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# An Improved Method for Transferring Nucleic Acids to Nylon Membranes

Bruce D. Parker
*Utah State University*

# AN IMPROVED METHOD FOR TRANSFERRING NUCLEIC ACIDS TO NYLON MEMBRANES

by

Bruce D. Parker

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biology

Approved:

| | |
|---|---|
| Major Professor | Committee Member |
| Committee Member | Committee Member |
| Committee Member | Dean of Graduate Studies |

**UTAH STATE UNIVERSITY**
**Logan, Utah**

**1987**

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I would like to thank my major advisor for giving me the opportunity to work in his lab and for the concern and confidence he has continually expressed in me.

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Most of all I wish to thank my wife, Jan, for her patience and love in helping me to succeed.

This work was supported by Utah Agricultural Experiment Station Projects 537 and 538.

Bruce D. Parker

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## ABSTRACT

**An Improved Method for Transferring Nucleic Acids to Nylon Membranes**

by

Bruce D. Parker, Master of Science

Utah State University, 1987

Major Professor: Dr. Joseph K.-K. Li
Department: Biology

Methods currently in use for the separation and identification of specific segments of nucleic acids involve long transfer periods or elaborate apparatuses and result in the production of a single blot. Contamination by organisms or enzymes is always a factor to be dealt with. An improved method for transferring nucleic acids from acrylamide or agarose gels for use in hybridization has been developed. This method uses NaOH as the blotting medium to improve the rate and efficiency of transfer to nylon membranes. As many as six blots can be obtained within one hour using this method. This method is effective for both viral double-strand RNA and single-strand RNA. By using 0.2 N NaOH as the transfer medium, and using nylon membranes for blotting, the nucleic acid appears to be covalently fixed to the membrane. These blots can be stripped of the probe and reused. In our studies with viral dsRNA, as little as 3.2 ng of total nucleic acid can be detected on the blot. This method provides a great improvement over previous methods for blotting and hybridization of both ss-and dsRNA and shows promise for use with dsDNA. (31 pages)

## INTRODUCTION

Much of the work done in the field of molecular biology relies upon the ability to identify specific segments of nucleic acids such as genes or specific mRNAs (1-12). Such identification depends upon the ability to: (a) separate the segment of choice from other such segments, and (b) confirm or detect the segment of interest. The advent of agarose and polyacrylamide gel electrophoresis made possible the separation of nucleic acids based upon size. However, the detection of segments of interest was impractical due to the nature of the gel matrix. Probes specific to the segment of interest would simply bind nonspecifically to the surface of the gel rather than specifically to the segment. Methods for the analysis of nucleic acids via hybridization to radioactively labelled probes have been developed for use on total DNA immobilized on filters or DNA eluted from gel bands in solution. Such methods lead to loss of the resolution provided by gel electrophoresis (1). In 1975, E.M. Southern (1) published a method whereby dsDNA was separated by agarose gel electrophoresis and the separated DNA bands transferred from the gel directly to the nitrocellulose filters. This transfer was accomplished by capillary flow in the presence of a high-salt buffer (see Fig.1 and Methods). This procedure revolutionized the work in molecular biology by meeting both criteria for identification of specific nucleic acid segments; the method used agarose gel electrophoresis to separate total genomic DNA and detection was performed on the DNA immobilized on the nitrocellulose. His method, referred to now as 'Southern blotting', has been modified only slightly since then. One such modification, 'Northern

blotting', was simply the application of the method to RNA (3,5,6). The major disadvantages of the method are: (a) the method is time-consuming, requiring 12-24 hours per transfer; (b) the method is inefficient since only a single blot is produced per gel; and (c) there is a risk of contamination of the transfer buffer (i.e. sodium phosphate) resulting in degradation of the nucleic acids (1,3).

A minor improvement was made with the development of electrophoretic blotting (7). This method employs the use of an electric field to transfer the nucleic acids from the gel to the membrane. The transfer in this method occurs in a shorter time frame (2-4 hours), which helps to avoid the problem of contamination. However, this method requires a rather expensive power supply and an elaborate blotting apparatus. Neither method provides the resolution required in many of the current molecular genetic analyses.

With both methods, capillary and electrophoretic blotting, the membrane-bound nucleic acids must be denatured on the membrane in order for detection via hybridization to occur. This is accomplished by treatment with alkali (usually sodium hydroxide), followed by neutralization before the membrane is subjected to hybridization (1).

In 1984, and again in 1985, two improved methods for transferring DNA to positively-charged nylon membranes were described (9,10). Both methods employ sodium hydroxide as the transfer 'buffer'. An advantage of this improvement, as described in the published methods (9,10), is the omission of the denaturation step prior to hybridization. This is accomplished by the use of sodium hydroxide as the transfer medium, because the DNA is denatured as it is being transferred. This also

results in greater transfer and greater sensitivity. However, the published methods produce only a single blot from an agarose gel after an overnight transfer (9,10).

Since much of the work done in our lab relies upon the ability to detect small amounts of single DNA or RNA bands by hybridization, it was desirable to develop an improved method for transfer of nucleic acids from gels which would be more efficient and sensitive than those now in use. It was particularly desirable to develop a blotting method which would apply to polyacrylamide gels since the resolution of the separation of nucleic acids obtainable on these gels is often far superior to that found with agarose. Specifically, we wanted to know if the improvements described for alkaline blotting would succeed with RNA separated on polyacrylamide gels (the previously described methods were developed for use with agarose).

This report details an improved method for blotting of nucleic acids, particularly ds- and ssRNA, which is an extension of the alkaline blotting method described above. This method is relatively easy and requires no expensive or elaborate materials or apparatuses. The improved efficiency and resolution provided by this method, combined with its relative ease, make this a valuable analytical tool for molecular biologists.

## MATERIALS AND METHODS

### Cells and Viruses

Bluetongue virus (BTV) serotypes 2, 10, 11, 13, and 17 were grown in BHK-21 monolayer cells in Dulbecco's Modified Eagle's Medium (DMEM,

Gibco) supplemented with 5% fetal bovine serum (HyClone). Reovirus type 3 (reo-3) was grown in mouse L-cells in the same medium.

## Nucleic Acids

Double-strand RNA was extracted from purified Reovirus virions as described by Li et al. (13), and from BTV-infected cell lysates using the method of Mertens et al. (14). Single-strand RNA from BTV was obtained during the purification of dsRNA by LiCl precipitation (14). Samples of double-strand RNA from virus-like particles of Endothia parasitica were obtained from Dr. Neal Van Alfen (Utah State University). Bacteriophage ø6 dsRNA was the kind gift of Dr. Helen Revel (University of Chicago). Isolated reo-1 dsRNA segments s3 and s4 were provided by Dr.Joseph K.-K. Li. Bovine rotavirus was provided by Dr. Bill Barnett.

## Gel Electrophoresis

Double-strand nucleic acids were separated on 10% polyacrylamide gels containing SDS (SDS-PAGE) using a modification of the method of Schuerch et al. (15). Glyoxalated ssRNAs were separated on either 1% electrophoresis grade agarose (Ultra Pure, Bethesda Research Labs) or 2% NuSieve agarose (FMC Bioproducts) using published methods (5,6,12). Individual segments of dsRNA were isolated from polyacrylamide gels, visualized with ethidium bromide and extracted following the procedure of Li et al. (13).

## Glyoxal Gel Electrophoresis

Pellets of ssRNA obtained during the extraction of dsRNA from BTV-

infected cells (14) were washed twice with 70% ethanol and resuspended in a small volume of 10 mM Tris-HCl,1 mM EDTA (TE) buffer pH 7. Samples were glyoxalated in the presence of dimethyl sulfoxide (DMSO) using established methods (5,6,12) and run on either 1% electrophoresis grade agarose or 2% NuSieve agarose in the presence of 10 mM sodium phosphate (pH 7.0). Glyoxal was from Kodak and DMSO was from EM Science.

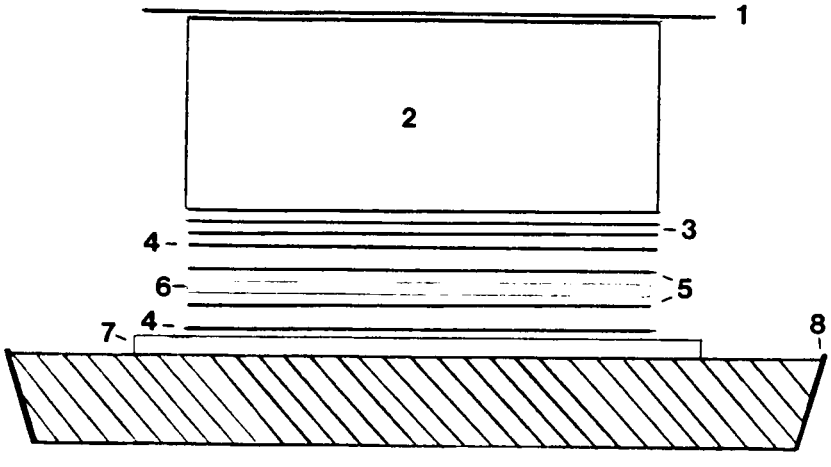
Radioactive Probes

Double-strand RNA probes were labelled at the 3'-ends using T4 RNA ligase and 3', 5'-bis-cytidine [$^{32}$P]-diphosphate according to the procedure of England (16). Individual dsRNA segments of BTV-11 and BTV-13 used for probes were obtained by elution from preparative 10% SDS-PAGE gels using a modified procedure of Li et al. (13) and 3'-end labelled as previously mentioned for dsRNAs. All probes were recovered from the labelling reactions by ethanol precipitation in the presence of 0.4 M LiCl and 0.04 mg per ml of glycogen as carrier (17).

Blotting

Following electrophoresis, gels containing double-strand nucleic acid were treated for ten minutes in 0.25 N HCl. Gels containing single-strand RNA were blotted without pretreatment. The HCl-treated gels were placed directly on the blotting apparatus without neutralization. All blotting was performed in a sandwich/capillary blotting apparatus as shown in Fig. 1 (12). Basically, a sponge was placed in a tray which was filled with blotting buffer (NaOH). The liquid level was maintained as close to the top surface of the sponge as possible. A

Fig. 1. An example of an alkaline blotting apparatus. In the diagram, membranes are shown above and below the gel. This represents the procedure generally used in producing multiple blots of the same gel (see Fig. 8.). When using buffers such as those used in standard Southern blotting, or when alkaline-blotting agarose gels, only a single membrane is used above the gel.




1- Glass plate
2- Stack of 8 blotting pads
3- 2 wet blotting pads
4- 3mm filter paper
5- Blotting membrane
6- Gel
7- Sponge
8- Tray with buffer

blotting pad (Fisher Scientific #E-D 320-200) and piece of 3MM filter paper were placed on this surface. The gel was placed on top of the filter paper followed by a blotting membrane, a second piece of 3MM filter paper, 2 pre-soaked blotting pads, 8 dry blotting pads, and finally a small weight (a glass plate is ideal). Two wet blotting pads were maintained above the blotting membrane at all times. Agarose gels were blotted essentially as described for acrylamide gels. All steps for both gel systems were performed at room temperature.

Following transfer, the blots were washed twice in 2X SSC (25X SSC is 3.7 M NaCl, 0.375 M sodium citrate pH 7.4) for 15 minutes each and blotted dry. The blots were then baked at 80$^{o}$C for 2 hours under vacuum or used directly after air drying.

Polyacrylamide gels were silver-stained directly after blotting to determine efficiencies of transfer and band separation in the gels. Gels were placed directly into the first acetic acid/methanol wash and stained following standard procedure (18). Agarose gels were also monitored after blotting by staining with ethidium bromide followed by visualization with UV irradiation.

Hybridization

Before prehybridization, the blots were washed at 65$^{o}$C in 0.1X SSC and 0.5% SDS for 1 hour. The membranes were blotted to dampness and placed in heat-sealable bags containing prehybridization solution. Prehybridization solution consists of 5X SSC, 50mM sodium phosphate (pH 6.5), 500 µg/ml of salmon sperm DNA, 0.1% SDS and 5X Denhardt's solution (2) which is 0.1% each of BSA, polyvinylpyrollidone and

Ficoll. Prehybridization was carried out at 42°C for at least 3 hours to overnight.

After prehybridization, the prehybridization solution was removed from the bag. Labelled probes were mixed with an equal volume of fresh prehybridization solution and boiled for 10 minutes. This hybridization mixture was diluted to a final volume of 1 ml per 10 $cm^2$ and added to the bag containing the blot. Hybridization was normally carried out at 55°C overnight. After hybridization, the blots were washed four times at room temperature in 2X SSC, 0.1% SDS, followed by two washes at 65°C with 0.1X SSC, 0.1% SDS. Membranes were then blotted to dampness, wrapped in plastic wrap and autoradiographed. Autoradiography was carried out at -70°C on Kodak XAR film using DuPont Cronex Lightning Plus intensifying screens. Following exposure, blots were stripped for reuse by incubation at 90°C in two changes of 0.1X SSC, 0.1% SDS for 20 minutes each. Blots were then prehybridized and hybridized as before.

## RESULTS

### Transfer Medium

Optimization of all blotting conditions was performed using dsRNA from reo-3 separated on 10% SDS-PAGE gels. The optimal NaOH concentration for use as a blotting medium was determined using Zeta-Probe (Bio-Rad) as the blotting membrane. 3'-labelled dsRNA was mixed with unlabelled dsRNA and applied to a SDS-PAGE gel. Each well contained 0.5 µg of unlabelled dsRNA plus 5 X $10^5$ cpm of [$^{32}$P]-labelled dsRNA. Following electrophoresis, the gel was treated with 0.25 N HCl for 10

minutes and cut into strips which were then immediately applied to separate blotting apparatuses containing various concentrations of NaOH. The NaOH concentrations used were: 0.05, 0.1, 0.2, 0.4, and 0.8 N. Strips of Zeta-Probe membrane, pre-wetted in distilled $H_2O$, were placed above the individual gel slices, and blotting was performed for 1 hour. The results (Fig. 2) indicate that more dsRNA is retained on the membrane blotted in 0.2 N NaOH. In addition, less degradation is seen at this concentration than at the higher concentrations as indicated by the increase in band diffusion at the higher concentrations. These data indicate that 0.2 N NaOH provides the best medium for transfer.

Membrane Comparison

Various blotting membranes were compared for optimal binding of nucleic acid during alkaline blotting. Membranes used were Zeta-Probe (Bio-Rad, lot #M1852), Nitrocellulose (Schleicher & Schuell BA85, 0.45 um, lot#4139/3), Magna 66A (Fisher Scientific, Cat. #E04HY00010, lot #10909) and Magna 66B (Fisher Scientific, Cat. #NJ4HY312, lot #S14616). A SDS-PAGE gel was run as before with each sample well containing 0.5 µg of dsRNA mixed with 5 X $10^5$ cpm of labelled dsRNA. The gel was pre-treated with HCl as described and the entire gel was placed upon a blotting apparatus using 0.2 N NaOH as the blotting medium. Strips of various membranes were placed above each track and blotting was carried out for one hour. The results (Fig. 3) indicate that only Zeta-Probe retained the nucleic acid during blotting. The other membranes allowed the labelled dsRNA to pass through them to the filter paper and pads

Fig. 2. Comparison of various NaOH concentrations used for blotting of dsRNA of reo-3 to Zeta-Probe membrane. Autoradiographs of dried blots are shown in (a) and of the dried gel (after transfers) in (b). The 10% SDS-PAGE gel contained 0.5 µg of unlabelled reo-3 dsRNA mixed with $5x10^5$ cpm of 3'-labelled reo-3 dsRNA per lane. Each lane was cut from the gel and blotted in the following NaOH concentrations: A=0.05 N, B=0.1 N, C=0.2 N, D=0.4 N, and E=0.8 N.

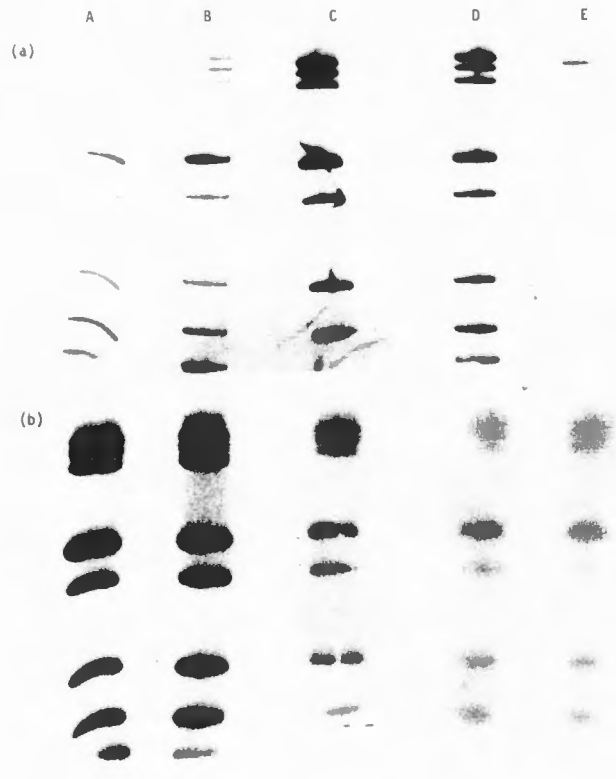
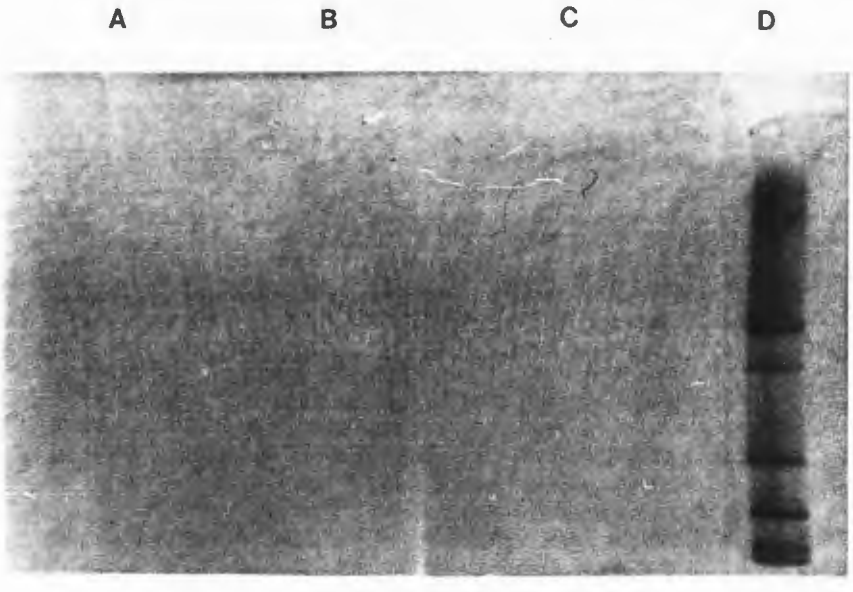

A
B
C
D
E
(a)
(b)

Fig. 3. Comparison of various membranes during blotting of reo-3 dsRNA using 0.2 N NaOH as the transfer medium. 0.5 µg of reo-3 dsRNA, mixed with $5x10^5$ cpm of 3'-labelled reo-3 dsRNA, was electrophoresed in each of 4 lanes of a 10% SDS-PAGE gel. The gel was blotted using 0.2 N NaOH with a strip of a different membrane above each lane. The figure shows an autoradiograph of the resulting blots. The membranes used were A=Magna 66A, B=Magna 66B, C=Nitrocellulose, and D=Zeta-Probe.


A
B
C
D

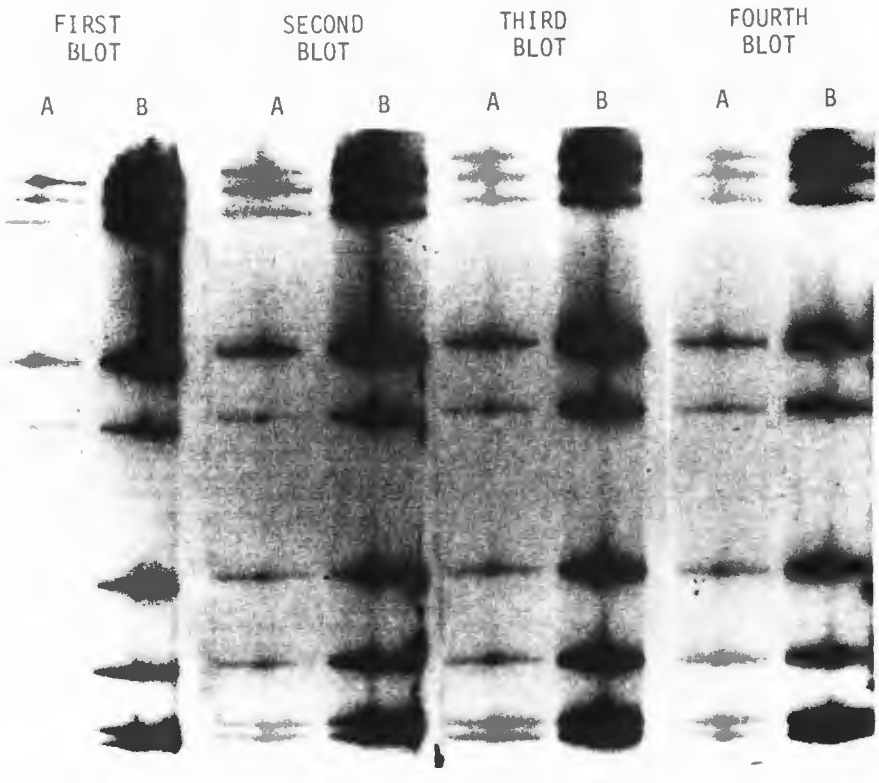
above (data not shown). Virtually no label passed through the Zeta-probe to the pads above. Similar results (data not shown).were obtained with the other nylon membranes such as Zetabind (AMF Cuno) and Gene Screen Plus (New England Nuclear).

Optimal Blotting Times

Using 0.2 N NaOH as the transfer medium, substantial amounts of nucleic acid were transferred to the membrane within a very short period of time. This effect was investigated using the other NaOH concentrations mentioned previously. The results indicate that significant amounts of dsRNA are transferred within the first 15 minutes using concentrations of NaOH of 0.2 N or less. NaOH concentrations greater than 0.2 N show substantial degradation of the dsRNA, as indicated by the diffusion of the bands remaining in the gel (Fig. 2). It was determined that, using 0.2 N NaOH, a sequence of 4 blots could be made at 15 minutes each over a period of 1 hour; each containing approximately the same amount of dsRNA (Fig. 4). This multiple blotting effect was not seen with lower (0.1 and 0.05 N) NaOH concentrations; the only blots containing substantial amounts of dsRNA were those made in the first 15 minutes. More time was required to transfer dsRNA to subsequent blots (data not shown). In addition, rapid multiple blotting was not successful for agarose gels of ssRNA (see 'ssRNA Blotting and Hybridization'). Due to the thicker nature of agarose gels, only a single blot was made in a 30 minute blotting period.

Using 0.2 N NaOH, a second set of blots could also be made during the same blotting times by placing a membrane below the gel. By expanding

Fig. 4. Multiple alkaline blotting of reo-3 dsRNA. A 10% SDS-PAGE gel containing, per lane, approximately 20 ng (A) and 100 ng (B) of reo-3 dsRNA, mixed with a small amount of 3'-end labelled sample, was blotted using 0.2 N NaOH. Four consecutive blots were made at 15 minutes each above the gel. The blots were dried and subjected to autoradiography. Note that each blot contains approximately the same amount of dsRNA.


FIRST BLOT
SECOND BLOT
THIRD BLOT
FOURTH BLOT
A
B
A
B
A
B
A
B

the blotting time under these conditions to 20 minutes per set, a total of 6 blots containing approximately the same amount of dsRNA were obtained within 1 hour (see"Practical Applications"). This transfer efficiency was not seen if a sandwich blot apparatus, using diffusion rather than capillary action, was used (data not shown).

No difference in the transfer efficiency of the dsRNAs of reo-3 and BTV-11 was detected (Fig. 5). Similar transfer efficiency was also seen using labelled rotavirus (data not shown).

Hybridization Sensitivity

A two-fold dilution series of reo-3 dsRNA from 0.5 ug down to 1 ng was prepared and electrophoresed on a 10% SDS-PAGE. Two blots were produced from this gel by blotting from the top of the gel in the presence of 0.2 N NaOH for 30 minutes each. Since it has been reported that approximately 20% of the input viral dsRNA remains associated with the gel after 1 hour of blotting under these conditions (19), each blot would have contained 40% of the input samples. The first blot was hybridized with total genomic reo-3 dsRNA (Fig. 6A) and the second with isolated reo-1 fragments s3 and s4 (Fig. 6B). As indicated in Fig. 6A, as little as 3.2 ng of total dsRNA can be detected when total homologous RNA is used as probe. Single bands from as little as 24 ng of membrane-bound dsRNA can be detected using the heterologous probe of isolated bands s3 and s4 from reo-1 (Fig. 6B).

ssRNA Blotting and Hybridization

Cellular and viral ssRNA from cells infected with BTV-2 was separated from dsRNA during the purification procedure by a LiCl precipitation

Fig. 5. Comparison of the blotting efficiency of dsRNA from two viruses: reo-3 and BTV-11. The 10% SDS-PAGE gel contained a two-fold dilution series of total dsRNA from 500 ng down to 30 ng, mixed with a small amount of 3'-end labelled dsRNA. The reo-3 dsRNA samples contained twice as much labelled sample as the BTV-11 samples ($1x10^6$ cpm versus $5x10^5$ cpm). Four consecutive 15 minute blots were made. The figure represents the first blot in the series. Note that transfer is equal for both viruses and for large and small segments.

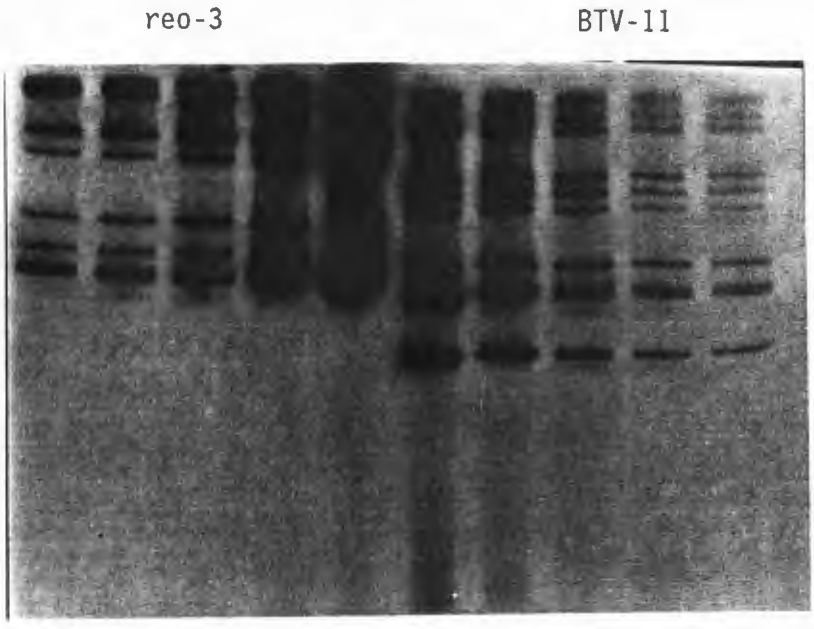
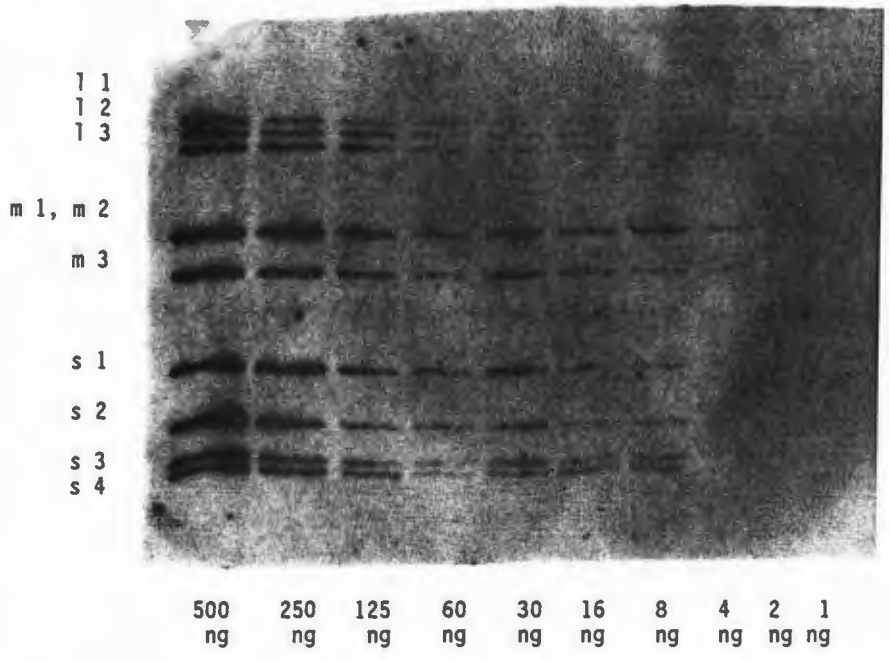
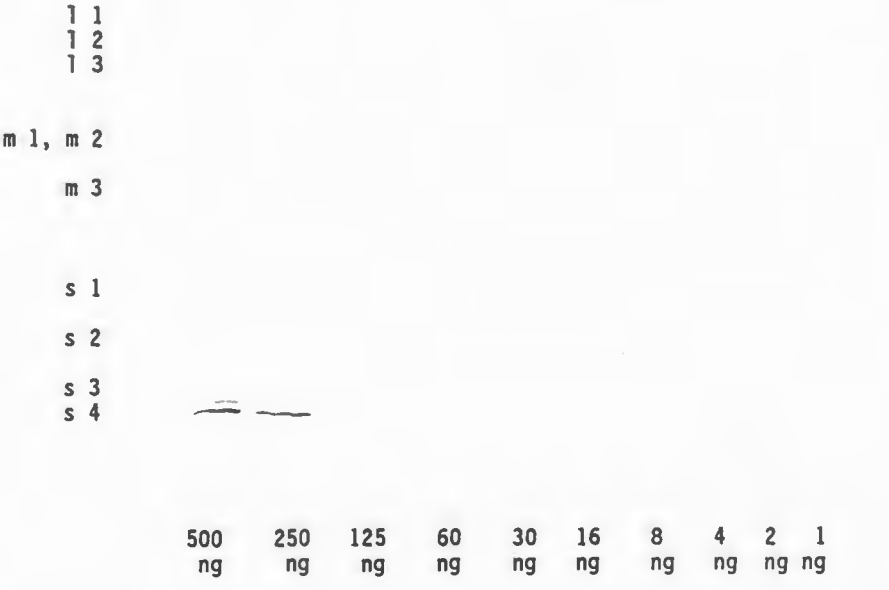

reo-3
BTV-11
31 ng
62 ng
125 ng
250 ng
500 ng
500 ng
250 ng
125 ng
62 ng
31 ng

Fig. 6. Comparison of hybridization of multiple-blotted reo-3 dsRNA probed with homologous and heterologous dsRNAs. A two-fold dilution series of reo-3 dsRNA starting with 500 ng was separated on a 10% SDS-PAGE gel. Two blots were made at 30 minutes, each above the gel, and probed with 3'-end labelled reo-3 dsRNA (A) or isolated segments s3 and s4 of reo-1(B). Blots were exposed overnight at -70$^{o}$C with intensifying screens. Note that, since 20% of the dsRNA remained assocciated with the gel and the remaining 80% was divided between the two blots, approximately 3.2 ng of total dsRNA is detected with the homologous probe and 24 ng is detected with the heterologous probe.


l 1
l 2
l 3
m 1, m 2
m 3
s 1
s 2
s 3
s 4
500 ng
250 ng
125 ng
60 ng
30 ng
16 ng
8 ng
4 ng
2 ng
1 ng
A

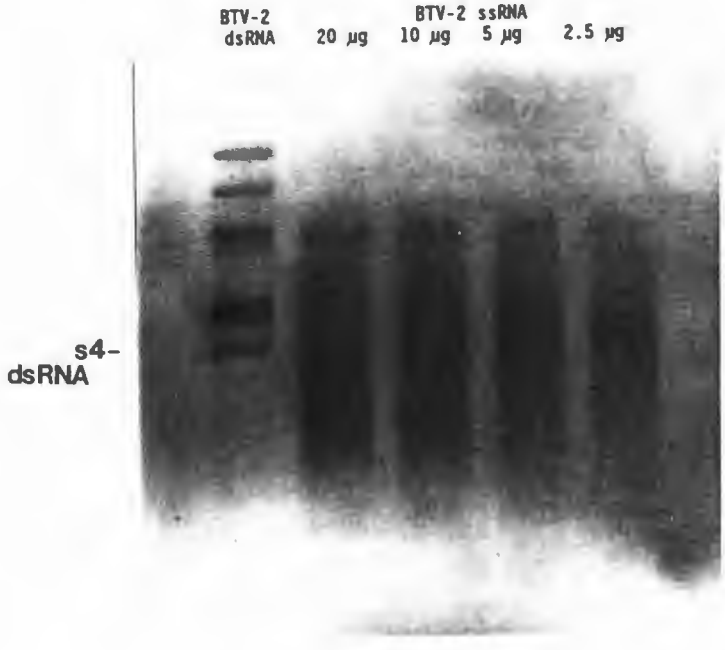

l 1
l 2
l 3
m 1, m 2
m 3
s 1
s 2
s 3
s 4
500 ng
250 ng
125 ng
60 ng
30 ng
16 ng
8 ng
4 ng
2 ng
1 ng
B

step (14). The ssRNA pellet was washed twice with 70% ethanol and re-suspended in a small volume of TE buffer. Samples of ssRNA were glyoxalated and electrophoresed on neutral agarose gels as described by Maniatis et al (12). Gels were either 1% regular agarose or 2% NuSieve agarose. Gels were blotted in 0.2 N NaOH without pretreatment with HCl. A 30-minute blot made in this manner was probed first with homologous genomic probe (Fig. 7A), stripped, and rehybridized using a heterologous single-fragment probe (Fig. 7B). The results indicate that glyoxalated ssRNA can be successfully blotted to Zeta-Probe from agarose gels using 0.2 N NaOH as the blotting medium. Attempts to separate glyoxalated ssRNA on SDS-PAGE were unsuccessful, presumably due to the dissociation of the glyoxal from the ssRNA in the presence of the high-pH buffer used in SDS-PAGE, resulting in aggregation of ssRNAs. Such aggregation would prevent the ssRNAs from entering the gel matrix.

Practical Applications

As an application of this method, the genomic relatedness of five dsRNA-containing viruses was determined. Approximately 0.2 ug of total genomic dsRNA from each of the following viruses was used: reo-3, BTV-11, BTV-13, bacteriophage ø6, and the virus-like particle from Endothia parasitica. These dsRNAs were separated on a 10% SDS-PAGE gel and blotted in 0.2 N NaOH. Six blots were made, two each every 20 minutes, and each blot was hybridized with a [$^{32}$P]-labelled total genomic dsRNA probe from a different virus. The results (Fig. 8) show no cross-hybridization among any of the viruses tested except between

Fig. 7. Differential hybridization of an alkaline blot of BTV-2 ssRNA. A two-fold dilution series of glyoxalated ssRNA, starting with 20 µg, was prepared and electrophoresed on a 2% NuSieve agarose gel. One lane contained 0.5 µg of BTV-2 dsRNA as markers. The gel was blotted for 30 minutes using 0.2 N NaOH. The blot was hybridized with 3'-end labelled dsRNA from BTV-2 and autoradiographed (A). The blot was then stripped of this probe, rehybridized with labelled s4 segment of BTV-13, and again autoradiographed (B). Both exposures were at -70$^{o}$C with intensifying screens.


BTV-2
dsRNA
20 µg
BTV-2 ssRNA
10 µg
5 µg
2.5 µg
A
s4-
dsRNA

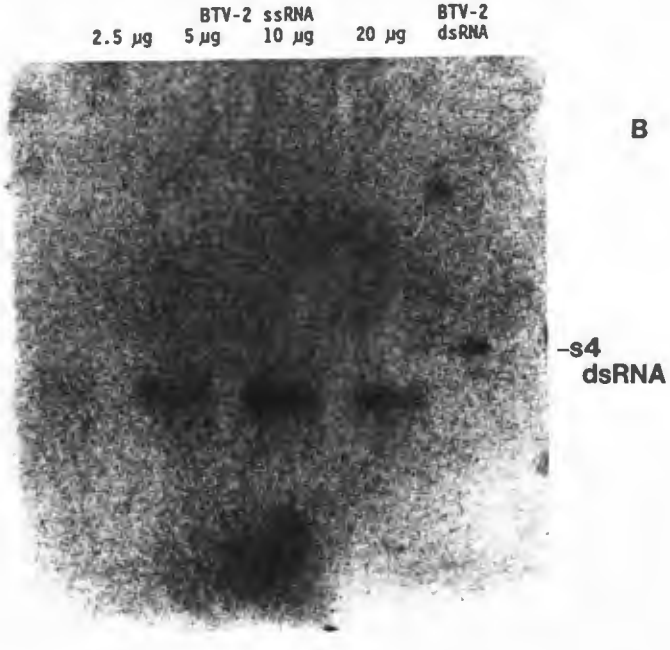
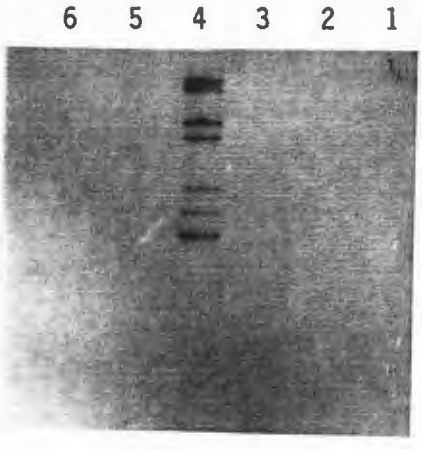
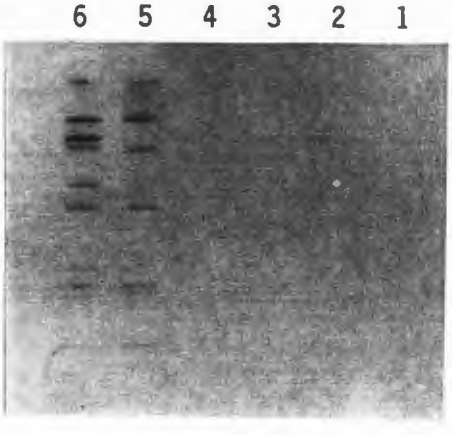

2.5 µg
BTV-2 ssRNA
5 µg
10 µg
20 µg
BTV-2
dsRNA
B
-s4
dsRNA

Fig. 8. A study of the genetic relatedness of five different dsRNA-containing viruses. A 10% SDS-PAGE gel containing 0.2 µg of each dsRNA sample ( 1 = bacteriophage ø6, 2 = dsRNA from E. parasitica, 3 = blank control, 4 = reo-3, 5 = BTV-13, 6 = BTV-11) was used to produce six blots (20 minutes per set). The gel was silver-stained after blotting (A). Each blot was probed with a different 3'-end labelled genomic dsRNA sample: B = reo-3, C = BTV-11, D = bacteriophage ø6. Data for the virus-like dsRNA of E. parasitica and for BTV-13 are not shown. Note that no homology exists between the various samples except for the genetically related BTV-11 and BTV-13.

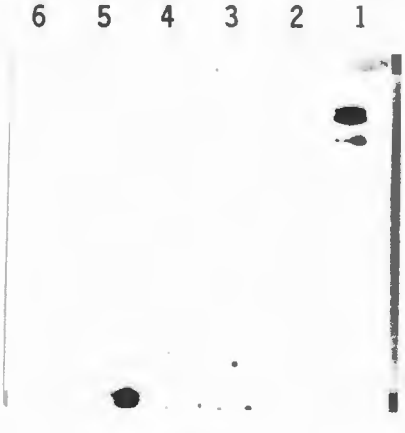

A




B




C




D

BTV-11 and 13. This confirms previous studies which have shown that genetic and protein homology exists between BTV serotypes (20,21), but no genetic homology exists between BTV, reovirus, and the virus-like particles from E. parasitica (20). In addition, this is the first report of a homology comparison of dsRNA from ϕ6 with the dsRNAs from these other viruses. No homology was detected between bacteriophage ϕ6 dsRNA and those of various other viral dsRNAs (Fig. 8D).

In conjunction with this study, a more complete study of the genetic relatedness of all the U.S. serotypes of BTV was undertaken using the methods of alkaline blotting described in this paper. The results of that study are reported elsewhere (20), and indicate that this alkaline blotting method is applicable to such a study to determine if limited homologies exist between closely related serotypes.

## DISCUSSION

E. M. Southern, in 1975, developed a method for analyzing DNA by coupling the resolving power of gel electrophoresis to the transfer of the nucleic acid bands from the gel to a nitrocellulose support membrane. In 1984, and again in 1985, two improved methods for transferring dsDNA to positively-charged nylon membranes from agarose gels were described (9,10). These methods employ sodium hydroxide as the transfer 'buffer'. However, each method produces only a single blot from an overnight transfer. An improved technique for blotting ds- and ssRNA has been developed from these alkaline blotting methods. This improved technique uses 0.2 N NaOH as the blotting medium and can produce as many as 6 blots from the same polyacrylamide gel within 1

hour. When four blots are made at 15 minutes intervals, each blot contains approximately 15% of the input nucleic acid. Approximately 20% is always retained in the gel after blotting for 1 hour under these conditions. This procedure requires no expensive equipment or special buffers, is simple and easy to perform and works on polyacrylamide and agarose gels. When using double-strand nucleic acid, a brief acid depurination step is preferred prior to blotting. The optimal NaOH concentration for blotting is 0.2 N (Fig. 2). When higher concentrations are used, the dsRNA bands become increasingly diffuse, indicating probable degradation by the NaOH. This degradation is also seen when transfers longer than 1 hour are performed using 0.2 N NaOH. Concentrations of NaOH lower than 0.2 N do not show significant degradation within 1 hour. However, transfer efficiency is greatly reduced, as shown by the binding of less of the labelled dsRNA to the membrane and the retention of more of the labelled dsRNA by the gel (Fig. 2).

Various lots and sources of blotting membranes were tested. Only the newer nylon membranes such as Zetabind, Zeta-Probe and Gene Screen Plus retain the dsRNA during and after blotting (Fig. 3). Other blotting membranes, including nitrocellulose and other nylon membranes, retain essentially no dsRNA but allow it to pass through to the blotting pads above the membrane. Different lots of Zetabind and Zeta-Probe also have shown variable background to signal ratios during hybridization although they appear to retain the same amount of dsRNA when used for blotting (data not shown).

The resolution and sensitivity of this method are a great improvement

over previous methods using sodium phosphate as the transfer medium. Using appropriately labelled homologous probes, it is possible to detect as little as 8 ng of total input dsRNA (Fig. 6) on a blot made in 15 minutes. Since it has been noticed that about 20% of the input dsRNA remains associated with the gel (19), approximately 3.2 ng of total genomic would therefore be detected on each blot in Fig. 6A. A lower sensitivity is seen when using a heterologous probe (Fig. 6B). This is in good agreement, however, with the reported low homology between reovirus types 3 and 1 (22,23). Blots have been stripped and reused as many as 10 times with no loss in sensitivity (19).

A final advantage to using 0.2 N NaOH as the blotting medium instead of the traditional buffers is that NaOH may be used without fear of contamination. It may, therefore, be stored at room temperature in a closed container (to prevent evaporation) and may be reused many times. Other buffers may become contaminated under similar circumstances and would require repeated autoclaving.

When blotting dsRNA from various viruses, no difference in blotting efficiency can be detected. There also appears to be equal transfer of large, medium and small segments (Fig. 5 and 8). Different sizes of dsRNA (0.5 - 8.0 Kb) appear to be transferred at approximately the same rate (Fig. 8). This even transfer allows for more exacting comparison of various genes without the problems created by unequal transfer previously reported using other methods (J. Li, personal communication).

This alkaline blotting method may also be used quite effectively on single strand RNA which has been glyoxalated and run on either NuSieve

or regular agarose. Due to the ease of degradation of ssRNA, no HCl wash should be performed prior to blotting. The efficiency of transfer appears to be less than that seen with polyacrylamide gels. This is presumably due to the greater thickness of the agarose. Although the resolution obtained from glyoxal gels is relatively poor, it is possible to detect single species of mRNA (Fig. 7). An additional advantage of using NaOH as the blotting medium for glyoxal gels is that the glyoxal dissociates from the nucleic acid during the blotting procedure and will, therefore, not interfere with subsequent hybridizations (5,12).

Initial experiments indicate that similar results might be achieved using dsDNA. Bacteriophage lambda DNA, which has been digested with Hind III, has been successfully separated on a 10% polyacrylamide gel, but blotting efficiencies appear to be reduced compared to those achieved with dsRNA. The optimal transfer conditions for DNA separated on SDS-PAGE gels appear to be different from the conditions for RNA. More work is required to optimize the separation of dsDNA on polyacrylamide gels, and subsequent alkaline blotting. These follow-up experiments may improve both resolution and sensitivity of standard Southern blotting.

Although the exact mechanism for the improved transfer and binding of nucleic acids to nylon membranes under these conditions is unknown, contributing factors can be noted: 1) The membrane is positively charged and, thus, binds negatively-charged nucleic acids more tightly than do other membranes. In the presence of NaOH, this binding seems to be enhanced, but is unaffected or decreased when using other membranes.

2) The nucleic acids can be denatured in the presence of alkali. This may enhance the transfer of the nucleic acids out of the gel. In addition, the sensitivity of the subsequent hybridizations is improved since hybridization to probes relies on the single-strand character of the membrane-bound nucleic acids. The difference in transfer efficiency between DNA and RNA may be a reflection of their respective sensitivities to denaturation by alkali. RNA is more sensitive to denaturation by alkali and is, therefore, transferred more efficiently than DNA under the same conditions.

The method developed in this study can potentially be used for all types of nucleic acid and with both polyacrylamide and agarose gels. The sensitivity of detection of input nucleic acid is greatly improved over previous methods. In addition, multiple blots can be obtained within a very short time for multiple comparison purposes. Therefore, all comparisons can be made from a single gel, as was done with the comparison of the genetic relatedness of various dsRNA-containing viruses (Fig. 8). If desired, these blots can be stripped and reused many times. The method described in this paper is very fast and reproducible and should prove to be very useful to those who are using hybridization as a major source of information.

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