 2001). The age of affected men is around 72 years, however, not all men with prostate cancer develop invasive disease. In fact, the slow growth of prostate cancer allows many men to die with the disease rather than from it.

PSA circulates in the serum in two forms: the free unbound form as well as the protease inhibitor bound form. Approximately 65-95% of total PSA is bound to α1-antichymotrypsin (ACT) and only 5-35% is the free form (Christensson A et al., 1990). Also, low amount of serum PSA is complexed to α2-macroglobulin. The binding of macroglobulin to PSA masks all its epitopes, inhibiting its antigenic activity, however ACT binding retains some of the PSA epitopes (Leinonen J et al., 1996). Thus, both forms of free and ACT bound PSA can be monitored for prostate cancer diagnosis and progression. Moreover, recent studies showed that the relative amount of free PSA tends to be increased in benign prostatic hyperplasia as compared to prostate cancer (Christensson A et al., 1993). The reason for this difference is not completely understood. However, it has been hypothesized that due to the loss of tissue architecture in prostate cancer, the intracellular active PSA gains quicker access to the circulation, allowing protease inhibitors to complex to PSA more easily (Stenman UH et al., 1999). Thus, the ratio of free to total PSA has become an important variable for distinguishing between benign and malignant prostatic disease.

Since women do not have a prostate gland, it was assume for many years that PSA is not detected in their blood. Recently, PSA immunoreactivity has been reported in healthy females as well as females with breast and lung cancer (Diamandis EP et al.,
1994; Levesque M et al., 1995). Despite these findings, PSA is still a valuable marker for prostate cancer screening, diagnosis, and especially post-surgical progression.

PSA is a member of the human kalleikrein family that shares considerable structural, functional homology, and gene location on the long arm of chromosome 19 (Reigman PH et al., 1992). Cloning of the 4 kb PSA cDNA (Lundwall A et al., 1987) has led to the development of qualitative and quantitative assays for the detection of PSA mRNA in order to identify circulating prostatic epithelial cells (Verhaegen M et al., 1998; Galvan et al., 1996).

4.2. Experimental Protocols

4.2.1. DNA oligonucleotides

The oligodeoxynucleotides (dA)$_{40}$ and a 17mer (5'-GTAGGCCTCAGCTGGAA-3'), both modified with a primary amino group at the 5' end, were synthesized by Oligos Etc, Inc. (Wilsonville, OR).

4.2.2. Preparation of poly(dT)-tailed DNA encoding firefly luciferase (poly (dT)-Luc DNA)

Luc-DNA plasmid containing the T7 RNA polymerase promoter upstream from the firefly luciferase gene, was linearized as follows: 100 µg of the plasmid were mixed with 200 units of Bgl I in 325 µL of 50 mmol/L Tris-HCl, pH 7.9, 100 mmol/L NaCl, 10 mmol/L MgCl$_2$, 1 mmol/L dithiothreitol. The reaction mixture was incubated at 37° C for 2 hours. The linearized DNA fragment was purified by ethanol precipitation. The 3' ends of the purified DNA were subsequently tailed with dTTP by using terminal deoxynucleotidyl transferase. The reaction mixture (20 µL) consisted of 10 µg of Luc DNA, 700 pmol dTTP, 25 units of terminal transferase, 25 mmol/L Tris-HCl, pH 6.6, 200 mmol/L potassium cacodylate, 5 mmol/L CoCl$_2$, and 250 mg/L BSA. The mixture was incubated at 37° C for 60 min, and the reaction was terminated by adding 2 µL of 2 mmol/L EDTA. The tailed DNA fragment was used without purification.
4.2.3. Preparation of the heterobifunctional linkers

The 5' amino group of (dA)₄₀ was first derivatized with N-succinimidy S-acetylthioacetate (SATA) in a 40 µL reaction mixture containing 2.5 nmol of (dA)₄₀, 250 nmol of SATA, and 0.1 mol/L carbonate buffer, pH 9.1. SATA was added to the mixture as a 20 mmol/L freshly prepared solution in 0.1 mol/L carbonate and 50% dimethylformamide. The mixture was incubated at room temperature for 60 min. The derivatized (dA)₄₀ was purified from excess SATA by size exclusion chromatography using a NAP column with an elution buffer containing 10 mmol/L ammonium carbonate, pH 6.8. The (dA)₄₀ solution was lyophilized to dryness and then reconstituted in conjugation buffer. The SATA-derivatized (dA)₄₀ (2.5 nmol) was mixed with 0.25 nmol of maleimide-activated avidin in the conjugation buffer (total volume 50 µL) and the reaction was initiated by deacetylation with hydroxylamine (final concentration 0.1 mol/L) to deprotect the sulphydryl group. The reaction was allowed to proceed for 60 min at room temperature. Afterwars, the volume was increased to 2mL with 0.1 mol/L sodium phosphate (pH 7.0) and the free oligo was removed by ultrafiltration using centricon-30. The purification step was repeated twice. The linker was stored at -20° C.

A streptavidin-(dA)₄₀ linker was prepared as follows: Streptavidin (0.96 nmol) was first derivatized with 38 nmol of sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) in 0.1 mol/L phosphate buffer, pH 7.0 (24 µL reaction mixture). After a 60-min incubation at room temperature, the free SMCC was removed by ultrafiltration using Centricon-30. The derivatized streptavidin was conjugated to the SATA-derivatized (dA)₄₀ as described above for avidin.

4.2.4. Application of the heterobifunctional linker to a sandwich-type immunoassay

Microwell plates were coated overnight at room temperature with 25 µL of 5 mg/L capture anti-PSA antibody, diluted in 50 mmol/L Tris, pH 7.8, and 0.5 g/L NaN₃. Before use, the wells were washed six times with wash solution. A 5 µL aliquot of PSA standard (or diluted serum sample) diluted in PSA buffer, and 20 µL of 0.5 mg/L biotinylated anti-PSA antibody (refer to section 3.2.3), were added into each well. The immunoreaction was allowed to proceed for 60 min with continuous shaking. At the end of the incubation, any unbound biotinylated anti-PSA was removed by washing the wells as above.
Afterwards, 25 μL of 8 nmol/L linker (diluted in blocking solution), was added into each well and incubated for 15 min followed by washing. Subsequently, 25 μL of 1.6 nmol/L poly(dT)-Luc DNA, diluted in hybridization buffer, were added into each well and incubated for 15 min. The wells were then washed eight times with wash solution and two times with 50 mmol/L potassium acetate. The bound firefly luciferase-coding DNA was expressed into active luciferase molecules by an in vitro coupled (one-step) transcription-translation reaction. 25 μL of transcription-translation mixture was added into each well and incubated at 30 °C for 90 min. The activity of synthesized luciferase was measured by mixing 2 μL of the transcription-translation reaction product with 50 μL of luciferase substrate solution (at room temperature) and monitoring the emitted luminescence for 1 min using the liquid scintillation counter.

4.2.5. Analysis of clinical specimens

Serum samples, obtained from Hotel Dieu Hospital (Windsor, Ontario), were diluted 10-fold in blocking solution prior to the analysis. The PSA concentration of the serum samples was determined by using a calibration curve constructed from PSA standards, whose concentrations were confirmed by the Abbott IMx PSA assay (Abbott Laboratories, IL).

4.3. Results and Discussion

The series of chemical reactions that are involved in the preparation of the heterobifunctional linker are summarized in Figure 4.1. In the first step, the N-hydroxy succinimide ester functional group of SATA reacts with the primary amino group at the 5’ end of (dA)₄₀ to produce a protected sulfhydryl derivative. The acetyl protecting group is then removed by a treatment with hydroxylamine and the released –SH group reacts with maleimide groups on (strept)avidin which were introduced by treatment with SMCC.

Figure 4.2 represents the configuration of a microtiter well-based sandwich-type immunoassay using the (strept)avidin-(dA)₄₀ conjugate as a heterobifunctional linker between a biotinylated detection antibody and an enzyme-coding reporter gene.
Figure 4.1. A schematic diagram of the series of reactions involved in the preparation of the heterobifunctional linker. In the first step, the free amino group of streptavidin (SA) reacts with SMCC to yield a maleimide-activated protein. The 5’ amino-modified oligonucleotide (dA)$_{40}$ reacts with SATA to yield an acetylated sulfhydryl group which in turn, is deprotected with hydroxylamine, and simultaneously conjugated to maleimide-activated (strept)avidin.
**Figure 4.2.** Sandwich-type immunoassay configuration using the heterobifunctional linker between the detection antibody and a reporter gene. After completion of the immunoreaction, the reporter gene is subjected to one-step *in vitro* transcription/translation and the activity of synthesized enzyme is monitored. B = biotin, Av = avidin.
For (strept)avidin-(dA)₄₀ conjugate to be suitable as a linker between an antibody and a reporter gene, the following requirements must be fulfilled; (a) (strept)avidin retains its affinity for biotin after conjugation, (b) the conjugation does not interfere with the hybridization of (dA)₄₀ to the poly (dT)-tailed reporter gene, (c) the bound reporter gene is accessible for in vitro transcription and translation and (d) the nonspecific binding of the linker and the reporter gene to the solid phase used in the assay is low.

A modified avidin (neutravidin) was chosen in this study because it has a significantly lower isoelectric point (pI=6.3). As a consequence, its nonspecific binding to the solid phase is relatively low.

In the first step, the SATA to (dA)₄₀ molar ratio required for complete derivatization was studied in the range of 20 to 400. The SATA-derivatized (dA)₄₀ was conjugated to avidin and the linker was used in the immunoassay as described in the Experimental Section. The highest signal was obtained with molar ratios greater than 100. The signal dropped by 60% when the ratio decreased to 20 because of the incomplete derivatization of the oligonucleotide.

The SATA-derivatized (dA)₄₀ to maleimide-activated avidin molar ratio was also studied in the range of 2 to 50 (Figure 4.3). Initially the signal increases when the SATA-(dA)₄₀ to avidin ratio is increased because the fraction of avidin molecules that are conjugated to (dA)₄₀ increases. The maximum signal is obtained at a ratio of 10. At higher ratios, the signal remains practically constant because the maximum number of LucDNA molecules that can be bound to the immunocomplex on the well is achieved. At molar ratios greater than 10 the signal/background ratio (S/B) drops sharply. This was attributed to an increase of the nonspecific binding of the linker to the solid phase.

For the preparation of streptavidin-(dA)₄₀ linkers, maleimide groups were first introduced to streptavidin by reacting with SMCC (Figure 4.1). The reaction was studied at molar ratios of SMCC to streptavidin ranging from 5 to 100. It was observed that the highest signal and S/B ratio were obtained at a molar ratio of 40. At higher SMCC concentrations the signal decreased because of the interference with the biotinstreptavidin interaction. Optimization experiments of the molar ratio of SMCC-derivatized streptavidin to SATA-(dA)₄₀ showed that the maximum signal was obtained at a ratio of 10 (similar to avidin results as above).
Figure 4.3. Optimization of the molar ratio of (dA)$_{40}$ to avidin required for the preparation of the linker. Linkers were prepared by reacting a constant amount of maleimide-activated avidin with various concentrations of the oligonucleotide (dA)$_{40}$ and then used in the sandwich type immunoassay of 25 pg of prostate specific antigen as described in the Experimental Section. The solid and dashed lines represent the luminescence and the signal-to-background ratio (S/B), respectively. cpm represents counts per minute.
The effect of the linker concentration used in the immunoassay was studied in the range of 0.4 to 80 nmol/L. The results are presented in Figure 4.4. The signal increases with the linker concentration and reaches a plateau at 8 nmol/L. At higher concentrations, the signal remains constant, but the S/B ratio decreases due to the increase of the nonspecific binding of the linker.

The time required for the binding of the linker to the immunocomplex was studied in the range of 5 to 80 min. The results are presented in Figure 4.5. It was observed that 40% of the linker binds to the immunocomplex in the first 5-min incubation. A 15-min incubation period generates the highest S/B ratio. With longer incubation periods, the S/B ratio drops due to the increased nonspecific binding of the linker.

The effect of the poly(dT)-Luc DNA concentration was studied in the range of 0.3 to 10 nmol/L (Figure 4.6). In this study, the linker concentration was kept constant at 8 nmol/L. A continuous increase of the S/B ratio is observed with increasing poly(dT)-Luc DNA concentration and a maximum is reached at 1.2 nmol/L. At higher concentrations, the S/B ratio decreases because of the increased nonspecific binding of Luc DNA.

We further studied the time required for hybridization of the poly(dT)-Luc DNA to the linker (Figure 4.7). Within 5 min, 50% of the Luc DNA is bound to the linker and the highest S/B ratio is obtained after 15-min incubation. Longer incubation times result in increased nonspecific binding of the poly(dT)-Luc DNA and lower S/B ratios.

The calibration curve for a “two-site” (sandwich-type) immunoassay for prostate specific antigen was constructed by analyzing PSA standard solutions. In Figure 4.8, the luminescence was plotted as a function of the PSA concentration in the assay mixture. As low as 20 ng/L of PSA were detectable with a S/B ratio of 2.6 and the linear range extended up to 20,000 ng/L. The linker was tested further by analyzing (after appropriate dilution) 24 serum samples with PSA concentrations ranging from 5 to 50 μg/L by the proposed method and another widely used method (Abbott IMx) (Figure 4.9). The linear regression equation was (Proposed method) = 1.16 + 1.12 (IMx) with a correlation coefficient of 0.98.
Figure 4.4. Effect of linker concentration used in the immunoassay. The sandwich-type Immunoassay was performed as described under Experimental Protocols using 25 pg of PSA and various concentrations of the linker. The signal (solid line) and the S/B ratio (dashed line) were plotted as a function of the linker concentration.
Figure 4.5. Time dependence of the binding of the linker to the biotinylated anti-PSA antibody. The immunoassays were carried out as described in the Experimental Section with 25 pg of PSA.
Figure 4.6. Optimization of the concentration of the poly(dT)-Luc DNA in the immunoassay. The sandwich immunoassay was performed as described under 'Experimental Protocols with 25 pg of PSA, and various concentrations of the poly(dT)-Luc DNA. S/B is plotted against the poly(dT)-Luc DNA concentration.
Figure 4.7. Time-dependence of the hybridization of the poly(dT)-tailed reporter gene to the linker. The immunoassay was performed as described in the Experimental Section with 25 pg of PSA.
Figure 4.8. Study of the linearity and sensitivity of the proposed immunoassay using the (dA)$_{40}$-avidin as a linker between the detection antibody and the reporter gene. The immunoassay was performed as described in the Experimental Section under optimum conditions. The luminescence (corrected for the background) is plotted as a function of the concentration of PSA present in the well.
Figure 4.9. Correlation of PSA concentrations obtained by the proposed immunoassay and the IMx method (n=24).
To assess the reproducibility of the immunoassay we analyzed serum samples containing 5.4, 15.5 and 49.9 μg/L PSA. The %CVs for all steps (immunocomplex formation, linker binding, Luc-DNA hybridization, in vitro transcription/translation and luciferase measurements) were 11.8, 9.5 and 5.0, respectively (n=9).

The performance of the avidin-(dA)$_{40}$ and streptavidin-(dA)$_{40}$ linkers was practically the same. We have also prepared an avidin-(dA)$_{25}$ linker by following the same protocol as described for the (dA)$_{40}$ in the Experimental Section. The luminescence decreased by 25% with the shorter oligonucleotide.

In addition, we have investigated whether the performance of the linker in the immunoassay would benefit by increasing the distance between the oligo(dA) sequence and avidin. A random 17mer oligo was derivatized with SATA at the 5' end and conjugated to avidin as described for (dA)$_{40}$ in the Experimental Section. The linker was then formed by tailing the conjugate (30 pmol) with dATP (3 nmol) using terminal transferase (25 units). Therefore, this linker contained a single stranded DNA spacer arm between avidin and the poly(dA) tail. Another linker was also prepared with a double stranded DNA spacer arm between avidin and poly(dA) to ensure a more rigid structure for the spacer. A 17bp double stranded DNA fragment was first prepared by annealing two complementary 17mer oligos, one of them containing a $-\text{NH}_2$ group at the 5' end. The DNA was derivatized with SATA, conjugated to maleimide-activated avidin and tailed with dATP. The results obtained with both linkers were practically the same as with the original (dA)$_{40}$ linker.

The preparation of the reporter gene used in the assay was carried out by simply linearizing the appropriate vector with a restriction enzyme (single cutter), preferably one that could create 3' protruding ends, and then tailing with dTTP using terminal transferase. Thus, both ends of the gene are tailed simultaneously. The poly(dT)-reporter gene can be used directly in the assay without purification. To simplify the assay protocol, we attempted to pre-form the complex of the poly(dT)-reporter gene with the linker and then add it into the well in a single step. However, a low signal was observed with the pre-formed complex. This was attributed to polymerization of the linker with the gene or the formation of star-like structures (several genes around each avidin molecule). These macromolecular structures subsequently interfere with the binding of
the linker to the biotinylated anti-PSA antibody. On the contrary, the two-step linking protocol ensures that the linker binds to the immunocomplex prior to the addition of the tailed reporter gene.

Stability studies showed that the linker does not lose its activity after storage for at least one year at -20°C.

Sequencing of the human genome has led to the identification of thousands new genes. The next challenge is to investigate gene function and regulation on a genome wide scale through the analysis of the encoded proteins under a variety of experimental conditions (proteomics). Differential profiling of proteins in patients and normal samples reveals potential markers for diagnosis and monitoring of disease. Sensitivity and specificity are the cornerstones of any proteomics technology. A recent study has shown that half of the yeast proteome was undetectable by using 2D electrophoresis followed by mass spectrometry (Gygi SP et al., 2000). The most sensitive protein assays are based on the interaction of the analyte with a specific binder (antibody, receptor or peptide) that is linked to a signal-generating molecule. In recent years there is an increased research activity on the selection of binders with high affinity and specificity for a large number of proteins. Various approaches include recombinant antibodies selected by phage-(deWildt RMT et al., 2000) or ribosome-display (Hanes J et al., 2000), RNA or DNA aptamers (Hesselberth J et al., 2000), and small organic compounds selected through combinatorial library methods (Han Y et al., 2000; Shuker SB et al., 1996). In most cases, the extent or the position of biotinylation of these binders can be controlled to avoid interference with the interaction between the binder and the protein. Thus, the proposed (Strept)avidin-(dA)$_{40}$ linker may be used as a universal reagent to attach a highly detectable reporter gene to practically any binder molecule targeted to a protein analyte. The microtiter well format facilitates automation and high-throughput analysis.
Chapter 5

Strategies for Immobilization of Oligonucleotides on Microtiter wells for Hybridization Assay Development
5.1. Introduction

Nucleic acid hybridization assays have become a fundamental analytical technique for detection of specific DNA or RNA sequences, especially since the advent of polymerase chain reaction (PCR). The basis of DNA identification by hybridization is the hydrogen bonding between a labeled DNA or RNA probe to its complementary sequence on target DNA, with Watson-Crick pairing restrictions. Application of hybridization assays have been reported in many areas such as detection of PCR products (Christopoulos TK, 2000), molecular diagnosis of disease (Laoboonchai A et al., 2001), analysis of point mutations (Orum H et al., 99), bacterial detection such as Salmonella and Escherichia coli strains (Namimatsu T et al., 2000, Cocolin T et al., 2000) as well as DNA fingerprinting in forensic science (Brinkmann B, 1998).

Traditionally, the analysis of nucleic acids by hybridization has employed radioactively labeled probes such as $^{32}$P or $^{35}$S. However, the problems associated with the use and disposal of such probes, have limited their routine use in hybridization assays. Current trends are towards sensitive and nonradioactive labels (Kricka LJ, 1999).

DNA labeling can be achieved either by direct incorporation of the label such as a fluorophore, or indirectly by exploiting a strong interaction such as the biotin-streptavidin, or digoxigenin-antidigoxigenin in which the DNA probe is labeled with the hapten and the protein is conjugated to an enzyme such as alkaline phosphatase (refer to section 1.4).

Hybridization assays may be carried out either in a homogeneous or a heterogeneous format. In the homogeneous assays there is no separation of the hybrids from the free (unreacted) probe. On the contrary, the heterogeneous hybridization assays involve a separation step to remove the excess of labeled probe prior to detection of the hybrids. For instance, in the "sandwich-type" hybridization assay, the target DNA is hybridized with two different probes that have complementary sequence to two different regions of the target. This results in the formation of a "sandwich" target-probe hybrid. One of the probes is used to capture the target on a solid phase and the other is used for detection (Chiu NHL et al., 1998). Alternatively, the target DNA can be labeled through PCR by either including a primer, which is labeled at the 5' end with a ligand (e.g.,
biotin), or by incorporating a nucleotide analogue such as digoxigenin-dUTP during the amplification process (Verhaegen M et al., 1998). After capturing the DNA target on the solid support, the label is detected as above.

A variety of approaches have been explored to increase assay sensitivity, e.g. using enzyme amplification combined with a chemiluminogenic substrate (Kricka LJ, 1991). Alkaline phosphatase is one of the commonly used enzymes as a reporter molecule since it is heat stable (Bronstein I et al., 1994) and offers high sensitivity.

Polystyrene microtiter wells are one of the most widely used solid supports for performing a hybridization assay. Many approaches have been developed for the immobilization of DNA probes on this type of wells. Some of these methods are time consuming, others have high cost. In order to coat microtiter wells, DNA probes with a complementary sequence to a specific region of the DNA target, are usually labeled with biotin or a hapten and coupled to a larger protein molecules such as streptavidin, or antibodies. This may lead to inconsistent DNA probe-protein stoichiometries, unfavorable orientation of the protein on the solid phase or the DNA probe on the protein, and/or well-to-well variation in the concentration of the DNA probe available to hybridize to the target DNA.

In the present work, we have compared five different protocols for immobilization of DNA probes to polystyrene microtiter wells. Some of these methods have been developed and used in our laboratory for many years such as coating with streptavidin or anti-digoxigenin antibody and capturing of an enzymatically labeled DNA probe with biotin or digoxigenin. Other methods are modifications of previously published protocols either for covalent immobilization of the probe to the wells (Running JA et al., 1990), or direct coating of probes by physical adsorption on polystyrene wells at high ionic strength (Hirayama H et al., 1996). An additional method was also developed and optimized in this study, in which albumin was first conjugated to a DNA probe and then evaluated for immobilization in hybridization assays. Detection was accomplished through indirect labeling, i.e., employing either the biotin-streptavidin or the digoxigenin-antidigoxigenin interaction. In all cases, alkaline phosphatase was used as the reporter, in combination with a chemiluminogenic substrate.
5.2. Experimental Protocols

5.2.1. DNA oligonucleotides

The following oligonucleotide sequences were used in the course of this work: 5'-GGTCGTGGCTGGAGTTCATCA-3', a 20-mer used as the downstream primer (d) in PCR, complementary to a sequence in exon 3 of the prostate specific antigen (PSA) gene; the same primer was synthesized with a biotin at the 5'-end for the preparation of biotinylated PSA target. 5'-CTCTCGTGGGCAGGGGCAGTCT-3', a 20-mer used as the upstream primer (u) in PCR, homologous to a sequence in exon 2 of the PSA gene; (Galvan B et al., 1997); 5'-ATCACGCTTTTGGTCTGATGCAG-3', the 24-mer probe used in the tailing reaction, and as the capture probe in the hybridization assay. Also the same probe was synthesized with an amino group at the 5'-end, and was used for the conjugation to albumin; 5'-(NH2)-GAAACAGGCTGGCCAGCCAGCAAGACATCG-GCTGTGTTCTCTGATGCAG-3', the extended probe used for the conjugation to albumin. The underlined sequence represents an extension at the 5'-end of the probe. All oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany).

5.2.2. Synthesis of PSA target DNA

The DNA target was synthesized starting with the recombinant plasmid pA75 that contains a 1.4 kb PSA cDNA insert (Reigman DHJ et al., 1998). The PCR mixture (total volume, 50 µL) contained 20 mmol/L ammonium sulfate, 75 mmol/L Tris-HCl (pH 8.8), 1mM/L Tween-20, 2.5 mmol/L MgCl2, 20 µmol/L of each dNTP, 25 pmol of each d and u primers, 5 million molecules of plasmid DNA, and 1.25 U of thermostable DNA polymerase. The primers were added to the mixture after the block temperature had reached 95°C. For preparation of biotinylated PSA target, 5'-biotin labeled primer was used and for the Dig-labeled target, 2.5 µmol/L of dig-dUTP was included in the PCR mixture. The cycling parameters were as follows: 15s, 95°C (denaturation), 15s, 55°C (annealing), and 30s, 72°C (extension). After the completion of 35 cycles, the mixture was incubated at 72°C for 10 min and cooled to 4°C. The PCR product was 233 bp. A 3 µL aliquot was electrophoresed on a 2% agarose gel, and the DNA was stained with
ethidium bromide to confirm the size and purity of the amplified product. The DNA target was quantified using the DC120 Kodak digital science camera and densitometry.

5.2.3. Conjugation of DNA probe to albumin

Bovine serum albumin (2.2 nmol) was first reacted with 88 nmol of sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) in 0.1 mol/L phosphate buffer, pH 7.4 (50 μL reaction mixture). After a 1-hr incubation at room temperature, the volume was increased to 2 mL with 0.1 mol/L sodium phosphate (pH 7.4) and the free SMCC was removed by ultrafiltration using centricon-30. This step was repeated twice. The SMCC activated albumin was used immediately for the conjugation reaction.

The 5' amino group of the probe was derivatized with N-succinimidyl S-acetylthioacetate (SATA) in a 85 μL reaction mixture containing 10 nmol of probe, 1 μmol SATA, and 0.1 mol/L carbonate buffer, pH 9.1. SATA was added to the mixture as a freshly prepared 20 mmol/L solution in 0.1 mol/L carbonate buffer (pH 9.1), 50% dimethylformamide. The mixture was incubated at room temperature for 1 hr. The modified probe was purified from excess SATA by size exclusion chromatography using spin-pure Sephadex G-25 columns. Subsequently, the SATA-derivatized probe (7 nmol) was mixed with 0.7 nmol of SMCC activated albumin in the conjugation buffer (total volume 60 μL) and the reaction was initiated by deacetylation with hydroxylamine (final concentration 0.1 mol/L) to deprotect the sulfhydryl group. The reaction was allowed to proceed for 1 hr at room temperature. Afterwards, the free oligonucleotide was removed by ultrafiltration using centricon-30 as above and the albumin-probe conjugate was stored at -20°C.

5.2.4. Enzymatic labeling of the probe at the 3' end

The 24-mer DNA probe was tailed with multiple B-dATP or Dig-dUTP. The tailing reaction was performed in 20 μL containing 50 mmol/L potassium acetate, 20 mmol/L Tris acetate (pH 7.9), 10 mmol/L magnesium acetate, 1 mmol/L DTT, 0.25 mmol/L CoCl₂, 100 pmol of the probe, 0.5 mmol/L dNTP, 0.05 mmol/L B-dATP or Dig-dUTP and 20U of terminal deoxynucleotidyl transferase. The reaction was carried out at
37° C for 1 hr. The labeled probes were purified from free dNTPs by size exclusion chromatography using spin-pure Sephadex G-25 columns.

5.2.5. Coating strategies and hybridization assay protocols

**Protocol A:** Probe immobilization through biotin-streptavidin interaction. Microtiter wells were coated overnight at room temperature with 50 μL of 1.4 mg/L streptavidin (SA) diluted in PBS. Initially, wells were washed three times with wash solution and 50 μL of 10 μmol/L biotinylated probe was added into each well and incubated at room temperature for 1 hr with mechanical shaking. Then, the wells were washed as before. Dig-labeled target DNA diluted in blocking solution was heat denatured at 95° C for 10 min and cooled on ice for 2 min. A 10 μL aliquot of the denatured target was added into each well containing 40 μL of blocking solution (preheated to 42° C) and the target was allowed to hybridize to the capture probe at 42° C for 60 min with continuous shaking. At the end of the incubation, wells were washed as above. Afterwards, 50 μL of 500 U/L antidig-AP conjugate (diluted in blocking solution) was added into each well and incubated for 30 min. The wells were washed three times and 50 μL of alkaline phosphatase substrate was added into each well and incubated for 30 min at 37° C in the dark. Finally, the luminescence was measured with 1s integration time.

**Protocol B:** Probe immobilization through digoxigenin-antidigoxigenin interaction. Microtiter wells were coated overnight at 4° C with 50 μL of 5 mg/L antidigoxigenin (antidig) antibody diluted in 0.1 mol/L carbonate buffer pH 9.6. Digoxigenin (dig) labeled probe (2 μmol/L) was immobilized on the well by incubating for 60 min. Biotinylated target DNA was denatured and hybridized with the immobilized probe as described under Protocol A. The hybrids were detected by incubation for 15 min with 50 μL of 200 U/L SA-AP conjugate (diluted in blocking solution) and measuring the luminescence as above.

**Protocol C:** Direct immobilization by physical adsorption and detection by using the digoxigenin-antidigoxigenin system: Wells were coated overnight at 4° C with 50 μL of 0.2 μmol/L probe diluted in 0.4 mol/L EDTA pH 7.8. A 10 μL aliquot of denatured
Dig-labeled target was added to each well and allowed to hybridize. Hybridization and detection with antidig-AP were carried out as in Protocol A.

**Protocol C**: Direct immobilization by physical adsorption and detection by using the biotin-streptavidin system. Microtiter wells were coated with the probe as in protocol C₁, followed by hybridization with biotinylated target DNA. Hybridization and detection with SA-AP were carried out as outlined in Protocol B.

**Protocol D**: Immobilization of albumin-probe conjugate. Microtiter wells were coated overnight at 4° C with 50 μL of 5 mg/L albumin-probe conjugate diluted in PBS containing 5 mmol/L EDTA. Wells were washed three times and denatured Dig-labeled target DNA was allowed to hybridize. Hybridization and detection were carried out as in Protocol A.

**Protocol E**: Probe immobilization by means of poly (Lys, Phe). Microtiter wells were coated by physical adsorption with 50 μL of 200 mg/L of poly (Lys-Phe) dissolved in sterilized water. The wells were left at room temperature for 30 min and then washed 3 times with wash solution. The 5’ amino group of the 24-mer probe was derivatized with Bis (sulfo succinimidyl) suberate (BS³) in a 120 μL reaction mixture containing 3 nmol of the probe and 1 μmol of BS³ in PBS. BS³ was added to the mixture as a freshly prepared 20 mmol/L solution in dimethylformamide. The mixture was incubated at room temperature for 15 min. The modified probe was purified from excess BS³ by size exclusion chromatography using Sephadex G-25 columns and then diluted in PBS to a final concentration of 1.3 μmol/L. 50 μL of the diluted derivatized probe was added to poly (Lys, Phe) coated wells and incubated for 30 min at room temperature followed by a washing step. The hybridization with the denatured Dig-labeled target DNA and the detection with antidig-AP conjugate were performed as in Protocol A.

**5.3. Results and Discussion**

We have compared five different strategies for immobilization of DNA probes to polystyrene microtiter wells as described under Experimental Protocols. The assay configurations are illustrated in Figure 5.1. Each set of coated microtiter wells was applied to a hybridization assay in which the DNA target was either labeled with digoxigenin or biotin through PCR, and the hybrid was detected with either
Figure 5.1. Schematic diagram showing the different strategies for immobilization of oligonucleotide: A. Biotin-streptavidin interaction, B. Digoxigenin-antidigoxigenin interaction, C. Direct adsorption of the probe to the well, D. Immobilization of an albumin-probe conjugate by adsorption, E. poly (Lys, Phe). Two different detection systems (digoxigenin-antidigoxigenin (C1), and biotin-streptavidin (C2)) are compared with alkaline phosphatase label. SA = streptavidin, B = biotin, Dig = digoxigenin, AP = alkaline phosphatase, BSA = bovine serum albumin, and Lys = lysine.
antidigoxigenin- or streptavidin-alkaline phosphatase conjugate, respectively. Two of these approaches have been used previously in our laboratory; the immobilization through biotin-streptavidin (SA) or dig-antidigoxigenin (antidig) interaction (Protocol A and B) (Verhaegen M et al., 1998; Chiu NHL et al., 1998, Galvan B et al., 1996) Furthermore, one coating strategy, albumin-probe conjugate, was developed and optimized during the course of this work (Protocol D). One of the obvious advantages of coating with albumin-probe conjugate rather than SA or antidig antibody is the decrease in assay time. This is because microtiter wells are first coated overnight with SA or antidig antibody. Then, an enzymatically labeled probe is added to each well and incubated for one hour. With the use of the albumin-probe conjugate, this extra step is eliminated since the oligonucleotide conjugated to albumin is ready to form a hybrid with the DNA target. Therefore, the assay time is decreased significantly.

The effect of SMCC to albumin molar ratio on the conjugation reaction was studied in the range of 5 to 400. SMCC derivatized albumin was conjugated to SATA activated probe as described under Experimental Protocols, and each conjugate was used to coat microtiter wells, followed by the hybridization assay (Protocol D). We observed that the signal was practically the same for all conjugates with different ratios of SMCC to albumin. This was attributed to the fact that regardless of the number of probes that are conjugated to albumin, only few of them are exposed for hybridization to the target. Therefore the ratio of SMCC to albumin did not matter as long as enough SMCC was used to have at least one probe per albumin. The molar ratio of 40 SMCC to albumin was used for further studies.

Secondly, the effect of the concentration of albumin-probe conjugate (used for immobilization) on the luminescence was studied in the range of 0.3 to 5 mg/L. The results are presented in Figure 5.2. We observed that the signal doubles as the concentration of conjugate doubles until a concentration of 2.5 mg/L. Higher concentration (5 mg/L) showed only a 12% increase in signal. The concentration used for further studies was chosen to be 5 mg/L.
Figure 5.2. Optimization of the albumin-probe conjugate concentration for coating on polystyrene microtiter wells. The hybridization assay was performed as described in the experimental section (Protocol D), with 200 pmol/L target DNA and various concentrations of the albumin-probe conjugate. The luminescence is plotted against the conjugate concentration. RLU = relative light units
In addition, we thought, that if an extended arm of nucleotides was incorporated to the probe at the 5' end between the amino group and the target complementary sequence, and is used for conjugation to albumin, the formation of the hybrid might be enhanced due the decrease of steric hindrance. We, thus, conjugated albumin to an amino-labeled probe, and to similar probe, but with an extended arm as described under Experimental Protocols, and we applied both to a hybridization assay (Protocol D). No difference in signal was observed between the conjugates. Therefore the extended arm on the oligonucleotide did not offer great advantage over the regular probe.

Furthermore, we investigated if the orientation of the probe in the conjugate influences the assay signal. Conjugates in which the probe is attached to albumin through the 5' or the 3' end were compared. Two different probes, one was labeled with an amino group at the 3'-end, and the other at the 5'-end, were used for the conjugation reaction. The hybridization assay was perfomed as in protocol D with 200 pmol/L PSA target DNA. No difference in signal was observed between the conjugates. Therefore albumin-probe conjugates that are reacted either through the 5' end or the 3' end are equivalent for coating for hybridization assay.

We also examined the use of a homobifunctional crosslinker (BS₃) for the preparation of albumin-probe conjugate. The conjugation reaction was performed as follows: 10 nmol of the 5'-amino-labeled probe was derivatized with 1 μmol of BS₃ in 0.1 mol/L carbonate buffer, pH 9.1 for 30 min at room temperature. The modified probe was purified from excess BS₃ using Sephadex G-25 columns and conjugated to 0.7 nmol of albumin in the conjugation buffer (final volume 60 μL). When these conjugates were used to coat the microtiter wells, we observed that the conjugate prepared with the heterobifunctional crosslinker (SMCC) gave similar background but three times better signal than the conjugate prepared with the homobifunctional crosslinker (BS₃). This was attributed to the fact that the conjugation with the heterobifunctional crosslinker is performed in two steps. Initially, the probe labeled with an amino group is derivatized with SATA, which yields a protected sulfhydryl group. In the second step, the sulfhydryl group is deprotected with hydroxylamine and simultaneously conjugated to SMCC activated albumin. Therefore, with this procedure, there is no chance for the formation of
homodimers as compared to the homobifunctional crosslinker where one BS$_3^1$ molecule might bridge two probes and therefore the conjugation between albumin and the probe is prohibited, which results in some free albumin that is not conjugated to the DNA probe.

Albumin-probe conjugate is suitable for the detection of two different target DNA in a single microtiter well (Larios E et al., 2000). In order to perform this type of hybridization assay, high sensitivity and binding of two different probes to a single microtiter well is required. Albumin can be easily conjugated to two different probes, then, equal amount of both conjugates can be used to coat the wells. This is more suitable for immobilization of DNA probes than to coat with a protein such as streptavidin, followed by the addition of two different probes that are labeled with biotin. Due to the competition of both biotinylated DNA probes to the binding to streptavidin, higher binding of one probe than the other can be observed. However when the albumin-probe conjugate is used for coating, equal amount of both conjugates can be mixed and adsorbed onto the solid phase simultaneously. Furthermore, with the albumin-probe coating, the biotin/streptavidin system becomes available for detection as indicated previously.

Direct coating of the probe to polystyrene microtiter wells (Protocol C) by physical adsorption was carried out using high ionic strength. Under these conditions, hydrophobic interaction between the oligonucleotide and the polystyrene plate are enhanced. Furthermore, high ionic strength decreases the electrostatic repulsion between the phosphate backbone of the oligonucleotide and the negatively charged groups on polystyrene, therefore enhancing hydrophobic interaction of oligonucleotide and polystyrene.

For protocol C, we compared coating overnight at 4° C or 37° C and 2 hr at 37° C. We applied the coated microtiter wells to hybridization assay of 200 pmol/L PSA DNA target as described in the experimental section (protocol C$_1$). We observed that all three sets gave similar results. We decided to use the overnight coating at 4° C for simplicity since all other coating protocols were performed in this manner.

The following conclusions were drawn after direct comparison of the immobilization strategies:
The detectability of the assay using the direct coating was four to five times less than the coating with the albumin-probe conjugate (Figure 5.3). This is probably due to the fact that the probe is attached to albumin through its end, therefore the oligonucleotide sequence is available for hybridization to the target. Also, coating with the albumin-probe conjugate provides a space between the solid phase and the probe, thus facilitating hybridization to the target. In contrast, the direct contact of the probe with the polystyrene (in the direct coating approach) may interfere to some extent with the hybridization. Moreover, direct coating by adsorption results in high loading, but at one point and therefore not all the bound material is capable of hybridization to complementary fragments (Running JA et al., 1990). It has been shown that dry-adsorption (coating at 37° C overnight, then heating to dryness at 60° C for 2 hr) results in binding of a DNA molecule at multiple points of the microtiter well, resulting in higher hybridization efficiency (Hirayama H et al., 1996). It would be interesting to compare this technique in future work.

Immobilization through the dig-antidig interaction gave similar results and sensitivity to the direct coating of probe by adsorption (Figure 5.3). We have observed in previous work that antidig antibody coated wells can capture a maximum of 100 fmols of digoxigenin labeled DNA probe (Chiu NHL et al., 1998). These results agree with previous publications, which state that only less than 10% of the potential capture sites of polyclonal antibodies remains functional after adsorption (Joshi KS et al., 1992). This low binding capacity is explained by that most of the coated antibodies are not able to interact with the antigen due to their orientation on the microtiter well. When using the dig-antidig interaction for immobilization of DNA probes, the hybridization must be performed the next day to coating. Longer coating time results in inefficient hybridization. With other coating strategies, such as streptavidin or albumin-probe conjugate, microtiter coated wells can be stored at least one week at 4° C and still be suitable for performance of hybridization assays.

Biotin-streptavidin interaction is one of the most widely used systems in hybridization assays. This is probably due the fact that streptavidin is a stable protein, and can bind up to four biotin molecules with a high association constant (Ka = 10^{15} M^{-1}, Diamandis EP et al., 1991). It is one of the strongest biological interactions reported and binding between
Figure 5.3. Study of the analytical range of the hybridization assay using the different protocols for DNA probe immobilization. The hybridization assays were carried out for all protocols as described under Experimental Protocols and the luminescence (corrected for the background) was plotted against the concentration of the PSA target DNA present in the well. (□) protocol A, (●) protocol B, (▲) protocol C, (☆) protocol C, (○) protocol D, (■) protocol E.
streptavidin and biotin is not disrupted even after incubation with 0.2 M NaOH at room temperature for 30 min (Laios E et al., 1998). Furthermore, we have observed in previous work that streptavidin saturated wells can capture up to 2.5 pmol of biotinylated DNA probe (Bortolin S et al., 1994). This is owing to the fact that regardless of the orientation of immobilized streptavidin, it will always be able to capture a biotinylated molecule. This leads to binding of probes at multiple points of the microtiter well, which in turn enhances the formation of the hybrid with the DNA target. Our results showed that immobilization of the DNA probe through biotin-streptavidin interaction, is two to three times less sensitive than albumin-probe conjugate. However, the former was observed to be eight times more sensitive than the immobilization through dig-antidig interaction (Figure 5.3).

Coating with poly (Lys, Phe) followed by conjugation to the DNA probe through the use of a homobifunctional crosslinker was found to perform in a similar manner to the albumin-probe conjugate. Both of these coating strategies gave the highest detectability of all protocols. It has been shown that with poly (Lys, Phe) coating, 1 – 5 pmol of probe are bound per well and nearly all the solid phase probe is available for hybridization (Running JA et al., 1990). This is probably the case with the albumin-probe conjugate since the same level of sensitivity is observed. For future work, it would be interesting to verify the amount of probes conjugated to albumin that are able to form a hybrid. This could be achieved by using a fluorophor labeled complementary oligonucleotide such as fluorescein followed by the detection of the label.

Furthermore, we also compared two commonly used indirect labeling methods based on the biotin-streptavidin and digoxigenin-antidigoxigen interactions by applying Protocol C (direct coating of probes by adsorption, Figure 5.1). For the biotin-streptavidin system, the target DNA was labeled through PCR by using a 5’ end-biotinylated primer which gives a single biotin molecule per target DNA. For the dig-antidig system, the nucleotide analogue dig-dUTP was incorporated during PCR, leading to multiple digoxigenin molecules per target DNA (refer to section 5.2.2). Alkaline phosphatase conjugates of streptavidin or antidigoxigenin were used for each corresponding system. As observed in Figure 5.3, the dig-antidig based detection system (protocol C1) was three to four times more sensitive than the biotin-streptavidin detection
system (protocol C₂). This was attributed to the fact that the digoxigenin labeled target DNA contains multiple labels. Therefore, the higher sensitivity is due to the binding of multiple antidig-AP conjugates per target DNA when protocol C₁ is applied. To achieve the same detectibility with protocol C₂, the nucleotide analogue biotin-dATP can be incorporated during amplification rather than a biotinylated primer. However, since streptavidin has four biotin biding sites, one molecule of SA-AP can bridge four biotin molecules on the target DNA, and therefore, no signifiant enhancement of signal is observed.

In conclusion, we can deduce the following: when performing a hybridization assay that requires high sensitivity, either albumin-probe conjugate or poly (Lys, Phe) coating protocols should be used in combination with the dig-antidig detection system. For detection of a PCR product of relatively high concentration, direct coating of DNA probe by adsorption under high ionic strength is suitable and is the simplest of all protocols, since no conjugation is necessary nor labeling of the capture probe. This will also cut down the overall cost of the assay, despite the fact that for direct adsorption on microtiter wells, higher concentration of DNA probe is required (0.2 μmol/L).

The calibration curve for a model hybridization assay for prostate specific antigen DNA was constructed by using all immobilization strategies, and by analyzing various concentrations of PSA target DNA solutions. In Figure 5.3, the luminescence was plotted as a function of the target DNA concentration in the assay mixture, for all hybridization assay configurations tested. As low as 3.9 pmol/L of PSA target could be detected with Protocol D (albumin-probe conjugate) and E (poly (Lys, Phe)) with a S/B ratio of 9.1 and 14.6 respectively. With Protocol A (biotin-streptavidin interaction), 7.8 pmol/L is detected with S/B of 5.6, and with Protocol C₁ (direct coating by adsorption and detection with dig-antidig system), 15.6 pmol/L can be detected with S/B of 6.8. However, with Protocol B (dig-antidig interaction) and Protocol C₂ (coating by adsorption of probe and detection with biotin-streptavidin system), only 62.5 pmol/L of PSA target DNA is detected with S/B of 9.7 and 4.4 respectively (Table 5.1). The analytical range of the hybridization assay extended up to 2 nmol/L of PSA target with all six protocols.

The reproducibility of each coating strategy was assessed by applying the immobilization strategies to a hybridization assay containing either, 40 pmol/L or 400
pmol/L of target DNA. Results are shown in Table 5.1. The % CVs for Protocols A, B, C₁, D, and E were 2.8, 6.7, 4.1, 5.5, 7.1% for 40 pmol/L and 7.1, 5.1, 8.1, 4.6 and 7.6 % for 400 pmol/L of target DNA respectively (n=5).

**Table 5.1.** Comparison of the detection limits and the reproducibility of the different strategies for immobilization of DNA probe to microtiter wells.

<table>
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<th>Strategy</th>
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<th>400 pmol/L</th>
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Chapter 6

Microtiter Well-Based Dual-Analyte Chemiluminometric Assay for Characterization of Point Mutations
6.1. Introduction

Single nucleotide polymorphisms (SNPs) are the most common DNA variation found in the human genome. Over the last decade the development of methods to detect and characterize SNPs has been rapidly increasing. These methods include gel-based SNPs analysis, allele-specific primer extension by the action of DNA polymerase, allele-specific hybridization (e.g. 5' nuclease assay, molecular beacons, and DNA microarray), and allele-specific oligonucleotide ligation assay (OLA) (for discussion on SNPs analysis, refer to Section 1.5). OLA is considered to be a very useful methodology for the detection and characterization of mutations, particularly for clinical purposes. Oligonucleotide ligation assay is based on the ability of DNA ligase to covalently join two adjacent oligonucleotide probes when they are perfectly hybridized to the DNA template. If a mismatch, however, is present at the junction, ligation fails to occur. OLA has been successfully applied to many diagnostic applications such as sickle cell anemia, cystic fibrosis (Nickerson DA et al., 1990), K-ras mutations (Rothschild CB et al., 1997), familial hypercholesterolemia (Baron H et al., 1996), and infantile neuronal ceroid lipofuscinosis (Romppanen E-L et al., 1998).

Current trends for ligation product detection include homogeneous as well as microtiter well-based (ELISA format) assays. The former is usually based on fluorescence resonance energy transfer (FRET) in which one oligonucleotide is labeled with a donor and the second with an acceptor dye. Upon ligation, intramolecular FRET is observed. Examples include the dye-labeled oligonucleotide ligation (DOL) (Chen X et al., 1998 and 1999), and europium cryptate-based time-resolved FRET assay (Lopez-Crapez E et al., 2001). For ELISA-based assays, two separate OLA reactions are usually performed, one for the normal allele, and another for the mutant allele. After ligation, the products are analyzed in two separate microtiter wells with a colorimetric detection system (Rotschild CB et al., 1997; Romppanen et al., 1999).

We developed a dual-analyte microtiter well-based chemiluminometric OLA for genotyping of single point mutations. The assay format enables simultaneous detection and genotype characterization of SNPs in a single microtiter well. The β-thalassemia (IVS-1-110) mutation is employed as a model, and the bioluminescent protein aequorin as well as the alkaline phosphatase with chemiluminogenic substrate are used for detection.
6.1.1. β-Thalassemia disorder

Thalassemias are hereditary disorders characterized by reduction or deficiency in the rate of synthesis of the globin chains. Hemoglobin is a tetrameric protein within erythrocytes, composed of structurally related α and β globin subunits (α₂β₂). Each subunit binds a single heme (iron-containing porphyrin), which is responsible for oxygen binding and transport from the lungs or skin to the capillaries for use in respiration (Wheatherall D et al., 1981). The heme group is also responsible for the characteristic red color of blood.

α-Thalassemia, reduction or deletion of α-globin chains, appears to be the most common human genetic disorder, affecting 30% of Asians and black Americans (Tietz NW, 1994). α-thalassemia affects Mediterraneans, but to a lower extent. However, β-thalassemia is the disorder historically associated with Mediterraneans. β-thalassemia is an autosomal recessive disorder characterized by reduction (β⁺) or absence (β⁰) of the β-globin chains. As with every other genetic disorder, it can be classified as homozygous or heterozygous. Homozygous β-thalassemia results in severe and often lethal anemia, accelerated hemolysis, growth retardation, bone malformations, and enlargement of the liver and spleen. The anemia is not only a result of β-chain deficiency, in fact, the imbalance between α and β-chains leads to precipitation of excess α-chains into the red blood cell membrane, resulting in membrane damaging and premature cell destruction (Cao A et al., 1994). β-thalassemia homozygote patient requires frequent blood transfusion and most likely is not compatible with life. Heterozygote β-thalassemia is depicted by elevated red blood cell count, however, it is very mild without any degree of anemia, and usually with unrecognized clinical symptoms.

6.1.1.1. Molecular pathology of β-thalassemia

Until today, approximately 200 mutations that cause β-thalassemia have been characterized (Appendix 1.4, Baysal E et al., 1998). Most of these defects are single nucleotide polymorphisms (Cao A et al., 2000) and can be separated in five different categories. (1) Mutations affecting promoter (TATA box) or enhancer (CACCC box)
sequences located upstream of the β-globin gene. All of these mutations cause β⁺-thalassemia. (2) Mutations causing defective mRNA translation. These SNPs include nonsense mutations in which a SNP converts a normal codon into a stop codon (causing early termination of protein synthesis), and frameshift mutations in which insertion or deletion of a nucleotide alters the DNA reading frame after the mutation site, resulting in a non-functional protein. Both of these types of mutations cause β⁺-thalassemia. (3) Mutations affecting mRNA splicing. These SNPs can be separated in three categories: (a) SNPs in the splice junction that prevent splicing of exons and proper excision of introns, leading to β⁺-thalassemia. (b) SNPs in the consensus codons adjacent to splice junctions that cause uncertainty in the processing of mRNA resulting in β⁺-thalassemia. (c) SNPs within exons or introns that cause impair in the processing of mRNA, leading to either β⁺-thalassemia or β⁺-thalassemia. (4) Mutations causing impaired mRNA cleavage and polyadenylation. These SNPs are within the polyadenylation signal, impairing the addition of the polyadenylate tail required for protection of mRNA (Voet D et al., 1990). This type of mutation is uncommon and could cause β⁺-thalassemia. (5) Mutations causing impairment in the 5’ cap formation on mRNA, which is required for proper translation start site. These mutations cause β⁺-thalassemia.

The most common β-thalassemia mutation in the mediterranean population is near the middle of intron 1 (IVS-1, position 110, Appendix 1.4). It is several nucleotides upstream from the exon 1/intron 1 splice junction. This SNP causes uncertainty in the site of mRNA splicing between exon 1 and intron 1, which results in β⁺-thalassemia as explained above for mutations affecting mRNA splicing. This SNP will be used as a model during the course of this project.

6.2. Experimental Protocols

6.2.1. DNA oligonucleotides

The following oligonucleotide sequences were used in the course of this work (Apexdit 1.4): 5’-GCCCATACAGCATCAGGAG-3’, a 20-mer used as the downstream primer (d) in the PCR, complementary to a sequence in exon 2 of the beta-globin gene; 5’-GAAGTTGGTGGTGAGGCCCT-3’, a 20-mer used as the upstream primer (u)
in the PCR, homologous to a sequence in exon 1 of the beta-globin gene; 5'-CTGACTCTCTCTGCCTATTG-3’, 20-mer wild type probe (wt) complementary to the normal IVS-1-110 sequence and synthesized with a biotin at the 5’ end; 5'-CTGACTCTCTCTGCCTATTA-3’, 20-mer probe complementary to the mutated IVS-1-110 sequence and synthesized with a digoxigenin at the 5’ end; 5'-GTCTATTTTCCCACCCCTTAGATAGGCCTCAGCTGGAA-3’ used as the common probe in the ligation assay and contains a 20-mer target specific sequence and a 17-mer sequence (underlined) complementary to the 17-mer probe used for albumin conjugation, and synthesized with a phosphate group at the 5'-end to allow ligation. 5'-TTCCAGCTGAGGCCTAT-3’, the 17-mer probe synthesized with an amino group at the 5'-end to allow conjugation with albumin. All oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany).

6.2.2. Synthesis of β-globin target DNA

Whole Blood was obtained from Patras General Hospital, Patras, Greece. After extraction of the genomic DNA, the β-globin DNA target was amplified with PCR. The PCR mixture (total volume, 50 µL) contained 10 mmol/L Tris-HCl (pH 8.3), 0.1% TritonX-100, 1.5 mmol/L MgCl₂, 50 mmol/L KCl, 50 umol/L of each dNTP, 25 pmol of each d and u primers, 5 µL of genomic DNA and 1.25 U of Taq DNA polymerase. The cycling parameters were as follows: 95° C for 15 sec, 65° C for 15 sec, and 72° C for 30 sec. After the completion of 35 cycles, the mixture was incubated at 72° C for 10 min and cooled to 4° C. The PCR product was 230 bp (Appendix 1.3). 10 µL aliquot were electrophoresed on a 2% agarose gel, and the DNA was stained with ethidium bromide to confirm the size and purity of the amplified product. The DNA target was quantitated using the DC120 kodak digital science camera.

6.2.3. Preparation of streptavidin-biotinylated aequorin complex (SA-BAeq)

Biotinylated aequorin (50 nmol/L) was mixed with 25 nmol/L of streptavidin in blocking solution and was incubated at room temperature for 15 min. The SA-BAeq complex was used without further purification.
6.2.4. Oligonucleotide ligation assay

In the ligation reaction, oligonucleotides specific to both the wild type and the mutant sequence of the IVS-1-110 β-globin gene are hybridized simultaneously and ligated to an upstream common probe. Ligation reactions (final volume, 25 µL) contained the PCR product of the β-globin gene (100 fmol), 400 fmol of each common, mutated and wild type probe, 1.5 units of Ampligase, 20 mmol/L Tris-HCl (pH 8.3), 25 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L NAD⁺ and 0.01% Triton X-100. The mixture was placed in the thermal cycler and heated to 95° C for 5 min followed by 12 cycles of 95° C for 30 sec and 57° C for 90 sec.

For the detection of the ligation products, microtiter wells were coated over night with 50 µL of 5 mg/mL albumin-17-mer conjugate in PBS with 5 mmol/L EDTA (refer to Section 5.2.3 for albumin-probe conjugation). The wells were washed 3 times with wash solution and 50 µL of 10 times diluted ligation reactions in blocking solution were heat denatured at 95° C for 10 min and immediately cooled on ice for 2 min. Afterwards, denatured ligation products were added to each well and incubated for 15 min at 37° C to allow the hybridization of the 17-mer to its complementary sequence in the common probe. The wells were washed six times and 50 µL containing 1 mmol/L of SA-BAeq complex (with respect to SA) and 750 U/L of antidig-ALP conjugate (diluted in blocking solution) was added to each well and incubated for 25 min. The wells were washed as above. To measure the bioluminescence of aequorin, 50 µL of triggering solution (100 mmol/L CaCl₂ in 100 mmol/L Tris-HCl, pH 7.5) was injected to each well and the signal was integrated over 10 sec. To measure the chemiluminescence from the bound alkaline phosphatase, the wells were washed as above and 50 µL of Lumi-phos alkaline phosphatase chemiluminescent substrate was added to each well and incubated at 37° C for 30 min in dark and the luminescence was measured using the luminometer.

6.3. Results and Discussion

The series of steps that are involved in the dual-analyte genotyping of point mutations are presented in Figure 6.1. In the first step, total DNA is extracted from whole blood and the β-globin gene (230 bp) is amplified with PCR. In the OLA reaction, three
**Figure 6.1.** The series of steps involved in the proposed dual-analyte assay for genotyping of point mutations. Genomic DNA is first extracted from whole blood and subjected to PCR to amplify the sequence of interest. Oligonucleotide ligation assay is then applied in which two allele-specific oligonucleotides competes to the same binding site on one strand of the PCR product, adjacent to the common probe binding site. Thermostable DNA ligase covalently joins the perfectly matched allele-specific oligonucleotide with the adjacent common DNA probe. If a mismatch occurs at the junction, ligation fails. The denaturation, competitive-annealing, and ligation steps of OLA are repeated 12 times. Ligation products are subsequently analyzed by a microtiter well-based assay in which the photoprotein aequorin and alkaline phosphatase are used for detection. The signal obtained from aequorin depicts a normal allele, and the signal from alkaline phosphatase represents a mutated allele.
oligonucleotides probes are included in equimolar amounts; a common oligonucleotide which consists 20 bases specific to the β-globin gene immediately upstream of the IVS-1-110 mutation site, plus 17 bases (non-target specific) at its 3’ end used for capturing on the solid phase, and two allele-specific oligonucleotides, one complementary to the wild-type allele labeled with biotin at the 5’ end and another to the mutant IVS-1-110 allele carrying a digoxigenin at the 5’ end. After heat denaturation of the PCR product, all three oligonucleotides are allowed to hybridize simultaneously in a way that the 3’ hydroxyl end of the allele-specific oligonucleotide is immediately adjacent to the 5’ end of the common DNA probe carrying a phosphate group. In this assay format, the two allele-specific oligonucleotides compete for the same hybridization site on one strand of the PCR product. Thermostable DNA ligase covalently joins the two perfectly matched oligonucleotides. However, if there is a mismatch at the junction, ligation fails to occur. The denaturation, competitive-annealing, and ligation are repeated several times (12 cycles).

For the detection step, ligation products are first heat denatured, and then added to microtiter wells that are coated with albumin-17-mer conjugate. The non-target sequence of the common probe is complementary to the 17-mer of the conjugate. The hybrid is then detected as discussed under Experimental Protocols with SA-BAeq and SA-ALP conjugates. For a normal sample, signal is obtained only from aequorin. For a homozygote (for the mutation) sample, signal is detected only from alkaline phosphatase. However, for a carrier (heterozygote) sample, both aequorin and alkaline phosphatase give signals. The dual-analyte assay enables detection and simultaneous characterization of the SNP genotype in a single microtiter well.

We have carefully optimized the critical parameters of the OLA reaction as well as the ligation product detection by carrying out experiments on genomic DNA extracted from normal, homozygote, and heterozygote individuals. The use of thermostable DNA ligase allowed the repetitive cycling of OLA, which proved to be an essential parameter for genotype discrimination in a single reaction as will be discussed shortly.
In the first step, it is important to ensure that upon capturing the ligation products (after heat denaturation) on albumin-17-mer coated wells, the target DNA does not re-annex to the OLA oligonucleotides, resulting in non-specific signals even in the absence of ligation. To prove specificity of the detection, we performed a series of OLA reactions on normal, homozygote, and heterozygote samples. For each sample, an enzyme negative control was included, which contained all the OLA reaction components except of the ligase. After capturing and detection, it was observed that the signal obtained from the non-ligated negatives is the same as the hybridization assay background (with no DNA target). These series of experiments were repeated several times and similar results were obtained. The background of the hybridization assay is due to the non-specific binding of the probes and detection reagents (streptavidin-aequorin and antidigoxigenin-alkaline phosphatase) to the microtiter well.

Since, the format of the proposed OLA reactions deals with competitive-annaeling of the two allele-specific probes (that differ in a single base at the 3' end) on the same target sequence, it is essential to optimize the molar ratio of the probes (wild-type, mutated and common) to the PCR product in order to obtain a clear discrimination of the sample genotype. This molar ratio was studied in the range of 0.25 to 64 for a normal, homozygote, and a heterozygote sample. OLA and analysis of ligation products were performed as described under Experimental Protocols with 100 fmol of amplified DNA used in the OLA reaction of each sample. The results for the normal and mutated alleles are plotted separately in Figures 6.2 and 6.3 respectively. As shown in Figure 6.2 and 6.3, the highest signal over background ratio (S/B) was obtained with a molar ratio (probe to amplified DNA used in the OLA) of 4. At higher ratios, S/B remained constant. However, at molar ratio of 0.25, the S/B decreased by an average of 5 fold for the normal and mutated allele due to insufficient amounts of oligonucleotides for binding on the DNA target. A molar ratio of 4 was used for further studies.
Figure 6.2. *Normal Allele*: Optimization of the molar ratio of probes to amplified DNA used in the OLA reaction. The signal to background ratio (S/B) of the carrier (solid line) and normal (dashed line) samples are plotted against the molar ratio of probes to amplified DNA. The background is defined as the signal obtained with no DNA target. The OLA was performed as described in the experimental section with 12 cycles.
Figure 6.3. *Mutated allele*: Optimization of the molar ratio of probes to amplified DNA in the OLA reaction. The signal to background ratio of the homozygote (solid line) and carrier (dashed line) samples are plotted against the molar ratio of probes to amplified DNA.
We have also studied the effect of annealing and ligation temperature of OLA in the range of 47° C to 65° C to determine the optimal reaction conditions. The results are presented in Figure 6.4 and 6.5 for the normal and mutated allele respectively. The highest S/B was obtained at 57° C. At higher temperature (65° C) S/B dropped by 7-10 fold for both normal and mutated alleles probably due to the melting temperature of the oligonucleotides. Furthermore, at lower OLA temperature (47° C) S/B also dropped by 1.5 fold for both alleles. 57° C was chosen as the annealing and ligation temperature for further studies.

The number of OLA cycles required to achieve the highest detectability was also studied in the range of 1 to 30 cycles (Figure 6.6). OLA and ligation product detection were performed as described under Experimental Protocols with previously optimized conditions. The highest detectability was obtained at 18 to 24 cycles. At higher cycle numbers, the signal remains constant probably due to the inactivation of the ligase. However, at lower cycle numbers (6 cycles), the luminescence dropped by 91% for aequorin and 97% for ALP. We also tried one OLA cycle of 60 minutes at 57° C to investigate if the cycling process is required, the signals obtained were 85% for aequorin and 91% for ALP lower than the optimum signal obtained at 24 cycles. Therefore, cycling is essential for genotype discrimination in a single reaction, because it increases the yield of the ligation product. Nevertheless, since our assay is qualitative, 12 cycles were sufficient for dual-analyte characterization of the single point mutation. However, if this assay format was to be applied for SNP detection in a few cells where sensitivity is essential, such as k-ras mutation detection (Rothschild CB et al., 1997), 24 OLA cycles should be used.

For the proposed dual-analyte assay, the genotype of each sample was depicted by calculating the ratio of alkaline phosphatase signal to the aequorin signal (both signals corrected for the assay background). This ratio is referred to as the “diagnostic ratio”. The data show that a diagnostic ratio of 10 denotes a normal sample, a ratio of 350 represents a carrier (heterozygote) sample, and a diagnostic ratio of 6500 denotes a homozygote (for the mutation) sample. One point worth mentioning is that the signal obtained from the negative allele was found to be the same as the hybridization assay background, depicting the specificity of the assay.
Figure 6.4. *Normal allele*: Study of the effect of annealing and ligation temperature on the specificity of OLA reaction. The signal to background ratio of the carrier (solid line) and normal (dashed line) sample are plotted against the OLA temperature. OLA was performed as described under Experimental Protocols with 12 cycles, 400 fmol of each probe, and 100 fmol of PCR product.
Figure 6.5. Mutated allele: Study of the effect of annealing and ligation temperature on the specificity of OLA reaction. The signal to background ratio for the homozygote (solid line) and carrier (dashed line) samples are plotted against the OLA temperature.
Figure 6.6. Optimization of the cycle number in the OLA mixture. Aequorin luminescence (solid line) and alkaline phosphatase luminescence (dashed line) are plotted against the OLA cycles number. The OLA was performed as described under Materials and Methods with 100 fmol of PCR product, 400 fmol of each probe, and annealing and ligation temperature of 57°C. AEQ = aequorin, ALP = alkaline phosphatase
As a final step, to prove that the proposed dual-analyte assay is suitable for screening, high throughput analysis, and genotyping in a single reaction/microtiter well, 23 blood samples were obtained anonymously and were tested for the β-globin IVS-1-110 mutation. Total DNA extraction, PCR, OLA and ligation product detection were performed as described in the experimental section under optimized conditions. The luminescence (corrected for the background) obtained from aequorin and alkaline phosphatase is plotted in Figure 6.7 for each sample. Figure 6.8 illustrates the diagnostic ratios calculated for each of the 23 samples. Comparing these results to the early diagnostic ratios definition for sample genotyping, we concluded that Sample 2 and 21 were homozygotes for the β-thalassemia IVS-1-110 mutation, samples 4, 5, 10, 13, 18, and 23 were heterozygotes, and all other samples were normal for this particular mutation.

To assess the reproducibility of the proposed assay including all steps (i.e., PCR, OLA, and product detection), we analyzed a normal, a homozygote, and a carrier sample as above. The % CV were found to be 7, 11.1 and 9.6% for the normal allele (luminescence obtained from aequorin), and 6.5, 10.5 and 7.9 for the mutated allele (luminescence obtained from ALP respectively (n=6).

In conclusion, a dual-analyte microtiter well-based chemiluminometric assay was developed for detection and characterization of single nucleotide polymorphisms. This assay has the advantage of simultaneously detecting and genotyping a SNP in a single OLA reaction followed by a single microtiter well assay. Moreover, this technique uses a dual chemiluminescent detection system based on aequorin and alkaline phosphatase, which offer a higher sensitivity and broader dynamic range of sensitivity over the traditional colorimetric detection, as well as great simplicity over the gel-based SNPs detection.
Figure 6.7. Application of the proposed dual-analyte assay for simultaneous genotyping of the \( \beta \)-globin IVS-1-110 mutation. Blood samples were obtained from 23 patients, and genomic DNA was extracted followed by PCR amplification of the \( \beta \)-globin gene sequence. The OLA reaction and the analysis of the ligation product were performed as described in the experimental section under optimal conditions. Luminescence (corrected for the background) obtained from aequorin (AEQ, shaded) and alkaline phosphatase (ALP, solid), are plotted against the sample number.
Figure 6.8. Diagnostic ratio obtained by the dual-analyte assay for each of the 23 samples. Alkaline phosphatase (ALP) over aequorin (AEQ) signals are plotted against the sample number. A ratio of 10 depicts a normal sample, a ratio of 350 denotes a carrier (heterozygote) sample, and a ratio of 6500 represents a homozygote (for the mutation) sample.
Closing Remarks

Whole-genome sequencing projects have led to the identification of thousands of new genes. The next challenge is to investigate gene function and regulation on a genome-wide scale through the analysis of the encoded proteins under a variety of experimental conditions (proteomics). Differential profiling of proteins in patients and normal samples reveals potential biomarkers for diagnosis, prognosis, and monitoring of disease progression. The challenge lies in the fact that proteins present at low concentrations are usually the ones that are involved in the early stages of pathological processes. Since, target amplification techniques analogous to polymerase chain reaction (PCR) that offer exquisite sensitivity to nucleic acid analysis, are not available for protein analytes, the trend is towards assays development for direct detection of a single protein molecule.

The most sensitive protein assays are based on the interaction of the analyte (antigen) with a specific binder (antibody) that is linked to a signal-generating molecule (label). The assay sensitivity is determined, mainly, by the detectability of the label and the affinity of the binder. In recent years, research efforts have focused increasingly on the identification of new labels with high detectability. The use of T7 RNA polymerase as a label with the ability to self-replicate proved to be a sensitive method for protein quantification. As seen in chapter 3, this system showed 1200 fold improvement over the traditional ELISA system. Furthermore, expression immunoassays have also proved to be more sensitive (10-100 fold) than enzyme-amplified, time-resolved fluorometric immunoassay, one of the most sensitive immunoassay systems available (Christopoulos TK et al., 1995).

The Human Genome Project has also stimulated the investigation of numerous DNA sequence variants in the human genome. Over 1.5 million single nucleotide polymorphism (SNP), the most common DNA variant, has been found until now (section 1.5). Genotyping of SNPs plays an essential role in disease diagnostics and treatment. Technologies to identify genetic polymorphisms have been rapidly increasing over recent years, however, the search for an ideal genotyping method is still in progress. An ideal system is mainly depicted by the simplicity of the reaction format, and the detection
methodology. Current trend is towards a reaction format that allows simultaneous detection of different mutations, or genotyping of a SNP in a single reaction. As presented in chapter 6, simultaneous genotype characterization of the IVS-1-110 mutation of the β-globin gene was achieved in a single reaction. The microtiter well-based hybridization assay facilitates automation and high throughput analysis. As discussed in chapter 5, there are many different ways for DNA immobilization on microtiter wells, which allow a simple and/or sensitive hybridization assay format. Furthermore, the use of a dual-analyte chemiluminescent detection system (the photoprotein aequorin and alkaline phophatase) offers a wide dynamic range of the assay and high analyte detectability.
Appendices

Appendix I. Circular Map of Luc-DNA Plasmid

4.3 kb containing the firefly luciferase coding gene under the control of T7 RNA polymerase promoter (Chiu NHL et al., 1999).

i. Sequence reference points:

1-17 T7 RNA polymerase promoter (pT7)
18 Transcription start site
69-1781 Firefly luciferase (Luc) coding gene
1719-1721 Translation stop codon
1788-1817 poly (A) tail
2463-3322 β-lactamase (Amp') coding gene
3136 Bgl I restriction site (GCCCTTCGCG)
4265-4322 lac operon sequences
ii. Firefly luciferase cDNA sequence  
(Genbank accession number E05448)

1  ATGGAAAAACG TGAGAACAGA TGAATATTT GTGATGTGTC CTGAACTATT TTACCCTATT
61  GAAGAGGGAT CTGCCTGAGC ACAATTCGCC AAGTATATGG ATCGATATGC AAAACTTGGA
121  GCAATTGGCT TTACTAAGGC ACTACTCCGGT GTGAGATATT ATCGCGCGA ATACTTGA
181  AAATCATGCT GTCTAGAGGA GCCTTGAAAG AATTATGGTT TGTTGCTGGA TGGAGAATT
241  GCGTTATGCA GTGAAACTGC TGAAGAATTC TTATATCTCT TATAGCAGGC TTATATTTAT
301  GGTGCGCTGT TGGCTCAACT TATGAGATT TACACTCTAC GTGAATTTGT TCAACTGTTT
361  GCATTCTCTA AGCCACATAT TGATATTTAT TCTAAAAAGG GATAGATAGA ATGTATATA
421  GTACAAAAAA CGTCATCTGC TATATAAACC ATTTTATAT AAGCAGCAAA ATGGGATTAT
481  AGAGGTGTATC ATCCAGACGA CACTTTATTT AAAAAAAACA TTCCACAGGA TTCCCAAAGG
541  TCAAGTTTTA AAGCTGTAAG AGTTAACCAG AAGAACAGA TTGTCTTTAT AATGAACTCT
601  TCGGGTTTCA ACGGTCTGAC TATAGAAACT ATGAAAATAC TACGCAGTAC TCGCTGATCA
661  TTTCCTCAAC CTAGAGATCC GATTTATGGA AACAAAGTTT CACGAGCAAC GGTATTATTT
721  ACTGTAAGTC CATTCCATCA TGTTTATTGT ATGTATCTAA TTTAGATTCA TTCAACTGTT
781  GGGTTTCTGA TGGTCAATGT CACGAAATTT GAPGAGAGAG GTTTTAAA AACTGTGCAA
841  GATTAAATAT GTCAGGCTT TATTTTTGTA CCGACTGTT GTGAGATATT TAATAGAAGT
901  GAATTTATCG AATAATATGA TTATCAATAT TATGTTGAAA TTGACCTGAG CGAGACCTT
961  TTATCTAAAG AAATGTGTGTG AGCTGTTTCT AGAGTTTTTT ATTACCGGG TGGTGCTCAA
1021  GCGTTATGTT TTAAGAGAAC AACTGCTGCA ATATATACCA CAGAGGACCG CAAATGAAAA
1081  CCAGGTGCTT CTGGCAAGGT TGGCCTATTA TTTAAGCAG AAGTTATCGA TCTGATACCT
1141  AAAAAACCTT TGCCGCGAGA CAGACTGTTA GAAGTTGTTA TAAAGGTTCC TATGTTTAGT
1201  AAAGGTTATG TAGATAATCC AGAAGCAACA AGAAAGATCA TAGATGAAGA AGGTTGTTTG
1261  CACACAAGAG ATATTGGTTA TACGATGAA AAAGAAATTT TTGGTATGTA GGTGTTTTG
1321  AGCTTTTATA TCATTACAGA AGGTATATCA GTACCTCCTG CGAATTATAG GTCTGCTCTT
1381  TTAGCATATC CAATAATTTT TACAGTGAGA GTTCGCTCCC CAAAATACGATT TTGCGATTGT
1441  GACGTTCTGG GAGCTCTTGT GTGATCTGAA AAGGAAATAT CAGATGTGAA AAAGAGGAT
1501  ATGGATTAGG TTGCTATGCA AGTTTCAACT GAAACCATTT CTGCTGTTGG TGCTGTTTTT
1561  GTGAGCAGAG TACCTAAAGG TTTCACTGAG TAAATGGTAC GTAAAGCAAT TAGAAGAAA
1621  CTGAAAGAAC CAGGGCTCAA GATG
Appendix II. Circular Map of T7RP-DNA Plasmid (pT7G1)

6.1 kb containing the T7 RNA polymerase coding gene under the control of its cognate promoter (Deng H et al., 1994).

i. T7RP-DNA plasmid contains:

1. T7 RNA polymerase promoter (pT7)
2. Encephalomyocarditis (EMC) untranslated sequence (592 bp)
3. T7 RNA polymerase coding gene (T7RP, 2652 bp)
4. 3’ β-globin gene (βgA_n, 180 bp).
5. β-lactamase coding gene (Amp', 860 bp)
ii. T7 RNA polymerase coding gene

3171-5822 DNA sequence of the bacteriophage T7
(Genbank accession number V01146).

3171
TTAAGACAGC TAAAGACGC TTTCTCAGA TCGAAGCTGG TGTAACTCCG TTTCAACACTC
3181 TGCGTCCAGA TTAGCTGTAG GGTGTTAGCT GCGAACAGTG GCGCTCTTGA CATGAGTTCTT
3241 ACGAGATGGG TGAAGACGC TCCGCGAGA GGTGGTACGC TCACTTAA AGCTGCTGAG
3301 TTGCGGATCA CGTCGCGGCA AACGCGTCTCA TACCTTTCAAT ATCACTGCTCAG
3361 GCATCCAGGA CGCGCGAGG GAAATGCGAG CTAGCGCTAG GACGCTGCTG
3421 ATGTCGCTCA AGATGACACT CCGAGCGCC TACGAGGCTA ATGACGCTGCTG
3481 CACTGTTGCC ATCGGCGAAT CGTCGCTCAG GCAAGCTGTA AAACATCCC TACGCTGCTG
3541 TTTTCGCTAAC CGACTGCAAT ACAAAGACTT TCTACGCTGAG CATGGAAGCC ACCACGCTGCTG
3601 CCATTGAGGA CGGCGGTCTC TCGGCTGCTA TACGCGGCTA AGCTGCGAG TACTGACGAG
3661 AAAACGGTGA GGAACAGCTC CCCAGGCGCT CGAGGCCGTA TACGACGCTGCTG
3721 AAGGTGCTGA GCGTACCGG TCCTCTAAGG TCTCTGCGT GCGCCAGCTG GCTGTCGCTG
3781 CGCCAGATAG AGACCTTATT CAGTGGGAGG TACGGTCTAC ACGAGTGTGC ATCAGTATGAA
3841 CGGAACTGCT TACGTTACGC CGCAAGAACT CTGCGGCTAG ATGACGCTGCTG
3901 TGCGTACGGC ATCGGCTGCTA AGCTGCTGCTG ACGAGGCTG TCGGCTGCTG
3961 TGCTCTCCAG ACGACCGATG TGGCGTGTG TCTGCAAGG TGGACGCTG TCTGACGCTG
4021 GGCTGGCTATG CAGTGCTCTC GGGCGCTGCTG GTCGATGAC ATGAAAGAG
4081 CATCGAGCGG CATTGTTAGTG ATGTCGCTAG GCAAGTGGCTA AACACAGC GCAGCTGCTG
4141 AACACAGAAG TACGAGCTGAG ATCGCTGCTG GCTGACGCTGCTG
4201 GGAGACCATG TCGGCTGCTG GACACCGCTG CAGATGACGCT TACGCTGCTG
4261 CGGAACTGCT CAGACCGCTG CCTGCGGCTG TACGCTGCTG TCGGCTGCTG
4321 TGCTACCGGA CGGAGCTGCT CAGACAAGCC CGCCAGTACG TCTGCAAGG TCTGACGCTG
4381 AAGCCAGATGTT GAGCTGCTGCTA ATGTCGCTGCTG CCTGACGCTGCTG
4441 CGCCAGATAG ATGTCGCTGCTG CCAGAGCGGT GCAAGTGGCTA AACACAGC GCAGCTGCTG
4501 TTACGCGGTA GAAATGCGAG ATGTCGCTGCTG ACGAGGCTG TCGGCTGCTG
4561 GTGCGGACAC TCGGCGTATG CTGCGGCTGCTG ACGAGGCTG TCGGCTGCTG
4621 AAAACAGCAGA GACACAGCTG CAGTGCTCTC GGGCGCTGCTG GTCGATGAC ATGAAAGAG
4681 AGCAAGATCC TCGGCTGCTG TCTGTCATGG TTAGCTCGTG ATCAGCCTGG
4741 AGCGCTGCTG GCTGACGCTGCTG TACGCTGCTG TCGGCTGCTG
4801 AGCGCTGCTG GCTGACGCTGCTG TACGCTGCTG TCGGCTGCTG
4861 TGACCGAGCT GACACGCTG CAGTGCTCTC GGGCGCTGCTG GTCGATGAC ATGAAAGAG
4921 AGCAAGATCC TCGGCTGCTG TCTGTCATGG TTAGCTCGTG ATCAGCCTGG
4981 AGCAAGATCC TCGGCTGCTG TCTGTCATGG TTAGCTCGTG ATCAGCCTGG
5041 GTGCGGACAC TCGGCGTATG CTGCGGCTGCTG ACGAGGCTG TCGGCTGCTG
5101 TGCGGCTGCTG TACGACGCTG CCTGACGCTGCTG
5161 GTGCGGACAC TCGGCGTATG ATGTCGCTGCTG CAGACAGCTG TACGCTGCTG
5221 CTGCGGACAC TCGGCGTATG ATGTCGCTGCTG CAGACAGCTG TACGCTGCTG
5281 AAGCGGCTGCTG TACGCTGCTG TACGCTGCTG TACGCTGCTG
5341 CTGCGGACAC TCGGCGTATG ATGTCGCTGCTG CAGACAGCTG TACGCTGCTG
5401 AGCAAGATCC TCGGCTGCTG TCTGTCATGG TTAGCTCGTG ATCAGCCTGG
5461 AGCAAGATCC TCGGCTGCTG TCTGTCATGG TTAGCTCGTG ATCAGCCTGG
5521 AGCAAGATCC TCGGCTGCTG TCTGTCATGG TTAGCTCGTG ATCAGCCTGG
5581 TGCGGCTGCTG ACGGCGGCTG TACGACGCTG ATCAGCCTGG
5641 ATGTCGCTGCTG CAGACAGCTG TACGCTGCTG TACGCTGCTG
5701 AACAGCGGCTGCTG TACGACGCTG ATCAGCCTGG
5761 TACGACGCTG CTGCGGCTGCTG ACGGCGGCTG TACGACGCTG ATCAGCCTGG
Appendix III. Human Prostate Specific Antigen cDNA Sequence
(Genbank accession number BC005307)

1 AGCCCCAGGC TTACCACCTG CACCCGGAGA GCTGTGTCAC CATGTGGGTC CCGTGTGCT
61 TCCCTCACCT GTGGGTGACG TGAGTTGGTG CTGGCACCCT CATCCTGCTGT CGGATTGTGG
121 GGAGCTGCTGGA GTGCCAGAAG CATTTCCAAC CCTGCCAGGT GCTGTGTGCC TCTGTTGGCA
181 TGCAAGGAGCA AAGGACTGCT TTTGCAGGCT TGGCACCCAC GTTTCCATCT GTAGACGAG
241 GCCAGCGATT ACTACAGTGT CAGTTCGAC CAACCCCAGG CACACTCTCT TACATATGAG ACCTCTGTGA
301 AGAATCGATT CCTCAGGGCCA GGTGATGACT CCGCCACAGA CTTCATGCTG CTCGGACTCT
361 CAGAGGCTGC CGAGCTCAGG GATGCTGTGA AAGTCTAGTA CCCTGCCCAC CAGAGGACAC
421 CACCTGGGAC CACCTGATAC GCCCTTGGGCT GGGGCAGCAT TGAAACCGAG GAGTTCTTTGA
481 CACCAAGGAAC ACTTCACTGT GTGGACTTCC ATTGTATTTT CAAATGACGTA TGGGACCAAG
541 TTTCACTCCA GAGGGTACCAG AGAGTTCTAG AAGTCTGCTG TGGGTGCTGG ACCTGAGCAAG
601 GCACCCGCTGC GGGTGTACCT GGGGAGCAGG TTGGCTGTAA TGGTGTGCTCT CATAGTCTACA
661 CGCGTGGGGCT GCTGCCGACG CTGAGGCGCT CACGAGGTGC TGGGACACAC TGGGGAAGTTTG
721 TGAGAGCTACG GAAGTGGATC AAGGACCAAC TGGGAGCAGA CCGCTGGCAG ACCCTATCAA
781 CTCTCTATTT AGTGAATATTT GAACTCCAGG GCCATGAGC AGCCTCCACCA CCCTTCTTCA
841 AGCTTCTTCTAC ACCCTTCTCC TTAGGTGTGA GTCCAGGGTG GCTAGGAAA AGAATGACG
901 AGACCGAGGT GTAGACGACA GTGGTTCTTA AATGCTGTAA TTTGCTTTCC TCTGTGTCCCT
961 GGGAATACCT GACAGATGTCT TCTAGGCAGT CACCAAAA CTCTGAGGAG ACAGATAGGA
1021 TTGGGTCATG GTGTTATTTG TGGGTCAGGC AGATGAAGAAC GGGTGTTAGG GACACAGTAG
1081 AGATCGAGAG TGGACATGTG CTGGCACTCT GCATGAGCAG ACCTGAGGCC ATGAGGACG
1141 ACAAGCAGCC AGACCTCCTAC AGCAGAGTAG GACTGGAATA CATACCCAC TACTGTCTGG
1201 AGCCACTGGG AAGCCGACAG AAGGCGTGG CAGCGAGAGG GAGGCTCCTG ATTTTCATG
1261 GAGATGGGAT GAGGCTGACG GAGGAGCTGG ACCCTGGTGA TCTGATTAAC TGGTGGGGG
1321 AGGATGAGTG AAGCTCTCCA GACAGACCAC AGATTTGTAG ATTCTCTAGT AGAATACACA
1381 GAAATAGAGA GCTGTATTAC TGTG

i. Sequence reference points:

170-189 Annealing sequence of the upstream PCR primer (bold), complementary to the template strand.

236-259 Annealing sequence of the wild-type probe (highlight), complementary to the non-template strand.

383-403 Annealing sequence of the downstream PCR primer (bold), complementary to the non-template strand.
Appendix IV. Common Mediterranean β-Globin Gene Mutations

Human β-globin gene cluster spans >70 kb on chromosome 11
(Gen accession number U01317)

61801 TTATTTGTGT GATCTTCGAT ATGCTTACCA AGCCTTGATT
61861 CCAATATTTA CATTTAGCTA AGGAGTTCGTT TGTATGCGAT
61921 GTGTATGGGCC CAGAGATATG ATCTATGAGG GAGGGCTGAG CAACTCTCTA
61981 AGCCAGTGCC AGAAGACCAA AGGACAGGTA TGCTGTCTAC CTAGTCCAG GCTGCCTCTG
   CD59∗(C-T) CD6(T-A) SICKLE (A-T) CD8(T-A) SICKLE (G-A)
62161 TTACTGACCA AGCCAAACTA GGTCACTCAG TGGACTGCG ACTGGTGCA
   IVS-1-5 (G-A) IVS-1-6 (T-C)
62221 TTACTGACCA AGCCAAACTA GGTCACTCAG TGGACTGCG ACTGGTGCA
62281 TTGGGATTCG ATGATGCTA AGGCTCTGAG AGCACTGAGC TGGAGAGCA
   IVS-1-110 (G-A)
62341 TGGAAGAATTC AGGAGTCCCA TCTGCATCTCT GCAATATCCCT GCAATATCCCT
   IVS-2 (C-G)
62581 CGTACTATTGA AACAGTCTGGTA CTGGATCTCG AGACGTCTGC ACTGACTCTGTA
62641 TTGGGATTCG ATGATGCTA AGGCTCTGAG AGCACTGAGC TGGAGAGCA
62701 GTGAGTTTAT AAGCTTTTAT TTTCTCTCTA CCTGCCTCTA ATGCTTACCA
62761 ATGCTTTTAG GGTCTATTTTT TTGCATTTCT TTGATCTGTT
62821 TTAATCTCTT ACTCTCTTCT AAGCTCTTCT TATGCTCTCT
62881 ATGCTTTTAG GGTCTATTTTT TTGCATTTCT TTGATCTGTT
62941 TTGCACTGGA ATGATGCTA AGGCTCTGAG AGCACTGAGC TGGAGAGCA
63001 ATGCTTTTAG GGTCTATTTTT TTGCATTTCT TTGATCTGTT
63061 ATGCTTTTAG GGTCTATTTTT TTGCATTTCT TTGATCTGTT
63121 GCTATTTTAG GGTCTATTTTT TTGCATTTCT TTGATCTGTT
63181 TTAATCTCTT ACTCTCTTCT AAGCTCTTCT TATGCTCTCT
63241 TTGCACTGGA ATGATGCTA AGGCTCTGAG AGCACTGAGC TGGAGAGCA
63301 ATGCTTTTAG GGTCTATTTTT TTGCATTTCT TTGATCTGTT
   IVS-2-745 (C-G)
63361 GCAGTACGTA TCCAGCTCC ATCTGCTCTT TTTATTTGAG TGGGCTGATT
63421 TCCGATCTTCA ATGCTTTTAT TTTTATTTTT TATATTATTT
   ATACATTCTACTTATTT
63481 GTGACTTGTGG ACAAGTGCTGT TCTGTGTGCTTC GGGGTCTCAG TGGAGAGCA
63541 ATGACTTGTGG ACAAGTGCTGT TCTGTGTGCTTC GGGGTCTCAG TGGAGAGCA
63601 GTGACTTGTGG ACAAGTGCTGT TCTGTGTGCTTC GGGGTCTCAG TGGAGAGCA

i. Sequence reference points:

62253-62272 Annealing sequence of the upstream PCR primer (underlined), complementary to the template strand.
62468-6248 Annealing sequence of the downstream PCR primer (underlined), complementary to the non-template strand.

Note: Exons 1, 2 and 3 are shown in highlights respectively.
Common Mediterranean β-globin gene mutations are shown in bold, and the corresponding mutation-change is shown above each one.
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Education

Ph.D. Clinical Chemistry University of Windsor Ontario, Canada 1998-2002
B.Sc. Honours Biochemistry University of Windsor Ontario, Canada 1996-1997
B.Sc. General Science University of Windsor Ontario, Canada 1992-1996
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Other Qualifications

Exchange Student University of Windsor, Ontario-University of Patras, Greece 2000-2001
Clinical Chemistry Hospital Internship Hotel Dieu Hospital, Windsor, Ontario, Canada Jan. - June 2000

Refereed Journal Publications

Tannous BA and Christopoulos TK. Strategies for Immobilization of Oligonucleotides on Microtiter wells for Hybridization Assays Development (manuscript in preparation).

Tannous BA and Christopoulos TK. Microtiter Well-Based Dual-Analyte Chemiluminometric Assay for Characterization of Point Mutations (manuscript in preparation)

Tannous BA, Laios E, and Christopoulos TK. T7 RNA Polymerase as a Self-Replicating Label for Antigen Quantification (submitted).

Abstracts


Other Contributions

Tannous BA. Comparison of the Vitros Chemistry System and the ACA Clinical Analyzer for the Determination of Salicylate, Ammonia, Urinary Protein, Acetaminophen, Alcohol, and Lactic Acid. Study performed at Hotel Dieu Hospital, Windsor, Ontario Canada (2000).

Presentations

May 2002 Novel Methods for Antigen and Nucleic Acid Analysis. Massachusetts General Hospital, Harvard Medical School, Boston, USA (Talk)


June 1997 Purification of large amount of DNA Fragments using Preparative Gel Electrophoresis. Canadian Society of Chemistry (CSC) conference, Windsor, Ontario (Poster).

Apr. 1997 Method for Large-Scale DNA purification. Metropolitan American Chemical Society, student affiliate meeting, University of Detroit Mercy, Michigan (Talk).


Awards

Post-Graduate Student Scholarship, University of Patras, Patras Greece (2001).


University of Windsor, Conference Travel Award (1998).
Chemical Institute for Chemistry (CIC), overall best presentation, Analytical Chemistry Divisional Award, at the 25th Southwestern Ontario Undergraduate Student Chemistry Conference (1997).

**Work Experiences**

Teaching Assistant, University of Windsor, Ontario, Canada (1997-2002).  
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