Photoproteins in Bioanalysis

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5
Photoproteins in Nucleic Acid Analysis

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5.1
Hybridization Assays

The highly specific and strong interaction between two complementary nucleic acid strands forms the basis for the development of hybridization assays. Nucleic acid hybridization has become a fundamental analytical technique for the detection and quantification of specific DNA or RNA sequences and is used extensively in research and diagnostics in laboratories. Major areas of application of hybridization assays include the detection of nucleic acid sequences that are related to neoplastic disease; the detection and/or determination of various pathogens in clinical, environmental, and food samples; the detection of mutations associated with disease; the analysis of chromosomal rearrangements associated with neoplasias; the detection of genetically modified organisms; and DNA fingerprinting.

DNA or RNA probes labeled with radioisotopes (^{32}P, ^{35}S or ^{3}H) in combination with autoradiography dominated the field of hybridization assays for more than two decades. The classical methodology for nucleic acid analysis includes electrophoretic separation, transfer to a suitable membrane (Southern or Northern transfer), and hybridization with radioactive probes. However, the health hazards and problems associated with the stability, use, and disposal of radioisotopes and the long exposure times (hours to days) required for detection by autoradiography have placed limitations on the routine use of hybridization assays. In recent years, nucleic acid analysis by hybridization has undergone a transition from radioactive labels to non-radioactive alternatives, which was driven by the need to improve detectability and facilitate automation and high-throughput analysis while avoiding the aforementioned limitations.

Bioluminometric hybridization assays [1–4] that use photoproteins as reporters offer higher detectability and wider dynamic range than spectrophotometric and fluorometric methods. This is due to the fact that in bioluminescence the excited species is formed during the course of a chemical reaction (an oxidation reaction). Consequently, bioluminometric measurements do not require excitation light,

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thereby avoiding the problems arising from the scattering of excitation radiation, fluorescence from other components of the sample, and photobleaching.

The photoprotein aequorin is an excellent reporter molecule because it can be detected down to the 10^{-18} mole level (1 atto mol) by the simple addition of excess Ca^{2+}. Furthermore, the reaction is completed within 3 s, a significant advantage over enzyme reporters (such as alkaline phosphatase) that require long incubation times.

The applications of hybridization assays that use a photoprotein as a reporter focus on the determination of nucleic acid sequences amplified by the polymerase chain reaction (PCR) or other exponential amplification techniques [5, 6]. PCR is a powerful technique for the *in vitro* exponential amplification of specific DNA sequences to levels that are several orders of magnitude higher than those in the starting material. PCR entails denaturation of the sample DNA, hybridization (annealing) of two oligodeoxynucleotide primers that flank the region of interest, and polymerization using a thermostable DNA polymerase. After repetitive cycles of denaturation, primer annealing, and enzymic extension, the DNA segment defined by the two primers is selectively amplified. Exponential amplification of specific RNA sequences can be achieved by first generating a complementary copy of DNA with reverse transcriptase.

Figure 5.1 presents general hybridization assay configurations for detection/quantification of nucleic acid sequences in a high-throughput format using photoproteins as reporters. In the first approach ("immobilized target" assay), the target DNA or RNA is immobilized on the appropriate solid surface (e.g., a microtiter well or beads), the one strand is removed by treating with NaOH, and the immobilized strand is hybridized with a specific probe that is linked to the photoprotein. In the second method ("immobilized probe" configuration), the target DNA is denatured and hybridized with a probe that is immobilized on the solid surface. The hybridized target is then linked with the photoprotein reporter. In the third configuration ("sandwich-type" assay), the target DNA is denatured and allowed to hybridize with two probes. One probe is immobilized on the solid surface and the other is linked with the photoprotein reporter. The

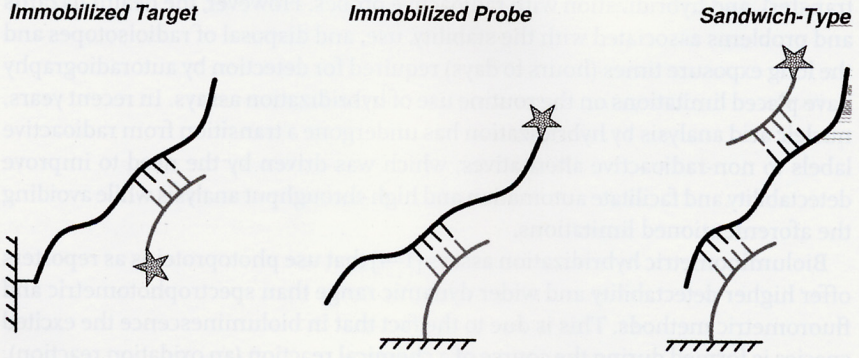

Fig. 5.1 Configurations of hybridization assays that use bioluminescent proteins as reporters.

third configuration offers higher specificity because two probes must hybridize in order to get a signal. The detectability of the hybridization assay is determined mainly by two factors: the detectability of the reporter molecule and the nonspecific binding of the detection reagents to the solid phase.

The immobilization of the target in the "immobilized target" assay is usually based on the strong and specific interaction between biotin and (strept)avidin. Polystyrene microtiter wells and polystyrene beads (including magnetic beads) are easily coated with (strept)avidin. The target DNA may be labeled through the polymerase chain reaction by using a primer biotinylated at the 5′ end or by incorporating biotin-modified dNTPs. Alternatively, the target DNA may be labeled with a hapten (e.g., digoxigenin) through PCR and captured by an anti-hapten antibody that is immobilized on the solid phase. Capture of the target DNA by a streptavidin-coated surface, however, offers the advantage that the binding withstands the NaOH treatment used for the removal of one strand. When an antibody is used for capture, the target is first heat-denatured and then added to the well.

The immobilization of the probe on the solid surface may also be accomplished by using the biotin/streptavidin or the hapten/anti-hapten antibody interaction. Oligonucleotide probes can be labeled with biotin or a hapten either during synthesis or by using the enzyme terminal deoxynucleotidyl transferase, a DNA polymerase that adds dNTPs and modified dNTPs to the 3′ end of any DNA molecule without the need of a template. Alternatively, the probe can be attached (chemically) to bovine serum albumin (BSA) and the conjugate used for coating of the polystyrene surface by physical adsorption. The probe also may be immobilized by covalent attachment to the solid surface.

The linking of a photoprotein to the hybrids is carried out either by direct covalent attachment of the photoprotein to the probe or by noncovalent bridging (with the probe or the target sequence) through biotin–streptavidin or hapten–antibody interaction as described above.

The detectabilities achieved with the above bioluminometric hybridization assays are in the low pmol range of target DNA (concentration of target DNA in the well), whereas fluorescent labels offer detectabilities in the nmol range. As a consequence, fewer PCR cycles are required for bioluminometric detection of the amplification product, and the possibility of sample contamination from amplified DNA is much lower.

The detectability of the aequorin-based bioluminometric hybridization assays can be enhanced by introducing (enzymically) multiple aequorin labels per DNA hybrid [7]. Heat-denatured DNA target is hybridized in microtiter wells with an immobilized capture probe and a digoxigenin-labeled detection probe. The hybrids react with anti-digoxigenin antibody conjugated to horseradish peroxidase. Peroxidase catalyzes the oxidation of a digoxigenin–tyramine conjugate by H_2O_2, resulting in the attachment of multiple digoxigenin moieties to the solid phase through the tyramine group, whereas the digoxigenin remains exposed. Aequorin-labeled anti-digoxigenin antibody is then allowed to bind to the immobilized haptens. The bound aequorin is determined by adding excess Ca^{2+}. The enzyme

amplification improves the detectability about 8–10 times compared to the assay that does not involve a peroxidase amplification step.

5.2 Quantitative Polymerase Chain Reaction

The exponential increase of the amplification product during PCR poses serious difficulties in the application of PCR as a quantitative method for determination of the starting quantity of target DNA. Quantification requires the establishment of a reproducible relationship between the analytical signal obtained from the amplification product and the number of target DNA molecules in the sample prior to amplification. The amount (P) of product accumulated after n cycles of exponential amplification is given by the equation

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

where T is the initial amount of target DNA and E is the average efficiency of the reaction for each cycle. The theoretical value of E is 1, i.e., the product doubles in each cycle. In reality, however, E has a smaller value depending on the reaction conditions and the nature of the sample. Variations in factors such as the concentration of polymerase, primers, dNTPs, Mg^{2+}, and cycling parameters (temperatures and times) affect the efficiency of amplification. In addition, the incorporation of primers into undesirable products leads to a decrease in the PCR yield. Furthermore, as the PCR enters a plateau phase at a high number of cycles (depending on the amount of starting template), there is a decrease in PCR efficiency, as a result of substrate saturation of the DNA polymerase and competition between strand reannealing and primer binding, as the concentration of amplified DNA increases.

A consequence of the exponential amplification is that small reaction-to-reaction variations in the efficiency have a profound effect on the quantity of the PCR product. For instance, a 5% decrease in E, from 1 to 0.95, results in a 50% decrease of the product (for $n = 25$).

One approach to quantitative PCR (QPCR) entails the co-amplification, in the same tube, of the target DNA sequence with a competitive synthetic DNA internal standard (DNA competitor) that closely resembles the target DNA [6, 8]. The internal standard (IS) uses the same primers as the target DNA and contains an insertion or deletion large enough to allow separation from the target by gel electrophoresis. Each sample is titrated with the IS. This is accomplished by adding increasing and known amounts of the IS to aliquots of the sample containing a constant amount of target DNA followed by PCR and electrophoresis. The equivalence point is determined either by inspection of the gel (same band intensities for target and IS) or, more accurately, by densitometric analysis, which should take into account the effect of the length of the DNA on the intensity of the bands. The use of an IS allows for compensation of the fluctuations of the

amplification efficiency. Also, any PCR inhibitors present in the sample will equally affect the amplification of target DNA and IS so that the ratio of the amounts of their PCR products gives the ratio of the initial amounts of the two sequences in the sample. The main disadvantage of competitive PCR is the low throughput, which is a result of the multiple amplification reactions required for titration of each sample, followed by gel electrophoresis and densitometry.

Another approach to QPCR is based on the continuous monitoring of product accumulation by a homogeneous fluorometric hybridization assay (real-time PCR) with detectability at the nanomolar range. The cycle at which the fluorescence signal attains a certain preset threshold value is inversely related to the starting amount of target DNA. The amplification products are measured at the beginning of the exponential phase [6, 9]. Quantification is based on external calibration curves constructed by serial dilutions of the target. Real-time PCR methods do not employ internal standards because a DNA competitor would suppress the yield of amplified target to levels that may be undetectable [10].

The high detectability (in the low pmol concentrations) of bioluminometric hybridization assays that employ photoproteins as reporters allows accurate and precise determination of the PCR products of target and competitor DNA, despite the suppression of amplification caused by their competition for the same primer set. This enables quantification of the target sequence without the need for titration of each sample with various quantities of competitor. Instead, the target DNA is co-amplified with a constant amount of competitor. Furthermore, bioluminometric hybridization assays of the PCR products are performed in microtiter wells, thus ensuring simplicity and high throughput. A typical hybridization assay configuration used in bioluminometric quantitative PCR [11, 12] is shown in Fig. 5.2. The microtiter wells are coated with a BSA–$(dT)_{30}$ conjugate. The oligonucleotide probes consist of a segment complementary to

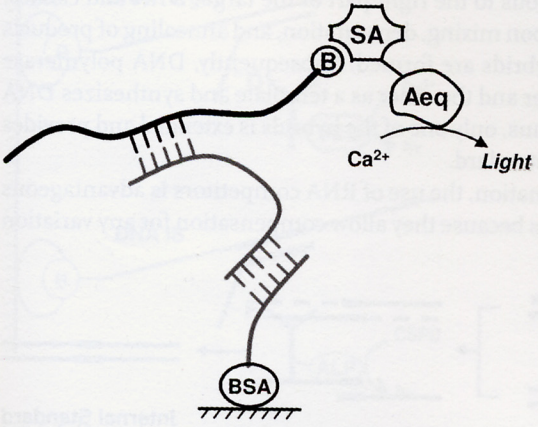

Fig. 5.2 Assay configuration for bioluminometric quantitative competitive polymerase chain reaction.
BSA: bovine serum albumin; B: biotin; SA: streptavidin; Aeq: aequorin.

the target or internal standard and a poly(dA) tail. The probes hybridize to both the biotinylated denatured PCR product and the immobilized $(dT)_{30}$ strands. A streptavidin–aequorin conjugate is used for determination of the captured hybrids. The ratio of the signals for target and internal standard is a linear function of the number of DNA molecules in the sample. The overall procedure including PCR and hybridization is complete in 2.5 h. Coating of the wells with BSA–$(dT)_{30}$ provides a universal solid phase for capturing both the target and IS.

Real-time PCR offers simplicity and automation but requires specialized and expensive equipment and reagents. The photoprotein-based quantitative PCR methods are endpoint assays (post-PCR detection) that use DNA competitors and are performed in a high-throughput format. The cost of the reagents and equipment are considerably lower than real-time PCR.

Contrary to competitive quantitative PCR methods that are based on electrophoretic separation of the amplification products, in bioluminometric quantitative PCR methods the DNA competitor is identical in size to the target sequence but is distinguishable by hybridization because it differs in a short (usually 20–25 bp) internal segment. DNA competitors may be prepared either by using appropriate vectors and standard cloning procedures or by faster and simpler approaches that employ PCR as a synthetic tool. The latter employs the target DNA sequence as a starting template and generates two short and overlapping DNA fragments through PCR. Subsequently, the two fragments are subjected to a PCR-like joining reaction to create the sequence of the DNA competitor. The procedure is illustrated in Fig. 5.3.

PCR-A and PCR-B are performed using primer sets a1, a2, and b1, b2. The downstream primer a2 and the upstream primer b1 carry extensions at the 5′ end that are complementary to each other and represent the new sequence that will be introduced into the DNA competitor. PCR-A gives a product that is identical to the left part of the target sequence and carries a new extension downstream. The product of PCR-B is homologous to the right part of the target DNA and carries, upstream, a new extension. Upon mixing, denaturation, and annealing of products A and B, two new types of hybrids are formed. Subsequently, DNA polymerase uses the one strand as a primer and the other as a template and synthesizes DNA in the 5′ to 3′ direction only. Thus, only one of the hybrids is extended and provides the sequence of the internal standard.

In the case of RNA determination, the use of RNA competitors is advantageous compared to DNA competitors because they allow compensation for any variation

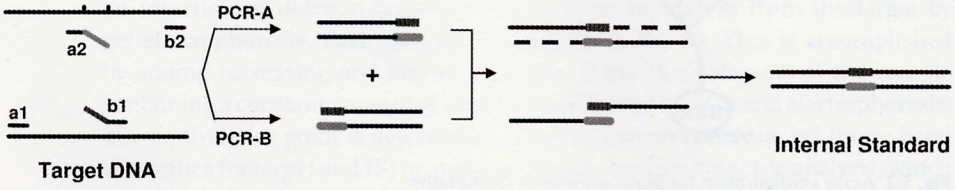

Fig. 5.3 Outline of the reactions used for the synthesis of a DNA internal standard (competitor) for quantitative PCR. Reprinted with permission from ref. 12.

in the efficiency of both the reverse transcription and the polymerase chain reaction. The RNA competitor is prepared by first synthesizing a DNA internal standard as described above and then introducing the T7 promoter. This is accomplished by subjecting the DNA competitor to PCR amplification using a primer with a T7 promoter sequence at its 5′ end. The DNA fragment is transcribed, *in vitro*, by T7 RNA polymerase to produce the RNA competitor [13].

Automation and throughput of quantitative PCR can be further enhanced by exploiting the variation in the kinetics of light emission from bio(chemi)luminescent reactions, thus allowing the development of dual hybridization assays for determination of target DNA/RNA and DNA/RNA competitor in the same reaction vessel (e.g., microtiter well) [11]. A rapid flash of light is generated from the aequorin reaction with a decay half-life of about 1 s, whereas a much slower emission (glow-type) that lasts from minutes to hours is produced by enzyme-catalyzed chemiluminescent reactions. Figure 5.4 presents the configuration of a dual-analyte assay for target and competitor that uses both aequorin and alkaline phosphatase as reporters. The two biotinylated PCR products from target DNA and IS are captured on a single microtiter well coated with streptavidin. The non-biotinylated strand is dissociated with NaOH and removed by washing. The immobilized single-stranded target DNA and IS hybridize simultaneously with their corresponding probes. The target- and IS-specific probes are labeled with the haptens digoxigenin and fluorescein, respectively. A solution containing aequorin-labeled anti-digoxigenin antibody and alkaline phosphatase-labeled anti-fluorescein antibody is added to the well and allowed to bind to the corresponding haptens. The excess of reagents is removed and the aequorin reaction is triggered with Ca^{2+}. The signal from the aequorin reaction is integrated for 3 s, followed by the addition of a chemiluminogenic substrate (CSPD) for ALP. The ALP reaction

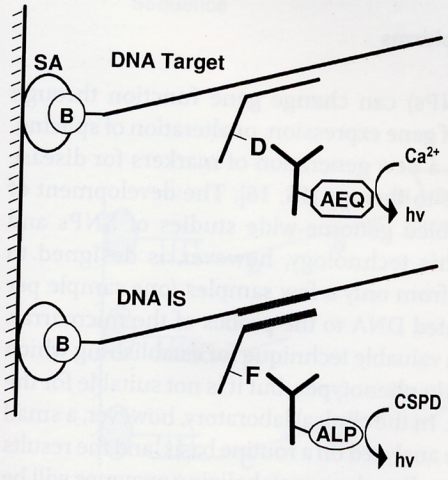

Fig. 5.4 Principle of quantitative competitive PCR based on a dual-analyte bio(chemi)luminometric assay for target and competitor (internal standard).
SA: streptavidin; B: biotin; IS: internal standard; D: digoxigenin;
F: fluorescein; Aeq: aequorin; ALP: alkaline phosphatase.

is allowed to proceed for 20 min followed by integration of the signal for 10 s. The ratio of the luminescence values for aequorin and ALP reactions is a linear function of the initial amount of the DNA (or RNA) in the sample prior to PCR. The linear range extends from 430 to 315 000 target DNA molecules and depends on the number of PCR cycles and the amount of DNA IS.

Besides facilitating high-throughput and automation, bioluminometric quantitative PCR methods eliminate a series of drawbacks of electrophoretic methods (including slab gel electrophoresis and capillary electrophoresis). For example, contrary to electrophoresis, hybridization methods provide sequence confirmation. In addition, it has been reported that PCR efficiency is inversely related to the size of the DNA [14]. This might create problems with electrophoretic methods that depend on size differences. The bioluminometric QPCR, however, employs an IS of a size identical to the target sequence. Finally, because of the high homology of the target and IS sequences, their co-amplification in the same reaction mixture leads to the formation of heteroduplexes, comprising a strand from the target DNA and a strand from the IS. The heteroduplexes usually migrate in a different way than the target and IS, causing errors in the determination of the amplification products, especially if they cannot be resolved from homoduplexes. Heteroduplex formation also may interfere with the digestion of the DNA in those QPCR methods that use competitors differing from the target sequence by a restriction site followed by electrophoresis of the digestion products. Heteroduplex formation is not a concern for the photoprotein-based QPCR methods described above, because they all rely on the denaturation of the amplified DNA and quantification of only one strand (the immobilized one).

5.3 Genotyping of Single-nucleotide Polymorphisms

Single-nucleotide polymorphisms (SNPs) can change gene function through amino acid substitution, modification of gene expression, or alteration of splicing. In recent years SNPs have emerged as a new generation of markers for disease susceptibility, prognosis, and response to therapy [15, 16]. The development of DNA microarray technology has enabled genome-wide studies of SNPs and correlation with various diseases. This technology, however, is designed to analyze thousands of polymorphisms from only a few samples (one sample per chip) by hybridization of the interrogated DNA to the probes of the microarray. Consequently, the DNA microarray is a valuable technique for establishing which SNPs are strongly associated with certain phenotypes, but it is not suitable for the screening of large numbers of samples. In the clinical laboratory, however, a small number of SNPs for each disease will be analyzed on a routine basis, and the results from disease-related genes or genes encoding drug-metabolizing enzymes will be utilized for disease prevention or for the design of effective therapeutic strategies. In this context, photoproteins may serve as reporters for high-throughput SNP genotyping performed in microtiter wells [17, 18].

The general procedure for SNP genotyping includes (1) the isolation of genomic DNA; (2) amplification, by PCR or other exponential amplification techniques, using primers flanking the locus of interest; (3) a genotyping reaction and; (4) detection of the products. The most commonly used genotyping reactions are the oligonucleotide ligation and the primer extension. The photoprotein aequorin has been used as a reporter in both genotyping reactions.

Figure 5.5 presents the configuration of a dual-analyte assay that combines flash- and glow-type reactions for the detection of the products of the oligonucleotide ligation reaction. The discrimination by DNA ligase against mismatches at the ligation site in two adjacently hybridized oligonucleotides (probes) is the basis for genotyping of SNPs by oligonucleotide ligation. The ligation methods make use of two recognition events between oligonucleotides and their targets, thus allowing these methods the required specificity for allele-specific SNP detection. Following PCR amplification, the DNA is denatured and hybridized simultaneously with three probes. Probes N and M are labeled at the 5′ end with biotin and digoxigenin, respectively. The last nucleotide at the 3′ end of N and M is specific for the normal and mutant allele, respectively. Probes N and M hybridize to the target DNA at a position adjacent to probe C. Upon perfect complementarity, a thermostable ligase joins the adjacent probes, giving two possible products depending on the allele that is present in the sample. If the normal allele is present, then biotinylated N-C is formed. Digoxigenin-labeled M-C is formed when the mutant allele is present. Probe C contains at the 3′ end an extension (about 17 nucleotides) that is irrelevant

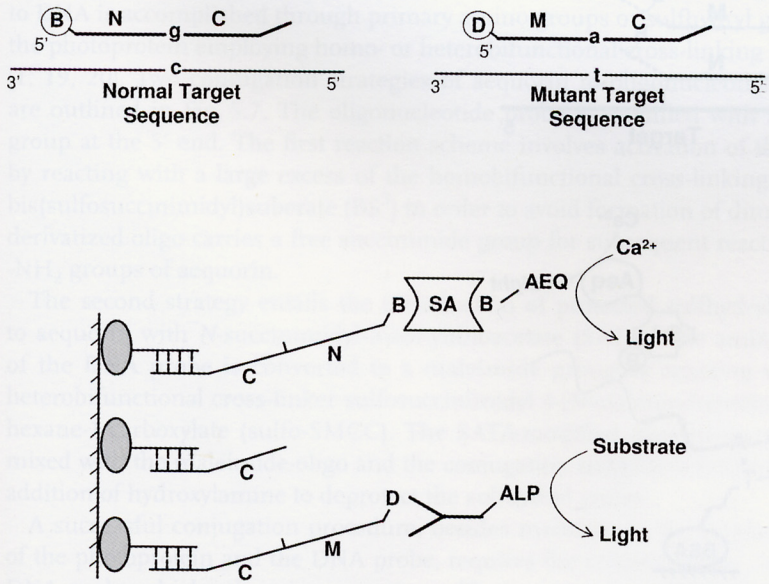

Fig. 5.5 Genotyping of SNPs by the oligonucleotide ligation reaction using the photoprotein aequorin and alkaline phosphatase as reporters. B: biotin; N: normal; M: mutant; C: common; SA: streptavidin; D: digoxigenin. Reprinted with permission from ref. 17.

to the target and enables the capture of the ligation products. Ligation products are heat-denatured and captured on the surface of a microtiter well through hybridization with an immobilized oligonucleotide that is complementary to the characteristic extension of the C probe. Aequorin-labeled streptavidin is added for detection of the N-C (normal allele) and ALP–anti-digoxigenin conjugate is used for detection of the mutant allele (M-C). The ratio of the luminescence signals obtained from aequorin and ALP gives the genotype for each sample. The microtiter well assay format is highly automatable and enables high-throughput genotyping of a large number of samples.

The principle of a bioluminometric assay that is based on a primer extension (PEXT) genotyping reaction and uses aequorin as a reporter is illustrated in Fig. 5.6. The distinction between the genotypes is based on the high accuracy of nucleotide incorporation by DNA polymerase. Two PEXT reactions are performed for each locus. Two allele-specific primers that hybridize with the target DNA adjacent to the mutation and have, at the 3′ end, a nucleotide complementary to the allelic variant are used in PEXT reactions. Only the primer with a perfectly matched 3′ end is extended by a thermostable DNA polymerase. Biotin is incorporated in the extended primer through the use of biotin-dUTP along with the dNTPs. Both genotyping primers N and M have at their 5′ end a $(dA)_{30}$ segment to enable affinity capture of extension products onto microtiter wells coated with a bovine serum albumin (BSA)-$(dT)_{30}$ conjugate. Prior to the bioluminometric assay, PEXT

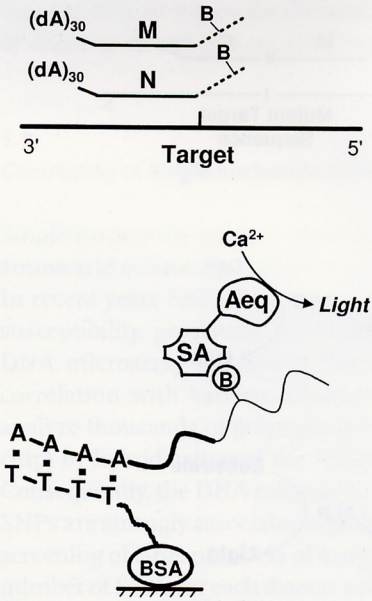

Fig. 5.6 Genotyping of SNPs by the primer extension (PEXT) assay using the photoprotein aequorin as a reporter. N: normal; M: mutant; B: biotin; SA: streptavidin; Aeq: aequorin; BSA: bovine serum albumin. Reprinted with permission from ref. 18.

products are heat-denatured to ensure separation of the extended strand of the genotyping primer from any biotinylated strands generated from extension of PCR primers. This denaturation step eliminates the need for purification of amplified fragments from unincorporated PCR primers. If primer extension has occurred, then the product carries incorporated biotin moieties that are detected by adding a streptavidin–aequorin conjugate. The PEXT reaction is completed in 10 min and the detection of the products takes less than 40 min.

The methods described above have been applied to the genotyping of the beta globin gene and the mannose-binding lectin gene.

5.4
Conjugation of Aequorin to Oligodeoxynucleotide Probes

A crucial step in the development of methods for detection and/or quantification of DNA/RNA is the linking between the recognition molecule (complementary DNA probe) and the reporter molecule (photoprotein). This linkage is carried out "directly" by chemical conjugation or "indirectly". In the indirect approach, a ligand (e.g., biotin or a hapten) is attached to the DNA probe and the hybrids are detected via a specific binding protein (streptavidin or an antibody), which is conjugated or complexed to the photoprotein. The advantage of the direct labeling approach lies in the fact that it eliminates an incubation step and a washing step, thus reducing considerably the assay time. The chemical attachment of aequorin to DNA is accomplished through primary amino groups or sulfhydryl groups of the photoprotein employing homo- or heterobifunctional cross-linking reagents [1, 19, 20]. Two conjugation strategies of aequorin to oligonucleotide probes are outlined in Fig. 5.7. The oligonucleotide probe is modified with an $-NH_2$ group at the 5′ end. The first reaction scheme involves activation of the probe by reacting with a large excess of the homobifunctional cross-linking reagent bis(sulfosuccinimidyl)suberate (BS^3) in order to avoid formation of dimers. The derivatized oligo carries a free succinimide group for subsequent reaction with $-NH_2$ groups of aequorin.

The second strategy entails the introduction of protected sulfhydryl groups to aequorin with *N*-succinimidyl-*S*-acetylthioacetate (SATA). The amino group of the DNA probe is converted to a maleimide group by reacting with the heterobifunctional cross-linker sulfosuccinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate (sulfo-SMCC). The SATA-modified photoprotein is then mixed with the maleimide-oligo and the conjugation reaction is initiated by the addition of hydroxylamine to deprotect the sulfhydryl group.

A successful conjugation procedure, besides maintaining the functionalities of the photoprotein and the DNA probe, requires the removal of the unreacted DNA probe which otherwise competes with the aequorin–DNA conjugate for hybridization to the target sequence and deteriorates the performance of the hybridization assays. The removal of the probe is usually accomplished by laborious chromatographic procedures followed by concentration steps.

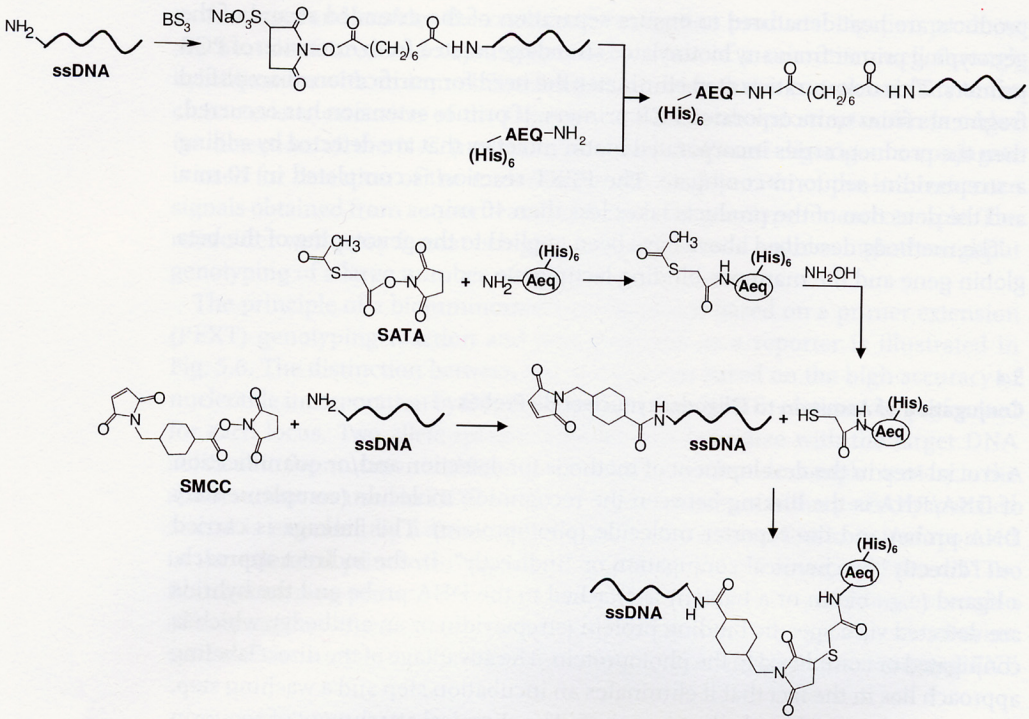

Fig. 5.7 Outline of two strategies used for conjugation of the photoprotein aequorin to oligonucleotide probes. Reprinted with permission from ref. 20.

In order to facilitate both the purification of recombinant aequorin from crude bacterial cultures and the preparation of aequorin conjugates, a suitable plasmid was constructed [21] in which a hexahistidine-coding sequence was fused upstream of the apoaequorin cDNA (Fig. 5.8). The $(His)_6$-apoaequorin fusion protein was overexpressed in *E. coli* under the control of the *tac* promoter. The inclusion bodies were solubilized by treating with 6 M urea, and the $(His)_6$-apoaequorin was purified in a single step by immobilized metal-ion affinity chromatography using a Ni^{2+}-nitrilotriacetate agarose column. Proper refolding of the photoprotein was achieved

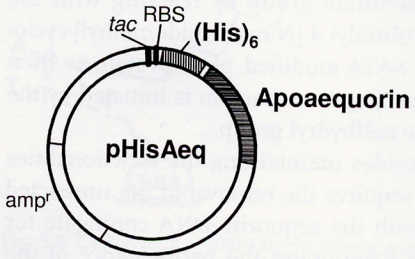

Fig. 5.8 Structure of the plasmid used for bacterial overexpression of recombinant hexahistidine–aequorin fusion protein.

by slow removal of urea using a gradient of 6–0 M. The fusion protein was eluted by imidazole and used for preparation of conjugates with DNA probes. Following the conjugation reactions described above, the $(His)_6$-apoaequorin was captured on a Ni^{2+}-nitrilotriacetate agarose column, the unreacted probe was washed away, and the conjugate was eluted as above. The presence of free aequorin in the conjugate solution does not interfere with the hybridization assay.

5.5 Development of New Recombinant Bioluminescent Reporters

The investigation and exploitation of new bioluminescent proteins as reporters in nucleic acid analysis (as well as in other applications) is an active area of research. The process of development and evaluation of new bioluminescent reporters involves the following steps:

1. isolation and cloning of the cDNA encoding the bioluminescent protein,
2. construction of suitable vectors for large-scale expression of the protein in a heterologous system (e.g., bacteria),
3. development of a purification method for the protein, and
4. linking of the bioluminescent protein to a DNA probe in order to prepare a recognition and detection reagent for nucleic acid analysis.

The luciferase of the marine copepod *Gaussia princeps* (*Gaussia* luciferase, GL) is a typical example of this process [22, 23]. GL is a single polypeptide chain of 185 amino acids (MW 19 900) and catalyzes the oxidative decarboxylation of coelenterazine to produce coelenteramide and light (470 nm). The cloning of the cDNA of GL was accomplished recently. In order to facilitate the purification of the protein and its subsequent use as a reporter in hybridization assays, a suitable vector was constructed (Fig. 5.9) that drives the expression of *in vivo* biotinylated GL in *E. coli*. The plasmid contains the sequence coding for the biotin acceptor peptide (bap) from *Propionibacterium shermanii* transcarboxylase positioned downstream of the *tac* promoter and a ribosome-binding site (RBS). The structure

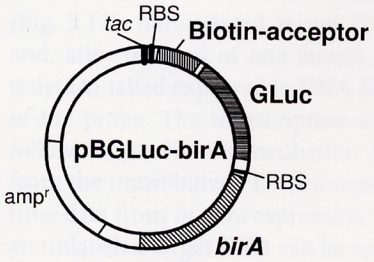

Fig. 5.9 Structure of the plasmid used for overexpression of *in vivo* biotinylated *Gaussia* luciferase in bacteria.

of the biotin domain of *P. shermanii* transcarboxylase is very similar to that of *E. coli* acetyl-CoA carboxylase, which is the physiological substrate of biotin protein ligase (BPL), the enzyme that is responsible for the *in vivo* biotinylation of bap at a unique site. The GL cDNA is positioned downstream of bap. Consequently, the expressed fusion is an *in vivo* biotinylated bap–GL sequence. Because the endogenous activity of BPL was found to be low, the gene birA, which codes for BPL, was also introduced in the same plasmid, thus achieving overexpression of both the BPL and the bap–GL fusion.

The *in vivo* biotinylated bap–GL was purified from the crude cellular extract by affinity chromatography using a monomeric avidin resin. Monomeric avidin has a much lower affinity for biotin ($k_d = 10^{-7}$) than tetrameric avidin ($k_d = 10^{-15}$), thereby allowing the binding of biotinylated GL and subsequent elution with free biotin. Moreover, biotinylation facilitates the linking of GL to streptavidin, avoiding chemical conjugation that may inactivate the protein. The complex of streptavidin (SA) and biotinylated GL (BGL) is prepared by precise optimization of the BGL: SA molar ratio. If BGL is present in excess, then the four biotin-binding sites of SA become saturated. If there is an excess of SA, then free SA competes with the SA-BGL complex for binding to the biotinylated hybrids on the well. The fact that a single biotin moiety is attached to the protein outside of the GL sequence is advantageous over chemical biotinylation methods that may alter functional groups of the luciferase. A typical (model) hybridization assay (Fig. 5.10) involves denaturation of a biotinylated DNA target and hybridization with an immobilized specific probe. The hybrids are detected by the addition of the SA-BGL complex. Coelenterazine is then added as a substrate. BGL is detectable down to 1 amol following light emission integration for 20 s, with linearity extending over 5 orders of magnitude. The hybridization assay has a linearity range of 1.6–800 pM of target DNA.

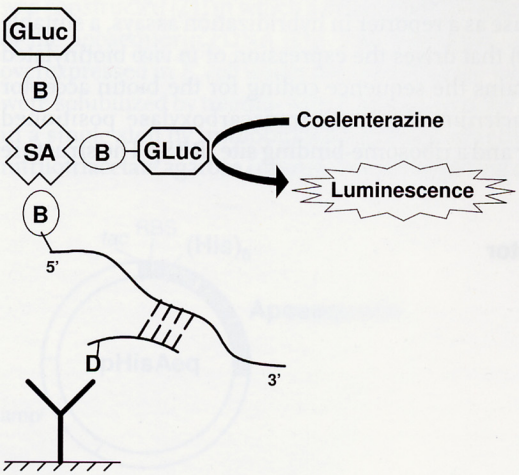

Fig. 5.10 Model hybridization assay using *Gaussia* luciferase as a reporter.
B: biotin; SA: streptavidin; GLuc: *Gaussia* luciferase; D: digoxigenin.

5.6 Signal Amplification by in Vitro Expression of DNA Reporters Encoding Bioluminescent Proteins

Cell-free expression of DNA fragments entails transcription of DNA to RNA followed by translation of RNA to protein. Several RNA molecules are synthesized from each DNA template during transcription, and more than one protein molecule is generated from each transcript. As a consequence, gene expression forms the basis of a signal amplification system. The detectability is further improved if the DNA template encodes a bioluminescent protein. Contrary to previously described hybridization assays in which only one reporter molecule is linked to the hybrid, a photoprotein-coding DNA fragment upon expression generates multiple bioluminescent molecules in solution [24–27].

DNA templates were engineered that contain all necessary elements to enable *in vitro* expression in wheat germ- or rabbit reticulocyte-coupled (one-step) transcription–translation systems under the control of the bacteriophage T7 RNA polymerase. The DNA template contains a T7 promoter, the cDNA of the bioluminescent protein, and a $(dA/dT)_{30}$ extension that enhances translation efficiency by facilitating translation initiation. When apoaequorin cDNA is used as a template, coelenterazine is added to the expression mixture to enable the formation of fully functional aequorin. The *in vitro* expression reaction typically proceeds for 90 min and then Ca^{2+} is added to trigger light emission. The wheat germ extract is preferred for expression of aequorin because the rabbit reticulocyte extract absorbs a significant portion of the emitted light. *In vitro* expression experiments have shown that although the transcription–translation process consists of a series of complex and not completely understood reactions that require the concerted action of numerous factors (RNA polymerase, initiation, elongation and termination factors, ribosomal subunits, aminoacyl-tRNA synthetases, etc.), the final outcome is a simple and reproducible linear relationship between the bioluminescence signal and the amount of the DNA template. As low as 5×10^3 molecules of aequorin DNA are detectable and the linearity extends up to 10^8 molecules. For application to hybridization assays, the DNA template (reporter) must be linked to a specific probe. This is carried out by tailing the template with dATP using terminal deoxynucleotidyl transferase. The probe is tailed with dTTP. In a typical expression hybridization assay configuration (Fig. 5.11), biotinylated target DNA is captured on streptavidin-coated wells and, after removal of one strand by NaOH, is hybridized with the probe. The poly(dA)-tailed expressible DNA fragment is then hybridized to the poly(dT) tail of the probe. The transcription–translation "cocktail" is then added (one step) followed by a 90-min incubation during which the photoprotein is synthesized from the immobilized DNA template. These studies [24–27] showed for the first time data from *in vitro* expression of immobilized DNA templates. Alternatively, an unlabeled target DNA can be sandwiched between an immobilized probe and a poly(dT) probe followed by the addition of the expressible DNA fragment and *in vitro* expression. The linear range of these hybridization assays extends from

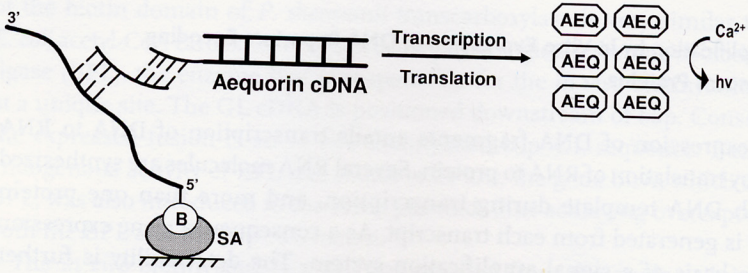

Fig. 5.11 Representative hybridization assay using, as a reporter, a DNA template encoding the photoprotein aequorin.

0.25 amol to 1500 amol target DNA. The above assays demonstrate that DNA, besides its wide use as a recognition molecule (probe), can also serve as a signal-generating molecule (reporter) providing an effective means for the generation of multiple aequorin molecules in solution (more than 150 molecules of the photoprotein per DNA template).

Luciferases, although highly detectable, have found only limited use as labels in DNA hybridization assays because of their significant loss of activity upon conjugation to other molecules. A distinct advantage, however, of using the luciferase-coding DNA as a reporter instead of the enzyme itself is that inactivation problems are avoided because the synthesized luciferase remains free in solution. The cDNA of firefly luciferase (FL, MW = 62 000) and *Renilla* luciferase (RL, MW = 36 000) have been used as reporters in expression hybridization assays. Furthermore, a microtiter well–based hybridization assay that allows simultaneous determination of two target DNA sequences has been developed [27]. The target DNAs were heat-denatured and hybridized with specific capture and detection probes. The capture probes were immobilized by physical absorption in the form of conjugates with bovine serum albumin. One detection probe was biotinylated and the other was tailed with dTTP. The hybrids were reacted with a mixture of SA-FL DNA complex and poly(dA)-tailed RL DNA. Subsequently, the transcription–translation mixture was added to initiate expression of the two immobilized reporter DNAs. It was shown that although the two DNA templates used the same promoter and shared the same transcription and translation machinery, they were expressed independently, i.e., the expression of one template does not affect the expression of the other in a large range of concentrations. Furthermore, the two luciferases (FL and RL) were co-determined in the expression mixture by a dual assay, exploiting the fact that the presence of Mg^{2+} is necessary for the activity of FL but is not required for RL. Thus, following expression, the substrate of FL was added (containing luciferin, ATP, and Mg^{2+}) and the luminescence was measured for 30 s. The FL reaction then was stopped by the addition of EDTA, the substrate of RL was injected, and the luminescence was measured for 10 s.

5.7 Conclusions

Bioluminescent reporters can be detected within a few seconds at the atto mol level using simple instrumentation. This is the basis for their application as non-radioactive reporters in nucleic acid analysis by hybridization with detectabilities in the low pmol range of target DNA. This is more than a 100-fold improvement over current hybridization assays that use fluorescent labels. In addition, photoproteins can be combined with glow-type chemiluminogenic reactions for the development of dual-analyte hybridization assays. The hybridization assays can be performed in microtiter wells, thus enabling automation and high-throughput analysis. Major application fields include the detection and determination of PCR products and the genotyping of single-nucleotide polymorphisms. One of the most important applications of photoprotein reporters is in the development of high-throughput quantitative competitive PCR methods that use synthetic DNA or RNA internal standards (competitors).

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