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Review

Non-radioactive detection methods for nucleic acids separated by electrophoresis

Klaus Düring

Center for Applied Plant Molecular Biology, University of Hamburg, Ohnhorststrasse 18, D-2000 Hamburg 52 (Germany)

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ABSTRACT

The different non-radioactive labelling and detection methods currently commercially available are compared and evaluated in this review. Minor factors such as electrophoresis and blotting techniques as well as choice of membrane and their impact on results are discussed. Two major labelling moieties, biotin and digoxigenin, and the various labelling methods are discussed in detail. A comparison of my own results and those from the literature favours application of the digoxigenin group as a routine label. Nevertheless, in several cases biotin will also lead to good results and may also serve as a second label. The most important factor within the non-radioactive systems is the detection of the targeted label. Colorimetric and chemiluminescent techniques are compared in terms of sensitivity, flexibility and applicability. Colorimetric detection can produce suitable results, but in most cases the major advantages of chemiluminescent techniques involving alkaline phosphatase and AMPPD or CSPD will make chemiluminescent detection the method of choice. A survey is given on applicability of the basic techniques to several important assay methods involving electrophoresis of nucleic acids. Finally, some examples of application of non-radioactive nucleic acid labelling and detection techniques in plant molecular biology and biomedicine are cited from the literature.

CONTENTS

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LIST OF ABBREVIATIONS

AMPPD	Disodium 3-[4-methoxyspiro(1,2-dioxetane-3,2′-tricyclo[3.3.1.1.^{3,7}]decan)-4-yl]phenyl-phosphate or 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl 1,2-dioxetane
AP	Alkaline phosphatase
bp	Base pair
BCIP	5-Bromo-4-chloro-3-indodyl-phosphate
BSA	Bovine serum albumin
CML	Chronic myelogenous leukaemia
CSPD	Disodium 3-[4-methoxyspiro(1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1.^{3,7}]decan)-4-yl]phenylphosphate
DIG	Digoxigenin
DMF	Dimethylformamide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
kb	Kilobase pair
Naphthyl-AS	3-Hydroxy-2-naphthoic acid anilide
NBT	4-Nitroblue-tetrazolium chloride
PAA	Polyacrylamide
PCR	Polymerase chain reaction
PPD	4-Methoxy-4-(phosphatephenyl)spiro(1,2-dioxetan-3,2′-adamantane)
PVDF	Polyvinylidenedifluoride
RFLP	Restriction fragment length polymorphism
TAE buffer	40 m*M* Tris acetate pH 8.0, 1 m*M* EDTA buffer
TBE buffer	90 m*M* Tris borate pH 8.3, 2 m*M* EDTA buffer

1. INTRODUCTION

Detection of specifically targeted nucleic acid sequences has evolved to be a highly versatile and useful method for different purposes in recent years. Techniques such as Southern and Northern hybridization have become routine methods for analysis of DNA and RNA. Several other more sophisticated methods rely on the same principles of detection. In molecular biological research as well as in biomedical analysis these techniques have proven to be of great potential, specificity and sensitivity. The major drawback inherent in these methods has been until recently the requirement of radioactivity for detection with highest possible sensitivity. Not all laboratories that could potentially take advantage of the newly developed methodologies, *e.g.* for applied biomedical routine analyses, have the facilities necessary for handling radioactivity. In addition to the facility, equipment, waste disposal and safety requirements there are the risks inherent in exposure of laboratory personnel to radioactive radiation. Widespread application or powerful new analytical methods with improved characteristics will be prevented by these factors. Also, small laboratories will not be able to establish isotope laboratories within their own rooms.

For many years, radioactive labelling of nucleic acids has been the only applicable method. No other efficient label was known until the mid-1980s. The emergence of molecular biological techniques with interest for a broad spectrum of fields of research and analysis has been a reason to initiate a search for new labelling techniques avoiding radioactivity. In recent years a highly innovative development has taken place, beginning with the first non-radioactive labelling and detection system (based on the interaction of biotin with avidin) for Southern hybridizations and achieving a well elaborated set of methods offering a broad spectrum of applicability and sensitivity equal to that of radioactive labelling.

Recent breakthroughs in genetic engineering and isolation of specific nucleic acid probes useful for clinical and research applications have led to a new field of DNA- and RNA-based analytical methods and procedures, removing the disadvantages inherent in previous analytical techniques. In addition, completely new techniques allowing improved or otherwise impossible investigations have been developed. Some of these techniques are already well established in forensic and clinical routine analysis. For instance restriction fragment length polymorphism (RFLP) analysis has revolutionized genetic maps of chromosomal loci and greatly improved knowledge about genetic correlations. Today, rather precise linkages of deficiencies to defined chromosomal positions have become possible using RFLP markers.

The method of choice for labelling of nucleic acids with ^{32}P at the beginning of the 1980s was nick translation. Since then several other labelling procedures providing greater flexibility and sometimes also improved yields or better handling have been developed.

In this review, the most advanced fundamental techniques of nucleic acid electrophoretic separation and transfer to blotting membranes as well as labelling protocols will be described. The second major part is devoted to the various non-radioactive detection methods for the introduced specific label. A critical evaluation of the commercially available techniques will be followed by a short comprehensive overview on the potential applications of already well established methods (formerly requiring radioactive labelling) as well as on new analytical nucleic acid-based techniques employing non-radioactive labelling and detection.

2. ELECTROPHORETIC SEPARATION OF NUCLEIC ACIDS

Nucleic acid (DNA and RNA) are commonly separated by electrophoresis using either agarose or polyacrylamide gels depending on their size ranges. Larger molecules [100 base pairs (bp) to 20 kilobase pairs (kb)] are well separated in 1.5–0.6% agarose gels and smaller molecules (up to about 200 bp) in 10–20% polyacrylamide gels [1]. Whereas DNA molecules are separated in the non-denatured state, RNA molecules have to be

separated in denatured form to avoid any disturbances in band separation originating from secondary structures. Several procedures have been developed for this purpose. Generally, for agarose gels a highly purified agarose with low electroendoosmosis has to be used in order to achieve good results, especially in respect of resolution. Common procedures are described in refs. 1 and 2.

2.1. DNA

Separation of double-stranded DNA can be routinely performed in 0.6–1.5% agarose gels in a suitable electrophoresis buffer. A versatile concentration is 0.8% agarose, which is applicable to most separation problems involving plasmid or genomic DNA restriction endonuclease digests or similar DNA preparations. Lower concentrations can be applied for better separation of larger fragments (> 3 kb) and higher concentrations for smaller molecules (100–1500 bp). TAE buffer (40 m*M* Tris acetate pH 8.0, 1 m*M* EDTA) has been used for a long time. A higher buffering capacity allowing faster gel runs is provided by TBE buffer (90 m*M* Tris borate pH 8.3, 2 m*M* EDTA). Both buffers are compatible with Southern blotting and hybridization. Agarose gels are run in so-called submarine electrophoresis tanks. Polyacrylamide (PAA) gels, which can be quite easily handled, should not be less concentrated than 7.5% total acrylamide, otherwise handling will become very sensitive to mechanical damage. This allows separation of nucleic acids up to some hundred base pairs depending on the band pattern that has to be resolved. Commonly, TBE buffer is used for PAA gels as a running buffer. Oligonucleotides (single-stranded molecules) can be separated without disturbances by secondary structures in gels containing 7 *M* urea [1,2].

For Southern hybridizations (detection of specific DNA sequences immobilized on a membrane by a labelled complementary strand of DNA), transfer of denatured DNA from the gel to the blotting membrane is required. Several methods have been developed in recent years. Choice of the optimial protocol is dependent on the type of membrane and covalent fixation of the DNA to the membrane as described in more detail in Section 3.

2.2. RNA

The performance of RNA separation by electrophoresis requires complete denaturation of the molecules prior to the gel run in order to obtain reproducible band patterns. Two mainly used methods are available: one employs formaldehyde and formamide for denaturation, the other glyoxal [1,2]. In my own studies I always use the formaldehyde–formamide system for RNA analysis. As RNAs are already present as single-stranded molecules, in a denatured state after gel electrophoresis no further treatment prior to blotting is necessary.

3. TRANSFER TO BLOTTING MEMBRANES

Transfer of nucleic acid from agarose gels to blotting membranes is usually performed by capillary blotting [1,2]. Apart from this method, several other techniques have been developed in recent years. Nevertheless, because of its efficiency and conveniency, capillary blotting overnight is still the method of choice in most cases.

Commonly, DNA is transferred to the hybridization membrane in a high-salt solution after alkaline denaturation and neutralization of the agarose gel according to a standard protocol [1–3]. This method was originally developed by Southern [4] and later improved [5]. A variant of this procedure is blotting under alkaline conditions [3]. Two other methods are vacuum blotting and electrophoretic transfer of DNA to the membrane, both requiring special equipment [3]. Neither of these techniques is widely used today because of experimental restrictions. For vacuum blotting gels have to be kept in a completely intact state. Minor damage to the gel may cause its breakdown and lead to unequal transfer of the nucleic acids owing to non-uniform vacuum. Electrophoretic blotting has been described to be inefficient in some cases. DNA may migrate through the membrane easily as a result of con-

tinuous current flow. In addition, conditions are widely dependent on the specific membrane. A comparison of different transfer methods has been reported previously [3].

RNA blots are performed under high-salt conditions similar to classical Southern transfer of DNA [1,2,6]. Electroblotting has also been reported [7,8].

A second important factor in transfer conditions is the choice of membranes. Nitrocellulose filters were first on the market but are no longer satisfying because they are difficult to handle and have limited stability. Nylon membranes are the most widely used type of filters employed for Southern and Northern blotting now. Polyvinylidenedifluoride (PVDF) membranes are also available for nucleic acid binding. Both types are available as neutral and positively charged membranes. The positively charged membranes provide a higher nucleic acid-binding capacity.

The third factor affecting sensitivity of the analysis is covalent immobilization of the transferred nucleic acids on the surface of the membrane. Originally, this was accomplished by drying the nitrocellulose filters and baking for 2 h at 80°C in a vacuum oven. Nylon membranes may also be baked under normal air conditions. Today, UV cross-linking of nucleic acids has become the most comfortable and efficient procedure. Wet or dry membranes are illuminated by UV light with a defined amount of energy [3,9]. This may be done simply by placing the membrane on a transilluminator or, better, by using specially designed UV ovens, which are commercially available from several companies. Usually, sensitivity obtained after UV cross-linking is higher than that obtained after baking.

The balance of all three factors will influence the final performance and sensitivity of the Southern or Northern hybridization protocol. In my own experiments I initially used nitrocellulose for studies of non-radioactive detection methods. After the introduction of the Immobilon-P PVDF membrane (Millipore, Bedford, MD, USA), this new type of support was tested, and good results have been achieved for a long time now by using a combination of alkaline capillary blot and UV cross-linking. On the other hand, the positively charged Immobilon-N membrane (Millipore) performs poorly in my experience (unpublished results). Nylon membranes are available as neutral (various suppliers), partially positive (Boehringer Mannheim, Mannheim, Germany) and strongly positive types (various suppliers). My own experience leads me to recommend use of the Boehringer nylon membrane in conjunction with UV cross-linking [9], although results of my own tests as well as from other laboratories demonstrate the applicability also of neutral nylon membranes for chemiluminescent detection of nucleic acids (described below and personal communications). With this procedure slightly improved sensitivity can be achieved in comparison with the alkaline Immobilon-P protocol.

When designing a personal protocol for DNA or RNA blotting these factors should be taken into consideration. Nylon membranes are not suitable for alkaline transfer in combination with UV cross-linking. Only baking will provide satisfying results in this case.

4. LABELLING OF NUCLEIC ACIDS

For a long time radioactive labelling of nucleic acids has been the only means of detecting specifically hybridizing DNA fragments and RNAs. At the beginning of the 1980s the first alternative non-radioactive detection methods evolved were based on the labelling of the nucleic acid probe with biotin and detection via interaction of the biotin with avidin or streptavidin. The reporter enzyme was alkaline phosphatase (AP) [10–14]. Usually, radioactive nucleotides were incorporated into probes by nick translation at that time [1,2,15]. This method has been adapted to incorporation of biotinylated nucleotides [10–14]. In the meantime various other methods of labelling DNA and RNA probes have been developed and have been commercialized by several suppliers. Some practical aspects are described in the following sections.

4.1. Choice of label

Initially, biotin, a small organic molecule, was chosen as a label for nucleic acids because of its highly specific interaction with avidin and streptavidin. Biotinylation of DNA probes has been used successfully for a long time now and has proven to be reliable and effective. Most companies today use biotinylated nucleotides (mostly bio-11-dUTP) as a label in their kits. However, Boehringer Mannheim have introduced another organic compound as a label, the digoxigenin molecule. It can be detected by a specific anti-digoxigenin-F_{ab} fragment and avoids the problems that sometimes occur because of the presence of biotin in many (often crude) biological tissue samples. The digoxigenin system is widely acknowledged as a standard method today. Its effectivity is similar to or even slightly better than that of the biotin system. As a result, two different efficient labelling systems are available, which enables detection of two different probes at the same time by using digoxigenin for the one and biotin for the other in combination with different detection systems. Amersham (Braunschweig, Germany) initially introduced another approach for labelling within the enhanced chemiluminescence (ECL) system. Via glutaraldehyde coupling, the nucleic acid probe is directly labelled with the reporter enzyme (horseradish peroxidase). This allows a reduction in the number of detection steps but, on the other hand, drastically reduces the flexibility of the assay system. The protein present in the hybridization solution means that the incubation temperatures are strictly limited to 42°C to prevent destruction of the activity of the enzyme. This is a major drawback in transferring previously used hybridization and washing conditions to non-radioactive detection systems and restricts applicability.

A striking advantage of non-radioactive labelling of nucleic acids with biotin or digoxigenin is the ability to store labelled probes for some years without loss of activity. This saves a lot of work in comparison with radioactive labelling, which requires probes to be prepared freshly and used within some days.

4.2. DNA probes

4.2.1. Nick translation

Nick translation of DNA probes by combined action of DNase and Klenow enzyme has been the method of choice for a long time [1,15]. By simply employing a biotinylated or digoxigenated nucleotide instead of a radioactive one the reaction protocol can be adapted [10]. The major drawback of this technique is the need for phenol extraction to remove the DNase and for ethanol precipitation to remove the phenol and the labelled nucleotide. In the case of radioactive labelling, the presence of the labelled DNA in the precipitation pellet can easily be monitored. This is not possible for non-radioactively labelled probes. Therefore, monitoring can only be performed by running a dot blot with the labelled probe including a complete cycle of detection. This is time-consuming and laborious.

4.2.2. Random primed labelling

In 1983 Feinberg and Vogelstein [16] published a new technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Single-stranded DNA is produced by heat denaturation and hybridized to random hexanucleotides. Labelled cDNA is synthesized by DNA polymerase I Klenow fragment extending from these primers. This method has been adapted to non-radioactive labelling, and reagents for biotinylation or digoxigenation are commercially available from most suppliers today. In my hands, this technique has proven to be reliable and efficient [9]. In the case of biotin and digoxigenin it is recommended that the degree of integration of labelled nucleotide into the newly synthesized probe should not be the highest possible. Instead, because of steric hindrance, it has been demonstrated that replacement of only 35% of the dTTPs by labelled dUTP provides optimal sensitivity (ref. 13 and unpublished results). It has been found that, in the case of random primed labelling, a further purification of the digoxigenated or biotinylated probe from the reaction mixture is not necessary. The crude extract can be used for hybridization. During colorimet-

ric or chemiluminescence detection procedures no specific problems have yet been detected (unpublished results). A general protocol for digoxigenin labelling of DNA probes has been described in ref. 9. This protocol can easily be modified for biotin labelling.

4.2.3. Polymerase chain reaction labelling

A rather new method for labelling of DNA probes is based on polymerase chain reaction (PCR) [17–23]. If specific oligonucleotide primers suitable for production of the required probe fragment are available this method offers a much higher versatility and reduced preparative work in comparison with nick translation and random primed labelling. Protocols have been described for introduction of biotin as well as digoxigenin. PCR directly amplifies DNA from crude sources requiring only minimal amounts of target DNA. High amounts of labelled probe can be synthesized by incorporating either biotinylated or digoxigenated nucleotides via Taq polymerase. In addition, full-length probes can be generated, which are obtained by the other two described methods only in very small amounts within the mixture of sequences of varying length.

Lanzillo [24] reported a comparison of PCR-generated digoxigenated and biotinylated probes with enzymatically labelled probes. He stated that digoxigenin-labelled probes with 37% substitution of dTTP by digoxigenin-labelled dUTP are the most sensitive probes available. Biotinylated nucleotides seem to be much less efficiently incorporated and do not lead to acceptable DNA probes using this labelling protocol. On the other hand, following enzymatic labelling, biotin and digoxigenin labels lead to rather equivalent sensitivities. This may be explained by the fact that biotinylated dUTP is an inefficient substrate for Taq polymerase.

For PCR labelling, isolation and purification of the probe fragment from a plasmid is no longer necessary. The required sequence is directly amplified and labelled in one reaction step from any source of DNA, even from genomic DNA. This also greatly extends the range of sources that may provide relevant DNA probes. In comparison with nick translation and random primed labelling, much higher amounts of labelled DNA can be produced within a short time. A second advantage of this technology is the ability to generate single-stranded DNA probes by using only one primer instead of two. Then, only one strand can be synthesized selectively.

Amplification combined with labelling is not as efficient as amplification alone but yield is still very efficient. The sensitivity of the detection reaction is not affected by the method of probe preperation. High numbers of different probes can be generated with one set of primers if the relevant DNA fragments are available within one vector, *e.g.* the pUC series [25]. For all these reasons, it is thought this method will be the method of choice in future.

4.2.4. Photolabelling

Photolabelling has been introduced as an alternative to enzymatic labelling for biotin and digoxigenin. Two examples shall be discussed briefly here. Photobiotin [26,27] (available from BRL, Gaithersburg, MD, USA) is a photoactivatable compound consisting of the biotin moiety linked to an arylazido group via a linker. The azido group is activated upon irradiation with UV light (260–300 nm), resulting in a highly active nitrene group that can react non-specifically with electron-deficient atoms [26,27]. Biotin labels can be introduced randomly by this method into any nucleic acid probe.

A similar system is offered by Boehringer Mannheim for introduction of the digoxigenin label. The digoxigenin moiety is again coupled by a hydrophobic linker to an azidophenyl group. Photoactivation of the azido group leads to labelling of DNA and RNA. Every 200th to 300th base will be labelled with a digoxigenin group. A limitation of this method is the achievable sensitivity of detection, which is inferior to that of random primed labelling by a factor of about 10.

Photolabelling may be useful as a specialized method for introduction of non-radioactive labels into DNA and RNA probes in case of special requirements but cannot be recommended as a routine technique.

4.2.5. Direct labelling with reporter enzyme

The ECL kit originally introduced by Amersham offers a new variant of non-radioactive labelling techniques. The reporter enzyme (horseradish peroxidase) was designed to be covalently linked directly to the DNA sample via glutaraldehyde cross-linking. Although the practical procedure is rather simple, a severe limitation is the presence of the reporter enzyme in the hybridization solution. Because of this, every incubation and washing step has to be performed at temperatures not higher than 42°C. The routine protocols employed for radioactive labelling and detection cannot be easily transferred in this case. Modification of the hybridization and washing conditions has to be done by changing the salt concentrations at fixed temperature. This major drawback complicates introduction of non-radioactive techniques in laboratories where radioactivity is routinely applied and protocols have been optimized to special requirements for a long time. In the meantime another supplier (Promega, Madison, WI, USA) has introduced a similar system with the difference that AP is used as a reporter enzyme.

4.3. RNA probes

Labelling of RNA probes will be discussed here using the example of digoxigenation. RNA probes can be easily generated from vectors bearing the required insert positioned between divergent SP6 and T7 RNA polymerase promoters. A defined RNA strand will then be produced using either SP6 or T7 RNA polymerase. Other equivalent systems are appropriate for this purpose. These polymerases accept digoxigenated nucleotides nearly as efficiently as non-modified ones. Efficient labelling can be obtained within 2 h of incubation. Single-stranded RNA probes can be of great value not only for mRNA detection but also for analysis of DNAs.

A linearized appropriate vector containing the insert is used for *in vitro* transcription. Again, a labelling density of about 35% is optimal and will be achieved by subsituting only a part of the TTP by DIG–UTP. A detailed protocol for this procedure has been published in ref. 9. I have successfully employed this method for chemiluminescence detection of specific low-abundance mRNAs from transgenic plants by Northern hybridization.

A similar labelling technique using biotinylated UTP instead of digoxigenated UTP is available. This modified nucleotide is offered by Clontech (Palo Alto, CA, USA).

4.4. Oligonucleotide probes

Short oligonucleotide probes are often used for reducing hybridization times or because of experimental design. Labelling of such probes can be performed by 3′-end labelling with terminal transferase. Protocols have been developed both for biotin and digoxigenin labelling [28–30]. The number of added labelled nucleotides can be determined by including either labelled dUTP or ddUTP. In the first case several labelled nucleotides are added, leading to a stronger signal than in the second case, where only a single labelled nucleotide is added. Generally, this labelling technique results in significantly lower sensitivities than those techniques employing longer DNA probes. Nevertheless, non-radioactive oligonucleotide labelling can be quite efficiently used for routine applications where the highest sensitivity is not necessary or for analysis of synthetic oligonucleotides themselves following electrophoretic band separation.

5. COLORIMETRIC DETECTION OF NUCLEIC ACIDS

When biotinylated nucleotides were developed as labels for nucleic acids in hybridization analyses colorimetric detection by AP and its substrate 4-nitroblue-tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) became the first standard methodology [12]. Various reporter enzymes with corresponding substrate dyes have been investigated to determine their sensitivity and performance in detection of specific DNA probes on Southern blots. Alkaline phosphatase emerged as the most sensitive one [12,31]. The NBT–BCIP substrate originated

from immunohistological studies. Using this system, including a biotinylated polymer of AP, already the first publication reported a limit of sensitivity of some picograms of homologous DNA in a Southern hybridization [12]. By introduction of some minor modifications Chan *et al.* [32] reported the extension of the detection limit into the range of subpicogram amounts of specific DNA. A Southern blot of HindIII-digested phage λ-DNA hybridized to biotinylated phage λ-DNA and detected by streptavidin followed by biotinylated poly(alkaline phosphatase) and NBT–BCIP is shown in Fig. 1 (see also Table 1). Signals could be detected down to about 100 fg of homologous DNA.

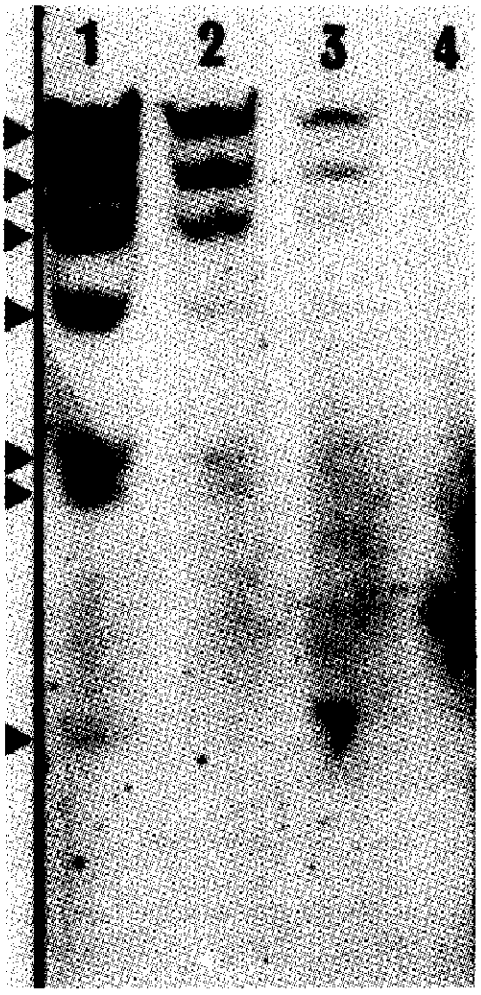

Fig. 1. Southern blot hybridization of HindIII λ phage DNA on nitrocellulose filters. The total amount of λ-DNA on each lane is 20 pg (lane 1), 6.6 pg (lane 2), 2.2 pg (lane 3) and 0.74 pg (lane 4). A 5-μg aliquot of sheared herring sperm carrier DNA was present in each lane. The size of each fragment is indicated in the table. Hybridization was performed at a probe concentration of 75 ng/ml for 17 h at 65°C. Reproduced from ref. 32 with permission.

Since this technique was first described, many experiments have been performed in order to optimize this system [14]. In the middle of the 1980s the first commercial kits became available for routine use in the laboratory. They were based on the above-mentioned system requiring a two-step incubation procedure. Although subpicogram detection limits had been described in the literature [31], in routine laboratory work I could only achieve reproducible detection of about 10 pg of homologous DNA in a Southern hybridization. Occasionally, bands representing only a few picograms of DNA could also be detected, but reproducibility of these results was not achievable routinely. Colorimetric detection by NBT–BCIP performed well with nitrocellulose and PVDF (Immobilon) membranes. In my own analyses, I have used the Immobilon-P membrane (Millipore) for a long time in combination with alkaline capillary blotting. This hydrophobic membrane performed well for single hybridization experiments. Rehybridization is not possible because PVDF is soluble in dimethylformamide (DMF), which is necessary to remove the color precipitate. Nitrocellulose membranes are highly fragile and tend to produce many unspecific background signals at sites where mechanical stress or damage has occurred. Only nylon membranes are suitable for removal of the dye precipitate by DMF.

The manufacturers have tried to enhance the efficiency of the new technique. A step forward has been the introduction of the covalent streptavidin–AP conjugates instead of separated streptavidin and biotinylated AP. Sensitivity could be raised to routine detection of some picograms of specific homologous DNA. Because in genomic Southern blots the specific DNA has to be detected within a mass of unspecific genomic DNA fragments, general sensitivity has to be about an order of magnitude higher than the calculated

amount of DNA which has to be detected. This is still not achievable with the colorimetric detection system.

Many researchers have claimed the flexibility of producing multiple hard copies of different intensity by varying exposure time. This problem cannot be solved with the colorimetric detection technique. Some experience is required to obtain optimal signal intensities, especially if bands of significantly differing strength have to be detected. My own experimental work trying to establish biotinylated probes in combination with streptavidin–AP and NBT–BCIP for detection of foreign (single-copy) genes within the genomic DNA of transgenic tobacco plants was clearly limited by the problems of reproducibility of the limit of sensitivity. Generally, single-copy foreign genes could be detected in some cases, but reproducibility could not be guaranteed (unpublished results). The sensitivity required for these assays is in the range of some picograms of specific DNA. Plant genomes are similar in size to the human genome or sometimes even larger. Therefore, experimental features are the same in biomedical applications.

As already outlined in the part dealing with labelling of nucleic acids, results obtained with digoxigenated nucleotides as a label are in general similar to those obtained with biotinylated nucleotides as a label. In some cases background problems can be reduced because biotin is a ubiquitous molecule in most tissues. Transfer of biotin present in the nucleic acid sample to the hybridization membrane may result in non-specific background staining by interaction with the streptavidin during the detection procedure. These problems are avoided with the digoxigenin label as this molecule is only present in a few plant species. Limits of sensitivity are essentially similar, although in some cases digoxigenin probes may be able to generate slightly stronger signals.

In conclusion, colorimetric detection of nucleic acids by biotinylation or digoxigenation has performed well for various types of nucleic acid analyses that do not require the full sensitivity which can be obtained with radioactive labelling. Although in most publications describing the aforementioned techniques equal sensitivity for non-radioactive and radioactive detection has been claimed, this could not be fully achieved in routine laboratory work under these conditions. Experiments for analysis of plasmid DNA or genomic DNA originating from organisms with genomes not larger than 10^8 nucleotides can easily be performed using the one-step AP NBT BCIP detection system. Experimental protocols involving random primed labelling, digoxigenin labelling and one-step detection are highly recommended for these purposes. A general procedure for colorimetric detection of DNA and RNA is described in ref. 9. Greater flexibility and higher sensitivity can be achieved by introducing the chemiluminescence detection technology (see Section 7.2.).

Recently, new dye substrates for AP have been adapted from immunohistochemistry for blotting techniques [33]. These so-called "fast colours" do not extend the limit of sensitivity but offer the advantage of being soluble in ethanol. Removal of the color precipitate is possible with significantly reduced hazard, and much more easily and more efficiently. A further advantage of these substrates in comparison to NBT–BCIP is their insensitivity to light and they do not fade upon drying of the membrane. Highest sensitivity is offered by naphthol-AS-phosphate in combination with fast blue B; the others are slightly less sensitive than NBT–BCIP [9,33]. Rehybridization has also become possible now with PVDF membranes and is generally simplified.

6. FLUOROMETRIC DETECTION OF NUCLEIC ACIDS

Labelling of nucleic acids with a fluorescent group can be achieved as easily as with biotin or digoxigenin. Only the appropriate modified nucleotide has to be incorporated into the labelling reaction. But fluorescent signals are not as easily detected as colour precipitates or luminescent signals. To obtain a fluorescent signal the target has to be irradiated with light of a suitable wavelength. Emission of fluorescent light has to be measured at a different wavelength. A compari-

son of different techniques revealed that in the case of the fluorescent labels a considerable loss of sensitivity is observed when comparing labelled probes with the labels alone. Another significant decrease in sensitivity is observed in hybridization assays [31]. A comparison of detection limits of several techniques is presented in Table 2. The requirement for specialized equipment and also the rather low sensitivity greatly limit the applicability of the fluorescent labelling and detection techniques for DNA and RNA analysis on membranes.

Only in automated DNA sequencing have fluorescent labels found a broad application. In membrane analysis significant improvements are not to be expected.

7. CHEMILUMINESCENCE DETECTION OF NUCLEIC ACIDS

The most important improvement in non-radioactive nucleic acid analysis techniques has been the development of highly sensitive chemiluminescent substrates for AP. At last, these new chemicals enable achievement of the same routine sensitivity as for radioactive labelling. The most advanced technique involves spiroadamantane–dioxetane compounds, which are dephosphorylated by AP followed by decomposition of the intermediate product. During this last step chemiluminescent light is emitted, which can be detected on standard X-ray films.

Two different systems are commercially available today. One is based on the horseradish peroxidase–luminol system and manufactured by a single supplier (Amersham). The other uses AP and disodium 3-[4-methoxyspiro(1,2-dioxetane-3,2′-tricyclo[3.3.1.1.^{3,7}]decan)-4-yl]phenylphosphate (AMPPD) (Tropix, Bedford, MA, USA) or Lumigen-PPD (Lumigen, Detroit, MI, USA) as substrate. This latter detection system is widely applied by several suppliers. It provides the most advanced and flexible technology.

TABLE 1

SENSITIVITY OF DETECTION ON SOUTHERN BLOTS

DNA Size (kb)	Quantity of DNA[a] (pg)	Signal[b]
23.61	3.20	+ve
	0.36	+ve
9.64	1.31	+ve
	0.15	+ve
6.64	0.90	+ve
	0.10	+ve
4.33	0.59	+ve
	0.07	+/− ve
2.26	0.31	+ve
	0.03	−ve
1.99	0.27	+ve
	0.03	−ve
0.56	0.08	+ve
	0.01	−ve

[a] These representative data are calculated from lanes 2 and 4 in Fig. 1. The fragment size in (HindIII) DNA digests (kb) and their corresponding quantity (pg) is correlated with their visibility on filters.

[b] Signal was scored as +ve, −ve or equivocal (−/−ve) depending on its visibility on wet filters viewed in ordinary light.

TABLE 2

DETECTION LIMITS OF SEVERAL NON-RADIOACTIVE LABELLING AND DETECTION TECHNIQUES

For detailed protocols see ref. 31. Abbreviations: ENH/LUM, enhanced chemiluminescence (luminol with *p*-hydroxycinnamic acid); LUM, luminol; NPP, *p*-nitrophenyl phosphate; OPD, *o*-phenylenediamine. Adapted from ref. 31 with permission.

Label (detection system)	Detection limit (fmol)		
	Label alone	Labelled probe	Probe in assay
Sandwich assay			
Fluorescein	20	100	500
Texas red	20	30	100
Rhodamine	5	20	100
Isoluminol	1	20	100
^{32}P	0.05	0.05	0.05
AP (NPP)	5	5	5
AP (NBT/BCIP)	0.2	0.2	0.5
HRP (OPD)	0.1	0.1	0.1
HRP (LUM)	1	1	1
HRP (ENH/LUM)	0.05	0.05	0.05
HBV assay			
Fluorescein	–	–	100
HRP (ENH/LUM)			0.02

7.1. Horseradish peroxidase–luminol-based systems

Luminol is a substrate for horseradish peroxidase in combination with hydrogen peroxidase that emits energy in form of chemiluminescence. Luminol is oxidized by hydrogen peroxide and produces *o*-aminophthalate under emission of light. This chemical reaction is catalyzed by horseradish peroxidase. The ECL kits offered by Amersham rely on this principle. Labelled DNA probes are detected by horseradish peroxidase either by direct labelling or by biotinylation of the probe and incubation with streptavidin–horseradish peroxidase. Incubation of the membrane with luminol and hydrogen peroxide results in the production of chemiluminescence and signals can be detected by exposure to an X-ray film. A specific feature of the horseradish peroxidase–luminol system as employed by Amersham is its high emittance, which is limited to a short time period only. Exposure times are of the order of less than 1 min. Re-exposure is possible only within some 10 min. Generally, handling of this system is sometimes tricky because of its kinetics.

The sensitivity of the ECL system is better than that of the NBT–BCIP colorimetric system but does not reach the levels which are obtained with AP and AMPPD. Therefore, the ECL system can be considered as an intermediate assay system which offers some improvements over the NBT BCIP system but does not provide ultimate routine sensitivity required for genomic Southern blots.

7.2. Alkaline phosphatase–dioxetane (AMPPD)-based systems

The development of new dioxetane-based AP substrates providing very high sensitivity has in recent years led to a significant improvement in the non-radioactive nucleic acid detection technology [34]. Dioxetanes are four-membered ring peroxides incorporating a "weak" oxygen–oxygen bond that easily decomposes to form strong carbonyl products [35]. This process is accompanied by a release of up to 400 kJ/mol of energy and is capable of producing a strong chemiluminescent signal. The currently available substrates incorporate several important functions: an energy source for chemiluminescence in the peroxy bond; stability provided by the spiroadamantane group; light emission properties defined by the aryl substituent; and, finally, an activation site consisting of an enzyme-cleavable group [36]. The enzyme-cleavable group confers a high stability, allowing luminescence to occur only when this group has been cleaved off by a suitable enzyme. AMPPD was the first chemical to be introduced. It contains a phosphate moiety bound to the aryl ring which can be removed by AP. The resulting intermediate product, the anion AMP^-D, decomposes in a second step to adamantone and the charge transfer excited-state methyl *m*-oxybenzoate anion, which emits light at a wavelength of 477 nm (Fig. 2). The half-life of decomposition of the intermediate is strongly dependent on the nature of its environment (*e.g.* the type of membrane used). The chemiluminescent light emission occurs in the form of a "glow". Breakdown of the intermediate anion is rather slow so that about 15 min are required to obtain steady-state chemiluminescence. The kinetics if light emission will determine the length of exposure time required to obtain appropriate signals [9].

Application of AMPPD in Southern or Northern blotting on nylon membranes results in increased-light emission within the first 24 h after incubation. On nylon membranes the intermediate anion AMP^-D accumulates before decomposition. The first step of enzymatic dephosphorylation is much faster than the breakdown of the intermediate. This leads to an increase in the intensity of light emitted from a specific signal within several hours of reaction time. Immediately after reaching steady-state kinetics of chemiluminescence an exposure time of about 2.5 h is necessary to obtain full sensitivity. The same strength of the signals is obtained within about 20–30 min exposure time after overnight incubation at room temperature (20–24 h). This means a decrease in exposure time by a factor of 5 or 6 [9]. Multiple exposures with optimized signals can

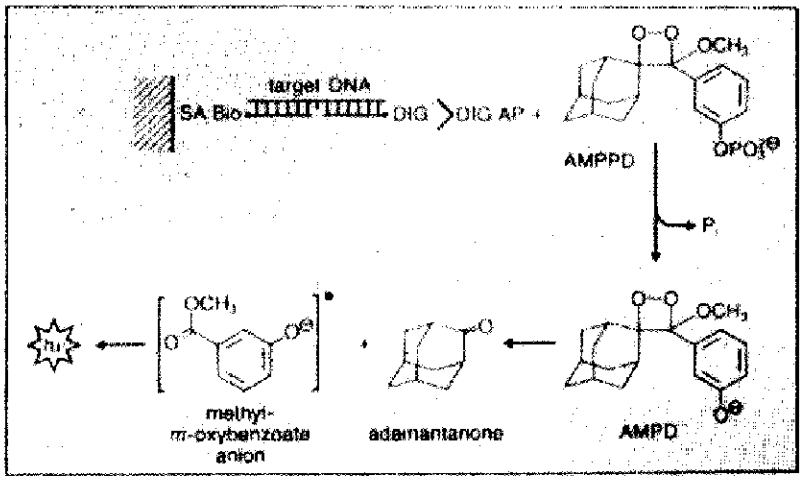

Fig. 2. Decomposition scheme of AMPPD. Incubation with alkaline phosphatase results in enzymatic dephosphorylation and formation of the moderately stable intermediate, AMP^-D. AMP^-D decomposes to adamantanone and the charge transfer excited methyl *m*-oxybenzoate anion, which emits light. Reproduced with permission from Boehringer Mannheim.

easily be obtained after overnight incubation, and require only minimal time and effort. This gives the AMPPD system an unbeaten flexibility amongst all the detection systems commercially available today.

As described, the nature of the surface of the membrane exerts a significant effect on the intensity of the chemiluminescent signal. Nylon membranes have to be considered to be an enhancer of the chemiluminescent signal. In comparison with properties in buffer solutions, AMPPD on nylon membranes produces a hypsochromic shift of the emission maximum and a dramatic decrease in the rates of signal generation. This suggests that the excited-state emitter resides in an aprotic environment [37]. This indicates that the intermediate anion is bound by hydrophobic interactions within the hydrophobic domains in the nylon membrane. The sharpness of the bands observed in several types of membrane assays coincides well with this assumption. Diffusion of the AMP^-D anion and the emitting molecule seems to be negligible [37].

AMPPD is offered by Tropix and under the trademark Lumigen PPD by Lumigen. In my own experiments I have used the digoxigenin DNA labelling and detection system manufactured by Boehringer Mannheim, which included until recently the Tropix AMPPD but has now been changed to Lumigen PPD, and the original Tropix chemicals. As described in ref. 9, there was originally a significant difference in the quality of AMPPD sold by different companies. Today, the Tropix AMPPD is still the purer compound because of inclusion of a final high-performance liquid chromatographic (HPLC) purification step. Tropix offers a range of related chemicals such as chemiluminescence enhancers (Sapphire, Emerald) and blocking reagents. With nylon membranes no additional effect can be observed when including these enhancers in the reaction mixture (unpublished results). The enhancers are useful for work with other solid supports than nylon membranes. Also, the wavelength of emission can be tuned by using different enhancers. Chemiluminescent detection on PVDF membrane (Immobilon-P) is possible by inclusion of Sapphire enhancer. Good results can be obtained with standard X-ray film (Kodak X-Omat AR). The Emerald enhancer shifts the emission maximum to green light and therefore preferably requires a green-sensitive X-ray film, which is difficult to obtain. Another enhancer is Nitro-Block (Tropix), which has been specifically designed for application with nitrocellulose membranes. All the enhancers provide a hydro-

phobic region surrounding the light-emitting molecule, which imitates the features of a nylon membrane. Nitrocellulose normally gives very poor signals in chemiluminescent assays. Nitro-Block converts this membrane into a high-performing support for AMPPD detection by covering its surface with a hydrophobic polymer. This leads to rather efficient production of signals [38]. Application of Nitro-Block to Immobilon-P and Immobilon-N membranes reveals that the positively charged N-membrane is not suitable for chemiluminescent detection, whereas Immobilon-P performs well [38]. I have had similar experiences with the two Immobilon membranes in my own experiments with colorimetric detection by NBT–BCIP (unpublished results).

Tropix recently introduced another substrate, disodium 3-[4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan)-4-yl]phenyl-phosphate (CSPD), which is a chlorinated derivative of AMPPD. This new molecule allows even faster generation of chemiluminescent signals than AMPPD. With this substrate exposure times can be shortened further. In addition, CSPD produces slightly sharper bands than AMPPD. The time period necessary to reach steady-state kinetics of light emission is significantly decreased in comparison with AMPPD [39]. In Fig. 3 structures and chemiluminescence kinetics of AMPPD and CSPD are displayed.

A procedure suitable for single-copy foreign gene detection in transgenic tobacco and potato plants has been developed for own experiments on the basis of the AP–AMPPD system. A detailed protocol can be found in ref. 9. The Boehringer nylon membrane that is partially positive was chosen and performes well during these investigations. Generally, other membranes can also be used. A specific comparison of the partially positively charged Boehringer nylon membrane with the widely used neutral nylon membranes Hybond-N membrane (Amersham) and Biodyne A (Pall, Dreieich, Germany) revealed that background staining is slightly lower with neutral membranes but sensitivity is clearly better with the Boehringer membrane (unpublished results). Therefore, the use of the Boehringer nylon membrane is recommended here. In each case, a decision has to be made between lower non-specific background or stronger signals. Following "classical" capillary blotting overnight and a short wash of the membrane, cross-linking of the nucleic acids is performed by UV treatment. Irradiation in a Stratalinker (Stratagene, Heidelberg, Germany) corresponding to about 2.5 min with fresh UV bulbs has been found to be the most efficient method of nucleic acid immobilization [9]. I perform all incubations with minimal amounts of solutions in order to decrease costs. This has proven to be a feasible technique. Hy-

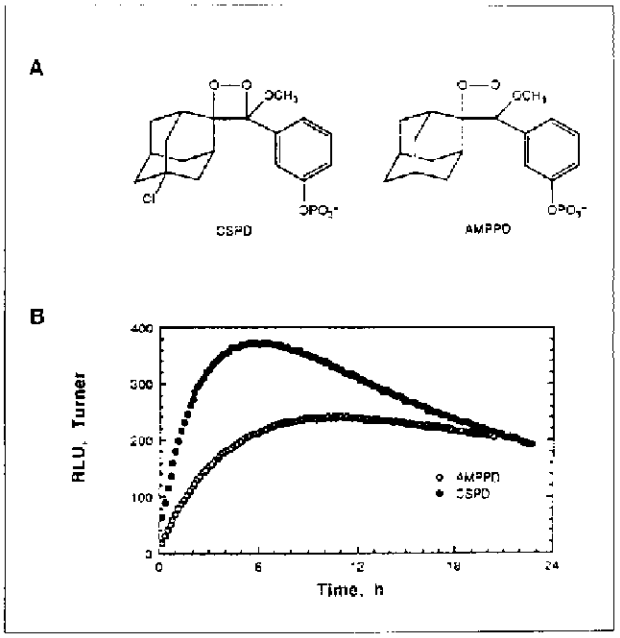

Fig. 3. (A) Structures of CSPD and AMPPD. (B) Chemiluminescence kinetics on nylon membranes: CSPD *versus* AMPPD. An aliquot 12.5 pg of biotin-pBR 322-35 mer was spotted on a nylon membrane, UV-fixed, blocked with 0.2% casein phosphate-buffered saline (PBS)–0.1% Tween 20, incubated with Avidx streptavidin–alkaline phosphatase conjugate, washed with 0.2% casein–PBS–0.1% Tween 20, washed with PBS–0.3% Tween 20, and rinsed in 0.1 *M* diethanolamine (DEA) 1 m*M* magnesium chloride, pH 10. The membranes were attached to the side of test tubes and incubated with either 0.25 m*M* AMPPD or 0.25 m*M* CSPD in DEA pH 10 buffer. Turner relative light units (RLUs) were measured using a Turner Model 20E luminometer (Turner Designs, Mountain View, CA, USA) equipped with a side-reading photomultiplier tube. Reproduced from ref. 39 with permission.

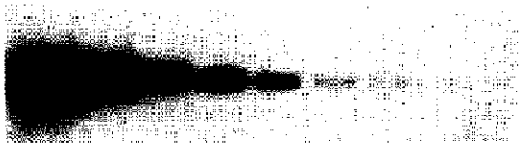

Fig. 4. Dilution series and hybridization of homologous DNA fragment (670 bp) in Southern blotting: 50, 25, 10, 5, 2.5 and 1 pg and 500, 250 and 100 fg. Reproduced from ref. 9 with permission.

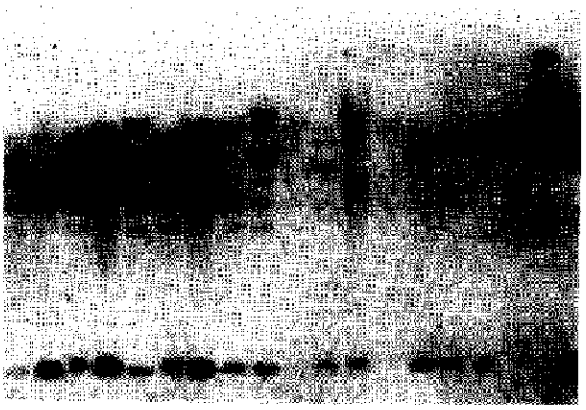

Fig. 5. Genomic Southern blot of transgenic potato plants: detection of foreign DNA at the single-copy level (10 μg of total genomic DNA per lane). Reproduced from ref. 9 with permission.

bridization is carried out with 50% formamide at 42°C overnight but conditions are dependent on the probe and may be adapted to those elaborated in earlier experiments using radioactive labelling. Southern blots can efficiently be hybridized with digoxigenin-labelled DNA probes. I found Northern blots were only successful when hybridized with digoxigenin-labelled RNA probes. Nevertheless, in the meantime there are also reports of efficient employment of digoxigenin-labelled DNA probes for Northern hybridizations (ref. 24 and C. Grogan, personal communication).

Following the procedure given in ref. 9, I was able to reproducibly obtain signals of about 100 fg of homologous DNA in a Southern hybridization using plasmid DNA (Fig. 4). In all experiments digoxigenin-labelled DNA probes generated by random primed labelling were employed. This means sensitivity is improved by a factor of 10–20 in comparison with the NBT–BCIP colorimetric detection protocol which was run in parallel. In dot blots usually still lower amounts of DNA can be detected. The level of sensitivity obtained with this technique is very similar to that which can be achieved by radioactively labelled DNA probes. Application of this protocol to genomic Southern hybridizations of tobacco and potato genomic DNA resulted in efficient detection of single or multiple copies of foreign genes in transgenic tissue. A 10-μg aliquot of genomic DNA is used for analysis. The amount of DNA which has to be detected specifically among the huge amounts of genomic DNA correspond to some picograms of DNA. Therefore, it is advisable to employ a methodology which is one order of magnitude more sensitive than required by pure calculation. The foreign DNA could be detected as fragments with a size of > 3 kb and 350 bp (Fig. 5). This demonstrates well the applicability of the AMPPD technique to high-sensitivity analyses.

Northern blotting has been performed with total RNA and purified poly(A)$^+$ RNA from the same transgenic plants. As already mentioned, digoxigenin-labelled RNA probes have been successfully used for this. Single-stranded digoxigenin-labelled antisense RNA probes have been generated by *in vitro* transcription from an SP6–T7 plasmid. This convenient procedure produces large amounts of RNA probes within 2 h of incubation [9]. The RNA has been blotted to the Boehringer nylon membrane and hybridized with the DIG-labelled RNA probe at 68°C in 50% formamide. The detection procedure is identical to that employed in Southern hybridizations. Specific RNA could be detected from 50 μg of total RNA with clear signals (Fig. 6). Similar results have been obtained with poly(A)$^-$ RNA isolated from 100 μg of total RNA [9,40]. Low-abundance mRNAs (≈0.001% of total mRNA) have been detected using this protocol. Signals of as low as 1 pg of specific *in vitro* transcribed RNA could be visualized by hybridization with a DIG-labelled RNA probe (unpublished results).

Detection of the chemiluminescent signal can

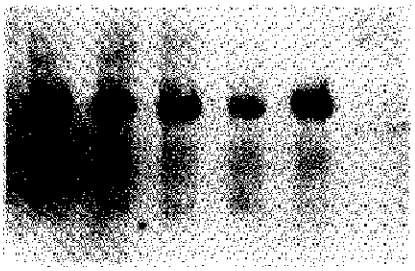

Fig. 6. Detection of mRNA in Northern blot: mRNA transcribed from foreign T4 lysozyme gene in transgenic potato plants (50 μg of total RNA per lane). Reproduced from ref. 9 with permission.

be achieved by exposure to either a standard X-ray film or a Polaroid instant film. In my own experiments for convenience I only used X-ray films. Kodak X-Omat AR and Kodak Ortho G films perform well. The XAR film provides highest possible sensitivity, but its spectrum is limited to the blue range. The light emitted by AMPPD on nylon membranes (477 nm) or in combination with the Sapphire (463 nm) and Emerald (emits at 542 nm) enhancers is in the green range. Therefore, perception of the chemiluminescent signal is limited to the extreme spectral end of sensitivity of the blue-sensitive film and does not enable the high sensitivity obtainable in the blue light range. The Ortho G film is green-sensitive but the overall sensitivity is much lower than that of the XAR film. Therefore, finally both films are suitable for nylon membranes and Sapphire and Emerald enhancers. To my knowledge, no highly sensitive X-ray film with maximal sensitivity in the green light range is available today.

In a system comprising nick translation labelling with biotin, transfer to Tropilon nylon membrane (Tropix), and detection by Avidx streptavidin–AP conjugate (Tropix) and AMPPD (Tropix) detection levels of 3 pg with oligonucleotide probes and of 380 fg with full-length probes could be obtained in a Southern blot. In dot blots a lower limit of 75 og for detection of plasmid DNA after hybridization has been reported [36]. Applying PCR amplified, digoxigenin-labelled probes, Lanzillo [24] reported a lower detection limit of only 23 fg of plasmid DNA after hybridization. The uncharged nylon membranes Tropilon (Tropix) and Hybond-N (Amersham) were found to give best results. The Boehringer nylon membrane (Boehringer Mannheim) was not included in this comparative experiment. A survey on applications of the digoxigenin nucleic acid labelling system in combination with chemiluminescent detection was also given by Höltke *et al.* [41]. In conclusion, several independent investigations report a maximum sensitivity which is very similar to that of radioactive nucleic acid labelling. As a result, today there is no longer any need to use radioactive nucleotides for DNA and RNA analysis.

The current methodology of chemiluminescent nucleic acid detection may be further improved by development of strictly adapted X-ray films with highest sensitivity in the green light range. Blocking reagents also seem to exert a significant influence not only on background staining but also on the intensity of the signals. I have found the Boehringer Blocking reagent (a fractionated and defatted milk powder; Boehringer Mannheim) and the Tropix I-Block Reagent (a highly purified casein; Tropix) to be very suitable. Bovine serum albumin (BSA) and gelatin also have been tested in early studies but resulted in higher backgrounds.

8. OTHER DETECTION METHODS

The only other technique apart from those already described here for nucleic acid detection that has become commercially available is the gold label detection system. A protocol was published some years ago by Tomlinson *et al.* [42]. Colloidal gold has been used for a long time in electron microscopy and more recently also light microscopy for detection of proteins or other antigens. The gold label also has been employed for detection of proteins on Western blots. In conclusion, the adaptation of this methodology has become possible with the advent of biotinylated or digoxigenated DNA or RNA probes. The basic feature of colloidal gold detection is the coating of the gold particles with a protein. Tomlinson *et al.* [42] used anti-biotin antibodies. Streptavidin-

gold or anti-DIG antibody–gold can also be used with equal efficiency. Generally, the colloidal gold detection method is slightly less sensitive than the AP–NBT–BCIP method. As a result it is not a serious alternative to the modern chemiluminescence methodology.

9. COMPARISON OF DIFFERENT DETECTION METHODS FOR SOUTHERN AND NORTHERN HYBRIDIZATION

As outlined in Sections 3, 4, 5–8, different methodologies for transfer of nucleic acids and labelling of nucleic acid probes and for detection of specifically hybridized probe are available today. Various combinations of these techniques are possible, and consequently can lead to significantly differing results. In the following paragraphs I will try to give a critical evaluation of the components involved in Southern and Northern blotting based on my own experience, on that of some colleagues who have also started working with these methods, and on what I could learn from the literature. It will follow the experimental steps. Literature references will not be given again. Please refer to the description and references given in the relevant sections.

In my opinion, capillary blot is still the most efficient and simplest way of transferring nucleic acids from an agarose gel to a hybridization membrane. All the other methods require special equipment and can also be susceptible, for example, to breakdown of the gel. Blotting overnight, in addition, fits very well into the "laboratory day". Alkaline blotting may provide a reduction in incubation times but is not compatible with UV cross-linking on nylon membranes. This has to be taken into account when designing a protocol. It has been shown to be highly efficient with Immobilon-P membrane. The choice of membrane is another critical feature of the assay system. Although I have worked for a long time efficiently with Immobilon-P (in combination with alkaline transfer) for colorimetric detection, I have switched to a high-quality nylon membrane (Boehringer Mannheim) because of the ease of handling. In addition, it is tested for compatibility with chemiluminescence detection. Other membranes are also suitable (Biodyne A, Hybond-N or Tropilon) or may be tested in advance for performance. For some time, strongly positively charged membranes have been viewed as preferential support, but it has become clear that the strong positive moieties lead to increased background with non-radioactive detection procedures. Therefore, a neutral membrane or a partially positive one such as the Boehringer nylon membrane should be chosen. This has been observed for nylon membranes as well as for PVDF (Immobilon) membranes. Nitrocellulose membrane is not recommended for routine assays because of its brittleness, which renders handling quite difficult.

Covalent fixation of the transferred nucleic acids to the membrane support has been performed for years by heating the nitrocellulose filters in a vacuum oven to 80°C for 2 h. This is now unnecessary since the introduction of UV cross-linking, which is compatible with all membranes, including nitrocellulose filters. It has been shown that in most cases even a higher sensitivity can be reached by UV treatment. Irradiation can simply be performed for 2–3 min on a UV transilluminator present in every laboratory working with DNA or in a more precise manner by using a UV illuminator, which allows energy dose to be controlled. In addition, there seems to be increased background when the membrane is allowed to dry before UV treatment and immersion into prehybridization buffer. This may be due to particles or compounds originating from the agarose or PAA gel adhering to the membrane which can be removed by the prehybridization buffer. Therefore, I recommend proceeding continuously from blotting to UV cross-linking and prehybridization with the wet membrane without drying.

Today, different recipes for hybridization buffers are available. The simple one described by Boehringer Mannheim for the digoxigenin system has proven to lead to good results in my hands. On the other hand, there is no reason not to continue with other well established buffer compositions as long as they are free from alkaline phosphatase.

With biotin- or digoxigenin-labelled probes hybridization and washing conditions can be easily transferred from radioactive procedures to non-radioactive ones, normally without any changes. Hybridization with 50% formamide seems to be beneficial to low background staining (F. Wolter, personal communication). The choice of label clearly should be restricted to biotin and digoxigenin, and I would give a slight preference to digoxigenin although I have also worked for a long time with biotin. My own experience and results from the literature show a tendency towards slightly better performance with digoxigenin in general. However, in case of simultaneous detection of two different probes on the same blot, both labels can be used without any problems. In this case, only colorimetric detection with two different substrates is possible with the inherent limitation in sensitivity.

The next parameter is the choice of the reporter enzyme. As outlined in Table 2, AP is the most sensitive reporter enzyme. Other enzymes produce inferior results. Gold labelling has also been shown to be less efficient. Therefore, AP clearly has to be considered to be the enzyme of choice.

Different levels of sensitivity and experimental details are inherent in the various substrates of AP. For experiments where only limited sensitivity is required (*e.g.* plasmid hybridization) colorimetric detection, perhaps using different "fast colours", is a simple and efficient method for Southern blotting. This is also true for analysis of bacterial genomic DNA. All other types of analyses, including those requiring ultrasensitive detection or rehybridization of the membranes, should be performed with the highly versatile chemiluminescent technique involving AMPPD or CSPD (Tropix) as substrates. It has been demonstrated that results equal to radioactive labelling can easily be obtained. Human or plant genomic Southern hybridizations as well as detection of rare mRNAs in Northern hybridizations can be performed well with this technique. Sensitivity is partly dependent on the type of labelling reaction and on the type of probe used. Oligonucleotides and probes with a very low number of labelled nucleotides always provide lower sensitivity.

In summary, a procedure based on all these considerations has been developed and successfully applied to plant molecular biology. A detailed protocol is published in ref. 9.

10. COLONY HYBRIDIZATION, FINGERPRINTING, RESTRICTION FRAGMENT LENGTH POLYMORPHISMS

The chemiluminescence detection technique can easily be applied to several other nucleic acid analysis techniques such as colony hybridization, fingerprinting or RFLP analysis. As the general principle of these methods is the same as for Southern hybridization, only differences in the probe preparation and the aim of the experiments are significant. Depending on the required sensitivity, colorimetric detection may also be applicable.

Haas and Fleming [43] reported a modified lysis procedure that is compatible with biotinylated DNA probes for hybridization and AP NBT BCIP colorimetric detection. Efficient determination of positive clones has been described. An even simpler procedure for lysis of *Escherichia coli* is described in ref. 9. As an alternative to nitrocellulose filters, which have been routinely used for colony hybridization for a long time, nylon membranes may display advantages in chemiluminescence detection because of better signal generation.

DNA fingerprinting involves hybridization of oligonucleotide probes specific for simple repetitive sequences to produce a highly characteristic pattern of signals. Non-radioactive labelling and detection would allow many laboratories to apply this method routinely for characterization of a given genomic DNA. Colorimetric detection of digoxigenin-labelled probes has been found to be an efficient method for detection on membranes and also in gels (without blotting) [44,45]. Comparison with biotinylated probes revealed difficulties with background problems when using samples from human tissue. A typical DNA fingerprint obtained by gel hybridization and colorimetric detection is shown in Fig. 7. The general feasibility of in-gel hybridization and detection has been confirmed in another publication [46].

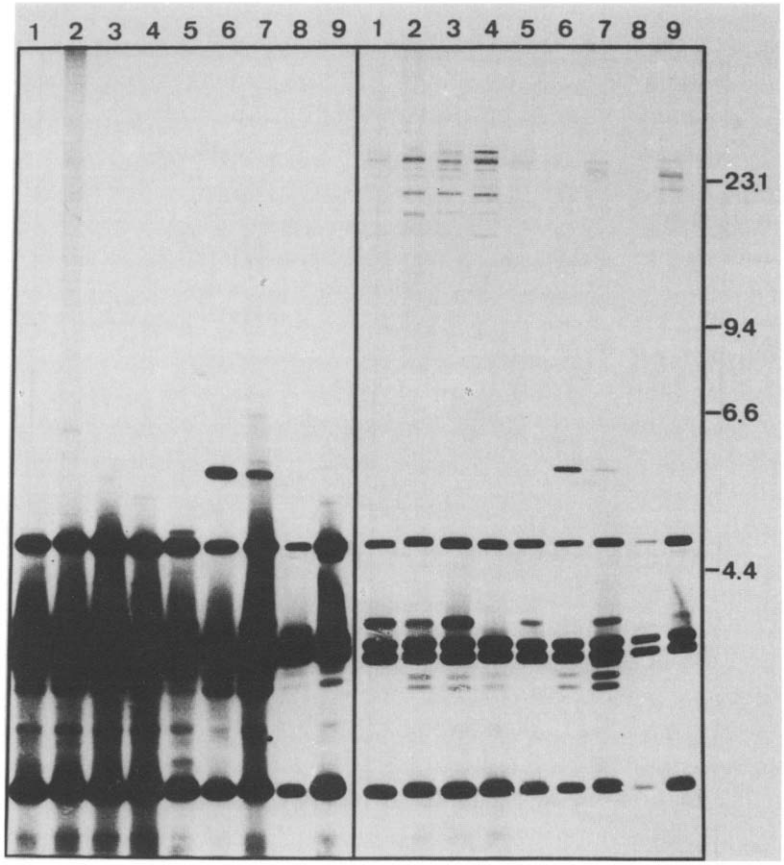

Fig. 7. Fingerprints of human DNA as obtained with the digoxigenin- and ^{32}P-labelled $(GACA)_4$ probes. DNA (10 μg) of each individual was HinfI digested and electrophoresed in 0.7% TAE gels at 45 V for 40 h. DNA was blotted onto PVDF membranes (Millipore, Bedford, MA, USA). Samples were from father (lane 1), mother (lane 2), child 1 (lane 3), child 2 (lane 4) and unrelated persons (lanes 5–9). Molecular mass markers are indicated in kilobases. Left, detection with ^{32}P; right, detection with digoxigenin–AMPPD. Reproduced from ref. 45 with permission.

As the samples were plasmid fragments in large amounts no conclusions can be drawn with respect to the sensitivity of this technique.

RFLP analysis technically is a Southern blotting and hybridization procedure. Therefore, the same requirements and limitations as discussed above are valid for this technique. Following introduction of non-radioactive labelling and detection to RFLP mapping is now possible without any problems. Two examples of plant RFLP mapping will be discussed in Section 13.

11. DNA-BINDING PROTEINS

Another interesting field of separation is the analysis of DNA-binding proteins, which has become a major tool in recent time. Usually, significant amounts of radioactivity are involved in gel retardation assays or "South-Western blots". A substitute for radioactivity would greatly facilitate performance of the experiments. An additional aspect has to be considered for introduction of non-radioactive labels into protein–DNA-binding assays. Radioactively labelled probes do not differ in any steric respect from natural, non-labelled DNA sequences. Therefore, DNA binding by the protein can be regarded as reliable. In the case of non-radioactive labelling all marker molecules will significantly change the three-dimensional structures of the DNA fragments.

In South-Western blotting protein extracts are

separated in a PAA gel and transferred to a membrane by electroblotting. This membrane is incubated with a labelled oligonucleotide containing the suspected interacting DNA sequence. Proteins that can interact with this DNA sequence will bind to the oligonucleotide and, after detection, generate a signal. Digoxigenin-labelled probes have been employed in the discussed investigations because of experimental presets. Biotin-labelled probes should generally be applicable as well. Dooley *et al.* [47] used a digoxigenin-labelled oligonucleotide probe generated by random primed labelling and were able to detect clear signals coinciding with those obtained on a radioactive blot performed in parallel. In addition, background problems inherent to the radioactive detection method have not been observed with this non-radioactive detection method (see Fig. 8).

A similar system has been used by Tan [48]. End-labelling and random primed labelling of an approximately 60-nucleotide probe were compared. Although one would expect the random primed probe to result in weaker signals or no signal at all (because labels are introduced randomly throughout the complete sequence) it performed even better than the end-labelled probe. With a shorter probe, end labelling gave only weak results whereas random primed labelling led to good signals. This may be explained in the following way: during random primed labelling, on average about every 20th or 25th nucleotide will be labelled. A population of heterologously labelled oligonucleotides will be generated. Some of these probes will be able to interact with the protein because of the favoured location of the labels away from the recognition site. In the case of end labelling the marker molecule always resides at the same position. If the recognition site is located near one of the ends this will necessarily lead to disruption of binding. In those cases randomly labelled oligonucleotides will be the only possible probes allowing a protein–DNA interaction.

Gel retardation assays represent another method of investigating DNA-binding by proteins. Suske *et al.* [49] employed a digoxigenin-end-labelled oligonucleotide probe. In this method protein extracts are incubated with the labelled probe and then eletrophoresed in a DNA gel. DNA–protein complexes will lead to a band shift in comparison with the free oligonucleotide. As the complex is much larger than the free DNA it migrates much slower in the gel. Clear signals can be obtained with this method (Fig. 9). In conclusion, the choice of labelling (random primed *versus* end labelling) will ultimately depend on the specific protein–DNA interaction and on the oligonucleotide used in each assay. Generally, colorimetric detection produces good results although chemiluminescence detection will further increase the flexibility of the method. Especially for this technique, the ability to label probes on a large scale and to store them is very useful because often larger sets of oligonucleotides have to be

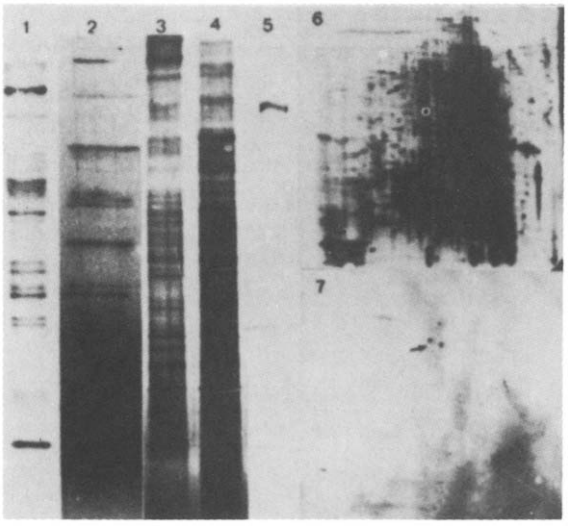

Fig. 8. South-Western analysis of DNA-binding proteins. Lanes 1–3, HT 1080 nuclear extracts, one-dimensional electrophoresis; 1 = South-Western blot using non-radioactive digoxigenin-labelled plasmid pc fos competed with 25 μg/ml *E. coli* DNA; 2 = same as 1 using a radioactive probe; 3 = silver stained gel. Lanes 4 and 5, mitochondrial (mt) extract; 4 = silver-stained; 5 = South-Western blot using a non-radioactive digoxigenin-labelled mt-DNA-specific oligonucleotide (43 bp, inhibited with 1 mg of *E. coli* DNA) showing a specific binding of this fragment to a 90 kDa protein. 6 = HT 1080 nuclear extract, two-dimensional electrophoresis, silver-stained; 7 = South Western blot according to 6 using the same probe as in 1. DNA-binding proteins are indicated by arrows. Reproduced from ref. 47 with permission.

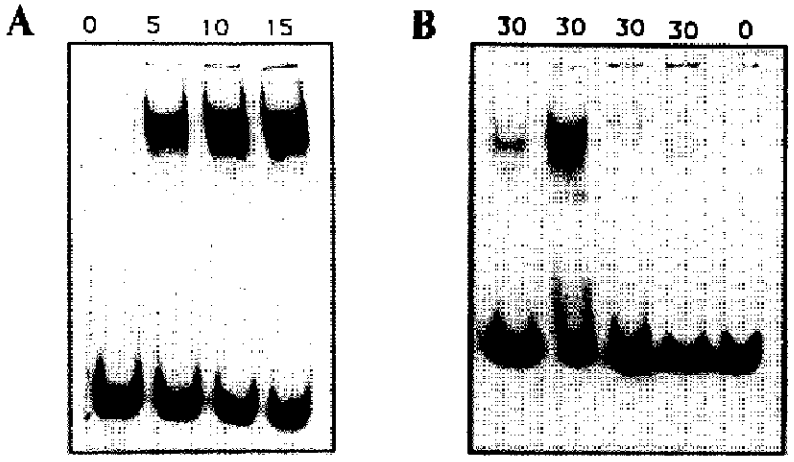

Fig. 9. Gel retardation assay for DNA-binding proteins. Stained Biodyne B membranes after electrophoresis and electroblotting. (A) Increasing amounts of rabbit progesterone receptor (5, 10, 15 ng) binding to the digoxigenin-labelled oligonucleotide. (B) Different preparations of glucocorticoid receptor (30 ng) binding to the digoxigenin-labelled oligonucleotide. Reproduced from ref. 49 with permission.

analyzed and compared first before extensive application of a single one is possible.

12. DNA SEQUENCING

DNA sequencing has become an important tool in molecular biology. Nucleotide sequences are essential information for many experiments. Radioactive labelling with ^{32}P or ^{35}S has been used since introduction of the two standard sequencing techniques (Sanger and Maxam–Gilbert sequencing methods). Some years ago, automated DNA sequencing was developed. Non-radioactive labels are involved in automatic detection (*e.g.* fluorescent labels). Without special equipment, detection of fluorescent labels is not possible so that this detection technique is restricted to automated sequencers.

Manual sequencing based on colorimetric detection of the bands has been reported for the Sanger as well as the Maxam–Gilbert method [50,51]. In the case of non-radioactive detection transfer of the DNA bands from the sequencing gel to a membrane support is required. This may be accomplished simply by vacuum blotting, electrophoretic transfer or in a more sophisticated manner using a direct blotting sequencer. During direct blotting electrophoresis the bands leaving the sequencing gel are immediately transferred to the membrane rotating with increasing speed along the end of the gel. This allows stretching of the larger fragments and enables more nucleotides to be read on one gel. Commercial equipment is now available from several suppliers. The chain-termination method (Sanger) needs primers to be extended. These primers can be labelled either with biotin or with digoxigenin. Detection can easily be performed by incubation of the sequencing membrane with the appropriate conjugate and colorimetric or chemiluminescence detection. In case of the chemical degradation method (Maxam Gilbert) the label has to be introduced during the second-strand synthesis step from single-stranded template DNA. It has emerged that conventional sequencing protocols are fully compatible with biotin- or digoxigenin-labelled nucleotides. Results are similar to those obtained by radioactive sequencing. The highest sensitivity is not required during these experiments. Therefore, only partial transfer of the DNA from the gel to the membrane will be sufficient to obtain clear signals.

The flexibility and compatibility of non-radioactive sequencing has been significantly extended

by introduction of the chemiluminescent detection technique. Results are now available on a hard copy (X-ray film) as for radioactive sequencing. Data processing by automatic systems may be performed as done usually. No difficulties will arise as perhaps possible with coloured signals on membranes.

Several publications are available describing sequencing protocols with biotin labels [39,50–53]. A protocol for sequencing using digoxigenin labels has also been published recently [41]. AMPPD-based chemiluminescent generation of an X-ray film hard copy needs only few minutes in comparison with the several hours to days necessary for radioactive sequencing. Resolution and sensitivity are unaltered in relation to established radioactive sequencing protocols [52]. A further improvement of the quality of sequencing films has been reported by using CSPD (Tropix) instead of AMPPD (Tropix) [39]. Exposure times can be further reduced so that a few minutes are sufficient instead of about half an hour. Bands are even sharper than with AMPPD, and after 24 h of incubation sharpness is retained excellently, whereas with AMPPD slight diffusion of the $AMP^{-}D$ anion is observed, resulting in broadening of the bands (Fig. 10). CSPD may be the optimal substrate available today. A comparison

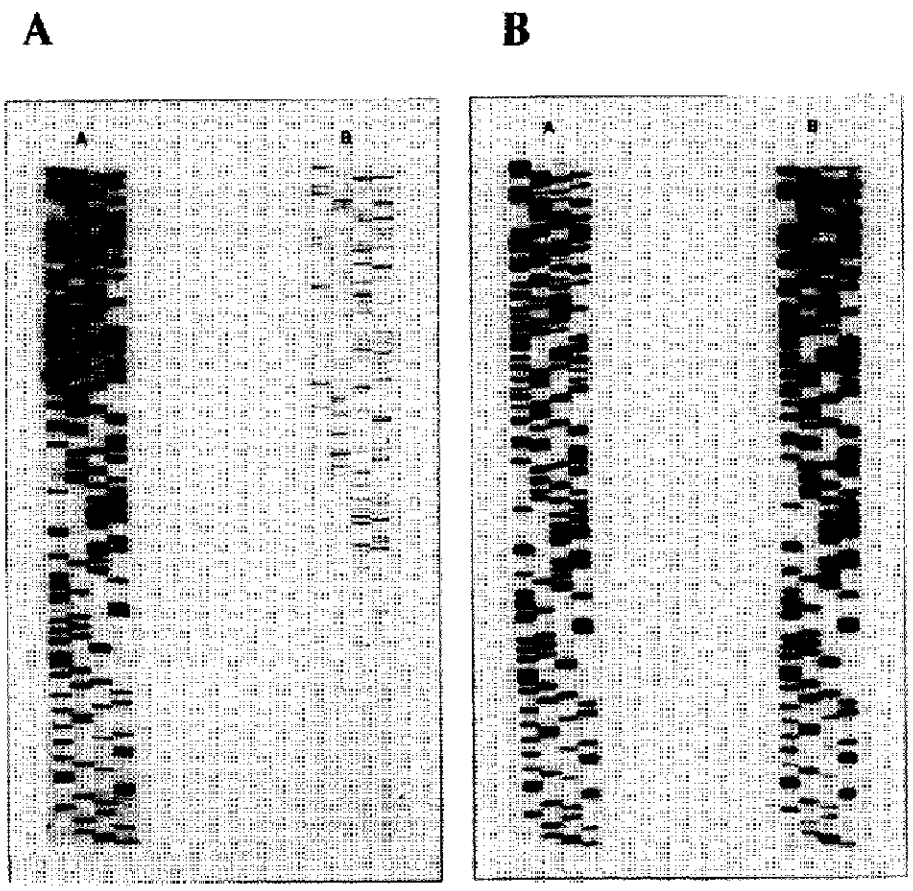

Fig. 10. Chemiluminescent DNA sequencing. (A) Comparison of light emission: CSPD *versus* AMPPD. Sequencing reactions were performed with biotinylated primers and separated on a 7.67 *M* urea ionic gradient PAA gel. DNA was transferred from the gel to nylon membrane by passive capillary transfer. Before adding substrate the membrane was divided into sections A and B. Section A was incubated with 0.25 m*M* CSPD and section B was incubated with 0.25 m*M* AMPPD. Sixty minutes after adding the substrate, both membranes were exposed to Kodak XAR film for 7 min. (B) Comparison of band resolution: CSPD *versus* AMPPD. Sequencing reactions and membrane treatment were as in A. Twenty-four hours after adding the substrates, both membranes were exposed to film for 1 min. Reproduced from ref. 39 with permission.

of biotin and digoxigenin labelling has not yet been published.

Membrane transfer and incubation with detection reagents may be considered a disadvantage of the non-radioactive sequencing methods described here. On the other hand, exposure times are drastically reduced, minimizing time to obtain the result. In my opinion, non-radioactive sequencing using the described methods is very valuable. Performing the gel run on a direct blotting electrophoresis apparatus offers the possibility of reading more nucleotides than is possible by direct exposure of the gel itself. Perhaps the most attractive field of application will be multiplex sequencing. In this technique not only a single DNA sample but a mixture of several samples is treated within the same tube. Following gel electrophoresis and transfer to a membrane, different hybridizations reveal the different sequences [54]. In this case, radioactive labels have only to be changed to non-radioactive ones without any significant additional work. Kits for chemiluminescent multiplex sequencing are available from Millipore/Biolabs for some time now.

13. APPLICATIONS IN PLANT MOLECULAR BIOLOGY

Non-radioactive nucleic acid labelling and detection has found use in research concerned with plant molecular biology for some years now. Many experiments have to be done with bacterial DNA during cloning and *Agrobacterium tumefaciens* transformation. Plasmids can easily be checked by hybridization with biotin- and digoxigenin-labelled probes and signals detected either by colorimetric or — if higher flexibility is required — by chemiluminescent substrates. Analysis of plant transformation vectors integrated into *Agrobacterium tumefaciens* can be performed rapidly in the same way. High sensitivity is not required for these experiments.

Another large part of nucleic acid analysis involved in plant molecular biological investigations and plant genetic engineering is detection of a specific endogenous or foreign gene within the plant genomic DNA and of its corresponding mRNA. Colorimetric detection of single-copy genes and low-abundance mRNAs has not been possible to be established routinely. As normally some picograms of specific DNA or mRNA have to be detected, the lower limit of sensitivity achievable with NBT BCIP staining (not less than 1 pg) is not sufficient for detecting similar amounts within the huge amount of non-specific DNA or RNA limiting the access. Nevertheless, good resolution and significant signals can sometimes be obtained with biotinylated probes and AP–NBT–BCIP staining in genomic Southern blots of transgenic tobacco plants (unpublished results).

Introduction of the chemiluminescent substrates AMPPD and CSPD for AP significantly improved the sensitivity of the non-radioactive assays. Now, by including digoxigenin-labelled DNA and RNA probes, analyses routinely can be performed. Detection of single-copy genes in 10 μg of genomic plant DNA is now possible, with the expected signals being one order of magnitude above the detection limits [9]. This technique has been successfully used for detection of foreign single-copy genes in transgenic tetraploid potato plants and its corresponding low-abundance mRNAs [40]. The simplicity of experimental work is largely dependent on the size of the different plant genomes, which may be larger than the human genome by up to about one order of magnitude.

RFLP mapping is evolving to become an important new method for plant breeding. Different cultivars may be distinguished by specific RFLP markers correlating with a desired genetic trait. The major drawback of this technique before introduction of the chemiluminescence technique was the need to use radioactivity for the Southern hybridizations. Now, non-radioactive labelling and chemiluminescence detection can easily be performed in every laboratory without any special expensive equipment. This will greatly expand the applicability of the method for plant breeders. Using digoxigenated DNA probes and AMPPD as substrate for AP, an optimized protocol has been published by Allefs *et al.* [55] and employed for RFLP screening of potato cultivars

[56]. A similar report has been published for rice RFLP analysis but still using colorimetric detection [57]. Especially within RFLP experiments the ability to reprobe blots easily is very important because large numbers of samples have to be screened with several different probes. If a new blot were necessary for each incubation the non-radioactive technology would not be accepted by scientists. With chemiluminescence detection, handling is exactly the same as with radioactive detection but without any hazard.

Nucleic acid detection techniques in plant disease diagnosis has been an expanding field for some years. The same arguments are valid in this field as for RFLP mapping because plant breeders and government laboratories are the most likely to use these diagnostic methods. An example for non-radioactive detection of potato virus X and cucumber mosaic virus has been published by Parent and Page [58]. Usually, most of the diagnostic assays in phytopathology are based on immunological techniques [mostly enzyme-linked immunosorbent assay (ELISA)] with their inherent difficulties in distinguishing related pathogens because of cross-reactivity of the antibodies. Using highly specific DNA probes distinction can be made much more decisive. Pathogen diagnosis will most probably become an important field of commercial application of non-radioactive nucleic acid detection.

14. APPLICATIONS IN BIOMEDICINE

Some examples of applications of non-radioactive colorimetric and chemiluminescence nucleic acid detection in biomedicine will be given in this section. In several of these reports the digoxigenin label is clearly preferred over the biotin label. This seems to be because of the presence of high amounts of endogenous biotin in clinical samples. Background problems with biotinylated probes have been reported to be prevented with digoxigenated probes.

Specific genes have been detected in some reports. Lanzillo [24] demonstrated the presence and expression of the gene encoding for angiotensin-converting enzyme in various tissue samples of different origin. Southern blotting using a cDNA probe and also Northern blotting with the same DNA probe were successful. The sensitivity of the Northern blot has not been reported but seems to be rather low in comparison with what can be achieved with RNA probes.

A specific DNA rearrangement (BCR/ABL) involved in chronic myelogenous leukaemia (CML) has been analysed by Lion *et al.* [59]. In previous investigations radioactive probes were employed for the verification of the rearrangement. Now, a PCR-amplified digoxigenin-labelled probe has been used in conjunction with colorimetric staining for hybridization of the PCR-amplified BCR/ABL region. Results were compatible with and equal to those obtained with radioactivity. A single nucleotide change in the mitochondrial DNA is responsible for Leber's hereditary optic neuropathy. This mutation can be detected using a special PCR amplification protocol and genomic Southern hybridization. Random primed digoxigenin-labelled probes detected heteroplasmy [60].

Viral DNAs have been detected in different experiments. Musiani and co-workers [61–63] established a system for detection of cytomegalovirus and B19 parvovirus DNA in clinical samples. Random primed probes detected 50–10 fg of viral DNA in a dot blot procedure using Lumiphos-530 as a substrate for AP. Digoxigenated probes performed much better than biotin-labelled ones, and chemiluminescence detection was five to ten times more sensitive than colorimetric detection. Another virus that has been extensively investigated in terms of non-radioactive detection is hepatitis B virus. Virus DNA is analyzed in dot blots of clinical samples. Several publications report successful application of colorimetric detection [64–67]. Hepatitis B virus could be detected at 250 fg with high sensitivity and specificity. Whereas biotinylated probes often generated false positives, the digoxigenated probes were much more reliable [64,65]. Some samples reproducibly led to false positives also with digoxigenated probes. This may be because of the immunological incubation step involved in non-radioactive detection methodology. This

problem may be overcome by improving sample preparation.

M protein of *Streptococcus* is crucial for infections. A specific oligonucleotide 30 nucleotides long hybridizing to the M protein gene detected the presence of the bacteria in a dot blot of isolated total DNA from clinical samples [68]. A comparison of biotinylated probes and chemiluminescent detection with the PhotoGene system (BRL) and digoxigenated probes and colorimetric detection using NBT–BCIP revealed equal sensitivity of both systems. Other bacteria identified by DNA hybridization were enterotoxic *Escherichia coli* by colony hybridization [69] and *Mycobacterium tuberculosis* by Southern hybridization [70]. *Escherichia coli* α-haemolysin genes have also been found in human faecal strains of *Enterobacter cloacae* [71]. Riley and Caffrey [69] identified the enterotoxic *Escherichia coli* strains by using digoxigenated DNA probes for heat-labile and heat-stable enterotoxins in a colony hybridization method. Digoxigenated probes performed better than biotinylated ones. In addition, there seems to be a non-specific binding of streptavidin to the membrane supports in this assay. With a non-radioactive RFLP method for typing of *Mycobacterium tuberculosis* strains distinction of different strains became possible. Hybridization was performed with digoxigenated *Mycobacterium tuberculosis* DNA [70]. Employing a Southern blot and a colony hybridization technique an *Escherichia coli* enterotoxin gene could also be detected in other bacteria, the first time also in *Enterobacter cloacae* [71].

Detection of minisatellite regions and of numbers of tandem repeat sequences have become very useful genetic markers in paternity determinations and forensic medicine. PCR amplification of the sequences including biotin labelling in the same step and subsequent electrophoresis and Southern blotting enables direct detection without any further hybridization step. Only incubation with the streptavidin AP conjugate and AMPPD is required. High-quality results can routinely be obtained during these investigations [72].

In conclusion, with clinical samples background problems have often been reported using biotinylated probes. These seem to be due to the high levels of endogenous biotin in many samples. As serial analyses require simple sample preparation procedures for ease of use, a case to case study has to be done for improvement of the sample preparation protocols. Raw extracts or serum samples often contain biotin. Therefore, digoxigenin, which only occurs in some special plant species, seems to be the preferred marker, at least for clinical samples.

15. PERSPECTIVES

As outlined in this review, in recent years powerful new non-radioactive nucleic acid detection techniques have been developed. Starting from the first biotin labelling system coupled to two incubation steps with streptavidin and biotinylated AP, and colour development using NBT and BCIP, significant steps forward have been made until the most advanced chemiluminescent detection system using CSPD as a substrate. The most promising technique today is digoxigenin labelling of DNA probes by random primed labelling or PCR labelling and of RNA probes by *in vitro* transcription. Hybridization on neutral or partly positive nylon membranes works well with chemiluminescence detection. Digoxigenated probes are detected by interaction with an anti-digoxigenin F_{ab} fragment that is covalently conjugated to AP. Chemiluminescence detection is the most sensitive and flexible method. AMPPD and CSPD are convenient substrates at an affordable price. Hard copies can be generated by exposure to X-ray film or instant Polaroid film.

This modern technology provides at least equal sensitivity as the commonly used radioactive labelling of nucleic acid probes and is easily applicable to a wide range of experimental techniques and protocols. The hazards involved in handling of radioactivity and the need for special laboratories and equipment are completely overcome. Experiments can easily be performed together with other routine laboratory work.

Although sensitivity similar to that of radio-

active labelling has been achieved with current protocols, a further enhancement of sensitivity might be possible by further improvement of the chemiluminescent substrates, as has been shown by the development of CSPD in comparison with AMPPD. An important problem will always be the signal-to-noise ratio. The more sensitive an assay becomes, the more problems will arise with background. When looking to the detection limits in liquid assays, which are more sensitive by some orders of magnitude, one can envisage the potential that still might be present also for solid phase techniques, *e.g.* Southern blots.

X-ray films available today are not optimal for chemiluminescence assays as described here in respect of their spectral sensitivity. This means that the range of wavelengths that are perceived by the film material is not optimal for the light emitted by chemiluminescence. Development of a specially adapted film combining the high sensitivity of Kodak X-Omat AR film with a suitable spectral range in the green region could drastically improve results and further enhance sensitivity of the assays.

Application of the new chemiluminescent detection techniques in my opinion will become very widespread in the next few years and will mostly replace the radioactive techniques used today. The environmental risks and irradiation of laboratory personnel inherent in the use of radioactivity are no longer necessary for most analytical techniques. This may also lead to a widespread application and the development of nucleic acid-based detection and analysis systems displacing immunological methods, which often lack ultimate sensitivity. In some cases, *e.g.* RFLP mapping, widespread application of new techniques will be enabled by suitable non-radioactive methods as users do not have the laboratory facilities required for radioactive work.

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