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In vitro expression as a signal amplification system.

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In Vitro Expression as a Signal Amplification System

by

Stephanie Rushbrook White

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Abstract

There is a growing demand for assay systems which permit detection of substances present at very low concentrations or in very small sample volumes. To this end we have used *in vitro* expression as part of an effective signal amplification system. *In vitro* expression contributes two levels of amplification; transcription of a DNA label to generate multiple mRNAs and translation of the mRNAs to generate multiple protein molecules which are used to initiate signal generation.

We used a DNA label encoding the α-peptide of β-galactosidase in an immunoassay. This label was linked to an analyte-specific antibody and, following immune complex formation, the immobilised label was expressed *in vitro* and α-complementation was performed. This assay facilitated detection of 3 fmol of analyte immobilised on the microtitre well surface. Various strategies were employed in attempts to increase the expression efficiency of the DNA label, including: i) switching from a prokaryotic to a eukaryotic expression system, ii) incorporation of a T7 promoter, iii) addition of 5'-untranslated leader sequences and iv) addition of downstream $[dA/dT]_{30}$ sequences. In no case was an improvement in expression observed.

A DNA label, containing the coding sequence for apoaequorin under the control of the T7 promoter and a downstream $[dA/dT]_{30}$ sequence, was engineered and used in sensitive nucleic acid hybridisation assays. Cell-free expression of this template in a eukaryotic system, followed by aequorin regeneration, permitted production of, on average, 156 aequorin molecules per DNA. The DNA label, linked to a DNA probe and used in captured target and sandwich hybridisation assays, allowed detection of 0.5 and 0.25 amol of target DNA, respectively.

We expanded the concept of expression as part of an amplification system in a technique for production of protein from traces of DNA. An isothermal, transcription-dependent amplification system was developed and linked to *in vitro* translation. The devised

system allowed production of approximately 21 million molecules of aequorin from each DNA template in two hours.

This work demonstrates that *in vitro* expression of an appropriate DNA can form part of an extremely efficient amplification system which may be successfully incorporated in a variety of applications.

– for Brad –
– Rosemary –
– my Mother and Father –

– and for Mom White –
– Those we hold most dear are never truly lost to us. –

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List of Abbreviations

AMPGD	3-(4-methoxyspiro[1,2-dioxetane-3,2′-tricyclo-[3.3.1 (3,7)]decan]-4-yl)phenyl-β-D-galactopyranoside
AMV	Avian Myeloblastosis virus
ATP	Adenosine 5′-triphosphate
bDNA	Branched chain DNA
β-Gal	β-Galactosidase
BSA	Bovine Serum Albumin
CITE	Cap Independent Translation Enhancer
CTP	Cytidine 5′-triphosphate
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme Linked ImmunoSorbent Assay
ExIA	Expression Immunoassay
IPTG	Isopropyl thiogalactopyranoside
GTP	Guanosine 5′-triphosphate
4-MU	4-Methylumbelliferone
4-MUG	4-Methylumbelliferyl-β-D-galactoside
NTP	Nucleotide triphosphate
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RLU	Relative Light Unit
RNA	Ribonucleic Acid
RNAP	RNA Polymerase

RT	Reverse Transcriptase
SSC	Sodium Chloride-Sodium Citrate Buffer
TCA	Trichloroacetic Acid
TdT	Terminal Deoxynucleotidyl Transferase
TE	Tris-Ethylenediamine Tetraacetic Acid
TTP	Thymidine 5′-triphosphate
UTP	Uridine 5′-triphosphate

1. General Introduction

1.1 Immunoassays

Protein binding assays are analytical methods which depend on the specific recognition and binding of an analyte by a detection molecule. Many kinds of high-affinity specific binding proteins exist in nature, including hormone receptors, lectins, protein A etc., but certain properties of antibodies have made them the most popular choice for detection molecules in these methods. Immunoassays are analytical techniques which exploit the strong affinity, specificity of antibody—antigen interactions for sensitive detection/determination of analytes. Immunoassays have become essential routine and research tools throughout the biochemical sciences, particularly in clinical analysis.

1.1.1 Antibody and Antigen Characteristics

Antigens, or immunogens, are substances which stimulate an immune response, either humoral (antibody) or cellular (sensitised cells) or both. Substances with low molecular weights can also stimulate an immune response when coupled to larger carrier proteins. These molecules are referred to as haptens. Antibodies are proteins which bind with high affinity and specificity to antigens or haptens. Antibodies have evolved to include variable regions which are responsible for molecular recognition and constant regions responsible for biological function.

The class of antibody predominantly used in immunoassays is IgG. It is a glycoprotein with an approximate molecular weight of 150 000 Da, comprised of two identical heavy (~ 420 residues) polypeptide chains and two identical light (~215 residues) polypeptide chains (see Figure 1-1). The chains are held together by non-covalent forces and covalent

disulphide bonds. Each chain contains domains, or regions of 100 - 110 amino acids with intrachain disulphide bonds. The N-terminal domain is responsible for antigen recognition and binding. This domain is highly variable from antibody to antibody while the subsequent domains are more constant.

The diversity of the variable region sequences is required for the enormous range of binding specificities observed. Two complementary processes in the B cells are responsible for this diversity; i) shuffling and splicing of genes coding for variable segments of heavy and light chains, and ii) facilitated mutations in the assembled variable regions which give rise to progressively improved strength and specificity of antibody binding. After initial immunisation the assembled variable genes of the heavy chain are combined with the constant regions of the μ chains to produce IgM antibodies. Class switching then occurs as the μ chain variable region recombines with gene segments for γ, α, ε, or δ constant regions to form cell lines which will synthesise IgG, IgA, IgE, or IgD class antibodies. In this way the immune response is tailored to the specific infectious threat.

Reagent antibodies are generally prepared in animals (for example: goats, rabbits, mice or horses) after repeated exposures to a foreign substance of interest. Under appropriate conditions the antibodies produced will be present in sufficient amounts that they are easily detected in the serum of the immunised animal. Collection of this antiserum produces a polyclonal antibody reagent since antibodies are produced, by the B cells of the animal, against multiple antigenic determinants of the foreign substance. In order to obtain monoclonal antibodies a single antibody-producing cell is fused with tumour cells to generate hybrid cells. The hybrid cells are isolated and used for the production of antibodies against a single antigenic determinant.

Selection of antibodies for use in immunoassay requires information about its strength (titre), affinity and specificity. Titre is the amount of antibody available for reactivity in a specific immunological method. It is the reciprocal of the maximum dilution of antibody that generates a detectable response using a specific method. Affinity refers to the strength of the binding reaction between the antibody and the antigen. Ideally only high

Figure 1-1: Diagrammatic Representation of IgG. IgG is made up of two identical light chains and two identical heavy chains. There are six distinct but structurally similar globular domains that form when these polypeptide chains come together. The two variable domains are made up of the variable regions of the light and heavy chains which come together to form the complementarity determining regions, or antigen binding sites. The other domains are less variable. V = variable; C = constant; L = light chain; H = heavy chain; CDR = complementarity determining region.

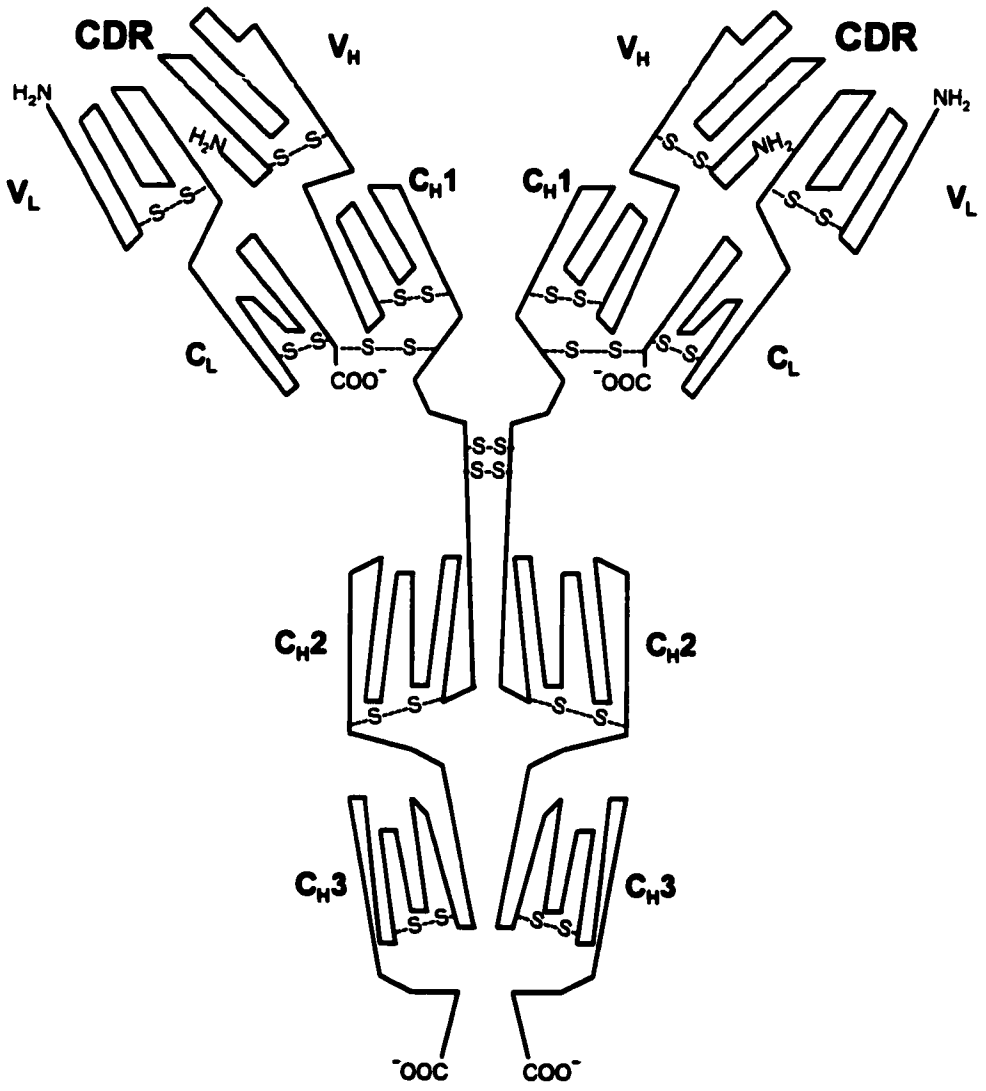

CDR
V_H
V_H
CDR
H_2N
H_2N
NH_2
NH_2
V_L
V_L
C_H1
C_H1
C_L
C_L
COO^-
^-OOC
S-S
S-S
C_H2
C_H2
C_H3
C_H3
^-OOC
COO^-

Figure 1-1

affinity antibodies should be used in immunoassays so that they do not limit the detection limit of the method. The strength of the antigen—antibody interaction is represented by the association (or affinity) constant which can be derived as follows:

$$Ag + Ab \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Ag \bullet Ab$$

$$\frac{[Ag \bullet Ab]}{[Ag][Ab]} = \frac{k_1}{k_2} = K_a$$

Ag is the antigen and Ab is the antibody, k_1 and k_2 are the rate constants for the forward and reverse reactions, respectively and K_a is the association constant. The K_a is typically between 10^9 and 10^{12} M^{-1}.

The reagents used in an immunoassay are the main determinants of sensitivity and selectivity, with the detection antibody being the most important component. Specificity is the ability of the antibody to restrict its reaction to a specifically defined group of molecules. In immunoassays the cross-reactivity, or the tendency of the antibody to combine with irrelevant analytes in the sample, must be minimised. The methods for obtaining excellent antibody reagents, those with high specificity and low cross-reactivity, are constantly improving. For instance, antibody engineering (1) and synthesis of multivalant or bispecific antibodies and fragments (2) are beginning to be employed in the production of highly effective immunoassay reagents.

1.1.2 Immunoassay Classification

Once an antibody has been selected it may be applied to the development of an immunoassay. Many different types of immunoassays exist, each with its own set of advantages and disadvantages. A review by Gosling places immunoassays into the classes briefly described here (3).

Precipitation Immunoassays

Precipitation immunoassays do not use a labelled antibody or antigen, rather they rely on the formation of insoluble immunocomplexes for direct detection. Soluble molecules

which possess multiple antigenic determinants that permit cross-linking will precipitate out of solution as they react with antibodies; first small aggregates are formed which eventually associate to form larger insoluble matrices. Agglutination occurs as specific antibodies aggregate particulate antigens bearing the antigenic determinant. Assays included in this class are precipitation, turbidometric and nephelometric immunoassays, gel diffusion assays, immunoelectrophoresis, particle or cell agglutination assays, particle counting assays and complement fixation assays.

Reagent Excess Immunoassays

Reagent excess assays are those in which the detection antibody is present in excess. All these assays have the potential for very low detection limits with maximisation of signal to noise ratios. As a result of the use of excess reagent precautions must be taken to reduce the non-specific binding of labelled reagents. Non-specific binding is the low unsaturable binding of the antibody reagent to the solid phase involved in the assay. Usually the principal reagent is a labelled antibody specific for the analyte of interest. Unbound label is most commonly removed from the bound label through the use of excess immobilised antibody which recognises a second antigenic determinant on the antigen (Figure 1-2). The use of this two-site immunoassay configuration has the disadvantage that it is not applicable to the detection of small peptides (15 - 20 amino acids), simple steroids or most drugs, since they are too small to have a second epitope. The sensitivity of two-site immunoassays can be enhanced with the use of labels with higher detectability and lower non-specific binding.

Reagent excess immunoassays for haptens have been developed. These assays depend on the use of a blocking reagent to occupy the remaining free analyte-specific binding sites after hapten in the sample has been bound by excess immobilised antibody. A second, labelled antibody is then used to mark the antibodies which have combined with the hapten.

Most antibody detection assays are reagent excess immunoassays. Also, immunoblotting and immunostaining techniques are reagent excess assays. The latter assays are generally

Figure 1-2: Two-Site Immunoassay Configuration. Antigen specific antibodies are immobilised on the surface of a solid phase. After completion of the immune reaction between the antigen and the labelled and immobilised antibodies, the unbound label is removed. Techniques for washing away unbound label depend on the type of solid phase employed in the assay. Microtitre wells are washed by alternatively aspirating and adding wash solution. Ab = antibody.

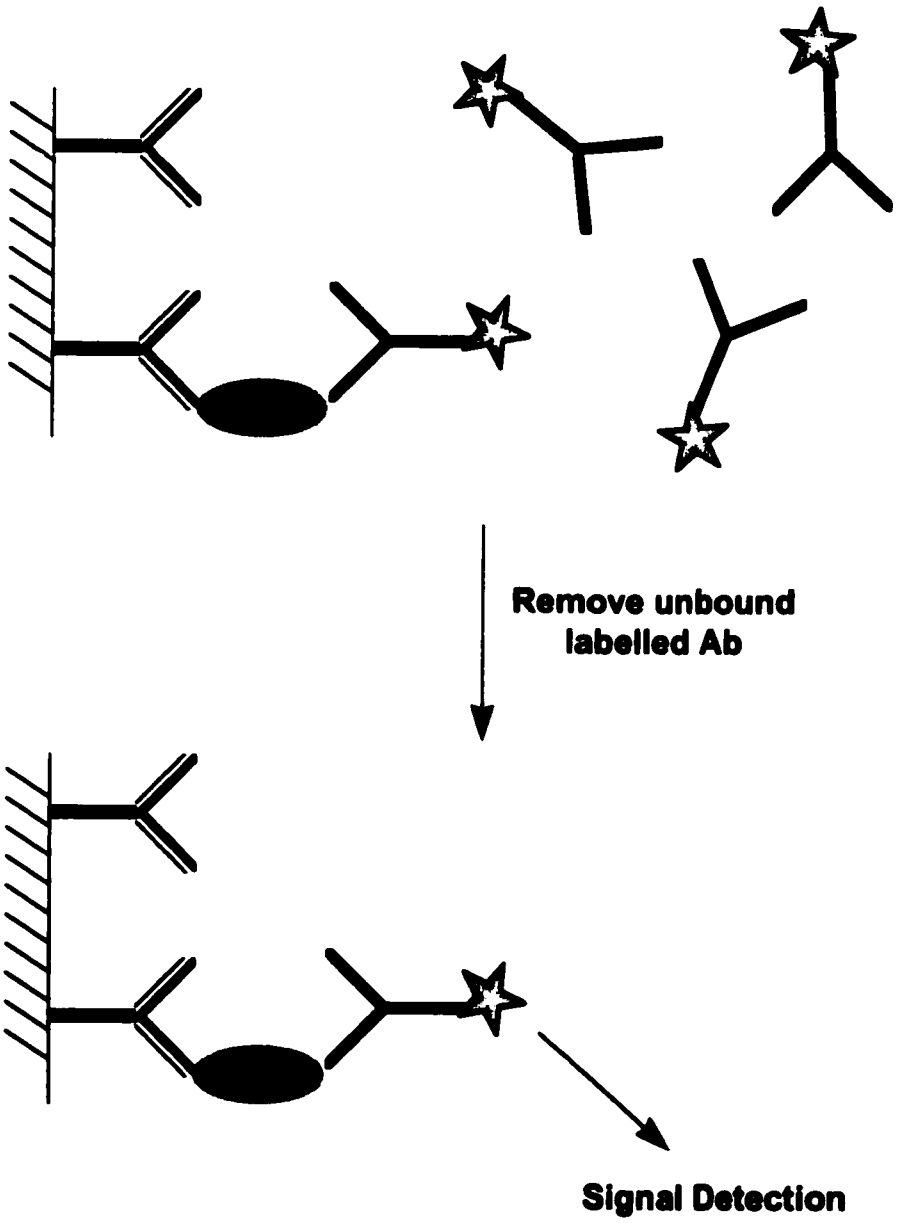


Figure 1-2

non-quantitative although they maybe made quantitative or semiquantitative with ancillary equipment such as densitometers or specialised fluorescence microscopes, depending upon the label used.

Reagent-Limited Immunoassays

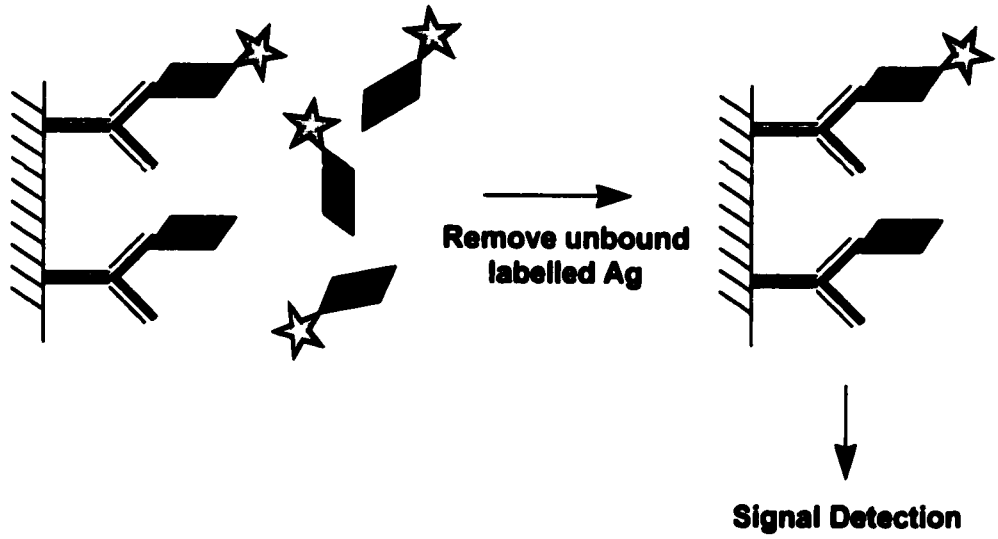
Reagent-limited assays, also referred to as competitive assays, are characterised by the use of limited antibody concentrations. Labelled antigen assays (Figure 1-3(A)) involve the use of a labelled antigen which competes with antigen present in the sample for immobilised antibody binding sites. The classic radioimmunoassay is an example of this type of assay. A plot of label bound to antibody as a function of analyte concentration, gives inverse, nonlinear standard curves. Labelled antibody assays (Figure 1-3(B)) make use of constant, limited amounts of analyte bound to the solid phase which compete with the analyte in the sample for labelled antibody binding sites. Again, inverse nonlinear standard curves are obtained from a plot of labelled antibody complexes against analyte concentrations. Although the detection limits of these assays may be improved with the use of labels with high specific activity, the smallest amount of analyte detected is ultimately limited by the affinity of the antibody employed.

Other reagent limited assays include immunoassays for specific antibodies. While most assays for specific antibodies are reagent excess assays, the use of limited reagent does offer advantages to specific applications; for example, in the determination of antibodies which bind only to a specific epitope of an antigen. In this assay, a monoclonal antibody, specific for the same antigenic determinant, is selected and labelled. A limited amount of this labelled antibody then competes with the specific antibodies in the sample for antigen immobilised on the solid phase. As the amount of specific antibodies in solution increases the label bound to the solid phase decreases.

Free analyte assays, used to measure hormones in their free form, are another form of reagent-limited assays. Since hormones are only active when not bound to carrier proteins, it is often useful to determine the amount of free hormone present in a sample.

Figure 1-3: Competitive Immunoassay Configurations. A) The labelled antigen configuration makes use of a labelled tracer molecule which is recognised by immobilised antibody. With the addition of analyte from the sample there is competition for the antibody binding sites, between the labelled tracer and the analyte. After completion of the immune complex formation, the unbound labelled tracer is removed. B) The labelled antibody configuration employs an immobilised antigen which competes with analyte in the sample, for binding sites on the labelled antibody. After completion of the immune complex formation, the unbound labelled antibody is removed. Ag = antigen; Ab = antibody.

A)


Remove unbound
labelled Ag
Signal Detection

B)

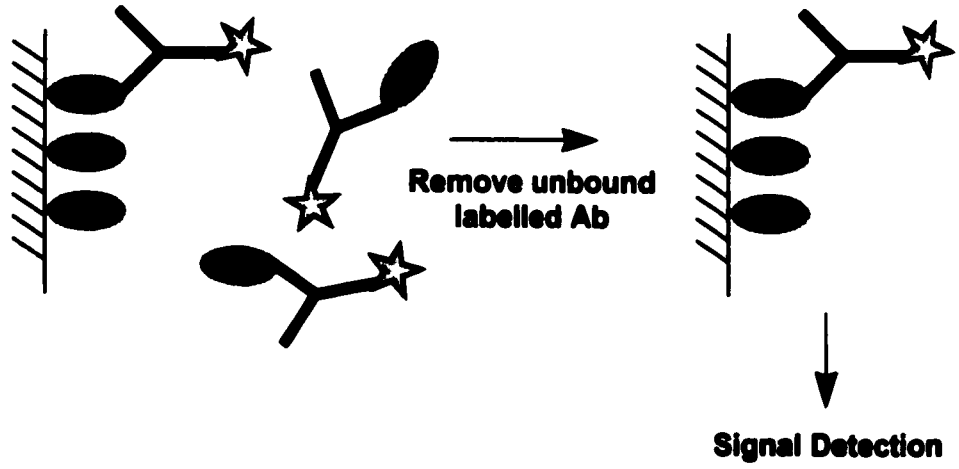

Remove unbound
labelled Ab
Signal Detection

Figure 1-3

The most accurate methods for detecting free hormones involve a separation step prior to analysis. However, newer competitive immunoassays are more convenient, less expensive and faster.

Homogeneous assays do not require a separation step prior to detection. These assays are simple and fast to perform. As a result they are often employed for monitoring levels of therapeutic drugs and drugs of abuse in blood and urine samples when low detection limits ($< 10^{-6}$ mol/L) are not required. Homogeneous assays are competitive assays which depend on the change in label activity as a consequence of antigen binding. Two types of enzyme-multiplied immunoassay techniques (EMITs) exist. In the first, enzyme activity is decreased with antigen binding (lysozyme or glucose-6-phosphate dehydrogenase labels) and in the second, enzyme activity is increased with antigen binding (malate dehydrogenase labels) (4). The cloned enzyme donor immunoassay (CEDIA) was developed on the basis of the well known α-complementation reaction of β-galactosidase (5, 6). Genetic engineering was used to generate and select appropriate inactive enzyme donor and enzyme acceptor molecules which spontaneously associate to form enzyme with full β-galactosidase activity. This is an example of a homogeneous competitive assay in which antigen is labelled with a molecule (the small enzyme donor molecule) which increases in activity with the presence of analyte in the sample.

Ambient Immunoassays

Ambient analyte assays are the fourth class of immunoassays. These are assays which make use of a primary antibody immobilised on microspots at such a low concentration that the degree to which the antibody is occupied by analyte is directly related to the analyte concentration. Both the immobilised "capture" antibody and the detection antibody are labelled and the ratio between the signal generated by the two labels is proportional to the amount of bound analyte.

1.1.3 Immunoassay Labels

The suitability of a labelling substance depends on its specific activity, solubility, low non-specific binding, ease of labelling, ease and reliability of detection, associated health and safety hazards and possibilities for incorporation into assay formulations. High specific activity of a label is essential for immunoassays with low detection limits. Specific activity is determined by:

i) the fraction available for detection, or the portion of label in each detection reaction that can be detected (eg. only a tiny percentage of an isotopic label decays during counting whereas chemiluminescent labels are all potentially available for detection)

i) the continuity of detection, or the ability of a label to be repeatedly detected (eg. chemiluminescent labels emit light only once whereas fluorescent labels may be repeatedly excited)

i) efficiency of detection, or the strength of the detection reaction (eg. an enzyme label is more efficiently detected using a chemiluminescent substrate than a colourimetric substrate)

i) the substitution ratio, or the number of labels incorporated per detection reagent.

Many different types of labels have been applied to immunoassay design since the first radioisotopic labels were introduced in 1959 (7). The current trend is to develop non-radioactive reporter molecules in order to avoid the disadvantages of using radioisotopes, including the need for proper licensing, the instability of the labels over time and the safety hazards. Although newer non-radioactive methods now comprise the majority of routine testing procedures, radioisotopic methods continue to play an important role in clinical and research testing. This is likely due to the fact that despite their problems, radiolabels offer easy, sensitive and reliable detection.

Fluorescence immunoassays all employ a fluorescent label. The most common methods in this type of immunoassay are the immunofluorometric assay (IFMA), the competitive binding fluoroimmunoassay (FIA), the fluorescence polarisation assay (FPIA), and the time-resolved fluoroimmunoassay (TRFIA). Reviews dealing with these techniques and other, less common fluorescent techniques, were recently published by Hemmila (8) and by Wood and Barnard (9). Advantages to assays which employ fluorescent labels are low detection limits with a wide analytical range. Time-resolved fluorescence determination of lanthanide chelate labels greatly reduces background fluorescence which is often observed with fluorescein and many other commonly used fluorescent labels (10).

Techniques based on chemiluminescent and bioluminescent labels are another group of non-radioactive immunoassays that are becoming more common in routine and research testing. The most common methods in this category are the competitive binding chemiluminescence immunoassay (CIA), the immunochemiluminometric assay (ICMA) and the electrochemiluminescence immunoassays. Chemiluminescence immunoassays were recently reviewed by Messeri (11). One difficulty that may be encountered in the development of luminescent immunoassays is the decrease in photoefficiency of some of these molecules when linked to large protein molecules, such as antibodies. This problem is not evident in all immunoassays and employing alternative chemiluminescent labels can often eliminate the difficulty (eg. acridinium esters may be used in place of isoluminol derivatives, which are adversely affected by linkage (12)). A number of bioluminescent immunoassays are actually enzyme assays. For example luciferase is an enzyme which catalyses a light producing reaction.

The first non-isotopic immunoassay, developed in 1971, employed an enzyme as a label (13, 14). Enzyme labels are the most commonly used labels in current routine immunoassays. A benefit of using enzymes as labels is the inherent signal amplification as a result of substrate turnover. Enzymes catalyse a wide range of reactions and a variety of naturally occurring and synthetic substrates allow the detection of enzyme activity through colourimetric, fluorimetric, time-resolved fluorimetric and luminometric assays. In this way enzymes can be used to develop assays with very low detection

limits. Unfortunately, the relatively large size of enzymes makes it impossible to attach more than one enzyme label to a detection antibody using standard conjugation methods. Also, some enzymes do not retain full activity with conjugation to a large protein molecule, such as an antibody.

One recurring theme for improvement of enzyme immunoassays is attachment of multiple enzyme labels to each immune complex or component to be detected. This has been achieved with various degrees of success through the use of the biotin—streptavidin affinity system (15). A biotinylated detection antibody or antigen can be linked to multiply labelled streptavidin. Streptavidin can also be used as a link between the biotinylated binding molecule and biotinylated reporter molecule. Since streptavidin has four biotin binding sites it can mediate the attachment of up to three reporter molecules to the binding molecule. The use of a polymeric biotin—streptavidin complex with residual biotin-binding ability has allowed the attachment of multiple reporter molecules.

A second approach to multiple labels is used in liposome immunoassays. These assays employ liposomes containing multiple detection molecules to label antibody reagents (16). After completion of the immune complex formation, the liposomes are lysed by complement, mellitin or phospholipase C, in homogeneous assays, or by detergent in heterogeneous assays. In each case, lysis of the liposome results in the release of multiple detection molecules into solution. Drugs, fluorophores, enzymes, enzyme substrates, RNA, DNA, ions and radioisotopes have all been used as marker molecules in liposome immunoassays. The difficult preparation of liposomes and the complications inherent in performing the detection assay in the presence of the lysing agent, are potential disadvantages to this method. This group of alternative immunoassays continues to be an area of active research and these disadvantages are quickly being remedied.

A novel approach for generating multiple detection molecules from a single immune complex has recently been proposed (17). The expression immunoassay makes use of an expressible DNA fragment as a label. Under appropriate conditions the label is expressed *in vitro* and multiple protein products are generated in solution. The expression

immunoassay has three potential levels of signal amplification; at transcription, at translation and, if the protein product is an enzyme, a third level is achieved with substrate turnover. The gene encoding luciferase under the control of the T7 promoter was used as a label in a sensitive expression immunoassay. Approximately 12-14 molecules of luciferase were produced from each DNA label which allowed significant signal amplification and, consequently, low detection limits.

As the repertoire of clinical tests continues to expand and the benefit of early detection of infectious agents and tumour markers is realised, the need for more sensitive detection methods coupled to specific immunoassays increases. One of the most active areas of research in the field of immunoassay design and development is the creation of more sensitive detection methods. Advances in this area are made every day but still a great deal of work remains.

1.2 Quantification of Specific Nucleic Acid Sequences

Knowledge of the molecular basis of many human diseases has resulted in the development of molecular biologic techniques for the purposes of diagnosis and prognosis using genetic information. In general these methods involve the quantification or sequencing of specific nucleic acid sequences. Several principles are common to all assays for quantification of specific DNA or RNA sequences. First a nucleic acid complementary to the sequence of interest is an essential component of all quantification techniques. This nucleic acid may be a DNA or RNA probe for solid phase or solution phase hybridisation assays, or it may be a target-specific oligonucleotide used to prime specific amplification of the sequence under study. The hybridising nucleic acid probe or primer is generally used in large molar excess over the target nucleic acid. The stringency of the conditions used for hybridisation is the second feature common to all quantification methods. In each case it is necessary to employ conditions which minimise hybridisation of the probe or primer to irrelevant sequences containing some degree of complementarity. Since nucleic acids do not have intrinsic properties to allow sensitive detection, a third component common to all quantification techniques is a

detection system. The detection system is designed to allow quantitation of the hybridised probe or of the amount of DNA or RNA produced from an amplification reaction. Parallel amplification or detection of a reference nucleic acid is a fourth component sometimes required for absolute or relative quantification of the target nucleic acid. A number of quantification methods for specific nucleic acids have been reviewed by Killeen (18). A brief description of these methods is provided here.

1.2.1 Quantification Techniques

Solid Phase Hybridisation Assays

All hybridisation assays exploit the inherent complementarity of DNA and RNA to allow detection of a specific target nucleic acid sequence. In solid-phase techniques the nucleic acids (target or probe) are immobilised before or during the hybridisation assays. The classic solid-phase assay, introduced by Southern in 1975 (19), involves electrophoresis of DNA followed by transfer to a solid support, typically a nylon membrane. The DNA must be size restricted prior to electrophoresis and it must be denatured before immobilisation on the solid support. The immobilised DNA is then probed by a labelled, complementary oligonucleotide in order to determine the position and the amount of the sequence of interest. Many different labels have been successfully incorporated into the detection probe, these will be discussed later. The Southern blot technique has been adapted for analysis of RNA using a similar procedure of electrophoresis, immobilisation and detection; this is referred to as a northern blot. Dot and slot blots involve the direct application of the RNA or DNA to a nylon membrane using a "dot" or "slot" manifold respectively. These techniques have been used extensively in research and routine testing laboratories.

Solid phase hybridisation assays can also be performed on the surface of microtitre wells. The principle of detection of a target sequence by a complementary probe is used in very small scale reactions (typically 25 - 100 μl) which allow sensitive quantification of the

specific nucleic acid sequence of interest. In these assays the limit of detection is ultimately a result of the detectability of the probe label.

Solution Hybridisation Assays

Solution assays do not employ a solid phase before or during the hybridisation step. The first example is the RNase Protection Assay. These types of assays were originally developed for mapping and sizing RNA molecules but have since been adapted for use as quantitative techniques. RNase protection assays rely on the ability of RNases A and T1 to hydrolyse regions of RNA that are not perfectly hybridised to the probe RNA. Radioactive labels are used to detect and quantify protected regions of the RNA target. This assay type is useful for determination of relative levels of expression of an RNA in various samples.

Other solution hybridisation formats have been used in clinical laboratories to produce quantitative results. These formats are often used to obtain information about microbial infections. One example is the detection of hepatitis B virus by using ^{125}I-labelled probes specific for the viral genome (20). The probes are allowed to hybridise to the target nucleic acid in solution before the hybrids are separated by size-exclusion column chromatography. This technique is not as sensitive as viral detection using PCR but it may be applied when extremely low detection limits are not required. The use of antibodies against RNA-DNA duplexes to capture hybrids formed between target RNA and complementary DNA probes has formed the basis of a novel hybridisation assay (18). Antibodies against DNA-DNA duplexes have also been used in hybridisation assays used to detect PCR products (21).

Amplification Techniques

Amplification techniques are used when it is necessary to detect and quantify a rare target sequence. It is only very recently that detection strategies have been developed which permit the direct detection of sub-attomole amounts of a specific nucleic acid. Amplification methods are used to generate multiple copies of the specific nucleic acid

before a detection step and thereby increase the sensitivity of the overall procedure. The polymerase chain reaction (PCR) (22) is the amplification method most commonly used for quantification of specific RNAs and DNAs. Determination of the amount of specific RNAs present in a sample requires an initial reverse transcriptase reaction to create cDNA that is used in the subsequent PCR; this technique is referred to as RT-PCR. The use of oligonucleotide primers designed to specifically amplify the sequence of interest, and conditions which minimise non-specific amplification allow PCR and RT-PCR to be extremely specific techniques.

Quantification of specific DNAs or RNAs by PCR requires the use of suitable internal controls. The theoretical yield of PCR is given by the equation:

$$P = T(1 + E)^n$$

where P is the number of molecules of PCR product, T is the number of copies of the initial target DNA sequence, n is the number of PCR cycles and E ($0 \leq E \leq 1$) is the overall efficiency of the PCR (18). The efficiency of a PCR process varies considerably between reactions and during reactions. As a result it is generally not acceptable to measure the concentration of final product in a single tube as a means of determining the concentration of starting target sequence. Instead, a suitable internal control is co-amplified in the same reaction. Then the ratio of product from the target sequence to product from the internal control is used to determine the amount of the specific nucleic acid sequence in the sample. The internal control may be amplified using the same primers used in amplification of the target sequence (competitive quantitative PCR) or using different primers (non-competitive quantitative PCR). In RT-PCR an RNA internal standard is also required to control for efficiency of the reverse transcription reaction. A number of quantitative analytical methods have been used to detect amplification products, for example: measurement of incorporated ^{32}P-labelled nucleotides, hybridisation assays, gel electrophoresis with staining and subsequent densitometry. In each case, amplification using PCR coupled with quantitative analysis of the amplification products has become a powerful tool in molecular diagnosis of disease.

A second amplification technique is referred to as Nucleic Acid Sequence Based Amplification (NASBA). This is an isothermal technique that makes use of the concerted activities of T7 RNA polymerase, avian myeloblastosis reverse transcriptase and ribonuclease H to amplify a target sequence, usually RNA (23, 24). Similar to PCR techniques, specificity of this reaction comes from the use of primers specific for the target sequence and the use of stringent conditions to minimise non-specific amplification. NASBA can generate a large increase in RNA target sequence (10^9 fold) which can then be detected using a number of methods. Similar to PCR techniques, internal controls are required to allow quantification of the original amount of target sequence.

Recently a third target amplification method has been applied to detection of HIV nucleic acid (25). Reverse transcription strand displacement amplification (SDA) is an isothermal reaction that can provide greater than 10^{10} fold amplification in 15 min (26). SDA is based on the ability of a restriction enzyme to nick a hemithioated recognition sequence and the ability of a DNA polymerase to initiate strand synthesis at the nick with simultaneous displacement of the downstream strand. The products are detected by measuring radioactivity incorporated during amplification and in RT-SDA the signal is related to the amount of RNA in the original sample..

Qβ replicase amplification methods allow quantification through amplification of a probe that hybridises to the target sequence of interest (27). Qβ replicase is an RNA-dependent RNA polymerase partially derived from Qβ bacteriophage. Another probe amplification technique recently adapted for determination of specific nucleic acid sequences, is rolling circle amplification. In this method a linear "padlock" probe (composed of two target-complementary segments connected by a linker sequence) hybridises to the target sequence such that its ends may be ligated and the probe circularised (28). The probe may only be circularised when it is bound to the target sequence. The circular DNA is then used as a template for isothermal rolling circle amplification to generate multiple concatenated copies of the probe through the action of a DNA polymerase. Detection of the products may be achieved with measurement of incorporated ^{32}P-labelled nucleotides

or through the use of a hybridisation assay. The signal is directly related to the amount of target nucleic acid present in the sample. The detection limit is determined by the stringency of the ligation reaction.

1.2.2 Detection Labels

As indicated above, many of the nucleic acid detection strategies require the use of a secondary detection system. The hybridisation assay is the most commonly used detection system. To date there is no consensus on the choice of a label for use in nucleic acid detection and no ideal label has been developed. A good label must exhibit good stability, low detection limit, ease of conjugation to detection probe without loss of activity, and speed and convenience of detection. Also, the reagents and equipment required for detection should be reasonably inexpensive. A great deal of research continues to focus on the creation of ultrasensitive, non-radioactive detection methods. The most recent advances in this area were recently reviewed by Kricka (29).

Just as radiolabels in immunoassays are being steadily replaced by non-isotopic labels, so too are radioactively labelled nucleic acid probes being replaced. Biotin and the hapten, digoxigenin, are two of the most commonly used non-radioactive tags developed. These labels serve to mediate efficient attachment of reporter molecules through biotin—streptavidin interaction and immunocomplex formation with anti-digoxigenin antibodies, respectively. Enzymes are the most common signal generating molecules used. As described for immunoassays, enzymes give rise to signal amplification through substrate turnover. Sensitive hybridisation assays have also been developed which rely on enzyme-amplified time-resolved fluorescence detection using alkaline phosphatase-labelled anti-digoxigenin antibodies (30). Other labels that have been used in hybridisation assays are fluorochromes such as fluorescein and rhodamine and lanthanide chelates (31).

Recent improvements in labels used for hybridisation assays include the use of acetate kinase as a reporter molecule which can be detected down to 8.6 zmol using a coupled bioluminescent assay; added luciferase is used to detect ATP from the acetate kinase

catalysed reaction to generate glow luminescence (29). Further advances have come as a result of protein engineering. Luciferase mutants that catalyse light emission at different wavelengths and mutants with improved stability have been used in sensitive hybridisation assays (29 and references therein). Further improvements in luciferase hybridisation assays have been made with the use of binding molecules—luciferase fusion proteins (eg. Protein A-luciferase (32), *Luciola* luciferase-biotin binding protein (33) and luciferase-RNA binding protein (34)). Fusion proteins of this type allow attachment of luciferase to a probe oligonucleotide without the loss of activity usually observed.

As described for immunoassay development, another strategy for improving assay sensitivity is through the attachment of multiple labels for detection of a single hybrid. Avidin or streptavidin reagents have been used to attach multiple labels using the techniques previously outlined. Also, DNA-tagged liposomes containing multiple dye molecules, have been incorporated into a rapid, dipstick-based hybridisation assay (35). The branched chain DNA (bDNA) technology introduces multiple labels to each hybrid and has achieved sensitivities that permit detection of rare target molecules without a prior amplification. In this case a probe is used which is bound to a preamplifier molecule to allow binding of multiple bDNA amplifiers. The bDNA amplifiers are synthetic branched DNA molecules which can each facilitate the binding of 45 alkaline phosphatase—conjugated labels (36). High background in these assays has been reduced through inclusion of novel nucleotides to prevent non-specific binding (37).

Rather than direct attachment of multiple enzyme labels to a detection probe, another approach has been to attach a label capable of creating multiple enzymes in solution. The expression detection system described for immunoassays has been extended to nucleic acid hybridisation assays (38). A gene for luciferase was incorporated into a label which allowed the detection of down to 0.1 fmol of target DNA (39). The sensitivity of this system is a consequence of the high detectability of the luciferase enzyme combined with the signal amplification derived from *in vitro* expression.

Molecular testing is being utilised more frequently for diagnosis of disease. Often early diagnosis plays a role in the outcome of the disease which requires that these tests are reliable and sensitive. For this reason and others a great deal of research is aimed at developing methods and labels which will permit ultrasensitive detection of specific nucleic acids.

1.3 Signal Amplification Using *In Vitro* Expression

Cell-free transcription and translation systems were valuable tools for the elucidation of the genetic code and for characterisation of the protein producing machinery. The first *in vitro* system, described by Zubay (40), was based on the use of relatively crude *E. coli* extracts as the source of expression machinery. Eukaryotic expression systems, which also couple transcription and translation, have been developed based on rabbit reticulocyte lysates and wheat germ extracts. Each system allows the use of defined DNA templates to direct the synthesis of protein which can be characterised as necessary. This characteristic has ensured that these systems remain valuable tools with a variety of applications. For example, cell-free transcription and translation methods are used in studies of regulation of gene expression (41), for the production of proteins that are poorly expressed *in vivo* (42) and in *in vitro* evolution studies (43). Kits are now commercially available which are optimised for efficient expression of either prokaryotic (*E. coli* S-30 systems) or eukaryotic (rabbit reticulocyte lysate and wheat germ extract systems) DNA templates.

In vitro expression offers the potential for signal amplification in immunoassays and nucleic acid hybridisation assays if an appropriate DNA template is employed as a label. Figure 1-4 illustrates the possible levels of amplification that occur during expression. Each DNA template is capable of producing multiple mRNA molecules through the action of an RNA polymerase. Each mRNA molecule is then able to generate multiple protein molecules by translation. Depending on the product, the protein can be used directly to generate signal or, if the product is an enzyme, it can act on substrate molecules to generate signal. If the product of *in vitro* expression is an enzyme an

Figure 1-4: Principle of Signal Amplification by *In Vitro* Expression. Transcription occurs *in vitro* to generate multiple mRNA molecules. Each mRNA molecule is translated *in vitro* to yield multiple protein products which can be detected directly or through their action on substrate molecules to generate detectable product. Both transcription and translation are considered to be amplification steps.

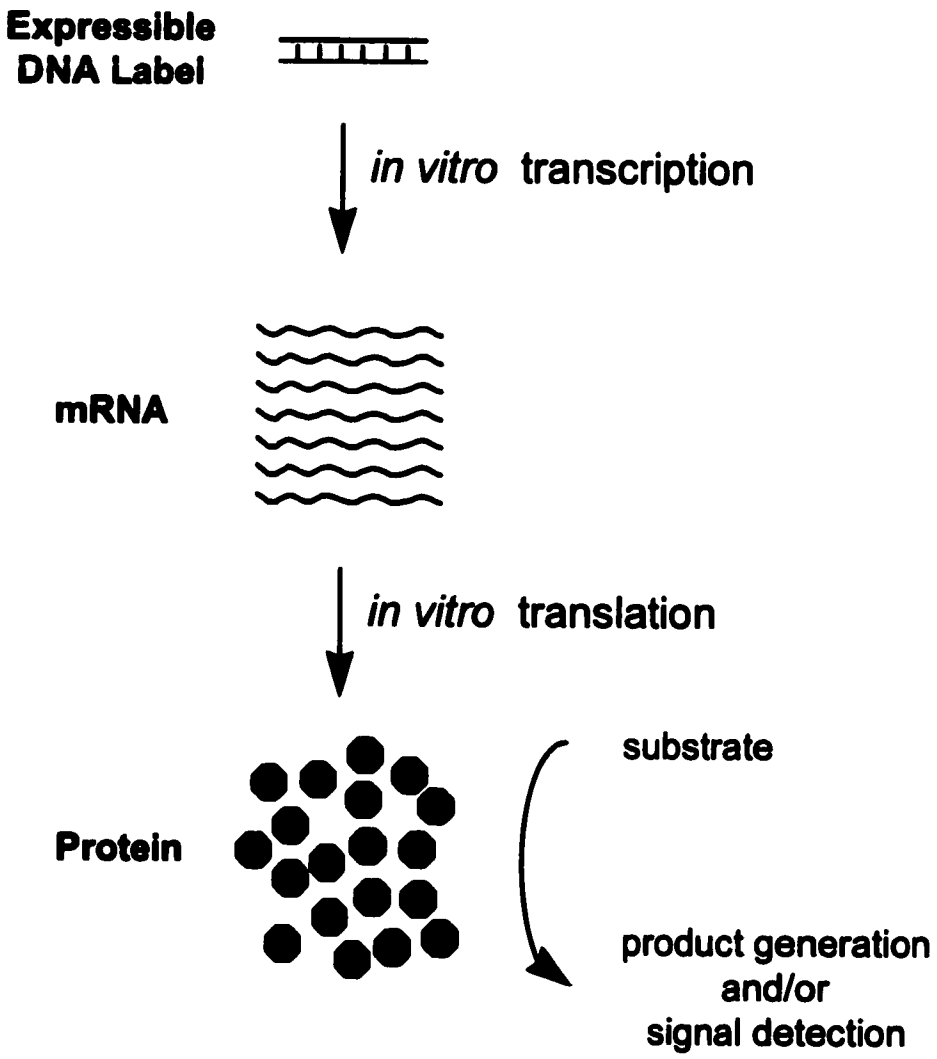

Expressible
DNA Label
in vitro transcription
mRNA
in vitro translation
Protein
substrate
product generation
and/or
signal detection

Figure 1-4

additional level of amplification is achieved through substrate turnover. As described previously, the gene for luciferase has been successfully incorporated as a label in both immunoassay and hybridisation assay procedures.

The design of the DNA label is critical to the development of a sensitive assay procedure and a number of criteria must be considered. The enzyme product of the *in vitro* transcription/translation reaction must be able to assume its active conformation in the expression mixture without the need for post-translational modifications or folding machinery which may not be present/active in the cell extracts. Also, enzyme products must be able to act on substrate molecules to generate easily and dependably detectable products within the expression reaction mixture. This serves to eliminate any need for purification or pretreatment procedures prior to substrate addition and signal detection. Ideally, the protein produced should be a monomer or an oligomer of identical subunits. In this way a single gene may be incorporated in the DNA label. Alternatively, if the active protein is an oligomer of non-identical subunits, the *in vitro* expressed polypeptide should be capable of spontaneous association with exogenously added subunits to form active multimeric enzyme. In addition to these considerations the DNA label itself should be amenable to attachment to the detection molecule while retaining its expressibility. For this reason the DNA should be relatively short to facilitate labelling and also because, in theory, shorter genes will yield more protein product. In construction of a DNA label for use in an expression detection system it is not always possible to meet all the above criteria, and in fact it may not always be necessary to do so.

The purpose of the present work was to develop novel DNA labels for use in sensitive expression detection systems. In particular the DNA labels studied code for proteins which are inactive themselves but are capable of triggering a signal generating system. The first area of research involved the use of a small DNA label encoding a peptide with no inherent enzymatic activity. The peptide was able to complement with a second exogenously added peptide to generate active enzyme. A number of DNA templates encoding this peptide were synthesised as potential labels for use in an immunoassay. The second area of research involved the use of a DNA label which encoded a peptide

capable of combination with a cofactor to create a bioluminescent signal generating system. Portions of this work was applied to the final study described here, in which an isothermal amplification system was developed for generation of protein from traces of DNA template.

2. Expression Immunoassay Using DNA Encoding the α-Fragment of β-Galactosidase as a Label

2.1 Introduction

The enzyme β-galactosidase (β-Gal) and its gene have been studied for many years and are among the most widely used tools in molecular biology. β-Gal has proved to be useful as part of a fusion protein, and as a marker or reporter molecule. Since the enzyme retains activity upon conjugation to antibodies, β-Gal has also played an important role as a label in enzyme linked immunosorbent assays (ELISAs). β-Gal catalyses the cleavage of β-glycosidic bonds (44) and may be easily detected through the use of substrates containing a glucose moiety which, upon cleavage, yield chromogenic, fluorogenic or luminogenic products. Depending on the substrate used this enzyme may be detected in extremely low amounts, for example, a chemiluminescent assay which makes use of a 1,2-dioxetane derivative containing a cleavable β-D-galactoside group, allows the detection of β-Gal in the femtogram range (45).

β-Gal is a large, tetrameric enzyme made up of identical subunits each with a molecular weight of 116 kDa (1029 amino acids) (46). Each subunit is composed of 5 domains with an additional ~50 residues at the N-terminal end which are relatively extended and are required for subunit association and catalytic activity (47). α-Complementation of β-Gal is an intracistronic complementation which can occur between inactive peptide fragments of β-Gal to form active enzyme (48, 49, 50). This phenomenon is the basis for the well known blue/white screening used to identify successful recombinant DNAs in host bacteria. A small N-terminal portion of β-Gal is referred to as the α-peptide and corresponds to the extended N-terminal residues of the native β-Gal subunits. α-

Complementation will occur between a mutant or truncated protein which contains the α-peptide and a second mutant peptide which has a deletion within the α-peptide region. These peptides are known as α-donors and α-acceptors, respectively, and neither exhibits any β-Gal activity. When α-donor and α-acceptor peptides are mixed together α-complementation occurs and active, complemented β-Gal is formed. The complemented enzyme is a pseudotetramer made up of four α-donors and four α-acceptors (Figure 2-1). In comparison to the wild-type enzyme, the complemented enzyme has full β-Gal activity, the same K_m value and differs only by a slightly lower stability at raised temperatures and high urea concentrations (49).

α-Complementation has an equilibrium constant of 1 to $2 \times 10^9\ M^{-1}$ (51). The α-acceptor proteins are able to form inactive dimers in solution but cannot associate to create active enzyme. The formation of active enzyme through α-complementation is a complex process involving fast association/dissociation equilibria between the dimeric α-acceptor and one or two monomeric α-donor molecules (52). The next step is a rate-determining first-order conformational change of the α-donor/α-acceptor complex. Finally, these intermediates associate in an extremely fast reaction to form the complemented enzyme with full β-Gal activity. This mechanism is summarised by the following equations:

$$a_2 + 2d \leftrightarrow a_2d + d \leftrightarrow (a_2d_2)'$$

$$2(a_2d_2)' \xrightarrow{k} 2(a_2d_2) \xrightarrow{\text{fast}} (a_2d_2)_2$$

where a is the α-acceptor and d is the α-donor. This reaction mechanism is analogous to the folding mechanism observed in the reactivation of native β-Gal from its dimeric precursors.

In order to generate wild-type β-Gal from a DNA label in an expression immunoassay it would be necessary to use the large lacZ gene (3069 bp) plus an appropriate promoter sequence as the label. In order to avoid the use of such an unwieldy label, a fragment of DNA capable of expressing the α-peptide may be used as a label. Through the use of α-complementation it is possible to generate a protein with β-Gal activity by *in vitro* expression of the α-peptide followed by the addition of an α-acceptor protein, such as the

Figure 2-1: Schematic of β-Galactosidase α-Complementation. A) Native β-galactosidase exists as a tetramer of identical subunits. B) Mutant β-galactosidase lacking N-terminal amino acids form inactive dimers in solution. When α-donor peptides are added the active, pseudotetrameric enzyme is formed.

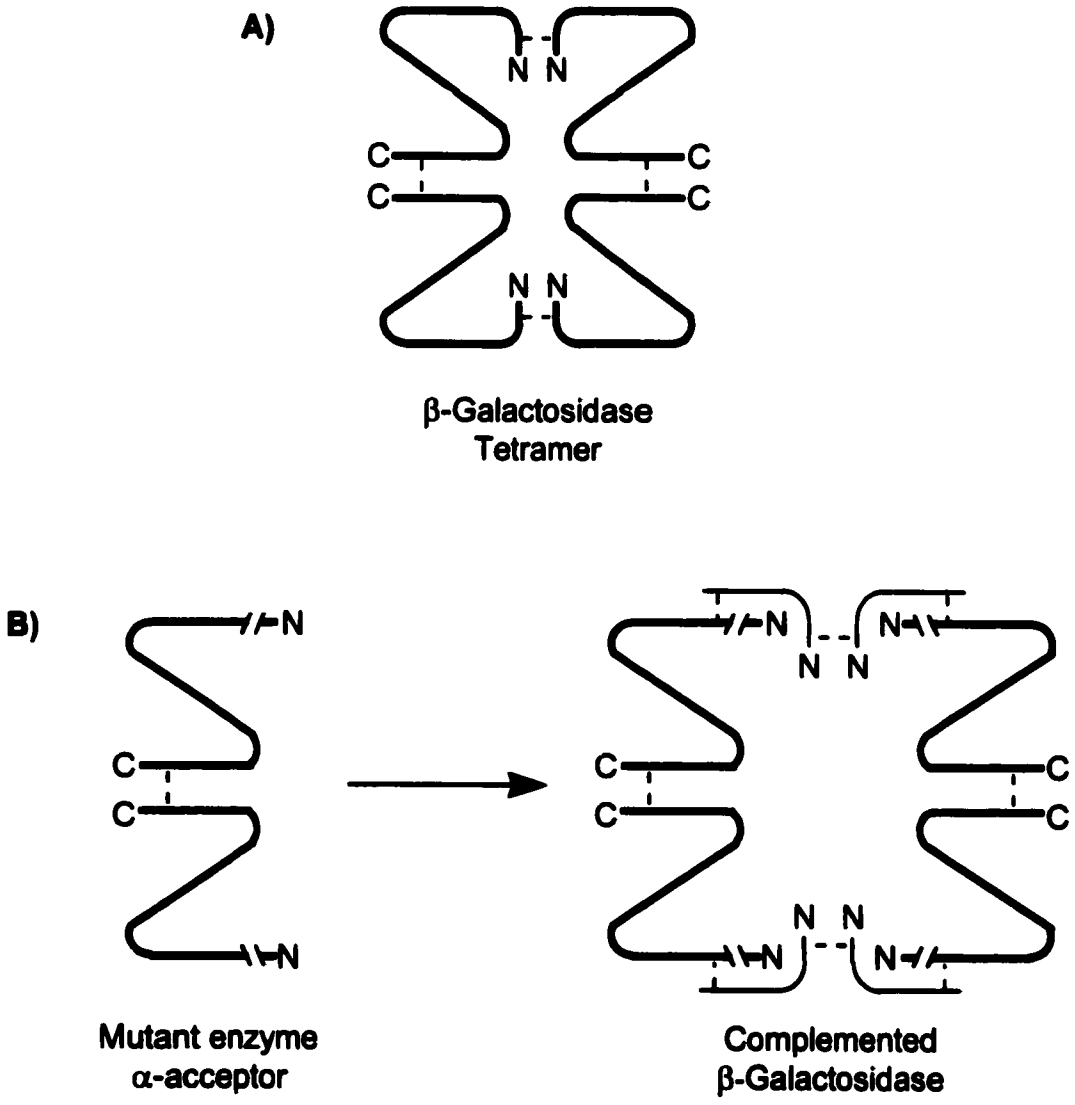

A)
N N
C
C
C
C
N N
β-Galactosidase
Tetramer
B)
N
C
C
N
N
N
N N
C
C
C
C
N N
N
N
Mutant enzyme
α-acceptor
Complemented
β-Galactosidase

Figure 2-1

M15 protein from the *E. coli* strain JM109. This protein is produced from the lacZΔM15 gene which produces mutant β-Gal with a deletion of amino acids 11-41 within the N-terminal portion of the protein (48). In solution the M15 proteins form inactive dimers which can each associate with two α-peptides. After a conformational change of the resulting complex the final pseudotetrameric enzyme is formed (as outlined above). This α-complementation can occur *in vitro* and is the basis of an ExIA which uses the α-peptide encoding DNA fragment as a label.

2.2 Experimental

2.2.1 Instrumentation

An imaging densitometer (Model GS-670, Bio-Rad Laboratories Ltd., Mississauga, ON), along with the Molecular Analyst™ version 1.2 software was used for the quantitation of DNA fragments after agarose gel electrophoresis. The miniature horizontal gel system MLB-06 from Tyler Research Corp. (Edmonton, AB) was used for electrophoresis. High performance liquid chromatography (HPLC) was performed using the Shimadzu system (Shimadzu Corp., Kyoto, Japan) with absorbance monitoring. The G24 environmental incubation shaker from New Brunswick Scientific (Edison, NJ, USA) was employed for culturing bacteria. The Fluoroskan II fluorescence plate reader from Labsystems (Ingram and Bell Scientific, Don Mills, ON) was used for all fluorescence measurements in microtitre wells.

2.2.2 Materials

Reagent	Supplier
Restriction enzymes Ngo MI and Afl III	New England Biolabs (Beverly, MA, USA)
Klenow fragment of DNA polymerase I (exonuclease free)	United States Biochemical (Cleveland, OH, USA)

Reagent	Supplier
Ultrapure 2′-deoxynucleotidyl 5′-triphosphates (dNTPs)	Pharmacia Biotech (Montreal, PQ)
Linear DNA markers, supercoiled DNA markers, streptavidin, β-galactosidase (isolated from *E. coli*), isopropyl thiogalactoside (IPTG), 4-methylumbelliferyl-β-D-galactoside (4-MUG), 4-methylumbelliferone (4-MU), ampicillin, diethylpyrocarbonate (DEPC)	Sigma (St. Louis, MO, USA)
Bio-Rad Protein Assay Reagent	Bio-Rad Laboratories (Hercules, CA, USA)
adenosine triphosphate (ATP), bovine serum albumin (BSA), blocking reagent (cat. # 1096 176)	Boehringer (Laval, PQ)
biotin-14-dCTP, U-bottomed polystyrene microtitre wells (Nunc, Maxisorp)	Life Technologies (Burlington, ON)
Microlite-2 (white, flat-bottomed polystyrene microtitre wells)	Dynatech Laboratories (Chantilly, VA, USA)
Monoclonal anti-thyrotropin antibody	Jackson Immunoresearch Laboratories (BioCan, Mississauga, ON)
Microcon-30 microconcentrators	Amicon (Beverly, MA, USA)
E. coli S-30 Extract system for Linear Templates, plasmid pGEM 13Zf(+), *E. coli* JM109, Wizard Maxipreps DNA purification system	Promega (Madison, WI, USA)

2.2.3 Solutions

10× Blocking Solution

1.0 g blocking reagent

10 ml maleic acid buffer

Microwave, turn microwave on and off to avoid boiling over, until the blocking reagent has dissolved. Store at 4°C.

Maleic Acid Buffer

5.80 g maleic acid

4.38 g NaCl

Dissolve in 400 ml ddH_2O and adjust pH to 7.5 using 10 M NaOH. Add ddH_2O to a final volume of 500 ml and store at 4°C

Antigen Coating Buffer

8.401 g $NaHCO_3$

Dissolve in 900 ml ddH_2O and adjust pH to 9.6. Add ddH_2O to a final volume of 1 l and store at 4°C..

Phosphate-buffered saline (PBS)

4 g NaCl

0.1 g KCl

1.43 g $Na_2HPO_4 \cdot 7H_2O$

0.12 g KH_2PO_4

Dissolve in 400 ml ddH_2O and adjust the pH to 7.4. Add ddH_2O to a final volume of 500 ml, autoclave and store at 4°C.

Tris-EDTA (TE) Buffer

0.61 g Tris base

0.19 g EDTA (sodium salt)

Dissolve in 400 ml ddH_2O and adust the pH to 8.0 using concentrated HCl. Add ddH_2O to a final volume of 500 ml, autoclave and store at 4°C.

10× Wash Solution

30.3 g Tris base

43.8 g NaCl

5 ml Tween-20

Disslove the Tris base and NaCl in 400 ml ddH_2O and adjust the pH to 7.4 using concentrated HCl. Add the Tween-20 and ddH_2O to a final volume of 500 ml. Note: dilute 10 fold for use in an assay.

M-9 plus thiamine-HCl agar plates

11.33 g $Na_2HPO_4 \cdot 7H_2O$

3.0 g KH_2PO_4

0.5 g NaCl

1.0 g NH_4Cl

Dissolve in 900 ml ddH_2O and adjust pH to 7.4. Add 15.0 g agar, bring the volume to 1 l with ddH_2O and autoclave. Cool the sterilised solution to 50°C and add:

2.0 ml 1 M $MgSO_4$

0.1 ml 1 M $CaCl_2$

10 ml 20 % glucose

1.0 ml 1 M thiamine-HCl

Mix well, pour the solution into culture plates and keep the plates at room temperature until the mixture has set. Store the plates at 4°C.

LB Broth and Agar Plates

10 g tryptone

5 g yeast extract

10 g NaCl

0.5 ml 4 M NaOH

Dissolve in 1 l ddH_2O and autoclave. Store the broth at 4°C.

LB agar plates are prepared by adding 15.0 g of agar per 1 l LB broth before autoclaving. If necessary, antibiotic is added after the suspension has cooled to at least 50°C. Pour the solution into culture plates and keep the plates at room temperature until the mixture has set. Store the plates at 4°C.

β-Galactosidase Assay Buffer

0.138 g NaH_2PO_4

0.584 g NaCl

20.3 mg $MgCl_2$

0.1 g NaN_3

0.1 g BSA

Dissolve in 90 ml ddH_2O and adjust the pH to 7.0. Add ddH_2O to a final volume of 100 ml and store at 4°C.

DEPC-Treated ddH_2O

Add 1 ml of diethylpyrocarbonate (DEPC) to 1 l of ddH_2O with stirring. Keep stirring at room temperature for at least one hour and let stand overnight before autoclaving. Store at 4°C.

2.2.4 Preparation of M15 Extract

The extract was prepared using a procedure based on that of Lin and Zabin (53). Briefly, *E. coli* JM109 cells {genotype: recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, λ-, Δ(lac-proAB), [F′ traD36, proAB, lacIqΔM15]} (54) were first grown on M-9 agar plates containing thiamine-HCl in order to select for the presence of the F′-episome which contains the M15 protein coding sequence. A colony of the JM109 cells was used to inoculate LB broth containing 0.5 mM IPTG, in order to stimulate production of the M15 protein. The resulting 6 l culture was harvested, in exponential growth phase at 37°C, by centrifugation (5000 × g, 10 min at room temperature) and was resuspended in 20 ml of 0.1 M NaH_2PO_4, pH 7.2. After lysis of the cells using a French pressure cell the cellular debris was removed by centrifugation at 49 000 × g for 40 min at 4°C. The supernatant solution containing approximately 17 mg/ml of protein (determined using Bio-Rad Protein Assay according to the procedure provided by the manufacturer) was divided into 1 ml aliquots and stored at –20°C until use.

2.2.5 Preparation of the α-Peptide Encoding DNA Template

For growth of *E. coli* JM109 cells, preparation of competent cells and transformation with the plasmid DNA standard procedures were followed (41). Transformed bacteria were grown overnight in LB broth containing 0.1 g/l ampicillin. The plasmid DNA was purified from a 1 l bacterial culture with the Wizard maxipreps DNA purification system according to the manufacturer's instructions. The size of the plasmid was confirmed by

agarose (1.2 %) gel electrophoresis and ethidium bromide staining, using the supercoiled markers. The plasmid concentration in solution was determined from its absorbance at 260 nm (1 Abs unit = 50 μg/ml of DNA).

The template containing the α-peptide coding sequence under the control of the lac operator and promoter was obtained by restriction digestion of the plasmid pGEM 13Zf(+). The plasmid was obtained in substantial amounts following transfection into *E. coli* JM109 cells, overnight culture in 1 l LB broth containing 0.1 g/l ampicillin and isolation and purification using Wizard maxipreps DNA purification system according to the manufacturer's directions. An aliquot of the purified plasmid DNA was first digested overnight at 37°C with Ngo MI:

pGEM 13Zf(+) (3.4 μg/μl)	60 μl
10× NEB buffer 4	8 μl
Ngo MI (10 U/μl)	10 μl
ddH_2O to final volume	80 μl

Subsequently a fill-in reaction was initiated by adding biotin-14-dCTP, dGTP and the Klenow fragment of the DNA polymerase I to the digestion reaction:

NEB buffer 4	2 μl
Ngo MI digestion mixture	80 μl
dGTP (0.1 mM)	9.6 μl
B-14-dCTP (0.4 mM)	2.4 μl
Klenow fragment (10 U/μl)	8 μl
	102 μl

After incubation at 37°C for 1.5 h the reaction was terminated by heating the mixture at 65°C for 10 min. A second digestion was then carried out by adding Afl III and incubating at 37°C overnight:

Fill-in reaction mixture	102 μl
NEBuffer 4	2 μl
Afl III (5 U/μl)	20 μl
	124 μl

After digestion the DNA fragments were separated by agarose (1.2 %) gel electrophoresis and stained with ethidium bromide. The band corresponding to 737 bp was excised and isolated by centrifugation using a modified version of the method described by Wu and Welsh (55). The agarose slices were placed in microcentrifuge tubes and frozen, 30 min at -80°C, then thawed at room temperature (1 h). The tubes were centrifuged 20 min at 14 000 × g and the supernatant was collected. Sterile ddH_2O (100 µl) was added to each tube before a second centrifuge for 20 min at 14 000 × g. The supernatant was collected and pooled with the first. The addition of H_2O and centrifugation was repeated and the supernatant was again collected and pooled with the first two. The DNA was obtained from the pooled supernatants by ethanol precipitation (41). The DNA pellet was dissolved in sterile ddH_2O and stored at -20°C. The concentration of the lacZα DNA template was determined by scanning densitometry of a photographic negative taken from an ethidium bromide stained agarose gel on which a portion of the DNA solution was electrophoresed along with a known amount of linear DNA markers (in a separate lane). The markers were used to generate a standard curve.

2.2.6 Preparation of Streptavidin-lacZα DNA Template Complex

The streptavidin-DNA template complex was prepared by mixing the biotinylated lacZα DNA with an excess of streptavidin:

biotinylated lacZα DNA (0.11 pmol/µl)	40 µl
streptavidin (1mg/ml)	25 µl
10× TE buffer	16 µl
	81 µl

This mixture was incubated for 30 min at room temperature. The complex was purified by HPLC using a size exclusion column (Bio-Sil Sec 400-5, 300 × 7.8 mm from Bio-Rad Laboratories) isocratically, using 0.5 M NaH_2PO_4, 0.5 M Na_2HPO_4 and 0.15 M NaCl, pH 6.8 as the mobile phase. The flow rate was maintained at 0.5 ml/min. Absorbance was monitored at 260 nm. A fraction, with an approximate volume of 2 ml, corresponding to a peak centred at 11 min was collected. Blocking solution (200 µl) was added, as a

carrier, and the mixture was concentrated using a Microcon™ 30 microconcentrator. Six microlitres of 10 mM EDTA was added to the concentrated DNA complex solution such that the final volume was 66 µl. A 5 µl aliquot of this preparation was electrophoresed and the DNA concentration was determined by scanning densitometry of the negative, as described above.

2.2.7 Fluorometric Assay of β-Galactosidase

A stock 60 mM solution of 4-MUG was prepared in dimethyl sulfoxide (DMSO). The working substrate solution for the β-Gal assay was prepared by diluting the stock 4-MUG solution to 0.2 mM in the β-Gal assay buffer. For the β-Gal assay, 50 µl of the sample was added to 50 µl of the substrate solution in a microcentrifuge tube and incubated at 37°C for 1 h. The reaction was terminated by adding 50 µl of a stop buffer containing 1 M glycine, pH 10.3. A 130 µl aliquot was removed and placed in a white, flat bottom microtitre well and the fluorescence was measured using the Fluoroskan II fluorometer, with excitation and emission wavelengths at 355 nm and 460 nm, respectively.

2.2.8 Quantitation of Immobilised Antigen by Expression Immunoassay

For the purpose of this work a monoclonal anti-thyrotropin antibody was used as the analyte. Solutions with various concentrations of this analyte were prepared by dilution in antigen coating buffer. Analyte was immobilised by pipetting 25 µl of each solution into separate 'U' bottom polystyrene microtitre wells and incubating overnight at 4°C. The coating solution was then aspirated and the remaining binding sites were blocked with 1× blocking solution for 90 min at room temperature. The wells were washed once with wash solution and 25 µl of a solution of biotinylated goat anti-mouse antibody (5 µg/ml), diluted in blocking solution, was pipetted into each well. After incubation for 30 min at room temperature the wells were washed four times with wash solution in order to remove excess biotinylated antibody. Then, 25 µl of the DNA-streptavidin complex (3.0 ng/µl with respect to DNA), diluted in 1× blocking solution, were added to each well.

The wells were incubated at room temperature for 15 min, to allow binding of the complex to the biotinylated antibody. The excess DNA complex was removed by washing five times with wash solution followed by three washes with TE buffer. Then, 25 μl, per well, of the *E. coli* S-30 extract transcription/translation mixture (prepared according to the manufacturer's directions) was added and the wells were incubated at 37°C for 90 min to allow expression of the DNA template bound to the immunocomplexes. Subsequently, 20 μl was transferred from each well into separate, labelled microcentrifuge tubes and incubated at 70°C for 30 min to destroy endogenous β-Gal activity. After cooling, 20 μl of the M15 extract was added to each tube and the complementation reaction was allowed to proceed for 1 hour at 37°C. The resulting mixture was diluted to 50 μl with the β-Gal assay buffer and β-Gal activity was determined as described above.

2.3 Results and Discussion

2.3.1 *In Vitro* Expression to Quantify DNA Encoding the α-Peptide

The DNA template used as a label in this study was a linear DNA fragment isolated from plasmid pGEM 13Zf(+) which contains the coding sequence for the α-peptide of β-Gal under the control of the lac operator and promoter. The plasmid also contains the β-lactamase gene which confers ampicillin resistance to the host bacteria and allows selection of *E. coli* which were successfully transformed with this plasmid. Isolated and purified pGEM 13Zf(+) was first digested with Ngo MI to produce linearised plasmid with recessed 3′ ends. Ngo MI cuts the plasmid downstream of the α-peptide coding sequence. In order to add a biotin tag to the DNA the recessed ends were "filled in" through the action of Klenow fragment of DNA polymerase in the presence of biotin-14-dCTP (biotin attached at the N^4 position of cytidine through a 14-atom linker) and dGTP. In this way both ends of the linearised plasmid were labelled with biotin. A second restriction digestion was performed on the biotinylated DNA, using Afl III which cuts upstream of the lac operator and promoter sequences. The product of this second

Figure 2-2: Preparation of 3′-Biotinylated lacZα DNA. The plasmid, pGEM 13Zf(+) was digested with Ngo MI and the resulting recessed ends were filled-in by the Klenow fragment in the presence of B-14-dCTP. After a second restriction digest, with Afl III, the biotinylated lacZα DNA was electrophoresed and purified.

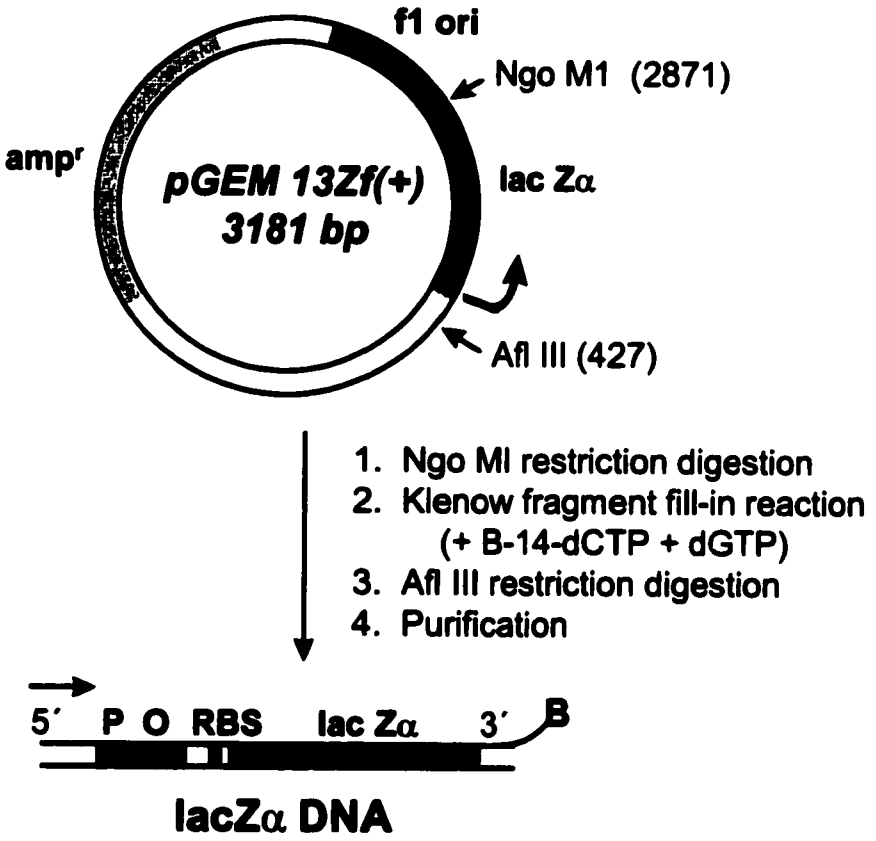

f1 ori
Ngo M1 (2871)
amp^r
pGEM 13Zf(+)
3181 bp
lac Zα
Afl III (427)
1. Ngo MI restriction digestion
2. Klenow fragment fill-in reaction
(+ B-14-dCTP + dGTP)
3. Afl III restriction digestion
4. Purification
5′ P O RBS lac Zα 3′ B
lacZα DNA

Figure 2-2

restriction digest was a 2444 bp fragment and the 737 bp fragment containing all of the sequences required for expression of the α-peptide. The 737 bp fragment also has a biotin moiety at its 3′ terminus. The 737 bp fragment was separated from the 2444 bp fragment by agarose gel electrophoresis and subsequently excised and purified. The resulting DNA fragment, lacZα DNA, contains all of the components necessary for expression of the α-peptide by cell-free transcription/translation using an *E. coli* based system.

The *E. coli* S-30 Extract system for linear templates, from Promega, was used for *in vitro* expression of the lacZα DNA. This kit is composed of a crude S-30 extract from *E. coli* which contains the ribosomes, transcription and translation factors etc., a premix which is a buffer containing the NTPs, tRNAs, an ATP generating system and IPTG, and amino acids. No exogenous RNA polymerase was added since this system makes use of the *E. coli* RNA polymerase. The mRNA produced from transcription of the lacZα DNA contains a ribosome binding site which allows translation in a prokaryotic system. By itself the α-peptide is inactive; however, after complementation with the M15 protein an active enzyme is formed (as described previously). The M15 protein is expressed in *E. coli* JM109 cells after induction with IPTG. A crude cell extract prepared from a large scale culture of these induced bacteria was used as the source of M15 protein for *in vitro* α-complementation.

Initial experiments indicated that the *E. coli* S-30 extract used in the *in vitro* transcription/translation mixture contained a significant amount of endogenous β-Gal activity. Heat inactivation was studied as a means of removing this activity the reaction mixture. Aliquots of *in vitro* expression reactions were heated to temperatures ranging from 65 to 75°C for 15 or 30 min. It was found that incubation for 30 min at 70°C was sufficient to eliminate the endogenous β-Gal activity without adversely affecting the α-peptide produced from expression of the DNA template.

After expression of the α-peptide, followed by heat inactivation of endogenous β-Gal activity, the M15 protein was added to allow α-complementation to occur. The time

dependence of this α-complementation reaction was studied by allowing aliquots of a cell-free transcription/translation containing 8.6 ng of lacZα DNA (heat inactivated to remove existing β-Gal activity) to react with M15 protein for various periods of time. In each case the complementation reaction was carried out at 37°C. After complementation the amount of β-Gal activity generated was determined using the fluorometric β-Gal assay. The results, shown in Figure 2-3, indicated that, under the conditions used, approximately 90 % of the α-complementation reaction was complete after a 1 hour incubation. This incubation time was used for α-complementation in all subsequent studies.

Further optimisation of the complementation reaction was performed by studying the amount of M15 extract added to the reaction mixture. In order for the expression of the lacZα DNA to act as a detection system it was essential that the amount of active enzyme formed is not limited by the amount of M15 extract added. Various dilutions of M15 extract with a total volume of 50 µl were added to an equal volume of the product of a transcription/translation of 8.6 ng of template DNA. After incubation of each complementation reaction for 1 h at 37°C the amount of β-Gal activity was determined. The results are illustrated in Figure 2-4 with fluorescence plotted as a function of protein concentration contributed by the addition of the M15 extract. For the purposes of this study undiluted extract was added to an equal volume of the expression reactions in all following experiments.

To assess the performance of the lacZα DNA as a label, various amounts of the DNA were subjected to expression in identical *in vitro* transcription/translation reactions (90 min at 37°C) followed by heat inactivation of endogenous β-Gal activity and α-complementation with the M15 protein. The β-Gal activity was determined using the fluorometric assay and in Figure 2-5 fluorescence was plotted against amount of DNA added to the expression reaction. It was found that the amount of DNA template introduced to the cell-free transcription/translation reaction was linearly related to the fluorescence over the range of 0.3 to 80 fmol of lacZα DNA.

Figure 2-3: Time Dependence of the α-Complementation Reaction. Aliquots (4 μl) of a typical *in vitro* expression reaction containing 8.6 ng of template DNA, were incubated with an equal volume of M15 extract for various times at 37°C. The reaction mixtures were then assayed for β-galactosidase activity.

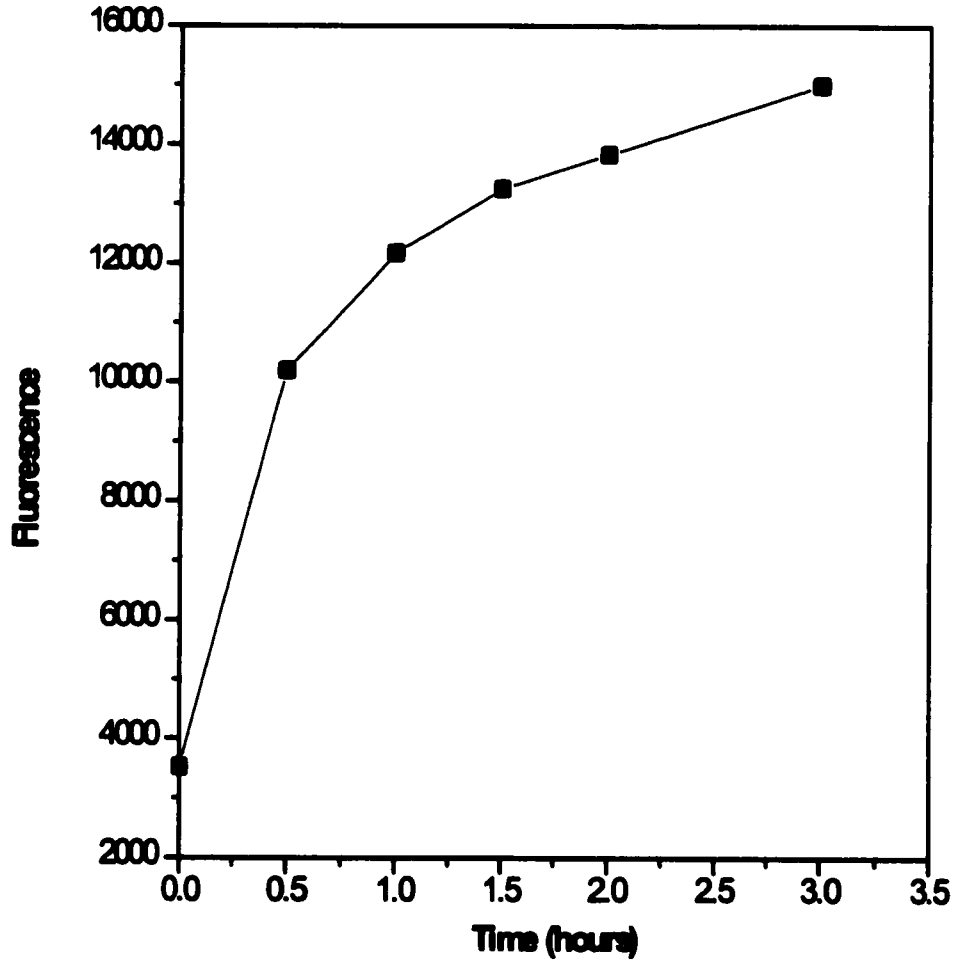

16000
14000
12000
10000
8000
6000
4000
2000
Fluorescence
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Time (hours)

Figure 2-3

Figure 2-4: Effect of the Concentration of M15 Extract Used in the α-Complementation Reaction. Aliquots (4 μl) of a typical *in vitro* expression reaction containing 8.6 ng of template DNA, were incubated with equal volumes of various dilutions of M15 extract. After 1 hour at 37°C the reaction mixtures were assayed for β-galactosidase activity.

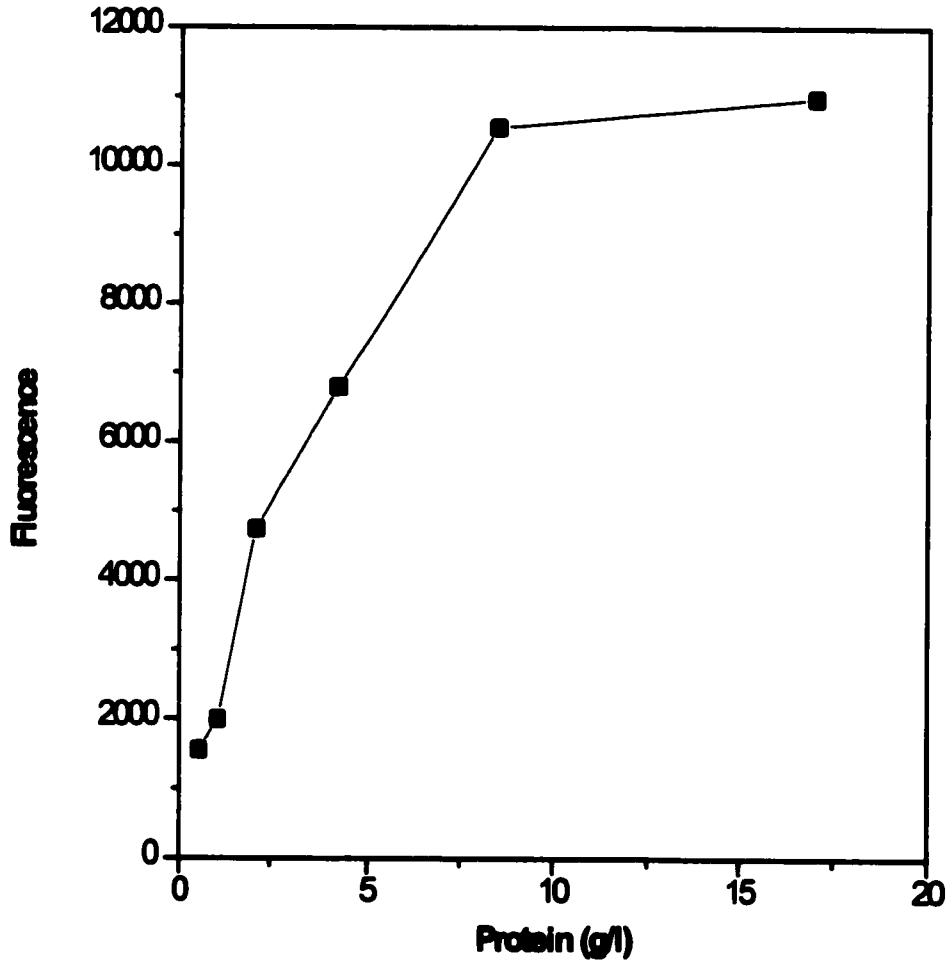

12000
10000
8000
6000
4000
2000
0
Fluoreseence
0
5
10
15
20
Protein (g/l)

Figure 2-4

Figure 2-5: Quantification of lacZα DNA. Various amounts of lacZα DNA were subjected to *in vitro* transcription/translation (25 μl reactions, 90 min at 37°C) followed by heat treatment (30 min at 70°C) and α-complementation with an equal volume of the undiluted M15 extract (1 hour at 37°C). The reaction mixtures were then assayed for β-galactosidase activity.

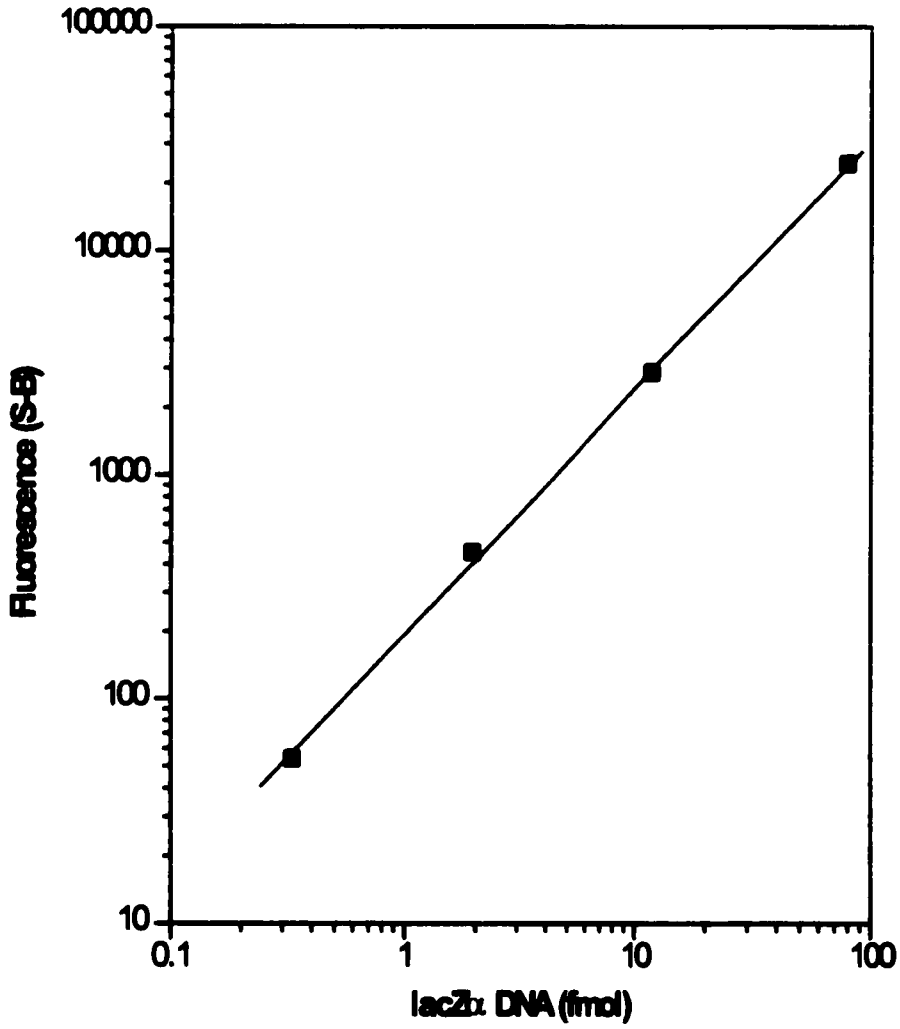

100000
10000
1000
100
10
Fluorescence (S-B)
0.1
1
10
100
lacZα DNA (fmol)

Figure 2-5

2.3.2 α-Peptide Expression Immunoassay

The configuration of the α-peptide expression immunoassay is illustrated in Figure 2-6. The DNA template was attached to a biotinylated antibody using streptavidin as a linker molecule. The first step was the preparation of a 1:1 complex of the biotinylated lacZα DNA and streptavidin. This was accomplished by mixing the DNA with a large excess of streptavidin and purifying the resulting streptavidin—DNA complex by size-exclusion HPLC. A typical chromatogram is shown in Figure 2-7. The fractions containing the streptavidin—DNA complex were collected, pooled, concentrated and stored at -20°C in the presence of 1mM EDTA. Quantification of the complex was performed by agarose gel electrophoresis followed by scanning densitometry, as described above.

The performance of the expression of this template in an expression immunoassay was assessed by first immobilising various amounts of analyte (anti-thyrotropin mouse monoclonal antibody) on the surface of microtitre wells. Biotinylated goat anti-mouse immunoglobulin was used for detection of immobilised antibodies. After completion of the immunoreaction and removal of the excess detection antibody, the streptavidin—lacZα DNA complex was added to the microtitre wells and allowed to bind to the immunocomplexes. The excess reagent was washed out and the transcription/translation mixture was added directly to the solid phase. The transcription/translation mixture was incubated for 90 min to allow expression of the bound DNA template and consequently the synthesis of α-peptide molecules. Aliquots of the reaction mixture were subjected to heat inactivation followed by α-complementation and measurement of the generated β-Gal activity. The results, presented in Figure 2-8, show the fluorescence (corrected for background) as a function of amount of immobilised analyte. The background is defined as the signal generated from an assay in which no analyte is present in the microtitre well (the zero standard). A linear relationship exists between the fluorescence and the number of immobilised antigen molecules in the well. It is possible to detect as few as 3 fmol of analyte using this immunoassay. The coefficients of variation (CV's) obtained at the 8 and 20 fmol levels were 10.7 and 9.3 %, respectively ($n = 4$).

Figure 2-6: Schematic Presentation of α-Peptide Expression Immunoassay. Analyte immobilised on the solid phase well is allowed to immunoreact with a specific, biotinylated antibody linked to the expressible lacZα DNA through biotin (B)—streptavidin (SA) interaction. The DNA bound to the solid phase is expressed *in vitro* to generate α-peptide molecules which subsequently complement with added M15 protein to form a pseudotetrameric enzyme with full β-galactosidase (β-Gal) activity. The complemented enzyme acts on 4-methylumbelliferyl galactoside (4-MUG) to produce the fluorescent 4-methylumbelliferone (4-MU).

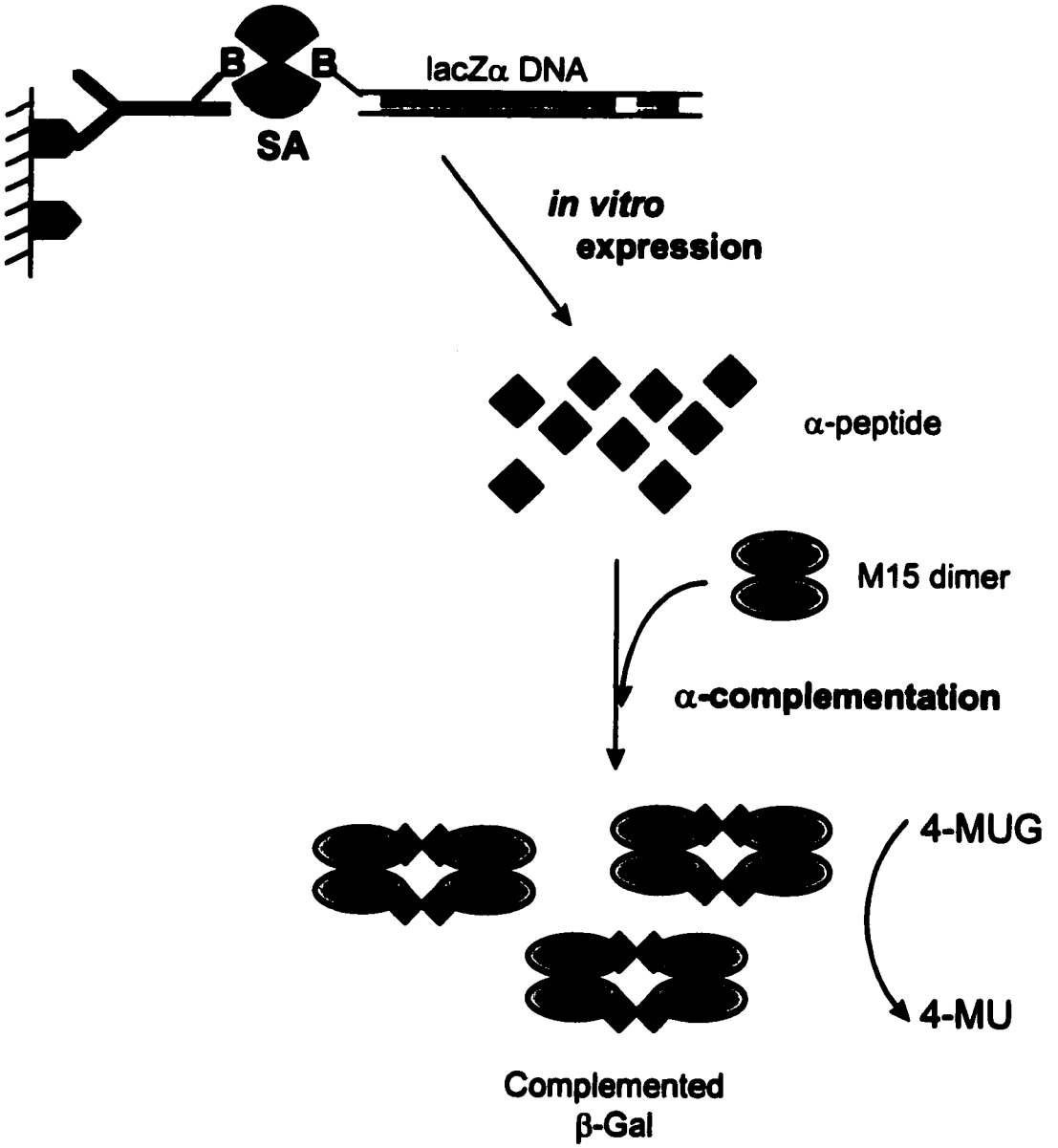

B
B
lacZα DNA
SA
in vitro
expression
α-peptide
M15 dimer
α-complementation
4-MUG
4-MU
Complemented
β-Gal

Figure 2-6

Figure 2-7: HPLC of the Streptavidin - lacZα DNA Complex. The typical chromatogram shown, was obtained using the conditions described in the text: flow rate 0.5 ml/min, isocratic elution in 0.5 M NaH_2PO_4, 0.5 M Na_2HPO_4 and 0.15 M NaCl, pH 6.8 and absorbance monitored at 260 nm. The peaks at 10.4 and 21.2 min correspond to the complex and the free streptavidin, respectively.

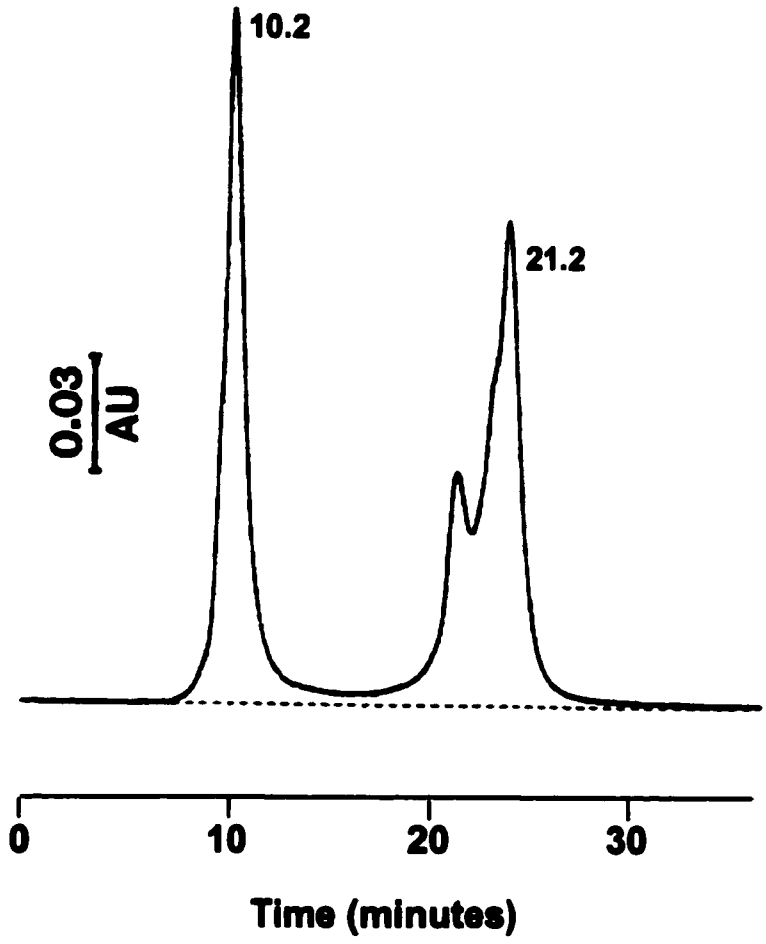


Figure 2-7

Figure 2-8: Quantification of Immobilised Antigen by α-Peptide Expression Immunoassay. Various amounts of antigen (anti-thyrotropin immunoglobulin) were immobilized on microtitre wells and detected by expression immunoassay using the lacZα DNA as a label.

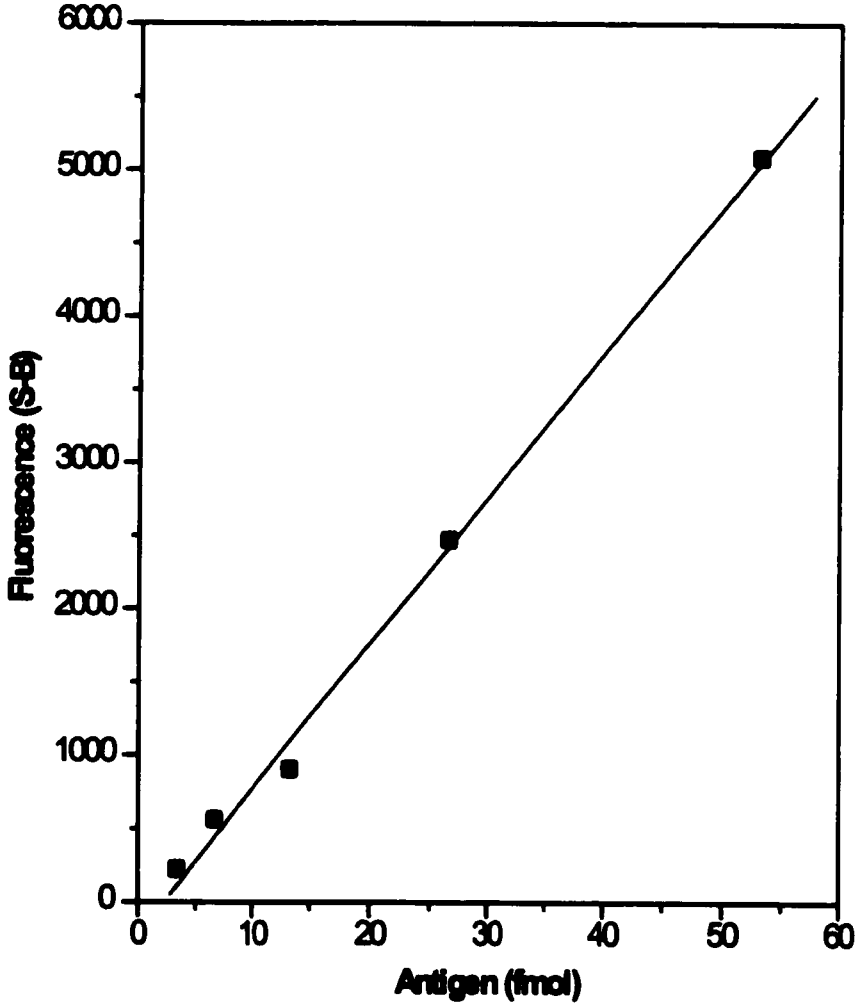

6000
5000
4000
3000
2000
1000
0
Fluorescence (S-B)
0
10
20
30
40
50
60
Antigen (fmol)

Figure 2-8

The lacZα DNA template, used in the α-peptide ExIA, has certain characteristics which make it a good label for use in expression detection systems. Specifically, the lacZα DNA is a relatively short DNA fragment and its expression protein does not require any post translational modification prior to complementation. As mentioned previously, because of its large size, it would be impractical to use the entire lacZ gene, which encodes native β-Gal. In the present study only a very small portion of the β-Gal protein is expressed, the N-terminal α-peptide (including amino acids 1-59), while the large remaining portion of the enzyme is added exogenously and allowed to complement with the synthesised peptide. Furthermore, this α-complementation was able to occur *in vitro*, without purification of the α-peptide from the expression mixture, and form fully active enzyme complexes.

The total time required for the detection step in the α-peptide ExIA is 4 hours. A possible improvement to this assay would be to use a transcription/translation extract which contains no endogenous β-Gal activity. The use of such an extract would eliminate the need for the heat inactivation step and may also permit the addition of purified M15 protein simultaneously with the transcription/translation reagents. In this way α-complementation would proceed simultaneously with the expression of the α-peptide. This procedure would reduce the time required for the assay to 135 min and may also increase the yield of complemented enzyme.

The sensitivity and practicality of the α-peptide ExIA is demonstrated. Although, two relatively complicated reactions (expression and α-complementation) are performed as part of the detection system, a linear relationship is observed between analyte and final signal. This was achieved without any optimisation of the DNA template. The template used in the current study was obtained as a restriction fragment of an expression vector, pGEM 13Zf(+), with no additional modification to the DNA sequence. However, the existing assay could, theoretically, be improved further by optimising the DNA sequence of the template. The lacZα DNA template still contains the polylinker site used for cloning in the original vector and it is unclear how these additional sequences affect the overall efficiency of *in vitro* transcription and translation. In addition, the template

makes use of the lac promoter which is sensitive to supercoiling; it is less effective in a linear template. Replacing the lac operator and promoter with alternative regulatory sequences may prove useful in improving the yield of *in vitro* expression.

The detection system developed in this work made use of a fluorescent substrate for the final enzymic determination. An obvious improvement to this would be to replace the 4-MUG with a chemiluminescent substrate such as the 1,2-dioxetane derivative, AMPGD (45). Whereas the fluorometric assay allows the detection of 20 zmol (12 000 molecules) of β-Gal (56), the chemiluminescent assay allows the detection of 4 zmol (2400 molecules) of β-Gal. Therefore, the use of the chemiluminescent substrate should improve the sensitivity of the α-peptide ExIA by a magnitude of approximately five.

β-Galactosidase α-complementation was chosen as a model in the expression detection system. This project demonstrated the feasibility of producing a small inactive peptide by *in vitro* expression of a DNA label and using it to generate detectable enzyme activity. This principle may be expanded to include any DNA label which encodes a small peptide which in turn is capable of triggering a signal generating system (eg. enzyme, photoprotein).

3. Alternative α-Peptide Encoding DNA Labels

3.1 Introduction

The previous chapter described the development of an expression immunoassay which exploited the *in vitro* expression of an α-peptide encoding DNA label as part of the detection system. It may be possible to increase the sensitivity of this system through the use of improved DNA labels which are more effectively expressed. This can be achieved either with or without switching to a eukaryotic *in vitro* expression system. The efficiency of expression of a DNA label may be increased through the use of a stronger RNA polymerase promoter and corresponding polymerase, addition of 5′-untranslated leader sequences (5′-ULSs), and the use of correct ribosome binding sites with the proper spacing. Each of these were incorporated into alternative lacZα DNA labels in an attempt to create a more sensitive α-peptide ExIA.

The lac promoter is known to be sensitive to supercoiling; it is more effective when the DNA template is supercoiled rather than linear. The short lacZα DNA label described in Chapter 2 is linear and too short to allow supercoiling which makes its lac promoter a poor choice for efficient expression of the α-peptide. Replacement of this promoter with the more effective tac promoter should improve the efficiency of expression of the α-peptide encoding DNA template. The tac promoter was engineered to contain the -10 region of the lacUV5 promoter and the -35 region of the trp promoter (57). This promoter has exhibited a five fold, or greater, increase in transcription yield over that obtained using the lacUV5 promoter. The lacUV5 promoter is, in turn, more efficient than the lac promoter used in the original lacZα DNA. The chimeric tac promoter is also less sensitive to supercoiling than the lac promoter. The use of the tac promoter in an alternative lacZα DNA would permit the use of an *E. coli* S-30 system for *in vitro* expression since it is an *E. coli* polymerase recognition sequence.

The T7 DNA dependent RNA polymerase (T7 RNAP) is routinely used in the *in vitro* synthesis of mRNA. This polymerase is well studied and commercially available. A particular advantage of phage polymerases, such as T7 RNAP, in *in vitro* transcription/translation reactions is the high degree of specificity of these polymerases for their promoter sequences. This reduces the transcription of other DNA molecules present as a part of the cell-free extract or introduced as a contaminant with the addition of the DNA of interest. In this way alone the efficiency of *in vitro* expression may be increased over that achieved using an endogenous polymerase since the components of the system are not wasted on expression of irrelevant DNAs. The known strength of phage expression machinery also aids in increasing expression efficiency in cell-free systems. The *in vitro* systems commercially available at the time of this study made use of T7 RNAP-mediated transcription in eukaryotic extracts (rabbit reticulocyte lysate and wheat germ extract). There were examples in the literature of systems which successfully made use of T7 RNAP in *E. coli* S-30 extracts (58, 59 and 60) but none were commercially available. Only recently have such systems come on the market. For this reason the T7 promoter-containing templates created in this study also contained sequences necessary for eukaryotic expression.

Three functional regions in the upstream portion of a DNA template are essential for expression to occur: i) a RNA polymerase-binding region (ie. promoter); ii) a ribosome binding site; and iii) a translation initiation codon. Since the templates which contain the T7 promoter were to be expressed in a eukaryotic system the ribosome binding site used was the Kozak consensus sequence (ACCATGG) (61). This is the sequence at -3 to +4 which is optimal for initiation by eukaryotic ribosomes. There is some evidence which suggests that two C's at positions -4 and -5 may also be part of the ribosome binding site. For the purposes of this study all sequences upstream and including the start codon were incorporated into the eukaryotic expression templates.

A fourth functional region found in many expression templates, although not absolutely necessary, is a 5′-untranslated leader sequence (5′-ULS) which is inserted downstream of the promoter and upstream of the Kozak sequence. In fact, there are cases where *in vitro*

translation may be dependent on the presence and characteristics of the 5′-ULS. For instance the efficiency of expression may be extremely poor if the start codon lies too close or too far from the 5′ end of the mRNA molecule (62), is inaccessible because of secondary structure of the mRNA (63) or if there are increased requirements for translation initiation factors (64). The presence of an appropriate upstream untranslated region may help correct these problems. The 5′-ULS may contain secondary structures that act as initiation signals and aid in the binding of eukaryotic initiation factor 2 (eIF2) (65). A number of particularly effective 5′-ULSs have been incorporated into DNA expression cassettes, often in place of the naturally occurring sequence. One example of this is the use of the 5′-ULS derived from the gene for the coat protein of the alfalfa mosaic virus (AMV) (66). Replacement of a native 5′-ULS with the AMV 5′-ULS increased translation efficiency 35 fold. This finding led researchers to incorporate the AMV 5′-ULS into a Universal Promoter used in expression-PCR (67, 68). They found that incorporation of the upstream untranslated sequence was necessary to provide a suitable context for ribosome binding and initiation of protein synthesis. In the work presented here a number of templates were synthesised by PCR to contain the AMV 5′-ULS, with the T7 promoter and Kozak sequence, upstream of the α-peptide coding sequence. These fragments were expressed in a rabbit reticulocyte based coupled transcription/translation system and the α-peptide produced was determined after α-complementation using the β-Gal fluorometric assay.

A second 5′-ULS that has been effective for *in vitro* expression of DNA templates is the so-called Cap Independent Translation Enhancer (CITE) 5′-ULS (69). This sequence includes portions of the upstream untranslated region from the encephalomyocarditis virus (EMCV). This is a large sequence, 502 nucleotides in length, which apparently directs internal translation initiation through its secondary structure (70). Ribosomes will bind to specific stem-loop structures within this sequence as opposed to the conventional binding of ribosomes to the 5′ terminal cap structure. The CITE sequence has been shown to enhance translation efficiency up to 16 fold in rabbit reticulocyte lysate based *in vitro* expression. Plasmid vectors, pCITE, which contain this 5′-ULS downstream from a

T7 promoter and upstream from a polycloning site are commercially available from Novagen. Simple cloning techniques were employed to add the α-peptide coding sequence downstream of the CITE 5′-ULS.

The pCITE vectors contain additional sequences which may be used to "tag" protein products with the S-peptide of ribonuclease S. Novagen has added this 45 base pair coding sequence which does not negatively affect the expression level of a variety of proteins when fused to the N-terminus. They have also incorporated the coding sequence for a thrombin cleavage site for specific proteolytic removal of the S-Tag peptide from the expressed protein, if necessary. The S-peptide (called S-Tag by Novagen) is a 15 amino acid peptide which will complement with the 103 amino acid S-protein to form active ribonuclease S (RNase S) (71, 72). The S-Tag and the S-peptide derived from ribonuclease A, have a strong affinity for one another and separately exhibit no ribonucleolytic activity. The affinity is strong enough to allow the use of the S-protein in a one-step affinity procedure for purification of proteins bearing the N-terminal S-Tag. The RNase S complementation can also be used in a quantitative assay and blot detection.

A number of α-peptide encoding templates were synthesised by cloning the coding sequence into the pCITE vectors. Templates were created both with and without the S-Tag and thrombin cleavage site coding sequences. The expression efficiency of these alternative templates were tested in a rabbit reticulocyte based coupled transcription translation system. Expression of these templates was measured using the β-Gal fluorometric assay. Expression levels of some of these templates were also monitored using the S-Tag rapid assay, described by Zimmerman and Sundeen (73), which makes use of the increase in absorbance at 280 nm as poly(C) substrate is broken down into acid soluble nucleotides by RNase S.

3.2 Experimental

Any instruments, materials, solutions or procedures not outlined here are described in Section 2.2.

3.2.1 Instrumentation

The 48-well Thermal Cycler from Perkin-Elmer Cetus (Norwalk, CT, USA) was used for all polymerase chain reactions. The Bead-Beater glass bead homogeniser used in the production of the S-30 extract was from Biospec Products (Bartlesville, OK, USA).

3.2.2 Materials

Reagent	Supplier
Pfu polymerase	Stratagene (La Jolla, CA, USA)
Wizard PCR preps DNA purification system, pSV-βGal, pSP64Poly(A), TNT T7 Coupled rabbit reticulocyte lysate system, beetle luciferin	Promega Corporation (Madison, WI, USA)
Bam HI	Boehringer Mannheim Corporation (Laval, PQ)
Taq polymerase, Hind III, Eco RI, Xho I	Bio/Can Scientific (Mississauga, ON)
Nde I, Bsr BI, Bsu 36I, Bgl I	New England Biolabs (Beverly, MA, USA)
Coenzyme A, calf intestinal alkaline phosphatase, T4 DNA ligase, Sac I	Pharmacia Biotech (Montreal, PQ)
pCITE-βGal, pCITE-3b, pCITE-4a(+) and S-Tag Rapid Assay Kit	Novagen (Madison, WI, USA)
E. coli A19 (CGSC strain # 5997)	*E. coli* Genetic Stock Center (Yale University, New Haven, CT, USA)

Reagent	Supplier
folinic acid, pyruvate kinase, phosphoenol pyruvate, amino acids, *E. coli* tRNA, yeast RNA, polyethylene glycol	Sigma (St. Louis, MO, USA)

3.2.3 PCR Primer Sequences

The following primers were purchased from DNAgency (Malvern, PA, USA):

Primer Name	Sequence
Uα-I	5′-ATG ACC ATG ATT ACG TCA CTG GCC GT-3′
Uα-II	5′-T AAT ACG ACT CAC TAT AGG GCC ACC ATG GTC GTT TTA-3′
Uα-III	5′- T AAT ACG ACT CAC TAT AGG GTT TTT ATT TTT AAT TTT CTT TCA AAT ACT TCC ACC ATG GTC GTT TTA-3′
UT7-I	5′-TAG GGC GAA TTC AAA TTA ATA CGA CTC ACT ATA G-3′
UT7-II	5′-TCT AGG CAA AGC TTC TAA TAC GAC-3′
UT7-III	5′- TCT AGG CAA AGC TTA ATT TAA TAC GAC-3′
Dα-I	5′-TCT AGG CAA AGC TTC TTA CCA TTC GCC-3′
Dα-II	5′-TTT GGG AGC TCG GAT CCC TTA CCA TTC GCC-3′
Extension	5′-TA GGG CGA ATT CAA ATT AAT ACG ACT CAC TAT AGG GTT TTT ATT TTT AAT TTT CTT TCA AAT ACT TCC ACC ATG ACC ATG ATT-3′
Dluc-I	5′-CCG GTG ATG CCA ATT CGG T-3′

The following primers were purchased from Biosynthesis (Lewisville, TX, USA):

Primer Name	Sequence
Uα-IV	5′-G GAA TTC CAT ATG GTC GTT TTA CAA CGT-3′
Utac	5′-GAG CTG TTG ACA ATT AAT CAT CGG CTC GTA TAA TGT GTG GAA TTG TGA-3′
UT7SD	5′-CTA ATA CGA CTC ACT ATA GGG AGA GCT AAG CTA AGC AGG AGG ATC CAA ATG GAA GAC-3′
Dα-III	5′-CGG GAT CCC TTA GGT TAC GTT GGT GTA-3′
Dα-IV	5′-CGG GAT CCC TTA AAC ATT AAA TGT GAG-3′

3.2.4 Solutions

2×YT Media

Tryptone	16 g
Yeast Extract	10 g
NaCl	5 g

Dissolve components in ddH_2O and adjust pH to 7.0 with NaOH. Adjust volume to 1 l with ddH_2O and sterilise by autoclaving. Store at 4°C.

Prepare 2×YT agar plates by adding 15.0 g of agar per 1 l LB broth before autoclaving. If necessary, add antibiotic after the suspension has cooled to at least 50°C. Pour the solution into culture plates and keep the plates at room temperature until the mixture has set. Store the plates at 4°C.

5× Low Molecular Weight Mixture

Mix the following components well, divide into 100 µl aliquots and store in microcentrifuge tubes at -80°C.

2.2 M HEPES/KOH, pH 8.2	13 µl
85 mM ATP, pH 7	5 µl
85 mM GTP, pH 7	5 µl

85 mM CTP, pH 7	5 µl
85 mM UTP, pH 7	5 µl
425 mM DTT	2 µl
64 mM cAMP, pH 7	5 µl
3 M magnesium acetate	0 - 3.3 µl
4.16 M potassium glutamate	12 - 30 µl
4.5 M ammonium acetate	1.1 - 5.6 µl
20 mg/ml tRNA (*E. coli*)	4.2 µl
8.5 mg/ml folinic acid	2 µl
75 U/ml pyruvate kinase	2 µl
55 mM amino acid mixture	4.6 µl
DEPC-treated ddH_2O to final volume	100 µl

This buffer must be optimised for each DNA template used for protein production and for each S-30 extract preparation.

Luciferin Substrate Solution

Dilute 5 ml of 100 mM Tricine in 21 ml of ddH_2O. Add 13 mg of $MgCO_3 \cdot 5H_2O$ and dissolve with gentle heating. Add 16.5 mg $MgSO_4$ and 0.25 ml of 10 mM EDTA, pH 8.0 and adjust the pH to 7.8. Add 0.1284 g DTT and repeat pH adjustment to 7.8.

Add the remaining components in the order shown:

5.54 mg Coenzyme A

8.02 mg ATP

150 µl luciferin solution (25 mg/ml)

Bring the final volume to 25 ml with ddH_2O and aliquot into separate microcentrifuge tubes (~ 400 µl/tube) for storage at -80°C.

3.2.5 Preparation of Alternative Expression Templates

tac Promoter-Containing Template

This prokaryotic expression template was prepared by PCR using the Utac primer which contained sequences necessary for the addition of the tac promoter sequence. The PCR was performed using pGEM 13Zf(+) as the initial template:

10× Pfu buffer	10 µl
Utac primer (10 µM)	5 µl
Dα-I primer (10 µM)	5 µl
dNTPs (2.5 mM)	6 µl
pGEM 13Zf(+) (4.3 ng/µl)	4.5 µl
ddH_2O to final volume	99 µl

The Pfu polymerase (2.5 U/µl, 1 µl) was added during the initial heating at 95°C (hot start). After the addition of the polymerase, PCR was carried out for 25 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 2 min. After the final incubation for 20 min at 72°C the reaction mixture was analysed by agarose (1.5%) electrophoresis to ensure that the 366 bp product was formed in good yield without additional unwanted products. The tac promoter-containing template was purified using Wizard PCR preps DNA purification system and quantitated by electrophoresis and scanning densitometry as described previously (see section 2.2.5).

A. Templates Containing No 5′-Untranslated Leader Sequence

Template A1: α-Peptide Codons 8-59

This expression template was prepared from two sequential PCR reactions with linearised pSV-βGal as the template. The plasmid was linearised by treatment with Eco RI:

10× Eco RI buffer	3 µl
pSV-βGal (396 ng/µl)	3 µl
Eco RI (12 U/µl)	2 µl
ddH_2O to final volume	30 µl

The reaction was incubated 1 hour at 37°C before dilution in sterile ddH_2O such that the concentration of linearised plasmid was 1.3 ng/μl. The diluted digestion reaction was then used in the first PCR:

10× Pfu buffer	10 μl
Uα-II primer (10 μM)	5 μl
Dα-II primer (10 μM)	5 μl
dNTPs (2.5 mM)	6 μl
linear pSV-βGal (1.3 ng/μl)	5 μl
ddH_2O to final volume	99 μl

The Pfu polymerase (2.5 U/μl, 1 μl) was added during the initial heating at 95°C (hot start). After the addition of the polymerase, PCR was carried out for 25 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. A final incubation for 20 min at 72°C was performed to allow Pfu polymerase to act on any unfinished PCR products. The PCR reaction was analysed by agarose (1.3 %) gel electrophoresis. Linear DNA markers were electrophoresed simultaneously with the PCR product. Although no clear band was visible, a slice of agarose was excised which corresponded to the expected location of the PCR product (199 bp).

A very small portion of the agarose slice was used in a reamplification PCR. This reaction was carried out exactly as described above except that a piece of agarose was used in place of the linear pSV-βGal. Agarose (1.3 %) electrophoresis of the PCR products and the linear DNA markers indicated that the 199 bp product was generated in good yield without additional, unwanted products. The band corresponding to the 199 bp product was excised from the gel for use in the next PCR.

The second PCR step was carried out with the 199 bp product from the first PCR step as the template. A master mix was prepared in order to carry out three identical PCRs:

10× Pfu buffer	30 μl
UT7-II primer (10 μM)	15 μl
Dα-II primer (10 μM)	15 μl
dNTPs (2.5 mM)	18 μl

ddH_2O to final volume	297 µl

The master mix was split into three equal aliquots of 99 µl and each was transferred to a separate PCR tube. A very small portion of the agarose slice containing the 199 bp DNA fragment was added to each tube. The PCR conditions were identical to those used for the first PCR step, including the addition of 1 µl of Pfu polymerase to each tube during the hot start. The PCRs were pooled and the DNA was purified using Wizard PCR preps DNA purification system. The concentration of template A1 was determined by electrophoresis and scanning densitometry as described previously.

Template A1[dA/dT]: Addition of Poly dA/dT Sequence

A poly dA/dT sequence was added downstream of the α-peptide sequence by cloning of template A1 into pSP64Poly(A) using a two step ligation procedure. Both template A1 and pSP64Poly(A) were first digested with Hind III:

	plasmid	insert
pSP64Poly(A) (1.9 µg/µl)	6 µl	—
template A1 (98 ng/µl)	—	25 µl
10× One-Phor-All buffer plus	5 µl	5 µl
Hind III (10 U/µl)	4 µl	1 µl
ddH_2O to final volume	50 µl	50µl

The restriction digestion reactions were incubated for 90 min at 37°C before inactivation of Hind III by heating the reaction mixtures to 65°C for 20 min. The linearised pSP64Poly(A) was dephosphorylated, in order to stop recircularisation in the next step, by mixing 48 µl of the digested pSP64Poly(A) (0.23 µg/µl) with 2 µl of calf intestinal alkaline phosphatase (0.05 U/µl) and incubating for 1 hour at 37°C. The alkaline phosphatase was heat inactivated by heating to 85°C for 15 min. No purification of the dephosphorylated linear plasmid was necessary. The digested template A1 was purified using Wizard PCR preps DNA purification system and quantitated by electrophoresis and scanning densitometry as usual.

Template A1 was attached to the pSP64Poly(A) vector by ligation at the Hind III overhangs left by the previous restriction digest. The ligation was performed using a 1:4 molar ratio between the plasmid and the insert:

linear pSP64Poly(A) (0.22 μg/μl)	5.6 μl
digested template A1 (36 ng/μl)	9.4 μl
ATP (20 mM)	2 μl
10× One-Phor-All buffer plus	4 μl
T4 DNA ligase (7 U/μl)	1 μl
ddH_2O to final volume	40 μl

The ligation was allowed to proceed for 4 hours at 10°C, followed by a 10 min heat inactivation step at 65°C.

The ligation reaction mixture was used directly, without any purification of the ligation product, in the second restriction digest. Bam HI was used to cut both ends of the long linear ligation product which contained the pSP64Poly(A) sequences attached to template A1:

ligation reaction	40 μl
10× One-Phor-All buffer plus	5.5 μl
Bam HI (10 U/μl)	2 μl
ddH_2O to final volume	50 μl

After 90 min at 37°C the entire reaction was loaded into an agarose (1.3 %) gel and electrophoresed to separate the products. The band corresponding to the 3220 bp product was excised and the DNA was purified using Geneclean according to the manufacturer's directions.

The ligated DNA fragment contained the template A1 attached to pSP64Poly(A) at the Hind III site within the polycloning region. After Bam HI digestion the DNA fragment had two complementary ends which allowed circularisation such that the template A1 was located directly upstream of the $[dA/dT]_{30}$ sequence:

purified 3220 bp fragment	20 μl
10× One-Phor-All buffer plus	3 μl

ATP (20 mM)	1.5 µl
T4 DNA ligase (7 U/µl)	1 µl
ddH_2O to final volume	30 µl

The ligation reaction was incubated 4 hours at 10°C followed by heat inactivation of the ligase at 65°C for 10 min.

The circularised DNA (1.5 µl of the ligation reaction) was used to transfect competent JM109 *E. coli* cells (200 µl) using standard procedures. The pSP64Poly(A) vector contains a β-lactamase gene which confers ampicillin resistance and which remained intact with the cloning of template A1 into the polycloning region. This allowed the selection of successfully transformed colonies of *E. coli* by plating the transfection mixture on agar plates containing 0.1 mg/ml ampicillin. One colony was used to inoculate 50 ml of LB broth containing 0.1 mg/ml ampicillin. After an overnight incubation at 37°C, with vigorous shaking, a stock solution of the transformed bacteria, for storage at -80°C, was prepared by mixing 850 µl of the culture with 150 µl of sterile glycerol. The remaining culture was scaled up in 1.4 l of LB broth containing 0.1 mg/ml ampicillin. Once this culture had reached its mid-log growth phase the cells were harvested by centrifugation and the DNA was purified using Wizard maxipreps DNA purification system. The concentration of the new plasmid, pSP64-A1, was determined by absorbance at 260 nm.

In order to obtain template A1[dA/dT], pSP64-A1 was digested with Hind III, which cuts upstream of the T7 promoter, and Eco RI, which cuts downstream of the $[dA/dT]_{30}$ sequence:

pSP64-A1 (1.7 µg/µl)	6 µl
10× Eco RI buffer	5 µl
Eco RI (12 U/µl)	2 µl
Hind III (10 U/µl)	2.4 µl
ddH_2O to final volume	50 µl

After incubation for 90 min at 37°C, the entire reaction mixture was loaded into an agarose (1.8 %) gel and electrophoresed to separate the products. The band

corresponding to the 245 bp product was excised and the DNA was purified using the Wizard PCR preps DNA purification system. The concentration of template A1[dA/dT] was determined by electrophoresis and scanning densitometry as usual.

Template A2: α-Peptide Codons 8-59

The difference between templates A1 and A2 was the sequence immediately upstream of the T7 promoter. In template A1 the promoter was immediately preceded by C whereas in template A2 it was immediately preceded by the sequence AATT. Template A2 was prepared using the method described for template A1. The 199 bp product of the first PCR described for production of template A1 was also used for the preparation of template A2. The second PCR was identical to that described for template A1 except that the UT7-III primer was used in place of the UT7-II primer. The resulting template A2 was also purified and quantitated as described for template A1.

Template A2[dA/dT]: Addition of Poly dA/dT Sequence

A poly dA/dT sequence was added downstream of the α-peptide sequence by cloning of template A2 into pSP64Poly(A) using the same two step ligation procedure described for the production of template A1[dA/dT]. The only difference between the two procedures is that template A2 was cloned into pSP64Poly(A) instead of template A1.

B. Templates Containing the AMV 5′-Untranslated Leader Sequence

Template B1: α-Peptide Codons 1-59

The first AMV-ULS containing template synthesised was prepared by a three step process. The first PCR was performed using the pGEM 13Zf(+) vector as the template and primers Uα-I and Dα-I:

10× Taq buffer	10 µl
$MgCl_2$ (25 mM)	10 µl
Uα-I primer (10 µM)	5 µl
Dα-I primer (10 µM)	5 µl

dNTPs (2 mM)	1 µl
pGEM 13Zf(+) (43 fg/µl)	2 µl
ddH_2O to final volume	99.5 µl

The Taq polymerase (5 U/µl, 0.5 µl) was added during the initial heating at 95°C (hot start). This was followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. A final 10 min incubation at 72°C was performed after the cycles were completed.

The PCR product was electrophoresed on a 1.4 % agarose gel and the band corresponding to the 192 bp product was excised and purified using Wizard PCR preps DNA purification system. The resulting DNA was dissolved in sterile ddH_2O and stored at 4°C. A 10 fold dilution of the purified 192 bp fragment was prepared in sterile ddH_2O for use in the extension PCR:

10× Taq buffer	30.8 µl
$MgCl_2$ (25 mM)	30.8 µl
dNTPs (2 mM)	3.5 µl
diluted 192 bp fragment	7 µl
Taq polymerase (5 U/µl)	1.8 µl
ddH_2O to final volume	290 µl

From this master mix 83 µl was added to three separate PCR tubes. The extension primer was added to each tube (5 µl of 0.1 µM) during the hot start. Four cycles of denaturation at 95°C for 30 sec, annealing at 40°C for 30 sec and extension at 72°C for 1 min were performed prior to the addition of the reagents for the final PCR. A master mixture containing the reagents for the next PCR was prepared:

10× Taq buffer	3.6 µl
$MgCl_2$ (25 mM)	3.6 µl
UT7-I primer (10 µM)	15 µl
Dα-I primer (10 µM)	15 µl

After the completion of the 4 cycles of extension PCR, the temperature was held at 80°C for 2 min. During this time 12.4 µl of the master mix was added to each of the three

identical PCR reactions. PCR was continued for 25 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min. The final incubation at 72°C for 10 min again allowed the enzyme to act on any unfinished products in the reaction mixtures. The PCR products from the three reactions were pooled and purified using the Wizard PCR preps DNA purification system. The concentration of template B1 (263 bp) was determined by electrophoresis and scanning densitometry as described previously.

Template B1-PCS: B1 + pGEM 13Zf(+) Polycloning site

The polycloning site from pGEM 13Zf(+) was not removed in this template, thus the procedure was much more straight forward than that required for the synthesis of template B1. A single PCR step was performed using the extension primer as the upstream primer:

10× Pfu buffer	10 µl
extension primer (10 µM)	5 µl
Dα-I primer (10 µM)	5 µl
dNTPs (2.5 mM)	6 µl
pGEM 13Zf(+) (4.3 ng/µl)	4.5 µl
ddH_2O to final volume	99 µl

The Pfu polymerase (2.5 U/µl, 1 µl) was added during the initial heating at 95°C (hot start). Following the addition of the polymerase, PCR was carried out for 25 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. After the final incubation for 20 min at 72°C a portion of the PCR mixture was loaded onto an agarose gel and electrophoresed simultaneously with linear DNA markers. Although no product was visible, an agarose slice was excised which corresponded to the expected location of the DNA (359 bp). A very small portion of this agarose slice was then used to reamplify the 359 bp DNA fragment. The PCR was carried out using the conditions outlined for the original reaction except that the small slice of agarose was used in place of the pGEM 13Zf(+). Template B1-PCS was then purified from the PCR

components using Wizard PCR preps DNA purification system and quantitated by electrophoresis and scanning densitometry as usual.

Template B2: α-Peptide 8-59

This expression template was prepared from two sequential PCR reactions with linearised pSV-βGal as the template. The linearised plasmid used as a template for the production of templates A1 and A2 was also used as the template for this PCR:

10× Pfu buffer	10 µl
Uα-III primer (10 µM)	5 µl
Dα-II primer (10 µM)	5 µl
dNTPs (2.5 mM)	6 µl
linear pSV-βGal (75 pg/µl)	5 µl
ddH_2O to final volume	99 µl

As described for the synthesis of template A1, the Pfu polymerase (2.5 U/µl, 1 µl) was added during the initial heating at 95°C (hot start). After the addition of the polymerase, PCR was carried out for 25 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. After the final incubation for 20 min at 72°C the PCR mixture was electrophoresed, simultaneously with linear DNA markers, on an agarose (2 %) gel. Although no product was visible, a slice of agarose corresponding to the expected size of the DNA (229 bp) was excised and a portion was used in a reamplification reaction. The initial PCR was repeated using identical conditions except that the pSV-βGal template was replaced with a very small amount of the agarose slice. Agarose (2 %) electrophoresis of the PCR mixture was carried out to determine that the product was obtained in good yield and the band corresponding to the 229 bp product was excised from the gel for use in the next PCR.

The second PCR step was carried out using the 229 bp product from the first PCR step as the template. A master mix was prepared in order to carry out three identical PCRs:

10× Pfu buffer	30 µl
UT7-II primer (10 µM)	15 µl

Dα-II primer (10 μM)	15 μl
dNTPs (2.5 mM)	18 μl
ddH_2O to final volume	297 μl

The master mix was split into three equal aliquots of 99 μl and each was transferred to a separate PCR tube. A very small portion of the agarose slice containing the 229 bp DNA fragment was added to each tube. The PCR conditions were identical to those used for the first PCR step, including the addition of 1 μl of Pfu polymerase to each tube during the hot start. The PCRs were pooled and the DNA was purified using Wizard PCR preps DNA purification system. The concentration of template B2 was determined by electrophoresis and scanning densitometry as described previously.

Template B2(no 5'): α-Peptide Codons 8-59

This template was identical to B2 except that there were no nucleotides upstream (5') of the T7 promoter. This template was prepared using the Eco RI linearised digest of pSV-βGal as in the preparation of template B2:

10× Taq buffer	10 μl
$MgCl_2$ (25 mM)	10 μl
Uα-III primer (10 μM)	5 μl
Dα-II primer (10 μM)	5 μl
dNTPs (2.5 mM)	0.8 μl
linear pSV-βGal (75 pg/μl)	1 μl
ddH_2O to final volume	100 μl

The Taq polymerase (0.5 μl, 5 U/μl) was added during the initial heating at 95°C (hot start). This was followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. A final 10 min incubation at 72°C was performed after the cycles were completed. The PCR mixture was electrophoresed (2 % agarose) with linear DNA markers in a separate lane. No product was visible, however, an agarose slice was excised which corresponded to the expected location of the DNA (229 bp). A very small portion of this agarose slice was then used to reamplify the 229 bp DNA fragment. The PCR was carried out using the conditions outlined for the

original reaction except that the small slice of agarose was used in place of the linear pSV-βGal and the annealing temperature was increased to 55°C. The template B2(no 5′) was not purified or quantitated.

Template B2[dA/dT]: Addition of Poly dA/dT Sequence

A poly dA/dT sequence was added downstream of the α-peptide sequence by cloning of template B2 into pSP64Poly(A) using the same two step ligation procedure described for the production of template A1[dA/dT]. The only difference between the two procedures was that template B2 was used in place of template A1.

Template B3: α-Peptide Codons 8-59

The difference between templates B2 and B3 was the sequence immediately upstream of the T7 promoter. In template B2 the promoter was immediately preceded by C whereas in template B3 it was immediately preceded by the sequence AATT. The preparation of template B3 was carried out in the same manner as that of template B2. The 229 bp product of the first PCR described for production of template B2 was also used for the preparation of template B3. The second PCR was identical to that described for template B2 except that the UT7-III primer was used in place of the UT7-II primer. The resulting template B3 was also purified and quantitated as described above.

Template B3[dA/dT]: Addition of Poly dA/dT Sequence

A poly dA/dT sequence was added downstream of the α-peptide sequence by cloning of template B3 into pSP64Poly(A) using the same two step ligation procedure described for the production of template A1[dA/dT]. The only difference between the two procedures was that template B3 was used in place of template A1.

Template B4: α-Peptide Codons 8-105

Template B4 was prepared using essentially the same procedure outlined for template B2, except the downstream primer and amount of initial template were altered:

10× Pfu buffer	10 µl
Uα-III primer (10 µM)	5 µl
Dα-III primer (10 µM)	5 µl
dNTPs (2.5 mM)	6 µl
linear pSV-βGal (1.3 ng/µl)	5 µl
ddH_2O to final volume	99 µl

The PCR conditions were the same as described for template B2. No reamplification reaction was required since analysis of the PCR mixture by electrophoresis indicated the presence of a strong band which corresponded to the expected 358 bp product. This band was excised from the gel for use in the next PCR.

The second PCR step was carried out using the 358 bp product from the first PCR step as the template:

10×Pfu buffer	10 µl
UT7-II primer (10 µM)	5 µl
Dα-III primer (10 µM)	5 µl
dNTPs (2.5 mM)	6 µl
358 bp fragment	gel slice
ddH_2O to final volume	99 µl

The PCR conditions were identical to those used for the first PCR step, including the addition of 1 µl of Pfu polymerase to each tube during the hot start. The PCR product was purified using Wizard PCR preps DNA purification system. The concentration of template B4 was determined by electrophoresis and scanning densitometry as described previously.

Template B5: α-Peptide Codons 1-105

Through the combination of the 5′ portion of template B1 with the 3′ portion of template C4 (see below) it was possible to synthesise template B5 containing the α-peptide coding sequence for the first 105 codons. This was achieved by first digesting both original templates with Bgl I:

Template B1 (83 ng/μl)	44 μl	—
Template C4 (84 ng/μl)	—	44 μl
10× NEBuffer 3	5 μl	5 μl
Bgl I (10 U/μl)	1 μl	1 μl
	50 μl	50 μl

The mixtures were incubated overnight at 37°C before the resulting fragments were separated by agarose (2 %) electrophoresis. The bands corresponding to the 235 bp product from template B1 and the 160 bp product from template C4 were excised from the gel and the DNA was purified from the agarose using Wizard PCR preps DNA purification system. No quantitation of the DNA fragments was necessary.

The restriction enzyme Bgl I cut both templates within the α-peptide coding sequence leaving cohesive ends which allowed the ligation of the 5′ fragment from template B1 to the 3′ fragment from template C4:

235 bp fragment	15 μl
160 bp fragment	15 μl
ATP (20 mM)	2 μl
10× One-Phor-All buffer plus	4 μl
T4 DNA ligase (6.2 U/μl)	1 μl
ddH_2O to final volume	40 μl

The ligation reaction was allowed to proceed for 10 hours at 10°C before the ligase was heat inactivated, 10 min at 65 °C. The ligation products were separated by agarose (2 %) electrophoresis and the band corresponding to the 395 bp product was excised. A very small portion of this slice was then used in a PCR:

10× Pfu buffer	10 μl
UT7-I primer (10 μM)	5 μl
Dα-III primer (10 μM)	5 μl
dNTPs (2.5 mM)	6 μl
395 bp DNA	gel slice
ddH_2O to final volume	99 μl

The Pfu polymerase (2.5 U/μl, 1 μl) was added during the initial heating at 95°C (hot start). After the addition of the polymerase, PCR was carried out for 25 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 2 min. After the final incubation for 20 min at 72°C the reaction mixture was analysed by agarose (2 %) electrophoresis to ensure that the 395 bp product was formed in good yield without additional irrelevant products. Template B5 was purified using Wizard PCR preps DNA purification system and quantitated by electrophoresis and scanning densitometry as usual.

C. Templates Containing the CITE 5′-Untranslated Leader Sequence

Template C1: α-Peptide Codons 8-59

This expression template was synthesised by a single PCR using pCITE-βGal as the template:

10× Pfu buffer	10 μl
UT7-II primer (10 μM)	5 μl
Dα-II primer (10 μM)	5 μl
dNTPs (2.5 mM)	6 μl
pCITE-βGal (2.7 ng/μl)	5 μl
ddH_2O to final volume	98 μl

As described previously, the Pfu polymerase (2.5 U/μl, 2 μl) was added during the initial heating at 95°C (hot start). After the addition of the polymerase, PCR was carried out for 25 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. After the final incubation for 20 min at 72°C the reaction mixture was analysed by agarose (1.7 %) electrophoresis to ensure that the 813 bp product was formed in good yield without additional unwanted products. Template C1 was purified using Wizard PCR preps DNA purification system and quantitated by electrophoresis and scanning densitometry as usual.

Template C2: α-Peptide Codons 8-105

Template C2 was prepared using the same procedure as C1 except the downstream primer Dα-II was replaced with Dα-III.

Template C3: α-Peptide Codons 8-130

This expression template was prepared using pCITE-βGal as the PCR template, however, it was necessary to linearise the plasmid for production of template C3:

10× Xho I buffer	5 µl
pCITE-βGal (1.34 µg/µl)	5 µl
Xho I (10 U/µl)	2.4 µl
ddH_2O to final volume	50 µl

After allowing the restriction digestion to proceed for 2 hours at 37°C the enzyme was heat inactivated for 20 min at 65°C. The reaction mixture was then diluted in sterile ddH_2O and used as a template in a PCR:

10× Pfu buffer	10 µl
UT7-II primer (10 µM)	5 µl
Dα-IV primer (10 µM)	5 µl
dNTPs (2.5 mM)	6 µl
linear pCITE-βGal (2.7 ng/µl)	3 µl
ddH_2O to final volume	99 µl

As described for the synthesis of template A1, the Pfu polymerase (2.5 U/µl, 2 µl) was added during the initial heating at 95°C (hot start). After the addition of the polymerase, PCR was carried out for 25 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. After the final incubation for 20 min at 72°C the reaction mixture was analysed by agarose (1.7 %) electrophoresis to ensure that the 1008 bp product was formed in good yield without additional unwanted products. Template C3 was purified using Wizard PCR preps DNA purification system and quantitated by electrophoresis and scanning densitometry as usual.

Template C4: α-Peptide Codons 8-105

Expression template C4 was identical to template C2 except the S-Tag and thrombin cleavage site coding sequences from the pCITE vector were removed. The first step in the synthesis of this template was the production of a fragment of DNA containing the α-peptide coding sequence (codons 8-105) with Nde I restriction site which incorporated the start codon (CATATG). This was accomplished by PCR:

10× Pfu buffer	10 µl
Uα-IV primer (10 µM)	5 µl
Dα-III primer (10 µM)	5 µl
dNTPs (2.5 mM)	6 µl
pCITE-βGal (2.7 ng/µl)	3 µl
ddH_2O to final volume	99 µl

As described previously, the Pfu polymerase (2.5 U/µl, 1 µl) was added during the initial heating at 95°C (hot start). After the addition of the polymerase, PCR was carried out for 25 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. After the final incubation for 20 min at 72°C the PCR mixture was electrophoresed, simultaneously with linear DNA markers, on an agarose (1.5 %) gel. Although no product was visible, a slice of agarose corresponding to the expected size of the DNA (314 bp) was excised and a portion was used in a reamplification reaction. The initial PCR was repeated using identical conditions except that the pCITE-βGal template was replaced with a very small amount of the agarose slice. The 314 bp product was purified from the PCR mixture using Wizard PCR preps DNA purification system and quantitated as usual.

The second step in this procedure was the ligation of the 314 bp PCR product at the Nde I site of pCITE-3b, which contains the CITE 5′-ULS upstream of the Nde I site. The S-Tag and thrombin cleavage site sequences are downstream of this site. Both the plasmid pCITE-3b and the 314 bp DNA fragment were cut using Nde I:

	plasmid	fragment
pCITE-3b (1.4 µg/µl)	8 µl	—

314 bp DNA	—	30 µl
10× NEBuffer 4	5 µl	5 µl
Nde I (20 U/µl)	2 µl	0.5 µl
ddH_2O to final volume	50 µl	50µl

The reactions were incubated 1 hour at 37°C before the enzyme was heat inactivated, 10 min at 65°C. The linearised plasmid was purified by ethanol precipitation, according to standard procedures (41), and dissolved in sterile ddH_2O. The digested DNA fragment was purified using Wizard PCR preps DNA purification system. Before ligation both DNAs were quantitated using electrophoresis and scanning densitometry as usual.

In order to avoid potential recircularisation during ligation the linear plasmid was dephosphorylated. Seventeen microlitres of the digested pCITE-3b (0.17 µg/µl) was mixed with 2 µl of calf intestinal alkaline phosphatase (0.05 U/µl) in One-Phor-All buffer plus and incubated for 1 hour at 37°C. The alkaline phosphatase was heat inactivated at 85°C for 15 min. No purification of the dephosphorylated linear plasmid was necessary. Next, the ligation reaction was performed using a 1:3 molar ratio of plasmid to DNA fragment:

linear pCITE-3b (56 ng/µl)	10.5 µl
digested DNA fragment (10.4 ng/µl)	14 µl
ATP (20 mM)	1.5 µl
10× One-Phor-All buffer plus	2 µl
T4 DNA ligase (6.2 U/µl)	1 µl
ddH_2O to final volume	30 µl

The reaction was incubated for 4½ hours at 10°C before the ligase was heat inactivated, 10 min at 65°C. The products of the ligation reaction were separated by agarose (1 %) electrophoresis and the band corresponding to the correct ligation product (4065 bp) was excised. The DNA was purified from the agarose using Geneclean according to the manufacturer's directions. The DNA was then dissolved in sterile ddH_2O and quantitated as usual.

The 4065 bp ligation product was used as the template in the final PCR step:

10× Pfu buffer	10 µl
UT7-II primer (10 µM)	5 µl
Dα-III primer (10 µM)	5 µl
dNTPs (2.5 mM)	6 µl
ligation product (2.8 ng/µl)	3 µl
ddH_2O to final volume	99 µl

The PCR conditions were exactly as described for the first PCR step. The PCR reaction was analysed by electrophoresis and the faint band, corresponding to the expected product of 858 bp, was excised and a portion of this was used in a reamplification reaction. The reamplification reaction was performed as described for the first PCR except the ligation product was replaced with a very small gel slice and the annealing temperature was increased to 60°C. The 858 bp template C4 was purified and quantitated as described previously.

Template C4[dA/dT](+T7term): Addition of Poly dA/dT and T7 Terminination Sequence

The 314 bp DNA fragment (used for the production of template C4), containing the α-peptide coding sequence with an Nde I restriction site incorporated at the start codon, was also used in the synthesis of template C4[dA/dT](+T7term). This fragment was cloned into pCITE-4a(+) which contains the CITE 5′-ULS upstream of the polycloning site and $[dA/dT]_{30}$ and T7 terminator sequences downstream of the polycloning site. As in the preparation of template C4 the S-Tag and thrombin cleavage site coding sequences were removed during the cloning procedure. Similar to the synthesis of template C4 the plasmid DNA was ligated with the fragment DNA after restriction digestion of both DNAs with Nde I:

	plasmid	fragment
pCITE-4a(+) (1.8 µg/µl)	7 µl	—
314 bp DNA	—	28 µl
10× NEBuffer 4	5 µl	5 µl
Nde I (20 U/µl)	2.5 µl	1 µl

ddH_2O to final volume	50 µl	50µl

The reactions were incubated 1 hour at 37°C before the enzyme was heat inactivated, 10 min at 65°C. The linearised plasmid was purified by ethanol precipitation, according to standard procedures (41), and dissolved in sterile ddH_2O. The digested DNA fragment was purified using Wizard PCR preps DNA purification system. Before ligation both DNAs were quantitated using electrophoresis and scanning densitometry as usual.

The linear pCITE-4a(+) was dephosphorylated as described for pCITE-3b in the preparation of template C4. The ligation reaction mixture was prepared as outlined above using a 1:3 molar ratio of plasmid DNA to fragment DNA. The ligation reaction was allowed to proceed for 6 hours at 10°C followed by heat inactivation of the ligase. The ligation products were separated by agarose (1 %) electrophoresis and the band corresponding to the 4017 bp product was excised. The DNA was purified from the agarose using Geneclean according to the manufacturer's directions. The DNA was dissolved in sterile ddH_2O and quantitated.

The S-Tag and thrombin cleavage site coding sequences were removed by restriction digestion of the ligation product with Bam HI. This restriction enzyme cuts downstream of the α-peptide coding sequence and just upstream of the $[dA/dT]_{30}$ sequence which left cohesive ends available for circularisation of the long DNA. The restriction digestion was performed as follows:

10× buffer B	3 µl
ligation product	20 µl
Bam HI (10 U/µl)	1 µl
ddH_2O to final volume	30 µl

The reaction was incubated for 90 min at 37°C before the products were separated by electrophoresis. The band corresponding to the 3922 bp fragment was excised and the DNA was purified using Geneclean. After the DNA was dissolved in sterile ddH_2O it was circularised by ligation:

3922 bp DNA	20 µl
ATP (20 mM)	1.5 µl

10× One-Phor-All buffer plus	3 µl
T4 DNA ligase (6.2 U/µl)	1 µl
ddH_2O to final volume	30 µl

The ligation reaction was incubated for 7 hours at 10°C before the ligase was heat inactivated, 10 min at 65°C. There was no need for purification of the resulting plasmid, pCITE-C4[dA/dT](+T7term).

The recombinant plasmid, pCITE-4a(C4), was transfected into *E. coli* JM109 bacteria, which were then selected, cultured and harvested. The recombinant plasmid was purified and quantitated as described for pSP64-A1 used in the production of template A1[dA/dT]. The template C4[dA/dT](+T7term) was obtained from a restriction digest of pCITE-4a(C4):

10× NEBuffer 2	5 µl
pCITE-4a(C4) (1.6 µg/µl)	5 µl
Bsr BI (10 U/µl)	8 µl
ddH_2O to final volume	50 µl

After a 1 hour incubation at 37°C the three resulting fragments were separated by agarose (1.5 %) electrophoresis. The band corresponding to the 1368 bp template C4[dA/dT](+T7term) was excised and the DNA was purified from the agarose using Geneclean. The DNA was then quantitated by electrophoresis and scanning densitometry as usual.

Template C5: α-Peptide Codons 8-79

This expression template was prepared by simply performing a single restriction digestion to linearise pCITE-βGal. The restriction enzyme, Bsu 36I, was used since it cuts the β-Gal coding sequence after codon 79:

10× NEBuffer 3	5 µl
pCITE-βGal (1.3 µg/µl)	5 µl
Bsu 36I (10 U/µl)	2.4 µl
ddH_2O to final volume	50 µl

After incubation of the reaction for 2 hours at 37°C the enzyme was heat inactivated, 20 min at 85°C. No purification of template C5 was required prior to *in vitro* expression.

Template C6: α-Peptide Codons 8-650

This expression template was prepared by simply performing a single restriction digestion to linearise pCITE-βGal. The restriction enzyme, Sac I, was used since it cuts the β-Gal coding sequence after codon 650:

10× One-Phor-All buffer plus	10 µl
pCITE-βGal (1.3 µg/µl)	20 µl
Sac I (8 U/µl)	12 µl
ddH_2O to final volume	100 µl

After incubation of the reaction for 1½ hours at 37°C the enzyme was heat inactivated, 20 min at 65°C. The fragments were separated by agarose (1 %) electrophoresis, the bands corresponding to the 5626 bp fragment were excised and the DNA was purified using Geneclean. Template C6 was dissolved in sterile ddH_2O and quantitated.

3.2.6 Fluorometric β-Galactosidase Assay in Rabbit Reticulocyte Lysate Based Expression Mixtures

After expression of the DNA template using the TNT T7 coupled rabbit reticulocyte lysate system (according to the manufacturer's directions), the 25 µl reaction was mixed with 25 µl of M15 extract and incubated for 1 hour at 37°C. Substrate was added (50 µl of 0.2 mM 4-MUG in β-Gal assay buffer) directly to the α-complementation reaction mixture and incubated for 1 hour at 37°C. The protein was precipitated by adding 25 µl of ice-cold 25 % (w/v) trichloroacetic acid (TCA) and the precipitate was removed by centrifugation for 10 min at 6000 × g. The supernatant was collected and mixed with 200 µl of stop buffer. A 130 µl portion of this solution was transferred to a white, flat bottomed microtitre well and fluorescence was measured using the Fluoroskan II fluorometer, with excitation and emission wavelengths at 355 nm and 460 nm, respectively.

3.2.7 S-Tag Rapid Assay

The S-Tag rapid assay was performed using the manufacturer's suggested protocol for assay of *in vitro* synthesised proteins. A series of reactions were prepared containing the following components:

	Blank	Standard	Unknown
sterile ddH_2O	338 µl	336 µl	338 µl
S-Tag standard (0.05 pmol/µl)	—	2 µl	—
Blank TNT reaction (- DNA)	12 µl	12 µl	—
Sample TNT reaction	—	—	12 µl
10× S-Tag Assay buffer	40 µl	40 µl	40 µl
S-Tag Grade S-protein	10 µl	10 µl	10 µl
	400 µl	400 µl	400 µl

The reactions were mixed well and incubated 5 min at 37°C before the reactions were terminated with the addition of 100 µl of ice-cold 25 % (w/v) TCA. The tubes were vortexed and, after a 5 min incubation on ice, centrifuged for 10 min at 14 000 × g at 4°C. The absorbance of the supernatants was read at 280 nm with the spectrophotometer zeroed using the supernatant from the blank reaction. The amount of S-Tag protein produced was determined using the following equation:

$$\text{pmol/µl} = (\text{Unknown } A_{280} \div 12\ \text{µl}) \times (0.1\ \text{pmol S-Tag standard} \div \text{Standard } A_{280})$$

3.2.8 Preparation of Luciferase Encoding DNA with T7 Promoter and Shine-Dalgarno Sequence

This expression template was prepared using pLucExp as the PCR template, however, it was necessary to linearise the plasmid prior to PCR:

10× Hind III buffer	2 µl
pLucExp (0.29 µg/µl)	4 µl
Hind III (10 U/µl)	1 µl
ddH_2O to final volume	20 µl

After allowing the restriction digestion to proceed for 2 hours at 37°C the enzyme was

heat inactivated for 20 min at 65°C. The reaction mixture was then diluted in sterile ddH_2O and used as a template in a PCR:

10× Pfu buffer	10 µl
UT7SD primer (10 µM)	5 µl
Dluc-I primer (10 µM)	5 µl
dNTPs (2.5 mM)	6 µl
linear pLucExp (9.6 ng/µl)	1 µl
ddH_2O to final volume	99 µl

The Pfu polymerase (2.5 U/µl, 1.5 µl) was added during the initial heating at 95°C (hot start). After the addition of the polymerase, PCR was carried out for four cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 5 min. After completion of the initial four cycles, PCR was continued for an additional 26 cycles of denaturation at 95°C for 30 sec, annealing at 65°C for 1 min and extension at 72°C for 5 min. The 1815 bp PCR product was purified using Wizard PCR Preps DNA purification system and quantitated.

3.2.9 Preparation of *E. coli* S-30 Extract

The *E. coli* S-30 extract was prepared using the method developed by Zubay (40, 74) and modified by Pratt (75). A ribonuclease I (RNase I) free strain of *E. coli*, strain A19 {geneotype: Hfr, λ^+, rna-19, gdhA2, his-95, relA1, spoT1, metB1}(76, 77), was used for the preparation of the extract. The first step in the procedure was to screen the *E. coli* A19 to ensure it had not reverted to rna^+, since the rna^-19 is a point mutation. This was accomplished by spotting eight colonies onto 2×YT agar plates, in duplicate, being careful to clearly label the colonies on each plate. These plates were incubated for 6 hours at 37°C before covering one of the plates with soft agar (7 g/L) containing 30 g/L yeast RNA and 0.1 M EDTA. The RNA-treated plate was incubated for 3 hours at 42°C while the second plate was kept at room temperature. The treated plate was then covered with a thin layer of 1 M HCl which precipitated uncut RNA. Colonies producing RNase I would be surrounded by a transparent "halo" which would indicate that the RNA proximal to these colonies had been destroyed by the enzyme. No A19 colonies

produced a "halo" after this procedure, therefore, a corresponding colony from the untreated plate was used to inoculate 50 ml of 2×YT media which was then incubated overnight at 37°C with vigorous shaking. The overnight culture was used to prepare stock solutions of A19 which were stored at -80°C (as described above).

A second overnight culture of A19 was prepared in 100 ml of 2×YT media. This culture was scaled up in a total of 6 l of 2×YT media which was incubated at 37°C with vigorous shaking. The growth was monitored at 30 min intervals by measuring the absorbance at 650 nm. The cells were ready for harvesting when the A_{650} had reached approximately 0.7. The cells were immediately chilled on ice, since it was important not to allow the cultures to overgrow because extracts prepared from stationary cells show little to no activity. The cells were harvested by centrifugation at 4°C. Meanwhile, the S-30 buffer (must be freshly made immediately before use) was prepared by mixing:

20 ml 1.4 M magnesium acetate

20 ml 6 M potassium acetate

20 ml 0.1 M DTT

20 ml 1.0 M Tris-CH_3COO, pH 8.2

1.92 l DEPC-treated ddH_2O

It was important that all solutions used in the preparation of the S-30 extract were RNase free which meant that all the solutions were prepared in DEPC-treated ddH_2O (see section 2.2) and sterilised either by autoclaving or filtering. The cells were washed three times with 500 ml cold S-30 buffer containing 0.05 % (v/v) mercaptoethanol. After the last wash the mass of the pellet was determined to be 14.26 g.

The cell pellet was slowly resuspended in 17.8 ml of S-30 buffer (1.25 ml/g) before the cells were disrupted using the Bead-Beater at 4°C with three, 1 min homogenisations. The glass beads were removed by centrifugation. The volume of the resulting preparation was determined (~12 ml) and 10 µl of 0.1 M DTT was added per ml of lysate. The mixture was immediately centrifuged at 30 000 × g at 4°C for 30 min. The centrifuge tubes were previously treated with DEPC to remove any RNases. The top four fifths of the supernatant was placed in a fresh, treated centrifuge tube and centrifuged for an

additional 30 min at 30 000 × g at 4°C. The volume of the top four fifths of the supernatant was determined (8.3 ml) and it was mixed with 2.5 ml of freshly prepared pre-incubation mixture (7.5 ml/25 ml of supernatant). The pre-incubation mixture was prepared by combining the following:

pyruvate kinase (1471 U/ml)	11.3 µl
2.2M Tris-acetate, pH8.2	333 µl
3.0 magnesium acetate	7.7 µl
40 mM ATP, pH 7.0	833 µl
0.42 M phosphoenol pyruvate, pH 7.0	499 µl
0.85 M DTT	12.9 µl
55 mM amino acid mix	5.5 µl
DEPC-treated ddH_2O to final volume	2.5 ml

The extract was incubated with the pre-incubation mixture for 80 min at 37°C with gentle mixing. The resulting solution was then dialysed against four changes of 50 volumes fresh S-30 buffer at 4°C, allowing 45 min between each change. The dialysed extract was centrifuged for 12 min at 4000 × g at 4°C and the supernatant was aliquotted into microcentrifuge tubes and stored at -80°C. This supernatant is the S-30 extract used in subsequent cell-free expression reactions.

3.2.10 *In Vitro* Transcription and Translation Using the S-30 Extract with T7 RNA Polymerase

The low molecular weight (LMW) mixture contained a buffer system, NTPs, tRNA, amino acids and an energy generating system. Certain components (eg. Mg^{2+}, K^+ and NH_4^+) required optimisation, however, the basic preparation is described in section 3.2.4. A typical *in vitro* expression reaction mixture is given here:

DEPC treated ddH_2O to final volume	x µl
S-30 Extract	6 µl
5× LMW Mix	5 µl
0.37 M phosphoenol pyruvate, pH 7	3.1 µl

40 % polyethylene glycol	2.5 µl
T7 RNA polymerase (69 U/µl)	0.5 µl
DNA template	y µl
	25 µl

The components were added in the order given, then incubated for 90 min at 30°C. In some cases 8.3 mg of rifampicin (1.7 µl of 5 mg/ml in 10 % methanol) was added to the reaction mixture before incubation at 30°C. The reaction mixture was then used in the appropriate detection reaction.

3.2.11 Bioluminescence Assay of Firefly Luciferase

After *in vitro* expression, 2 µl of the reaction mixture was mixed with 50 µl of luciferin substrate solution at room temperature in a microcentrifuge tube (1.5 ml). The tube was immediately placed in a glass scintillation vial and the luminescence was measured for 1 min using a liquid scintillation counter in the single photon monitoring mode. The measurement obtained after 0.75 min elapsed time was used for the reported signal.

3.3 Results and Discussion

The tac promoter is a chimeric promoter composed of part of the trp promoter and part of the lacUV5 promoter. As illustrated in Figure 3-1, PCR was used to synthesise a DNA template with a tac promoter, in place of the lac promoter, upstream of the α-peptide coding sequence in pGEM 13Zf(+). The upstream primer contained the sequence corresponding to the trp portion of the tac promoter plus 10 nucleotides from the lacUV5 promoter/operator (present in the PCR template). The downstream primer contained the last three codons of the α-peptide coding sequence plus the stop codon. In each template synthesised in this study the stop codon was UAA, which is the most efficient terminator of translation (78). The stop codon was immediately followed by G. Studies have shown that the first nucleotide following the stop codon also has a strong effect on translation termination and G was found to be the most effective (78). The expression of the tac

Figure 3-1: Preparation of the tac Promoter-Containing lacZα DNA. pGEM 13Zf(+) was used as the initial template in PCR which used an upstream primer containing tac promoter sequences to replace the lac promoter. The downstream primer was complementary to the 3′ end of the α-peptide coding sequence. tac = tac promoter; amp^r = β-lactamase gene.

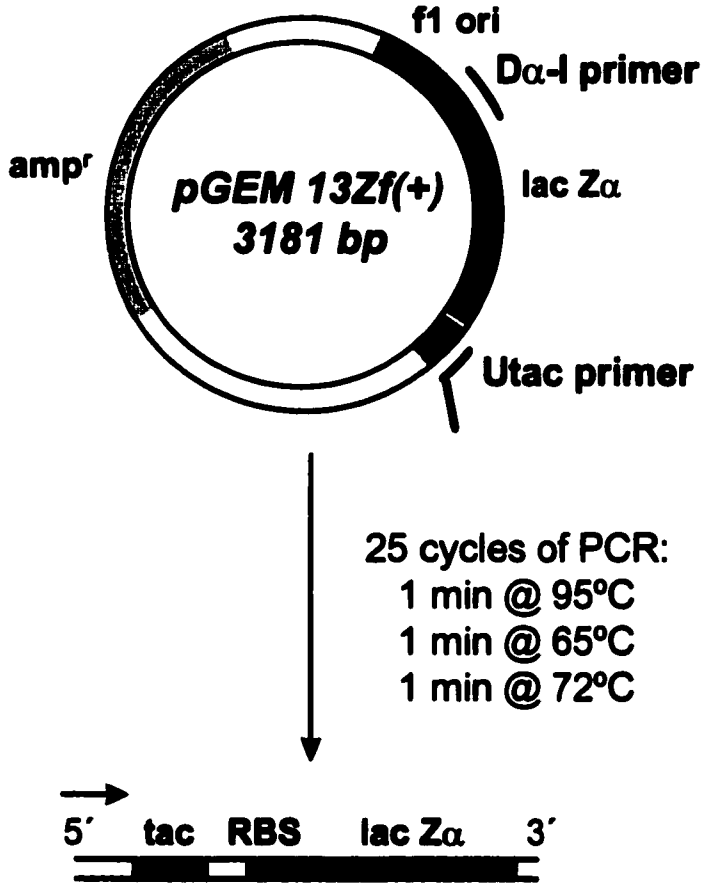

f1 ori
Dα-I primer
amp^r
pGEM 13Zf(+)
3181 bp
lac Zα
Utac primer
25 cycles of PCR:
1 min @ 95°C
1 min @ 65°C
1 min @ 72°C
5′
tac
RBS
lac Zα
3′

Figure 3-1

promoter containing lacZα DNA was compared to the lac promoter containing template using the *E. coli* S-30 extract expression system from Promega. Unfortunately, the original lac promoter-containing fragment was approximately three times more efficient. This suggested that in order to improve the efficiency of α-peptide expression it may be necessary to switch to a more effective RNA polymerase.

As discussed above, the T7 RNAP is well studied and known to work well in cell-free translation of DNA templates with the appropriate promoter sequence. This promoter sequence was incorporated into a number of alternative lacZα DNA templates. The first set of templates described here is the most simple. These templates contain the T7 promoter sequence, a Kozak consensus sequence, the α-peptide coding region and a stop codon (as described previously). In this set of templates the α-peptide coding region contained codons 8 to 59 only, which is the region spanned by most prokaryotic α-donor peptides. The plasmid, pSV-βGal, was used as the template in the first PCR step. This plasmid contains the lacZ gene (codons 8-1023) which encodes the active β-Gal monomer. The absence of the first 7 amino acids does not affect the activity of the wild type enzyme, so it was reasoned that it would, similarly, have no effect on the complemented enzyme.

Two α-peptide encoding templates, A1 and A2, were prepared containing the sequences just described, by a two-step PCR procedure (Figure 3-2). The presence of a C directly upstream of the T7 promoter has been shown to increase the yield of T7 RNAP mediated transcription (79). Template A2 contained an AATT sequence, in place of the C in template A1, 5′ of the T7 promoter. The two templates were identical in all other aspects. Further, both templates were modified with the addition of a $[dA/dT]_{30}$ sequence downstream of the α-peptide coding sequence. A 3′-poly(A) sequence in mRNA is thought to contribute to translation initiation factor binding and consequently increase translation initiation efficiency (80, 81). The 3′-poly(A) tail was added to the templates by simply cloning the templates into pSP64-Poly(A). During the second PCR step used to create templates A1 and A2, restriction sites were added, Hind III at the 5′end and Bam

Figure 3-2: Synthesis of lacZα Templates A1 and A2. A two-step PCR procedure was used to prepare these templates. The 199 bp intermediate was prepared by PCR of the pSV-βGal template using an upstream primer containing the T7 promoter upstream of the 5′ sequence of the α-peptide coding sequence and a downstream primer which was complementary to the 3′ end of the α-peptide coding sequence. After isolation of this DNA it was used as the template for the next PCR step. The UT7-II primer was used for the preparation of template A1 and the UT7-III primer was used for the preparation of template A2. These primers contained the 5′ sequences of the T7 promoter, and added specific nucleotides immediately upstream of the promoter and Hind III restriction sites to the 5′ end of the templates. The downstream, Dα-II, primer was used for all PCRs and added a Bam HI restriction site to the 3′ end of the templates. T7 = T7 promoter; rbs = Kozak consensus sequence; amp^r = β-lactamase gene.

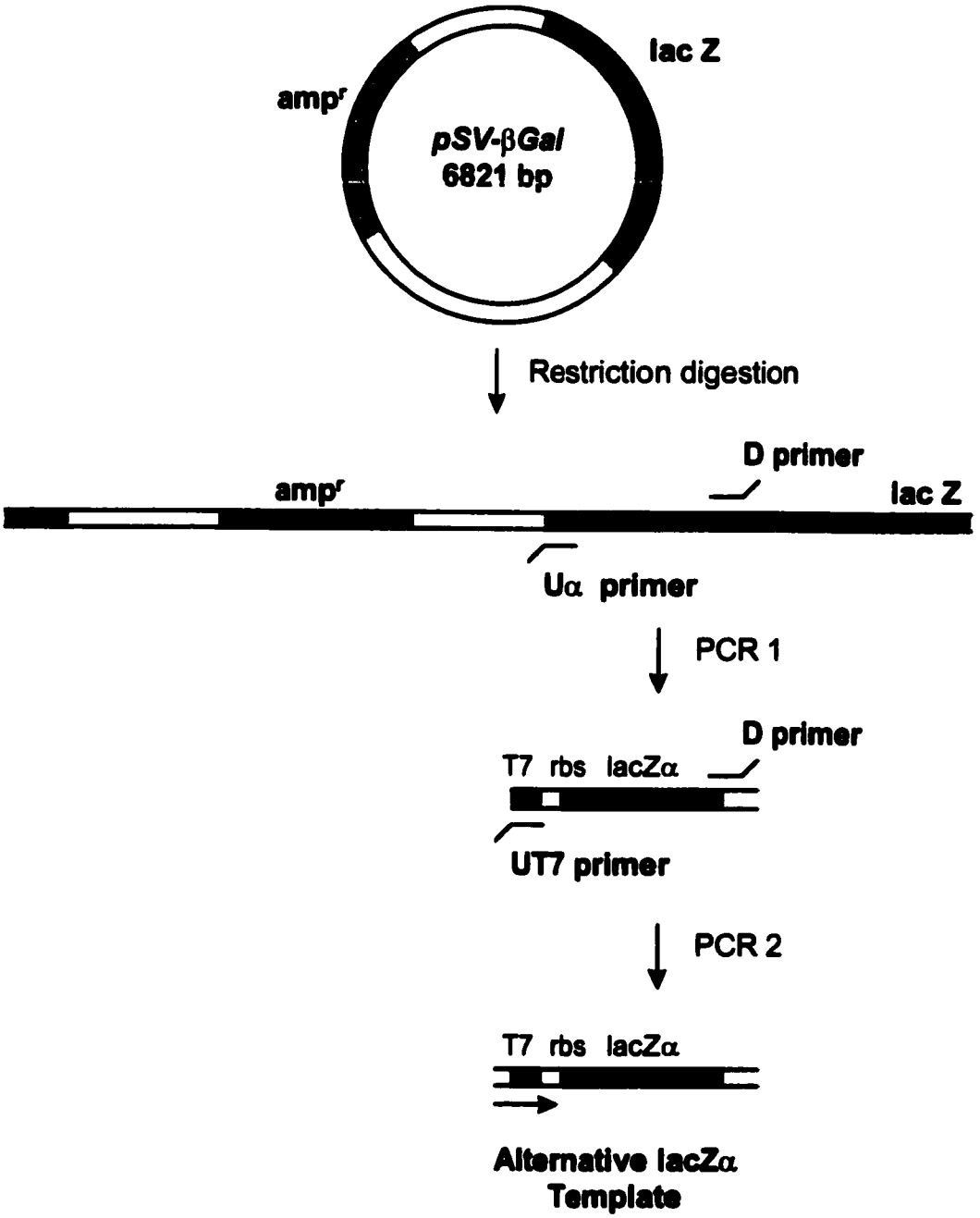

lac Z
amp^r
pSV-βGal
6821 bp
Restriction digestion
D primer
amp^r
lac Z
Uα primer
PCR 1
D primer
T7 rbs lacZα
UT7 primer
PCR 2
T7 rbs lacZα
Alternative lacZα
Template

Figure 3-2

HI at the 3′end. These sites facilitated the insertion of the templates with the correct orientation and position in the pSP64Poly(A) polycloning site (Figure 3-3). After the recombinant plasmids were obtained the templates A1[dA/dT] and A2[dA/dT] were isolated following restriction digestion with Hind III and Eco RI (Figure 3-3).

The four templates, A1, A2, A1[dA/dT] and A2[dA/dT] , were expressed *in vitro* using the T7 TNT coupled rabbit reticulocyte lysate system and, following α-complementation, the β-Gal activity was determined using the fluorometric assay. In each case no appreciable production of active complemented β-Gal was observed. Since studies indicated that α-complementation proceeds well in the rabbit reticulocyte lysate (results not shown), this lack of activity was attributed to low expression of α-peptide from these templates. At this point it was thought that it may be necessary to include a 5′-ULS to the templates to facilitate expression in the eukaryotic system.

A series of potential lacZα templates was prepared incorporating the AMV 5′-ULS. As outlined previously, there is evidence that this upstream untranslated region can improve ribosome binding and increase the yield of *in vitro* translation. The first template, B1, contained codons 1-59 of the α-peptide coding region. This template was created to test the need for the first seven amino acids in successful α-complementation and/or β-Gal activity of the complemented enzyme. The absence of these amino acids was a possible cause for lack of activity observed from expression of the "A" templates. Template B1 was synthesised by PCR using pGEM 13Zf(+) as the initial PCR template. The first PCR was performed using an upstream primer containing sequences surrounding the polycloning site of pGEM 13Zf(+). In this way, the polycloning site was removed from the template (Figure 3-4). An extension PCR was subsequently carried out to add the entire T7 promoter and AMV 5′-ULS. Since the yield from this reaction was presumed to be significantly low, upstream and downstream primers were added directly to the reaction mixture to amplify the extension product. The 263 bp template B1 was obtained in good yield. Expression of template B1 indicated that the presence of the first seven codons did not improve the yield of active complemented enzyme. In fact, *in vitro* expression did not yield any appreciable activity. Interestingly, template B1-PCS,

Figure 3-3: Addition of $[dA/dT]_{30}$ to Alternative lacZα Templates. The template and pSP64Poly(A) were digested with Hind III which cleaved the alternative lacZα template upstream of its T7 promoter and cleaved the plasmid within its poly cloning site. After ligation with T4 ligase the resulting DNA was digested with Bam HI which cut downstream of the α-peptide coding sequence and within the poly cloning site of the original plasmid. T4 ligase was used to circularise the DNA and the resulting recombinant plasmid was amplified in *E. coli* JM 109 and purified. Double restriction digestion of the isolated plasmid using Hind III and Eco RI (cuts downstream of the $[dA/dT]_{30}$ sequence) was used to isolate the final expression template. T7 = T7 promoter; rbs = Kozak consensus sequence; amp^r = β-lactamase gene.

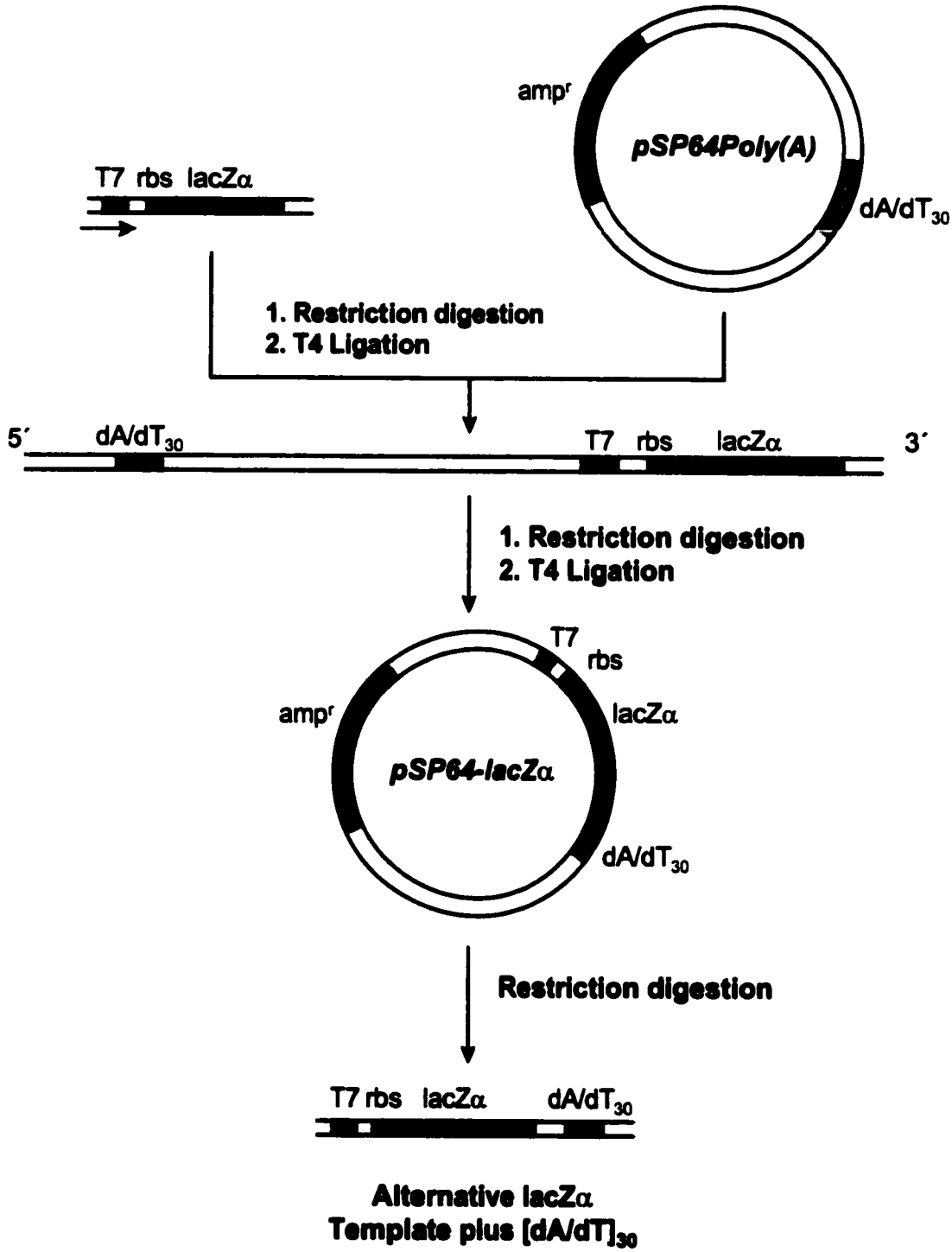

amp^r
pSP64Poly(A)
dA/dT$_{30}$
T7 rbs lacZα
1. Restriction digestion
2. T4 Ligation
5′
dA/dT$_{30}$
T7 rbs lacZα
3′
1. Restriction digestion
2. T4 Ligation
T7
rbs
amp^r
lacZα
pSP64-lacZα
dA/dT$_{30}$
Restriction digestion
T7 rbs lacZα
dA/dT$_{30}$
Alternative lacZα
Template plus [dA/dT]$_{30}$

Figure 3-3

Figure 3-4: Synthesis of lacZα Template B1. pGEM 13Zf(+) was used as the initial template in PCR which used an upstream primer containing the coding sequence for amino acids 1-8 of the α-peptide. This sequence surrounds the poly cloning site of the plasmid so this PCR step effectively removes this irrelevant sequence. The intermediate product was purified and used as the template in the next PCR using an extension primer to add the T7 promoter, the AMV 5′-ULS and a eukaryotic ribosome binding site. The primers used to amplify the PCR product were added directly to the reaction mixture and PCR was continued to obtain the final template B1. T7 = T7 promoter; rbs = Kozak consensus sequence; ULS = 5′ untranslated leader sequence from AMV.

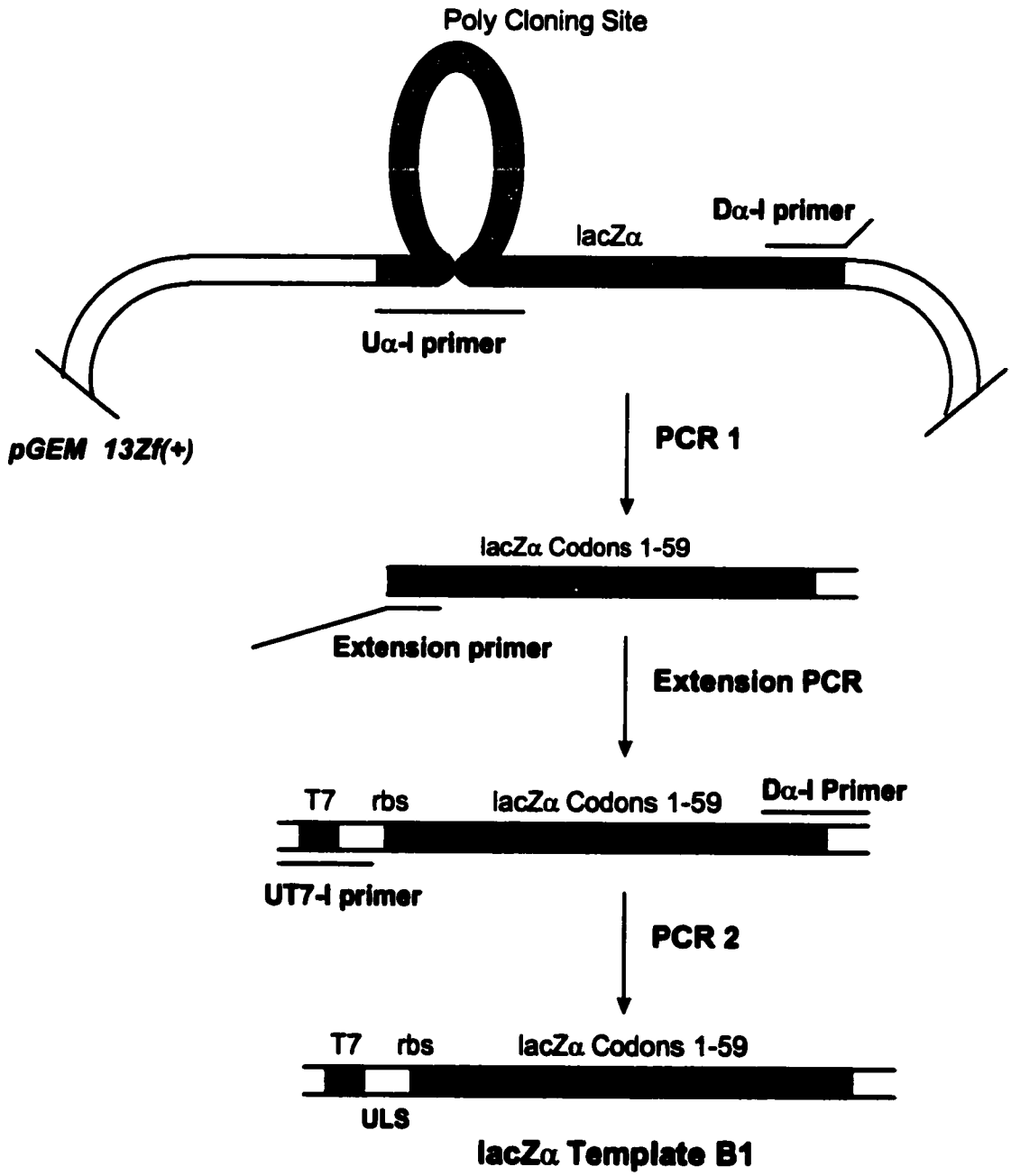

Poly Cloning Site
lacZα
Dα-I primer
Uα-I primer
pGEM 13Zf(+)
PCR 1
lacZα Codons 1-59
Extension primer
Extension PCR
T7
rbs
lacZα Codons 1-59
Dα-I Primer
UT7-I primer
PCR 2
T7
rbs
lacZα Codons 1-59
ULS
lacZα Template B1

Figure 3-4

which was identical to B1 except that the polycloning site was not removed, did exhibit a very low level of *in vitro* expression. Perhaps the length of the α-peptide is important for α-complementation.

A schematic illustrating the components of the "B" templates is given in Figure 3-5. In each case the templates were prepared by PCR, except B2[dA/dT] and B3[dA/dT] which were prepared after insertion of B2 and B3, respectively, into pSP64Poly(A). None of these templates resulted in the production of active complemented β-Gal following *in vitro* expression and α-complementation. It was thought that by increasing the length of the templates it may be possible for successful expression and α-complementation to occur. The first example of α-complementation in mammalian cells, published in 1996 (82), indicated that longer α-donor peptides were required for successful α-complementation in mammalian systems than in *E. coli* systems. However, in the present study it was found that increasing the length of the templates to include up to codon 105 did not result in the formation of active enzyme.

The CITE 5′-ULS was the second 5′-ULS incorporated into lacZα DNA templates to attempt to increase the yield of *in vitro* expression. Three templates were prepared which differed only in the length of the α-peptide coding sequence; C1 contained codons 8-59, C2 contained codons 8-105 and C3 contained codons 8-130. Each was prepared by PCR using the plasmid pCITE-βGal as the initial template (Figure 3-6). Each template was expressed *in vitro* in order to determine the amount of active complemented β-Gal that could be produced from each template. Expression of templates C1 and C3 followed by α-complementation, resulted in no detectable β-Gal activity. Template C2 did show a small amount of active enzyme produced from *in vitro* expression and α-complementation; corresponding to 0.03 α-peptide molecules produced per DNA molecule. This was calculated from a β-Gal calibration curve prepared by diluting wild-type β-Gal in a typical rabbit reticulocyte based expression mixture (no DNA template) and measuring fluorescence generated from incubation with 4-MUG, followed by precipitation using 25 % TCA and collection of the supernatant.

Figure 3-5: Schematic of lacZα Templates Containing the AMV 5′-ULS. T7 = T7 promoter; rbs = Kozak consensus sequence; AMV-ULS = 5′ untranslated leader sequence from AMV; PCS = poly cloning site from pGEM 13Zf(+).

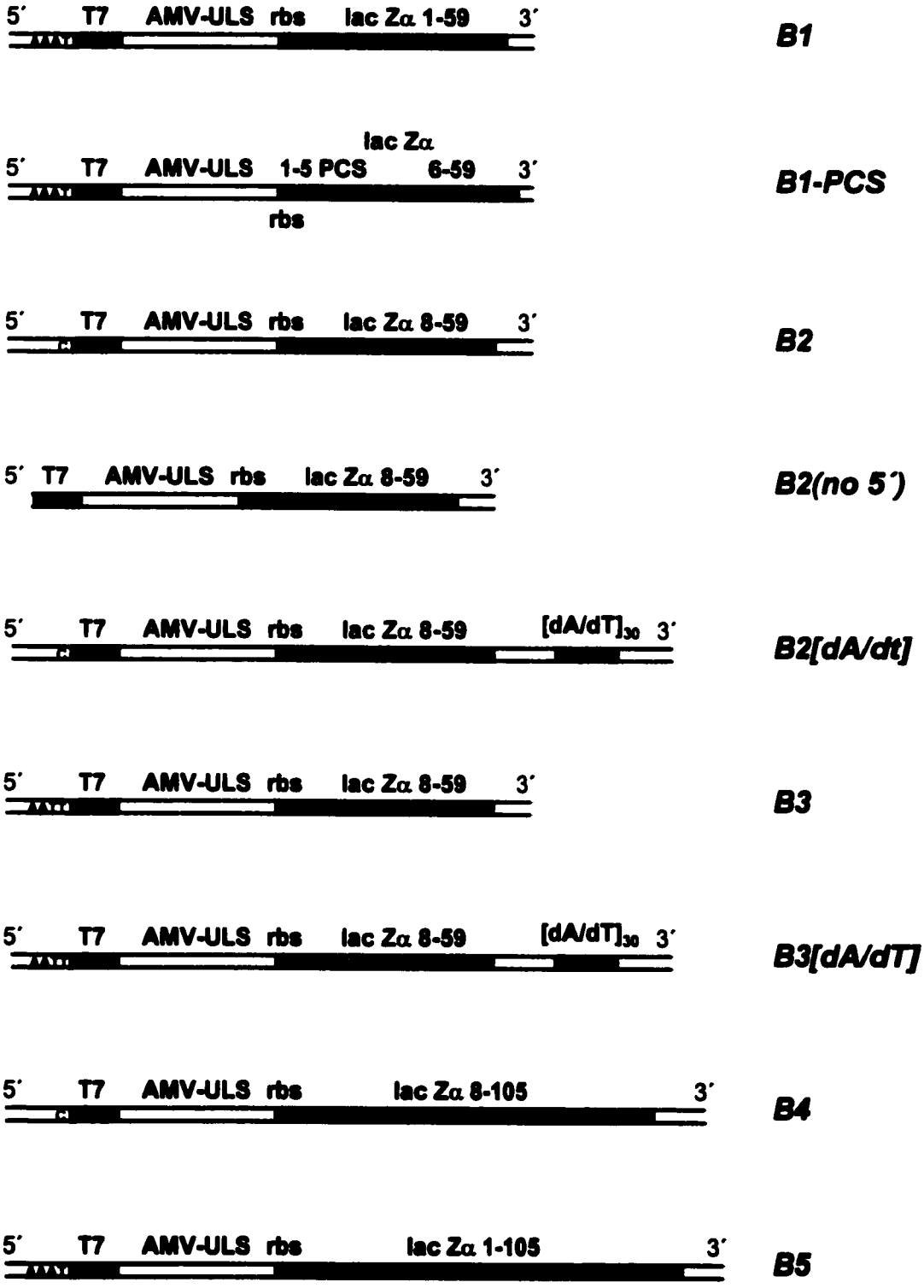

5′ T7 AMV-ULS rbs lac Zα 1-59 3′
B1
lac Zα
5′ T7 AMV-ULS 1-5 PCS 6-59 3′
rbs
B1-PCS
5′ T7 AMV-ULS rbs lac Zα 8-59 3′
B2
5′ T7 AMV-ULS rbs lac Zα 8-59 3′
B2(no 5′)
5′ T7 AMV-ULS rbs lac Zα 8-59 [dA/dT]30 3′
B2[dA/dt]
5′ T7 AMV-ULS rbs lac Zα 8-59 3′
B3
5′ T7 AMV-ULS rbs lac Zα 8-59 [dA/dT]30 3′
B3[dA/dT]
5′ T7 AMV-ULS rbs lac Zα 8-105 3′
B4
5′ T7 AMV-ULS rbs lac Zα 1-105 3′
B5

Figure 3-5

Figure 3-6: Synthesis of lacZα Templates C1, C2 and C3. The templates were prepared from a single PCR step using pCITE-βGal as the initial template and primers as indicated in the figure. For preparation of template C3, the plasmid was linearised by restriction digestion with Xho I prior to PCR. T7 = T7 promoter; CITE-ULS = Cap Independent Translation Enhancer 5′ untranslated leader sequence; amp^r = β-lactamase gene; STag = S-peptide and thrombin cleavage site coding sequences.

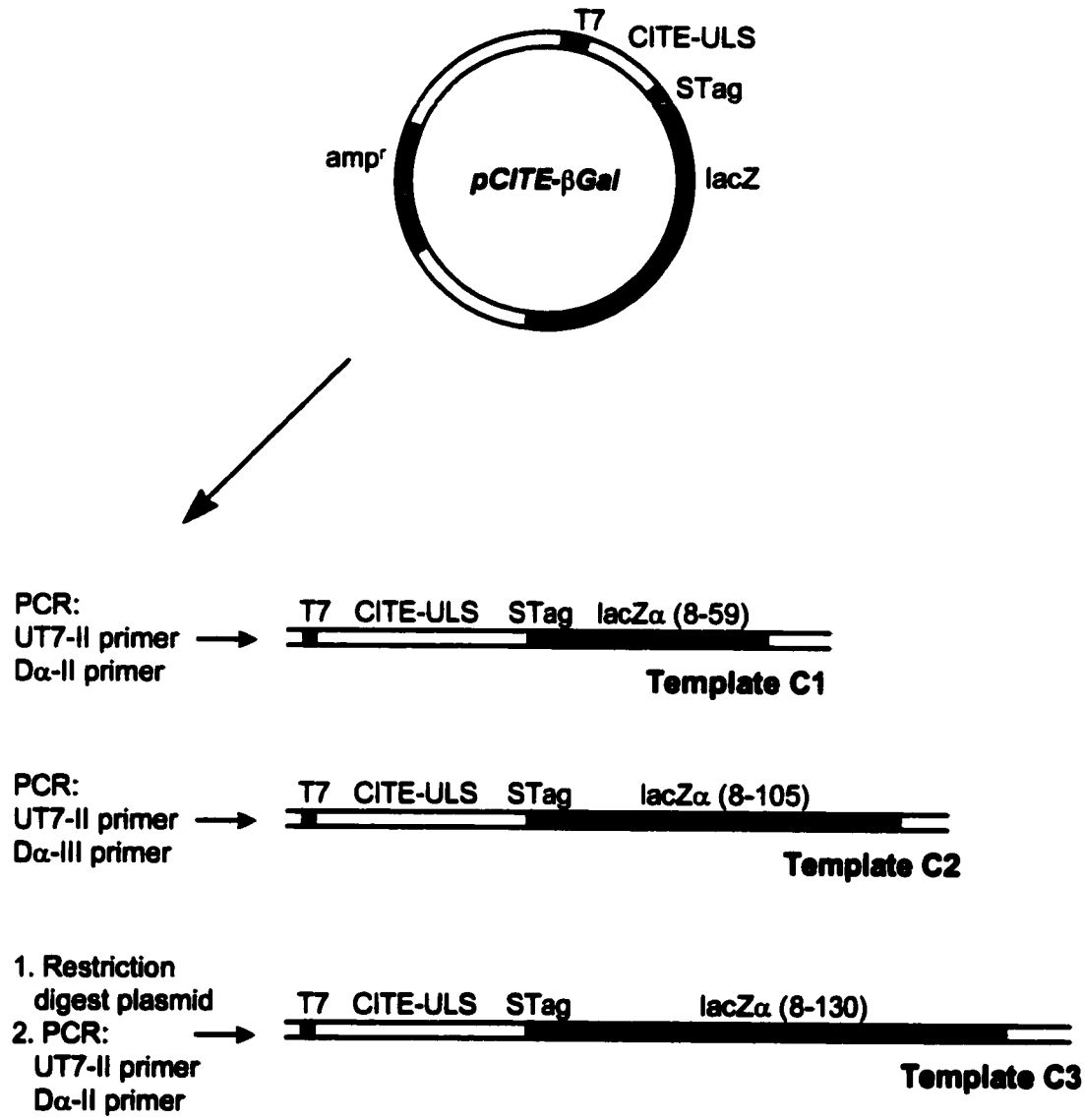

T7
CITE-ULS
STag
amp^r
pCITE-βGal
lacZ
PCR:
UT7-II primer
Dα-II primer
T7 CITE-ULS STag lacZα (8-59)
Template C1
PCR:
UT7-II primer
Dα-III primer
T7 CITE-ULS STag lacZα (8-105)
Template C2
1. Restriction digest plasmid
2. PCR:
UT7-II primer
Dα-II primer
T7 CITE-ULS STag lacZα (8-130)
Template C3

Figure 3-6

In an attempt to improve this expression the S-Tag and thrombin cleavage site coding sequences were removed from template C2 (Figure 3-7). This template, C4, was prepared by first using PCR to add an Nde I restriction site at the start codon of the α-peptide coding sequence. By cutting both pCITE-3b and the PCR product with Nde I followed by ligation at the resulting cohesive ends a template for the last PCR step was formed. The final PCR for production of template C4 was performed using an upstream primer containing T7 promoter sequences and a downstream primer containing the 3′-terminal codons of the α-peptide coding sequence. The resulting template C4 was produced in good yield and used in an *in vitro* expression reaction. Analysis of the *in vitro* α-complementation product indicated that the removal of the S-Tag and thrombin cleavage site coding sequences did improve either the expression or complementation efficiency, or both. It was found that expression followed by complementation produced 0.1 α-peptide molecules from each C4 DNA template. Surprisingly, the addition of a 3′ poly(A) tail followed by a T7 terminator (by cloning into pCITE-4a(+) instead of pCITE-3b as shown in Figure 3-8) actually decreased the amount of α-peptide produced to 0.02 molecules per DNA template.

The final CITE 5′-ULS containing templates studied were prepared by simply creating linear pCITE-βGal with restriction enzymes that cut the within the β-Gal coding sequence. Although these templates do not end with a stop codon, translatable, run off transcripts should be formed through the action of T7 RNAP. Following *in vitro* transcription/translation and α-complementation the amount of active complemented β-Gal was determined. It was found that template C5, which contained codons 8-79, produced less β-Gal activity (0.01 α-peptides per DNA molecule) than template C2, which contained codons 8-105. It should be noted that in the absence of the α-complementation reaction no β-Gal activity was observed, indicating that the truncated lacZ gene could not produce active β-Gal. Expression of template C6 followed by α-complementation produced the highest amount of complemented β-Gal activity. This template contained codons 8-650 which makes it the longest of all the templates tested. Again, expression of template C6 without α-complementation did not result in any β-Gal

Figure 3-7: Synthesis of lacZα Template C4. pCITE-βGal was used as the initial template for PCR using an upstream primer which added an Nde I restriction site at the start codon of the α-peptide coding sequence and a downstream primer which was complementary to the 3′ end of the α-peptide coding sequence (to codon 105). After restriction digestion of the PCR product and pCITE-3b with Nde I the two DNAs were ligated. The resulting DNA was the template for a second PCR step using an upstream primer containing T7 promoter sequence and the downstream primer which was complementary to the 3′ end of the coding sequence. T7 = T7 promoter; CITE-ULS = Cap Independent Translation Enhancer 5′ untranslated leader sequence; amp^r = β-lactamase gene; STag = S-peptide and thrombin cleavage site coding sequences.

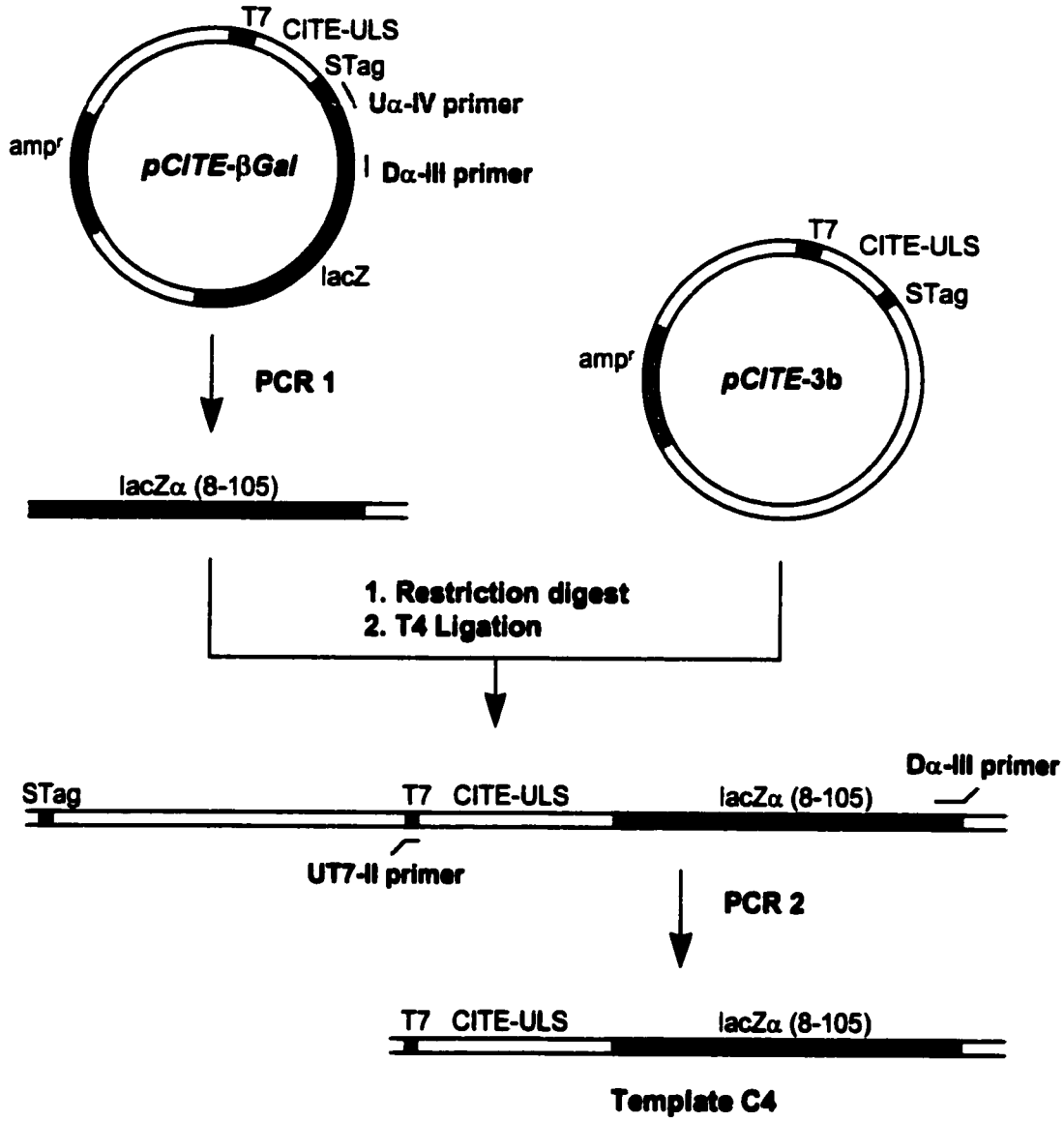

T7
CITE-ULS
STag
Uα-IV primer
ampr
pCITE-βGal
Dα-III primer
lacZ
PCR 1
lacZα (8-105)
T7
CITE-ULS
STag
ampr
pCITE-3b
1. Restriction digest
2. T4 Ligation
Dα-III primer
STag
T7 CITE-ULS
lacZα (8-105)
UT7-II primer
PCR 2
T7 CITE-ULS
lacZα (8-105)
Template C4

Figure 3-7

Figure 3-8: Addition of $[dA/dT]_{30}$ and T7 Termination Sequences to Template C4. pCITE-βGal was used as the initial template for PCR using an upstream primer which added an Nde I restriction site at the start codon of the α-peptide coding sequence and a downstream primer which was complementary to the 3′ end of the α-peptide coding sequence (to codon 105) and added a Bam HI restriction site. After restriction digestion of the PCR product and pCITE-4a(+) with Nde I the two DNAs were ligated. A second restriction digest using Bam HI cut the DNA downstream of the α-peptide coding sequence and removed the S-Tag and thrombin cleavage site coding sequences. The DNA was circularised by ligation using T4 ligase and the resulting recombinant plasmid was amplified in *E. coli* JM109 and purified. The plasmid was used for preparation of template C4[dA/dT](+T7term) by restriction digestion with Bsr BI which cut upstream of the T7 promoter and downstream of the T7 terminator. T7 = T7 promoter; CITE-ULS = Cap Independent Translation Enhancer 5′ untranslated leader sequence; amp^r = β-lactamase gene; STag = S-peptide and thrombin cleavage site coding sequences.

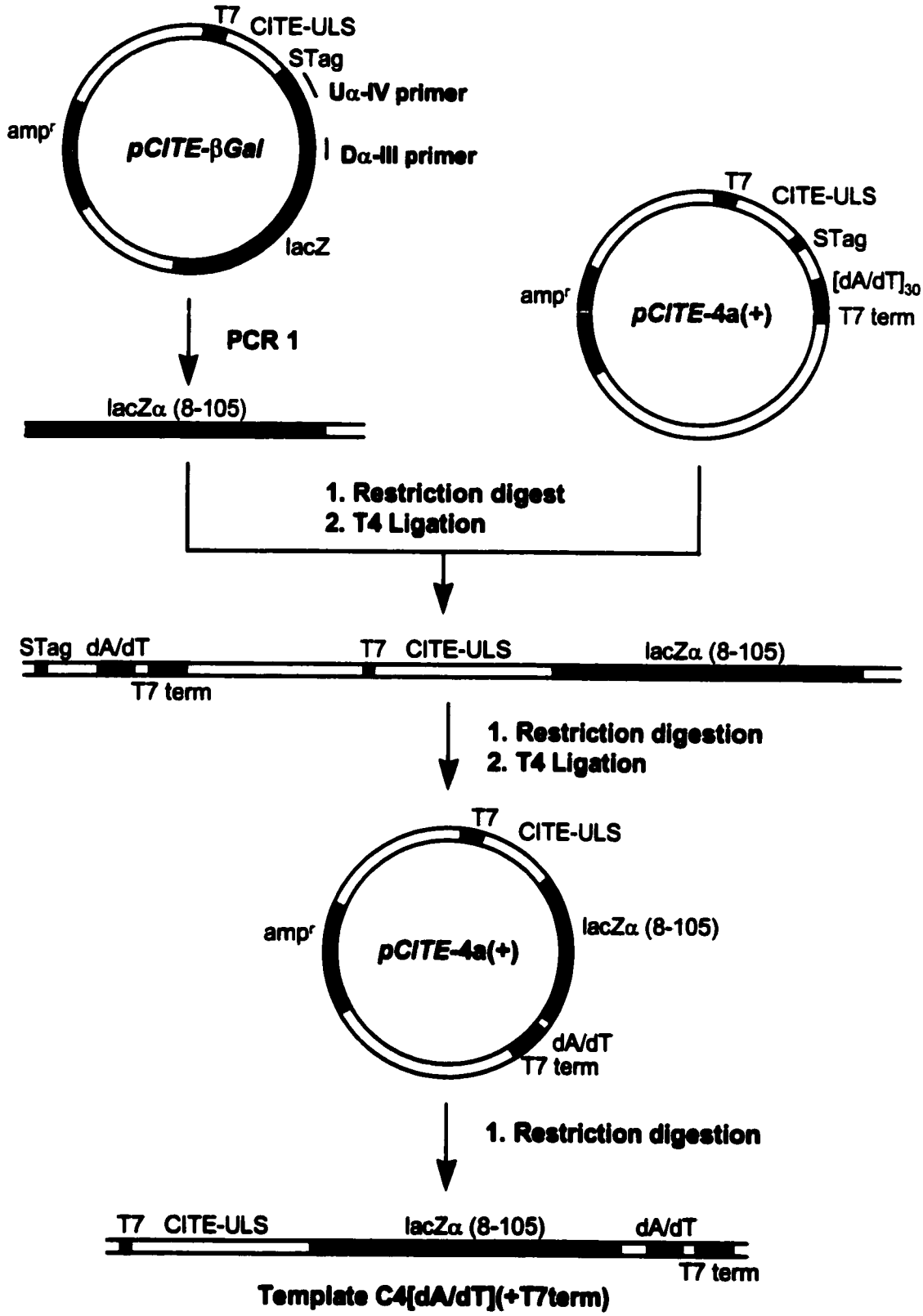

T7
CITE-ULS
STag
Uα-IV primer
ampr
pCITE-βGal
Dα-III primer
lacZ
PCR 1
lacZα (8-105)
T7
CITE-ULS
STag
[dA/dT]30
ampr
pCITE-4a(+)
T7 term
1. Restriction digest
2. T4 Ligation
STag dA/dT
T7 term
T7 CITE-ULS
lacZα (8-105)
1. Restriction digestion
2. T4 Ligation
T7
CITE-ULS
ampr
pCITE-4a(+)
lacZα (8-105)
dA/dT
T7 term
1. Restriction digestion
T7 CITE-ULS
lacZα (8-105)
dA/dT
T7 term
Template C4[dA/dT](+T7term)

Figure 3-8

activity. However, after α-complementation it was calculated that each DNA template was able to produce 0.28 α-peptides.

In each case discussed we were unable to create a eukaryotic α-peptide expression template which exhibited better expression efficiency than the original lacZα DNA used in Chapter 2. It may be that an α-peptide is produced but it is unable to complement with the added M15 protein. To answer this question the "C" templates which contained the S-Tag coding sequence were expressed as before and the products were analysed using the S-Tag rapid assay rather than the β-Gal fluorometric assay. In most cases each template molecule was able to produce approximately 14 molecules of the S-peptide. The only exception to this was template C1, which produced only 3.5 molecules of S-peptide per DNA molecule. The reason for this discrepancy was not determined. These data suggest that the DNA templates are being effectively expressed but the resulting peptide is not capable of α-complementation. A possible reason for this may be the lack of the proper folding machinery (eg. ref 83) and other conditions which are present in the *E. coli* S-30 system.

To address the potential folding problem while making use of the T7 RNAP and promoter system the next step was to develop an *E. coli* S-30 expression system for use with the T7 RNAP. An *E. coli* S-30 extract was prepared using the method described by Pratt (75) with minor modifications. The *E. coli* strain A19, used in the production of the S-30 extract, is RNase I deficient. An RNase I deficient strain is necessary for production of an S-30 extract which will not degrade RNA produced from transcription of added (foreign) DNA. The extract produced here exhibited good activity for the expression of DNA templates using its endogenous *E. coli* RNAP, as observed from expression of the original lacZα DNA template used in Chapter 2.

To test the activity of the extract with added T7 RNAP, a luciferase encoding DNA template containing a T7 promoter sequence and a Shine-Dalgarno ribosome binding site was employed. This template was used since its expression product is easily and reliably detected and efficient expression of luciferase from a template with a T7 promoter is known. The template was successfully prepared by PCR using the appropriate upstream

and downstream primers. Unfortunately, no expression was observed. This was not remedied with the addition of rifampicin to inhibit the endogenous RNAP and allow exclusive expression of the gene under the control of T7 promoter. It is well known that this type of *in vitro* expression system can require a great deal of optimisation before appreciable expression yields may be obtained. This may explain the lack of activity observed when the S-30 extract was used for expression of T7 promoter-containing templates. Also, it may be useful to use an *E. coli* strain which contains endogenous T7 RNAP. One study has shown that the protein-synthesising activity is slightly higher in an S-30 extract containing endogenous rather than exogenous T7 RNAP (59).

Since the time that this work was carried out, T7 RNAP dependent S-30 extract transcription/translation systems have become commercially available. It may be advantageous to test these systems with alternative lacZα DNA templates designed for expression using T7 RNAP in a prokaryotic system.

4. Expression Hybridisation Assay Using an Apoaequorin Encoding DNA as a Label

4.1 Introduction

The Ca^{2+}-dependent photoprotein aequorin is a complex of the apoprotein, called apoaequorin (a single polypeptide chain of 189 amino acids), coelenterazine and molecular oxygen (84). When traces of Ca^{2+} come in contact with aequorin blue light is emitted. This occurs from a Ca^{2+} binding induced conformational change in the protein, which causes oxidation of coelenterazine by the bound oxygen to produce coelenteramide, CO_2 and light (λ_{max}=470 nm) (85). Although analysis of the primary structure of apoaequorin indicates the presence of three putative Ca^{2+} binding sites, titration studies suggest that only two bound Ca^{2+} ions are required to trigger this luminescent reaction (86). The active group of aequorin is apparently peroxidised coelenterazine and the light emitter is the excited state of coelenteramide bound to apoaequorin which is released as it goes to its relaxed state. A remarkable property of apoaequorin is its ability to undergo regeneration to form fully functional aequorin when incubated with coelenterazine in the presence of O_2 and dithiothreitol (DTT) or 2-mercaptoethanol (87). The mechanism of luminescence and regeneration is illustrated in Figure 4-1.

Aequorin, originally isolated from the bioluminescent jellyfish *Aequoria*, has been used for decades as a biological Ca^{2+} indicator. In fact, it was the first indicator used to monitor changes in cytosolic free Ca^{2+} (88). Early studies were limited by the need to microinject the purified protein into the cellular space under study. Despite this, its wide dynamic range continued to make aequorin a valuable tool for following physiological fluctuations in free Ca^{2+} concentrations. In 1985 the cDNA for the apoaequorin gene in

Figure 4-1: Mechanism of Luminescence and Regeneration of Aequorin.

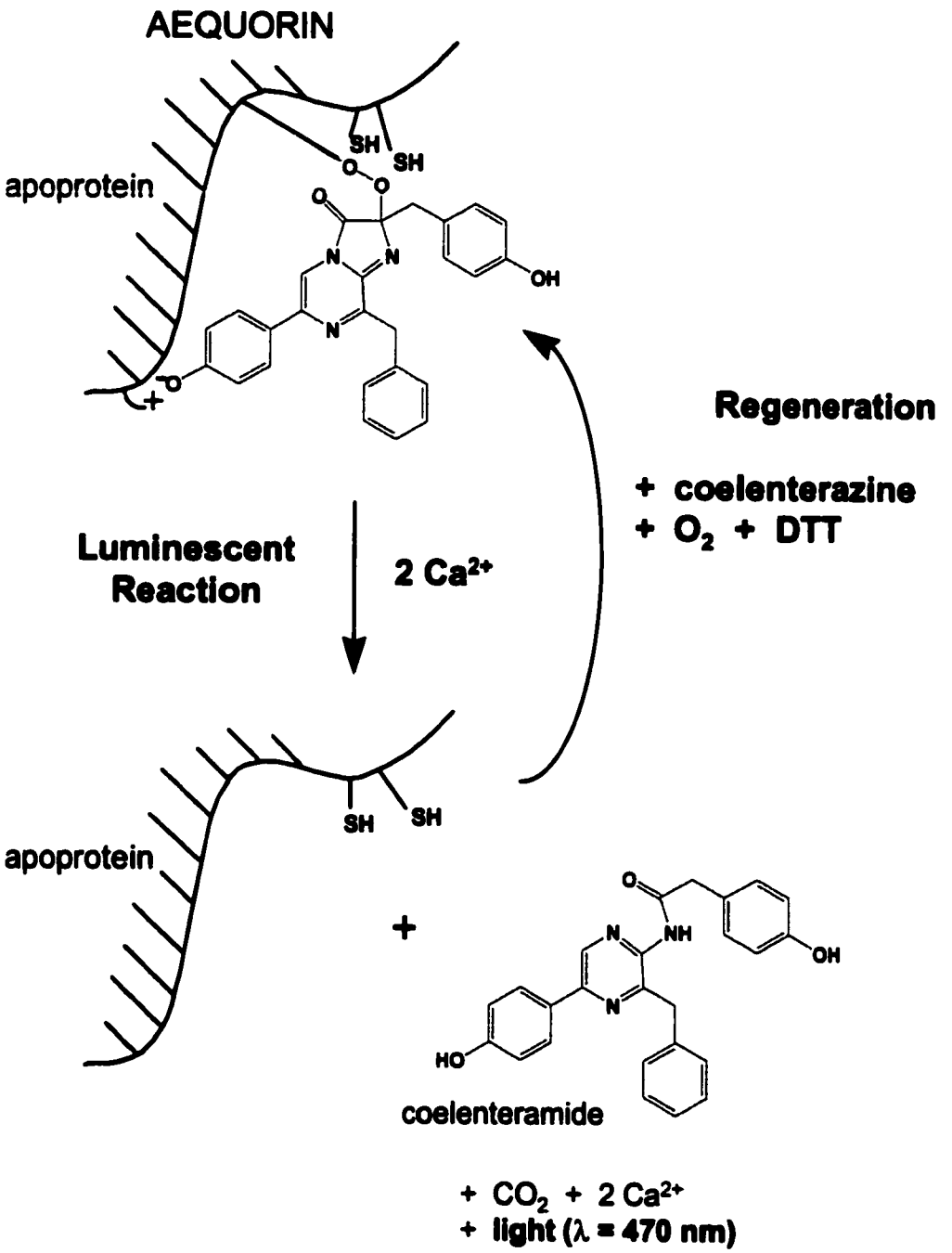

AEQUORIN
apoprotein
SH
SH
O
O
O
N
N
N
OH
O
+
Regeneration
+ coelenterazine
+ O_2 + DTT
Luminescent Reaction
2 Ca^{2+}
apoprotein
SH
SH
+
O
NH
N
N
OH
HO
coelenteramide
+ CO_2 + 2 Ca^{2+}
+ light (λ = 470 nm)

Figure 4-1

Aequoria victoria was isolated and analysed (89). This finding made it possible to transiently transfect cells with cDNAs encoding apoaequorin and thereby circumvent the need for microinjection. Since then aequorin has been expressed in a variety of cell types (90 and references therein) and chimeras have been created for targeting of aequorin to discrete cellular sub-domains (91 and references therein). In each case the use of aequorin to monitor Ca^{2+} was a vast improvement over the alternative of fluorescent dyes that are uniformly distributed over the cytosol.

The cDNA for apoaequorin was cloned into expression vectors and expressed in *E. coli* (92). Using the recombinant apoprotein it was possible to regenerate aequorin with the addition of coelenterazine, 2-mercaptoethanol and EDTA. Later apoaequorin was over expressed in *E. coli* and purified (93, 94, 95). Aequorin is an excellent reporter molecule since it can be detected at the attomole level with the addition of excess Ca^{2+} to trigger the light emitting reaction (96) and the production of the recombinant complex has expanded its use. Recombinant aequorin has been used as a label in immunoassays (97) and other binding assays (94, 98). Fusion proteins consisting of aequorin and IgG heavy chain were also prepared for use in immunoassays (99). Conjugates of recombinant aequorin with streptavidin or anti-digoxigenin antibody have been used in hybridisation assays and quantitative polymerase chain reaction (100, 101).

In this work we developed an expression signal amplification system which takes advantage of aequorin bioluminescence. First it was necessary to engineer an expressible DNA label encoding apoaequorin. A number of plasmids containing the apoaequorin coding sequence have become commercially available and can act as a source for the coding sequence. Since it is known that apoaequorin is efficiently expressed in a wide variety of cell types it is reasonable to assume that an apoaequorin encoding DNA label can be efficiently expressed in a eukaryotic cell-free expression system. To facilitate high yield of protein production the T7 RNAP and promoter are used to mediate *in vitro* transcription. As found in early studies on the photoprotein aequorin regeneration can occur *in vitro* (84). The remaining question was whether or not regeneration occurs most

efficiently when performed subsequent to expression or simultaneously with protein production.

We used the apoaequorin encoding DNA label in sensitive expression hybridisation assays. Similar to the expression immunoassay, the DNA label is attached to the detection probe. After completion of the hybridisation reaction between the probe and target DNA in microtitre wells, the apoaequorin DNA label is subjected to *in vitro*, one-step transcription and translation, thus generating multiple apoaequorin molecules in solution. The apoaequorin is converted into fully active aequorin in the transcription/translation mixture. The aequorin produced is determined by adding a Ca^{2+}-containing triggering solution and measuring the characteristic bioluminescence. The relationship between the amount of DNA label and the analytical signal (bioluminescence) obtained after expression was used to develop two hybridisation assay configurations. These assays were compared to a hybridisation assay which utilised the photoprotein aequorin as a label and an expression hybridisation assay which used a DNA label encoding firefly luciferase .

4.2 Experimental

Any instruments, materials, solutions or procedures not outlined here are described in previous chapters.

4.2.1 Instrumentation

Bioluminescence was measured using the MLX Microtitre Plate Luminometer from Dynex Technologies (Chantilly, VA, USA).

4.2.2 Materials

Reagent	Supplier
NAP-5 columns	Pharmacia Biotech (Montreal, PQ)

Reagent	Supplier
TNT T7 coupled wheat germ extract system	Promega Corporation (Madison, WI, USA)
Terminal deoxynucleotidyl transferase (TdT), digoxigenin-11-2′-deoxyuridine-5′-triphosphate (Dig-dUTP)	Boehringer Mannheim Corporation (Laval, PQ)
pSVAEQN, aequorin (Aqualite aequorin, recombinant biotinylated)	Molecular Probes (Eugene, OR, USA)
QIAquick gel extraction kit	Qiagen Inc. (Mississauga, ON)
'U'-bottom, transparent, polystyrene lock wells (Nunc, Maxisorp)	Life Technologies (Burlington, ON)
Primer A_1 (5′-GCT CTA GAC TAA TAC GAC TCA CTA TAG GGA GAA TGG TCA AGC TTA CA-3′), Primer A_2 (5′-CGA GCT CCT TAG GGG ACA GCT CCA CC-3′), Oligo 1 (5′-AATT CGC CAC ACA CAC TCC-3′), Oligo 2 (5′-TGT GTG TGG CG-3′)	Biosynthesis Inc. (Lewisville, TX, USA)
Coelenterazine, Anti-digoxigenin antibody labelled with aequorin	Sealite Sciences Inc. (Norcross, GA, USA)
Xba I	New England Biolabs (Beverly, MA, USA)

4.2.3 Solutions

Coelenterazine Stock Solution

Dissolve 0.65 mg of coelenterazine in 0.62 ml of 0.1 M HCl in deoxygenated methanol (supplied by the manufacturer). This solution is stable for 6 months at -20°C. The working solution of coelenterazine is prepared by diluting this stock solution in sterile ddH_2O immediately before use. Dilute the stock solution in

DEPC-treated H_2O if the coelenterazine is to be added to an *in vitro* expression reaction.

Aequorin Dilution Buffer

0.1 M Tris-HCl, pH 7.5	1 ml
0.1 M EGTA, pH 7.5	1 ml
2.5 M KCl	4 ml
1 M $MgCl_2$	100 µl
bovine serum albumin	10 mg
NaN_3	10 mg

Mix the components and add sterile ddH_2O to bring the final volume to 10 ml. Store at 4°C.

20× Sodium Chloride-Sodium Citrate (SSC) Buffer

17.53 g NaCl

8.823 g Na(citrate)·$2H_2O$

Dissolve in 90 ml of sterile ddH_2O and adjust pH to 7.0. Add sterile ddH_2O to a final volume of 100 ml.

Hybridisation Buffer

10× blocking reagent	1 ml
20× SSC	2 ml

Mix well and add sterile ddH_2O to a final volume of 10 ml.

A-T Annealing Buffer

10× blocking reagent	1 ml
20× SSC	3 ml

Mix well and add sterile ddH_2O to a final volume of 10 ml.

Aequorin Luminescence Triggering Solution

Mix together 5 ml of 0.2 M $CaCl_2$ and 5 ml of 0.2 Tris-HCl, pH 7.5.

4.2.4 Preparation of Target DNA

The target DNA was a 233 bp fragment produced by reverse transcriptase-polymerase chain reaction of the prostate specific antigen mRNA (as described in reference 102).

4.2.5 Preparation of the Apoaequorin Encoding DNA Fragments

A. Aeq DNA Template

The apoaequorin encoding expression template was prepared from pSVAEQN by PCR:

10× Pfu buffer	10 µl
A_1 primer (10 µM)	5 µl
A_2 primer (10 µM)	5 µl
dNTPs (2.5 mM)	6 µl
linear pSVAEQN (5 ng/µl)	1 µl
ddH_2O to final volume	98 µl

The Pfu polymerase (2.5 U/µl, 2 µl) was added during the initial heating at 95°C (hot start). After the addition of the polymerase, PCR was carried out for 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min. A final incubation for 20 min at 72°C was performed to allow Pfu polymerase to act on any unfinished PCR products. The PCR reaction was analysed by agarose (2 %) gel electrophoresis. Linear DNA markers were electrophoresed simultaneously with the PCR product. A slice of agarose, corresponding to the expected location of the PCR product (613 bp), was excised although no clear band was visible.

The initial PCR was repeated using identical conditions except that the pSVAEQN template was replaced with a very small amount of the agarose slice, only 1 µl of Pfu polymerase was used instead of 2 µl and the annealing temperature was increased to 65°C. The PCR product was purified using Wizard PCR Preps DNA purification system and quantitated by electrophoresis followed by scanning densitometry as described in previous chapters.

B. Aeq[dA/dT] DNA Template

A poly dA/dT sequence was added downstream of the apoaequorin coding sequence by cloning of the Aeq DNA into pSP64Poly(A) using a two step ligation procedure. Both the Aeq DNA and pSP64Poly(A) were first digested with Xba I:

	plasmid	insert
pSP64Poly(A) (1.9 μg/μl)	2 μl	—
purified Aeq DNA	—	40 μl
10× NEBuffer 2	4 μl	5 μl
Xba I (10 U/μl)	2 μl	1 μl
ddH_2O to final volume	40 μl	50μl

The restriction digestion reactions were incubated for 90 min at 37°C before heat inactivation of Xba I, 65°C for 20 min. The linearised pSP64Poly(A) was purified by ethanol precipitation according to standard procedures and quantified as usual. The purified plasmid DNA was dephosphorylated, in order to stop recircularisation in the next step, by mixing 11.5 μl of the digested pSP64Poly(A) (0.305 μg/μl) with 2 μl of calf intestinal alkaline phosphatase (0.05 U/μl) and incubating for 1 hour at 37°C. The alkaline phosphatase was heat inactivated by heating to 85°C for 15 min. No purification of the dephosphorylated linear plasmid was necessary. The digested Aeq DNA was purified using Wizard PCR preps DNA purification system and quantitated by electrophoresis and scanning densitometry as usual.

The Aeq DNA was attached to the pSP64Poly(A) vector by ligation at the Xba I overhangs left by the previous restriction digest. The ligation was performed using a 1:4 molar ratio between the plasmid and the insert:

linear pSP64Poly(A) (70.2 ng/μl)	20 μl
digested Aeq DNA (134 ng/μl)	8.4 μl
ATP (20 mM)	2.5 μl
10× One-Phor-All buffer plus	3 μl
T4 DNA ligase (6.2 U/μl)	1 μl
ddH_2O to final volume	50 μl

The ligation was allowed to proceed for 8 hours at 10°C, followed by a 10 min heat inactivation step at 65°C.

The ligation reaction mixture was used directly, without any purification of the ligation product, in the second restriction digest. Sac I was used to cut both ends of the long linear ligation product which contained the pSP64Poly(A) sequences attached to the Aeq DNA template:

ligation reaction	50 µl
10× One-Phor-All buffer plus	1 µl
Sac I (8 U/µl)	3.2 µl
ddH_2O to final volume	60 µl

After two hours at 37°C the restriction enzyme was heat inactivated, 20 min at 65°C. The entire reaction mixture was loaded into an agarose (1.0 %) gel and electrophoresed to separate the products. The band corresponding to the 3623 bp product was excised and the DNA was purified using Geneclean according to the manufacturer's directions.

The ligated DNA fragment contained the Aeq DNA attached to pSP64Poly(A) at the Xba I site within the polycloning region. After Sac I digestion the DNA fragment had two cohesive ends which allowed circularisation such that the apoaequorin coding sequence is located directly upstream of the $[dA/dT]_{30}$ sequence:

purified 3625 bp fragment	16 µl
10× One-Phor-All buffer plus	2 µl
ATP (20 mM)	1 µl
T4 DNA ligase (6.2 U/µl)	1 µl
	20 µl

The ligation reaction was incubated 8 hours at 10°C followed by heat inactivation of the ligase at 65°C for 10 min.

The circularised DNA (10 µl of the ligation reaction) was used to transfect competent JM109 *E. coli* cells (200 µl) using standard procedures (41). The pSP64Poly(A) vector contains a β-lactamase gene which confers ampicillin resistance and which remained intact with the cloning of Aeq template into the polycloning region. This allowed the

selection of successfully transformed colonies of *E. coli* by plating the transfection mixture on agar plates containing 0.1 mg/ml ampicillin. One colony was used to inoculate 50 ml of LB broth containing 0.1 mg/ml ampicillin. After an overnight incubation at 37°C, with vigorous shaking, a stock solution of the transformed bacteria, for storage at -80°C, was prepared by mixing an 850 µl aliquot with 150 µl of sterile glycerol. The remaining culture was scaled up in 1.5 l of LB broth containing 0.1 mg/ml ampicillin. Once this culture had reached its mid-log growth phase the cells were harvested by centrifugation and the DNA was purified using Wizard maxipreps DNA purification system. The concentration of the new plasmid, pSP64-Aeq, was determined by absorbance at 260 nm.

In order to obtain template Aeq[dA/dT], pSP64-Aeq was digested with Xba I, which cuts upstream of the T7 promoter, and Eco RI, which cuts downstream of the $[dA/dT]_{30}$ sequence:

pSP64-Aeq (1.1 µg/µl)	40 µl
10× Eco RI buffer	22 µl
Eco RI (12 U/µl)	10 µl
Xba I (10 U/µl)	12 µl
ddH_2O to final volume	220 µl

After the reaction was incubated for 2½ hours at 37°C the entire reaction was loaded into an agarose (1.0 %) gel and electrophoresed to separate the products. The bands corresponding to the 644 bp product were excised and the DNA was purified using the Wizard PCR preps DNA purification system. The concentration of template Aeq[dA/dT] was determined by electrophoresis and scanning densitometry as usual.

4.2.6 Tailing of Aeq[dA/dT] DNA with dATP

In order for Aeq[dA/dT] to be used as a label it was tailed with dATP using terminal deoxynucleotidyl transferase (TdT). The initial step in this procedure was the creation of a 3′-overhang to act as a substrate for the TdT. The pSP64-Aeq recombinant plasmid was first digested with Eco RI (note 2× One-Phor-All buffer plus was required for Eco RI

to act effectively):

pSP64-Aeq (1.1 μg/μl)	120 μl
10× One-Phor-All buffer plus	34 μl
Eco RI (12 U/μl)	16 μl
	170 μl

After a two hour incubation at 37°C, Eco RI was heat inactivated, 20 min at 65°C. The entire restriction digest was then used in a ligation reaction in which an adapter nucleotide was attached to the linear pSP64-Aeq at the 5′-overhang left from the Eco RI digestion. The adapter DNA was prepared by mixing equal volumes of 100 μM solutions of Oligo 1 and Oligo 2, heating the resulting solution to 55°C for 10 min and then gradually cooling to room temperature. The ligation reaction was carried out using a 4:1 molar ratio of adapter to plasmid DNA:

Eco RI digestion reaction	170 μl
Eco RI adapter DNA (50 pmol/μl)	4.5 μl
ATP (20 mM)	9.5 μl
T4 DNA ligase (6.2 U/μl)	3 μl
ddH_2O to final volume	190 μl

The ligation was allowed to proceed for 10 hours at 10°C before the ligase was heat inactivated, 10 min at 65°C. The DNA was purified from the ligation reaction mixture by ethanol precipitation using standard procedures (41).

The next step in the preparation of the Aeq[dA/dT] DNA label was the second restriction digest to remove the irrelevant plasmid sequences:

purified DNA	80 μl
10× NEBuffer 2	10 μl
Xba I (20 U/μl)	10 μl
	100 μl

After a 2½ hour incubation at 37°C the resulting DNA fragments were separated by electrophoresis. The bands corresponding to the 657 bp product were excised and the DNA was purified from the agarose using the QIAquick gel extraction method according

to the directions supplied by the manufacturer. The DNA solution was quantitated by electrophoresis and scanning densitometry as usual.

The purified Aeq[dA/dT] DNA with the added 3′-overhang was enzymatically tailed with dATP:

Aeq[dA/dT] DNA (3′-overhang, 0.3 pmol/µl)	10 µl
5× TdT buffer	4 µl
5× $CoCl_2$ solution	4 µl
dATP (0.3 mM)	1 µl
TdT (25 U/µl)	1 µl
	20 µl

After a 1 hour incubation at 37°C the reaction was terminated by the addition of 2 µl of 0.2 M EDTA, pH 8.0. No purification of the tailed DNA template, Aeq[dA/dT]-A_x was required. The solution was stored at -20°C until it was diluted for use in a hybridisation assay.

4.2.7 Labelling of Oligonucleotide Probes

A. Capture Probe

The oligonucleotide used as a capture probe in the sandwich type hybridisation assays was tailed with biotin-14-dATP. Similar to the addition of the dATP tail to the Aeq[dA/dT] DNA the oligonucleotide was tailed enzymatically:

$Probe_2$ (100 µM)	1 µl
5× TdT buffer	4 µl
5× $CoCl_2$ solution	4 µl
dNTPs (1.25 mM)	2 µl
B-14-dATP (0.4 mM)	2.5 µl
TdT (25 U/µl)	1 µl
ddH_2O to final volume	20 µl

After a 1 hour incubation at 37°C the reaction was terminated by the addition of 2 µl of

0.2 M EDTA, pH 8.0. The tailed $Probe_2$ was purified from the excess nucleotides using a NAP-5 column, according to the manufacturer's directions. A 100 % recovery of tailed DNA was assumed for the calculation of the final probe concentration. The resulting DNA solution was stored at -20°C until it was diluted for use in a hybridisation assay.

B. Detection Probe

The oligonucleotide used as the detection probe was enzymatically tailed with dTTP in the same way as the Aeq[dA/dT] DNA was tailed with dATP:

$Probe_1$ (100 µM)	1 µl
5× TdT buffer	4 µl
5× $CoCl_2$ solution	4 µl
dTTPs (5 mM)	2 µl
TdT (25 U/µl)	1 µl
ddH_2O to final volume	20 µl

After a 1 hour incubation at 37°C the reaction was terminated by the addition of 2 µl of 0.2 M EDTA, pH 8.0. No purification of the dTTP tailed $Probe_1$ ($Probe_1$-T_x) was required. The solution was stored at -20°C until it was diluted for use in a hybridisation assay.

4.2.8 Hybridisation Assays Based on *In Vitro* Expression of Aeq[dA/dT]-A_x DNA

A. Captured Target Assay Configuration

'U' bottomed wells were coated overnight, at room temperature, with 25 µl of 1.4 mg/l streptavidin in filter sterilised PBS. Prior to use the wells were washed three times with wash solution. A 25 µl aliquot of biotinylated target DNA (diluted in PBS containing 0.5 % (v/v) Tween-20) was pipetted into each well and allowed to bind for 30 min at room temperature with shaking. The wells were washed as above and then one strand of the target DNA was removed by incubating for 20 min with 25 µl 0.2 M NaOH. The wells were washed and 25 µl of 1.75 nM of $Probe_1$-T_x, diluted in hybridisation buffer, were

added into each well and allowed to hybridise for 30 min at 42°C with shaking. The excess of probe was removed by washing with wash solution containing 2 mM EDTA and 25 µl of 0.125 nM Aeq[dA/dT]-A_x DNA, diluted in A-T annealing buffer, were added to each well then allowed to anneal for 10 min at room temperature. The wells were washed five times with wash solution containing 2 mM EDTA and twice with 50 mM potassium acetate, 2 mM EGTA. The Aeq[dA/dT] DNA bound to the hybrids was transcribed and translated in a cell-free, one-step reaction by adding to each well 25 µl of a wheat germ TNT mixture (prepared according to the manufacturer's directions) supplemented with 8.3 µM coelenterazine. The expression was allowed to proceed for 90 min at 30°C followed by the addition of 10 µl 0.25 M DTT. The mixtures were transferred to opaque flat bottom wells and 50 µl of the Ca^{2+}-containing-triggering solution were added into each well and the light emission was integrated for 3 s.

B. Sandwich-Type Assay Configuration

A 25 µl aliquot of 3.5 nM biotinylated $Probe_2$, diluted in PBS containing 0.5 % (v/v) Tween-20, was pipetted into each streptavidin-coated well. Following a 30 min incubation (at room temperature with shaking), the wells were washed three times. A 20 µl aliquot of 0.625 nM $Probe_1$-T_x (diluted in hybridisation buffer and preheated to 42°C) was pipetted into each well. The target DNA (diluted in 10 g/l blocking reagent) was denatured by heating at 95°C for 10 min and cooled on ice. Subsequently, 5 µl of the denatured target DNA was added into each well and allowed to hybridise with the two probes for 1 hour at 42°C. The wells were washed three times with wash solution containing 2 mM EDTA and 25 µl of 0.125 nM Aeq[dA/dT]-A_x DNA, diluted in A-T annealing buffer, were added per well then allowed to anneal to the immobilised detection probe for 10 min at room temperature. The wells were washed five times with wash solution containing 2 mM EDTA and then twice with 50 mM potassium acetate, 2 mM EGTA. The bound Aeq[dA/dT] DNA was then expressed and the generated aequorin was measured as described under Captured Target Assay Configuration.

4.2.9 Hybridisation Assay Using the Photoprotein Aequorin as a Label

Flat-bottom microtitre wells were coated with streptavidin as described above for the 'U' bottom wells, except that 50 µl of coating solution was used instead of 25 µl. The capture of biotinylated target DNA and removal of one strand were carried out as described under Captured Target Assay Configuration, except that 50 µl volumes were used instead of 25 µl. Subsequently, 50 µl of 1.75 nM Dig-tailed $Probe_1$, diluted in hybridisation buffer, were added and the probe was allowed to hybridise for 30 min at 42°C. The wells were washed three times with wash solution containing 2 mM EDTA, followed by the addition 50 µl of 10 µg/l aequorin-labelled anti-digoxigenin antibody diluted in 1× blocking solution plus 2 mM EGTA (pH 7.5). The immunoreaction was allowed to proceed for 30 min at room temperature and the excess antibody was removed by washing the wells with wash solution containing 2 mM EDTA. To each well, 50 µl of the Ca^{2+}-containing luminescence-triggering solution were added and the signal was integrated for 3 s.

4.2.10 Hybridisation Assay Based on *In Vitro* Expression of Luc-A_x DNA

The 4.3 kbp plasmid pLucExp (38, 39), containing the T7 RNAP promoter, the firefly luciferase coding sequence and a downstream $[dA/dT]_{30}$ extension, was linearised with Bgl I (39) and tailed enzymatically with dATP as described above for the apoaequorin DNA. The resultant DNA (Luc-A_x) was used as a label in the hybridisation assay. The assay was carried out exactly as described under Captured Target Configuration except that 0.125 nM Luc-A_x DNA was used instead of the Aeq[dA/dT]-A_x DNA during the A-T annealing step. After incubating for 10 min at room temperature, the wells were washed five times with wash solution containing 2 mM EDTA and then twice with 50 mM potassium acetate, 2 mM EGTA. The bound Luc DNA was subjected to *in vitro* transcription/translation by adding 25 µl of the rabbit reticulocyte TNT mixture (prepared according to the manufacturer's directions) and incubating for 90 min at 30°C. The activity of generated luciferase was determined by mixing 2 µl of the reaction mixture

and 60 µl of luciferase substrate buffer (103) and measuring the luminescence with a delay time of 2 s and an integration time of 30 s.

4.3 Results and Discussion

4.3.1 Optimisation of *In Vitro* Formation of Aequorin

An *in vitro* expressible DNA fragment (Aeq DNA), consisting of a T7 RNAP promoter upstream of apoaequorin coding sequence, was prepared by PCR as illustrated in Figure 4-2 and described in the previous section. A second template was prepared with the addition of a $[dA/dT]_{30}$ extension to Aeq DNA, downstream of the apoaequorin-coding sequence. The primers A_1 and A_2 contained the sequences for restriction sites Xba I and Sac I which were incorporated, through PCR, at the 5′ and 3′ end of the Aeq DNA, respectively. This ensured the correct position and orientation of the DNA when inserted into pSP64Poly(A), which was used as the source of the $[dA/dT]_{30}$ sequence. The Aeq DNA was inserted between the Xba I and Sac I sites in the polycloning site of pSP64Poly(A) to create the new plasmid pSP64-Aeq (Figure 4-3). After isolation of the recombinant plasmid the 645 bp Aeq[dA/dT] DNA was obtained by restriction digestion. Cleavage of pSP64-Aeq with Xba I and Eco RI produced Aeq[dA/dT] with the $[dA/dT]_{30}$ sequence positioned downstream of the apoaequorin gene.

In order to be used as a label in hybridisation assays, the Aeq[dA/dT] DNA was modified to allow its linkage to the detection probe. By adding a poly dTTP "tail" to the probe DNA and a poly dATP "tail" to the DNA label it was possible to link the two molecules through A-T annealing. The Aeq[dA/dT] DNA was tailed enzymatically with dATP to produce the Aeq[dA/dT]-A_x. However, it has been reported that tailing of 3′ recessive ends (such as those produced from the Eco RI digestion) by TdT is not efficient (104). In order to create a 3′ protruding end, a suitable adapter was ligated to the Aeq[dA/dT] DNA. The adapter had an upstream cohesive end with the Eco RI cleavage site of Aeq[dA/dT] DNA and a downstream non-specific 3′ overhang (see Figure 4-3). The

Figure 4-2: Preparation of Aeq DNA. Aeq DNA was prepared by a single PCR reaction step using an upstream primer which contained the T7 promoter sequence and the first five codons of the apoaequorin coding sequence and a downstream primer which contained the last five codons of the coding sequence plus a stop codon.

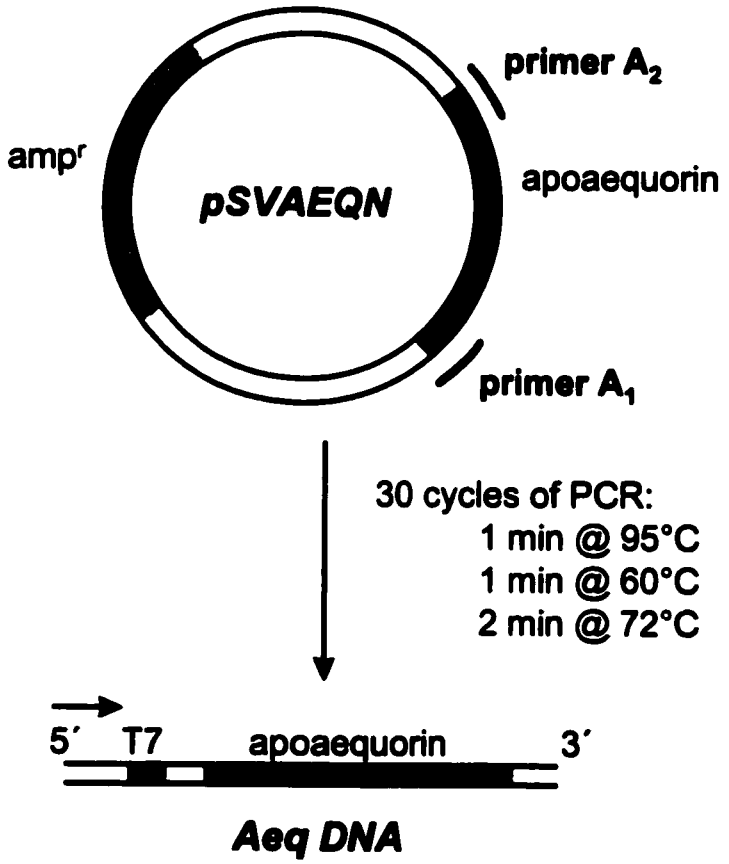

primer A_2
amp^r
pSVAEQN
apoaequorin
primer A_1
30 cycles of PCR:
1 min @ 95°C
1 min @ 60°C
2 min @ 72°C
5′ T7
apoaequorin
3′
Aeq DNA

Figure 4-2

Figure 4-3: Preparation of Aeq[dA/dT]-A_x. Aeq DNA and pSP64Poly(A) were digested with Xba I. After ligation using T4 ligase the resulting linear DNA was digested with Sac I and a second ligation reaction was performed to circularise the DNA. The resulting recombinant plasmid was amplified in bacteria and purified. A portion of the pSP64-Aeq was digested with Eco RI and ligated to adapter DNA which introduced a 3′ overhang. After a final digestion using Xba I the DNA fragments were separated by electrophoresis and the Aeq[dA/dT] with a 3′ overhang was purified. The DNA was tailed using TdT to form Aeq[dA/dT]-A_x

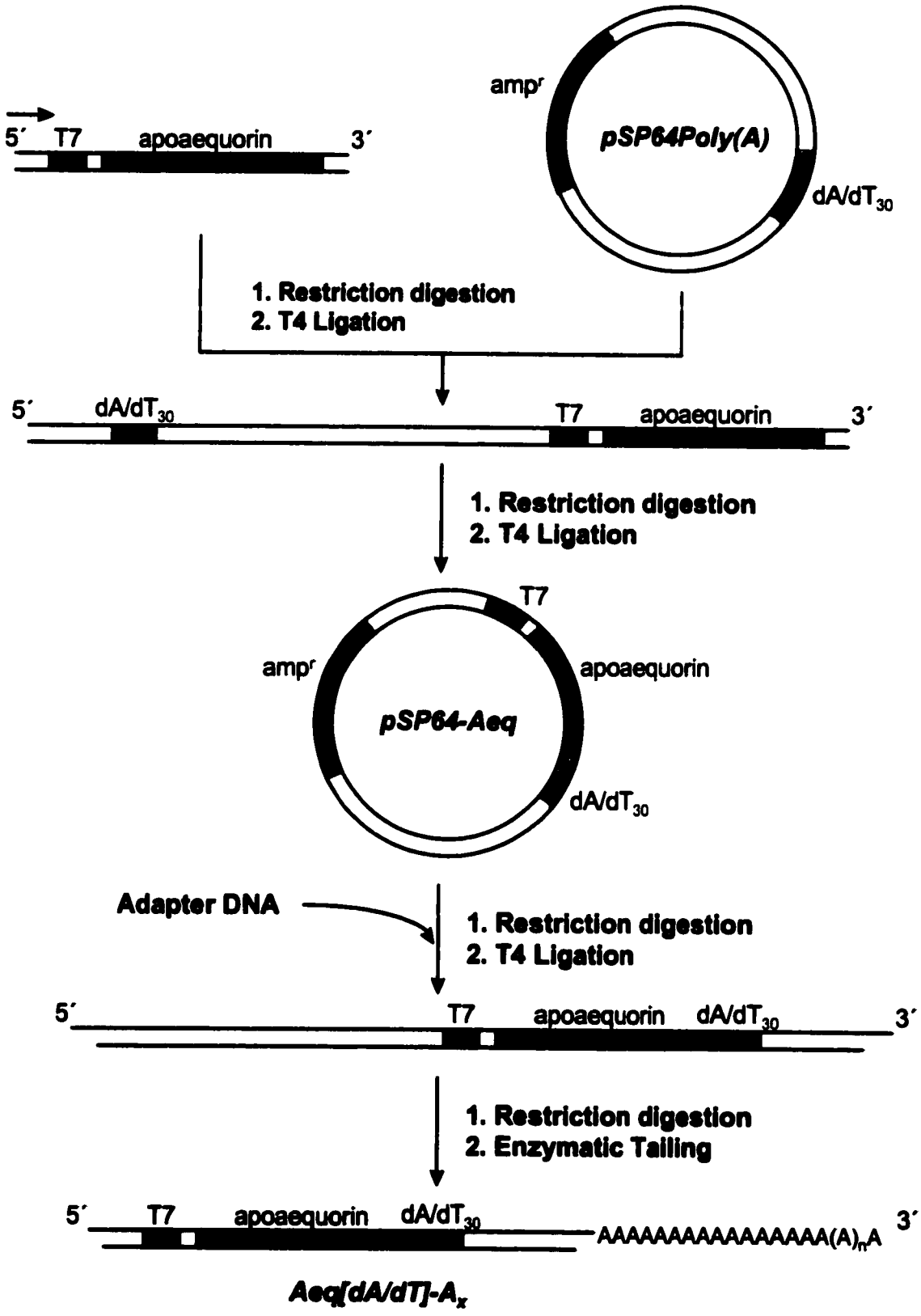

5′ T7 apoaequorin 3′
ampr
pSP64Poly(A)
dA/dT30
1. Restriction digestion
2. T4 Ligation
5′ dA/dT30 T7 apoaequorin 3′
1. Restriction digestion
2. T4 Ligation
T7
ampr
apoaequorin
pSP64-Aeq
dA/dT30
Adapter DNA
1. Restriction digestion
2. T4 Ligation
5′ T7 apoaequorin dA/dT30 3′
1. Restriction digestion
2. Enzymatic Tailing
5′ T7 apoaequorin dA/dT30 AAAAAAAAAAAAAAAA(A)nA 3′
Aeq[dA/dT]-Ax

Figure 4-3

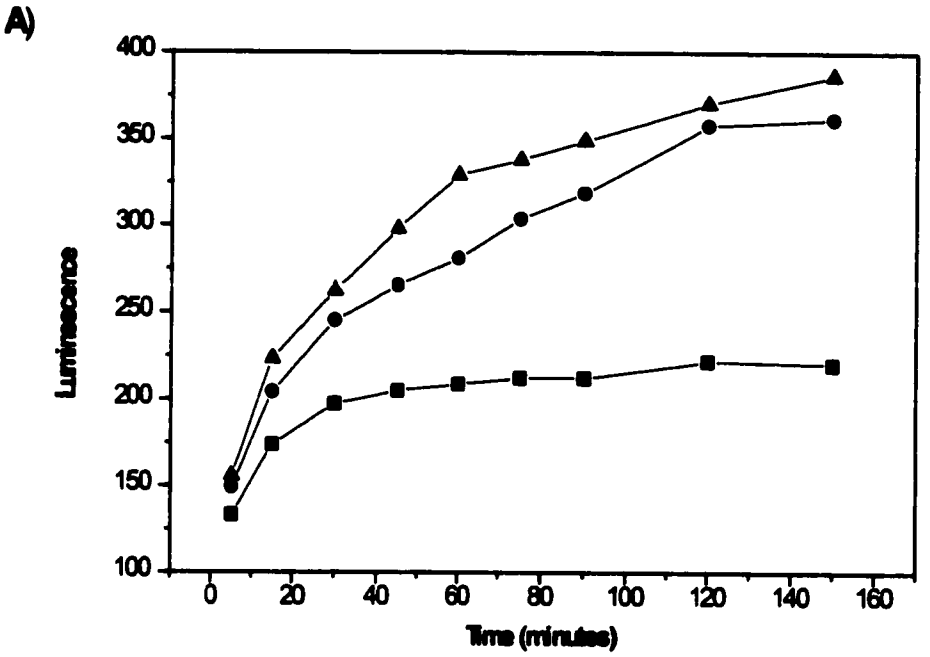
resultant DNA was purified and tailed. The Aeq[dA/dT]-A_x DNA was used directly in hybridisation assays without further purification.

The expression of the apoaequorin encoding DNAs was first tested using the TNT T7 coupled rabbit reticulocyte lysate system according to the instructions supplied by Promega. The difficulty with this system was that a component of the expression mixture was found to interfere with the bioluminescent signal of aequorin. However, it was possible to monitor aequorin production by diluting 1 µl of the expression mixture in 50 µl of aequorin dilution buffer before reading the light emission from the addition of 50µl of Ca^{2+}-containing triggering solution. The aequorin regeneration reaction was studied both subsequent to and during the expression reaction. First, three identical expression reactions were carried out (each containing 96×10^6 molecules of Aeq[dA/dT] DNA) before coelenterazine was added. After addition of coelenterazine, the regeneration reactions were incubated at 4°C, 22°C and 30°C, respectively. At various time intervals aliquots were removed from the reactions and aequorin was measured. The results in Figure 4-4(A) indicate that aequorin regeneration proceeds most efficiently at 30°C.

Since aequorin regeneration did not require lower temperatures the next study was carried out to determine whether regeneration could occur during expression. A single expression reaction was carried out containing 96×10^6 molecules of Aeq[dA/dT] DNA (as in the previous study) and 25 µM coelenterazine. The reaction was incubated at 30°C and at various time intervals aequorin was measured. The results, shown in Figure 4-4(B) indicate that, although regeneration does occur, the amount of aequorin produced was approximately three times lower than that produced with regeneration carried out after *in vitro* expression.

A study was done to determine the optimum incubation times required for *in vitro* expression and for aequorin regeneration. Three identical expression reactions of Aeq[dA/dT] DNA were incubated at 30°C for 30 min, 60 min and 90 min, respectively. Coelenterazine was added to each reaction and incubation was continued at 30°C. At various time intervals aliquots were removed from the reactions and aequorin was

Figure 4-4: Time Dependence of Aequorin Regeneration from Apoaequorin Synthesised *In Vitro* Using a Rabbit Reticulocyte Lysate Based System. A) Three 25 μl expression reactions (96 × 10^6 molecules of Aeq[dA/dT] DNA) were incubated for 90 min at 30°C before addition of 2.4 μl of 0.25 mM coelenterazine. The regeneration reactions were incubated at 4°C (-■-), 22°C (-●-) and 30°C (-▲-). B) A 25 μl rabbit reticulocyte lysate based expression reaction, containing 25 μM coelenterazine and 96 × 10^6 molecules of Aeq[dA/dT] was incubated at 30°C. In both experiments, A and B, 2 μl aliquots were removed from the reactions at various time intervals, diluted 50 fold in aequorin dilution buffer and aequorin bioluminescence was measured in 50 μl following addition of 50 μl of Ca^{2+}-containing triggering solution.


A)
Luminescence
400
350
300
250
200
150
100
0
20
40
60
80
100
120
140
160
Time (minutes)

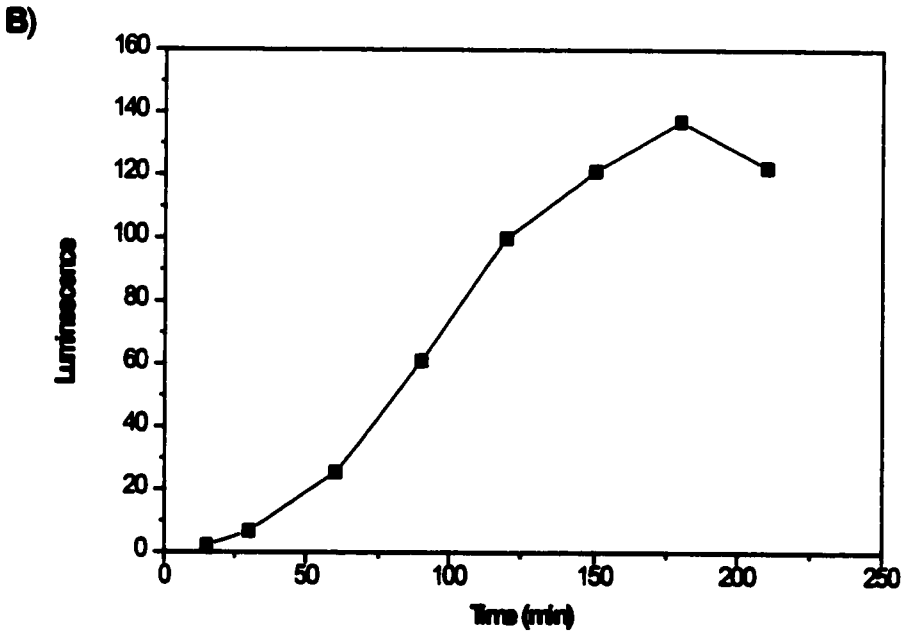

B)
Luminescence
160
140
120
100
80
60
40
20
0
0
50
100
150
200
250
Time (min)

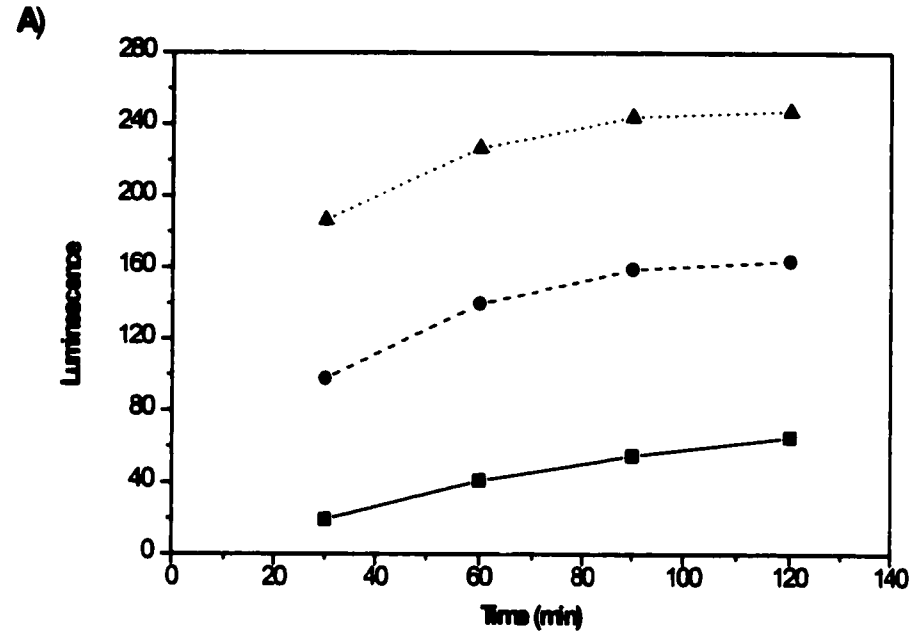
Figure 4-4

measured. The results (Figure 4-5(A)) indicate that a 90 min incubation is required for a good yield of protein from the expression reaction. Also, the yield from the regeneration reaction appears to plateau after 90 min with approximately 75 % of the maximum yield obtained after 30 min. Therefore, a 90 min expression reaction followed by a 30 min regeneration reaction were used in future studies with the rabbit reticulocyte lysate system.

The last condition to be studied was the amount of coelenterazine used in the regeneration reaction. Seven *in vitro* transcription/translation reactions were performed. After the 90 min incubation 1 µl of various concentrations of coelenterazine was added to each reaction and incubated for 30 min. Finally, the amount of aequorin produced was measured as usual. An obvious plateau is observed after the concentration of coelenterazine reached 11.4 µM in the final reaction mixture (Figure 4-5(B)).

To assess the performance of Aeq DNA and Aeq[dA/dT] DNA as labels, various amounts of each DNA fragment were subjected to *in vitro*, one step transcription/translation using the rabbit reticulocyte lysate based expression mixture (total volume 25 µl). Following the 30 min regeneration reaction at 30°C (15 µM final concentration of coelenterazine) the aequorin produced was determined by adding the Ca^{2+}-containing triggering solution and measuring the luminescence immediately. In Figure 4-6 the luminescence was plotted against the number of DNA molecules introduced in the expression mixture. It was observed that the luminescence is a linear function of the input Aeq DNA and Aeq[dA/dT] DNA. Moreover, the Aeq[dA/dT] DNA gave about 125 times higher expression yield than the Aeq DNA. As low as 1.6×10^5 Aeq[dA/dT] DNA molecules were detected with a signal/background ratio of 2.4 and linearity extending up to 5×10^8 molecules (over three orders of magnitude). From the signals obtained at various DNA levels and an aequorin calibration curve, prepared by diluting commercially available recombinant aequorin in 50 % rabbit reticulocyte lysate and then further diluting the samples 50 fold in aequorin dilution buffer (as the expression mixtures are treated), it was estimated that 1.1 molecules of aequorin were synthesised from each Aeq DNA and 142 aequorin molecules were generated from each Aeq[dA/dT] DNA molecule. It has been

Figure 4-5: Optimisation of Aequorin Formation After Expression in a Rabbit Reticulocyte Lysate Based System. A) Three 15 µl expression reactions (58×10^6 Aeq[dA/dT] molecules) were incubated for 30 (-■-), 60 (-●-) and 90 min (-▲-) at 30°C before addition of 1.5 µl of 0.25 mM coelenterazine. Incubation was continued at 30°C and 2 µl aliquots of each reaction were removed at various times. After a 50 fold dilution in aequorin dilution buffer, 50 µl was used for aequorin measurement (after addition of the Ca^{2+}-containing triggering solution. B) Seven 10 µl expression reactions (38×10^6 Aeq[dA/dT] molecules) were incubated for 90 min at 30°C before addition of 1 µl of various concentrations of coelenterazine. Incubation was continued at 30°C for 30 min before measurement of aequorin (as in A).


A)
Luminescence
Time (min)
0
40
80
120
160
200
240
280
0
20
40
60
80
100
120
140

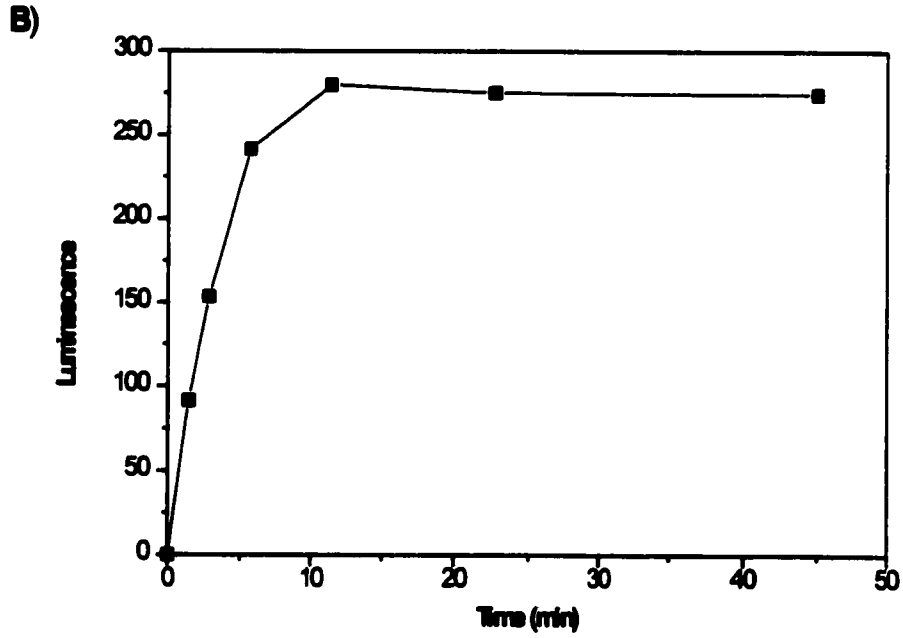

B)
Luminescence
Time (min)
0
50
100
150
200
250
300
0
10
20
30
40
50

Figure 4-5

Figure 4-6: Quantification of Apoaequorin Encoding DNAs by *In Vitro* Expression in a Rabbit Reticulocyte Lysate Based System. Various amounts of Aeq DNA (—■—) and Aeq[dA/dT] DNA (—●—) were subjected to 90 min, cell-free transcription/translation reactions at 30°C. Coelenterazine was added to each reaction (2.5 μl of 167 μM) and incubations were continued for 30 min prior to aequorin measurements (as described in the text).

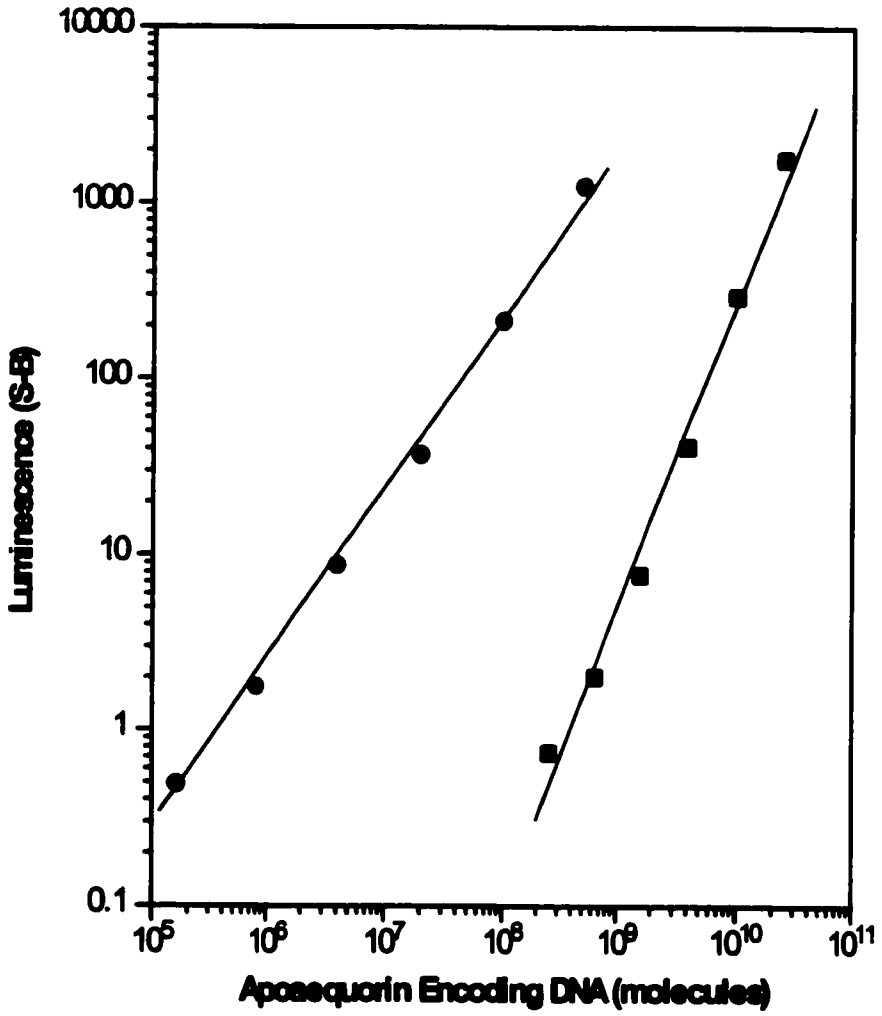

10000
1000
100
10
1
0.1
Luminescence (S-E)
10^5
10^6
10^7
10^8
10^9
10^{10}
10^{11}
Apoaequorin Encoding DNA (molecules)

Figure 4-6

reported that the poly(A) tail enhances the translation efficiency *in vivo* by facilitating the translation initiation and controlling the mRNA stability.

A highly sensitive hybridisation assay, based on the *in vitro* expression of an apoaequorin-coding DNA label, presupposes that the aequorin produced can be measured readily in the transcription/translation mixture without prior purification. Unfortunately, the reticulocyte lysate caused a 40 fold decrease in the signal when a rabbit reticulocyte-based expression mixture was spiked with recombinant aequorin (final concentration 55 pM) and the luminescence was compared with a 55 pM aequorin solution in the aequorin dilution buffer. This was attributed to the presence of hemoglobin in the reticulocyte mixture whose Soret band is close to λ_{max} of aequorin bioluminescence. As a consequence, light emitted from aequorin is absorbed by hemoglobin. Various methods were attempted for the removal of hemoglobin from the reaction mixture but none were able to eliminate the hemoglobin without also affecting the apoaequorin. As described above the expression reaction mixtures must be diluted 50 fold and only 50 µl of this dilution can be used in aequorin measurements without reintroducing the signal interference. Essentially this means that the aequorin in only 1 µl of the 25 µl reaction is detected and contributing to the signal. If it were possible to measure the signal from the entire sample it should be possible to detect much fewer Aeq[dA/dT] DNA labels.

A TNT T7 coupled wheat germ extract system is also commercially available from Promega. The one disadvantage to wheat germ extract based systems is their tendency to produce incomplete products due to premature termination and release of peptidyl-tRNA. This is generally a problem only when the protein product is in excess of 60 kDa. Apoaequorin is only 22.5 kDa so there should be no difficulty in the *in vitro* expression of apoaequorin encoding DNA using a wheat germ extract based system. To investigate possible interference from components of this expression mixture with the aequorin measurement, a wheat germ based expression mixture was spiked with recombinant aequorin (final concentration 55 pM) and the luminescence was compared with a 55 pM aequorin solution in aequorin dilution buffer. Since no interference was observed the wheat germ extract system was used in all subsequent studies.

The time course of the formation of fully active aequorin, from coelenterazine and apoaequorin synthesised *in vitro*, using a wheat germ extract based system, was studied by first subjecting Aeq[dA/dT] DNA to a transcription/translation reaction for 90 min. At the end of this period the aequorin regeneration was initiated by addition of coelenterazine at a final concentration of 8 μM. Aliquots were removed at various time intervals (up to 4 h from the beginning of expression) and the luminescence of aequorin was measured immediately upon addition of the Ca^{2+}-containing light-triggering solution. In Figure 4-7 (dashed line), the luminescence was plotted versus time (the zero time corresponds to the start of the transcription/translation reaction). The luminescence increased with time and reached a maximum after 60 min from the addition of coelenterazine. A second experiment, performed in parallel, had coelenterazine included in the expression mixture before the addition of Aeq[dA/dT] DNA. Aliquots of the reaction mixture were removed at various time intervals and the luminescence was measured immediately. The results, also presented in Figure 4-7 (solid line), indicated a continuous increase in luminescence and a plateau was reached at 150 min. A significant finding is that the maximum luminescence values obtained were the same for the two experiments. Consequently, coelenterazine does not interfere with the transcription/translation reaction in the wheat germ based expression. This permitted simultaneous apoaequorin synthesis and aequorin formation. In order to achieve a high yield of active aequorin without compromising the practicality of the proposed assays, coelenterazine was included in the expression mixture which was allowed to proceed for 90 min at 30°C; this incubation corresponded to 81 % of the maximum luminescence signal obtained.

The effect of the concentration of coelenterazine on the formation of aequorin was studied by performing transcription/translation of Aeq[dA/dT] DNA with various concentrations of coelenterazine included in the expression mixture. At the end of expression the Ca^{2+}-containing light-triggering solution was added and the luminescence was measured. The results presented in Figure 4-8 illustrate a continuous increase of the luminescence with coelenterazine concentration until the maximum was reached at 8 μM.

Figure 4-7: Time Course of Aequorin Formation from Coelenterazine and Apoaequorin Synthesised *In Vitro* Using a Wheat Germ Extract Based System. Aeq[dA/dT] DNA (1.5×10^8 molecules) was subjected to *in vitro* transcription/translation at 30°C. Coelenterazine (8 μM final concentration) was either included in the expression mixture (—■—), or it was added after the expression reaction had proceeded for 90 min (- -●- -). In each case 2 μl aliquots were removed at various time intervals, mixed with 25 μl aequorin dilution buffer and aequorin bioluminescence was measured immediately after the addition of 50 μl of Ca^{2+}-containing light triggering solution.

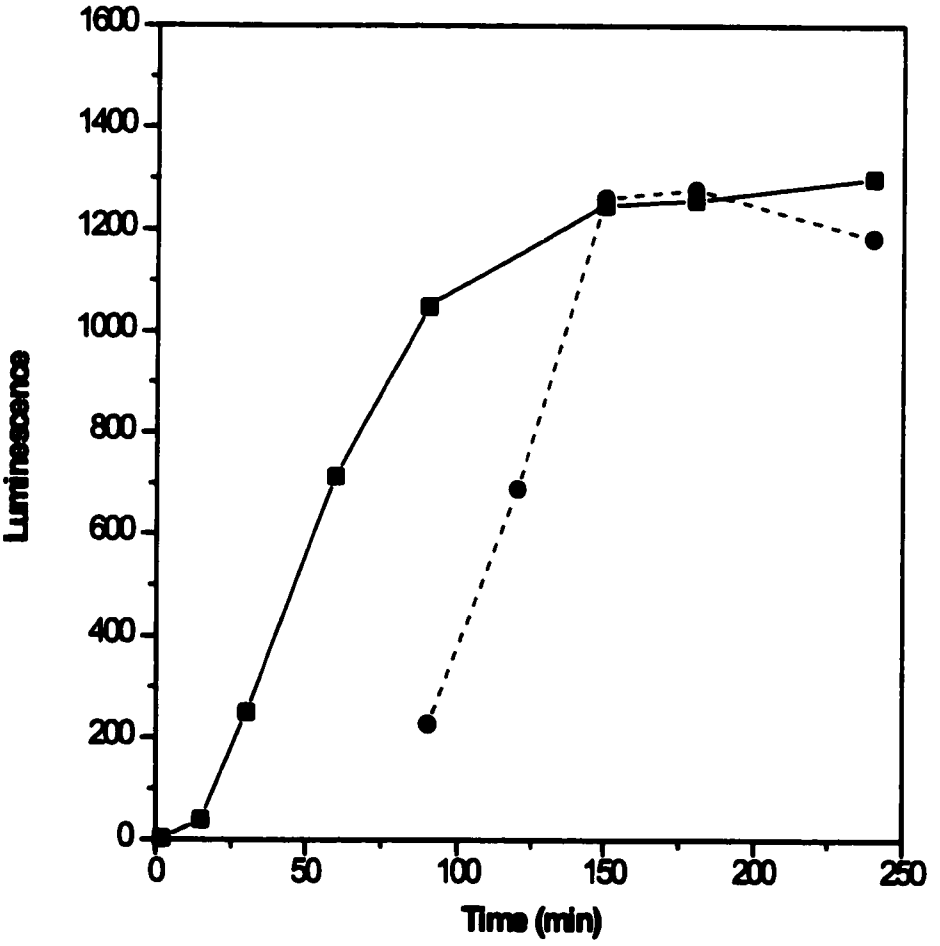

1600
1400
1200
1000
800
600
400
200
0
Luminescence
0
50
100
150
200
250
Time (min)

Figure 4-7

Figure 4-8: Effect of Coelenterazine Concentration on Aequorin Formation. Aeq[dA/dT] DNA (1.7×10^7 molecules) was subjected to *in vitro* transcription/translation for 90 min at 30°C with various concentrations of coelenterazine contained in the wheat germ extract based expression mixture. Aequorin was measured immediately after the addition of 50 µl of Ca^{2+}-containing triggering solution (as described in the text).

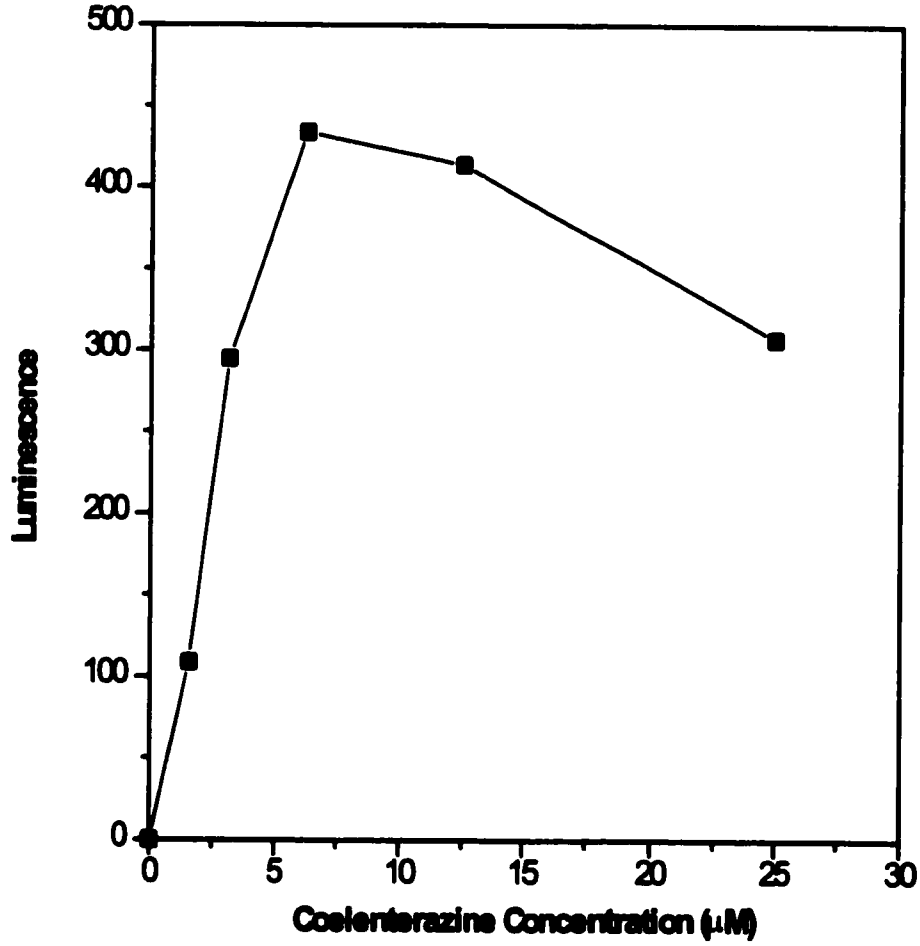

500
400
300
200
100
0
Luminescence
0
5
10
15
20
25
30
Coelenterazine Concentration (μM)

Figure 4-8

The decrease of the luminescence at higher coelenterazine concentrations might be due to the presence of an inhibitor in the coelenterazine solution (perhaps coelenteramide) (105).

To assess the performance of Aeq DNA and Aeq[dA/dT] DNA as labels, various amounts of each DNA fragment were subjected to *in vitro*, one step transcription/translation using a wheat germ-based expression mixture (total volume 25 µl) which contained 8.3 µM coelenterazine. The aequorin produced was determined by adding the Ca^{2+}-containing triggering solution and immediately measuring luminescence. In Figure 4-9, the luminescence was plotted against the number of DNA molecules introduced to the expression mixture. The luminescence was found to be linearly related to the input Aeq DNA and Aeq[dA/dT] DNA. Similar to expression in the rabbit reticulocyte based system, the Aeq[dA/dT] DNA produced approximately 25 times higher expression yield than the Aeq DNA. As low as 5000 Aeq[dA/dT] DNA molecules were detected with a signal/background ratio of 2.0 and a linear range extended up to 10^8 molecules (over four orders of magnitude). From the signals obtained at various DNA levels and an aequorin calibration curve, prepared by diluting commercially available recombinant aequorin in aequorin dilution buffer, it was estimated that 6.5 molecules of aequorin were synthesised from each Aeq DNA and 156 aequorin molecules were generated from each Aeq[dA/dT] DNA molecule. Therefore, the Aeq[dA/dT] DNA was used as a label for the development of hybridisation assays.

4.3.2 Development of Aequorin Expression Hybridisation Assays

The relative positions of the oligonucleotide probes used in the hybridisation assays are shown in Figure 4-10(A). Schematic presentations of the two expression hybridisation assay configurations studied, are also shown in Figure 4-10 (B and C). These configurations were based on the *in vitro* expression of the apoaequorin-encoding DNA (Aeq[dA/dT]). In the captured target configuration (B), biotinylated target DNA is first bound to streptavidin-coated wells. Then, one strand is removed by NaOH treatment and the other is hybridised with $Probe_1$ that contains a dTTP-tail at the 3′ end ($Probe_1$-T_x). The dTTP tail allows for subsequent binding of the Aeq[dA/dT]-A_x DNA to the hybrids.

Figure 4-9: Quantification of Apoaequorin Encoding DNA by *In Vitro* Expression in a Wheat Germ Extract Based System. Various amounts of Aeq DNA (—■—) and Aeq[dA/dT] DNA (—●—) were subjected to 90 min, cell-free transcription/translation reactions at 30°C. Coelenterazine was included in each reaction at a final concentration of 8.3 μM. Aequorin was determined from luminescence measurements taken immediately after the addition of 50 μl of Ca^{2+}-triggering solution (as described in the text).

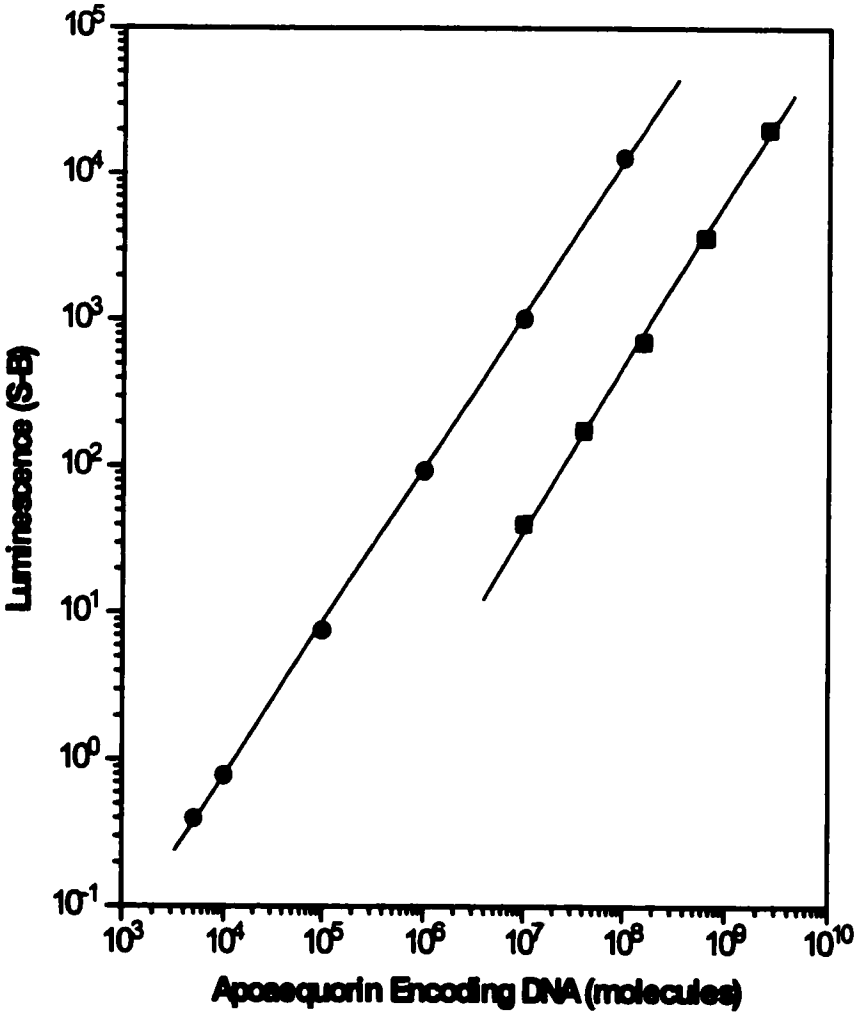

Luminescence (S-B)
Aposequorin Encoding DNA (molecules)
10^5
10^4
10^3
10^2
10^1
10^0
10^-1
10^3
10^4
10^5
10^6
10^7
10^8
10^9
10^10

Figure 4-9

Figure 4-10: Aequorin Expression Hybridisation Assays. A) The target DNA (233 bp) is shown with the relative positions of $Probe_1$ and $Probe_2$. B) Captured target configuration based on the *in vitro* expression of the apoaequorin encoding DNA label (Aeq[dA/dT]-A_x). C) Sandwich type configuration based on the *in vitro* expression of the apoaequorin encoding DNA label (Aeq[dA/dT]-A_x). The conditions of each assay are given in the text. SA = streptavidin; B = biotin.

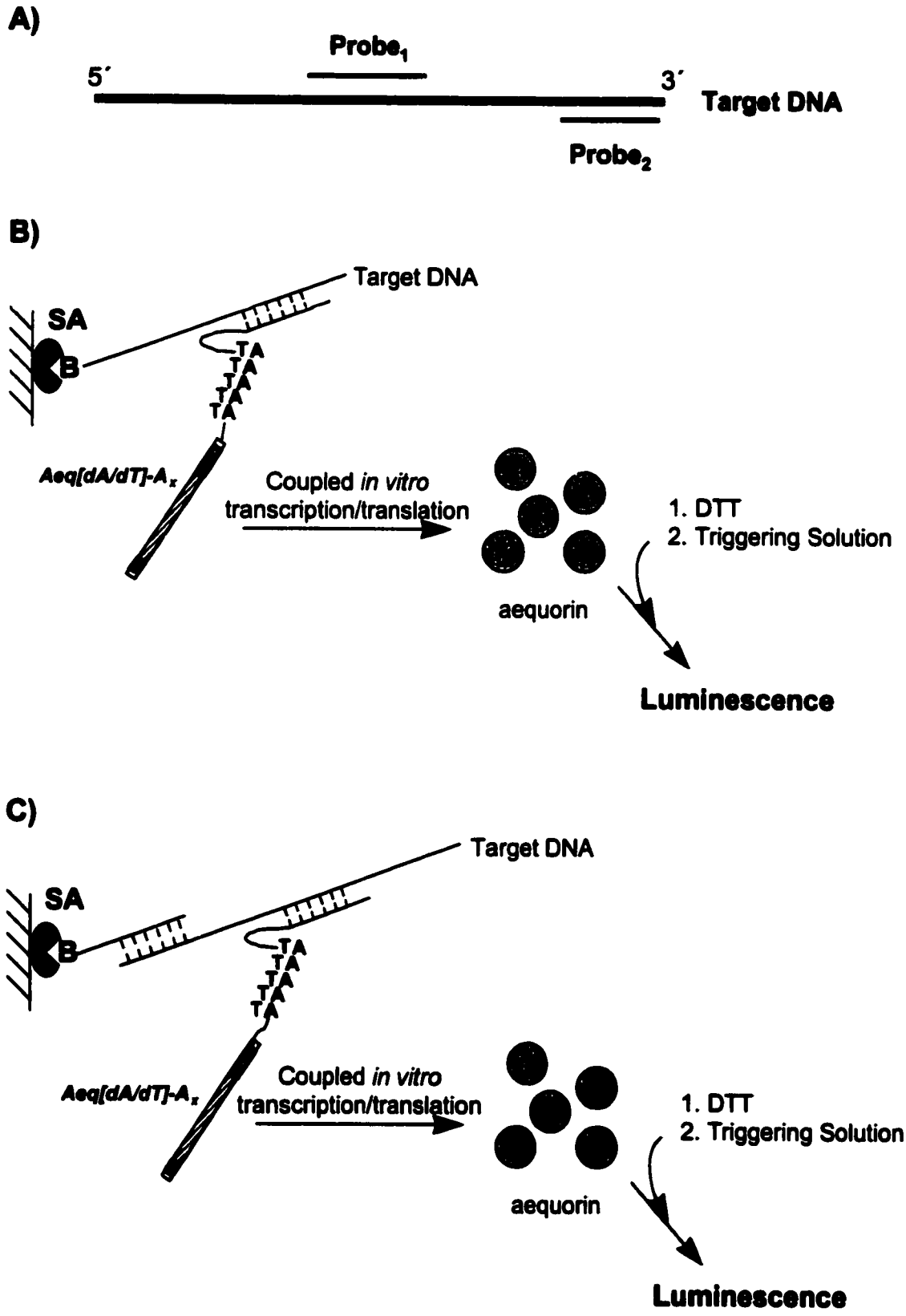

A)
Probe1
5′
3′
Target DNA
Probe2
B)
Target DNA
SA
B
Aeq[dA/dT]-Ax
Coupled in vitro
transcription/translation
1. DTT
2. Triggering Solution
aequorin
Luminescence
C)
Target DNA
SA
B
Aeq[dA/dT]-Ax
Coupled in vitro
transcription/translation
1. DTT
2. Triggering Solution
aequorin
Luminescence

Figure 4-10

Biotinylated target DNA may be prepared by PCR, using the appropriately biotinylated upstream primer (102) (as performed in the current study), when the target is rare or by ligation of a biotinylated adapter DNA to restriction digested target DNA.

Previous studies have shown that aqueous solutions of coelenterazine exhibit a low level luminescence which has been attributed to the auto-oxidation of coelenterazine (106). In the work summarised here it was found that a 25 µl aliquot of wheat germ-based expression mixture supplemented with 8.3 µM coelenterazine (the solution used in the hybridisation assays) and containing no apoaequorin-coding DNA gave a typical luminescence value of 2.34 RLU with the instrument settings of aequorin measurement. In the absence of coelenterazine, the luminescence was only 0.35. This luminescence was not dependent on the addition of the Ca^{2+}-containing triggering solution. Furthermore, it was found that this luminescence was gradually decreased when increasing concentrations of DTT were added to the expression mixture. When DTT was added to the expression mixture to a final concentration of 71 mM the luminescence dropped to less than 0.4. This addition of DTT did not interfere with the subsequent aequorin measurement.

The effect of the concentration of apoaequorin-coding DNA was studied by preparing various dilutions of Aeq[dA/dT]-A_x in A-T annealing buffer and using them in the hybridisation assay of 50 amol of target DNA. In Figure 4-11(A) the luminescence and the signal/background ratio are plotted versus the Aeq[dA/dT]-A_x concentration. The background is defined as the luminescence observed when no target DNA is present in the well and is mainly due to the non-specific binding of the detection probe and the Aeq[dA/dT]-A_x DNA to the solid phase. It was observed that the luminescence increased with the Aeq[dA/dT]-A_x concentration until a plateau was reached at 0.375 nM. However, a peak in the signal/background ratio occurred at 0.125 nM. At higher concentrations the non-specific binding of Aeq[dA/dT]-A_x DNA increased and the signal/background ratio dropped.

Figure 4-11(B) shows the effect of the concentration of the detection probe, Probe$_1$-T_x, on the luminescence and the signal/background ratio of the hybridisation assay. The

Figure 4-11: Optimisation of the Aeq[dA/dT]-A_x and Probe$_1$-P_x Concentrations. A) Captured target assays were performed using 50 amol of immobilised target DNA. The Probe$_1$-T_x concentration was kept at 1.75 nM and the Aeq[dA/dT]-A_x was varied. At each Aeq[dA/dT]-A_x concentration a blank well (no target DNA) was also included. B) Similar to A, the captured target assays were performed using 50 amol of immobilised target DNA. Various Probe$_1$-T_x concentrations were used and the Aeq[dA/dT]-A_x concentration was kept at 0.5 nM. At each Probe$_1$-T_x concentration a blank well (no target DNA) was also included. Luminesecence (—■—); Signal/Background ratio (- -●- -).

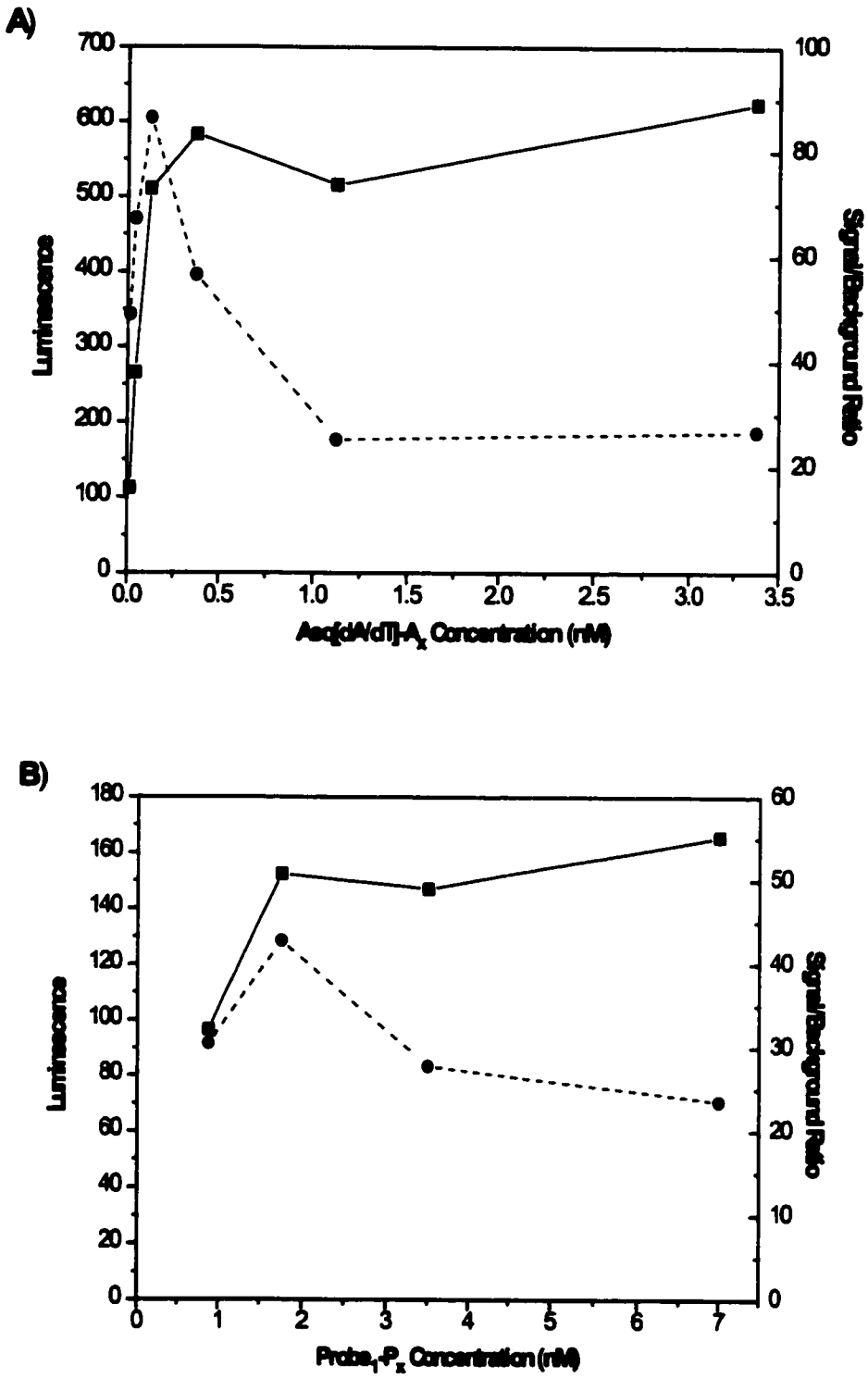

A)
Luminescence
Signal/Background Ratio
Aeq[dA/dT]-A$_x$ Concentration (nM)
B)
Luminescence
Signal/Background Ratio
Probe$_1$-P$_x$ Concentration (nM)

Figure 4-11

luminescence reached a maximum at 2 nM probe and then remained constant, whereas the signal/background ratio decreased because of the increased non-specific binding of the probe at high concentrations. A final probe concentration of 1.75 nM was chosen as the optimum for the captured target assay configuration.

In order to assess the performance of the optimised captured target hybridisation assay (Figure 4-10(B)), various dilutions of target DNA were analysed and the luminescence (corrected for the background) was plotted as a function of the amount of DNA. The results are presented in Figure 4-12. The assay is linear in the range of 0.5 – 7812 amols of target DNA, a range spanning over four orders of magnitude. The signal/background ratio at the level of 0.5 amols is 1.9.

In the sandwich type hybridisation assay (Figure 4-10(C)), the target DNA (unlabelled) hybridises simultaneously with two probes, i.e., $Probe_2$, which is immobilised on the well through biotin/streptavidin interaction, and $Probe_1$-T_x. The Aeq[dA/dT]-A_x DNA is bound to the hybrids followed by *in vitro* expression. An alternative configuration, with $Probe_1$ immobilised on the well through biotin/streptavidin interaction and $Probe_2$-T_x used as the detection probe, was also tested but it was found to be less effective than the first configuration. Optimisation studies similar to those outlined above, for the captured target assay configuration, were carried out and it was found that the highest signal/background ratios were obtained using 3.5 nM probe $Probe_2$, 0.625 nM $Probe_1$-T_x and 0.125 nM Aeq[dA/dT]-A_x DNA. Data pertaining to the sensitivity and linearity of the sandwich-type hybridisation assay are presented in Figure 4-12. The linearity extends from 0.25 – 1562 amols of target DNA. The signal/background ratio at the level of 0.25 amols is 1.4.

The reproducibility of the proposed sandwich-type hybridisation assay was assessed by analysing samples containing 5, 50 and 500 amols of target DNA. The CVs were 10.9 %, 9.1 % and 4.2 %, respectively (n = 4). The captured target hybridisation assay gave CVs of 6.0 %, 12.7 % and 11.5 % for 5, 500 and 2500 amols of target DNA, respectively (n = 4).

Figure 4-12: Quantification of Target DNA Using Aequorin Expression Hybridisation Assays. Various amounts of target DNA were detected using the captured target expression assay configuration (—■—) and the sandwich type assay configuration (- - ● - -), as described in the text.

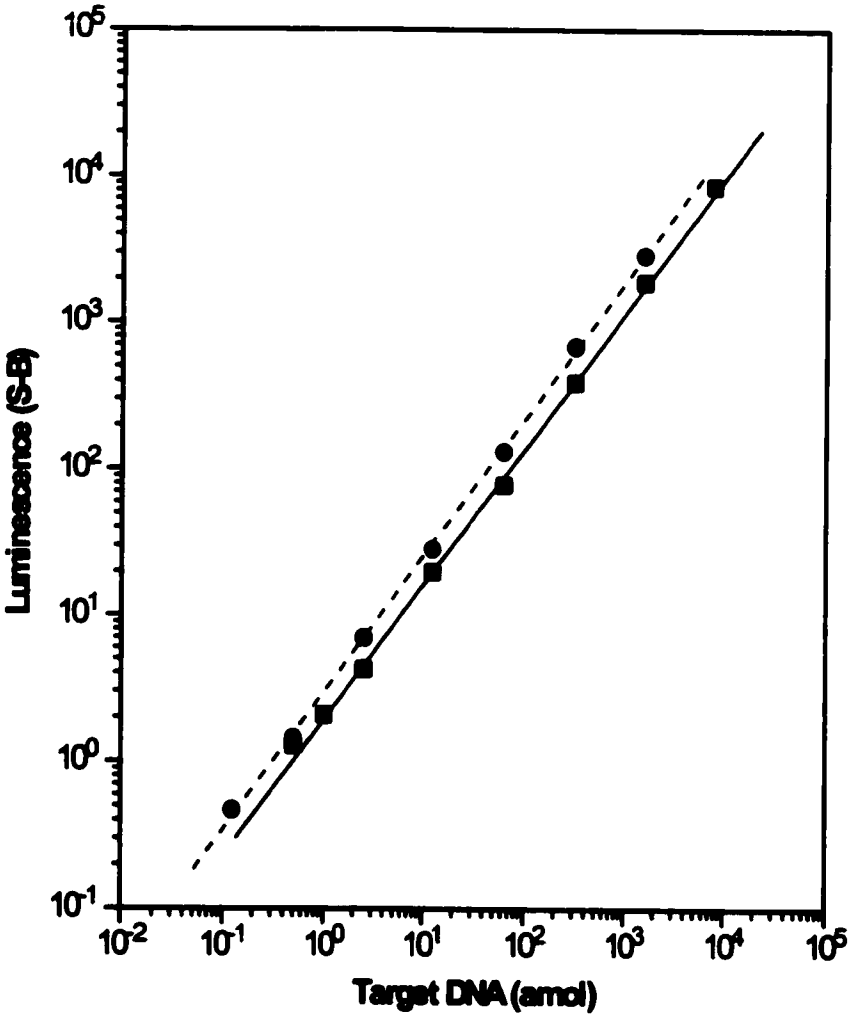

Luminescence (S-B)
Target DNA (amol)
10^5
10^4
10^3
10^2
10^1
10^0
10^-1
10^-2
10^-1
10^0
10^1
10^2
10^3
10^4
10^5

Figure 4-12

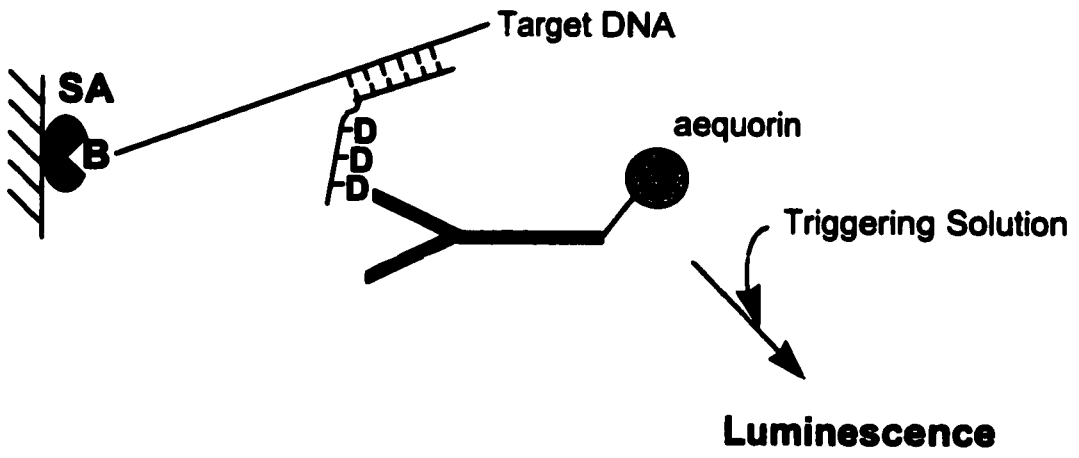
The photoprotein aequorin has been used as a reporter molecule in hybridisation assays and immunoassays (97, 99-101). In order to compare the sensitivity of the proposed method, which uses a DNA label encoding apoaequorin, with the method that employs the protein aequorin as a label, a captured target hybridisation assay was performed using the same detection probe ($Probe_1$) tailed with Dig-dUTP at the 3′ end. The hybrids were determined by reacting with an anti-digoxigenin antibody labelled with aequorin (see Figure 4-13(A)). The results are presented with molecules of target DNA plotted as a function of luminescence (Figure 4-13(B)). This aequorin-based assay can detect 25 amols of target DNA per well with a signal/background ratio of 1.6. This corresponds to a 50 fold improvement in sensitivity when using the DNA encoding apoaequorin as a label rather than the aequorin complex.

The proposed system was compared with a previously described hybridisation assay (38, 39), which was based on a DNA label encoding firefly luciferase. A captured target hybridisation assay was performed (see Figure 4-14(A)) using $Probe_1$ with a dTTP-tail at the 3′ end ($Probe_1$-T_x). The hybrids were treated then with Luc-A_x DNA. The bound Luc DNA was expressed and the activity of generated luciferase was measured. The luminescence was plotted against the amount of target DNA (see Figure 4-14(B)). It was observed that the luciferase based assay can detect 20.5 amols of target DNA with a signal/background ratio of 1.9. Thus, there is a 40 fold improvement in sensitivity by using the DNA encoding apoaequorin as a label.

The dramatic improvement in sensitivity observed when a DNA encoding apoaequorin is used as a label instead of aequorin, is due to the amplification introduced by the *in vitro* expression of apoaequorin DNA into several active aequorin molecules. This may also account for the observed improvement over the use of the luciferase encoding DNA label. Earlier work indicated that 12 - 14 molecules of luciferase are produced from each DNA molecule whereas the present work shows that more than 150 molecules of aequorin are produced from each Aeq[dA/dT] DNA. Thus, despite the fact that luciferase can be detected at lower levels than aequorin, the Aeq[dA/dT]-A_x DNA can act as a more effective label than the Luc-A_x DNA.

Figure 4-13: Captured Target Hybridisation Assay Using Aequorin as a Label. A) A schematic diagram of the assay configuration. SA = streptavidin; B = biotin; D = digoxigenin. B) Quantification of target DNA using the assay configuration shown in A and described in the text.

A)



B)

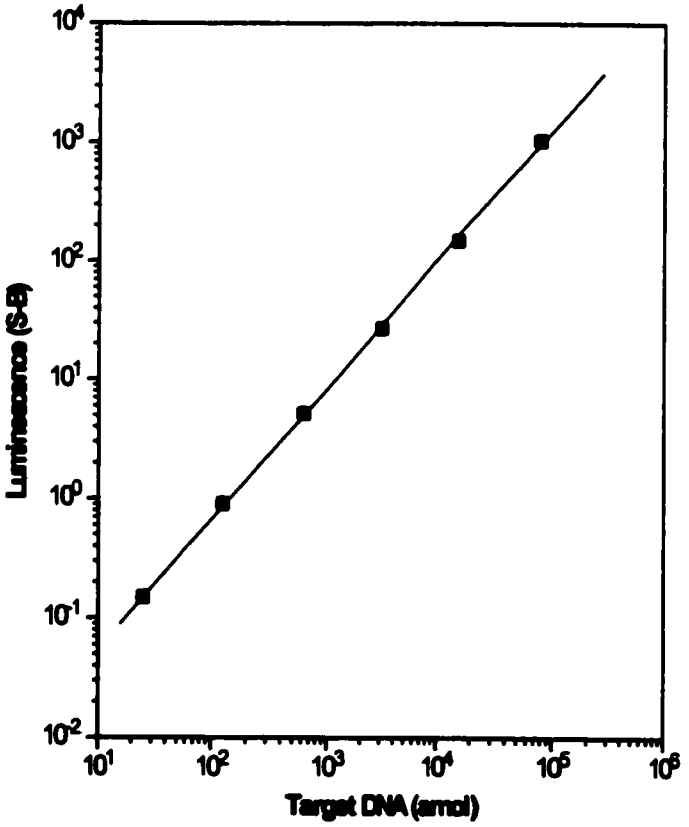
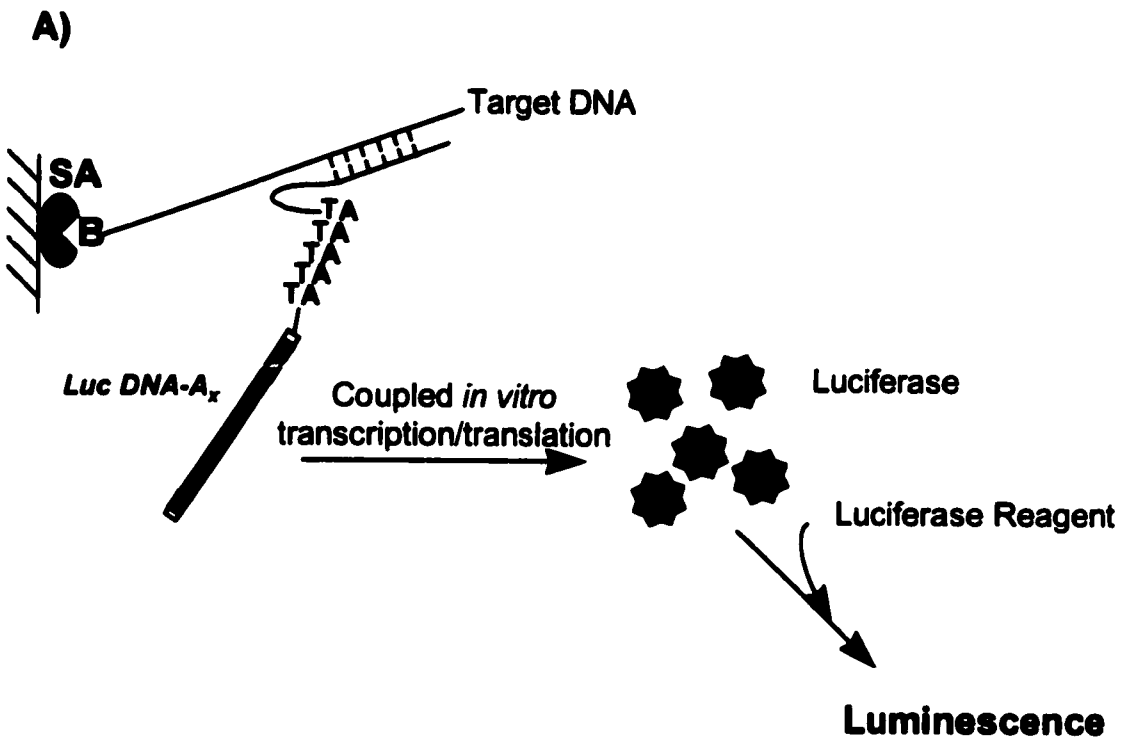


Figure 4-13

Figure 4-14: Captured Target Hybridisation Assay Based on the Expression of Luciferase Encoding DNA Label. A) A schematic diagram of the assay configuration. SA = streptavidin; B = biotin; D = digoxigenin. B) Quantification of target DNA using the assay configuration shown in A and described in the text.


A)
Target DNA
SA
B
TA
TA
TA
TA
Luc DNA-A$_x$
Coupled *in vitro*
transcription/translation
Luciferase
Luciferase Reagent
Luminescence

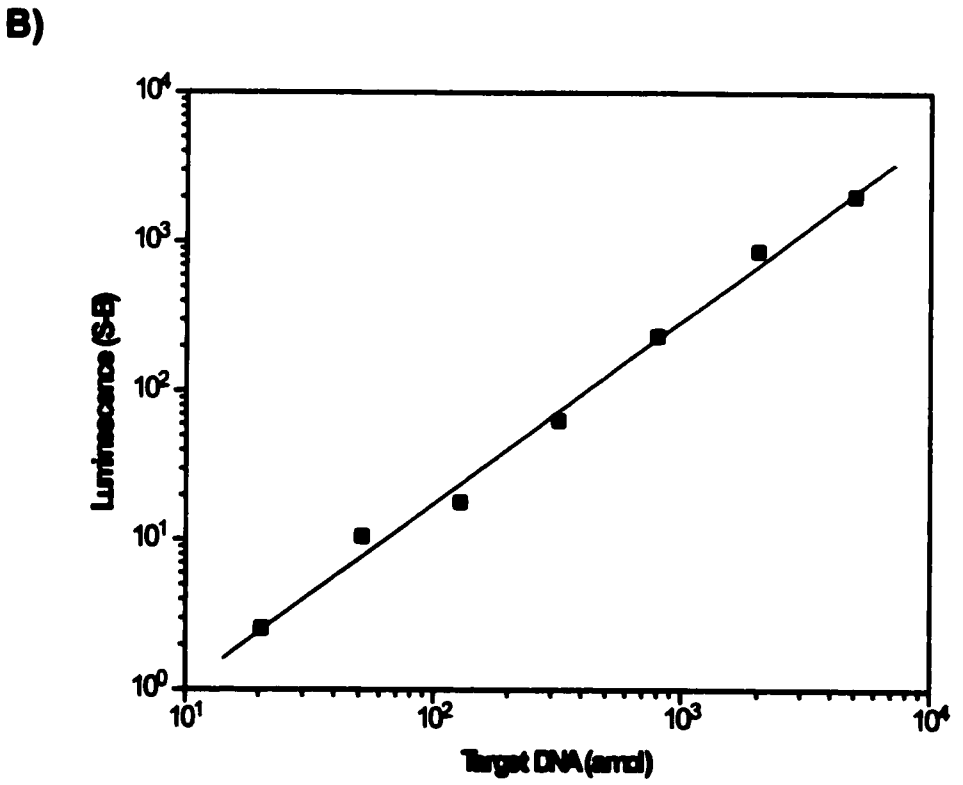

B)
10^4
10^3
10^2
10^1
10^0
Luminescence (S-B)
10^1
10^2
10^3
10^4
Target DNA (amol)

Figure 4-14

This is the second example of a system which produces a small inactive peptide by *in vitro* expression of a DNA label and uses this peptide to generate signal. In this case the apoaequorin produced by expression cannot catalyse a luminescent reaction without prior regeneration with its coelenterazine cofactor, under the appropriate conditions. Using this *in vitro* expression/regeneration detection system it was possible to develop two reliable hybridisation assays able to detect less than 1 amol of target DNA.

5. Isothermal Amplification System for Protein Production from Trace Amounts of DNA

5.1 Introduction

Protein expression by *in vitro* transcription and translation has proven to be a powerful tool with many potential uses. For example, it has proven extremely valuable for examination of the structure-function relationships of proteins. Standard methods for preparation of DNA for *in vitro* expression include cloning of the sequence of interest into a plasmid containing the appropriate regulatory sequences, bacterial amplification of the recombinant plasmid, DNA extraction from the culture and restriction digestion followed by purification of the expressible DNA fragment. This complicated and tedious procedure leading to the *in vitro* expression often limits the proteins and polypeptides which may be studied. A relatively recent advance in this area came with the use of PCR to successfully generate amplified DNA capable of efficient expression *in vitro* (107, 108, 109, 110, 111). This tool has been valuable in the generation of combinatorial protein libraries from PCR of single DNA molecules followed by *in vitro* expression (112), and the construction of an expressible DNA library encoding a single-chain antibody repertoire (113).

Combinatorial protein libraries are useful in screening for proteins with desired properties and/or functions. For example, antibodies or other binding proteins with increased or altered affinities. They can also be used for selection of enzymes with increased activity or altered specificities. The difficulty is finding a reliable cell-free method of library production, thereby avoiding *in vivo* techniques which tend to prolong the procedure and limit the number of library members. PCR has been used as a means of creating amplified DNA, containing all the regulatory sequences required for *in vitro* expression,

from small amounts of original DNA. Combined with current *in vitro* transcription/translation methods, this permits cell-free production of protein in amounts sufficient for analysis and/or screening.

An expressible DNA library may be used in ribosome display (114, 43) or RNA-peptide fusion display (115, 116) for selection of desirable proteins with their corresponding mRNAs. The disadvantages of using PCR for the synthesis of expressible DNA are the time required for each amplification and the need for specific equipment. PCR from a single DNA molecule followed by *in vitro* expression has been suggested to be potentially useful for high-throughput generation of protein libraries (112). The difficulty in this system arises from the amplification of a single DNA molecule. Under such extreme conditions the formation of by-products such as primer dimers, even in small amounts, will severely inhibit amplification of the DNA template. To reduce this effect it was necessary to perform nested PCR which increased the complexity of the system and the time required for amplification. This may be a particular problem when it is necessary to perform multiple amplifications as in molecular evolution studies.

Alternative nucleic acid amplification strategies exist which include transcription dependent methods such as Nucleic Acid Sequence-Based Amplification (NASBA) (117, 118) and Self-Sustained Sequence Replication (3SR) (119). The main product of these reactions is RNA rather than DNA (as produced by PCR) although either DNA or RNA may be used as the original template. Transcription dependent amplification methods do not require temperature cycling or the use of thermostable enzymes. These systems are capable of amplification typically in the range of 10^6 to $\geq 10^9$ (120) in 30 min to one hour, depending upon the template and the enzymes incorporated in the reaction. Each PCR cycle roughly doubles the template copies through the action of the DNA polymerase. The transcription dependent amplification strategies are able to increase the template copies by 10 to several hundred fold through the action of T7 RNAP which can transcribe many RNA products from each T7 promoter-containing DNA. This allows significant amplification to occur without the length of time that is required by PCR.

To date neither NASBA nor 3SR have been used to produce expressible amplification products. The aim of the current study is to develop a transcription dependent isothermal amplification system capable of generating mRNA amplicons which can be expressed by *in vitro* translation. It should then be possible to produce protein from very small amounts of DNA template using a simple isothermal amplification method linked to *in vitro* translation, in place of PCR and *in vitro* coupled transcription and translation. Similarly, this amplification method should prove useful when combined with *in vitro* display technologies, such as ribosome display.

Transcription dependent amplification systems rely on the concerted action of three enzymes; RNA polymerase, reverse transcriptase (RT) and RNase H. This type of amplification is similar to the general scheme used during retroviral replication. Figure 5-1 depicts the steps involved in isothermal amplification of a DNA template by T7 RNAP, avian myeloblastosis virus reverse transcriptase (AMV-RT) and RNase H. The DNA template contains a T7 promoter sequence which allows the T7 RNAP to produce multiple copies of mRNA from each DNA template. Subsequently, the downstream primer anneals to the 3′-end of the mRNA molecules. This allows the AMV-RT to start extending DNA in the 5′ to 3′ direction. The RNA strand of the resulting RNA-DNA duplex is then cleaved through RNase H activity. This allows the upstream primer to hybridise and AMV-RT is then able to form the DNA duplex which is identical to the original DNA template. In this way both DNA and RNA are produced by the isothermal amplification reaction. The digestion of the RNA-DNA duplexes is the rate limiting step which in turn reduces the AMV-RT activity and consequently favours the action of T7 RNAP. As a result, the major product of the isothermal amplification is RNA. In two-enzyme systems which make use of the RNase H activity of AMV-RT, the ratio of RNA to DNA product is further increased. In the present work the DNA template is designed to incorporate the T7 promoter sequence along with a start codon, a protein coding sequence and a stop codon. In this way the mRNA product should be capable of acting as a template for protein generation when added to an *in vitro* translation reaction (Figure 5-1).

Figure 5-1: Isothermal Amplification of DNA Template to Produce Translatable RNA Product. Double stranded template DNA containing a T7 promoter is transcribed by T7 RNAP to produce multiple RNA molecules. With hybridisation of a downstream primer AMV-RT can act to generate DNA in the 5′ to 3′ direction. RNase H activity digests the RNA of the DNA-RNA hybrids and allows hybridisation of the upstream primer (containing the T7 promoter sequence). Again, AMV-RT acts to generate DNA. The product of this reaction is identical to the starting template. T7 = T7 RNA polymerase; RT = avian myeloblastosis virus reverse transcriptase; RNase = ribonuclease; D primer = downstream primer; U primer = upstream primer.

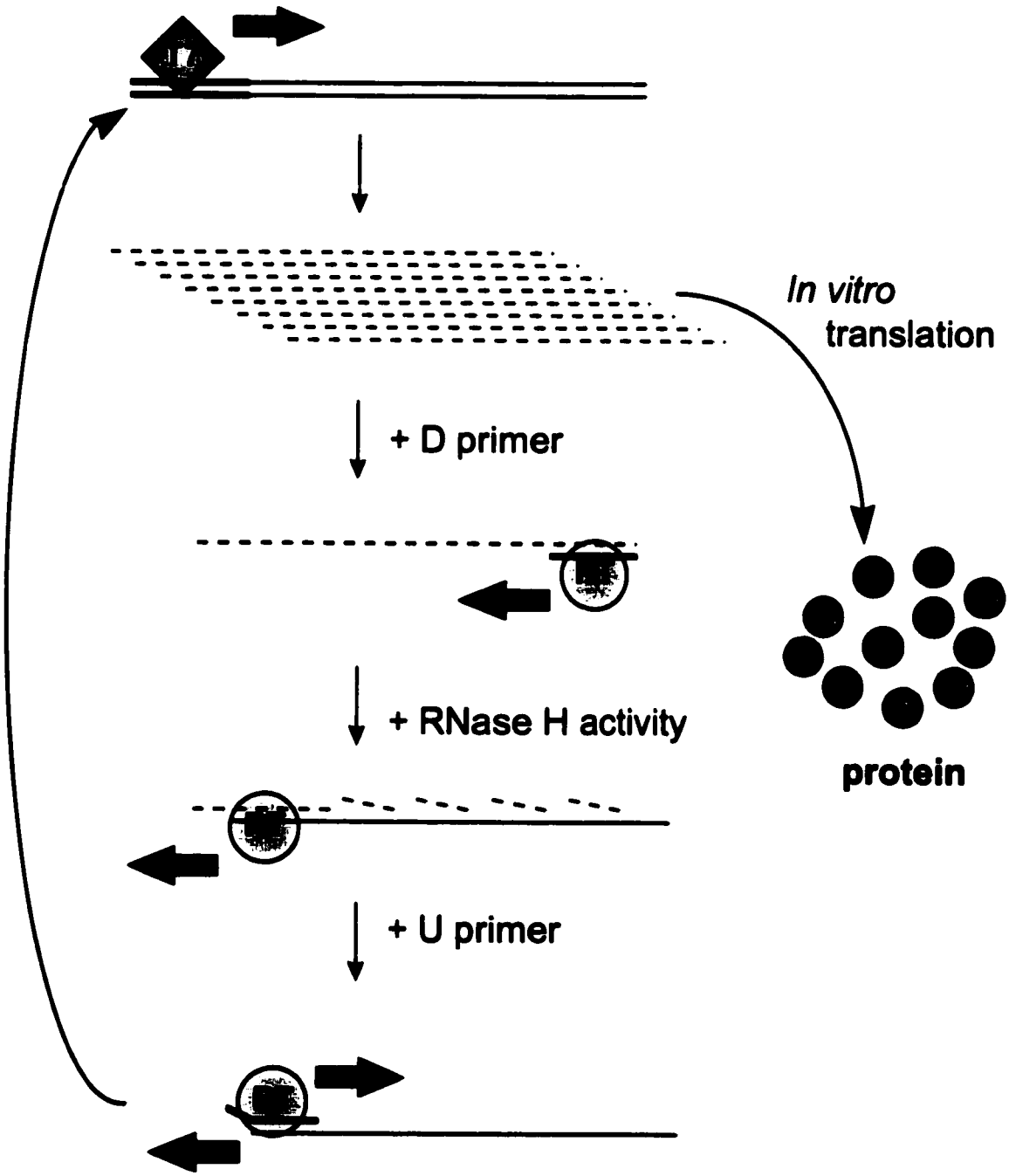

In vitro
translation
+ D primer
+ RNase H activity
protein
+ U primer

Figure 5-1

Previous attempts have been made to couple the isothermal amplification with *in vitro* transcription/translation (121). The results show only limited success in that relatively large amounts of DNA template (0.01 - 1 pmol) were required to generate protein from a 2 hour incubation. The present work is the first example of transcription dependent amplification linked to *in vitro* translation for the production of detectable protein from very small amounts of DNA template.

5.2 Experimental

All instruments, materials, solutions or procedures not outlined herein are described in the experimental sections of previous chapters.

5.2.1 Materials

Reagent	Supplier
E. coli RNase H	Pharmacia Biotech (Montreal, PQ)
AMV Reverse transcriptase	Seikagaku Corporation (Ijamsville, MD, USA)
Wheat Germ Extract	Promega Corporation (Madison, WI, USA)
Oligo(dT) 12-18 primer	Life Technologies (Burlington, ON)
Analytical grade DMSO, sorbitol	Sigma (St. Louis, MO, USA)

5.2.2 Solutions

All solutions, preparations and materials used in this chapter were RNase free. Sterile RNase free ddH_2O was prepared by DEPC treatment as described in Chapter 2.

2.5× Amplification Buffer

2.2 M Tris-acetate, pH 8.2	45.5 µl
0.85 M DTT	29.4 µl
3 M magnesium acetate	26.7 µl

4.2 M mM potassium glutamate	99.2 µl
0.1 mM Primer A_1	5 µl
0.1 mM Oligo(dT) 12-18 primer	5 µl
0.1 M dATP, dTTP, dGTP, dCTP	25 µl (each)
0.1 M ATP, UTP, CTP, GTP	100 µl (each)
DEPC-treated ddH_2O to final volume	1 ml

Mix all the components well and store in 200 µl aliquots at -20°C until ready for use. Note that, before use, the solution may need gentle warming to dissolve the precipitate that forms after freezing.

Amplification Enzyme Buffer

1 M Tris-acetate, pH 8.2	8 µl
0.25 M DTT	8 µl
DEPC-treated ddH_2O to final volume	200 µl

Mix the components well and store at -20°C until ready for use.

Amplification Enzyme Mix

Enzyme buffer	9.9 µl
AMV-RT (26.84 U/µl)	3.7 µl
T7 RNAP (46.8 U/µl)	21.4 µl
	35 µl

Mix the components by vortexing and store at -20°C until ready to use. The mixture should be kept on ice throughout the preparation of the amplification reaction mixture.

Also, the concentration of enzyme units (U/µl) may vary between preparations so it is also necessary to adjust the volumes of enzyme stock solutions added to the enzyme mixture in order to keep the units of enzymes used constant between reactions.

5× Transcription Buffer

1 M Tris-HCl, pH 8.0	100 µl
1M $MgCl_2$	25 µl

0.85 M DTT	29.4 µl
spermidine	1.6 µl
1 M NaCl	25 µl
10 mg/ml BSA	12.5 µl
DEPC-treated ddH_2O to final volume	500 µl

Mix the components well, split into 100 µl aliquots and store at -20°C.

5.2.3 Isothermal Amplification of Apoaequorin mRNA

The amplification reaction components were added to a microcentrifuge tube in clean area, well removed from the template addition and product analysis areas:

2.5× Amplification buffer	10 µl
68.2 % sorbitol	3.5 µl
DMSO	2.5 µl
Enzyme mixture	3.5 µl
DEPC-treated ddH_2O to final volume	25 - x µl

In the template addition area, which was well removed from the product analysis area, x µl of Aeq[dA/dT] DNA was added to the reaction mixture. The amplification reaction was then incubated for 1 hour at 45°C. After amplification, the reaction tube was kept closed until the products were analysed in the product analysis area. Note that the DMSO used in the amplification reaction should be analytical grade and fresh. Immediately upon receipt of new DMSO it should be aliquotted and stored at -20°C. Freeze-thaw cycles of the DMSO should be minimised in order to avoid the formation of decomposition products which may inhibit the amplification reaction.

5.2.4 *In Vitro* Transcription of Apoaequorin Encoding DNA

The transcription reaction was prepared according to the following:

5× Transcription buffer	5 µl
2.5 mM NTPs	5 µl
Aeq[dA/dT]	x µl

T7 RNAP (69 U/μl)	0.8 μl
DEPC-treated ddH_2O to final volume	25 μl

The reaction was incubated for 30 min at 37°C before it could be used as a source of mRNA for use in an *in vitro* translation reaction. This reaction mixture did not require dilution before use in the translation reaction.

5.2.5 *In Vitro* Translation of Apoaequorin mRNA

Prior to use in an *in vitro* translation reaction the amplification reaction was diluted 10 fold in DEPC treated water. Three microlitres of the diluted reaction were used in the translation reaction. Promega's wheat germ extract was used according to a scaled down version of the manufacturer's directions. Essentially, 3 μl of the diluted amplification reaction was mixed with the translation mixture supplemented with 8.7 μM coelenterazine, the final volume was brought to 12.5 μl with DEPC-treated water. The reaction was allowed to proceed for 1 hour at 30°C before the generated aequorin was determined. The mixture was transferred to an opaque flat bottom microtitre well, then 50 μl of the Ca^{2+}-containing luminescence-triggering solution were added and the light emission was integrated for 3 seconds (as described in Chapter 4).

5.2.6 Coupled *In Vitro* Transcription and Translation of the Amplification Products

Prior to use in an *in vitro* transcription/translation reaction the amplification reaction was diluted 10 fold in DEPC treated water. Three microlitres of the diluted reaction were used in the expression reaction. Promega's T7 TNT coupled wheat germ expression system was used according to a scaled down version of the manufacturer's directions. The diluted reaction (3 μl) was added to the TNT mixture supplemented with 8.7 μM coelenterazine, the final reaction volume was brought to 12.5 μl with DEPC-treated water. Expression was allowed to proceed for 1 hour at 30°C before determination of the aequorin generated. Luminescence detection was performed as described above.

5.3 Results and Discussion

5.3.1 Optimisation of Isothermal Amplification Reaction

The DNA fragment chosen as a model template for the development of the isothermal amplification reaction was Aeq[dA/dT]. The template is an expressible DNA fragment encoding apoaequorin which has previously been shown to be an effective substrate for *in vitro* transcription/translation. The production of the expressible DNA fragment, Aeq[dA/dT], was described in section 4.2.5 and elsewhere (122). In the current study the aim was to develop a system capable of producing detectable protein from minute amounts of template DNA. In the previous work each Aeq[dA/dT] DNA was able to generate, on average, 156 molecules of aequorin by *in vitro* expression using the TNT coupled wheat germ system with a 90 minute incubation. This corresponds to detectable protein produced from as little as 5000 DNA molecules. Using a transcription dependent isothermal amplification, prior to *in vitro* translation, it is possible to make use of much less DNA to generate detectable protein.

The transcription dependent isothermal amplification system relies on three enzyme activities, T7 RNAP, AMV-RT and RNase H. The difficulty in developing such a system lies in the determination of ideal buffer conditions which will allow the three enzyme activities to work effectively in a concerted reaction. The amplification must be reproducible, selective and capable of producing large amounts of full length RNA molecules. The concentration of specific anions and cations, as well as the overall ionic strength, have a considerable effect on amplification accuracy and efficiency. The isothermal amplification protocol described in section 5.2.3 is a result of experiments designed to determine the optimum concentrations of Mg^{2+}, K^{+}, nucleoside triphosphates, primers, enzymes and organic additives as well as the optimum incubation time and temperature. In each experiment, amplification was followed by *in vitro* translation and detection of aequorin as a means of monitoring the amplification reaction efficiency. The aequorin determination was carried out as described earlier with the addition of 50 µl of

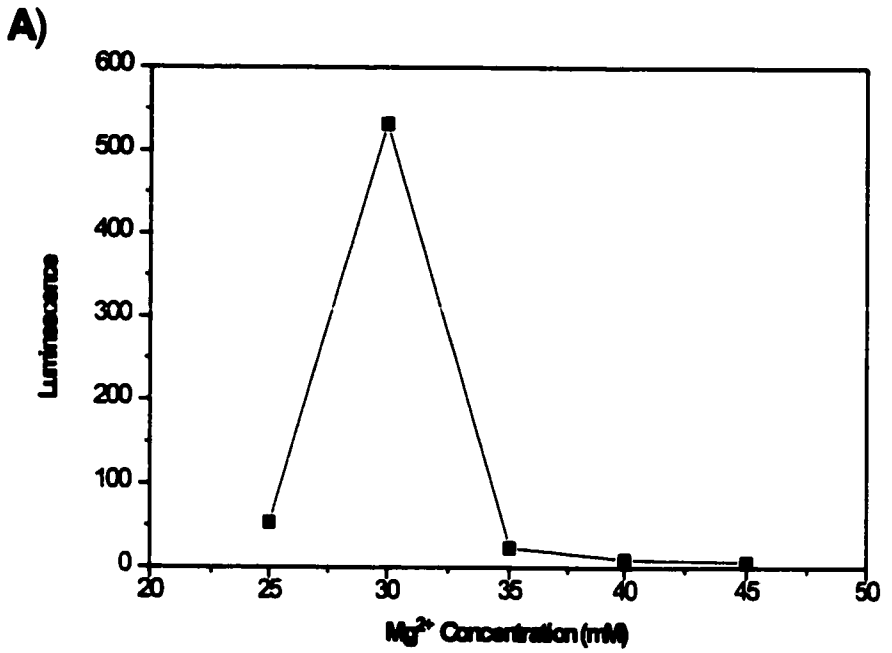
the Ca^{2+}-containing light triggering solution to the sample followed immediately by light measurement integrated over 3 s.

It is well known that Cl^- ions have an adverse affect on RNA polymerases, in particular T7 RNAP (123). For this reason no Cl^- was added to the amplification buffer. Instead, acetate or glutamate were used as counter ions for added cations. It is also well known that the concentrations of free divalent and monovalent cations in solution have a dramatic effect on the activity of the enzymes employed in the amplification reaction. In fact Mg^{2+} levels that are not within a relatively narrow range will completely abolish amplification.

Therefore, the first buffer component to be optimised was magnesium acetate. Several identical amplification reactions were performed using varying amounts of magnesium acetate over a relatively broad concentration range (25 – 45 mM). The results (Figure 5-2 (A)) indicated that the optimum concentration was close to 30 mM. Since very small changes in Mg^{2+} concentration can have a significant effect on enzyme activity it was necessary to narrow the range of magnesium acetate concentrations studied. A second experiment was carried out varying Mg^{2+} concentrations in 2 mM increments over a 26 – 34 mM range. The results, shown in Figure 5-2 (B), indicated that the ideal concentration was 32 mM. Similar experiments were performed to determine the ideal potassium glutamate concentration. The results, shown in Figure 5-3 (A and B), indicated that the ideal K^+ concentration was 160 mM. These concentrations represent the added concentrations of Mg^{2+} and K^+, not the total concentrations. A small amount of Mg^{2+} and K^+ is contributed by the enzyme stock solutions.

Initial studies were carried out to determine the ideal enzyme complement for efficient amplification. Organic additives have been shown to increase productivity of transcription dependent amplification. Therefore, all studies were performed in the presence of sorbitol. No difference in amplification was observed when sorbitol levels were varied from 9.5 to 15 %. Spermidine and DMSO were also tested as additives in the amplification reaction. Three reverse transcriptases have been used successfully in

Figure 5-2: Optimisation of Mg^{2+} Concentration. Various concentrations of magnesium acetate were included in 25 µl amplification reactions which contained 40 mM Tris-acetate, pH 8.2, 10 mM DTT, 100 mM potassium glutamate, 0.1 µM primer A_1, 0.1 µM Oligo(dT) primer, 1 mM dNTPs, 6 mM NTPs and 15 % sorbitol. Aeq[dA/dT] (1×10^6 molecules) was added to each reaction followed by addition of the enzymes (7.5 U AMV-RT, 0.5 U RNase H, and 25 U T7 RNAP). The reactions were incubated for 1½ hours at 42°C before portions of the reactions were used in separate *in vitro* translation reaction (1½ hours at 26°C). Aequorin was determined by measuring luminescence immediately after addition of 50 µl of Ca^{2+}-containing triggering solution and integrating over 3 s. A) A wide range of magnesium acetate concentrations were studied, 25 to 45 mM in 5 mM increments. A 1 µl portion of undiluted amplification reactions were used in the translation reactions. B) A narrow range of magnesium acetate concentrations were studied; 26 to 34 mM in 2 mM increments. The amplification reaction was diluted 10 fold in DEPC-treated ddH_2O and 1 µl of the dilution was used in the translation reactions.


A)
600
500
400
300
200
100
0
Luminescence
20
25
30
35
40
45
50
Mg2+ Concentration (mM)

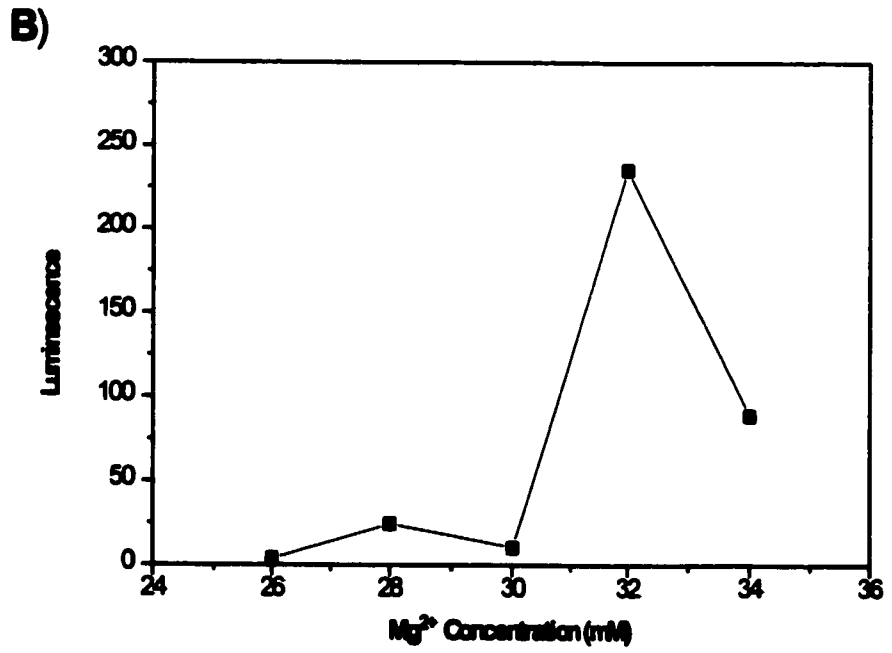

B)
300
250
200
150
100
50
0
Luminescence
24
26
28
30
32
34
36
Mg2+ Concentration (mM)

Figure 5-2

Figure 5-3: Optimisation of K^+ Concentration. Various concentrations of potassium glutamate were included in 25 μl amplification reactions which contained 40 mM Tris-acetate, pH 8.2, 10 mM DTT, 32 mM magnesium acetate, 0.1 μM primer A_1, 0.1 μM Oligo(dT) primer, 1 mM dNTPs, 6 mM NTPs and 15 % sorbitol. Aeq[dA/dT] (1×10^6 molecules) was added to each reaction followed by addition of the enzymes (7.5 U AMV-RT, 0.5 U RNase H, and 25 U T7 RNAP). The reactions were incubated for 1½ hours at 42°C. Aliquots (1 μl) of 10 fold dilutions of the amplification reactions were used in separate *in vitro* translations (1½ hours at 26°C). Aequorin was determined by measuring luminescence immediately after addition of 50 μl of Ca^{2+}-containing triggering solution and integrating over 3 s. A) A wide range of potassium glutamate concentrations were studied, 130 to 170 mM in 10 mM increments. B) A narrow range of potassium glutamate concentrations were studied; 145 to 165 mM in 5 mM increments.

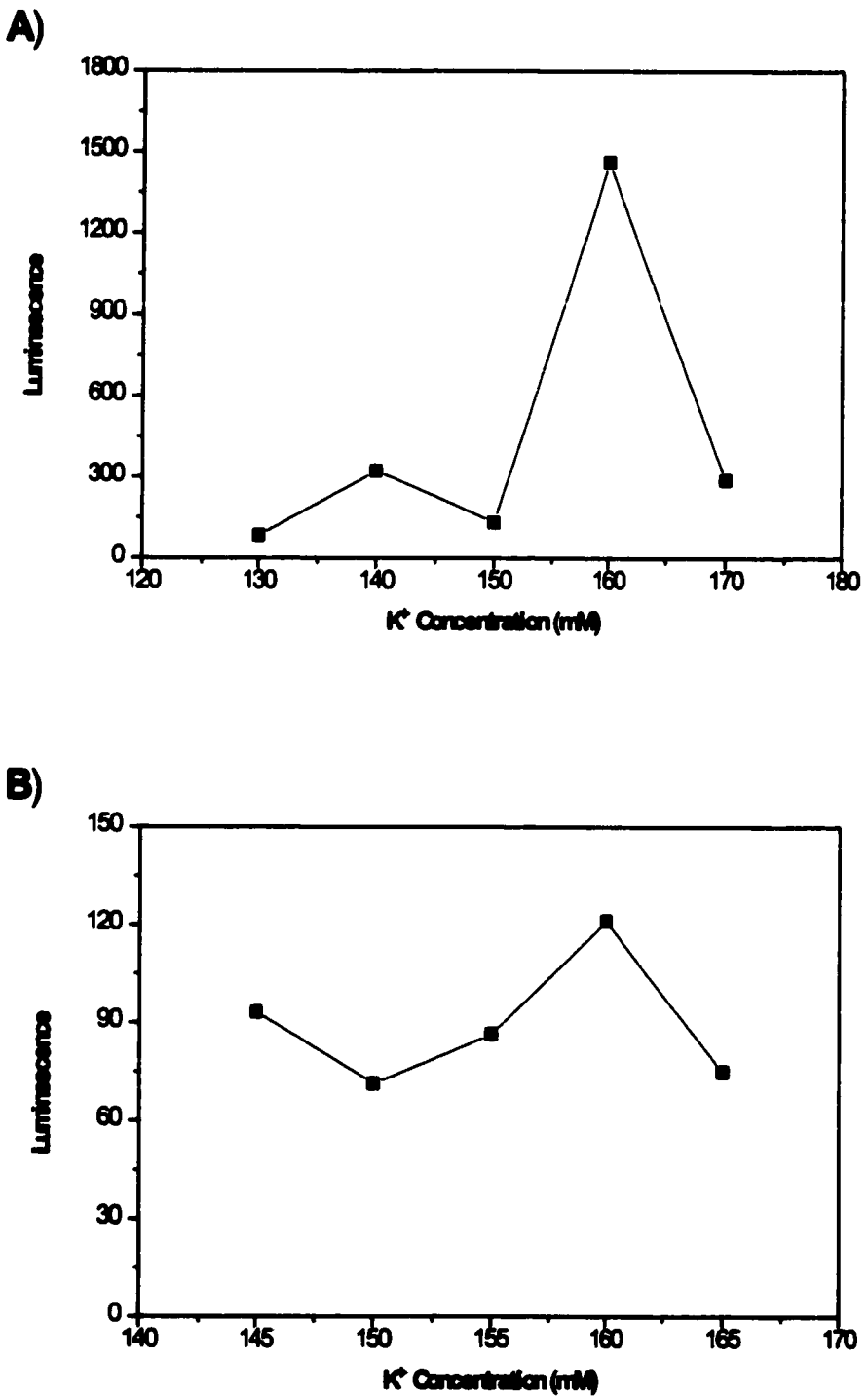

A)
1800
1500
1200
900
600
300
0
Luminescence
120
130
140
150
160
170
180
K+ Concentration (mM)
B)
150
120
90
60
30
0
Luminescence
140
145
150
155
160
165
170
K+ Concentration (mM)

Figure 5-3

transcription dependent amplifications; Moloney Murine Leukemia Virus (MMLV), Human Immunodeficiency Virus (HIV) and AMV. The most commonly used RT is the AMV-RT which has been employed in both three-enzyme and two-enzyme systems.

Figure 5-4 illustrates the difference in amplification yield from reactions containing various components; including two-enzyme and three-enzyme reactions. In the present study it was found that the two-enzyme system containing 10 % DMSO was approximately 6 times more efficient than the three enzyme reaction. The addition of 10 % DMSO apparently enhances the intrinsic RNase H activity of AMV-RT (124) which allows amplification to occur without addition of *E. coli* RNase H. The two-enzyme system also increases the ratio of RNA to DNA as the T7 RNAP activity is favoured over the RT activity (as explained in section 5.1). Once it was determined that a two-enzyme system would be used it was then necessary to determine the optimum amounts of each enzyme required for efficient amplification. The yield was highest with the use of 100 units of T7 RNAP plus 10 units of AMV-RT in a reaction containing sorbitol and 10 % DMSO (results not shown).

The major product of the amplification reaction is RNA, so it is not surprising that the NTP to dNTP ratio must reflect this bias. However, the ideal concentrations and ratio between the two must be determined empirically. An experiment was performed in which NTP concentrations were varied at two concentrations of dNTPs, low (0.2 mM) and high (1 mM), such that ratios of 1:2 to 1:6, dNTPs to NTPs, were studied. The graph in Figure 5-5(A) (low dNTP and NTP concentrations) shows an increase in product with increasing NTP concentration. This suggests that at these concentrations the NTPs and dNTPs are acting as limiting reagents. The higher concentrations of dNTPs and NTPs are required and the best ratio of dNTPs to NTPs was found to be 1:4 (Figure 5-5(B)). Since nucleoside triphosphates chelate Mg^{2+}, it was necessary to confirm the optimum concentration of magnesium acetate, with the new nucleoside triphosphate levels, by again varying the amount of magnesium acetate added to otherwise identical amplification reactions. It was found that the previously determined optimum, 32 mM, remained correct. The optimum concentration of potassium glutamate was also re-

Figure 5-4: Comparison of Enzyme Mixtures. Five identical amplification reactions containing 40 mM Tris-acetate, pH 8.2, 10 mM DTT, 32 mM magnesium acetate, 160 mM potassium glutamate, 0.1 μM primer A_1, 0.1 μM Oligo(dT) primer, 1 mM dNTPs, 6 mM NTPs and 15 % sorbitol were prepared. Aeq[dA/dT] (1×10^6 molecules) was added to each reaction followed by A) 10 % DMSO, 2.5 U AMV-RT and 5 U T7 RNAP; B) 10 % DMSO, 7.5 U AMV-RT and 25 U T7 RNAP; C) 10 % DMSO, 7.5 U AMV-RT, 25 U T7 RNAP and 0.5 U RNase H; D) 2 mM spermidine, 7.5 U AMV-RT, 25 U T7 RNAP and 0.5 U RNase H; E) 7.5 U AMV-RT, 25 U T7 RNAP and 0.5 U RNase H. Each reaction was incubated for 1½ hours at 42°C. A 1 μl aliquot of 10 fold dilutions of the amplification reactions were used in separate *in vitro* translations (1½ hours at 26°C). Aequorin was determined by measuring luminescence immediately after addition of 50 μl of Ca^{2+}-containing triggering solution and integrating over 3 s.

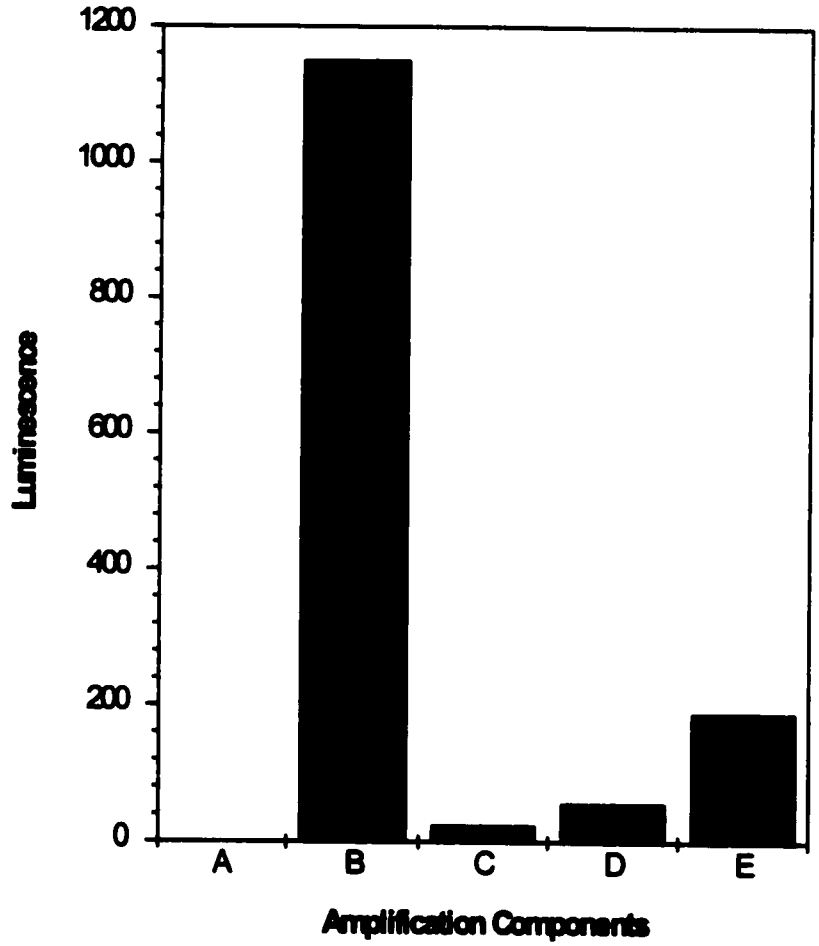

1200
1000
800
600
400
200
0
Luminescence
A
B
C
D
E
Amplification Components

Figure 5-4

Figure 5-5: Optimisation of NTP and dNTP Concentrations. A) Five identical 12.5 µl amplification reactions containing 40 mM Tris-acetate, pH 8.2, 10 mM DTT, 32 mM magnesium acetate, 160 mM potassium glutamate, 0.1 µM primer A_1, 0.1 µM Oligo(dT) primer, 0.2 mM dNTPs, 15 % sorbitol and 10 % DMSO were prepared. One microlitre of various concentrations of NTPs was added to each reaction. Aeq[dA/dT] (5×10^4 molecules) was then added to each reaction followed by 5 U AMV-RT and 50 U T7 RNAP. The reactions were incubated for 1½ hours at 42°C. Aliquots (1 µl) of 10 fold dilutions of the amplification reactions were used in separate *in vitro* translations (1½ hours at 26°C). Aequorin was determined by measuring luminescence immediately after addition of 50 µl of Ca^{2+}-containing triggering solution and integrating over 3 s. B) The experiment was performed exactly as in A except 1 mM dNTPs was included in the amplification reactions and different concentrations of NTPs were tested.

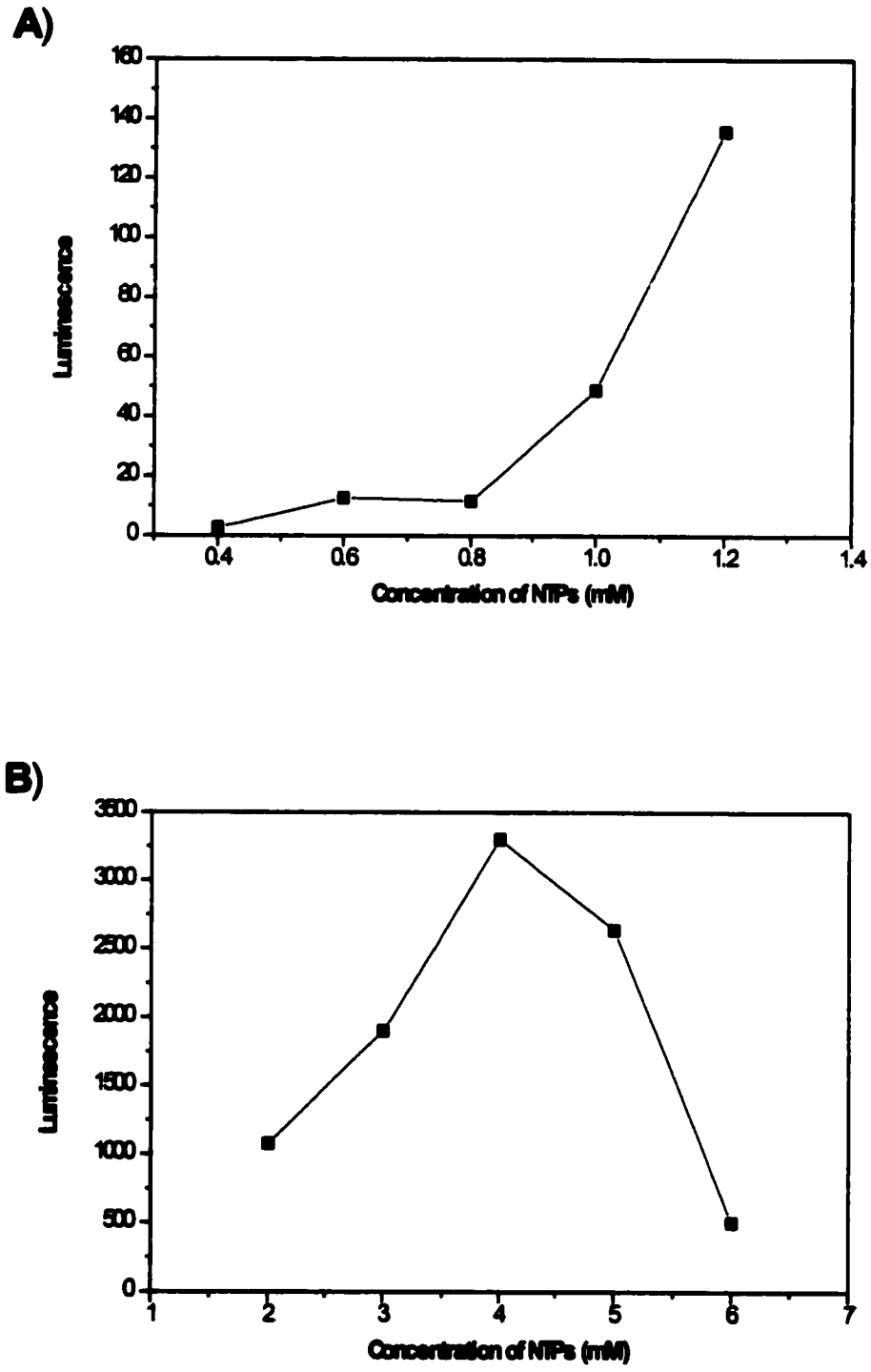

A)
Luminescence
Concentration of NTPs (mM)
B)
Luminescence
Concentration of NTPs (mM)

Figure 5-5

evaluated. Using the new amplification conditions it was found that slightly more K^+ was required; 165 mM potassium glutamate was used in all subsequent amplification reactions.

The primers were the final essential components of the amplification reaction to be studied. The structure and the concentration of the primers have a significant effect on the specificity and the yield of the amplification reaction. The 5′-primer (primer A_1, sequence given in section 4.2.2) used in this study contains the T7 promoter sequence and the first five codons of the aequorin coding sequence. Changes in the structure of this upstream primer were not studied, although two downstream primers were tested. One contained the last five codons of the aequorin coding sequence plus the stop codon and the second was an oligomer of 12 to 18 dTTP which hybridises to the poly(ATP) sequence at the 3′ end of the RNA. Use of the oligo(dT) primer resulted in the production of approximately 30 fold more aequorin protein (results not shown). This may be attributed to the increased expressibility of templates which contain a 3′ poly A tail (see section 4.3.1). Earlier work in the development of 3SR and NASBA technologies suggested that the concentration of the primers should be approximately 0.1 to 0.2 μM. In the system developed here 0.2 μM was found to result in the highest yield of translatable RNA, as determined by measurement of aequorin generated from *in vitro* translation of the amplification products.

From the optimisations summarised above, a 2.5× amplification buffer was prepared which contained the Tris-acetate buffer, magnesium acetate, potassium glutamate, 5′- and 3′-primers, nucleoside triphosphates and DTT. In order to perform the amplification this buffer was mixed with DMSO, sorbitol, an enzyme mixture and finally the DNA template such that the final concentrations of the buffer components and the enzymes were at the optimised levels in a 25 μl reaction.

In addition to the reaction components the incubation temperature was also examined. It was found that the optimum temperature for amplification was 45°C. This temperature allows the correct balance between efficient primer hybridisation and temperature-

dependent enzyme degradation. Unlike previous studies (125), it was found that it was not necessary to denature the DNA template prior to amplification. This is likely due to the fact that the initial DNA template contains a T7 promoter sequence. Therefore, the first step in the amplification cycle is transcription by the T7 RNAP rather than primer annealing and product extension through reverse transcriptase activity, as occurs in NASBA and 3SR techniques.

The time required for amplification of sufficient mRNA production was studied by preparing three amplification reactions containing 10, 100 and 1000 molecules of template DNA and following the production of RNA over time. Aliquots of the reactions were removed at appropriate time intervals and diluted 10 times (as described in the methods) before incubation in an *in vitro* translation mixture with subsequent aequorin determination. The results of this time study are illustrated in Figure 5-6. In each case a peak in aequorin levels was reached; at 90 minutes for 100 and 1000 DNA molecules and at 120 minutes for 10 DNA molecules. After this peak the levels of aequorin produced appear to drop. This may be attributed to depletion of nucleoside triphosphates, degradation of amplification enzymes or inhibition of either the amplification reaction or the translation reaction (or both) by the presence of large amounts of RNA. For the purposes of this study, a one hour incubation for amplification was found to be sufficient.

In order to generate protein from the amplification products, *in vitro* translation was used. It was thought that since the amplification reaction generates DNA amplicons along with the RNA products, albeit to a lesser extent, that the use of coupled *in vitro* transcription and translation may result in increased protein production. To study this, an amplification reaction was diluted 10 fold in RNase free water and aliquots were used in expression reactions using either the wheat germ extract for *in vitro* translation only or the wheat germ based coupled TNT system for *in vitro* transcription and translation. There was no significant difference in the amount of protein generated from the two expression reactions. Even when using the coupled system the protein was predominantly produced from translation of the amplified RNA and the DNA produced did not produce measurable amounts of protein. This is in fact a fortunate observation as

Figure 5-6: Time Dependence of the Isothermal Amplification Reaction. Three 25 μl amplification reactions were performed using the optimised reaction conditions (40 mM Tris-acetate, pH 8.2, 10 mM DTT, 32 mM magnesium acetate, 165 mM potassium glutamate, 0.2 μM primer A_1, 0.2 μM Oligo(dT) primer, 1 mM dNTPs, 4mM NTPs, 15 % sorbitol, 10 % DMSO, 10 U AMV-RT and 100 U T7 RNAP). Aeq[dA/dT] DNA was included in each reaction: 10 molecules (—■—), 100 molecules (- - ● - -), and 1000 molecules (—▲—). The reactions were incubated at 45°C and 2 μl aliquots were removed at various time intervals. Each aliquot was diluted 10 fold in DEPC-treated ddH_2O and 3 μl of each dilution was used in an *in vitro* translation reaction (1½ hours at 26°C). The aequorin produced was determined by adding 50 μl of Ca^{2+}-containing triggering solution and integrating light emission over 3 s.

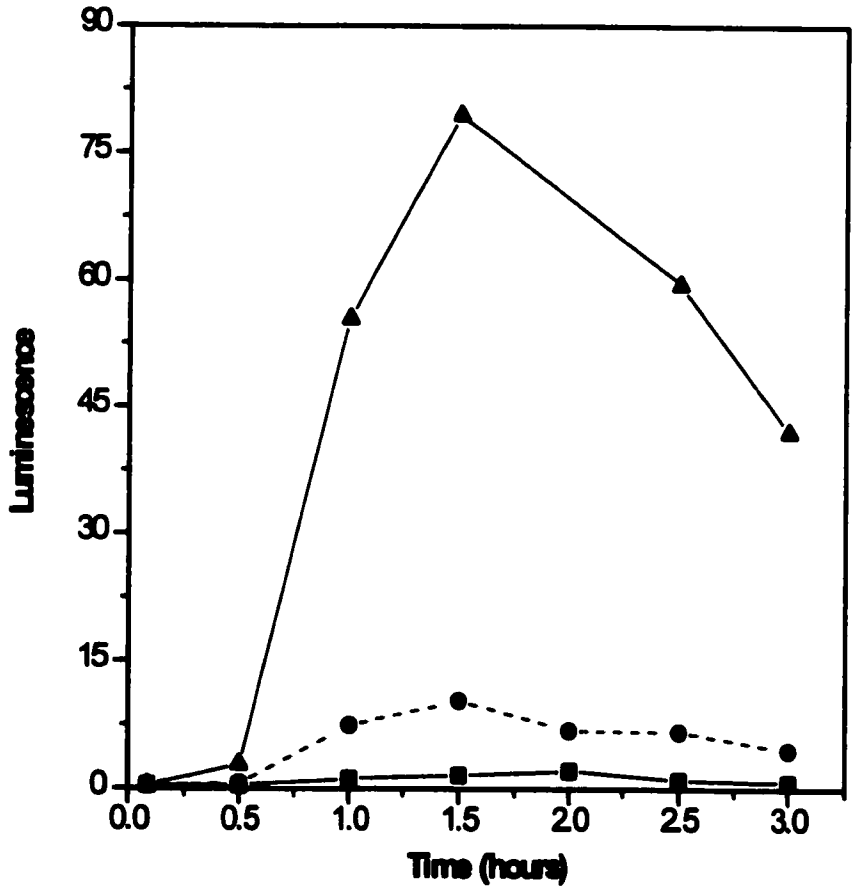


Figure 5-6

it is much more economical to use an *in vitro* translation system, rather than a coupled system, for expression. This also confirms that RNA is the major product of amplification.

5.3.2 Optimisation of *In Vitro* Translation of Amplified RNA

The translation reaction was studied in order to ensure maximum protein production from the produced RNA. It was found that reaction could be performed using a total volume of 12.5 µl using a scaled-down version of the manufacturer's protocol and retaining the component concentrations suggested. No additional Mg^{2+} or K^{+} was required. Similarly, no additional amino acids were required despite the concern that they may act as limiting reagents when large amounts of RNA are translated. The best incubation temperature was 30°C which is higher than the suggested temperature of 25°C. A study (presented in Figure 5-7) in which identical translations were performed (containing aliquots of the same, diluted, amplification reaction) for various incubation times indicated that a plateau is reached after approximately 90 minutes. After incubation for one hour, greater than 80 % of the maximum protein was produced. This was a sufficient incubation for the purposes of this work.

It was observed that the translation reaction was inhibited by components of the amplification reaction. When only 1 µl of a typical amplification reaction (no DNA template) was added to a 12.5 µl translation reaction containing Aeq[dA/dT] mRNA, a 60 % decrease in translation was observed. This is not surprising since the amplification reaction contains organic additives and high salt concentrations which may both adversely affect translation efficiency. To determine which components affected the *in vitro* translation a 25 µl *in vitro* transcription reaction was performed using 1×10^8 molecules of Aeq[dA/dT] DNA. One microlitre aliquots of this reaction were added to separate translation reactions which contained various components of the amplification reaction. It was found that the DMSO and the high Mg^{2+} and K^{+} concentrations contributed significantly to inhibition of translation. There are two possible methods for eliminating or reducing this inhibition. The most effective method would be to purify the

Figure 5-7: Time Dependence of *In Vitro* Translation of the Amplification Products. An amplification reaction (40 mM Tris-acetate, pH 8.2, 10 mM DTT, 32 mM magnesium acetate, 165 mM potassium glutamate, 0.2 μM primer A_1, 0.2 μM Oligo(dT) primer, 1 mM dNTPs, 4mM NTPs, 15 % sorbitol, 10 % DMSO, 10 U AMV-RT and 100 U T7 RNAP) containing 500 molecules of Aeq[dA/dT] DNA was incubated for 1 hour at 45°C. An 87.5 μl (7 × 12.5 μl) *in vitro* translation mixture was prepared containing 21 μl of a 10 fold dilution of the amplification reaction. The reaction was incubated at 30°C and 10 μl aliquots were removed at various time intervals. The aliquots were immediately used for aequorin determination by addition of 50 μl of Ca^{2+}-containg triggering solution and luminescence measurement.

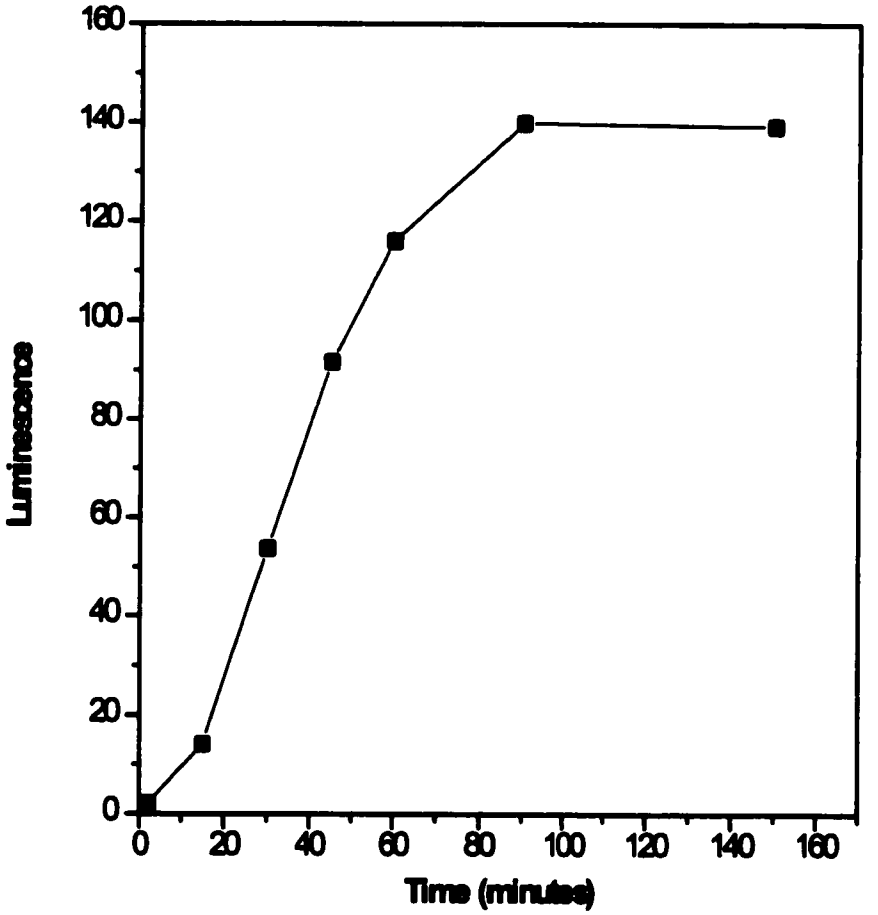

160
140
120
100
80
60
40
20
0
Luminescence
0
20
40
60
80
100
120
140
160
Time (minutes)

Figure 5-7

amplification products prior to expression but this would increase both the time required and the complexity of the overall procedure. The more practical alternative was to use only a portion of the amplification reaction for *in vitro* expression and thereby reduce the amount of inhibitors added to a level tolerated by the translation reaction.

The next study was performed to find the maximum amount of amplification mixture that could be added to the *in vitro* translation reaction without purification of the amplification products. While attempting to maximise the amount of amplification product introduced it was equally important to minimise the amount of inhibitors added to the translation reaction. The best translation yield was observed with 4 μl of a 10 fold dilution of the amplification mixture used for protein production. There was very little difference in the yield of aequorin obtained from addition of 3 μl or 4μl of the amplification dilution to the translation reaction. This study was carried out with amplification of only 100 molecules of Aeq[dA/dT] DNA. The concern was that if more DNA template was used, and consequently much larger amounts of RNA product were produced, the RNA may overload the translation machinery and inhibit expression. For this reason only 3 μl of diluted amplification reactions were added to subsequent translation reactions.

To demonstrate the utility of the amplification system various amounts of DNA template were subjected to isothermal amplification for one hour using the conditions determined above. After amplification each reaction was diluted 10 fold and 3 μl of each dilution were used in separate translation reactions, as described in section 5.2.5. The results are illustrated in Figure 5-8. There is a linear relationship between the amount of input DNA template and the amount of aequorin bioluminescence detected. From the signals obtained at the various DNA levels and an aequorin calibration curve, prepared by diluting commercially available recombinant aequorin in aequorin dilution buffer, it was estimated that a single Aeq[dA/dT] DNA template can produce 2.1×10^7 molecules of aequorin.

It was then necessary to prove that the isothermal amplification reaction increased the yield of RNA over that produced by regular *in vitro* transcription of the DNA by T7

Figure 5-8: Quantification of Aeq[dA/dT] DNA by Isothermal Amplification Linked to *In Vitro* Translation. Various amounts of Aeq[dA/dT] DNA were used in typical amplification reactions (40 mM Tris-acetate, pH 8.2, 10 mM DTT, 32 mM magnesium acetate, 165 mM potassium glutamate, 0.2 μM primer A_1, 0.2 μM Oligo(dT) primer, 1 mM dNTPs, 4mM NTPs, 15 % sorbitol, 10 % DMSO, 10 U AMV-RT and 100 U T7 RNAP) which were incubated for 1 hour at 45°C. The reactions were diluted 10 fold in DEPC-treated water and 3 μl of each dilution was used in *in vitro* translations (1 hour at 30°C). Aequorin production was determined by luminescence measurement after addition of 50 μl of Ca^{2+}-containing triggering solution.

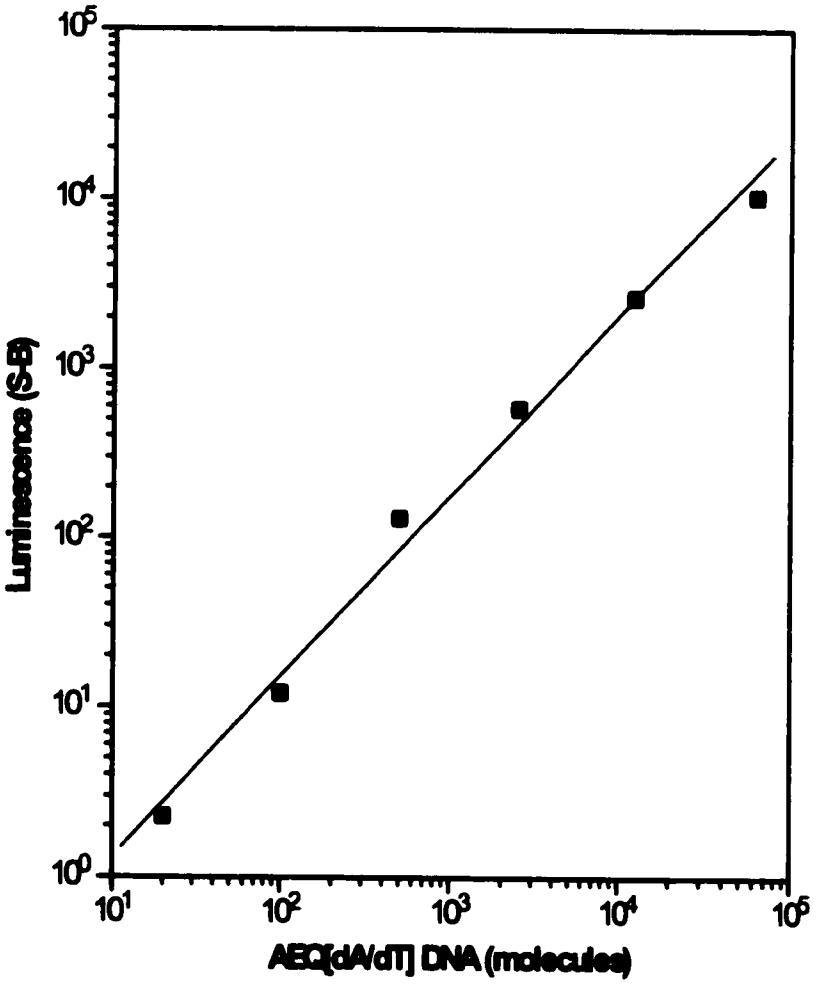

Luminescence (S-B)
AEQ[dA/dT] DNA (molecules)
10^0
10^1
10^2
10^3
10^4
10^5

Figure 5-8

RNAP. Aeq[dA/dT] (1×10^6 molecules) was transcribed *in vitro* as described in the experimental section except that the incubation was performed at 37°C for 1½ hours. Next, 1 µl of this reaction was added to a typical 12.5 µl translation reaction which was incubated for 1½ hours at 26°C before determining the amount of aequorin produced. The results indicated that approximately 250 molecules of aequorin were produced from each DNA template using this procedure. The increased incubation time of the translation reaction should more than compensate for the non-optimum incubation temperatures. Therefore, the large aequorin yield per Aeq[dA/dT] observed using the isothermal amplification linked to *in vitro* translation cannot be attributed to simple transcription of the DNA template. The high yield of aequorin is due to the concerted action of the T7 RNAP and the AMV-RT with its inherent RNase H activity during the isothermal amplification.

Despite the fact that only 3 µl of a ten fold dilution of the amplification reaction was used in the *in vitro* translation reaction, detectable protein was generated from less than 20 molecules of Aeq[dA/dT] template DNA. An important consideration is that this was accomplished in only 2 hours using an isothermal amplification system. This eliminates the need for a thermocycler, which is required for PCR amplifications. Furthermore, the amplification occurs in only one hour whereas PCR amplifications can take much longer, depending on the length and number of cycles used. The product of the isothermal reaction is translatable RNA which also eliminates the need for transcription after amplification.

5.3.3 Potential Applications

The system presented here is the first example of an isothermal amplification system linked to *in vitro* translation for production of protein from traces of DNA. The potential applications of this system are numerous. An obvious extension, which could make use of the work presented in previous chapters, would be the adaptation of this technique as a signal amplification system (again using Aeq[dA/dT] as a label) for use in extremely sensitive immunoassays and nucleic acid hybridisation assays. High backgrounds may be

one difficulty which should be anticipated with this application. The non-specific binding of even a few molecules of DNA label will give rise to significant luminescence as a result of the large signal amplification.

The isothermal amplification procedure described here generates large amounts of translatable RNA from each DNA template. This can be useful for the preparation of protein from genes which are available in only very small quantities. The procedure may be adapted to amplification of rare mRNA molecules and subsequent protein production, as long as the upstream primer incorporates a T7 promoter sequence. Since this method, from DNA to protein, is entirely cell free it also provides an attractive alternative to *in vivo* techniques when the protein product is cytotoxic. If large amounts of protein are required for analysis, the RNA amplification products may be purified prior to translation which should allow more product to be incorporated in the expression reaction. Further, the *in vitro* translation reaction may be increased from 12.5 μl to allow expression of larger portions of the amplification reaction and, as a result, the production of larger quantities of protein.

Molecular evolution or evolutionary molecular engineering is a new approach in modern biotechnology for synthesis of biomolecules with novel functions or improved properties. This has been successfully applied to synthetic nucleic acids where the phenotype and genotype are the same molecule (126). However, when the biomolecule is a protein the phenotype and genotype are distinct. Until recently molecular evolution of proteins has required an *in vivo* step for screening and selection of protein library members along with their corresponding genetic material (eg. phage display). New techniques such as ribosome display and RNA-peptide fusion have allowed this selection to be performed *in vitro*.

In each case of molecular evolution the gene of interest is subjected to mutagenic amplification to create a library of mutants, usually by PCR. Using appropriate conditions transcription dependent amplification has been used for mutagenic amplification and evolution of catalytic RNAs (127). The system described here could be used to amplify RNAs to create an mRNA library. By following the amplification with

ribosome display using an *in vitro* translation system rather than coupled transcription/translation it may be possible to select proteins, with desired properties or improvements, along with their corresponding RNAs. These RNA molecules can then be used in the next round of mutagenic isothermal amplification followed by ribosome display and selection. The rounds of amplification, expression and selection can be repeated until the protein product reaches the required level of evolution. Alternatively, PCR may be used to create a DNA mutant library then the isothermal amplification linked to *in vitro* translation (as described here) may be used to generate protein. Dilution of the DNA such that, statistically, only one molecule of DNA is available for use as a template in the transcription dependent amplification would result in protein produced from only one library member. Since the method developed here is fast, does not require additional equipment and can result in the production of relatively large amounts of protein from traces of DNA, this may be a useful alternative to other expression and selection techniques.

6. Conclusions

We have demonstrated that *in vitro* expression can be used as a powerful signal amplification system. *In vitro* expression of a DNA label introduces at least two levels of amplification. Each DNA label can be transcribed by an appropriate RNA polymerase to generate multiple copies of mRNA. Each mRNA is subsequently translated to yield multiple protein products. The product molecules are detected in solution or, if the product is an enzyme, a third level of amplification is achieved through substrate turnover and the products of the enzymatic reaction are detected. Although the process of transcription and translation are complex, the advent of cell-free coupled transcription-translation systems has allowed the expression to be performed in a single *in vitro* reaction.

We have applied a cell-free expression system to a sensitive immunoassay that utilised a DNA label which encoded the α-peptide of β-galactosidase. The lacZα DNA label used in this immunoassay was prepared from restriction digestion of the plasmid, pGEM 13Zf(+), with no optimisation of its sequence, and was expressed in a prokaryotic system using the endogenous RNA polymerase. This assay was able to detect as few as 3 fmol of immobilised antigen. Numerous attempts to create a template for efficient production of functional α-peptide in a eukaryotic *in vitro* expression system failed to produce an improved DNA label.

We expanded the potential of the expression detection system by introducing a new DNA label. A DNA encoding apoaequorin was used to develop a very sensitive bioluminescent nucleic acid hybridisation assay. Similar to the α-peptide assay, the product of expression was a polypeptide capable of activating a signal generating system. Using the aequorin expression detection system we were able to detect 0.5 amol of target DNA in a captured target hybridisation assay configuration and 0.25 amol of target DNA

in a sandwich assay configuration. The assays were found to be significantly more sensitive than captured target assays which employed either the aequorin protein complex or a luciferase encoding DNA as a label.

In addition to detection procedures, amplification as a result of *in vitro* expression can be useful for fast and reliable production of protein without the use of *in vivo* techniques. We devised a system for isothermal amplification of the apoaequorin encoding DNA to generate large quantities of expressible RNA. Linking this procedure to *in vitro* translation allowed production of protein from traces of DNA in only two hours. Many potential applications of the isothermal amplification linked to cell-free translation exist and examples were reviewed in Chapter 5.

Sensitivity is a major focus of research in the areas of immunoassay and nucleic acid hybridisation assay design and development. The detection/determination of analytes and specific DNA or RNA sequences in small biological samples, or in samples containing low concentrations of the substance of interest, is a common and challenging analytical problem. Examples include the early detection and quantification of infectious agents when they are present at minute amounts in human body fluids and other clinical and environmental samples. Also, the diagnosis and monitoring of neoplastic disorders is frequently based upon the analysis of very low concentrations of tumour markers or DNA/RNA sequences, that are specific and characteristic of malignant cells. In nucleic acid determinations one approach to these problems involves exponential amplification of the target nucleic acid sequence (e.g. by PCR) to levels that are several orders of magnitude higher than that in the starting material. Although target amplification techniques provide the required sensitivity, a serious disadvantage is the potential contamination of the samples from previously amplified sequences. An additional disadvantage is the requirement of internal standards to control for fluctuations in amplification efficiency. Signal amplification methods provide sensitivity through the high detectability of the reporter molecule (label) while the amount of target nucleic acid remains constant, thus eliminating the above drawbacks.

The *in vitro* expression detection system has a number of advantages over other detection methodologies. DNA molecules are more stable labels than proteins which can be sensitive to degradation over time and under some assay conditions. The presence of nucleases in the sample under investigation will not affect the DNA label as they are removed with the excess label during the wash steps. Since the protein product of the expression reaction is in solution there is no restriction to proteins that are amenable to conjugation to the recognition molecule. Also, solution phase kinetics exist rather than solid phase kinetics which are limited by the accessibility of the enzyme active site and progressively reduced local substrate concentrations. Since the assays are performed in microtitre wells and blotting procedures are avoided, the technique of signal amplification by *in vitro* transcription and translation is amenable to automation which should allow it to become useful in clinical situations as well as research laboratories. The sensitivity attained through the use of the *in vitro* expression detection system with an appropriately designed DNA label, is the result of the significant amplification which can be achieved.

References

1. Hayden,M.S., Gilliland,L.K. and Ledbetter,J.A. (1997) *Curr. Opin. Immunol.*, **9**, 201-212.

2. Pluckthun,A. and Pack,P. (1997) *Immunotechnology*, **3**, 83-105.

3. Gosling,J.P. (1994) In Gosling,J.P and Basso,L.V. (eds.) *Immunoassay: Laboratory Analysis and Clinical Application*, Butterworth-Heinemann, Boston, MA, pp. 1-30.

4. Jaklitsch,A. (1985) In *Enzyme-mediated Immunoassay*, Plenum Press, New York, NY, pp. 33-55.

5. Henderson,D.R., Friedman,S.B., Harris,J.D. and Manning,W.B. (1986) *Clin Chem.*, **32**, 1637-1641.

6. Khanna,P.L., Dworschack,R.T., Manning,W.B. and Harris,J.D. (1989) *Clin. Chim. Acta*, **185**, 231-240.

7. Yalow,R.S. and Berson,S.A. (1959) *Nature*, **184**, 1648-1649.

8. Hemmila,J.A. (1997) In Johnson,A.P., Turner,MW. (eds.) *Immunochemistry 1*, IRL Press, Oxford, UK, pp 193-214.

9. Wood,P.J. and Barnard,G. (1997) In Price,C.P., Newman,D.J., (eds.) *Principles and Practice of Immunoassay*, 2nd edn., Stockton, New York, NY, pp 389-424.

10. Diamandis,E.P. and Christopoulos,T.K. (1990) *Anal. Chem.*, **62**, 1149A-1157A.

11. Messeri,G. (1998) *Am. Clin. Lab.*, **17**, 6-8.

12. Weeks,I. and Woodhead,J.S. (1984) *J. Clin. Immunoassay*, **7**, 82-89.

13. Engvall,E. and Perlmann,P. (1971) *J. Immunol. Methods*, **10**, 161-170.

14. Van Weeman,B.K. and Schuurs,A.H.W.M. (1971) *FEBS Lett.*, **15**, 232-236.

15. Diamandis,E.P. and Christopoulos,T.K. (1991) *Clin. Chem.*, **37**, 625-636.

16. Rongen,H.A.H., Bult,A. and van Bennekom,W.P. (1997) *J. Immunol. Methods*, **204**, 105-133.

17. Christopoulos,T.K. and Chiu,N.H. (1995) *Anal. Chem.*, **67**, 4290-4294.

18. Killeen,A.A. (1997) *Clin. Lab. Med.*, **17**, 1-19.

19. Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503-517.

20. Kuhns,M.C., McNamara,A.L., Perrillo,R.P., Cabal,C.M. and Campbel,C.R. (1989) *J. Med. Virol.*, **27**, 274-281.

21. Mantero,G., Zonaro,A., Albertini,A., Bertolo,P. and Primi,D. (1991) *Clin. Chem.*, **37**, 422-429.

22. Saiki,R.K., Scharf,S., Faloona,F.A., Mullis,K.B., Horn,C.T., Erlich,H.A. and Arnheim,N. (1985) *Science*, **230**, 1350-1354.

23. van Gemen,B., Kievits,T., Schukkink,R., van Strijp,D., Malek,L.T., Sooknanan,R, Huisman,H.G. and Lens,P. (1993) *J. Virol. Meth.*, **43**, 177-188.

24. Morré,S.A., Sillekens,P., Jacobs,M.V., van Aarle,P., de Blok,S., van Gemen,B., Walboomers,J.M.M., Meijer,C.J.L.M., and van den Brule,A.J.C. (1996) *J. Clin. Microbiol.*, **34**, 3108-3114.

25. Nycz,C.M., Dean,C.H., and Haaland,P.D. (1998) *Anal. Biochem.*, **259**, 226-234.

26. Spargo,C.A., Praiser,M.F., Van Cleve,M., Wright,D.J., Nycz,C.M., Spears,P.A. and Walker,G.T. (1996) *Mol. Cell. Probes*, **10**, 247-256.

27. Chu,B.C.F., Kramer,F.R. and Orgel,L.E. (1986) *Nucleic Acids Res.*, **14**, 5591-5603.

28. Lizardi,P.M., Huang,W., Zhu,Z., Bray-Ward,P, Thomas,D.C. and Ward,D.C. (1998) *Nature Genetics*, **19**, 225-232.

29. Kricka,L.J. (1999) *Clin. Chem.*, **45**, 453-458.

30. Bortolin,S. and Christopoulos,T.K. (1995) *Clin. Chem.*, **41**, 693-699.

31. Hurskainen,P., Dahlen,P., Ylikoski,J., Kwiatkowski,M., Siitari,H. and Lövgren,T. (1991) *Nucleic Acids. Res.*, **19**, 1057-1061.

32. Lindbladh,C., Mosbach,K. and Bulow,L. (1991) *J. Immunol. Methods*, **137**, 199-207.

33. Tatsumi,H., Fukuda,S., Kikuchi,M. and Koyama,Y. (1998) In Hastings,J.W., Kricka,L.J. and Stanley,P.E. (eds.) *Bioluminescence and chemiluminescence: molecular reporting with photons*, Wiley, Chichester, UK, pp. 232-235.

34. Kajita,Y., Iwai,T., Ikariyama,Y. and Aizawa,M. (1995) *J. Biotechnol.*, **43**, 63-70.

35. Rule,G.S., Montagna,R. A. and Durst,R.A. (1996) *Clin. Chem.*, **42**, 1206-1209.

36. Kern,D., Collins,M., Fultz,T., Detmer,J., Hamren,S., Peterkin,J.J., Sheridan,P, Urdea,M.S., White,R., Yeghiazarian,T., and Todd,J. (1996) *J. Clin. Microbiol.*, **34**, 3196-3202.

37. Collins,M.L., Irvine,B., Tyner,D., Fine,E., Zayati,C., Chang,C., Horn,T., Ahle,D., Detmer,J., Shen,L-P., Kolberg,J., Bushnell,S., Urdea,M.S. and Ho,D.D. (1997) *Nucleic Acids Res.*, **25**, 2979-2984.

38. Chiu,N.H.L., Christopoulos,T.K. (1996) *Anal. Chem.*, **68**, 2304-2308.

39. Laios,E., Ioannou,P.C. and Christopoulos,T.K. (1998) *Clin. Biochem.*, **31**, 151-158.

40. Zubay,G. (1973) *Annu. Rev. Genet.*, **7**, 267-287.

41. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

42. Köhrer,C., Mayer,C., Gröbner,P. and Piendl,W. (1996) *Eur. J. Biochem.*, **236**, 234-239.

43. He,M. and Taussig,M.J. (1997) *Nucleic Acids Res.*, **25**, 5132-5134.

44. Wallenfels,K., and Weil,R. (1972) In Boyer, P.D. (ed.) *The Enzymes*, 3rd edn., vol. 7, Academic Press, New York, NY, pp. 617-663.

45. Jain,V.K. and Magrath,I.T. (1991) *Anal. Biochem.* **199**, 119-124.

46. Fowler,A.V., and Zabin,I. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 1507-1510.

47. Jacobson,R.H., Zhang,X-J., DuBose,R.F., and Matthews,B.W. (1994) *Nature*, **369**, 761-766.

48. Langley,K.E., Villarejo,M.R., Fowler,A.V., Zamenhof,P.J., and Zabin,I. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 1254-1257.

49. Langley,K.E., and Zabin,I. (1976) *Biochemistry*, **15**, 4866-4875.

50. Zabin,I. (1982) *Mol. Cell. Biochem.*, **49**, 87-96.

51. Langley,K.E. and Zabin,I. (1976) *Biochemistry*, **15**, 4866-4875.

52. Nichtl,A., Buchner,J., Jaenicke,R., Rudolph,R., and Scheibel,T. (1998) *J. Mol. Biol.*, **282**, 1083-1091.

53. Lin,S. and Zabin,I. (1972) *J. Biol. Chem.*, **247**, 2205-2211.

54. Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103-119.

55. Wu,W. and Welsh,M.J. (1995) *Anal. Biochem.*, **229**, 350-352.

56. Imagawa,M., Hashida,S., Ohta,Y. and Ishikawa,E. (1984) *Ann. Clin. Biochem.*, **21**, 310-317.

57. deBoer,H.A., Comstock,L.J. and Vasser,M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 21-25.

58. Nevin,D.E. and Pratt,J.M. (1991) *FEBS Lett.*, **291**, 259-263.

59. Köhrer,C., Mayer,C., Gröbner,P. and Piendl,W. (1996) *Eur. J. Biochem.*, **236**, 234-239.

60. Kim,D-M., Kigawa,T., Choi,C-Y. and Yokoyama, S. (1996) *Eur. J. Biochem.*, **239**, 881-886.

61. Kozak,M. (1986) *Cell*, **44**, 283-292.

62. Struhl, K. (1989) In Ausubel, R.M.,Brent,R.,Kingston,R.E., Moore,D.D.,Seidman,J.G., Smith,J.A. and Struhl,K.J. (eds.) *Current protocols in Molecular Biology*, Suppl. 8, Greene/Wiley-Interscience,New York, NY, pp. 10.17.1-10.17.5.

63. Pelletier,J. and Sonenbert,N. (1985) *Cell*, **40**, 515-526.

64. Browning,K.S., Lax,S.R., Humphreys,J., Ravel,J.M., Jobling,S.A. and Gehrke,L. (1988) *J. Biol. Chem.*, **263**, 9630-9634.

65. Liarakos,C.D., Theus,S.A., Watson,A.S., Wahba,A.J. and Dholakia,J.D. (1994) *Arch. Biochem Biophys.*, **315**, 54-59.

66. Jobling,S.A. and Gehrke,L. (1987) *Nature*, **325**, 622-625.

67. Kain,K.C., Orlandi,P.A. and Lanar,D.E. (1991) *BioTechniques*, **10**, 366-374.

68. Lanar,D.E. and Kain,K.C. (1994) *PCR Methods and Applications*, **4**, S92-S96.

69. Elroy-Stein,O., Fuerst,T.R. and Moss,B. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6126-6130.

70. Duke,G.M., Hoffman,M.A. and Palmenberg,A.C. (1992) *J. Virol.*, **66**, 1602-1609.

71. Richards,F.M. and Vithayathil,P.J. (1959) *J. Biol. Chem.*, **234**, 1459-1465.

72. Richards,F.M. (1992) *Protein Sci.*, **1**, 1721-1730.

73. Zimmerman,S.B. and Sundeen,G. (1965) *Anal. Biochem.*, **10**, 444-449.

74. DeVries,J.K. and Zubay,G. (1969) *J. Bacteriol.*, **97**, 1419-1425.

75. Pratt, J.M. (1984) In Hames, B.D. and Higgins, S.J. (eds) *Transcription and Translation: A Practical Approach*, IRL Press, Oxford, UK, pp.179-209.

76. Gesteland,R.F. (1966) *J. Mol. Biol.*, **16**, 67-84.

77. Reiner,A.M. (1969) *J. Bacteriol.*, **97**, 1522-1523.

78. Stansfield,I., Jones,K.M. and Tuite,M.F. (1995) *Trends Biol. Sci.*, **20**, 489-491.

79. Baklanov,M.M., Golikova,L.N. and Malygin,E.G. (1996) *Nucleic Acids Res.*, **24**, 3659-3660.

80. Jackson, R.J. and Standart, N. (1990) *Cell*, **62**, 15-24.

81. Munroe, D. and Jacobson, A. (1990) *Gene*, **91**, 151-158.

82. Moosmann,P. and Rusconi,S. (1996) *Nucleic Acids. Res.*, **24**, 1171-1172.

83. Chattopadhyay,S., Pal,S., Pal,D., Sarkar,D., Chandra,S. and Das Gupta,C. (1999), *Biochim. Biophys. Acta*, **1429**, 293-298.

84. Shimomura,O. and Johnson,F.H. (1975) *Nature*, **256**, 236-238.

85. Shimomura,O. and Johnson,F.H. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2611-2615.

86. Shimomura,O. (1995) *Biochem. Biophys. Res. Commun.*, **211**, 359-363.

87. Shimomura,O., Kishi,Y. and Inouye,S. (1993) *Biochem. J.*, **296**, 549-551.

88. Ashley,C.C. and Ridgway,E.B. (1968) *Nature*, **219**, 1168-1169.

89. Inouye,S., Noguchi,M., Sakaki,Y., Takagi,Y., Miyata,T., Iwanaga,S., Miyata,T. and Tsuji,F.I. (1985) *Proc. Natl., Acad., Sci. USA*, **82**, 3154-3158.

90. Badminton,M.N., Sala-Newby,G.B., Kendall,J.M. and Campbell,A.K. (1995) *Biochem. Biophy. Res. Commun.*, **217**, 950-957.

91. Nakahashi,Y., Nelson,E., Fagan,K., Gonzales,E., Guillou,J-L. and Cooper,D.M.F. (1997) *J. Biol. Chem.*, **272**, 18093-18097.

92. Inouye,S., Sakaki,Y., Goto,T. and Tsuji,F.I. (1986) *Biochemistry*, **25**, 8425-8429.

93. Inouye,S., Aoyama,S., Miyata,T., Tsuji,F.I. and Sakaki,Y. (1989) *J. Biochem.*, **105**, 473-477.

94. Stults,N.l., Stocks,N.F., Rivera,H., Gray,J., McCann,R.O., O'Cane,D., Cummings,R.D., Cormier,M.J. and Smith,D.F. (1992) *Biochemistry*, **31**, 1433-1442.

95. Shimomura,O and Inouye,S. (1999) *Protein Exp. Purif.*, **16**, 91-95.

96. Lewis,J.C., Feltus,A., Ensor,C.M., Ramanathan,S. and Daunert,S. (1998) *Anal. Chem.*, **70**, 579A-585A.

97. Witkowski,A., Ramanathan,S. and Daunert,S. (1994) *Anal. Chem.*, **66**, 1837-1840.

98. Ramanathan,S., Lewis,J.C., Kindy,M.S. and Daunert,S. (1998) *Anal. Chim. Acta*, **369**, 181-188.

99. Erikaku,T., Zenno,S. and Inouye,S. (1991) *Biochem. Biophys. Res. Commun.*, **174**, 1331-1336.

100. Galvan,B. and Christopoulos,T.K. (1996) *Anal. Chem.*, **68**, 3545-3550.

101. Verhaegen,M. and Christopoulos,T.K. (1998) *Anal. Chem.*, **70**, 4120-4125.

102. Galvan,B. and Christopoulos,T.K. (1997) *Clin. Biochem.*, **30**, 391-397.

103. Titus,D.E. (1991) *Promega Protocols and Applications Guide*, 2nd ed.; Promega Corp., Madison, WI.

104. Deng,G-R. and Wu,R. (1983) *Methods Enzymol.*, **100**, 96-116.

105. Watkins,N.J. and Campbell,A.K. (1993) *Biochem. J.*, **293**, 37-43.

106. Teranishi,K. and Shimomura,O. (1997) *Anal. Biochem.*, **249**, 37-43.

107. Mackow,E.R., Yamanaka,M.Y., Dang,M.N. and Greenberg,H.B. (1990), *Proc. Natl. Acad. Sci. USA*, **87**, 518-522.

108. Kain,K.C., Orlandi,P.A. and Lanar,D.E. (1991) *BioTechniques*, **10**, 366-374.

109. Resto,E., Iida,A., Van Cleve,M.D. and Hecht,S.M. (1992) *Nucleic Acids Res.*, **20**, 5979-5983.

110. Lanar,D.E. and Kain,K.C. (1994) *PCR Methods Applic.*, **4**, 592-596.

111. Martemyanov,K.A., Spirin,A.S. and Gudkov,A.T. (1997) *FEBS Lett.*, **414**, 268-270.

112. Ohuchi,S., Nakano,H. and Yamane,T. (1998) *Nucleic Acids Res.*, **26**, 4339-4346.

113. Makeyev,E.V., Kolb,V.A. and Spirin,A.S. (1999) *FEBS Lett.*, **444**, 177-180.

114. Hanes,J. and Plückthun,A. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 4937-4942.

115. Nemoto,N., Miyamoto-Sato,E., Husimi,Y. and Yanagawa,H. (1997) *FEB Lett.*, **414**, 405-408.

116. Roberts,R.W. and Szostak,J.W. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 12297-12302.

117. Compton,J. (1991) *Nature*, **350**, 91-92.

118. Kievits,T., van Gemen,B., van Strijp,D., Schukkink,R., Dircks,M., Adriaanse,H., Malek,L., Sooknanan,R. and Lens,P. (1991) *J. Virolog. Methods*, **35**, 273-286.

119. Fahy,E., Kwoh,D.Y. and Gingerase,T.R. (1991) *PCR Methods Applic.*, **1**, 25-33.

120. Fahy,E., Biery,M., Goulden,M., Ghosh,S.S. and Gingeras,T.R. (1994) *PCR Methods Applic.*, **3**, S83-S94.

121. Joyce,G.F. (1993) *Pure Appl. Chem.*, **65**, 1205-1212.

122. White,S.R. and Christopoulos,T.K. (1999) *Nucleic Acids Res.*, **0**, 00-00.

123. Chamberlain,M. and Ring,J. (1973) *J. Biol. Chem.*, **248**, 2245-2250.

124. Golom,M. and Grandegenett,D.D. (1979) *J. Biol. Chem.*, **254**, 1606-1613

125. Malek,L., Sooknanan,R. and Compton,J. (1994) *in Methods in Molecular Biology*, **28**, 253-260. Ed. P.G. Isaac, Humana Press Inc. Totowa, NJ.

126. Gold,L., Polisky,B., Uhlenbeck,O. and Yarus,M. (1995) *Annu. Rev. Biochem.*, **64**, 763-797.

127. Wright,M.C. and Joyce,G.F (1997) *Science*, **276**, 614-617.

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- S.R. White, N.H. Chui, T.K. Christopoulos, **Expression Immunoassay**, *Methods: A Companion to Methods in Enzymology*, 0, 00-00 (1999).
- S.R. White, N.H. Chui, T.K. Christopoulos, **Expression Immunoassay Based on Antibodies Labeled with a DNA Fragment Encoding the α-Peptide of β-Galactosidase**, *Analyst*, 123, 1309 - 1314 (1998).
- R.D. Dinnen, S.R. White, S. Elsayed, Y.I. Yeh, K. Ebisuzaki, **An Endogenous Signal Triggering Erythroid Differentiation - Identification as Thyroid Hormone**, *Cell Growth Diff.*, **5**, 855 - 861 (1994).

PRESENTATIONS

- T.K. Christopoulos, S.R. White, **Highly Sensitive Bioluminescence Hybridization Assays Based on *in vitro* Expression of DNA Reporter Molecules Encoding Apoaequorin**, *International Federation of Clinical Chemistry Annual Conference*, Florence, Italy, June 1999. Poster.
- S.R. White, T.K. Christopoulos, **Highly Sensitive Bioluminescence Hybridization Assays Based on *in vitro* Expression of DNA Reporter Molecules Encoding Apoaequorin**, *Annual San Diego Conference*, San Diego, California, USA, November 19-21, 1998. Poster.
- S.R. White, **A Sensitive Hybridization Assay Using an Apoaequorin Encoding DNA as a Label**, *Research and Education Forum by the Michigan Section of the American Association for Clinical Chemistry*, Ypsilanti, Michigan, USA, May, 1998. Talk.
- S.R. White, T.K. Christopoulos, **Expression Immunoassay using a DNA Encoding the α-Fragment of β-Galactosidase as a Label**, *Annual Meeting of the Canadian Society of Chemistry*, Windsor, Ontario, Canada, June 1 - 4, 1997. Talk.
- S.R. White, **Expression Immunoassay using a DNA Encoding the α-Fragment of β-Galactosidase as a Label**, *Research and Education Forum by the Michigan Section of the American Association for Clinical Chemistry*, Ypsilanti, Michigan, USA, May 21, 1997. Talk.
- S.R. White, T.K. Christopoulos, **Expression Immunoassay using a DNA Encoding the α-Fragment of β-Galactosidase as a Label**, *Joint Annual Meetings of the American Association for the American Chemical Society and the Canadian Society of Clinical Chemists*, Chicago, Illinois, USA, July 28 - August 1, 1996. Poster.

- S.R. White, **Preparation of a Eu^{3+}-Diethylenetriaaminepentaacetic Acid Derivative of dATP and its Application in Time Resolved Fluorometric Hybridization Assays,** *Research and Education Forum by the Michigan Section of the American Association for Clinical Chemistry,* Ypsilanti, Michigan, USA, May 25, 1995. Talk.