

## REVIEW

## Hybridization of Nucleic Acids Immobilized on Solid Supports

JUDY MEINKOTH AND GEOFFREY WAHL

*The Salk Institute, Post Office Box 85800, San Diego, California 92138*

## I. INTRODUCTION

Two decades have elapsed since the development of methods for immobilizing DNA on nitrocellulose paper (49,50) and for detecting the fixed nucleic acid with radioactive probes (18,25). Since this technology (which we will refer to as mixed phase hybridization) made it possible to process many samples simultaneously, it led to a rapid demise of the cumbersome and tedious centrifugation (45) and column techniques (3,5,11) commonly used for the separation of single- and double-stranded nucleic acids. While the mixed phase hybridization technology was limited in its applications initially by the inability to obtain a diversity of gene-specific hybridization probes, molecular cloning techniques have now eliminated this problem. Within the last seven years, mixed phase hybridization is no longer a technology in search of a problem to solve; rather, it forms the cornerstone of the gene (and gene product) detection methods which have revolutionized our understanding of gene structure, genome organization, and the control of gene expression. The sensitivity (<1 pg of complementary sequence), speed (<24 h), and convenience (simple machines and inexpensive materials are required) of the nucleic acid hybridization procedures have enabled them to be applied not only to basic research problems, but also to the diagnosis of heritable diseases and to the detection of a wide variety of microbial and viral pathogens.

KEY WORDS: DNA blot hybridization; RNA blot hybridization; nucleic acid probes.

Many variations on the central theme of detection of immobilized nucleic acids have appeared in the past several years. To the novice first encountering these techniques, the variations between procedures can be bewildering and result in the adoption of needlessly complicated methods. To those experienced in the art, the most expedient methods may not always be employed. Our aim in this review is to describe methods for detecting electrophoretically fractionated nucleic acids either after transfer to solid supports or directly in the gel matrix, and for detecting sequences in unfractionated nucleic acids applied directly to solid supports. We will emphasize the most sensitive and rapid techniques, simplifying the procedures on the basis of published observations or where our experience and that of many colleagues permit.

## II. HYBRIDIZATION PARAMETERS

The kinetics of hybridization of RNA or DNA probes with DNA tethered to nitrocellulose (NC)<sup>1</sup> or free in solution are very similar (25,49,50,72), suggesting that parameters which influence nucleic acid reannealing in solution will have similar effects in mixed phase systems. In this section, we discuss the

<sup>1</sup>Abbreviations used: NC, nitrocellulose; SSC (1×), 0.15 M NaCl, 0.015 M Na citrate, pH 7.0; SSPE (1×), 0.18 M NaCl, 0.01 M NaPO<sub>4</sub>, pH 7.7, 0.001 M EDTA; MS, multiple sclerosis; BSA, bovine serum albumin, Fraction V; SDS, sodium dodecyl sulfate; DPT, diazophenylthioether; EtBr, ethidium bromide; NT, nick translation; Pol I, *E. coli* polymerase I; DTT, dithiothreitol; PEG, polyethylene glycol.

parameters which affect hybridization rate and hybrid stability. An understanding of these parameters enables one to derive hybridization conditions which should yield the optimal signal to noise ratios.

### A. Hybridization Rate

The rate of hybrid formation for single-stranded probes in mixed phase hybridizations should follow first-order kinetics since the concentration of probe is in vast excess over that of target sequences. For complementary probes (e.g., those generated by nick translation as described in Section IVA), the situation is more complicated since probe reannealing in solution decreases the concentration of probe available for hybridization with the target. For illustration, we will consider the case for single-stranded probes.

The time required for half of the probe to anneal with the tethered DNA is

$$t_{1/2} = \frac{\ln 2}{k \cdot C} \quad [1]$$

where  $k$  is the first-order rate constant (expressed as liters mol nucleotide<sup>-1</sup> s<sup>-1</sup>) for formation of a hybrid molecule and  $C$  is the probe concentration (mol nucleotides/liter). The hybridization rate constant ( $k$ ) is a function of probe strand length ( $L$ ), molecular complexity ( $N$ , the total number of base pairs in a nonrepeating sequence), temperature, ionic strength, viscosity, and pH (the latter four factors are included in the nucleation rate constant  $k'_N$ ) (84)

$$k = k'_N L^{0.5} N^{-1}. \quad [2]$$

Ionic strength has little effect on the rate constant as long as it is kept above 0.4 M (e.g.,  $k$  increases 1.5-fold between 0.4 and 1.0 M NaCl) and pH effects are small (i.e., <1.3-fold) in the range pH 5.0 to 9.15 when the salt concentration is above 0.4 M NaCl (84). The maximum rate of hybridization in solution (at 1.0 M NaCl) has been determined empirically to occur at 25°C below the  $t_m$  of the duplex (12) ( $t_m$  is the temperature at which half of the hybrids are dissociated). This ob-

servaion also pertains to mixed phase hybridizations which utilize probes longer than approximately 150 nucleotides (17). However, the temperature giving the optimal hybridization rate is depressed further in mixed phase hybridizations for probes shorter than 150 nucleotides (7,79).

Under the optimal hybridization conditions, the Wetmur and Davidson relationship (84) pertains to nucleic acids of all sizes measured [even oligonucleotides as short as 14 bp (80)] and can be written

$$k = 3.5 \times 10^5 L^{0.5} N^{-1}. \quad [3]$$

Combining Eqs [1] and [3] yields an expression for the time (in seconds) required to anneal half of the probe to the target:

$$t_{1/2} = \frac{N \ln 2}{3.5 \times 10^5 \cdot L^{0.5} \cdot C}. \quad [4]$$

This equation indicates that the hybridization time is minimized by using probes of low complexity at high concentrations. Thus, a probe with a complexity (and size) of 15 bp has a  $t_{1/2}$  of 250 s at 10 ng/ml ( $3 \times 10^{-8}$  mol nucleotides/liter). On the other hand, the prediction from the equation that longer probes should decrease hybridization times is not likely to be true for some mixed phase hybridizations since some immobilized target sequences may not be accessible to long probe molecules (7,17).

The rate of probe reannealing can be enhanced in solution (83) and in mixed phase hybridizations using anionic dextran polymers (e.g., dextran sulfate 500) (78). In mixed phase hybridization, the effect of dextran sulfate is most pronounced for polynucleotides longer than about 250 nucleotides (G. Wahl, unpublished observations) and has no effect on oligonucleotides 14 bases long (R. B. Wallace, personal communication). The increase in the apparent rate of hybridization is approximately 3-fold for mixed phase hybridizations which utilize single-stranded probes and up to 100-fold for hybridizations utilizing nick-translated probes (78) (see below for a description of nick translation.) This effect has been attributed to the accelerated rate of for-

mation of probe "networks" or "hyperpolymers" between the partially overlapping sequences of the molecules generated by nick translation (78). It is important to emphasize that the ability to form such networks is critically dependent on probe size since the overlaps in small probes are not likely to be able to initiate or maintain the formation of stable networks.

### B. Hybrid Stability

The formation of nucleic acid hybrids is a reversible process and an understanding of the parameters which affect their stability enable one to derive the optimal conditions for discriminating between perfect and imperfect hybrids. The melting temperature ( $T_m$ ) is affected by ionic strength ( $M$ , in mol/liter), base composition (% G + C), the length of the shortest chain in the duplex ( $n$ ), and the concentration of helix destabilizing agents such as formamide (e.g., see (43,66)). The following equation has been derived from analyzing the influence of these factors on hybrid stability:

$$T_m = 81.5^\circ\text{C} + 16.6 \log M + 0.41(\% \text{ G} + \text{C}) - 500/n - 0.61(\% \text{ formamide}). \quad [5]$$

This equation pertains to probes longer than approximately 50 nucleotides. Hybrids between oligonucleotides (14–20 bp) and immobilized DNA show decreased stability (79) and an empirical formula has been determined to define the optimal conditions for their hybridization (81). The temperature at which 50% of these short duplexes dissociate ( $T_d$ ) when the hybridization is performed under standard conditions (e.g., 0.9 M NaCl) is:

$$T_d(^\circ\text{C}) = 4(G + C) + 2(A + T) \quad [6]$$

where  $G$ ,  $C$ ,  $A$ , and  $T$  indicate the number of the corresponding nucleotides in the oligomer. A temperature 5° below the  $T_d$  is used to detect hybridization between perfectly matched molecules (74,79). Further considerations for hybridization with oligonucleotides are given below (see Section IVC).

The stability of duplexes formed between strands with mismatched bases is decreased according to the number and location of the

mismatches and is especially pronounced for short (e.g., 14 bp) oligonucleotides. For hybrids longer than 150 bp, the  $T_m$  of a DNA duplex decreases by 1°C with every 1% of base pairs which are mismatched (6). For hybrids shorter than 20 bp, the  $T_m$  decreases by approximately 5°C for every mismatched base pair (79–81). In order to minimize the hybridization of probe to related but nonidentical sequences, hybridization reactions must be performed under the most stringent conditions possible. From the discussion above, hybridization stringency can be altered by adjusting the salt and/or formamide concentrations and/or by changing the temperature. The stringency can be adjusted either during the hybridization step, or in the posthybridization washes (see Section VII). It is often convenient to perform the hybridization at low stringency and wash at increasing stringencies, analyzing the results after each wash. This enables the detection of related sequences and the monitoring of the effectiveness of the washes in removing these sequences. This strategy also enables one to obtain an estimate of sequence relatedness.

### III. DETECTION OF NUCLEIC ACIDS IMMOBILIZED ON SOLID SUPPORTS OR IN AGAROSE GELS

Techniques are now available for immobilizing both DNA and RNA on solid supports consisting of NC (1,2,25,71,75), diazotized cellulose (1,2,67), Ecteola cellulose (65), DEAE-cellulose (29), and nylon [e.g., see (8)]. We review below the blotting applications best served by these solid supports, some new applications for immobilized nucleic acids, and some of the parameters affecting transfer efficiency.

#### A. Uses of Different Solid Supports

The mechanism of binding of nucleic acids to NC is unknown, but has been assumed to be noncovalent. This assumption led to the belief that immobilized nucleic acids might be eluted from nitrocellulose during stringent washing procedures and provided one incentive for developing diazotized paper supports which bind nucleic acids covalently [e.g., see

(1,2,48,67)]. However, in numerous independent experiments with RNA or DNA bound to NC, it has been possible to remove the majority of the hybridized probe molecules (see Section VII) without eluting significant quantities of the bound nucleic acids. The hybridization signal does not change significantly after at least six probe removal washes (J. Meinkoth and G. Wahl, unpublished observation). Due to the relatively low cost, convenience of use, and reusability, NC is clearly the solid support of choice for most mixed phase hybridization applications. Since fragments smaller than about 200–300 bp bind poorly to NC but are covalently bound to diazotized papers (62,73), the latter are recommended for experiments requiring the binding of small fragments.

A novel technique has recently been described for the transfer of RNA fractionated in agarose gels to Ecteola paper (65). The binding of RNA to Ecteola paper is noncovalent and can be reversed, allowing for its subsequent translation *in vitro*. Alternatively, the RNA can be translated *in situ* on the paper and the protein products immobilized at their site of synthesis. This procedure is more rapid and sensitive than others which require RNA elution from gels prior to translation. RNA immobilized on NC has also been reported to be a substrate for *in vitro* translation and for reverse transcriptase (9). Alternatively, RNA may be transiently bound to commercially available DEAE membranes and eluted in a biologically active form using strong denaturants (e.g., 6 M guanidine thiocyanate) (29).

#### B. Techniques for Detecting Nucleic Acids after Electrophoresis through Gels

Southern's technique (71) for "blotting" electrophoretically fractionated DNA from an agarose gel to NC by passive diffusion established that transfers were faithful replicas of the high-resolution gel patterns. Passive transfer techniques consist of four steps: electrophoresis, transfer [with the attendant DNA fragmentation (optional), denaturation, and neutralization steps], fixation onto NC, and

detection of specific immobilized sequences by hybridization. Commonly encountered problems in DNA electrophoresis have been the subject of a recent paper and will not be discussed here (70). Early reports indicated that efficient transfer of large DNA was difficult to achieve, leading to the development of chemical (78) or uv light treatments (41) to fragment the DNA prior to transfer to ensure equal transfer efficiencies of all DNA molecules. The chemical fragmentation (depurination) method has been modified by decreasing the depurination time (in 0.25 M HCl) to 7.5–10 min. Longer treatments generate smaller fragments which do not bind well to NC and result in lower hybridization signals. Solarization of uv filters over time reduces uv transmission to the gel and results in irreproducible fragmentation. Fragmentation of DNA to average single-strand lengths of approximately 1000 bp enables the efficient transfer and retention of DNA molecules regardless of their original size or conformation (e.g., supercoil, linear, etc.). Fragmented DNA also transfers very rapidly; only 1–2 h are required to transfer >90% of the DNA from a 1.5-cm-thick gel. A good rule of thumb is that transfer can be stopped when the gel thickness decreases to approximately 1 mm since the gel concentration at this point is sufficiently high to prevent further transfer. Procedures for electroeluting DNA from agarose gels are therefore unnecessary since they require as much time as passive diffusion of fragmented DNA. However, in order to resolve small DNA fragments (i.e., <0.5 kb), it is necessary to use polyacrylamide or composite acrylamide-agarose gels (4,62). Electroelution may be advantageous for the transfer of small DNA molecules since DNA diffuses slowly from acrylamide gels and diazotized paper, the solid support of choice, has a limited time over which it can bind DNA (e.g., see (4) for examples). Denaturation of small DNA fragments has been reported to promote their retention on nitrocellulose (75). The use of large volumes of transfer buffer (e.g., 20× SSC, 20× SSPE, or 1 M NH<sub>4</sub>OAc) is unnecessary since efficient transfer can be obtained from a simple

apparatus consisting of a tray with two to four pieces of buffer-saturated blotting paper (e.g., Whatman 3MM or S&S No. 470 WH) or a sponge.

While most protocols specify a 2-hr baking step to fix nucleic acids to NC, we have found that it is only necessary to bake for as long as is required to completely dry the paper. This is usually 10–20 min at 80°C in a vacuum oven evacuated to 30 mm Hg. Baking times in excess of 24 h can produce brittle NC which shatters upon further manipulations. Nylon supports have been developed which remain pliable and easy to handle after baking. However, the signals obtained from genomic DNA transferred to some nylon supports by the Southern technique can be as much as 25-fold less than those obtained for the same DNA transferred to NC (J. Meinkoth, unpublished observations) and the background can be much higher. A new durable support, Genetran (manufactured by Plasco in Woburn, Mass.), has given signals equivalent to NC with low noise levels in preliminary experiments.

A recent modification of the original Southern transfer protocol is the “bidirectional blot” which allows two identical blots to be produced from a single gel (69). This technique is valuable for restriction mapping studies (i.e., enabling one to hybridize with two different probes simultaneously) and for checking the specificity of multiple cloned probes. DNA fragmentation is required for the transfer to be efficient since the gel diminishes in thickness very rapidly using this procedure (transfer from a 0.7% gel is complete after 1 h). Small DNA fragments can also be transferred bidirectionally from polyacrylamide gels (69). With 10–20 µg of DNA, bidirectional blotting can be used to detect unique sequence genes in mammalian genomic DNA (G. Wahl, unpublished observations).

An alternative to the conventional Southern transfer technique is to hybridize radioactive probes directly with DNA in dried agarose gels (58,68,76). The sensitivity is the same as that of standard DNA blots with nick-translated probes and is at least 5-fold greater with

oligonucleotide probes (79) (see Section IVC). Hybridization in the presence of 50% formamide for extended periods (e.g., >36 h at 42°C) can solubilize the gel. DNA fragments 1–50 kb long are quantitatively retained in the dry gel, but there is some loss of small fragments (<312 bp) during drying and/or hybridization. Dried gels can be rehybridized with different probes. Elution of hybridized DNA has been reported (58), but in our experience it is often incomplete (G. Wahl, unpublished observations).

The “Northern transfer” technique (1,2) was designed to transfer RNA from agarose gels to solid supports. The sensitivity is sufficient to detect low abundance (<0.01% of total mRNA) eukaryotic RNA molecules. A number of improvements have been made in the original procedure to make it more sensitive and easier to perform. The denaturant originally used, methylmercuric hydroxide (1), has been replaced by less toxic ones such as glyoxal (44,75) or formaldehyde (59) and NC has replaced diazotized paper as the solid support of choice (75). Fragmentation of the RNA is not required prior to transfer when glyoxal or formaldehyde are used as denaturants. Under these conditions, it is possible to quantitatively transfer RNA molecules 10 kb or larger (55; J. Meinkoth, unpublished observations). The sensitivity of detection of formaldehyde denatured RNA transferred to NC is greater than that obtained using other combinations of denaturants and solid supports (85). Bidirectional transfer of RNA from a 0.75% methyl mercury agarose gel to diazotized paper has been reported (69) and so it is likely that the same methodology can be applied to glyoxal or formaldehyde agarose gels for transferring RNA to nitrocellulose paper. *In situ* detection of RNA in agarose gels has also been achieved (76).

### C. Techniques for Detecting DNA and RNA Sequences in Unfractionated Nucleic Acid Samples

It is often sufficient to be able to measure the amounts of DNA or RNA sequences with-

# Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

## Real-Time Litigation Alerts



Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

## Advanced Docket Research



With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

## Analytics At Your Fingertips



Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

## API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

## LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

## FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

## E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.