Journal of Chromatography, 618 (1993) 133–145
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6820

Review

Southern and Northern analysis

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(First received November 27th, 1992; revised manuscript received March 28th, 1993)

ABSTRACT

This review on Southern and Northern analysis, rather than providing step by-step protocols, focuses on a critical evaluation of the existing experimental methods and modifications thereof. Principal parameters influencing electrophoresis of DNA/RNA in agarose gels are outlined and at the same time alterations in these parameters for optimal resolution of DNA of varying length are discussed. Further, methods for evaluating the quality of DNA/RNA size separation in agarose gels are described. Since efficient transfer of DNA/RNA from the gel onto a membrane support is critical in both methods, several experimental approaches for transfer are compared. Also discussed in this review are alternative methods for radioactive and non-radioactive labelling of DNA probes. Finally, detailed protocols are provided for an effective hybridization of Southern and Northern blots.

CONTENTS

LIST OF ABBREVIATIONS

bp	Base pairs	kbp	Kilobase pairs
DEPC	Diethyl pyrocarborate	PCR	Polymerase chain reaction
dNTP	Deoxynucleoside triphosphate	PEG	Polyethylene glycol
ds	Double-stranded	SDS	Sodium dodecyl sulphate
EtBr	Ethidium bromide	SSC	Sodium salt citrate
		SSPE	Sodium salt phosphate EDTA

0378 4347/93/$24.00

1. INTRODUCTION

Southern analysis allows the detection of a given DNA sequence in a complex mixture of DNA sequences. The method was developed to identify homologous sequences in genomic DNA and to facilitate gene mapping. Southern blotting is also commonly used in a variety of DNA cloning techniques, since it allows the identification of correct DNA fragment(s) in analytical restriction enzyme digestions of cloned DNA material. The method is similarly applied for the verification of products obtained by the polymerase chain reaction (PCR) technique. Northern analysis allows the detection of a given RNA sequence in a complex mixture of RNA sequences. It is used to detect and quantitate a given mRNA species in cells and tissues.

The basic principles of Southern and Northern analysis techniques are very similar. First, the nucleic acid (DNA or RNA) has to be purified from eukaryotic cells or bacteria. In a second step the (charged) nucleic acid is separated according to length in a gel matrix placed into an electrical field. Following size separation, the DNA (denatured to single strands) or RNA is transferred onto a Nylon membrane and fixed to it. Subsequently, the immobilized DNA or RNA is hybridized to a homologous, labelled single-stranded nucleic acid ("probe"). The detection of the hybridized probe depends on the labelling method used. With ^{32}P-labelled probes the signal is detected and quantified using X-ray films for autoradiography. Standard molecular biology manuals [1–3] provide step-by-step protocols for Southern and Northern analysis. It is suggested that readers familiarize themselves with these protocols before reading this review. Here, the aim is to critically evaluate methodological variations of essential steps in both techniques and to provide an overview of recent technical developments.

2. SOUTHERN AND NORTHERN BLOTTING

2.1. Principal factors influencing electrophoretic mobility of nucleic acids in agarose gels

Nucleic acids are negatively charged at neutral pH and can therefore be separated according to length using the sieving properties of agarose (a highly porous polysaccharide) and the driving force of an electrical field. In a system where the electrical resistance of all components remains constant (low-voltage conditions), linear nucleic acid molecules will move in agarose at a velocity proportional to the voltage applied. It should be emphasized that the driving force for nucleic acids in an agarose gel is not the voltage set on the power supply, but the *voltage gradient* per unit length effective in the gel matrix. In a typical electrophoresis system this voltage gradient is mainly dependent on the geometry of the electrophoresis chamber, on the geometry and composition (*i.e.* the total resistance) of the gel, and on the volume and ionic strength (*i.e.* the total resistance) of the buffer used. At a set voltage the following rules apply: The greater the distance between the electrodes of the electrophoresis chamber, the lower the effective voltage gradient and the velocity of nucleic acid migration. An increase in gel thickness or buffer volume also leads to a lower effective voltage gradient in the gel matrix. Theoretically, the actual voltage gradient in the gel could be measured with a high-resistance voltmeter, but in practice this is not done. In many publications the parameter V/cm (voltage set at the power supply per cm distance between the two electrodes — *not* the voltage gradient per unit length!) is indicated — this is obviously not sufficient to define exactly an electrophoresis system. (For a more detailed discussion of physical parameters influencing agarose electrophoresis see ref. 4.) In practice, one should keep the gel geometry as well as the buffer volume and composition constant and determine the optimal voltage for a given application empirically. Ideally, the agarose gel should be covered by 3–4 mm of buffer. The maximal voltage applicable is limited by the heat generated in the

gel. With higher effective voltage gradients, constant buffer recirculation is recommended to prevent temperature gradients. Increase in voltage will eventually lead to melting of the agarose gel. However, since optimal separation of nucleic acids is usually achieved at low-voltage conditions, overheating of the gel is not a limitation with the commonly used low-salt buffers.

As a general rule, double-stranded (ds) linear DNA or RNA migrates through a gel matrix at a velocity approximately inversely proportional to the $\log_{10}$ of the number of base pairs [5]. Conditions used to achieve optimal size separation vary depending on whether DNA or RNA is analysed. Electrophoresis parameters for both techniques are therefore discussed in detail in the following chapters.

2.2. Agarose gel electrophoresis and blotting of DNA

Southern analysis was developed by E. M. Southern in 1975 [6]. It is used to detect a given DNA sequence in a complex mixture of DNA sequences, *e.g.* in restriction mapping of genes or in the detection of restriction fragment length polymorphism. Since its original description, the principle of Southern analysis has remained essentially unchanged. The main modifications have been the introduction of Nylon membranes instead of cellulose nitrate for binding of blotted DNA, and the establishment of vacuum blotting instead of capillary transfer [7,8]. Today, agarose electrophoresis allows separation of DNA ranging from 200 base pairs (bp) to $10 \cdot 10^6$ bp. However, no single gel system allows the separation of nucleic acids over this range of length. The "classical" Southern analysis can resolve DNA between 200 bp and 20 kilobase pairs (kbp). For separation of DNA shorter than 200 bp or for optimal resolution in the range of 10 bp to 1 kbp, polyacrylamide gel systems are applied [9–11]. DNA larger than 20 kbp is analysed using the pulsed-field gel electrophoresis technique [12].

By far the majority of applications of Southern analysis require the discrimination of DNA in the range of 0.2–20 kbp, and this is discussed below. In "classical" Southern analysis various parameters are modified to achieve optimal resolution in the desired range of DNA length. Two important parameters are the percentage and the composition of the agarose gel used. When resolving DNA of 0.2–1 kbp, good results are achieved with 2–4% agarose gels using a 3:1 mixture of low-melting agarose and standard agarose (product information provided by FMC BioProducts, Rockland, ME, USA). These high-percentage gels are typically used for Southern analysis of PCR products or cloned DNA. Since the amount of target DNA is not limiting in these applications, sufficient amounts of size-separated DNA can be transferred onto a membrane support in spite of the high density of the agarose matrix. For the analysis of *genomic* DNA, 0.7–1.2% agarose gels are used. Gels below 0.7% are difficult to handle, while transfer efficiencies with high-percentage gels (> 1.2%) are unsatisfactory in the analysis of single-copy genes where the amount of target DNA is the limiting factor. Table I provides a guideline for the percentage of agarose gels used to achieve an optimal resolution in the 0.2–20 kbp range, as well as the approximate migration of two commonly used dyes, relative to DNA length standards, in gels of various agarose percentage.

TABLE I

OPTIMAL RESOLUTION OF DNA OF VARYING LENGTH IN AGAROSE GELS[a]

Size of DNA fragment (kbp)	Agarose concentration (w/v%)	Bromophenol blue dye migration[b]
0.1–0.5	4% agarose blend[c]	35 bp
0.5–1.0	3% agarose blend	Not determined
0.4–6	1.2% agarose	400 bp
1–20	0.7% agarose	700 bp

[a] Partly adapted from ref. 2 and product information provided by FMC BioProducts (Rockland, ME, USA).

[b] Approximate dye migration in TAE buffer (0.04 *M* Tris–acetate, 1 m*M* EDTA, pH 8.0) relative to DNA standards, varies slightly with various agarose brands.

[c] Agarose blend of three parts low-melting agarose and one part standard agarose (FMC BioProducts).

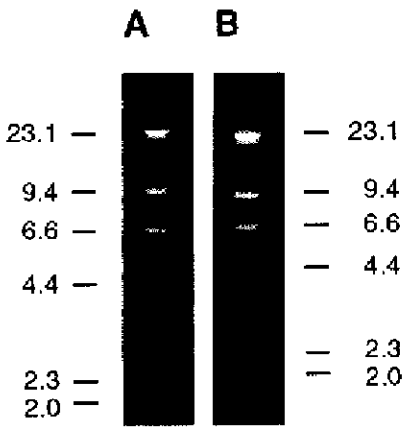

Fig. 1. Effect of voltage conditions on size separation of dsDNA of various lengths. *Hind*III-cut lambda DNA was separated electrophoretically at 28 V/37 mA for 16 h (A) or at 70 V/97 mA for 5.25 h with buffer recirculation (B); all other parameters were identical. It is apparent that low-voltage conditions favour size separation of DNA between 2.0 and 9.4 kbp, whereas DNA between 9.4 and 23.1 kbp migrates relatively faster at high voltage conditions.

A second parameter in DNA electrophoresis, which has major effects on resolution, is the voltage applied. High voltage combined with short run times gives optimal size separation of DNA 10–20 kbp in length, whereas low voltage combined with long run times gives good resolution between 1 and 10 kbp. An example is given in Fig. 1. As mentioned before, the application of high voltage requires a constant buffer recirculation to avoid uneven heat distribution in the gel/buffer system, whereas this is not necessary with low-voltage runs.

For the analysis of single-copy genes most standard protocols suggest the use of 10 μg of DNA. By changing the geometry of the sample wells in the gel, the same signal can in my experience be achieved with 2.5 μg of DNA, an important aspect in all applications where the amount of DNA is limiting. To this end I use 2.7 mm × 1.5 mm combs to generate gel pockets with a volume of 13 μl. Electrophoresis and blotting are performed according to standard protocols. An important prerequisite in the analysis of single-copy genes is an optimal restriction enzyme digestion of genomic DNA. Statistically, if 80% of restriction enzyme sites are cut, only 64% (0.8 × 0.8) of fragments with the correct size are generated. When digesting mini-prep genomic DNA, which is often less pure than standard preparations, the addition of spermidine (5 m*M* final) to intermediate- or high-salt enzymatic reactions considerably improves the final result [13–15].

Staining of the electrophoresed DNA is very informative. This can easily be achieved with the use of dyes binding to DNA (and RNA), such as ethidium bromide (EtBr). The gels can be stained *after* electrophoresis, but the results are suboptimal. Best staining of DNA is achieved with EtBr present during electrophoresis. EtBr is added to a final concentration of 0.5 μg/ml, both to the melted agarose (cooled to 55°C) *and* to the electrophoresis buffer. This procedure allows a constant monitoring of DNA migration and also a final photographic documentation of the size-separated DNA. Staining of restriction enzyme-digested genomic DNA is particularly informative. When size-separating enzyme-restricted genomic DNA I use 15-cm-long gels and electrophoresis conditions under which short DNA fragments are still retained in the lower portion of the gel. After electrophoresis, distinct bands become visible in this lower portion of the gel. These bands result from restriction site repeats in genomic DNA and are characteristic of the restriction enzyme used (Fig. 2). Their appearance confirms an adequate enzymatic digestion of the genomic DNA as well as a correct size separation during gel electrophoresis. After photographing, I cut off the lower 5 cm of the gel and use the remaining gel for Southern blotting (when analysing DNA > 2.5 kbp). It should be noted that EtBr is a powerful mutagen. The use of EtBr requires appropriate inactivation of the mutagenic properties of the dye before disposal [2].

After electrophoresis, the DNA is transferred onto a membrane support. Many protocols still suggest the use of nitrocellulose for this purpose. In my opinion, Nylon membranes are clearly superior to nitrocellulose because of their higher DNA/RNA binding capacity and their greater mechanical strength on stripping and reprobing. A systematic comparison of a large number of

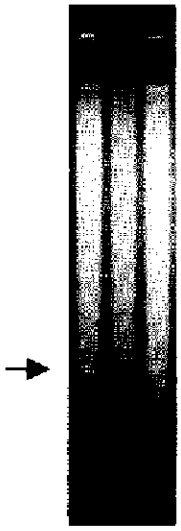

Fig. 2. Restriction site repeats in genomic DNA. Human genomic DNA was digested with the restriction enzyme *Hind*III and size-separated by agarose (0.7%) gel electrophoresis at 28 V for 16 h in the presence of ethidium bromide (0.5 μg/ml). Note the appearance of a band characteristic of a *Hind*III digestion (arrow) caused by restriction site repeats in genomic DNA.

Nylon membranes for use in Southern and Northern analysis has been conducted [16,17], however, the hybridization protocols used may not have been optimal for all membrane types tested. I have compared Nylon membranes from several major manufacturers under identical blotting/hybridization conditions and found only insignificant differences. Before DNA transfer I strongly recommend "soaking" the Nylon membrane in the transfer buffer used (typically 20× SSC; 1× SSC is 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7.0) for more than 30 min, since this prevents a "moth-eaten" appearance of transferred DNA on hybridization.

Several parameters are critical to achieve an optimal transfer of DNA from a gel to a membrane support. First of all, the dsDNA has to be denatured to single-stranded DNA, and this is achieved by soaking the gel in 0.5 *M* sodium hydroxide plus 1.5 *M* sodium chloride for 45 min. For the analysis of DNA >5 kbp many protocols recommend depurinating the DNA prior to denaturation by soaking the gel in 0.2 *M* hydrochloric acid for 5–15 min [1]. The depurination by hydrochloride, upon subsequent denaturation by sodium hydroxide, leads to nicks in the DNA strands, resulting in a breakdown of long DNA fragments into shorter pieces. These shorter fragments are more efficiently transferred in the blotting process. In my experience the depurination step introduced by Wahl *et al.* [18] is *not* required. However, if used, the duration of the exposure of the gel to 0.2 *M* hydrochloric acid should be carefully determined, since extensive depurination and nicking of DNA will drastically reduce the hybridization signal finally obtained. Obviously, the size of target DNA as well as gel thickness and percentage have to be taken into account in this procedure. For high-molecular-mass DNA, as used in pulsed-field gel electrophoresis, nicking of DNA is an absolute requirement and can also be achieved by exposing the gel to short-wave UV light [19]. In contrast to the depurination step, the denaturation step with 0.5 *M* sodium hydroxide plus 1.5 *M* sodium chloride is obligatory. After a subsequent neutralization step (soaking of the gel in 1 *M* Tris, 1.5 *M* sodium chloride, pH 7.4, for 45 min), the DNA is transferred onto a membrane support.

For the physical transfer of DNA onto the membrane support various methods are used. Southern [6] described "ascending" capillary transfer, which is still the most widely used method. Recently, a modification of this procedure, designated "descending" capillary transfer, has been introduced. In this modification there is gravitational flow of the transfer buffer which, according to the observations of the authors, allows a more rapid blotting of DNA [20,21]. I have routinely used "ascending" capillary transfer with 20× SSC as transfer buffer and have determined that a transfer time of 12 h clearly gives better results than transfers over 4 or 8 h. When using this "classical" method one should avoid an early compression of the gel matrix, which prevents an efficient diffusion of DNA onto the membrane support. The weight on top of the stack of blotting paper should therefore be limited to approximately 350 g for standard-size gels.

Regarding the transfer buffer, Southern has determined that retention of DNA on cellulose

nitrate filter supports is best with high-salt buffers, *e.g.* 20 × SSC [6]. Later, Reed and Mann [22] introduced 0.4 *M* sodium hydroxide as an alternative transfer buffer applicable with Nylon membranes. This was the basis for several modifications of "alkaline blotting" [23,24]. In my experience, however, the use of 0.4 *M* sodium hydroxide for transfer leads to a diminished specific signal and to increased non-specific background on hybridization, an observation also made by others [16,25]. The use of 0.025 *M* sodium phosphate, pH 6.5, for transfer onto Nylon membranes in my hands also yielded suboptimal results when compared with 20 × SSC. In this context, one should keep in mind that optimal transfer of DNA onto a membrane support does not necessarily lead to an optimal hybridization signal. In fact, only a fraction of the membrane-bound DNA (or RNA) is accessible to the hybridizing probe. The size of this fraction is determined by the degree of cross-linking achieved by baking or UV fixation (see below), by the strength of DNA binding to the membrane and by other ill-defined factors. Saluz and Jost [26] have obtained optimal hybridization signals with blotting/fixation conditions under which only 30% of the DNA bound to the membrane. When comparing two methods for Southern blotting it is therefore critical to compare the final hybridization signals obtained and not the transfer rates of ^{32}P-labelled DNA onto a membrane support.

Recently, vacuum blotting has been introduced as an alternative to capillary transfer [7,8] and commercial apparatuses are available for this purpose. With vacuum blotting, the gel is pretreated in the same manner as for capillary transfer and is also blotted with 20 × SSC. Major advantages of vacuum blotting are the rapidity and reproducibility of the transfer. The degree and duration of negative pressure applied to the gel have to be determined experimentally and depend on the agarose percentage and the thickness of the gel. With a 1% agarose, 1-cm-thick gel, vacuum blotting at −40 to −50 cmH_2O for 4 h is a reasonable starting point. Owing to its speed, vacuum blotting is convenient for blotting of single gels. If several gels have to be subjected to Southern blotting in parallel, the method becomes more cumbersome. When using vacuum blotting, we recommend replacing the usual rubber mask with an appropriate silicone material. This simple modification significantly improves the seal, and soothes the nerves of the experimenter! Electroblotting, which was described as an alternative to capillary transfer and vacuum blotting [27], never gained popularity because of its poor reproducibility and the equipment required.

After transfer, the DNA has to be "fixed" onto the membrane support. This is achieved by baking the still damp (!) membrane at 80°C for 2 h or by UV light irradiation for a few minutes, followed by air drying. Both methods are equally effective for fixation of DNA to a Nylon membrane support. With UV irradiation the optimal exposure times have to be determined experimentally (for details see the following section on blotting of RNA). The baked or UV-fixed Southern blots can be stored for prolonged periods of time at room temperature. After capillary or vacuum transfer I suggest restaining the gel in 500 μg/ml EtBr for several hours or overnight and determining the efficiency of DNA transfer under UV light.

2.3. Agarose gel electrophoresis and blotting of RNA

Northern analysis allows detection of a given RNA species in a mixture of heterogeneous RNA, and is therefore another basic technique in molecular biology. For standard protocols the reader is again referred to molecular biology manuals [1–3]. In contrast to electrophoresis of DNA, where separation of nucleic acids varying between 100 bp and $10 \cdot 10^6$ bp in length is performed, electrophoresis of RNA typically requires separation in the 0.5–6.0 kbp range. Consequently, to obtain optimal resolution, the use of 1.2% agarose gels is standard practice. Compared with DNA, RNA is more prone to degradation. The reagents used to prepare RNA have to be kept free from ubiquitous RNAses, which is achieved by treatment of most reagents with

diethyl pyrocarbonate (DEPC [2,28]). Equally important but less frequently observed is the fact that RNA will be chemically degraded at pH values >7.5 or <4.0. A meticulous adjustment of the pH of all solution coming into contact with the RNA is therefore critical.

Several methods are used to extract RNA from cells and tissue. The method described by Chirgwin *et al.* [29] and Glisin *et al.* [30] needs ultracentrifugation equipment. Another drawback of this method is that (without special modifications) one requires a relatively high amount of starting material (*e.g.* $50 \cdot 10^6$ primary T-cells) to recover the RNA. However, the quality of the RNA obtained by the method of Chirgwin *et al.* [29] is to date unsurpassed, making it the standard method for RNA extraction in most laboratories, including my own. A quick RNA preparation method recently introduced by Chomczynski [21] yields RNA of acceptable quality for Northern analysis, but the quantitation of RNA following extraction is difficult owing to the presence of unidentified material absorbing in the 230–280 nm UV range. In our view this latter method is optimal for obtaining RNA from limited number of cells, as is often required for PCR analysis.

Since single-stranded, native RNA tends to form secondary structures, it has to be denatured before electrophoresis and kept in a denatured state during electrophoresis. Before electrophoresis, denaturation is achieved by heating the sample to 55°C in the presence of formaldehyde and formamide. During electrophoresis of RNA, addition of formaldehyde to the gel prevents re-formation of secondary structures [31]. As an alternative to formaldehyde denaturation, modification of RNA with glyoxal was introduced by McMaster and Carmichael [32], yielding excellent results. However, the glyoxal products have to be removed from the RNA after blotting, and this requires an additional experimental step. Denaturation of RNA by mercury has also been described [33], but did not gain broad acceptance because of the inherent toxicity of the system. As a result, formaldehyde denaturation of RNA for Northern analysis is the standard method in most laboratories.

Standard protocols for Northern blotting suggest the use of 10 μg of RNA for analysis. I have downscaled the amount of RNA loaded to 5 μg without loss of signal. The RNA is denatured (see Table 2) and loaded into small gel pockets (2.7 mm × 1.5 mm). Using an appropriate comb, up to 30 RNA samples can be analysed at the same time. After loading, RNA is size-separated at 70 V for 3.5 h with buffer recirculation (for details see ref. 28).

RNA can be stained by addition of EtBr to the

TABLE 2

DENATURATION, STAINING AND ELECTROPHORETIC SEPARATION IN NORTHERN BLOTTING

(1) The RNA sample finally loaded onto the gel is composed of:
5.5 μl RNA (5 μg) in water
19.5 μl premix
5.0 μl loading solution
1.0 μl EtBr of 0.5 mg/ml stock
31.0 μl

(2) The sample is heated to 55°C for 15 min (denaturation of RNA).

(3) The sample is briefly quenched on ice, loaded onto a 1.2% agarose–1.1% formaldehyde gel. The RNA is separated electrophoretically at 70 V for 3.5 h with buffer recirculation (for further details see ref. 28).

Premix: 1.3× MOPS buffer, 3 *M* formaldehyde, 64% formamide.

Loading solution: 1 m*M* EDTA, pH 8.0, 0.25% saturated bromophenol blue solution, 0.25% saturated xylene cyanol solution, 50% glycerol.

1× MOPS buffer: 0.02 *M* 4-morpholinepropanesulphonic acid, 5 m*M* sodium acetate, 1 m*M* EDTA, pH 5.5–7.0.

sample prior to heat denaturation [28,34]. Alternatively, EtBr can be added to the gel, but this gives unsatisfactory results. After electrophoresis the gel is photographed on an UV transilluminator. The image obtained provides information on the size separation of the RNA, on RNA integrity and on the relative positions of the 28S (5.0 kb [35]), 18S (1.87 kb [36]) and 5.8S (0.16 kb [37]) ribosomal RNA bands that serve as size markers. At the same time, the amount of RNA loaded per lane can easily be assessed (Fig. 3A and ref. 34).

After electrophoresis the size-separated RNA has to be transferred onto a membrane support, preferentially a Nylon membrane. As with Southern analysis, capillary blotting or vacuum blotting can be used. We have directly compared both principles for Northern blotting and obtained a clearly better hybridization signal with vacuum blotting (-60 cmH_2O for 4 h), compared with overnight capillary transfer [28]. Like transferred DNA, RNA has to be fixed onto the membrane. With RNA, fixation by UV light irradiation is clearly superior to baking at 80°C [16,28,38]. The optimal UV irradiation dose has to be determined experimentally, since overexposure leads to a decrease in hybridization signal (author's own observation) thought to result from extensive cross-linking of the RNA. Prior to UV exposure the membrane is briefly air-dried. The degree of moisture still contained in the filter has to be kept constant since water significantly absorbs UV light. Another potential variable is the ageing of UV light bulbs. Commercial suppliers of UV fixation ovens try to check the decrease of UV light intensity over time by directly measuring UV light over a given nanometer range, or by measuring total light energy, which is less satisfactory. UV fixation of RNA can also be performed by placing the Nylon membrane, RNA side down, onto a UV transilluminator covered with plastic wrap. Since the EtBr is still bound to the blotted RNA, the lanes containing size-separated RNA light up. By taking a photograph of the UV-exposed membrane one can easily document both the quality and quantity of transfer (Fig. 3B). The use of EtBr to stain RNA in Northern analysis decreases the final hybridization signal by 12–18% compared with unstained RNA [28,34]. However, in my view this moderate decrease in signal is clearly outweighed by the critical information available when stained RNA is used. After UV fixation the membrane is completely air dried and can be stored at room temperature for prolonged periods of time.

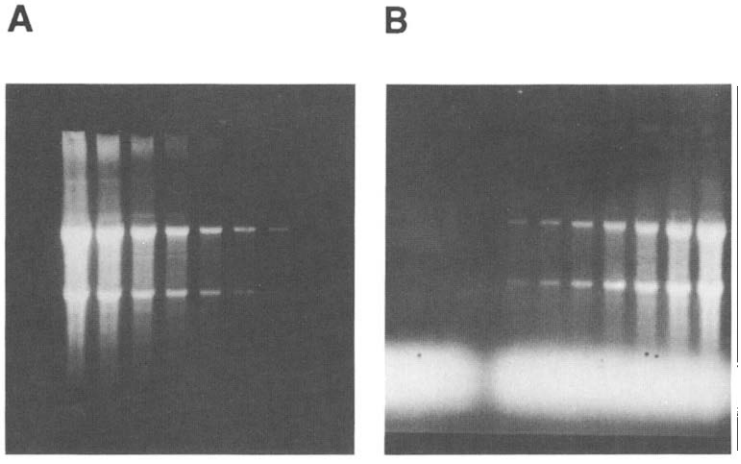

Fig. 3. Effect of ethidium bromide staining of RNA in Northern blotting. Various amounts of total RNA (20–0.3 μg) were stained with ethidium bromide, separated electrophoretically (for details see ref. 28) and photographed (A). The RNA was then blotted onto a Nylon membrane by vacuum transfer. The photograph shown in (B) was obtained during UV fixation of the blotted RNA. Note that the picture is side-reversed, since the blot was photographed with the RNA facing down. The bright fluorescence signal in the lower part of the blot was caused by the concomitantly transferred bromophenol blue dye.

3. PROBE LABELLING AND HYBRIDIZATION

3.1. General considerations

Since many aspects of signal detection are common to both Southern and Northern analysis, they are discussed together in this section. In principle, a labelled, denatured single-stranded probe is hybridized to membrane-bound homologous DNA or RNA. Temperature, salt and solvents (*e.g.* formamide) are important parameters influencing the kinetics of this reaction and the relative binding strength of DNA–RNA and RNA–RNA duplexes. These parameters are extensively reviewed elsewhere [39].

Conventionally, a dsDNA probe is labelled, using ^{32}P-modified deoxynucleoside triphosphates (dNTP), by "nick translation" [40] or by "random-primed" DNA labelling [41] to a specific activity of $> 1 \cdot 10^8$ cpm/μg DNA. In the nick translation method, DNAse I first nicks one strand of the DNA, then *Escherichia coli* polymerase I incorporates labelled dNTPs at this site. In random-primed labelling, the dsDNA is denatured and random hexamers or nanomers are hybridized to the template DNA strands. The annealed primers are then extended by the Klenow enzyme or by the T7 DNA polymerase utilizing labelled dNTPs. With random-primed labelling the probe can be labelled to a higher specific activity, however, the resultant probe is statistically shorter than the nick-labelled probe (Fig. 4). Theoretically, one should achieve a stronger hybridization signal with random-primed probes. In practice the signals obtained are often similar in both methods (author's own observation) — the shorter random-primed probes apparently hybridize less efficiently to target DNA/RNA. I routinely use the nick translation reaction because labelling of DNA is more consistent. However, random-primed labelling has one important advantage over nick translation. It allows the labelling of short (> 100 bp) dsDNA fragments that are inefficient substrates for the nick translation reaction. Recently, non-radioactive labelling methods have been designed [42–48]. They offer the advantage of long storage times of the labelled probe and avoid radiation hazards. Unfortunately, the lower sensitivity obtained with non-radioactive labelling methods does not easily allow a routine detection of single-copy genes on Southern blots [49,50]. In Northern analysis the signals generated with most of the available non-radioactive detection systems are not linearly related to the amount of RNA present on the membrane, a major drawback for a method designed to quantitate target mRNA [47]. In addition, background signals are often high with these non-radioactive methods. These shortcomings have to date prevented a general use of non-radioactive probe labelling in Southern and Northern analysis.

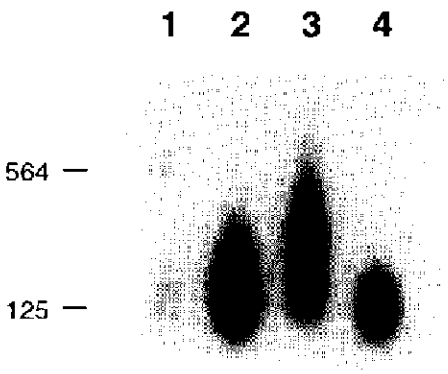

Fig. 4. Length of single-stranded, ^{32}P-labelled DNA fragments obtained with the "nick translation" and the "random-primed labelling" methods. Lane 1 = ^{32}P labelled size marker (564 and 125 bp); lane 2 = random-primed labelled DNA (commercial kit A); lane 3 = nick translation labelled DNA; lane 4 = random-primed labelled DNA (commercial kit B). For size analysis the DNA was separated on an alkaline 1% agarose gel [2].

Once labelled, the DNA probe is denatured at 95°C for 4 min and is hybridized to the membrane-fixed DNA or RNA at $1–2 \cdot 10^6$ cpm/ml hybridization solution for 18–24 h. For hybridization, we strongly recommend the use of commercially available hybridization ovens since hybridization performed in plastic bags, as still suggested in some modern molecular biology manuals, pose radiation hazards and the danger of radioactive spills. After hybridization, the blots are washed to remove non-specifically bound probe. In spite of theoretical predictions, the temperature of the washing solution in the

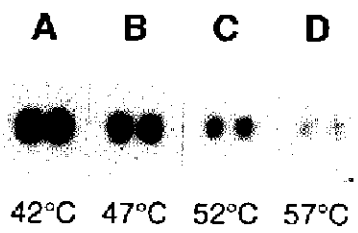

Fig. 5. Effect of the hybridization temperature on the final hybridization signal. Total RNA from HeLa cells (5 μg per lane) was separated on a 1.2% agarose–1.1% formaldehyde gel. After vacuum transfer onto a Nylon membrane the lanes were cut apart and hybridized at varying temperatures but otherwise standard conditions (see Table 4) to a rat glyceraldehyde phosphate dehydrogenase (GAPDH) probe that is 85% identical to human GAPDH. All hybridized blots were washed under the conditions outlined in Table 4.

"stringent" washing step [low salt, high sodium dodecyl sulphate (SDS) concentration] has only a moderate effect on the specificity of the signal obtained. The effect of the washing temperature is dramatic only in hybridization experiments with labelled *short* synthetic oligonucleotides. Rather, the specificity of the hybridization signal in Southern and Northern analysis is mainly determined by the *hybridization* temperature (Fig. 5). The higher the hybridization temperature, the more stringent the hybridization reaction. With a given hybridization protocol the temperature limits should be determined empirically.

After hybridization and washing, the blots are exposed to appropriate X-ray films. Intensifying screens achieve an approximately ten-fold amplification of the signal, when applied at −70°C. For intensifying screens we recommend modern tungstate-based screens, which replaced the less effective rare earth screens.

After film exposure, both Southern and Northern blots can be stripped multiple times and rehybridized with different probes. The stripping procedure differs for the two methods and is indicated in the appropriate sections below. Regardless of the stripping procedure used, care should be taken that the blots are only briefly air-dried and sealed in a humid state in a plastic bag. Drying of the membrane would result in unacceptably high background on rehybridization. The sealed blots can be stored at 4°C for extended periods of time without loss of signal.

3.2. Hybridization of Southern blots

The baked or UV-fixed Southern blot is "prehybridized" with an appropriate solution to reduce non-specific binding of labelled DNA upon hybridization. Subsequently, the prehybridization solution is replaced by the hybridization solution and the labelled probe added. After extensive testing I recommend protocols that use formamide to lower the hybridization temperature [51–53]. Since there are several formamide-based prehybridization solutions, I provide a protocol, which I have successfully used with Zetabind (Cuno, Meriden, CT, USA), Zetaprobe (Bio-Rad, Cambridge, MA, USA), and Biodyne (Pall, Glen Cove, NY, USA) membranes for Southern analysis. The composition of the prehybridization and hybridization solutions, as well as the hybridization temperature used are given in Table 3. I use high-molecular-mass polyethylene glycol (PEG 35 000) to increase probe concentration [54]. PEG 35 000 is an alternative to the addition of the expensive dextran sulphate [18], which is similarly effective (Fig. 6).

After hybridization, the blots are washed to remove non-specifically bound probe. Again, several equally effective variations of this procedure exist. My protocol for washing of Southern blots is indicated in Table 3. Stripping of Southern blots is best achieved with 0.4 *M* sodium hydroxide followed by a neutralization step (Table 3).

3.3. Hybridization of Northern blots

For Northern analysis I routinely use a modification of a recently introduced hybridization method [55]. The blot is pretreated for only 10 min and is then immediately hybridized with the labelled probe (Table 4). The high percentage of SDS prevents high background signals and concentrates the probe. The addition of PEG 35 000 to this hybridization solution leads to background problems without increasing the specific signal (author's own observation). The stripping

TABLE 3

HYBRIDIZATION AND STRIPPING OF SOUTHERN BLOTS

(1) The Nylon membrane is prehybridized in 50% deionized formamide, 0.6 *M* sodium chloride, 0.04 *M* sodium dihydrogenphosphate, pH 7.4, 4 m*M* EDTA, 1% SDS, 1 mg/ml yeast RNA, for 8–16 h at 42°C.

(2) Hybridization is performed in 50% deionized formamide, 0.6 *M* sodium chloride, 0.04 *M* sodium dihydrogenphosphate, pH 7.4, 4 m*M* EDTA, 1% SDS, 5% PEG 35 000, for 24 h at 42°C.

Alternatively, the membrane is prehybridized and hybridized according to the protocol used for Northern blots (see Table 4).

(3) The labelled DNA probe is denatured for 4 min at 98°C, briefly quenched on ice and added at the beginning of the hybridization at $1–2 \cdot 10^6$ cpm/ml hybridization solution.

(4) Following hybridization the membrane is rinsed in 2 × SSC and vigorously washed in 2 × SSC–0.1% SDS, 0.5 × SSC–0.1% SDS and 0.1 × SSC–0.1% SDS for 15 min each at room temperature, followed by a final wash in 0.1 × SSC–1% SDS for 30 min at 50°C.

(5) After brief air-drying the membrane is sealed in a plastic bag and exposed to an autoradiography film.

(6) For stripping of the probe the membrane is washed in 0.4 *M* sodium hydroxide for 30 min at 42°C and subsequently in 0.1 × SSC, 0.1% SDS, 0.2 *M* Tris · HCl, pH 7.4, for 30 min at 42°C (neutralization step).

of Northern blots is also performed differently from Southern blots, since use of 0.4 *M* sodium hydroxide would lead to degradation of the RNA. Instead, melting of DNA–RNA hybrids is achieved by raising the temperature at low-salt conditions (Table 4). As with Southern blots, stripping of Northern blots allows multiple re-hybridization experiments. Autoradiography of Northern blots is performed as described previously.

The protocol given above is the standard method for the detection and quantitation of target mRNA in cells and tissue. In the case that the analysed cell population contains only low amounts of the target RNA species, more sensitive detection methods must be applied. Instead of DNA probes obtained by nick translation or random priming one can generate single-stranded RNA probes by *in vitro* transcription of dsDNA templates cloned into appropriate vec-

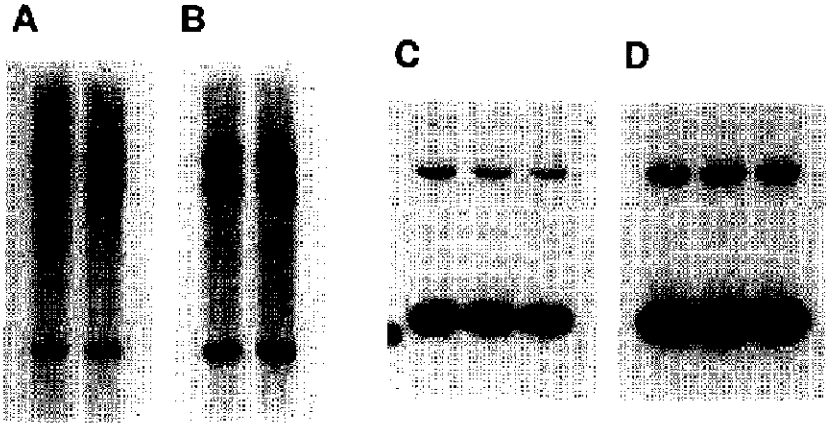

Fig. 6. Effect of dextran sulphate and PEG 35 000 on the final hybridization signal. Human genomic DNA was digested with appropriate restriction enzymes and hybridized to a human T-cell receptor β-chain probe. Hybridization was in 50% formamide, 4 × SSPE, 1% SDS (see Table 3) without dextran sulphate (A), in the presence of 5% dextran sulphate (B and C) or in the presence of 5% PEG 35 000 (D).

TABLE 4

HYBRIDIZATION AND STRIPPING OF NORTHERN BLOTS

(1)	The Nylon membrane is prehybridized in 0.1 × SSC, 0.5% SDS, pH 7.4 for 10 min at 65°C.
(2)	Hybridization is performed in 50% deionized formamide, 0.12 *M* sodium hydrogenphosphate, pH 7.2, 0.25 *M* sodium chloride, 7% SDS (w/v) for 24 h at 47°C.
(3)	The labelled DNA probe is denatured for 4 min at 98°C, briefly quenched on ice and added at the beginning of the hybridization at $1–2 \cdot 10^6$ cpm/ml of hybridization solution.
(4)	Following hybridization the membrane is rinsed in 2 × SSC and vigorously washed in 2 × SSC–0.1% SDS, 0.5 × SSC–0.1% SDS and 0.1 × SSC–0.1% SDS for 15 min each at room temperature, followed by a final wash in 0.1 × SSC 1% SDS for 30 min at 50°C.
(5)	After brief air-drying the membrane is sealed in a plastic bag and exposed to an autoradiography film.
(6)	For stripping of the probe the membrane is washed in 12 m*M* Tris–HCl, pH 7.4, 0.2% SDS for 1.5 h at 70°C.

tors [2]. These RNA probes ("riboprobes") can be labelled to very high specific activity. In addition, because of the higher stability of RNA–RNA duplexes, RNA probes give stronger signals on hybridization than DNA probes of equal specific activity. If even this approach fails to detect the target mRNA, the experimenter has to resort to the PCR method performed on reverse-transcribed mRNA [56,57]. This type of analysis offers exquisite sensitivity at the cost of an, at best, semiquantitative determination of mRNA abundance.

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