Zeta-Probe®
Blotting Membranes

Instruction Manual

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BIO-RAD

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Section 1
Introduction

Zeta-Probe blotting membranes are nylon membranes which have unique binding and handling properties that make them ideally suited for nucleic acid, and some protein, blotting applications.

Zeta-Probe membranes possess a high tensile strength. They won't shrink, tear, or become brittle during transfer, baking, hybridization, or reprobing. Zeta-Probe membranes are heat-resistant, nonflammable, and autoclavable. Zeta-Probe membranes are naturally hydrophilic with no added wetting agents. These membranes are resistant to a wide variety of chemicals, including 100% formamide, 2 M NaOH, 4 M HCl, acetone, most alcohols, DMSO, DMF, and chlorinated aliphatic hydrocarbons. The nominal porosity of Zeta-Probe membranes is 0.45 µm. When stored at 23–25°C, Zeta-Probe membranes are stable for at least 1 year.

When handling Zeta-Probe membranes, always wear gloves or use forceps. After blotting, do not allow wet membranes to come in contact with each other. Contact may result in the transfer of blotted nucleic acids or proteins from one membrane to the other.

Stock buffers are listed in the appendix. It is suggested that you read the entire protocol before proceeding.

Section 2
Nucleic Acid Blotting Protocols

Several nucleic acid blotting methods are presented in this section. Capillary blotting (Sections 2.1through 2.3) is generally used with agarose gels, and electrophoretic transfer (Section 2.4) is used with polyacrylamide gels. Dot blotting (Sections 2.5 and 2.6) is used for

nucleic acids in solution. DNA alkaline blotting (Section 2.3) is an alternative to Southern blotting. DNA alkaline blotting results in higher resolution and greater sensitivity in many applications. DNA alkaline fixation (Section 2.7) can be used to denature and covalently fix DNA to Zeta-Probe membranes after transfer.

2.1 Southern Blotting[1,2] (DNA Capillary Transfer)

1. Depurinate the DNA by soaking the gel in 0.25 M HCl for 10–15 min (be sure that the gel is floating free in all baths).

   Note: Acid depurination is only recommended for fragments >4 kb.
2. Denature the DNA by placing the gel in a bath of 0.5 N NaOH, 1 M NaCl. Place the container on a moving platform for 30 min at room temperature.
3. Neutralize the gel by bathing it in 1.5 M Tris-HCl, pH 7.4, 1.5 M NaCl for 30 min at room temperature on a moving platform. Prepare a Whatman 3MM paper wick. Hang two sheets, prewetted with 10x SSC hung over the sides and into the bottom of the capillary transfer apparatus containing 800 ml 10x SSC. On top of the wick, place two additional sheets of 3MM paper cut to the size of the gel prewetted with 10x SSC.
4. Invert the gel and place it on the wick. Roll a 10 ml plastic pipet, over the gel to remove any bubbles. Trim off the wells from the gel using a spatula.
5. Place membranes labeled side against the gel above the lanes to be transferred. Trim edges of the gel as required with a spatula, or cover exposed areas with Parafilm. Roll with the pipet to remove any air bubbles. It is important to remove air bubbles from underneath the blotting membrane as they will block transfer. To avoid trapping bubbles, place the Zeta-Probe membrane onto the gel surface by first bowing the membrane diagonally and aligning the opposite corners with the gel corners. Then lower the Zeta-Probe membrane onto the gel.
6. Cut two pieces of 3MM paper to the size of the gel. Place both sheets on top of gel. Wet paper with small amount of transfer buffer. Roll with the pipet to remove air bubbles.
7. Flood the surface of the gel with buffer. Carefully place paper towels over the Whatman paper. Stack the paper towels about 15 cm high.
8. Cover the paper towel stack with a glass or plastic plate. Keep the pressure on the paper towel stack at a minimum. Excessive weight will compress the gel, retarding capillary transfer.
9. Keep an excess of buffer in the dish, but do not cover the top of the sponge. Continue transferring for 2–24 hr, depending on the gel concentration and fragment size.
10. After transfer, separate the membrane from the gel, rinse the membrane briefly in 2x SSC, and briefly blot the membrane with filter paper. The DNA can then be fixed onto the Zeta-Probe membrane by baking it at 80°C for 30 min in a vacuum oven. Alternatively, the DNA can be UV-crosslinked to the membrane using 5,000 μJ/cm^2 radiation. Higher levels, although they increase the absolute retention of the nucleic acid on the membrane, can lead to a reduction in signal intensity. The membranes can be stored dry between two pieces of filter paper in plastic bags at 23–25°C.

2.2 Northern Blotting (RNA Capillary Transfer)

Follow the Southern blotting protocol (Section 2.1), omitting steps 1–3. No pretreatment of RNA gels is necessary.[5]

If gels contain glyoxal, remove glyoxal adducts by vacuum baking Zeta-Probe membrane for 1 hour at 80°C after transfer. Alternatively,

pour 95°C 20 mM Tris-HCl, pH 8.0, 1 mM EDTA onto the blotted membrane, then gently agitate at room temperature until the solution cools. After removal of glyoxal adducts, proceed to hybridization or store the membranes dry.

2.3 Alkaline Blotting[3] (DNA Capillary Transfer)

1. Depurinate the DNA by soaking the gel in 0.25 M HCl for 10–15 min. Rinse the gel several times with distilled water.

   Note: Acid depurination is only recommended for fragments >4 kb.

2. Cut four sheets of Whatman 3MM paper so they overhang the bottom of the gel tray by 5 cm on each end. Prewet the Zeta-Probe membrane in distilled water.
3. Place the four sheets of 3MM paper on an inverted gel casting tray. Place the 3MM/tray in the bottom of a deep dish. Then saturate the 3MM paper with 0.4 M NaOH. Remove the bubbles by repeatedly rolling a glass pipet over the saturated 3MM paper. Pour enough NaOH into the deep dish so that the 3MM wick ends are immersed in NaOH.
4. Pour more NaOH onto the 3MM wick to saturate it, then carefully place the gel on the wick. Make sure that no bubbles are trapped beneath the gel. Cover the gel with a small amount of NaOH.
5. Place plastic wrap (such as Saran wrap) over the entire gel/3MM stack. Cut out a window with a clean razor blade, allowing only the gel to be exposed.
6. Lower the sheet of pre-wetted Zeta-Probe membrane onto the gel surface, making contact first in the center, then allowing the edges to gradually fold down. Carefully flood the filter surface with NaOH. Make sure that no bubbles are present between the gel and the Zeta-Probe membrane.

7. Cut two pieces of 3MM exactly to the gel size. Wet a sheet of precut 3MM paper in water and place it on the Zeta-Probe membrane/gel stack, then repeat with the second sheet. Remove any bubbles from beneath each layer of 3MM paper.
8. Place a stack of precut paper towels on the 3MM/Zeta-Probe membrane/gel stack. Cover the paper towel stack with a plastic or glass plate. Keep the pressure on the paper towel stack at a minimum. Excessive weight will compress the gel, retarding capillary transfer.
9. Continue transferring for 2–24 hours, depending on the gel concentration and fragment size. Note: Higher background may appear if transfer is longer than 24 hr.
10. After transfer, remove the stack of paper towels. Gently peel the Zeta-Probe membrane from the surface of the gel, rinse it in 2x SSC, and air dry. DNA is fixed to the membrane during transfer, eliminating the need for subsequent fixation. The dried membranes are stable at room temperature. The membranes can be stored dry between two pieces of filter paper in plastic bags at 23–25°C.

2.4 Electrophoretic Transfer

The following protocol was developed for maximum efficiency of electrophoretic transfer. It affords the greatest mobility of DNA and RNA, and the most complete transfer from gel to membrane without excessive heat generation. The buffer (ionic strength and pH) and field strength have been optimized for electrophoretic blotting of DNA and RNA from both agarose and acrylamide gels. For electrophoretic transfer from agarose gels, a heat exchanger must be used, because increased temperatures could melt the agarose gel. The protocol was developed using the Trans-Blot® electrophoretic transfer system with a heat exchanger.

1. Prepare the stock electrophoretic transfer buffer, 20x TAE or 5x TBE.
2. Prepare gels for transfer immediately after electrophoresis:
   A. Electrophoresis Under Denaturing Conditions

      If gel electrophoresis was done under denaturing conditions (*e.g.*, agarose/formaldehyde gels), equilibrate the gel in 0.5x transfer buffer for 10–15 min prior to electrophoretic transfer.

   B. Electrophoresis Under Nondenaturing Conditions

      1. Soak the gel in 0.2 N NaOH, 0.5 M NaCl for 30 min. For polyacrylamide gels, be sure not to exceed 30 min, since limited gel hydrolysis may occur with subsequent swelling during transfer.

         Note: Zeta-Probe membranes will bind nondenatured nucleic acids. Therefore, denaturing is not mandatory before transferring. Yet, after transferring, the blotted Zeta-Probe membrane must be treated with NaOH. Refer to the DNA alkaline fixation procedure (Section 2.7).

      2. After base treatment, neutralize the gel by washing in 5x transfer buffer two times, 10 min each. Then wash the gel once in 0.5x transfer buffer for 10 min.
      3. While gels are being equilibrated, soak the Zeta-Probe membrane at least 10 min in 0.5x transfer buffer.
      4. Fill the electrophoretic transfer cell to half full with 0.5x transfer buffer, and circulate 4°C coolant through the heat exchanger. If possible, place the cell on a magnetic stirring plate and add a stirbar. Circulate buffer in the cell by stirring to maintain uniform temperature during the run.
      5. Prepare the transfer assembly.

      Soak one fiber pad by squeezing it while it is submerged in 0.5x transfer buffer. Lay the soaked pad on the open gel holder. Soak a piece of thick filter paper (*e.g.*, slab gel dryer type paper cut to the size of the fiber pad) in the transfer buffer and place it on the fiber pad. Place the gel on the filter paper. Hold the presoaked Zeta-Probe membrane with both hands so that the middle of the membrane is sagging or bowed downward. Allow the middle of the membrane to contact the gel first. Gradually lower the ends of the membrane onto the gel. This process will expel most bubbles from between the gel and the membrane. If there are any remaining bubbles between the gel and membrane, remove them by sliding a test tube or extended gloved finger across the surface.

      Note: Maintaining uniform physical contact between the gel and membrane is of critical importance in electrophoretic transfer.

      Place a presoaked piece of thick filter paper on the membrane followed by a presoaked fiber pad. Close the gel holder and place it in the transfer cell so that the membrane is on the anode side of the gel (red pole). Add more 0.5x transfer buffer, if necessary, to bring the buffer level to 1 cm below the electrode post.

6. Transfer at 80 V for 4 hours.

   Note: For comprehensive electrophoretic transfer instructions, including protocols, technical discussion, and troubleshooting guide, refer to the Trans-Blot cell operating manual.

7. After transfer, separate the membrane from the gel, rinse the membrane briefly in 1x transfer buffer, and briefly blot

the membrane with filter paper. Fix nucleic acids onto the Zeta-Probe membrane by baking it at 80°C for 30 min. The membranes can be stored dry between two pieces of filter paper in plastic bags at 23–25°C.

2.5 DNA Dot Blotting

When Zeta-Probe membrane is used, it is not necessary to extract DNA from tissue samples for dot blot analysis. Regardless of whether the sample is purified DNA (covalently closed circular DNA, double-stranded DNA, single-stranded DNA), whole blood, tissue, or cultured cells, it can be heated in alkali, then filtered directly onto the Zeta-Probe membrane.

1. Heat the sample in a total volume of 0.5 ml with a final concentration equal to 0.4 M NaOH, 10 mM EDTA at 100°C for 10 min.[16] The sample may be purified or crude DNA (≤5 µg), whole soft tissue, e.g., liver (≤0.5 mg), whole blood (≤10 µl), cultured cells (≤10^5 cells).
2. Wet a sheet of Zeta-Probe membrane by immersing it in distilled water.
3. Assemble the microfiltration apparatus with the prewetted Zeta-Probe membrane. Make sure that all screws and clamps have been tightened under vacuum to prevent contamination between wells. Rinse wells with 0.5 ml TE or H_2O. Apply vacuum until wells are empty but not dry.
4. Apply a 0.5 ml DNA sample into each appropriate well without vacuum.
5. Start vacuum until the wells are just dry.
6. Rinse all wells by placing 0.5 ml of 0.4 M NaOH in each, then apply vacuum until all wells are quite dry.

7. Disconnect the vacuum, disassemble the apparatus, and rinse the membrane briefly in 2x SSC. UV-crosslink the DNA to the membrane or vacuum dry the blotted Zeta-Probe membrane at 80°C for 30 min. The membranes can be stored dry between two pieces of filter paper in plastic bags at 23–25°C.

2.6 RNA Dot/Slot Blotting

Both native and denatured RNA are retained quantitatively by Zeta-Probe membrane. However, to insure optimal hybridization, RNA samples must be totally denatured before fixing onto the Zeta-Probe membrane.

Glyoxal RNA Denaturation and Fixation

1. Add RNA sample to the following final concentrations:
   50% dimethyl sulfoxide (DMSO)
   10 mM sodium phosphate, pH 7
   1 M glyoxal
2. Incubate sample for 1 hr at 50°C. Then cool the RNA sample on ice.
3. Wet a sheet of Zeta-Probe membrane by immersing it in distilled water.
4. Assemble the microfiltration apparatus with the prewetted Zeta-Probe membrane. Make sure that all the screws or clamps have been tightened under vacuum to prevent cross well contamination.
5. Place a 0.5 ml RNA sample into each appropriate well without vacuum.
6. Apply vacuum until the wells are just dry, then release vacuum.
7. Rinse all wells with 0.5 ml TE, and apply vacuum until the wells are completely dry.

8. Disconnect the vacuum. Remove the blotted Zeta-Probe membrane.
9. Remove the glyoxal by rinsing the membrane in 2x SSC and letting it airdry. Fix RNA onto Zeta-Probe membrane by baking the membrane at 80°C for 1 hr.

2.7 DNA Alkaline Fixation

After transfer, place the Zeta-Probe membrane (DNA surface facing up) on a pad of 3MM paper saturated with 0.4 M NaOH for 10 min. Rinse in 2x SSC and air dry. The dried membranes are stable at room temperature. The membranes can be stored between two pieces of filter paper in plastic bags at 23–25°C.

Section 3
Probe Recommendations

The specific activity, concentration, size range, and purity of the probe all have an important effect on signal-to-noise ratio during hybridization. For hybridization on Zeta-Probe blotting membranes, the following is recommended:

Probe specific activity	10^8 cpm/µg probe
Probe concentration in the hybridization mixture	10^6 cpm/ml (10–50 ng/ml)
Probe length	200–1,000 bp

Probe length is an important parameter to control. DNA probes prepared by random priming tend to be small. Small probes can cause lane specific background during low stringency hybridizations. DNA probes prepared by nick translation are generally long. Probe fragments longer than 1 kb decrease hybridization specificity.

Alternative hybridization protocols are necessary when probe lengths vary outside this recommended range (refer to Oligonucleotide Protocol, Section 4.4).

Template purity is essential during probe synthesis, especially probes made by random primer extension. Small amounts of contaminating DNA templates can cause lane background or extra bands due to the high specific activity of random priming.

Optimal probe specific activity and concentration can vary according to available hybridization sites and exposure time. Probe cleanup is essential to minimize background. Unincorporated nucleotides present after probe preparation contribute to hybridization background. The most effective cleanup method is desalting by column separation. This can be done in a column (1 to 5 ml bed volume) using Bio-Gel® P-30 gel (catalog #150-1340) or with Bio-Spin® 30 columns (catalog #732-6004).

After cleanup, denature the double-stranded probe by increasing temperature to 95–100°C for 5 min. Then cool rapidly on ice. Use the probe as soon as possible after preparation.

Section 4
Hybridization Protocols for DNA Probes

There are several hybridization protocols that will give high quality results. The key to successful nucleic acid blotting is proper blocking of the Zeta-Probe membrane. The most important blocking reagent in the hybridization solution is sodium dodecylsulfate (SDS). SDS is most effective when used at concentrations $\geq$1% (w/v). The Standard Protocol (Section 4.1) uses 7% (w/v) SDS, which has been shown to give extremely low background and high signals. The protocol described in Section 4.2 includes formamide, which allows

hybridization to be performed at a lower temperature. The protocol in Section 4.4 is recommended for oligonucleotide probes. The Alternative Protocol (Section 4.3) should be used only when extreme sensitivity is needed.

The final volume of hybridization solution is important in reducing background. For prehybridization, use 150 µl solution/cm^2 Zeta-Probe membrane. For washes, use at least 350 µl solution/cm^2 Zeta-Probe membrane.

One of the most significant advantages offered by Zeta-Probe membrane over conventional membranes is that target nucleic acids of all sizes can be fixed irreversibly. The stringency of hybridization can therefore be optimized for detection of specific target sequences. There is no need to use high ionic strength and low temperature to minimize the loss of nucleic acids from the membrane during hybridization or washing procedures.

Hybridizations should be conducted at 20–25°C below the melting temperature (T_m) of the probe duplex to insure optimal rates of specific hybridization while minimizing interaction with partially homologous sequences.[10] The stringency of post-hybridization washes is less critical, but a good rule of thumb is to conduct the most stringent wash at 10–15°C below T_m.[11] The protocols described below are suitable for probes having a (G+C) content representative of the mammalian genome, i.e., 0.42. If desired, conditions can be varied in accordance with the following empirical formula:

T_m (DNA/DNA) = 81.5 + 16.6 x log [Na] – 0.65 x (% formamide) + 41 x (G + C).11

T_m (RNA/RNA) = 79.8 + 18.5 x log [Na+] – 0.35 x (% formamide) + 58.4 x (G + C) + 11.8 x (G + C)2 12

T_m (DNA/RNA) = approx. mean of T_m (DNA/DNA) and T_m (RNA/RNA)

The T_m is decreased approximately 1.5°C for every 1% decrease in homology.[10, 11]

The T_m is decreased as the fragment length of the probe decreases; the appropriate correction factor is approximately –500 / (# bp in probe fragment)°C.[10, 11]

The rate of hybridization increases as the salt concentration increases.[10]

The rate of hybridization decreases as the formamide concentration increases.[10, 13]

The hybridization temperature (TH) appropriate to synthetic oligomeric DNA probes in 1 M Na+ can be approximated by the following:

T_H + 2 x (no. of A-T bp) + 4 x (no. of G-C bp) –5.14

4.1 Standard Protocol

Prehybridization

1. Seal the blotted Zeta-Probe membrane inside a heat-sealable plastic bag.
2. Cut one corner of the plastic bag and pipet prehybridization solution in:

   0.5 M Na_2HPO_4, pH 7.2
   7% (w/v) SDS
3. Incubate briefly at 65°C for 5 min. The goal is to evenly and completely coat the membrane with this solution.

Hybridization

1. Cut one corner of the plastic bag, remove the prehybridization solution, and replace it with the same buffer.

2. Add the denatured probe and remove all bubbles before resealing the bag. Hybridize for 4–24 hours at 65°C with agitation.
3. Carefully remove the hybridization solution by cutting one corner. Remove hybridized Zeta-Probe membrane plastic bag.

   Note: At no stage before washing should the membranes be permitted to dry.

Washes

1. Wash the membrane at 68°C, 2 times for 10 min each, in the following:

   1x SSC

   0.1% (w/v) SDS

   The first wash should be conducted at room temperature; the second wash should be conducted in the hybridization oven.
2. Wash the membrane at 65°C, 2 times for 30–60 min each, in the following:

   0.1x SSC

   0.1% (w/v) SDS

   These washes should be conducted in the hybridization oven.
3. After washing, the blotted membranes are ready for autoradiography. If no further cycles of hybridization are to be done on the membrane, the membrane can be dried. When reprobing, do not allow the membrane to dry between hybridizations. Expose moist membranes between plastic wrap or enclosed in a sealable plastic bag. Do not allow a wet membrane to come in contact with the film, because wet Zeta-Probe membrane will stick to the film.

4.2 Formamide Protocol

Prehybridization

1. Seal the blotted Zeta-Probe membrane inside a heat-sealable plastic bag. Prepare the following solution for prehybridization:

   50% formamide
   0.12 M Na_2HPO_4, pH 7.2
   0.25 M NaCl
   7% (w/v) SDS
   1 mM EDTA
2. Cut one corner of the plastic bag and pipet the prehybridization solution in, then reseal the bag.
3. Incubate at 43°C for 5 min.

Hybridization

1. Cut one corner of the bag, remove the prehybridization solution, and replace it with the same buffer.
2. Add probe, then seal the open corner (taking care to exclude all air bubbles). Mix the contents of the bag thoroughly. Incubate at 43°C for 4–24 hr with agitation.

   Note: At no stage before washing should the membranes be permitted to dry.

Washes

1. At the completion of hybridization, remove membranes from their hybridization bags and place them in 2x SSC. Rinse briefly, then wash them successively by vigorous agitation at room temperature for 15 min in each of the following solutions:

   2x SSC/0.1% SDS
   0.5x SSC/0.1% SDS
   0.1x SSC/0.1% SDS

Note: For single-copy detection or high stringency, conduct the last wash at 65°C.

2. After washing, the blotted membranes are ready for autoradiography. If no further cycles of hybridization are to be done on the membrane, the membrane can be dried. When reprobing, do not allow the membrane to dry between hybridizations. Expose moist membranes between plastic wrap or enclosed in a sealable plastic bag. Do not allow a wet membrane to come in contact with the film, because wet Zeta-Probe membrane will stick to the film.

4.3 Alternative Protocol

In this section two hybridization protocols using hybridization accelerators are presented. When extreme hybridization sensitivity is needed, these accelerators will help to increase the target signal by acting as volume excluders. Hybridization accelerators will also decrease the hybridization time needed. In some applications, hybridization accelerators can reduce the hybridization time from overnight to 4 hr. It is suggested that you first work with the standard hybridization protocol (Section 4.1) and determine if your experiments require a hybridization accelerator before using the following protocols.

1. Polyethylene glycol (PEG)[15]— follow the instructions for standard hybridization (Section 4.1) or formamide hybridization (Section 4.2) except add 10% (w/v) PEG 8,000 MW into the hybridization solution in step 1.

   Conduct post-hybridization washes as described in Section 4.1 or 4.2, without PEG.

2. Dextran sulfate — follow the instructions for formamide hybridization (Section 4.2) except increase the hybridization temperature to 65°C and substitute the following prehybridization and hybridization solutions in step 1:

   2x SSPE
   1% (w/v) SDS
   0.5% (w/v) BLOTTO
   10% (w/v) dextran sulfate
   0.5 mg/ml nonhomologous carrier DNA

4.4 Oligonucleotide Protocol[6]

Prehybridization

1. Seal the blotted Zeta-Probe membrane inside a heat-sealable plastic bag. Prepare the following solution for prehybridization:

   5x SSC
   20 mM Na_2HPO_4, pH 7.2
   7% SDS
   1x Denhardt's
   100 µg/ml denatured herring sperm DNA

   The carrier DNA must be denatured before adding it to the hybridization solution by heating at 100°C for 5 min, followed by rapid cooling on ice.

2. Cut one corner of the plastic bag and pipet prehybridization solution in, then reseal the bag.

3. Incubate at 50°C for 0.5–24 hr.

Hybridization

1. Immediately before use, fragment and denature the probe and carrier DNA as follows. Dissolve the radiolabeled probe in 0.1 ml of 0.2 M NaOH, add carrier DNA, mix, and centrifuge briefly to consolidate the solution. Pierce a fine hole in the tube cap and place the tube in a heating block at 100°C for 5 min, followed by rapid cooling on ice.

2. Cut one corner of the bag, remove the prehybridization solution, and replace it with the same buffer.
3. Add probe, then seal the open corner (taking care to exclude all air bubbles). Mix the contents of the bag thoroughly. Incubate at 50°C for 4–24 hr.

   Note: At no stage before washes should the membranes be permitted to dry.

Washes

1. Wash the membrane twice at 50 °C for 30 min in the following:

   3x SSC
   10x Denhardt's
   5% SDS 25 mM NaH_2PO_4, pH 7.5
2. Wash the membrane once at 50 °C for 30 min in the following:

   1x SSC
   1% SDS
3. After washing, the blotted membranes are ready for autoradiography. If no further cycles of hybridization are to be done on the membrane, the membrane can be dried. When reprobing, do not allow the membrane to dry between hybridizations. Expose moist membranes between plastic wrap or enclosed in a sealable plastic bag. Do not allow a wet membrane to come in contact with the film, because wet Zeta-Probe membrane will stick to the film.

Section 5
Hybridization Protocol for RNA Probes

Prehybridization

1. Seal the blotted Zeta-Probe membrane inside a heat-sealable plastic bag. Prepare the following solution for prehybridization:

   50% formamide
   1.5x SSPE
   1% SDS
   0.5% BLOTTO
   0.2 mg/ml carrier RNA
   0.5 mg/ml carrier DNA

   The carrier DNA must be denatured before adding it to the hybridization solution by heating at 100°C for 5 min, followed by rapid cooling on ice.
2. Cut one corner of the plastic bag and pipet prehybridization solution in, then reseal the bag.
3. Incubate at 50°C for 0.5–24 hr.

Hybridization

1. Immediately before use, fragment and denature the probe and carrier DNA as follows. Add to the precipitated RNA probe 0.1 ml of yeast RNA (20 mg/ml), 0.5 ml of carrier DNA (10 mg/ml), and 0.6 ml of deionized formamide, mix thoroughly, and heat at 70°C for 5 min.
2. Cut one corner of the bag, remove the prehybridization solution, and replace it with hybridization buffer:

   50% formamide
   1.5x SSPE

1% SDS
0.5% BLOTTO

3. Add probe, then seal the open corner (taking care to exclude all air bubbles). Mix the contents of the bag thoroughly. Incubate at 50°C for 4–24 hr.

   Note: At no stage before washing should the membranes be permitted to dry.

Washes

1. At the completion of hybridization, remove the membranes from their hybridization bags and place them in 2x SSC. Rinse briefly, then wash them successively by vigorous agitation for 15 min at room temperature in the following solutions:

   2x SSC/0.1% SDS
   0.5x SSC/0.1% SDS
   0.1x SSC/0.1% SDS

2. After washing, the blotted membranes are ready for autoradiography. If no further cycles of hybridization are to be done on the membrane, the membrane can be dried. When reprobing, do not allow the membrane to dry between hybridizations. Expose moist membranes between plastic wrap or enclosed in a sealable plastic bag. Do not allow a wet membrane to come in contact with the film, because wet Zeta-Probe membrane will stick to the film.

   Note: To increase the rate of hybridization, include 10% dextran sulfate (final concentration) in the hybridization solution. Prewarm the hybridization solution to 50°C. Denature the probe and carrier as above. Special care must be taken to insure uniform mixing of the denatured probe with the hybridization solution, since the solution is quite viscous at 50°C.

Section 6
Probe Stripping and Rehybridization

If reprobing is desired, do not allow the Zeta-Probe membrane to dry between hybridizations.

The Zeta-Probe membrane should be stripped as soon as possible after autoradiography.

Wash the membrane twice for 20 min each in a large volume of 0.1x SSC/0.5% SDS at 95°C.[2] Check membrane by overnight exposure.

Section 7
Zeta-Probe Membrane Troubleshooting Guide

7.1 Nucleic Acids Problem

Problem	Solution
1. Fragments greater than ~1,000 bp cannot be electrophoretically transferred from polyacrylamide gels after base denaturation, even at increased volts/hours	We have observed that fragments >~1,000 bp can become trapped in polyacrylamide gels if they are base denatured and neutralized after electrophoresis, whereas nondenatured fragments will transfer completely up to at least 2,000 bp. Solve this problem in one of three ways: 1. Omit pretreatment, and transfer ds DNA. Alkaline fix post-blotting. 2. Run gel electrophoresis under denaturing conditions, and omit base denaturation step and neutralization step prior to transfer. 3. Omit base denaturation step, and denature gel instead with 1 M glyoxal, in 25 mM sodium phosphate, pH 6.5, 50% DMSO for 1 hr at 50°C. Then transfer directly.
2. Very large fragments cannot be electrophoretically eluted from agarose gels	1. Solutions 1 and 2 to problem #1 can also be applied to agarose gels. 2. Depurinate prior to transfer by soaking the gel in 0.25 M HCl for 20 min,[6] then soak the gel in transfer buffer for 10–15 min.

Nucleic Acids (Continued)

Problem	Solution
3. High background observed throughout membrane on autoradiograph	The major contributors to background are unincorporated label, small radioactive decay products, and small probe fragments resulting from nick translation or random priming. 1. Use a desalting gel column to remove unincorporated label. Bromophenol Blue is a useful indicator. The peak of unincorporated label overlaps with, and slightly precedes the Bromophenol Blue in a desalting column. 2. Use the probe as soon as possible after preparation, since decay results in fragmentation. 3. Reduce exposure of the probe to DNase I during nick translation to increase average probe length. 4. Use a different heterologous nucleic acid in the prehybridization and hybridization mixtures. Sonicate it thoroughly and denature it before use. 5. For RNA probe, post-hybridization washes: remove SDS by washing 3x in 100 mM sodium phosphate (pH 7.2), then wash with 10 µg/ml RNase A, 0.3 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM Na_2EDTA at 37°C for 15 min.

Nucleic Acids (Continued)

Problem	Solution
4. Localized high background observed on autoradiograph	1. Make sure the membrane is free-floating within the plastic bag during hybridization. Membrane/bag contact during hybridization can cause background. Add more hybridization solution. 2. Make sure not to pinch the membrane when sealing the plastic bag prior to hybridization. 3. Be sure no bubbles exist in the hybridization bag.
5. Lane background or extra bands	1. Indicates contaminated template. Make sure the probe is synthesized with the pure template of choice.
6. Low autoradiograph signal	This problem may occur when total genomic DNA is probed for single-copy or low copy number genes. 1. Incorporate 10% dextran sulfate in the hybridization mixture. This polymer effectively reduces the solvent volume, thereby increasing the concentration of the solutes and enhancing hybridization. Refer to Section 4.3. 2. Increase exposure time to increase signal-to-noise ratio. 3. Increase sample load on the gel. 4. If low signal is accompanied by low background, probe concentration can be increased 2- to 4-fold.

Problem	Solution
7. No autoradiograph signal	1. After transfer, stain the gel to check that transfer was complete. If not, increase transfer time and/or voltage of transfer, or see solution to problem #1 above. 2. Be sure probe is denatured by boiling or heating to 65°C for 5 min in 50% formamide prior to hybridization.

Section 8
Appendix

20x TAE	MW	g/L
0.8 M Tris	121.1	96.9
0.4 M base sodium acetate	82.04	32.8
20 mM EDTA	372.2	7.45

pH to 7.4 with glacial acetic acid

5x TBE	MW	g/L
0.5 M boric acid	61.8	30.9
0.5 M Tris base	121.1	60.5
10 mM EDTA	372.2	3.73

20x SSC	MW	g/L
3 M NaCl	58.44	175.0
0.3 M trisodium citrate	294.1	88.2

20x SSPE	MW	g/L
3.6 M NaCl	58.44	210.0
0.2 M $Na_2HPO_4 \bullet 7\ H_2O$	268.07	53.6
20 mM EDTA	372.2	7.44

TE

10 mM Tris-HCl, pH 8.0
1 mM EDTA, pH 8.0

100x Denhardt's Solution	MW	g/100 ml
2% bovine serum albumin		2
2% polyvinylpyrrolidone	360,000	2
2% Ficoll	400,000	2

10% BLOTTO	g/100 ml
Nonfat powdered milk	10
0.2% sodium azide	0.2

Store at 4°C

20% SDS	MW	g/L
20% sodium dodecyl sulfate	288.38	200

Heat to 65°C to get into solution

1 M Na_2HPO_4, pH 7.2	MW	g/L
1 M $Na_2HPO_4 \bullet 7\ H_2O$	268.07	268.07

Add 4 ml 85% H_3PO_4 [1 M in Na^+, see Reference 4]

50% Dextran Sulfate	MW	g/100 ml
50% dextran sulfate	500,000	50
0.2% sodium azide	65.01	0.2

Store at 4°C

50% Formamide	g/100 ml
50% formamide	50

Store at 4°C. Immediately before use, deionize the required volume by stirring gently for 1 hr with 1 g mixed-bed ion exchange resin (AG® 501-X8(D) resin, catalog #142-6425) per 10 ml of formamide. Filter through coarse filter paper.

6 M Glyoxal (Deionized)

Deionize 6 M glyoxal by pouring over a small mixed-bed resin column (AG 501-X8 mixed bed ion exchange resin, catalog #142-6424). Store at –20°C in small aliquots. Once an aliquot has been exposed to air, it cannot be reused.

Section 9
References

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Section 10
Ordering Information

Catalog Number	Product Description
162-0153	**Zeta-Probe Membranes,** 9 x 12 cm, 15 sheets
162-0154	**Zeta-Probe Membranes,** 10 x 15 cm, 15 sheets
162-0155	**Zeta-Probe Membranes,** 15 x 15 cm, 15 sheets
162-0156	**Zeta-Probe Membranes,** 15 x 20 cm, 15 sheets
162-0157	**Zeta-Probe Membranes,** 20 x 20 cm, 15 sheets
162-0158	**Zeta-Probe Membranes,** 20 x 25 cm, 3 sheets
162-0159	**Zeta-Probe Membranes,** 30 cm x 3.3 m roll
162-0165	**Zeta-Probe Membranes,** 20 cm x 3.3 m roll
165-5000	**Model 785 Vacuum Blotter**
165-5031	**GS Gene Linker UV Chamber,** 120 VAC
165-5052	**PowerPac™ 200 Power Supply,** 100/120 VAC
170-6545	**Bio-Dot® Microfiltration Apparatus**
170-6542	**Bio-Dot SF Microfiltration Apparatus**
162-0133	**Molecular Biology Certified Agarose,** 100 g
162-0134	**Molecular Biology Certified Agarose,** 500 g
161-0733	**10x Tris/Boric Acid/EDTA,** 1L
161-0301	**SDS (Sodium Dodecyl Sulfate),** 100 g
161-0302	**SDS (Sodium Dodecyl Sulfate),** 500 g
732-6000	**Bio-Spin 6 Columns,** 10
732-6004	**Bio-Spin 30 Columns,** 10
142-6425	**AG 501-X8 (D) Resin,** 500 g