CHAPTER 9

Gene Assays Based on Bio(Chemi)luminescence

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9.1 DNA Hybridization Assays

One of the first reports describing the use of recombinant aequorin as a reporter molecule in a bioluminometric nucleic acid hybridization assay was in 1996.[1] Microtiter wells were coated with anti-digoxigenin antibody. The target DNA was hybridized simultaneously with an immobilized digoxigenin-labeled capture probe and a biotinylated detection probe. The hybrids were determined using an aequorin–streptavidin conjugate followed by the measurement of luminescence in the presence of excess Ca^{2+} (Figure 9.1). The linearity of the assay was in the range 0.1–200 pM (5 amol $well^{-1}$ to 10 fmol $well^{-1}$) and the S/B ratio at 0.1 pM (5 amol $well^{-1}$) was 5.3. A configuration in which the aequorin–streptavidin conjugate was replaced by a preformed complex of biotinylated aequorin to streptavidin resulted in equivalent signals and detectability. An advantage of using the preformed complex is that it was conveniently prepared by simply mixing the two components (streptavidin and biotinylated aequorin), thus providing a practical alternative to the use of aequorin–streptavidin conjugates prepared by covalent crosslinking techniques. The aequorin–biotin and the aequorin–streptavidin conjugates in this study were obtained commercially (the same group later reported the construction of a plasmid suitable for bacterial expression of *in vivo*-biotinylated aequorin, facilitating further the development of highly sensitive hybridization assays,[2] as well as a method for

Chemiluminescence and Bioluminescence: Past, Present and Future
Edited by Aldo Roda

Published by the Royal Society of Chemistry, www.rsc.org

Figure 9.1 Bioluminometric hybridization assay based on aequorin. Denatured DNA is hybridized with an immobilized capture probe and a biotinylated (B) detection probe. Probe immobilization is accomplished through digoxigenin (Dig)–anti-digoxigenin interaction. Hybrids react with aequorin (AEQ)–streptavidin (SA) conjugate. AEQ is determined by Ca^{2+} addition.

rapid conjugation of streptavidin to aequorin,[3] as discussed in Section 9.5). The proposed assay was applied to the detection and semi-quantitative assessment of the mRNA for prostate-specific antigen (PSA) as a potential marker for the staging of prostate cancer. PSA mRNA from a single cell in the presence of one million non-PSA expressing cells was detected with an S/B ratio of 2.5.

Through these studies it was shown that although the aequorin reaction does not entail substrate turnover it does provide high sensitivity, comparable to alkaline phosphatase (ALP) using chemiluminogenic substrates.[4] The sensitivity of aequorin-based hybridization assays was further enhanced by enzymically introducing multiple aequorin labels per DNA hybrid.[5] The target DNA was hybridized simultaneously, in streptavidin-coated microtiter wells, to an immobilized biotinylated capture probe and a digoxigenin-labeled detection probe. The hybrids reacted with horseradish peroxidase (HRP) conjugated to anti-digoxigenin antibody. A digoxigenin-tyramine (Dig-Tyr) conjugate was used as the hydrogen donor. In the presence of hydrogen peroxide, peroxidase catalyzed the oxidation of Dig-Tyr, resulting in the covalent attachment of multiple Dig-Tyr molecules to the solid phase, through the tyramine group, with the digoxigenin moiety remaining exposed.[6] The immobilized digoxigenins were then reacted with aequorin conjugated to anti-digoxigenin antibody (Figure 9.2). As low as 20 fM (1 amol $well^{-1}$) target DNA was detected with an S/B ratio of 2.7 and the analytical range extended to 2.6 pM. The proposed assay was directly compared to an assay that used only aequorin conjugated to anti-digoxigenin antibody, without the peroxidase amplification step. An S/B ratio of 2 was obtained for 160 fM target DNA, indicating that the enzyme amplification resulted in an eight-fold increase in sensitivity as compared to the assay that used only aequorin conjugated to anti-digoxigenin antibody.

Chemiluminometric hybridization assays for the detection of PCR products are also applicable to testing for genetically-modified organisms (GMO).[7] DNA is the preferred analyte in GMO testing due to its superior stability

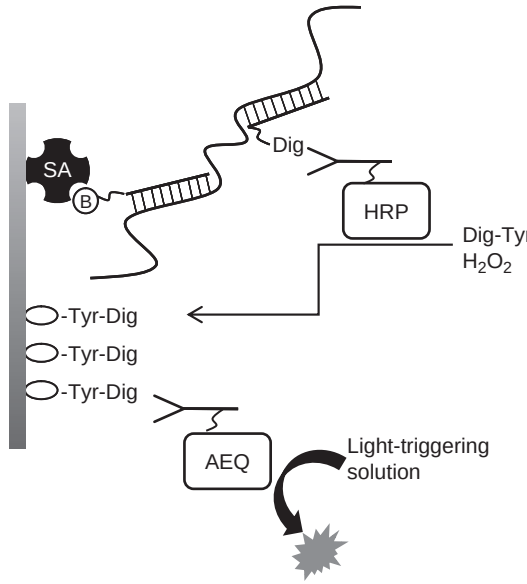


Figure 9.2 Enzyme-amplified aequorin-based bioluminometric hybridization assay. Denatured DNA is hybridized, in streptavidin (SA)-coated wells, with an immobilized biotinylated (B) probe and a digoxigenin (Dig)-labeled detection probe. Hybrids react with horseradish peroxidase (HRP) conjugated to anti-digoxigenin antibody. HRP catalyzes the oxidation of Dig-tyramine (Dig-Tyr) by H_2O_2, resulting in attachment of multiple Dig moieties to the solid phase. Aequorin (AEQ) conjugated to anti-digoxigenin antibody is then allowed to bind to the immobilized Dig. AEQ is determined by Ca^{2+} addition.

compared to the expressed proteins encoded by the transgenic sequences, especially in processed foods, and because DNA-based methods offer detectability superior to that of protein methods.[8–10] Target DNA sequences frequently used for GMO screening are the 35S promoter of the cauliflower mosaic virus and the nopaline synthase (NOS) terminator from *Agrobacterium tumefaciens* since these are the most commonly used regulatory elements for the production of transgenic plants.[11] The challenge is to detect the low number of genetically modified genome copies in a background of unaltered genome. Hence, DNA amplification by PCR constitutes an essential step of GMO detection methods. In one report, target DNA was biotinylated through PCR and was added to streptavidin-coated microtiter wells.[7] After removal of the non-biotinylated strand by NaOH treatment, the target DNA was hybridized to the detection probe, an oligonucleotide containing a target-specific sequence and a poly(dT) tail. Determination of the hybrids was accomplished through the interaction of the poly(dT) tail with an aequorin–$(dA)_{30}$ conjugate (Figure 9.3).[11] Three targets were detected by this approach (each in a separate well): the 35S promoter, the NOS terminator and the endogenous, soybean-specific, lectin gene, which is a

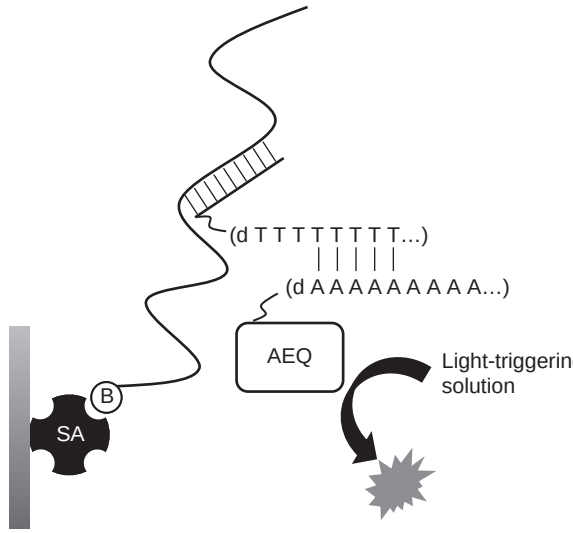


Figure 9.3 Bioluminometric hybridization assay based on a universal aequorin-labeled oligonucleotide probe. Biotinylated (B) target DNA is captured on streptavidin (SA)-coated wells and denatured by NaOH. Immobilized single-stranded DNA is then hybridized with a poly(dT) tailed-detection probe. Hybrids react with the universal aequorin (AEQ)–$(dA)_{30}$ conjugate. AEQ is determined by Ca^{2+} addition.

reference gene for confirmation of the integrity of extracted DNA. For all three targets, as low as 2 pM (100 amol well^{-1}) of amplified DNA was detected with an S/B ratio of about 2. The analytical range extended up to 2000 pM. As low as 0.05% GMO content in soybean was detectable with an S/B ratio of 8.2. This method offered 80-times higher detectability than agarose gel electrophoresis and ethidium bromide staining of PCR-amplified DNA, a commonly used GMO detection method. GMO analysis by real-time PCR has also been reported but the cost is much higher than the proposed assay.[12] Biosensors have also been proposed for GMO detection.[13,14] The simple bioluminometric assay is advantageous because it is based on a universal aequorin-labeled oligonucleotide probe, which can be used with other target sequences in combination with appropriate poly(dT)-tailed specific probes. The assay may be used for the semi-quantitative assessment of GMO content since luminescence increases with GMO content. But, to obtain accurate quantitative results, an internal standard should be used that contains the same primer binding sites and is distinguishable from the target by hybridization. This is addressed in Section 9.2 (quantitative PCR).[15]

Another application of hybridization assays as detection methods for PCR products is the area of molecular diagnostics. An assay using PSA mRNA as the model target has already been presented in this section.[1] The mRNAs for PSA and prostate-specific membrane antigen (PSMA) are the most commonly

investigated markers for identification of prostate cancer cells in blood, lymph nodes and bone marrow by the use of reverse transcriptase PCR (RT-PCR). However, the routine clinical application of PSA mRNA or PSMA mRNA assays for the molecular diagnosis and monitoring of prostate cancer is prohibited by false-negatives and false-positives, *i.e.*, the marker may not be detected in patients with diagnosed metastatic cancer or may be detected in normal blood samples and in non-prostate cell lines. Combined screening of PSA and PSMA mRNAs has been proposed as a more useful marker than the separate PSA mRNA or PSMA mRNA markers. The method involves two separate nested RT-PCR steps employing primers specific for each target mRNA. The limitation of nested RT-PCR is its complexity and high risk of contamination. Detection of amplification products is usually performed by agarose gel electrophoresis and ethidium bromide staining. The result is confirmed by Southern transfer using target-specific probes carrying signal-generating labels. Enhanced sensitivity is achieved by either increasing the number of PCR cycles or using sensitive methods for the detection of the amplification products. The need for a rapid and simple assay for both PSA and PSMA mRNAs has been addressed with the development of a sensitive method for the simultaneous detection of both markers using duplex RT-PCR and a chemiluminometric microtiter well-based DNA hybridization assay. [16] Total RNA from peripheral blood was reverse-transcribed using oligo$(dT)_{20}$ primer followed by duplex PCR in the presence of two pairs of primers specific for PSA and PSMA. The biotinylated amplification products were determined through a hybridization assay. Specifically, after thermal denaturation, biotinylated PCR products were captured on microtiter wells coated with PSA- or PSMA-specific probes conjugated to bovine serum albumin (BSA). The captured single-stranded DNA was then detected by reacting with an ALP–streptavidin conjugate. Luminescence was measured with a chemiluminogenic substrate (Figure 9.4). Using the duplex PCR, 50 copies of PSA DNA and 5 copies of PSMA DNA were detected with S/B ratios of 9.7 and 22, respectively. The detectability of cancer cell equivalents was assessed by performing RT-PCR in samples containing total RNA corresponding to 0.04–400 LNCaP cells in the presence of 1 μg of total RNA from healthy subjects isolated from whole blood. The detectability was one cancer cell equivalent in 10 mL of blood and was comparable to that achieved by nested RT-PCR. The assay offers several advantages. Sensitivity is achieved without using nested PCR, therefore minimizing contamination problems, while specificity is accomplished through the use of gene-specific probes. The assay is suitable for high-throughput screening as opposed to the commonly used labor-intensive methods of electrophoresis and Southern blotting. Furthermore, the assay can be applied to the simultaneous mRNA detection of other marker combinations.

Another application of hybridization assays is the detection of parasites. A novel bioluminescence DNA hybridization assay has been developed for the detection of *Plasmodium falciparum*, the most prevalent and deadly species of malaria.[17] The gold standard for malaria detection is light microscopy but low-throughput, high cost and high skill limit its applicability, especially in

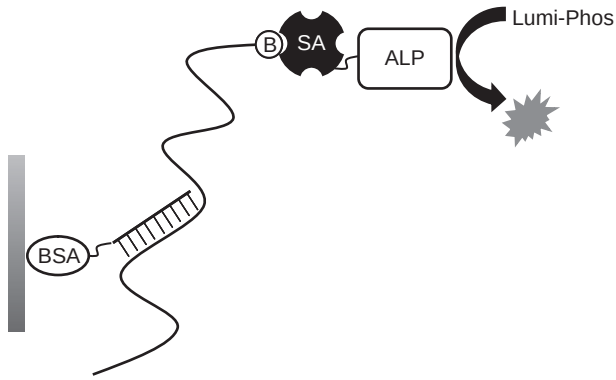


Figure 9.4 Chemiluminometric hybridization assay. Denatured biotinylated (B) DNA is hybridized with an immobilized probe [oligonucleotide conjugated to bovine serum albumin (BSA)]. Hybrids react with an alkaline phosphatase (ALP)–streptavidin (SA) conjugate. ALP is determined by adding a chemiluminogenic substrate.

developing regions where malaria detection is mostly needed. Promising alternatives to light microscopy, including hybridization assays involving PCR amplification, have emerged. However, there is still a need for assays suitable for the conditions typically encountered in developing areas with limited resources. An assay was developed that employed aequorin as a label and did not require PCR amplification of the sample.[17] Sensitivity was accomplished by using a genetically engineered mutant aequorin that contained a unique cysteine at position 5 and was characterized by greater bioluminescence activity and activity after conjugation to streptavidin.[18] The assay was based on the competition between the target DNA and the probe (B-probe) for hybridization with an immobilized probe. Specifically, the assay was performed by first immobilizing a probe on neutravidin-coated microtiter wells. Target DNA was then allowed to hybridize with the immobilized probe. Next, the B-probe (complementary to the immobilized probe) was added to the well to hybridize with any immobilized probe that was left unbound by the target DNA. Aequorin–streptavidin conjugate was then employed for interaction with the biotin of the B-probe and quantitation of the amount of B-probe, which in turn was related to the amount of the target *Plasmodium falciparum* DNA (Figure 9.5). The assay showed a detection limit of $3\,\mathrm{pg}\,\mu\mathrm{L}^{-1}$ and was used for the detection of target DNA in spiked human serum samples.

A different direction in the detection of PCR products is the recent development of a flow-through chemiluminescence microarray read-out system not only for the detection but also for the quantification of *E. coli* DNA after PCR amplification.[19] The method has applications in the field of water monitoring and quality control since the most dangerous water contaminants are pathogenic microorganisms. Traditionally, these pathogens are detected by labor-intensive, time-consuming microbiological methods that require bacterial

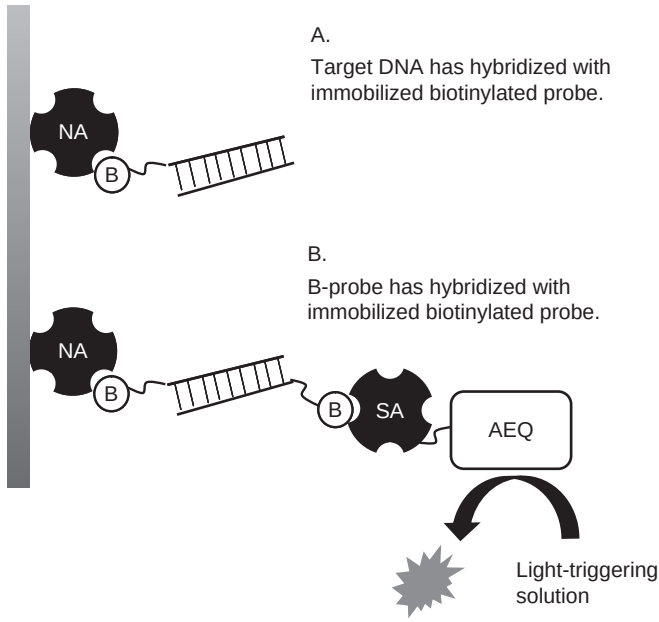


Figure 9.5 Bioluminometric hybridization assay for the detection of *Plasmodium falciparum*. The assay is based on the competition between the target DNA and the probe (B-probe) for hybridization with an immobilized probe. Target DNA is hybridized in neutravidin-coated wells with an immobilized probe (schematic A). Next, the B-probe (complementary to the immobilized probe) is added to hybridize with any immobilized probe that was left unbound by the target DNA (schematic B). Aequorin–streptavidin (SA) conjugate is used for interaction with the biotin of the B-probe and quantitation. AEQ is determined by Ca^{2+} addition.

cultures. However, DNA microarrays can also be used for the quantification of microorganisms in water samples. In fact, fluorescence, electrochemical, chemiluminescence and label-free microarray read-out systems have been developed. Although sensitive, microarrays still require a preceding PCR amplification step. For this purpose, the authors have developed a stopped-PCR method that overcomes the drawback of the sigmoidal amplification curve of end-point PCR and allows the sensitive quantification of target DNA after PCR by means of a chemiluminometric DNA microarray.[19] The principle of stopped-PCR is to end amplification in the logarithmic phase of the reaction, at the point where the spread between different starting amounts of target DNA is at a maximum. As a consequence, the amplification product is strongly dependent on the initial concentration of the target DNA. The chemiluminometric hybridization assay involved microarrays constructed on a poly(ethylene glycol)-modified glass substrate. A NH_2-modified capture probe, which was covalently immobilized onto the microarray surface *via* the free amine, served for capturing the target and HRP–streptavidin conjugate was used as the reporter molecule. The target

DNA was biotinylated through PCR and was captured on the microarray surface by the immobilized probes. The hybrids were detected by HRP–streptavidin conjugate. HRP activity was determined by adding a chemiluminogenic substrate (luminol and H_2O_2). The chemiluminescence intensity was recorded by a sensitive charge-coupled device (CCD) camera. All assay steps, including the addition of denatured biotinylated target DNA, HRP–streptavidin conjugate and HRP substrates, were conducted with the flow-through chemiluminescence read-out system. The method was applied to the detection of the uidA gene (β-galactosidase) of *E. coli*. The detection limit for the uidA gene was 1.1×10^5 copies mL^{-1}. Detection and quantification of *E. coli* was feasible in the range from 10^6 to 10^9 copies mL^{-1}. The assay time was 7 h and the limiting factor was the double-stranded DNA analyte. The authors observed that the assay was very fast (15 min) and sensitive (detection limit 40 copies mL^{-1}) if the analyte was single-stranded DNA. This means that the two strands of the amplification product should be separated before the microarray hybridization assay, *i.e.*, not only denatured to form single strands but also physically separated such that only one strand is added to the microarray. One possible configuration would be biotinylation of the target DNA through PCR, removal of the biotinylated strand by using streptavidin-labeled microbeads, addition of the single-stranded target DNA to the microarray (this strand is now the analyte and it should be designed such that it carries a label incorporated through PCR) and detection of the hybrids through an HRP–antibody conjugate that recognizes the label on the single-stranded DNA analyte. This treatment might improve the sensitivity of the proposed assay by over three orders of magnitude and reduce assay time to 15 min, making the proposed flow-through chemiluminescence microarray read-out system extremely superior to traditional microbiological techniques, which take 18 h for *E. coli* detection and 10 days for *Legionella*.

A novel ultrasensitive flow injection chemiluminometric hybridization assay has been reported that is based on probes linked to copper sulfide (CuS) nanoparticles in combination with chemiluminescence of the luminol-H_2O_2–Cu^{2+} system.[20] The DNA probe was labeled with CuS nanoparticles and the target DNA was immobilized on a glass-carbon electrode. After hybrid formation, cupric ions (Cu^{2+}) were released from dissolution of the CuS nanoparticles on the probe, and the target was determined by the chemiluminescence intensity of luminol–H_2O_2–Cu^{2+}. The chemiluminescence was proportional to the concentration of the dissolved Cu^{2+}. To increase the sensitivity of the DNA biosensor, a Cu^{2+} preconcentration process was performed by using the anodic stripping voltammetry process, *i.e.*, the Cu^{2+} was retained temporarily by electrochemical preconcentration on a platinum electrode placed in an anodic stripping voltammetric cell. The assay gave a linear response curve in the range of 2–100 pM target DNA. The detection limit was estimated to be 0.55 pM. The selectivity of the DNA biosensor was investigated by allowing hybridization of the labeled DNA probe with the complementary target DNA, a two-base mismatched target DNA and a non-complementary target DNA. A well-defined chemiluminescence signal was obtained for the complementary

sequence whereas the chemiluminescence intensity of the two-base mismatched sequence was significantly weaker, and the non-complementary sequence showed no response. The drawbacks of this method are the requirement of additional steps due to the dissolution of the nanoparticles and the Cu^{2+} preconcentration, as well as the relatively time-consuming labeling of the probe with the CuS nanoparticles (about 13 h). One way to simplify the assay and increase sensitivity is to investigate more suitable nanoparticle tags and chemiluminescent reactions.

In another publication the authors observed that the catalytic activity of gold nanoparticles (AuNPs) on luminol–H_2O_2 chemiluminescence is greatly enhanced after aggregation in the presence of 0.5 M NaCl.[21] This observation led them to the development of a homogeneous label-free chemiluminescence detection system for sequence-specific DNA hybridization. This is the first label-free chemiluminometric hybridization assay. The method is based on the fact that single- and double-stranded oligonucleotides have different tendencies for adsorption onto AuNPs. In the absence of target DNA (single-stranded DNA due to denaturation), the probe (single-stranded) is adsorbed on the surface of AuNPs and does not allow the aggregation of AuNP in 0.5 M NaCl. The dispersed AuNPs induce a weak chemiluminescence signal of the luminol–H_2O_2 system. In contrast, if hybridization occurs between the single-stranded target DNA and the single-stranded probe (resulting in a double-stranded conformation) then aggregation of AuNPs at 0.5 M NaCl occurs because the probe is no longer on the surface of the AuNPs. The aggregated AuNPs, in turn, induce a strong chemiluminescence signal of the luminol–H_2O_2 system. The detection limit of target DNA was estimated to be 1.1 fM. The sensitivity was more than six orders of magnitude higher than an AuNP-based colorimetric method. The method was also satisfactorily applied to human plasma samples. In this assay, both hybridization and detection occur in homogeneous solution, thereby eliminating the need for covalent attachment of the AuNP to the probe or the target DNA. The assay avoids the stripping procedure of metal nanoparticles, which would result in a high chemiluminescence background. However, it is not known whether longer target DNA molecules in the presence of excess unrelated DNA would interfere with the detection.

A different direction in the development of DNA hybridization assays is the use of magnetic beads. A magnetic bead-based DNA hybridization assay using either conventional chemiluminescence detection or chemiluminescence imaging has been developed.[22] In this method, a sandwich DNA hybridization assay was performed. The assay consisted of a NH_2-modified capture probe immobilized onto carboxylated magnetic beads, a biotinylated detection probe, and HRP–streptavidin conjugate as the reporter molecule. Hybridization occurred in one step by mixing the magnetic beads (which carry the capture probe) with the target DNA and the biotinylated detection probe. The hybrids were detected by adding HRP–streptavidin conjugate. HRP activity was determined by its chemiluminescent reaction (luminol/oxidant/enhancer system). The chemiluminescence intensity was measured both conventionally (with a luminometer), as well as with chemiluminescence imaging. The method has

been applied to the detection of sequence-specific DNA related to the avian influenza A H1N1 virus; conventional chemiluminescence detection and chemiluminescence imaging had similar sensitivities (as low as 10 amol target DNA, *i.e.*, 0.1 pM). The assay with chemiluminescence detection was linear in the range 0.3–300 pM target DNA. The assay with chemiluminescence imaging had a wider linear range, from 0.1 to 1000 pM target DNA. The sensitivity of the magnetic bead-based assay is five-times better than that of the CuS nanoparticle assay,[20] but the AuNP assay remains the most sensitive (100-times better than the magnetic bead assay).[21] The magnetic bead-based assay with chemiluminescence detection was also used for genotyping of single nucleotide polymorphisms (SNP). The assay was able to distinguish between perfectly complementary sequences and single-base mismatched sequences by optimizing the stringency of the hybridization and washing steps.

Another recent application involving DNA hybridization has been the development of a multiplex chemiluminescence microscope imaging method for identification and classification of cervical intraepithelial neoplasia (CIN) lesions.[23] Classification of CIN lesions into low-grade (CIN1) or high-grade (CIN2-3) lesions is critical for optimal patient management. A shortcoming of current conventional histological diagnosis on biopsy samples is inter-observer variability. The authors developed a method for two complementary biomarkers that combined (a) immunohistochemical localization and quantitative detection of $p16^{INK4A}$ (a protein marker of high-grade CIN lesions) and (b) *in situ* hybridization for the localization of human papillomavirus (HPV) DNA (in low-grade CIN lesions HPV DNA is an important indicator of the risk of progression to a higher grade lesion). Both determinations were performed in the same tissue biopsy section, sequentially. Different enzyme labels were employed to avoid any interference between the two assays. The enzyme label for detection of $p16^{INK4A}$ was ALP and enzyme activity was measured by using a luminol/oxidant/enhancer system. The enzyme label for detection of HPV DNA was HRP and the enzyme activity was measured by using a dioxetane-based substrate. In fact, HPV DNA localization was performed by using a pool of digoxigenin-labeled DNA probes that recognize the 6, 11, 16, 18, 31, 33 and 35 HPV genotypes. The hybrids were detected by using HRP conjugated to anti-digoxigenin antibody. The light emission of each chemiluminescent reaction remained stable for 20–30 min, thus allowing optimal handling of the sample and the signal and enabling the acquisition of several images from different areas of the same section.

In the field of biosensors, a DNA detection approach that has received great attention is electrogenerated chemiluminescence; the electrogenerated chemiluminescence is obtained from the excited state of a luminophore generated at the electrode surface during an electrochemical reaction. CdS nanocrystals are an example of a semiconductor nanocrystal that provides electrogenerated chemiluminescence in the presence of co-reactant.

In a recent report, a simple electrogenerated chemiluminescence biosensor for the highly sensitive and specific detection of DNA was developed.[24] The biosensor featured CdS:Mn nanocrystals as the luminophore and gold

nanoparticles (AuNPs) as both the electrogenerated chemiluminescence quencher and enhancer. AuNPs have made excellent building blocks for the construction of DNA biosensors because of the following features: (1) AuNP-enhanced Raman scattering and AuNP-enhanced surface plasmon resonance, (2) fluorescence quenching due to fluorescence resonance energy transfer (FRET) when the fluorophore and the AuNPs are in close proximity, and (3) AuNP-enhanced fluorescence due to interaction of the excited fluorophore with surface plasmons when the fluorophore and the AuNPs are largely separated.

The features of the biosensor and the assay steps are as follows:

1 a CdS:Mn nanocrystal film (it functions as the luminophore) on a glassy carbon electrode – the nanocrystals have carboxylic acid groups due to modification with 3-mercaptopropionic acid;
2 AuNPs (function as both electrogenerated chemiluminescence quencher and enhancer) covalently attached to one end of a DNA hairpin probe (specific for the target DNA) through 6-mercapto-1-hexanol;
3 crosslinking of the hairpin probe–AuNP conjugates to the CdS:Mn film through *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) (through the second end of the DNA hairpin probe);
4 addition of the target DNA.

There are three stages at which electrogenerated chemiluminescence was measured. In the first stage, the CdS:Mn nanocrystal film on the glassy carbon electrode exhibited electrogenerated chemiluminescence in the presence of the co-reactant $S_2O_8^{2-}$ ions. In the second stage, the hairpin probe–AuNP conjugates were brought close to the CdS:Mn nanocrystal film by EDC/NHS crosslinking, in which case the electrogenerated chemiluminescence peak height decreased by 25% in comparison with that of the CdS:Mn nanocrystal film before assembly; the decrease indicated the quenching of electrogenerated chemiluminescence due to FRET between the CdS:Mn nanocrystal film and the AuNPs (close proximity). In the third stage, the AuNPs were largely separated from the CdS:Mn nanocrystal film because the addition of target DNA (5 fM) caused unfolding of the hairpin probe due to hybridization between the target DNA and the probe. The peak height increased by 55% in comparison with that before assembly; the increase indicated the enhancement of electrogenerated chemiluminescence due to the interactions of the excited CdS:Mn nanocrystals with electrogenerated chemiluminescence-induced surface plasmon resonance in AuNPs (large separation). This combination of quenching and enhancement of electrochemiluminescence by AuNPs together in one assay provided high sensitivity for target DNA detection (50 aM). The assay was linear in the range of 50 aM to 5 fM target DNA. The specificity of the biosensor was studied by using one-base mismatched target DNA, three-base mismatched target DNA and a non-complementary target DNA.

DNA has been commonly used as a recognition molecule (probe) in hybridization assays but not as a signal-generating molecule (reporter). However,

DNA is an excellent candidate for a reporter molecule since it is much more stable than enzyme reporters, which must maintain activity during (a) isolation from the corresponding cell/organism, (b) conjugation to molecules that are required for binding to the analyte and (c) storage. A novel direction in hybridization assays has been introduced by using a DNA fragment (DNA template) coding for an enzyme, as the reporter molecule. In these assays, after hybrid formation, the solid-phase-bound DNA template is expressed by *in vitro* transcription/translation and the activity of the synthesized enzyme is measured, resulting in a highly sensitive analytical system. Expressible DNA fragments encoding firefly luciferase as well as *Renilla* luciferase were used as labels. The application is not limited to enzymes, but has also been extended to photoproteins such as aequorin, in which case the expressible DNA label encodes apoaequorin.

A hybridization assay that utilizes as a label an expressible enzyme coding DNA fragment was designed for the first time in 1996.[25] The DNA label contained a firefly luciferase coding sequence downstream from a T7 RNA polymerase promoter. The DNA label was expressed by *in vitro* transcription/translation, leading to the production of luciferase. Because the T7 promoter sequence was present only in the DNA label, the transcription/translation process used the luciferase-coding DNA exclusively, and not the probe-target hybrids. In this assay configuration, the capture probe was tailed with digoxigenin-dUTP and was bound to microtiter wells coated with anti-digoxigenin antibody. Denatured target DNA was hybridized simultaneously with the immobilized capture probe and a detection probe tailed with biotin–dATP. The hybrids reacted with a preformed streptavidin–luciferase DNA complex. The complex was prepared by mixing biotinylated luciferase-coding DNA with a large excess of streptavidin. The assay was completed by expressing the solid-phase-bound DNA label by coupled (one-step) *in vitro* transcription/translation. The activity of the synthesized luciferase was then measured. Luciferase catalyzed the luminescent reaction of luciferin, O_2 and ATP to produce oxyluciferin, AMP, pyrophosphate and CO_2 (Figure 9.6).[26,27] As low as 5 amol target DNA was detected with an S/B ratio of 2. The luminescence was linearly related to the amount of target DNA up to about 5000 amol. The high sensitivity achieved was a result of the combined amplification due to transcription/translation and the substrate turnover. The proposed method was compared directly with a fluorometric and a chemiluminometric hybridization assay in which the streptavidin–DNA complex was replaced by an ALP–streptavidin conjugate for detection of the hybrids. The activity of solid-phase-bound ALP was then measured by using a fluorogenic substrate (4-methylumbelliferyl phosphate) or a chemiluminogenic substrate (dioxetane derivative, CSPD). The fluorometric assay detected 200 amol target DNA with an S/B ratio of 2.4. The chemiluminometric assay detected 100 amol target DNA with an S/B ratio of 3.2. Therefore, the assay utilizing the expressible streptavidin–DNA complex resulted in a 20- to 40-fold increase in sensitivity. Notably, luciferases have found only limited use as enzyme labels in DNA hybridization assays and immunoassays due to the significant loss of activity upon conjugation.[28]

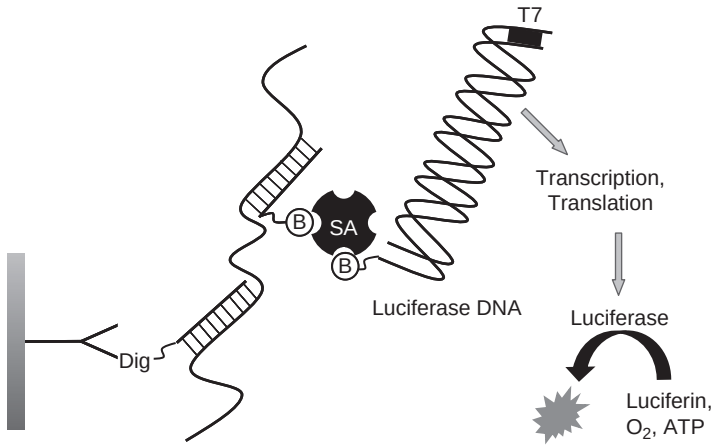


Figure 9.6 Bioluminometric hybridization assay based on an expressible DNA fragment encoding firefly luciferase as a label. Denatured DNA is hybridized with an immobilized capture probe and a biotinylated (B) detection probe. Probe immobilization is accomplished through digoxignenin (Dig)–anti-digoxigenin interaction. Hybrids react with a streptavidin (SA)–firefly luciferase DNA complex. Solid-phase-bound firefly luciferase DNA is measured by coupled *in vitro* transcription/translation. The activity of synthesized luciferase is measured.

The luciferase-coding DNA was selected as the expressible enzyme coding DNA fragment because luciferase is a monomeric protein, it requires no post-translational modification and its activity can be readily measured in the transcription/translation mixture without prior purification. Because of these attractive properties, the luciferase cDNA has been used extensively as a reporter gene to monitor gene expression in various tissues. The proposed methodology was the first that used luciferase as a reporter molecule in hybridization assays and represents a novel approach for introducing multiple enzyme molecules in the system, because from a single expressible DNA fragment several enzyme molecules (12–14 luciferase molecules) can be synthesized by *in vitro* transcription/translation.[29]

Subsequently, the investigation of novel hybridization assay configurations based on the *in vitro* expression of DNA reporter molecules was extended by exploiting the biotin–streptavidin interaction for the capture of hybrids to the solid phase.[30] This modification enhanced system versatility as it allowed two types of hybridization assay configurations to be performed. In configuration A (captured target hybridization assay) the target DNA was end-labeled with biotin (through PCR) and captured on streptavidin-coated wells (Figure 9.7). The one strand was removed by NaOH treatment and the remaining strand was hybridized with a poly(dA)-tailed oligonucleotide probe. Configuration B (sandwich-type hybridization assay) involved simultaneous hybridization of heat-denatured target DNA with a biotinylated capture probe (immobilized on

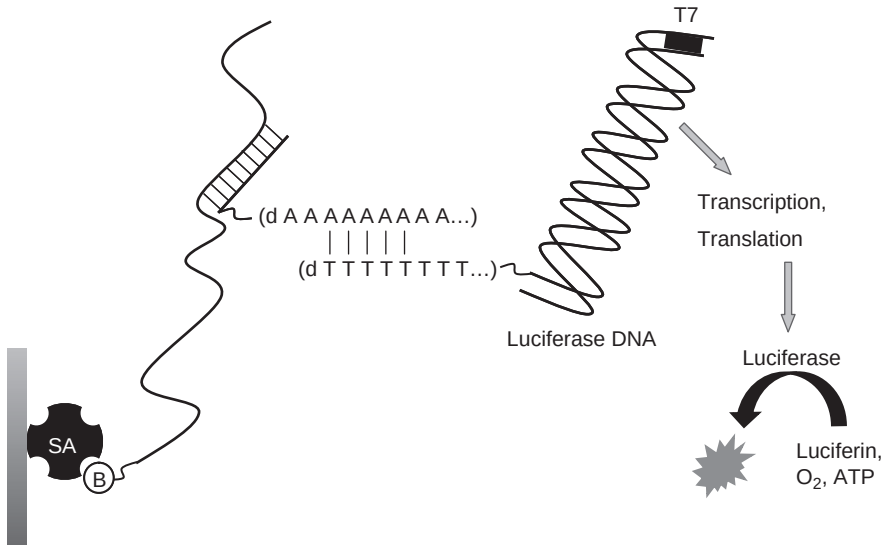


Figure 9.7 Bioluminometric hybridization assay based on an expressible DNA fragment encoding firefly luciferase as a label (captured target configuration). Biotinylated (B) target DNA is captured on streptavidin (SA)-coated wells and denatured by NaOH. Immobilized single-stranded DNA is then hybridized with a poly(dA) tailed-detection probe. Hybrids react with a poly(dT)-tailed DNA fragment encoding firefly luciferase. Solid-phase-bound luciferase DNA is measured by *in vitro* expression (either coupled or separate transcription/translation). The activity of synthesized luciferase is measured.

streptavidin-coated wells) and a poly(dA)-tailed detection probe (Figure 9.7). In both configurations the hybrids reacted with a poly(dT)-tailed luciferase-coding DNA fragment followed by *in vitro* expression of the bound DNA on the solid phase. Expression was accomplished either by using a commercially available coupled (one-step) transcription/translation or by performing sequential transcription and translation reactions that were optimized separately, leading to an increased expression yield (Figure 9.7). For configuration A, at the level of 0.93 fmol target DNA the S/B ratios were 2.6 and 16.7 with the coupled and the separate transcription/translation protocols, respectively. As low as 0.1 fmol target DNA was detected with the separate expression protocol, with an S/B ratio of 2.7, providing at least nine-times higher detectability than the coupled protocol. The reproducibility of configuration A was tested at the level of 3.1 fmol target DNA. For configuration B, at the level of 0.1 fmol target DNA the S/B ratios were 2.2 and 4.6 using the coupled and the separate transcription/translation protocols, respectively. In addition, the assays were directly compared to the sandwich hybridization assay in which the label was a streptavidin–luciferase DNA complex (Figure 9.6). The signals obtained with configuration B using the coupled and the separate expression protocols were

10- and 25-times, respectively, higher than those obtained with the configuration in Figure 9.6. An S/B ratio of 2.4 was obtained for 0.3 fmol target DNA using the streptavidin–luciferase DNA complex as the detection reagent. Configuration B offered higher sensitivity than configuration A but the latter was simpler to perform and potentially automatable because it avoided the heat denaturation step of the target DNA and had a shorter incubation time for hybridization.

A significant improvement of the assay involved the preparation of the firefly luciferase DNA label.[30] In the original use of an expressible enzyme coding DNA as a label,[25] the detection reagent was a complex of streptavidin with a biotinylated luciferase-coding DNA fragment. The preparation of the complex is tedious and time consuming, involving restriction enzyme digestion of the appropriate plasmid to produce three fragments with recessed 3′ ends, a filling-in reaction with the Klenow fragment of DNA polymerase I in the presence of biotin–dATP to create DNA fragments that are biotinylated at both termini, purification of the DNA by ethanol precipitation and another digestion to remove the one biotinylated end, therefore, leaving a 2.1 kbp fragment labeled with biotin only at one terminus. After electrophoretic separation, the DNA is excised, purified and complexed with an excess of streptavidin. Finally, the complexes are purified by HPLC and concentrated. The yield of the entire procedure is 10–20%. In contrast, the subsequent procedure was simpler, involving a single digestion to linearize the plasmid (the entire 4.3 kbp plasmid is used as the label), purification by ethanol precipitation, enzymatic tailing of the DNA with dTTP and no additional purification.[30] The detection probe was a poly(dA)-tailed oligonucleotide. The luciferase-coding DNA fragment is attached through its 3′ terminus to the detection probe to avoid steric hindrance during transcription.

In addition to firefly luciferase as a DNA label for hybridization assays,[25,30] an apoaequorin DNA label has also been reported.[31] The constructed label contained the T7 RNA polymerase promoter, the apoaequorin coding sequence and a downstream $(dA/dT)_{30}$. Two hybridization assay configurations were developed. In configuration A (captured target assay), biotinylated target DNA was captured on streptavidin-coated microtiter wells. The one strand was removed by NaOH treatment and the remaining strand was hybridized with a poly(dT)-tailed detection probe. In configuration B (sandwich-type assay), the target DNA was hybridized simultaneously with an immobilized capture probe (through biotin/streptavidin) and a poly(dT)-tailed detection probe. In both configurations, the hybrids reacted with poly(dA)-tailed apoaequorin DNA. The DNA label was subjected to coupled *in vitro* transcription/translation to produce multiple apoaequorin molecules in solution, which were converted into fully active aequorin in the transcription/translation reaction mixture. Generated aequorin was determined by its characteristic Ca^{2+}-triggered bioluminescence (Figure 9.8). Configuration A was linear in the range 0.5–7812 amol target DNA. The S/B ratio at the level of 0.5 amol was 1.9. In addition, the assay was directly compared to a hybridization assay in which the photoprotein aequorin was used as the label (Figure 9.10 top), as well as to a hybridization

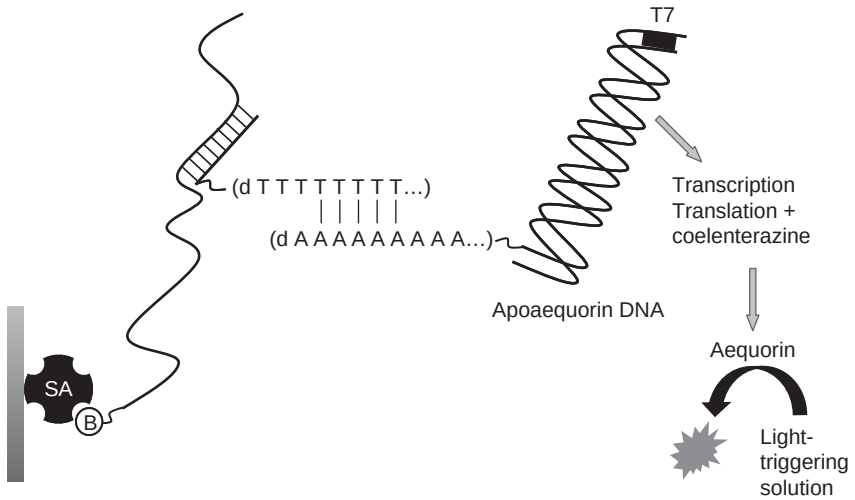


Figure 9.8 Bioluminometric hybridization assay based on an expressible DNA fragment encoding apoaequorin as a label (captured target configuration). Biotinylated (B) target DNA is captured on streptavidin (SA)-coated wells and denatured by NaOH. Immobilized single-stranded DNA is then hybridized with a poly(dT) tailed-detection probe. Hybrids react with poly(dA)-tailed apoaequorin DNA. Solid-phase-bound apoaequorin DNA is measured by coupled *in vitro* transcription/translation to produce apoaequorin molecules in solution, which are converted into fully active aequorin in the reaction mixture. Generated AEQ is determined by Ca^{2+} addition.

assay that uses poly(dT)-tailed luciferase DNA as the label (Figure 9.7 configuration A). The aforementioned assays were able to detect 25 amol (S/B = 1.6) and 20.5 amol (S/B = 1.9) target DNA, respectively. Thus, there was more than a 40-fold improvement in sensitivity when using the DNA encoding apoaequorin as a label. The dramatic improvement in sensitivity observed in configuration A, as compared to the assay in which aequorin was the label, was due to the amplification introduced by the *in vitro* expression of apoaequorin DNA into several active aequorin molecules. Each DNA label was estimated to produce 156 aequorin molecules. Configuration B was linear in the range 0.25–1562 amol target DNA. The S/B ratio at the level of 0.25 amol was 1.4.

The development of hybridization assays based on firefly luciferase-coding or apoaequorin-coding DNA labels was followed by a novel dual-analyte expression hybridization assay. Two DNAs encoding firefly luciferase (FLuc) and *Renilla* luciferase (RLuc) were used as labels for the development of a microtiter well-based expression hybridization assay that allowed simultaneous determination of two target DNA sequences in the same well.[32] The RLuc DNA was chosen because RLuc, like FLuc, is a monomeric protein, it requires no post-translational modification and its activity can be readily measured in the transcription/translation reaction without prior purification. The constructed FLuc

label contained the T7 RNA polymerase promoter, the firefly luciferase coding sequence and a single biotin at the 3′ terminus. Preparation of the streptavidin–FLuc complex involved a single digestion to linearize the FLuc plasmid (the entire 4.3 kbp plasmid was used as label), purification by ethanol precipitation, enzymic labeling with biotin-ddUTP, ethanol precipitation, complexing with streptavidin, and purification of the protein–DNA complex by electroelution. The yield was 40–60%, and the overall procedure was much simpler and faster than previously.[25] The constructed RLuc label contained the T7 RNA polymerase promoter, the *Renilla* luciferase coding sequence and a poly(dA) tail. Preparation of the poly(dA)-RLuc label involved a single digestion to linearize the RLuc plasmid (the entire 4.0 kbp plasmid was used as the label), purification by ethanol precipitation and enzymic tailing with dATP. In the model assay, the target DNAs (target DNA A and target DNA B) were heat-denatured and hybridized simultaneously with the specific immobilized capture probes and the detection probes. The capture probes were conjugated to BSA and used for coating the well. One detection probe was biotinylated (with a single biotin at the 3′ end) while the other detection probe was poly(dT)-tailed. The hybrids reacted with the streptavidin–FLuc DNA complex and the poly(dA)-tailed RLuc DNA. Subsequently, the DNA labels were expressed *in vitro* simultaneously and independently in the same transcription/translation reaction mixture. The activities of the generated firefly and *Renilla* luciferases were co-determined in the same sample based on the differential requirements of their characteristic bioluminescent reactions for Mg^{2+}. Firefly luciferase was measured first. The firefly luciferase reaction was then terminated by the addition of EDTA to measure *Renilla* luciferase with its coelenterazine substrate (Figure 9.9). The S/B ratio obtained for 66 amol of target A in the presence of 10 fmol of target B (a 150-fold excess) was 2.7. The S/B ratio obtained for 66 amol of target B in the presence of 10 fmol of target A was 6.

The following requirements must be fulfilled for the simultaneous determination of two target DNA sequences by an expression hybridization assay:

1 The reporter genes can be expressed independently in the same reaction mixture (the transcription and translation of one gene does not affect the expression of the other) and in a broad range of concentrations, despite the fact that they use the same promoter and share the same transcription and translation machinery.
2 The gene products can be co-determined in the same well, without splitting the sample (the presence of one enzyme and its substrate does not interfere with the assay of the other enzyme). In this work, enzyme determination is based on the fact that, contrary to FLuc, RLuc does not require the presence of Mg^{2+} for full activity.
3 There is no cross-reactivity between probes and target DNA sequences during hybridization.

The dual-analyte expression hybridization assay opens the way for the possible application of multiple enzyme-coding DNA labels for the simultaneous

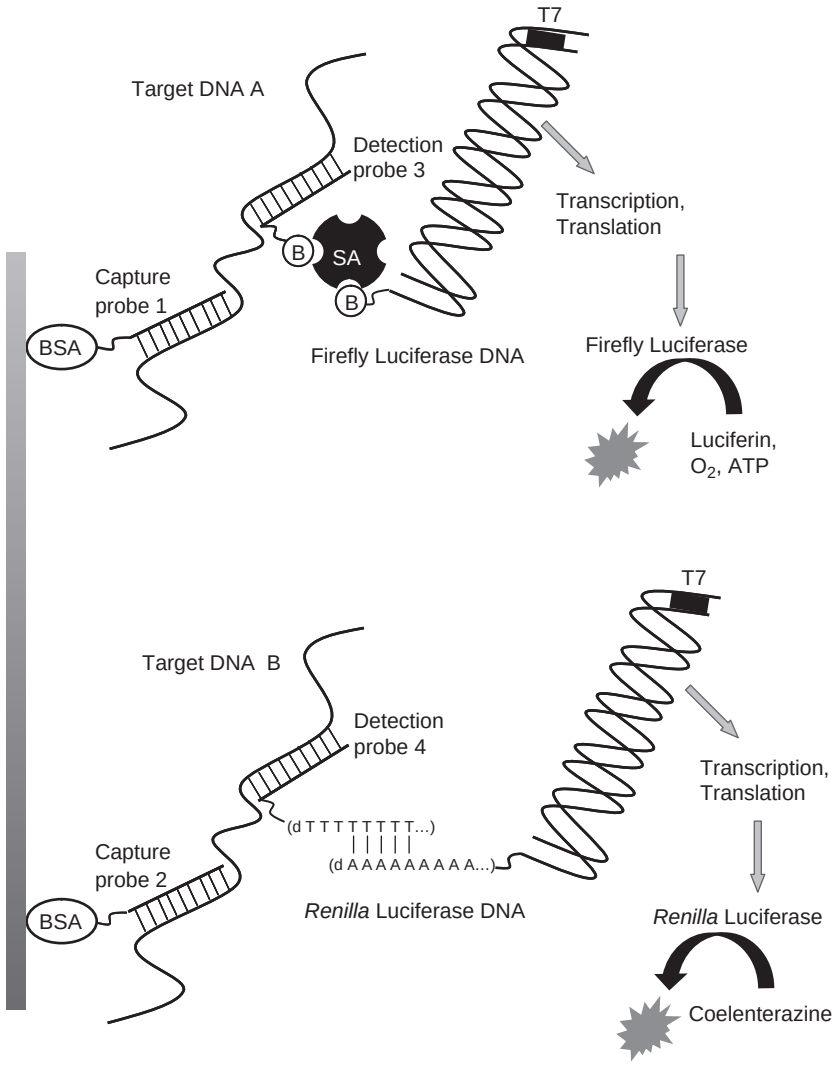


Figure 9.9 Dual-analyte bioluminometric hybridization assay based on expressible DNA fragments encoding firefly luciferase and *Renilla* luciferase as labels. Wells are coated with bovine serum albumin (BSA)-probe conjugates. Denatured DNAs (targets A and B) are hybridized with immobilized capture probes (probes 1 and 2) and detection probes [biotinylated (B) detection probe 3 and poly(dT)-tailed detection probe 4]. Hybrids react with a streptavidin (SA)–firefly luciferase DNA complex and a poly(dA)-tailed *Renilla* luciferase DNA. Solid-phase-bound luciferase DNAs are measured by coupled *in vitro* transcription/translation. The activity of synthesized luciferases is measured sequentially.

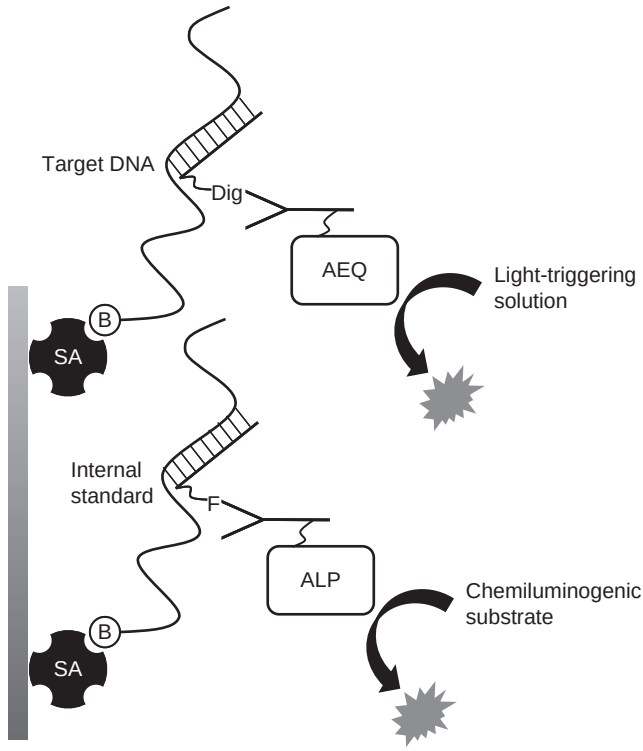


Figure 9.10 Dual-analyte bio(chemi)luminometric hybridization assay for quantitative competitive PCR. Biotinylated (B) target DNA and biotinylated internal standard (competitor) are captured on a single streptavidin (SA)-coated well and denatured by NaOH. Immobilized single-stranded target DNA and internal standard are simultaneously hybridized with their respective specific probes, a digoxigenin (Dig)-labeled probe and a fluorescein (F)-labeled probe. Hybrids react with a mixture of aequorin (AEQ) conjugated to anti-digoxigenin antibody and alkaline phosphatase (ALP) conjugated to anti-fluorescein antibody. AEQ is determined first by Ca^{2+} addition and ALP is determined by adding a chemiluminogenic substrate.

determination of many target DNAs in the same well. The color of emitted light can also serve as an extra parameter to distinguish between the synthesized luciferase reporters.

9.2 Quantitative PCR

In quantitative PCR the goal is to relate the signal obtained from the amplified DNA to the initial number of copies of the target sequence in the sample prior to amplification. The challenge is to compensate for sample-to-sample variation of the amplification efficiency due to the presence of inhibitors and the

variability in reaction conditions. This is achieved by co-amplifying, in the same reaction tube, the target sequence with a synthetic DNA or RNA competitor (internal standard, IS) that has the same primer binding sites and similar size to the amplified target (competitive PCR). As a result, the ratio of the two amplification products (target/competitor) is linearly related to the initial amount of target in the sample.[33,34]

Competitive PCR requires highly sensitive assays for the amplification products. This is because the amplification of the target suppresses the amplification of the competitor to undetectable levels (if the target is much higher than the competitor) or *vice versa*, *i.e.*, the amplification of the competitor overpowers the target amplification (if the target is present in minute amounts with respect to the competitor). Thus, the use of an IS is only possible in assays that are highly sensitive in detecting the amplified products.[35,36]

Electrophoresis-based competitive PCR (slab gel or capillary electrophoresis) requires internal standards differing in size (insertion or deletion) to enable electrophoretic separation of the amplification products from target DNA and IS. However, sequence length is also a major determinant of the amplification efficiency. Differences in size cause differences in amplification efficiency.[37] Alternatively, the IS may have the same size as the target but contains a new restriction site, allowing product digestion prior to electrophoresis. The use of HPLC may facilitate the separation and quantification of the two products.[38] It has been observed, however, that co-amplification of substantially homologous DNA sequences, such as target and IS, yields heteroduplexes during PCR due to hybridization of target strands with IS strands (even if their sizes are different).[37] Heteroduplexes may cause errors in electrophoresis if they cannot be resolved from the homoduplexes. In the case of an IS having the same size as the target, but differing only in a restriction site, the heteroduplexes interfere because they are resistant to digestion.

Below, we discuss quantitative PCR methods based on the detection of amplification products by hybridization. Following PCR, the target DNA and the IS are quantified by hybridization to specific probes allowing confirmation of the amplified sequences and high sensitivity. The IS has the same size as the target DNA, thus circumventing the problem of variation of amplification efficiency. In addition, heteroduplex formation is not a concern because only one strand of DNA (target or IS) is captured on the microtiter well through hybridization.

In one report the authors developed a dual-analyte chemiluminescence hybridization assay for quantitative PCR, which allowed simultaneous determination of both amplified target DNA and IS in the same microtiter well.[39] The target DNA from the sample was co-amplified with a constant amount of a recombinant DNA IS that had the same size and primer binding regions as the target DNA, differing only by a 24-bp sequence, located between the primers. Biotinylated PCR products from target DNA and IS were captured on a single microtiter well coated with streptavidin. The non-biotinylated strands were removed by NaOH treatment. The immobilized single-stranded target DNA and IS were allowed to simultaneously hybridize with their respective specific

probes, *i.e.* a digoxigenin-labeled probe and a fluorescein-labeled probe. The hybrids were determined by allowing aequorin conjugated to anti-digoxigenin antibody and ALP conjugated to anti-fluorescein antibody (in the same mixture) to bind to their corresponding haptens. Aequorin was determined by adding Ca^{2+}. ALP was measured by the subsequent addition of CSPD (without prior washing of the wells) (Figure 9.10). The ratio of the luminescence values obtained from the target DNA and IS amplification products was linearly related to the number of target DNA molecules present in the sample prior to amplification. The linear range of the assay extended from 430 to 315 000 target DNA molecules. The S/B ratios observed at 430 molecules of target DNA in the presence of 10 000, 20 000 and 40000 molecules of IS were 4.4, 2.4 and 2.7, respectively. Since only 5% of the initial PCR mixture was used in the assay, the luminescence signal was essentially obtained from amplification product corresponding to 22 target DNA molecules.

A bioluminescence hybridization assay using aequorin was applied to the detection of PSA mRNA as discussed in Section 9.1.[1] The detection of PSA mRNA was extended to the quantification of PSA mRNA in a subsequent paper. The authors reported a simple, rapid and sensitive assay protocol for the quantification of PSA mRNA in peripheral blood (a potential marker for molecular staging of prostate cancer) by using an IS, RT-PCR and a chemiluminometric hybridization assay.[40] The key to quantification was the recombinant RNA IS, which had the same primer binding sites and size as the amplified PSA mRNA but differed only in a 24-bp segment. Amplified sequences were labeled with biotin during PCR by using a 5′ biotinylated upstream primer. The products were heat-denatured and hybridized with oligonucleotide-specific probes (for PSA and IS) that were immobilized in separate microtiter wells. The hybrids were measured using ALP–streptavidin conjugate and a chemiluminogenic substrate (Figure 9.4). The ratio of the luminescence values obtained for the PSA mRNA and the RNA IS was a linear function of the initial amount of PSA mRNA present in the sample prior to RT-PCR. As few as 50 copies of PSA mRNA were detected with an S/B ratio of 2. The linearity of the assay extended up to 500 000 copies of PSA mRNA. Samples containing total RNA from PSA-expressing LNCaP cells gave luminescence ratios that were linearly related to the number of cells in the range 0.04–400 cells.

Another marker for the molecular staging of prostate cancer is prostate-specific membrane antigen (PSMA) mRNA. PSMA mRNA expression is restricted to the prostate. This tissue-specificity renders PSMA mRNA an excellent candidate marker for molecular staging of prostate cancer. A quantitative competitive RT-PCR method has been reported, in which the biotinylated PCR products from amplified target and IS were captured on streptavidin-coated wells and detected by using conjugates of oligonucleotide probes with ALP.[41] The probes were specific for PSMA and IS. The use of probes that are directly labeled with the reporter molecule made the assay procedure shorter (streptavidin: biotinylated target: probe-reporter conjugate) as opposed to indirect labeling in which the reporter molecule is conjugated to a

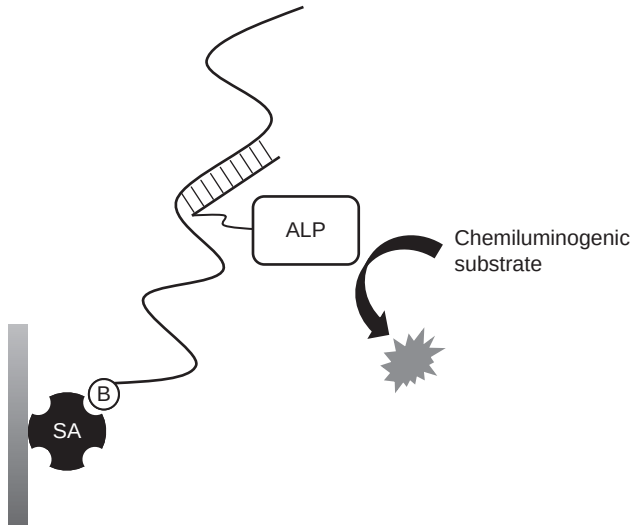


Figure 9.11 Chemiluminometric hybridization assay for quantitative PCR. Biotinylated (B) target DNA and biotinylated internal standard are captured in separate streptavidin (SA)-coated wells and denatured by NaOH. Immobilized single-stranded target DNA and internal standard are each hybridized with alkaline phosphatase (ALP) conjugated to target-specific probes.

specific antibody or a hapten (streptavidin: biotinylated target: probe: antibody-reporter conjugate) (Figure 9.11). The ratio of the luminescence values obtained for PSMA mRNA and the RNA IS was a linear function of the initial amount of PSMA mRNA copies initially present in the sample, before RT-PCR. As few as 500 copies of PSMA mRNA were detected with an S/B ratio of 5. The linear range extended from 500 to 5 000 000 PSMA mRNA copies. Samples containing total RNA from PSMA-expressing LNCaP cells gave luminescence ratios linearly related to the number of cells in the range 0.5–5000 cells.

An interesting application of the above principles of quantitative competitive PCR with chemiluminometric detection is in the field of quantification of genetically modified organisms (GMO) in food. A high-throughput double competitive quantitative PCR method has been developed for quantification of the transgene and a plant reference gene.[15] The latter compensates for differences in the total amount and integrity of DNA between samples. The advantage of double competitive PCR (compared to real-time PCR) is that any potential PCR inhibitors equally affect the amplification of target and IS, so that the ratio of their PCR products gives the ratio of their initial amounts in the sample, providing absolute quantification of the GMO-specific sequence and the reference gene. The conventional double competitive PCR method for GMO quantification entails the co-amplification of each target sequence with a

competitive synthetic DNA IS that closely resembles the target DNA and shares the same primers. The IS also contains an insertion or deletion, large enough to allow separation from the target by slab gel electrophoresis. Each sample is titrated with the IS, *i.e.*, increasing, and known, amounts of IS are added to aliquots containing a constant amount of target, followed by PCR and electrophoresis. When the band densities are the same, the starting quantities of target and IS are equal. Internal standards are required for both transgene and reference gene. The conventional double competitive PCR is a low-throughput and labor intensive method due to multiple PCRs required for titration of each sample, electrophoresis and densitometry. A recent improvement in double competitive PCR is the introduction of capillary electrophoresis with laser-induced fluorescence detection for faster and automatable separation of the amplification products.

To address the drawbacks of conventional double competitive PCR, a high-throughput double quantitative competitive PCR (HT-DCPCR) method has been developed for GMO quantification.[42] In HT-DCPCR, electrophoresis and densitometry were replaced by a rapid, microtiter well-based bioluminometric hybridization assay and there was no need for titration of each sample. Instead, the target was co-amplified with a constant amount of IS. The determination of GM soya was chosen as a model. Internal standards (competitors) were constructed both for the transgene (35S promoter sequence) and the reference gene (lectin). Each IS had the same primer binding sites and size with the target sequences but differed in short internal segment. Each target sequence (35S and lectin) was co-amplified with a constant amount of the respective IS. For the hybridization assay, a universal solid phase coated with BSA–$(dT)_{30}$ conjugate was used. Each specific probe consisting of a 24-nt region complementary to its respective analyte and a poly(dA) tail was added to a separate well (four probes each in a separate well and each specific for one analyte). The four analytes (biotinylated amplified fragments of 35S target, 35S IS, lectin target and lectin IS) were denatured and hybridized with their respective specific probes. The hybrids were determined by using aequorin–streptavidin conjugate (Figure 9.12). The ratio of the luminescence values obtained for the target and the IS competitor was linearly related to the starting amount of target DNA. The detectability and linear range of each of the four hybridization assays were established. The limits of quantification were 4 pM amplified 35S target (200 amol $well^{-1}$), 6 pM amplified 35S IS (300 amol $well^{-1}$), 5 pM amplified lectin target (250 amol $well^{-1}$) and 13 pM amplified lectin IS (650 amol $well^{-1}$). The analytical range of the assays extended up to 1000 pM. For the quantitative competitive PCR of 35S promoter, the limit of detection and the limit of quantification were found to be 3 and 24 copies of 35S DNA, respectively. HT-DCPCR was evaluated by determining the GMO content of soybean powder certified reference materials. GMO contents determined by HT-DCPCR were in close agreement with the nominal values of the certified reference materials. In addition, HT-DCPCR was compared to real-time PCR (TaqMan assay) in various real samples. The results obtained by the two methods were in good agreement.

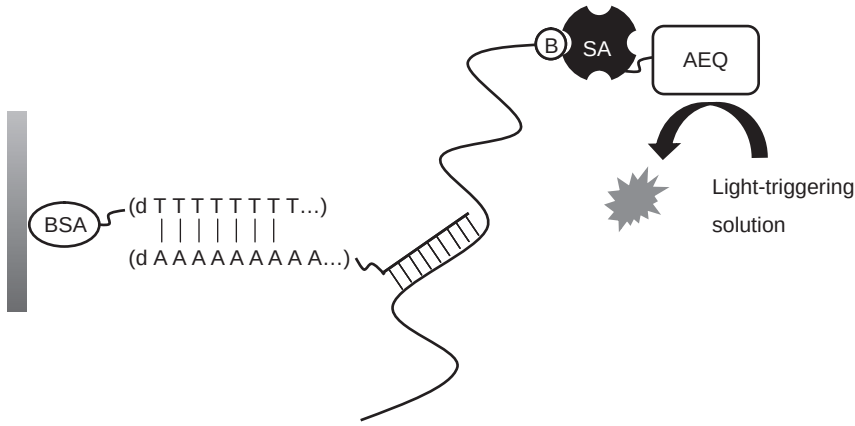
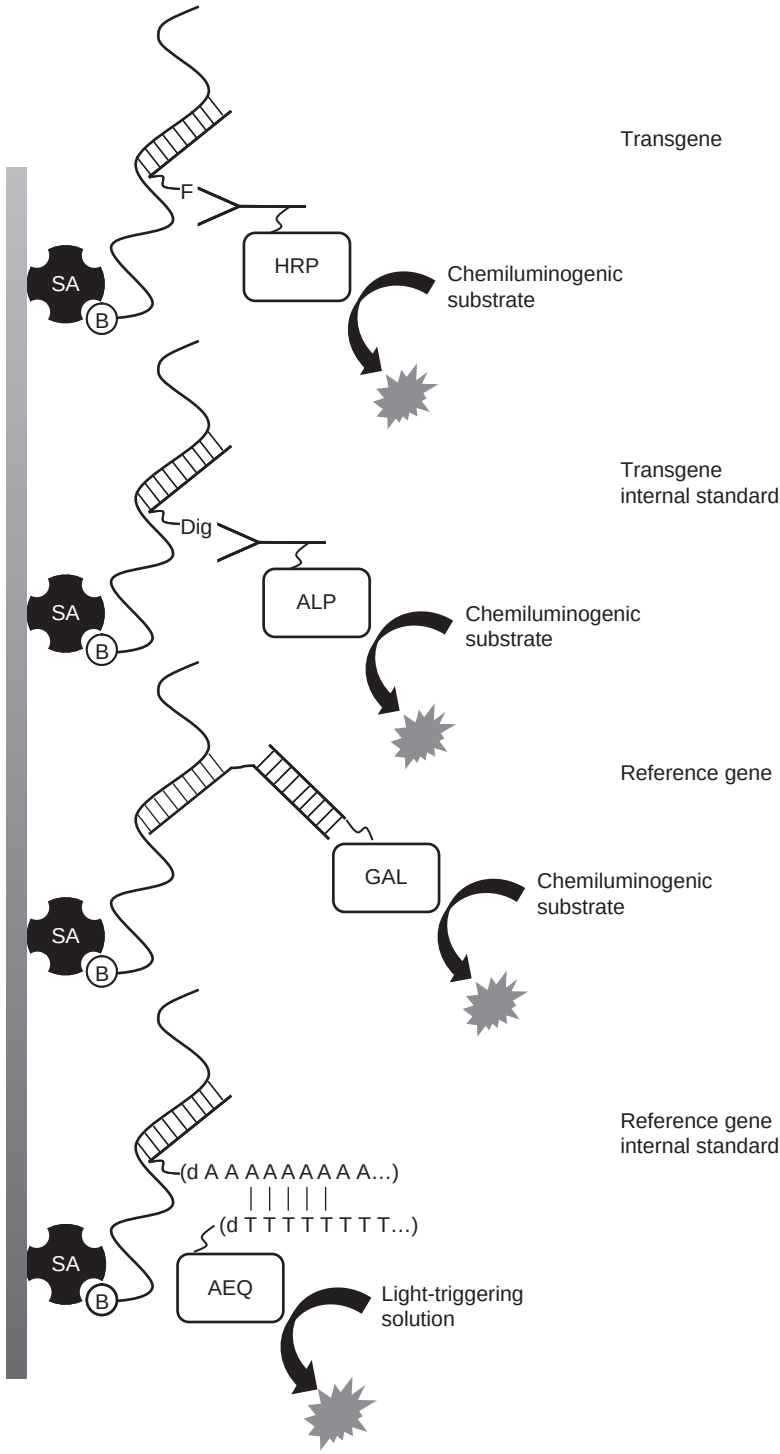


Figure 9.12 Bioluminometric hybridization assay for double quantitative competitive PCR. Wells are coated with bovine serum albumin (BSA)–$(dT)_{30}$ conjugate. For each analyte (35S target, 35S internal standard, lectin target or lectin internal standard), a specific probe consisting of a 24-nt region complementary to the analyte and a poly(dA) tail is added to a separate well. Each denatured biotinylated (B) target DNA is hybridized with its specific probe. Hybrids react with aequorin (AEQ)–streptavidin (SA) conjugate. AEQ is determined by Ca^{2+} addition.

The advantages of the HT-DCPCR assay are as follows. First, the high detectability that arises from the bioluminometric hybridization assay allows accurate and precise determination of the PCR products of each target and its respective IS despite the suppression of amplification due to their competition for the same primer set. This in turn enables quantification of the target sequence without the need for titration of each sample with various quantities of IS. Second, the hybridization assay is performed on a universal solid phase to ensure simplicity and high-throughput. The overall procedure (including PCR and hybridization assay) was completed in 2.5 h. Third, the amplified sequences for target and IS have identical sizes. This is an advantage over electrophoretic based double competitive PCR which requires internal standards differing in size, leading to differences in amplification efficiency. Fourth, heteroduplexes (as formed during co-amplification of substantially homologous DNA sequences) are not an issue because only one strand is captured on the microtiter well through hybridization.

The next challenge is the development of multi-analyte hybridization assays for simultaneous quantification of several target sequences in the same sample. Advantages include higher sample throughput, reduced consumption of reagents, smaller sample volume and lower cost of analysis compared to single-analyte assays. In principle, multi-analyte configurations require either the use of a single reporter along with spatial separation of the assays or the successful combination of several labels in a single assay. Spatial separation along with a single label has been employed in a multi-analyte hybridization assay for detection of various pathogens by constructing microtiter plates containing


Transgene
F
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Chemiluminogenic substrate
Transgene internal standard
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Light-triggering solution

main wells with built-in sub-wells, with each sub-well corresponding to a single assay.[42] Few reports describe the combination of two labels in a single hybridization assay with applications to the quantification of PCR products.[4,32] These assays are based on the combination of two chemiluminescent labels (detection of two targets in the same sample and in one microtiter well).

A more recent advance is the development of a quadruple-analyte chemiluminometric hybridization assay for simultaneous quantification of four nucleic acid sequences.[43] As a model, the assay was applied to double quantitative competitive PCR for the determination of GMOs. The transgene is denoted NK, the reference gene is denoted IVR (invertase), and their respective internal standards (competitors) are NK-IS and IVR-IS. The four targets were amplified by a single PCR. The four biotinylated PCR products were then captured in the same microtiter well, which is coated with streptavidin. The non-biotinylated strands were removed by NaOH treatment, and the immobilized single-stranded DNA fragments were allowed to hybridize with a mixture of four specific probes. Each probe contained a sequence complementary to its respective target and a sequence or a hapten that allowed linkage with a unique chemiluminescent reporter. Specifically, NK and NK-IS probes were labeled at the 3′ end with the haptens fluorescein and digoxigenin, respectively, to allow recognition by HRP-anti-fluorescein and ALP-anti-digoxigenin antibodies. The IVR-IS probe consisted of a segment at the 3′ end complementary to IVR-IS and a sequence at the 5′ end complementary to the oligonucleotide that was conjugated to β-galactosidase (GAL). In the middle of the IVR-IS probe, a 14-nt random spacer was introduced to avoid interference with hybridization. The IVR probe carried a poly(dA) tail at the 3′ end, which was recognized by aequorin–$(dT)_{30}$ conjugate. Next, a mixture containing the four chemiluminescent reporters was added to the well (HRP conjugated to anti-fluorescein antibody, ALP conjugated to anti-digoxigenin antibody, GAL-oligo conjugate and aequorin–$(dT)_{30}$ conjugate). The four chemiluminescent reactions were triggered sequentially. The flash-type reaction of aequorin was triggered first by adding Ca^{2+} and decayed in a few seconds. The glow-type chemiluminescent reactions catalyzed by HRP, GAL and ALP were then triggered sequentially by adding appropriate substrates (Figure 9.13). Regarding the order in which

Figure 9.13 Quadruple-analyte bio(chemi)luminometric hybridization assay for double quantitative competitive PCR. The four biotinylated (B) target DNAs (transgene, transgene internal standard, reference gene and reference gene internal standard) are captured in a single streptavidin (SA)-coated well and denatured by NaOH. Immobilized single-stranded DNAs are hybridized with specific probes (a mixture of four probes is added to the well). Each probe contains a sequence complementary to its respective target and a sequence or a hapten [fluorescein (F) or digoxigenin (Dig)] that allows linkage with its unique reporter. Hybrids react with a mixture of horseradish peroxidase (HRP) conjugated to anti-fluorescein antibody, alkaline phosphatase (ALP) conjugated to anti-digoxigenin antibody, β-galactosidase (GAL)–oligonucleotide conjugate, and aequorin (AEQ)–$(dT)_{30}$ conjugate. The four reporters are determined sequentially in the same well.

HRP, GAL and ALP activities were measured, the authors observed that the GAL signal decreased considerably when measured after ALP (possible GAL inactivation due to exposure to the alkaline solution of the ALP substrate). The measurement order of HRP activity did not affect GAL. Based on these findings, the measurement order followed in the quadruple-hybridization assay was aequorin, HRP, GAL and ALP. A successful quadruple-analyte hybridization assay requires the absence of signal cross-talk between the reporters. A washing step was introduced after each measurement to remove one substrate before addition of the next. The signals were linearly related to the concentration of target sequences. The limits of quantification were 9 pM amplified IVR DNA (0.45 fmol well^{-1}), 3 pM of IVR-IS DNA (0.15 fmol well^{-1}), 6 pM of NK DNA (0.3 fmol well^{-1}) and 5 pM amplified NK-IS DNA (0.25 fmol well^{-1}). The analytical range extended up to 1000 pM DNA. For quantitative competitive PCR of NK or IVR, the S/B ratio at 50 copies of NK and IVR target DNA was 1.6 and 2.8, respectively. This assay combined the high sensitivity, wide dynamic range and simple instrumentation of chemiluminometric assays with the ability to simultaneously quantify four nucleic acid sequences in a single microtiter well. The assay is universal since the same reporter conjugates can be used for multi-analyte quantification of any sequences with properly designed sequence-specific probes.

9.3 Genotyping of Single Nucleotide Polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs) constitute the most common form of human genetic variation. The method chosen for SNP detection depends on whether we are screening for unknown or known mutations. In this section, we focus on the detection of known mutations and particularly on methods that are suitable for the routine molecular diagnosis laboratory. DNA microarray technology plays an important role in genome-wide association studies of various SNPs with certain diseases because each chip enables parallel genotyping of thousands of SNPs in a single sample. However, it is expected that only a small number of SNPs will be routinely analyzed in the clinical setting. For instance, SNPs in disease-related genes will be tested for diagnosis and monitoring of disease; SNPs in genes encoding drug-metabolizing enzymes will be detected to design improved therapeutic strategies. Consequently, in the routine clinical laboratory high sample-throughput is much more useful than high SNP-throughput.

SNP genotyping methods, in general, include isolation of genomic DNA, exponential amplification (usually by PCR) of the region that spans the SNP, a genotyping reaction to distinguish the alleles and, finally, the detection of the genotyping reaction products. Sequencing of the amplified fragment is the reference method, but the relatively high cost of this technique prohibits its wide use in the clinical laboratory. Most genotyping techniques are based on one of the following principles. PCR combined with restriction fragment length

polymorphism (RFLP) analysis is a commonly used method, but its low-throughput is a major drawback. Several genotyping methods for known mutations rely on the hybridization of allele-specific oligonucleotide probes (ASOs) under conditions that allow discrimination of a perfect match from a mismatch. ASOs may also be used as PCR primers because DNA polymerase amplifies only when the 3′ end of the primer perfectly complements the target. This approach, however, requires two PCRs per SNP. Enzyme-based genotyping assays, such as primer extension (PEXT), oligonucleotide ligation reaction (OLR) and invasive cleavage, have proven to be more robust and specific than ASO hybridization. The PEXT reaction is the most widely used because it is the simplest, it requires the least number of probes, and it can be easily optimized. Mini-sequencing, pyrosequencing, flow cytometry and mass spectrometry have been used for the detection of PEXT products. Here we discuss the development of cost-effective, rapid, automatable high-throughput bio(chemi)luminometric assays for SNP detection using PEXT or OLR.

One such method is based on the fact that the kinetics of light emission of chemiluminescent reactions varies considerably. The flash-type emission from an acridinium ester or the aequorin reaction has a decay half-life of about 1 s. The glow-type emission from enzyme-catalyzed chemiluminescent reactions may last several minutes or hours. The variation in emission kinetics allows the development of multi-analyte hybridization assays.

To this end, the difference in light-emission kinetics between the aequorin bioluminescent reaction and the ALP-catalyzed chemiluminescent reaction has been exploited for the analysis of bi-allelic polymorphisms in a single microtiter well.[44] The genotyping of the IVS-1-110 locus of the human β-globin gene was chosen as a model. Genomic DNA, isolated from whole blood, was first subjected to PCR using primers flanking the polymorphic site. A single OLR employing two allele-specific probes (normal probe, N; mutant probe, M) and a common probe (C) carrying a characteristic tail was then performed.[45] Probe N was labeled at the 5′ end with biotin whereas the 3′ end had a nucleotide specific for the normal allele. Probe M was labeled at the 5′ end with digoxigenin whereas the 3′ end had a nucleotide specific for the mutant allele. Probes N and M were designed to anneal to the target DNA at a position adjacent to probe C. Probes that are perfectly matched to the target sequence are covalently joined by the ligase. In contrast, a mismatch at the junction inhibits ligation. Thus, two different ligation products, depending on allele composition of the sample, may be formed. Biotin-labeled N-C was formed when the normal allele was present and digoxigenin-labeled M-C was formed when the mutant allele was present (Figure 9.14a). Next, the ligation products were captured in a single microtiter well through hybridization of the tail of the C probe with an immobilized complementary oligonucleotide (prior to capturing, the ligation products were heat-denatured to ensure separation of the non-ligated probes). The ligation products were detected by adding a mixture of biotinylated aequorin complexed to streptavidin and ALP conjugated to anti-digoxigenin antibody. The characteristic Ca^{2+}-triggered bioluminescence of aequorin (flash-type) was measured first, followed by the addition of the dioxetane aryl

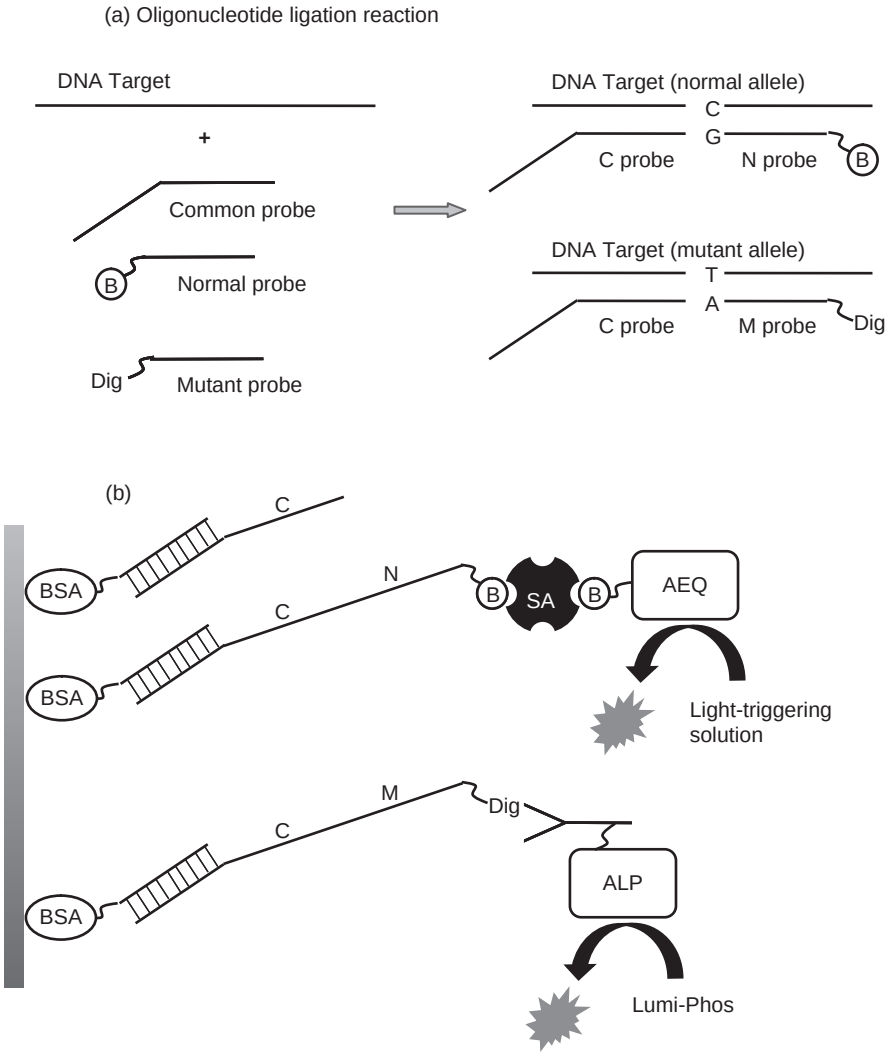

Figure 9.14 (a) Illustration of the principle of the oligonucleotide ligation reaction (OLR). The assay employs two allele-specific probes (normal probe, N; mutant probe, M) and a common probe (C) carrying a characteristic tail. N is 5′ labeled with biotin (B) and the 3′ end has a nucleotide specific for the normal allele. M is 5′ labeled with digoxigenin (Dig) and the 3′ end has a nucleotide specific for the mutant allele. N and M probes anneal to the target DNA at a position adjacent to C. Probes that are perfectly matched to the target sequence will be covalently joined by the ligase. (b) Dual-analyte bio(chemi)luminometric hybridization assay based on combined flash- and glow-type reactions for SNP genotyping by OLR. Denatured ligation products are hybridized in a single well with an immobilized probe [oligonucleotide conjugated to bovine serum albumin (BSA)]. Hybrids react with a mixture of biotinylated (B) aequorin (AEQ) complexed to streptavidin (SA) and alkaline phosphatase (ALP) conjugated to anti-digoxigenin antibody. AEQ is determined first by Ca^{2+} addition and ALP is determined by adding a chemiluminogenic substrate.

phosphate substrate for ALP (Figure 9.14b). The ratio of the luminescence signals obtained from ALP (signified mutant allele) and aequorin (signified normal allele) gave the genotype of each sample. A heterozygote for the mutation gave both signals. Free C probe (not ligated) was also captured to the well but not detected. The dual-analyte bio(chemi)luminometric genotyping assay provided clear distinction of the three genotypes with signal ratios differing by more than an order of magnitude. The assay is an excellent candidate for high-throughput genotyping of a large number of individuals for bi-allelic polymorphisms.

The previously mentioned assay used two different reporters for detection of the oligonucleotide ligation products in a single well.[44] An alternative genotyping assay was subsequently developed in which the high specificity of OLR was again combined with the simplicity and sensitivity of chemiluminometric detection but this time the assay employed a universal detection approach that allowed simultaneous analysis of several samples for various SNPs in the same microtiter plate.[46] The method was applied to the genotyping of four SNPs within the genes of histamine H2 receptor (HRH2), serotonin receptor (HTR2A1 and HTR2A2) and b3 adrenergic receptor (ADRB3). SNPs in these neurotransmitter receptor genes form the basis of pharmacogenetic studies on the efficacy of anti-psychotic agents.

Genomic DNA, isolated from whole blood, was first subjected to PCR using primers flanking the polymorphic site. A single OLR employing two allele-specific probes (normal probe, N; mutant probe, M) and a biotinylated common probe (C) was then performed. Probe N was labeled at the 3′ end with digoxigenin whereas the 5′ end contained a nucleotide specific for the normal allele. Probe M was labeled at the 3′ end with fluorescein whereas the 5′ end contained a nucleotide specific for the mutant allele. Thus two different ligation products were formed. Digoxigenin-labeled N–C was formed when the normal allele was present and fluorescein-labeled M–C was formed when the mutant allele was present. Next, the ligation products were captured in streptavidin-coated microtiter wells through the biotin moiety of the C probe and denatured by NaOH treatment. The ligation products were detected by adding either ALP conjugated to anti-digoxigenin antibody or ALP conjugated to anti-fluorescein antibody (for each SNP to be analyzed there was one OLR whose products were then split into two wells for capturing) (Figure 9.15). The ratio of the luminescence signals obtained from ALP conjugated to anti-digoxigenin antibody (signified normal allele) and ALP conjugated to anti-fluorescein antibody (signified mutant allele) gave the genotype of each sample. A heterozygote for the mutation gave both signals. Free C probe (not ligated) was also captured in the well but not detected.

The use of aequorin as a reporter molecule in the detection of oligonucleotide ligation products as applied to SNP genotyping[44] was followed by the development of a hybridization assay for the detection of primer extension (PEXT) products by aequorin.[47] As discussed in the previous sections, conjugates of aequorin with antibodies or streptavidin had already been successfully used as reporter molecules for the detection of PCR products and for quantitative PCR.[4,42]

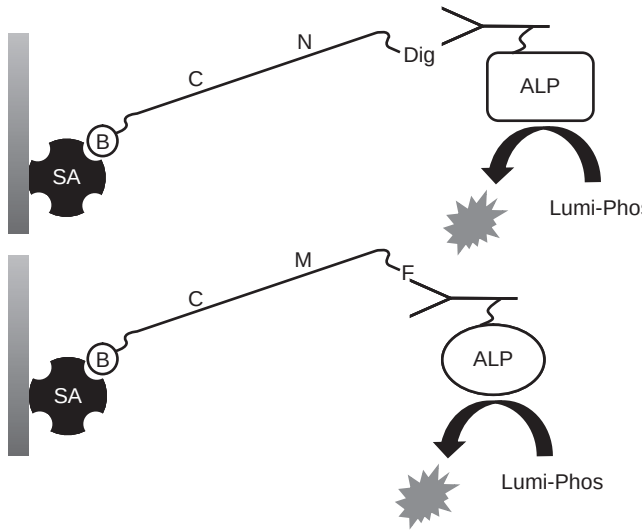

Figure 9.15 Chemiluminometric hybridization assay based on a universal detection approach for SNP genotyping by OLR (oligonucleotide ligation reaction). Following OLR, ligation products are captured on streptavidin (SA)-coated wells through the biotin moiety of the C probe and denatured by NaOH. Ligation products react with either alkaline phosphatase (ALP) conjugated to anti-digoxigenin antibody or ALP conjugated to anti-fluorescein antibody (for each SNP to be analyzed there is one ligation reaction whose products are then split into two wells for capturing).

The developed assay was applied to SNP genotyping of the mannose-binding lectin 2 (MBL2) gene.[47] Since MBL2 SNPs have been associated with the functional deficiency of MBL (a key component of the innate immune system), there is a growing need to develop genotyping methods for screening MBL2 allelic variants. The method involves the following: (a) a single PCR to amplify the genomic region of interest encompassing all six variant nucleotide sites, (b) PEXT reactions (using unpurified PCR products) in the presence of biotin-dUTP and a DNA polymerase lacking 3′-5′ exonuclease activity and (c) a microtiter well-based assay of the extension products with an aequorin–streptavidin conjugate. Two PEXT reactions were performed for each site. For each variant, two primers were designed for PEXT (normal and mutant primer) and they were extended in two separate reactions. Each of these primers contained a poly(dA) segment at the 5′ end and an allele-specific nucleotide at the 3′ end. The distinction between genotypes is based on the high accuracy of nucleotide incorporation by DNA polymerase. Biotin-dUTP (along with the other dNTPs) was incorporated in the extended primer only when the primer was a perfect match to an allele (Figure 9.16a). The products were captured by hybridization on the surface of microtiter wells that were coated with BSA–$(dT)_{30}$ conjugate. Only the extended primers (with the incorporated biotins)

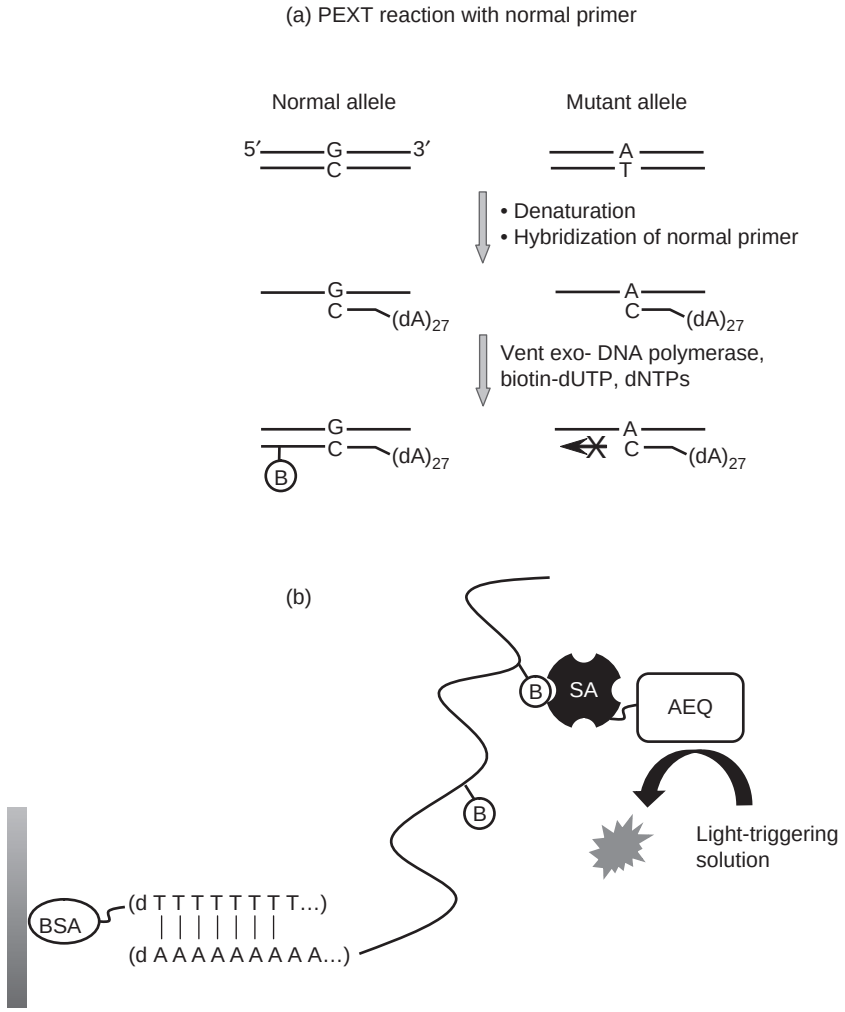

Figure 9.16 (a) Primer extension (PEXT) reaction with normal primer. Two primers (normal and mutant) are designed for PEXT and they are extended in two separate reactions. Each of these primers contains a poly(dA) segment at the 5′ end and an allele-specific nucleotide at the 3′ end. Biotin-dUTP (along with the other dNTPs) is incorporated by DNA polymerase in the extended primer only when the primer is a perfect match to an allele. (b) Bioluminometric hybridization assay based on aequorin for SNP genotyping by PEXT reaction. The products of each PEXT reaction are captured on wells coated with bovine serum albumin (BSA)–$(dT)_{30}$ conjugate. The extended primers [with the incorporated biotins (B)] are detected by reaction with an aequorin (AEQ)–streptavidin conjugate. AEQ is determined by Ca^{2+} addition.

were detected by reaction with an aequorin–streptavidin conjugate (Figure 9.16b). Genotypes were assigned by the signal ratio of the normal-specific primer to the mutant-specific primer. The PEXT reaction was completed in 10 min and the detection of the products in less than 40 min. The assay is a cost-effective, rapid, robust and automatable method for detecting all known allelic variants of MBL2, introducing for the first time aequorin as a reporter in genotyping by PEXT reaction.

A method that combines the high detectability and dynamic range of chemiluminescence with the high allele-discrimination ability of PEXT has been developed for the simultaneous characterization of 15 SNPs in a high-throughput microtiter well-based assay in a dry-reagent format.[48] As a model for the development and validation of the method, the 15 most common β-hemoglobin (*HBB*) gene mutations found in the populations of the Mediterranean basin were detected. Although various methods for *HBB* genotyping have been reported there is still a need for a cost-effective method (in terms of both equipment and reagents) with high sample-throughput, for simultaneous screening of several mutations. The method consist of: (a) duplex PCR to amplify the genomic region of interest producing two fragments encompassing all 15 mutations, (b) PEXT reactions (using unpurified PCR products) in the presence of fluorescein-dCTP and (c) a microtiter well-based assay of extension products with HRP conjugated to anti-fluorescein antibody and a chemiluminogenic substrate. Two PEXT reactions were performed for each mutation. Two primers were designed for PEXT (normal and mutant primer) and they were extended in two separate reactions. Each of these primers contained a biotin at the 5′ end and an allele-specific nucleotide at the 3′ end. Fluorescein-dCTP was incorporated in the extended primer only when the primer was a perfect match to an allele. The products were captured by hybridization on the surface of microtiter wells coated with streptavidin. Only the extended primers (with the incorporated fluoresceins) were detected by reaction with HRP conjugated to anti-fluorescein antibody (Figure 9.17). Genotypes were assigned by the signal ratio of the normal-specific primer to the mutant-specific primer. A significant advantage is that the assay used lyophilized reagents for PCR and PEXT reactions, as well as dried streptavidin-coated wells. The lyophilized and dried reagents had a long shelf-life when stored at 4 °C, and the use of pre-prepared lyophilized and dried reagents in a microtiter plate format made the entire assay much faster (about 3.5 h including PCR). This feature makes the method attractive for the routine molecular diagnostic laboratory.

The previously mentioned methods for SNP detection by PEXT require spatial separation during the capture of the PEXT products (two separate wells followed by two separate assays for detection).[47,48] A major step was the subsequent development of a dual-analyte bio(chemi)luminometric assay for SNP genotyping by PEXT.[49] The method consists of: (a) one PCR to amplify the genomic region of interest, (b) PEXT reactions in the presence of digoxigenin-dUTP (normal primer) and biotin-dUTP (mutant primer) and (c) a microtiter well-based assay of extension products with ALP conjugated

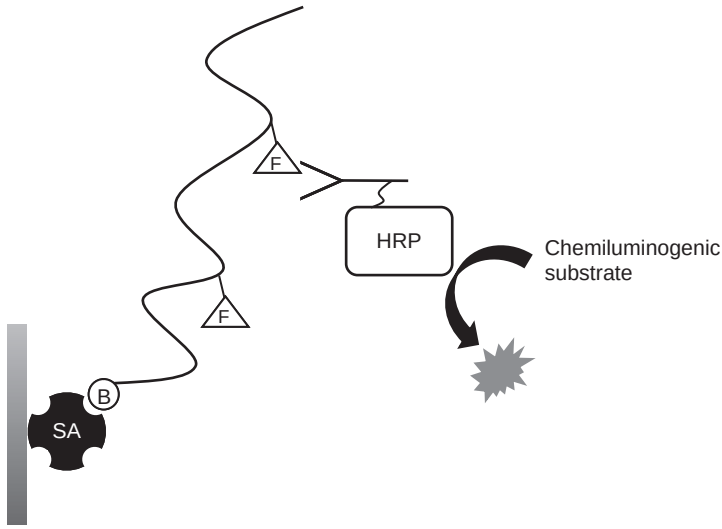

Figure 9.17 Chemiluminometric hybridization assay based on a dry-reagent format for genotyping of 15 SNPs by PEXT reaction. The products of each PEXT reaction are captured on streptavidin (SA)-coated wells. Only the extended primers [with the incorporated fluoresceins (F)] are detected by reacting with horseradish peroxidase (HRP) conjugated to anti-fluorescein antibody.

to anti-digoxigenin antibody (for detection of the normal allele) and an aequorin–streptavidin conjugate (for detection of the mutant allele). For each SNP, two primers were designed for PEXT (normal and mutant primer) and they were extended in two separate reactions. Each of these primers contained a poly(dA) segment at the 5′ end and an allele-specific nucleotide at the 3′ end. The PEXT reaction of the normal primer was performed in the presence of digoxigenin-dUTP. The PEXT reaction of the mutant primer was performed in the presence of biotin-dUTP. The digoxigenin-labeled product (extension of normal primer) and the biotin-labeled product (extension of mutant primer) were captured by hybridization on the surface of a single microtiter well coated with BSA–$(dT)_{30}$ conjugate (for each SNP to be analyzed there were two separate PEXT reactions whose products were then captured on a single well). Detection was performed by adding a mixture of ALP conjugated to anti-digoxigenin antibody and an aequorin–streptavidin conjugate. First, aequorin was determined by its characteristic Ca^{2+}-triggered bioluminescence. ALP was then measured by adding a chemiluminogenic substrate (Figure 9.18). The ALP/aequorin signal ratio gave the genotype of the SNP.

In the above papers,[47–49] all assays used PEXT reactions for allele discrimination and, in all cases, both extended and non-extended primers were captured to the solid phase either through their poly(dA) segment[47,49] or their

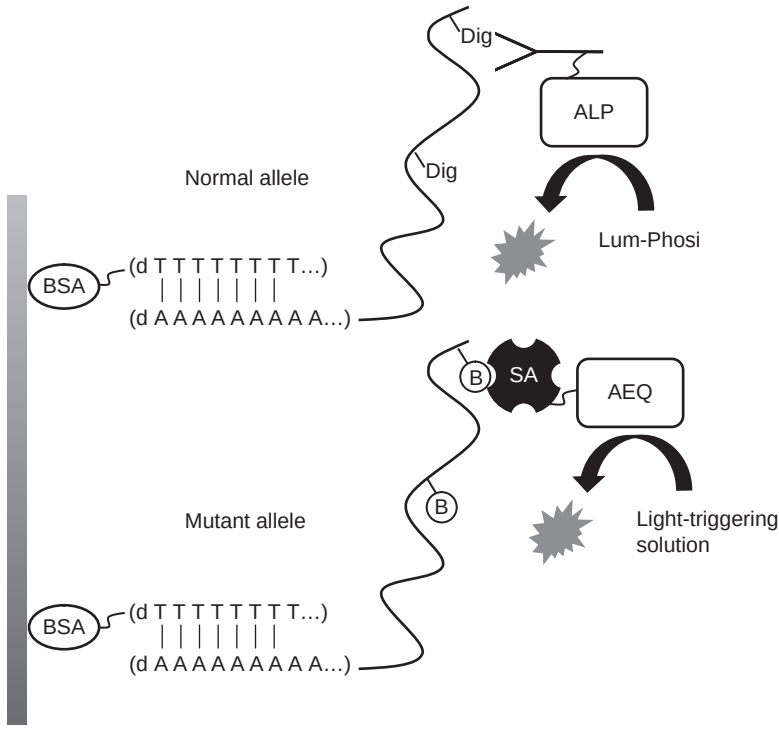

Figure 9.18 Dual-analyte bio(chemi)luminometric hybridization assay based on combined flash- and glow-type reactions for SNP genotyping by PEXT reaction. The products of both PEXT reactions [a digoxigenin (Dig)-labeled product from extension of normal primer and a biotin (B)-labeled product from extension of mutant primer] are captured in a single well coated with bovine serum albumin (BSA)–$(dT)_{30}$ conjugate. Hybrids react with a mixture of alkaline phosphatase (ALP) conjugated to anti-digoxigenin antibody and an aequorin (AEQ)–streptavidin conjugate. AEQ is determined first by Ca^{2+} addition and ALP is determined by adding a chemiluminogenic substrate.

biotin moiety.[48] The difference in a subsequent paper is that only the extended primers were captured to the solid phase through the incorporated biotins.[50] The genotyping of two SNPs (A896G and C1196T) in the toll-like receptor 4 gene was chosen as a model. Toll-like receptors (TLRs) play a fundamental role in pathogen recognition and activation of innate immunity and SNPs in TLR have been associated with reduced host immune response to TLR ligands. The method consists of: (a) PCR to amplify the genomic region of interest, (b) PEXT reactions (using unpurified PCR products) in the presence of biotin-dUTP and (c) a microtiter well-based assay of extension products with an aequorin–$(dT)_{30}$ conjugate. Two PEXT reactions were performed for each SNP. Two primers were designed for PEXT (normal and mutant primer) and

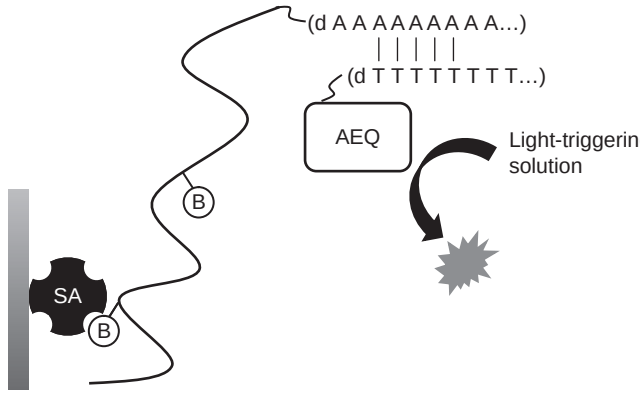

Figure 9.19 Bioluminometric hybridization assay based on aequorin for SNP genotyping by PEXT reaction. The products of each PEXT reaction are captured on streptavidin (SA)-coated wells. Only the extended primers [with the incorporated biotins (B)] are captured on the wells and detected by adding an aequorin–$(dT)_{30}$ conjugate. AEQ is determined by Ca^{2+} addition.

they were extended in two separate reactions. Each of these primers contained a poly(dA) segment at the 5′ end and an allele-specific nucleotide at the 3′ end. Biotin-dUTP was incorporated in the extended primer only when the primer was a perfect match to an allele. The extended products were captured on streptavidin-coated wells and detected by using an aequorin–$(dT)_{30}$ conjugate (Figure 9.19).[13] Genotypes were assigned by the allelic ratio (AR), which was calculated from the equation $AR = L_N/(L_N + L_M)$, where L_N and L_M are the luminescence signals obtained from PEXT reaction with the N and M primer, respectively. The theoretical values of AR for a normal sample (N/N), a heterozygote and a mutant homozygote are 1, 0.5 and 0, respectively. The authors also investigated and presented the possibility of screening pooled samples to reduce cost and increase throughput, a feature that makes this already cost-effective assay (compared to homogeneous fluorometric assays and mass spectrometry) even more attractive. The method is also rapid, as opposed to other time-consuming methods reported for TLR4 SNPs (RFLP analysis), and amenable to automation.

The high-throughput PEXT-based bioluminometric assay for genotyping A896G and C1196T SNPs in the TLR4 gene was followed by the development of a quadruple-analyte PEXT-based bio(chemi)luminometric assay for the simultaneous genotyping of the two TLR4 SNPs (simultaneous detection of four alleles).[51] This became possible by successfully combining four reporters, namely, aequorin, HRP, β-galactosidase (GAL) and ALP. A quadruple PEXT reaction was first performed in the presence of two pairs of allele-specific primers and biotin-dUTP (PCR products were used without purification). For each SNP, two primers were designed for PEXT (normal and mutant primer,

N and M) with allele-specific nucleotides at the 3′ end. Each primer carried a characteristic label (hapten or oligonucleotide) at the 5′ end to enable detection through a specific reporter (enzyme or photoprotein). The N and M primers for the 896 SNP were 5′-labeled with fluorescein and digoxigenin, respectively. The N primer for the 1196 SNP contained a characteristic oligonucleotide sequence at the 5′ end, which enabled detection through a complementary oligonucleotide that is conjugated to GAL. The M primer for the 1196 SNP contained a $(dA)_{21}$ sequence at the 5′ end that allowed detection through an aequorin–$(dT)_{30}$ conjugate. Biotin–dUTP was incorporated in the extended primer. The biotin-labeled products were captured by hybridization on the surface of a single microtiter well coated with streptavidin (for the two SNPs to be analyzed there was one PEXT reaction whose products were then captured on a single well). Detection was performed by adding a mixture of HRP conjugated to anti-fluorescein antibody (detection of 896 N allele), ALP conjugated to anti-digoxigenin antibody (detection of 896 M allele), GAL conjugated to a characteristic oligonucleotide (detection of 1196 N allele) and aequorin–$(dT)_{30}$ conjugate (detection of 1196 M allele). PEXT products were used without purification. The four reporters were determined in the same microtiter well (Figure 9.20). The HRP/ALP signal ratio gave the genotype of the 896 SNP whereas the GAL/aequorin signal ratio gave the genotype of the 1196 SNP. The PEXT reaction was completed in 15 min and the detection of the products in 75 min. The cost of the method was considerably reduced by performing four PEXT reactions in the same tube followed by detection of four PEXT products in the same well. The microtiter plate assay format facilitates automation of the genotyping method and provides high sample-throughput. PCR for the TLR4 gene was designed in such a way that both SNPs were present in the same amplified fragment. However, the loci of the two SNPs need not be contiguous, since a duplex PCR can be performed, providing two amplified fragments with the loci of interest. In this report, multi-analyte detection is accomplished through the use of four different reporters. A different chemiluminometric assay achieves multi-analyte detection through spatial separation.[42] The two concepts are not competitive but rather can be combined to further enhance the multiplicity of DNA targets.

9.4 Determination of Allele Burden

Thus far, we have discussed methods for detection of SNPs as applied to mutations that are inherited. Unlike inherited mutations, which are present in all cells, somatic (acquired) mutations occur only in certain cells of the body. Thus, somatic mutations are considered the primary cause of human cancer. An example is the somatic point mutation V617F of the JAK2 kinase, a recently discovered diagnostic marker for myeloproliferative neoplasms. The challenge with somatic point mutations is to develop sensitive, robust and practical methods to quantify the mutant allele while discriminating from a

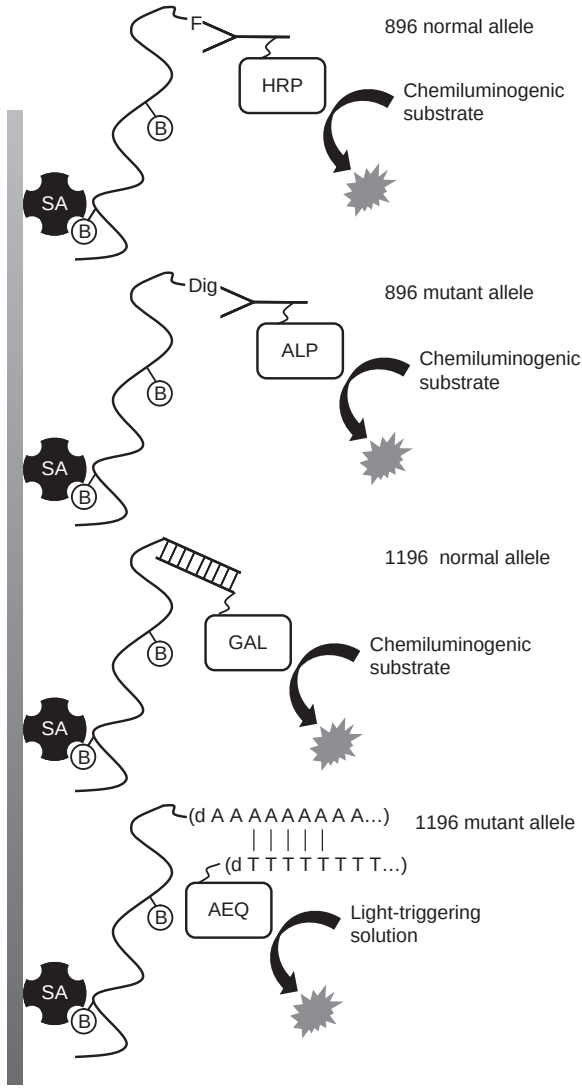

Figure 9.20 Quadruple bio(chemi)luminometric hybridization assay for SNP genotyping by PEXT reaction. A quadruple PEXT reaction is performed. Normal and mutant primers for the 896 SNP are 5′-labeled with fluorescein (F) and digoxigenin (Dig), respectively. Normal primer for the 1196 SNP has a characteristic oligonucleotide sequence at the 5′ end. Mutant primer for the 1196 SNP has a $(dA)_{21}$ sequence at the 5′ end. Products of PEXT are captured on a single streptavidin (SA)-coated well. Only extended primers [with incorporated biotins (B)] are captured on the well and detected by reaction with a mixture of horseradish peroxidase (HRP) conjugated to anti-fluorescein antibody, alkaline phosphatase (ALP) conjugated to anti-digoxigenin antibody, β-galactosidase (GAL)–oligonucleotide conjugate and aequorin–$(dT)_{30}$ conjugate. The four reporters are determined sequentially in the same well.

large excess of the normal allele that differs only in a single base-pair. Quantification of mutant allele burden (percentage of the mutant allele) is critical for diagnosis, therapeutic monitoring and detection of minimal residual disease. Sequencing has been used for allele burden studies but the detectability is low (15–20% of mutant allele). Current methods are based on real-time PCR. These homogeneous fluorometric assays are advantageous because amplification and detection are simultaneous. However, the assays require costly equipment and expensive reagents.

The bio(chemi)luminometric genotyping assays described in Section 9.3[44,46–51] have focused on the detection of alleles in the case of inherited mutations where the goal is the discrimination between the normal homozygote, mutant homozygote or heterozygote genotypes. The above methods provide no quantitative estimate of the percent of each allele (normal and mutant) in the DNA sample. However, in the case of somatic mutations, quantification of the allele burden is a crucial requirement. We next discuss a bioluminometric assay for quantification of allele burden as applied to JAK2 V617F mutation (model assay).[52]

The method involved a single PCR of the JAK2 genomic region of interest. PCR was followed by two PEXT reactions. The PEXT primers for the normal and mutant allele contained a poly(dA) segment at the 5′ end and an allele-specific nucleotide at the 3′ end (Figure 9.16a). Biotin–dUTP was incorporated in the extended primer only when the primer was a perfect match to an allele. The extended products were captured on streptavidin-coated wells and detected by using an aequorin–$(dT)_{30}$ conjugate (Figure 9.19). The assay was completed within 50 min after PCR. The allele burden was given by the ratio (expressed as percentage) of the signal due to the mutant allele over the total luminescence obtained from both alleles. The values of allele burden ranged from 0 (only the normal allele is present) to 100 (only the mutant allele is present). The authors demonstrated the linear relationship between the allele burden and the percent (%) luminescence signal due to the mutant allele. As value of as low as 0.85% of mutant allele was detected, and the linearity was extended to 100%. Therefore, the bioluminometric assay enabled the relative determination of the mutant allele burden.

9.5 Conjugation Strategies

In this section we discuss conjugation strategies aimed at reducing the cost of commercially available conjugates and/or improving the sensitivity, ease and practicality of assays.

In one report, the need for either costly commercial aequorin–biotin and aequorin–streptavidin conjugates or for chemical crosslinking of aequorin (to biotin or streptavidin) was replaced by the construction of a plasmid suitable for bacterial expression of *in vivo*-biotinylated aequorin.[53] The biotin tag facilitated both the isolation of aequorin from the crude cell extract as well as direct complexing of aequorin with streptavidin for utilization as a reporter

molecule in the development of highly sensitive bioluminometric hybridization assays. The plasmid contained a biotin-acceptor coding sequence fused to the apoaequorin gene. The *birA* gene, encoding biotin protein ligase (BPL), was inserted downstream of the apoaequorin sequence. BPL post-translationally biotinylated the aequorin acceptor domain at a unique position. Functional aequorin was generated by incubating the lysate with coelenterazine followed by purification by affinity chromatography using immobilized monomeric avidin. After elution, the biotinylated aequorin was complexed with streptavidin. Purified aequorin was detected down to 1.6 amol with an S/B ratio of 4.4 and a linear range extending over three orders of magnitude. Moreover, the *in vivo* biotinylated aequorin was compared to a commercial aequorin and found to have identical performance. In addition, streptavidin-biotinylated aequorin complex was used as a reporter molecule in a microtiter well-based hybridization assay. Microtiter wells were coated with anti-digoxigenin antibody. A digoxigenin-labeled capture probe was then added to the well followed by hybridization of the heat-denatured biotinylated target DNA. The hybrids were determined using the streptavidin-biotinylated aequorin complex. The linearity of the assay ranged from 80 amol to 40 fmol target DNA. An S/B ratio of 2.1 was obtained at 80 amol. It was calculated that *in vivo*-biotinylated aequorin produced from 1 L of culture was sufficient for 300 000 hybridization assays. The entire process, including cell culturing, extraction of total soluble protein and generation and purification of fully active biotinylated aequorin, was completed in 2 days.

The availability of recombinant aequorin was further enhanced by developing a simple method for expression and purification of recombinant aequorin based on a commercially available starting vector and a common *E. coli* strain.[54] The paper reported a purification method for aequorin in one step based on immobilized metal-ion affinity chromatography, a method for purification of proteins with engineered poly-histidine tags. These tags form high affinity complexes with immobilized divalent metal ions, thereby allowing isolation of tagged proteins from a crude cellular extract. An appropriate plasmid was constructed in which a hexahistidine $(His)_6$-coding sequence was fused upstream of the apoaequorin cDNA. Overexpression of heterologous proteins in the *E. coli* cytoplasm is often accompanied by misfolding and segregation into inclusion bodies (insoluble and inactive aggregates). In the case of apoaequorin, inclusion bodies were solubilized by urea treatment and purification was accomplished in one step using a Ni^{2+}-nitrilotriacetic acid (Ni-NTA) agarose column in a way that proper protein refolding was ensured. Purified aequorin was detected down to 0.5 amol with an S/B ratio of 1.8 and a linear range extending over six orders of magnitude. The one-step purification procedure of $(His)_6$–aequorin lasted only a few hours.

The $(His)_6$- tag greatly facilitated the preparation of conjugates of aequorin to DNA probes as demonstrated in this next report.[55] Conjugates of aequorin to DNA probes represent the ''direct labeling'' approach in bioluminometric hybridization assays. This approach is advantageous over the ''indirect

labeling'' approach because it eliminates an incubation step and a washing step, thus reducing the time required for assay completion. In the ''indirect labeling'' approach, a ligand is attached to the DNA probe, and the hybrids are detected by using a specific binding protein conjugated or complexed to aequorin.[1,4] However, even though direct labeling is preferred, the preparation of aequorin–DNA conjugates requires laborious chromatographic procedures followed by concentration steps, to remove the free (unreacted) DNA probe, which otherwise competes with the conjugate for hybridization to the target sequence. The authors reported a general procedure for the preparation of aequorin–DNA conjugates, and central to the conjugation protocols is the use of $(His)_6$–aequorin.[55] Conjugates were prepared by using either homobifunctional or heterobifunctional crosslinking reagents.

In one protocol, an amino-modified oligonucleotide was treated with a homobifunctional crosslinker carrying two N-hydroxysuccinimide ester groups, and the derivative was allowed to react with $(His)_6$–aequorin. Following synthesis of the conjugate, the effective removal of the free oligonucleotide was crucial because it competes with the aequorin–oligo conjugate for hybridization to the complementary target DNA. The aequorin–oligo conjugates were purified either by (a) both affinity capture on a Ni-NTA agarose column and anion-exchange HPLC or (b) only by anion-exchange HPLC. The performances of the purified aequorin–oligo conjugates were similar when tested in a hybridization assay, in which biotinylated target DNA was captured on streptavidin-coated wells. After removal of the non-biotinylated strand by NaOH treatment, the target DNA was hybridized to the aequorin–oligo conjugate. As low as 2 pM target DNA was detected using the conjugate purified both by affinity capture and HPLC (S/B ratio = 1.8). Moreover, it was found that conjugates purified by a single rapid affinity-capture step offered the same detectability and analytical range as those purified both by affinity capture and HPLC. This simplified the preparation of conjugates by eliminating the need for HPLC purification.

In the second protocol, protected sulfhydryl groups were introduced into $(His)_6$–aequorin (to avoid aequorin inactivation due to derivatization of cysteine groups that play a role in the bioluminescent reaction) followed by reaction with a heterobifunctional crosslinker containing a N-hydroxysuccinimide and a maleimide group. The aequorin–oligo conjugates were purified either by (a) both affinity capture on a Ni-NTA agarose column and anion-exchange HPLC or (b) only by affinity capture. The performances of the purified aequorin–oligo conjugates were similar when tested in hybridization assays. As low as 2 pM target DNA was detected using the conjugate purified both by affinity capture and HPLC (S/B ratio = 2.9). The linearity of the assay extended to 2000 pM target DNA for both conjugates. The conjugate obtained from a reaction of 10 nmol of $(His)_6$–aequorin was sufficient for about 5000 hybridization assays.

$(His)_6$–aequorin[54] has been exploited not only for the conjugation of oligonucleotides to aequorin[55] but also for the conjugation of streptavidin to aequorin.[56] The aequorin–streptavidin conjugate may be exploited as a

universal reporter molecule for bioluminometric DNA hybridization assays because streptavidin non-covalently binds to biotin with high affinity and biotin can be easily attached to practically any biomolecule. Protected sulfhydryl groups were introduced into $(His)_6$–aequorin whereas streptavidin was derivatized with maleimide groups. The conjugate was purified in a single step by immobilized metal-ion affinity chromatography. The performance of the aequorin–streptavidin conjugate was tested by using it as a reporter molecule in a microtiter well-based DNA hybridization assay. After thermal denaturation, biotinylated PCR products were captured on microtiter wells coated with target-specific probe conjugated to BSA. The captured single-stranded DNA was then detected by reacting with the aequorin–streptavidin conjugate. The limit of detection was found to be 0.29 pM (14.5 amol $well^{-1}$). The limit of quantitation was 0.50 pM (25 amol $well^{-1}$). The analytical range of the assay extended up to 500 pM. The conjugate obtained from 10 nmol of $(His)_6$–aequorin was sufficient for about 5000 hybridization assays.

Aside from aequorin, conjugation strategies have been employed for *Gaussia* luciferase. The cDNA for *Gaussia* luciferase (GLuc), the enzyme responsible for the bioluminescent reaction of the marine organism *Gaussia princeps*, was cloned in 2001.[57] The substrate for GLuc is coelenterazine. GLuc presented as an excellent candidate for a potential new bioluminescent reporter molecule for DNA hybridization assays since it is a monomeric protein and it gives high levels of light emission when transfected into mammalian cells. The first quantitative analytical study of GLuc was performed in 2002 and involved bacterial expression of *in vivo* biotinylated GLuc.[58] A plasmid encoding both a biotin acceptor peptide-GLuc fusion protein and the enzyme biotin protein ligase (BPL) was engineered. Purification of GLuc was then accomplished by affinity chromatography using immobilized monomeric avidin. Complex formation with streptavidin eliminated the need for chemical conjugation reactions, which are known to inactivate luciferases. Purified GLuc was detected down to 1 amol with an S/B ratio of 2 and a linear range extending over five orders of magnitude. Furthermore, the GLuc–streptavidin complex was used as a reporter molecule in a microtiter well-based DNA hybridization assay. Microtiter wells were coated with anti-digoxigenin antibody. A digoxigenin-labeled capture probe was then added to the well followed by hybridization of the heat-denatured biotinylated target DNA. The hybrids were determined using the GLuc conjugate (biotinylated GLuc complexed to streptavidin). Luminescence was measured in the presence of excess coelenterazine. The linearity of the assay ranged from 1.6 to 800 pM (80 amol $well^{-1}$ to 40 fmol $well^{-1}$) target DNA. The S/B ratio at 80 amol was 1.4. This detectability was similar to the one exhibited in assays using *in vivo*-biotinylated aequorin.[2] It was calculated that *in vivo*-biotinylated GLuc produced from 1 L of culture was sufficient for 150 000 hybridization assays. The entire process, including cell culturing, extraction of total soluble protein and generation and purification of fully active biotinylated GLuc was completed in 2 days.

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