Zeta-Probe® GT (Genomic Tested) Blotting Membranes

Instruction Manual

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Section 1
Introduction

Zeta-Probe GT blotting membranes are quaternary amine derivatized nylon membranes which have unique binding and handling properties that make them ideally suited for nucleic acid blotting applications. Zeta-Probe GT membranes possess a high tensile strength. They will not shrink, tear, or become brittle during transfer, baking, hybridization, or reprobing. Zeta-Probe GT membranes are heat resistant, non-flammable, and autoclavable. Zeta-Probe GT membranes are naturally hydrophilic with no added wetting agents. These membranes are resistant to a 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 GT membranes is 0.45 mm. When stored at 23–25 °C, Zeta-Probe GT membranes are stable for at least 1 year.

When handling Zeta-Probe GT 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 from one membrane to the other.

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

Section 2
Nucleic Acid Blotting Protocols

Several nucleic acid blotting methods are presented in this section. Capillary or vacuum blotting (Section 2.1, 2.2, 2.3, 2.4, and 2.5) is generally used with agarose gels, and electrophoretic blotting (Section 2.4) is used with polyacrylamide gels. Dot blotting (Section 2.7 and 2.8) is used for nucleic acids in solution. DNA alkaline blotting is an alternative to Southern blotting and has shown higher resolution and greater sensitivity in many applications (Alkaline Blotting, Section 2.3). RNA alkaline blotting reduces experimental time and is presented in Section 2.4. DNA alkaline fixation (Section 2.8) can be used to denature and fix DNA to Zeta-Probe GT 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 minutes (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 minutes at room temperature.

3. Neutralize the gel by bathing it in 0.5 M Tris-HCl, pH 7.4, 3 M NaCl for 30 minutes at room temperature on a moving platform. Cut five sheets of Whatman 3MM paper and one sheet of Zeta-Probe GT membrane to the exact size of the gel. Place the cut sheet of Zeta-Probe GT membranes into distilled water for 5 minutes before using.

4. Place a sponge larger than the gel in the bottom of a deep dish or tub (two smaller sponges can be aligned together snugly without sacrificing transfer efficiency). Some commercial sponges have been presoaked in detergent and should be washed well before using. Place enough 10x SSC buffer in the transfer dish to immerse the sponge about half way up its side.

5. Place three sheets of the pre-cut 3MM Whatman paper on the sponge. Flood the paper surface with buffer and place the gel on it. Remove any bubbles from beneath the gel by pressing them away to the sides.

6. Spread plastic wrap over gel/3MM/sponge stack. Cut out a window with a new razor blade, allowing only the gel to emerge from the window. This procedure will insure capillary action only through the gel.

7. Flood the surface of the gel with buffer and place the pre-wetted Zeta-Probe GT membrane on it. Carefully remove bubbles from underneath the blotting membrane, as they will block transfer. To avoid trapping bubbles, place the Zeta-Probe GT membrane onto the gel surface by first bowing the membrane diagonally and aligning the opposite corners with the gel corners. Then lower the membrane onto the gel. Remove any bubbles from beneath the membrane by pressing them away to the side.

8. Flood the blotting membrane surface with buffer. Wet the two remaining pre-cut sheets of Whatman paper and place them on top of the blotting membrane. Align them carefully so that they completely cover the blotting membrane and do not touch the gel directly. Remove any bubbles from beneath each layer of filter paper.

9. Carefully place paper towels over the Whatman paper. Stack paper towels about 15 cm high.

10. 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.

11. Keep an excess of buffer in the dish, but do not cover the top of the sponge. Continue transferring for 2–24 hours, depending on the gel concentration and fragment size.

12. After transfer, separate the membrane from the gel, rinse the membrane briefly in 2x SSC, and air dry the membrane. Dry the blotted Zeta-Probe GT membrane at 80 °C for 30 minutes or crosslink the DNA to the membrane in a GS Gene Linker. The dried membranes are stable at room temperature. The membrane can be stored dry between two filter papers in plastic bags at 23–25 °C.

2.2 Northern Blotting (RNA Capillary Transfer)

Follow Southern Blotting protocol (Section 2.1), omitting steps 1–3. If large RNA doesn't transfer, treat the gel with 10 mM NaOH for 10–15 minutes to facilitate transfer to the membrane. In this case, continue on with Step 3 of Section 2.1.

2.3 Alkaline Blotting[3] (DNA Capillary Transfer)

1. Depurinate the DNA by soaking the gel in 0.25 M HCl for 10–15 minutes.

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

2. Cut four sheets of Whatman 3MM paper so that they overhang the bottom of the gel tray by 5 cm on each end. Pre-wet Zeta-Probe GT membrane in distilled water for 5 minutes.

3. Place the four sheets of Whatman 3MM paper on an inverted gel casting tray. Place the 3MM/tray in the bottom of a deep dish. Then saturate the 3MM with 0.4 M NaOH. Remove bubbles by repeatedly rolling a test tube over the saturated 3MM. 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 onto the saturated filter paper. Make sure that no bubbles are trapped beneath the gel. Cover the gel with a small amount of NaOH.

5. Place plastic wrap over the entire gel/3MM stack. Cut a window out with a new razor blade, allowing only the gel to emerge.

6. Lower the sheet of pre-wetted Zeta-Probe GT 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 gel and membrane.

7. Cut two pieces of 3MM exactly to the gel size. Wet a sheet of pre-cut 3MM paper in water and place it onto the Zeta-Probe GT membrane/gel stack, then repeat with the second sheet. Remove any bubbles from beneath each layer of filter paper.

8. Place a stack of pre-cut paper towels on the 3MM/membrane/gel stack. Cover the paper towel stack with a 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.

10. After transfer, separate the membrane from the gel, rinse the membrane briefly in 2x SSC, and air dry the membrane. Dry the blotted Zeta-Probe GT membrane at 80 °C for 30 minutes or crosslink the DNA to the membrane in a GS Gene Linker. The dried membranes are stable at room temperature. The membranes can be stored dry between two filter papers in plastic bags at 23–25 °C.

2.4 Alkaline Northern Blotting (RNA Capillary Transfer)

The following protocol uses a mild alkali (NaOH) as an RNA transfer solvent. This low NaOH concentration will cause limited hydrolysis of RNA which permits quantitative elution of large RNA molecules. This protocol is not recommended for RNA below 5 kb. The NaOH also removes glyoxal from RNA and insures single stranded RNA binding.

1. Glyoxal gel—follow instructions for DNA Alkaline Blotting (Section 2.3) except:
    a. Omit step 1.
    b. Use 10 mM NaOH instead of 0.4 M NaOH as a transfer solvent in steps 3–6.
    c. Transfer for 3–8 hours in step 9. Less time is better to minimize hydrolysis.

2. Formaldehyde gel—follow instruction for DNA Alkaline Blotting (Section 2.3) except:
    a. Omit step 1.
    b. Use 50 mM NaOH instead of 0.4 M NaOH as a transfer solvent in steps 3–6.
    c. Transfer for 3–8 hours in step 9. Less time is better to minimize hydrolysis.

2.5 Vacuum Blotting (RNA or DNA)

1. Set up the Model 785 Vacuum Blotter according to the manual.

2. Cut a Zeta-Probe GT membrane 0.5 cm bigger than each border of the precut window on the Window Gasket. Cut a sheet of filter paper the same size as the membrane.

Note: The larger the membrane/filter paper, the easier it is to center the Window Gasket on top of the membrane.

3. When cutting a customized window from the blank Window Gasket, make sure the window's dimensions are at least 0.5 cm smaller than the gel's dimensions all around, *i.e.* if the gel is 15 x 10 cm, then the maximum window size should be 14 x 9 cm.

4. Fill the wells of the agarose gel with melted agarose gel of equal concentration. Allow agarose to dry before continuing.

5. There are two transfer procedures listed, the Standard Transfer Procedure and the Rapid Transfer Procedure. The Standard Transfer Procedure is for detecting a single copy gene in genomic DNA. On the other hand, the Rapid Transfer Procedure is for fast identification of DNA inserts from various cloned vectors. For RNA transfers, follow the procedure outlined in RNA Transfer Procedure.

Standard Transfer Procedure:

1. Depurinate the gel in 0.25 N HCl for 5–15 minutes in a tray. Cover the gel with 0.25 N HCl and shake gently.
2. Remove the 0.25 N HCl solution. Rinse the gel twice with deionized distilled water.
3. Denature the gel in 0.5 N NaOH for 30 minutes. Cover the gel with 0.5 N NaOH and shake gently.
4. Transfer the gel in 10x SSC for 90 minutes at 5 inches Hg. Skip to step 6.

Rapid Transfer Procedure:

1. Depurinate the gel in 0.25 N HCl for 5–15 minutes in a tray. Cover the gel with 0.25 N HCl and shake gently.
2. Remove the 0.25 N HCl solution. Rinse the gel twice with deionized distilled water.
3. Immediately transfer in 0.5 N NaOH + 0.6 N NaCl for 90 minutes at 5 inches Hg. Skip to step 6.

RNA Transfer Procedure:

1. After electrophoresis, rinse the RNA gel in deionized distilled water.
2. Soak the RNA gel in 50 mM NaOH for 5–15 minutes depending on fragment size.

3. Transfer in 10x SSC for 90 minutes at 5 inches of Hg. Continue with Step 6.
6. Wet the pre-cut Zeta-Probe GT membrane in double distilled water by slowly lowering the membrane at a 45 degree angle to the water. Then, wet the membrane and the filter paper in the appropriate transfer solution from the section above.
7. Make sure that the Porous Vacuum Plate lays flush on the Vacuum Stage. Place the wetted filter paper on the Porous Vacuum Plate. Lay the filter paper in the area where the cut window of the Window Gasket will be. Place the wetted membrane on top the filter paper. Remove any air bubbles by rolling a 10 ml glass pipet over the membrane.
8. Wet the Reservoir Seal O-ring with water.
9. Put the Window Gasket on top of the membrane/filter paper. Make sure the Window Gasket covers the entire O-ring on the Vacuum Stage. At the same time, make sure the membrane/filter paper is overlapping the Window Gasket. Realign as necessary.

Alternatively, place the Window Gasket on the Porous Vacuum Plate first. Adjust the Window Gasket to cover the entire O-ring. Then, hold one end of the Window Gasket and slowly peel the other end back until the window area is up off the Porous Vacuum Plate. Place the wet membrane/filter paper on the Porous Vacuum Plate under the window area and under the

Window Gasket. Finally, replace the partially peeled back Window Gasket over the membrane/filter paper. Make sure the Window Gasket covers the entire O-ring and overlaps the membrane/filter paper on all sides.

10. Gently place the gel, well side up, on top of the Window Gasket. The gel must overlap the window. Remove air bubbles by using a 10 ml glass pipet. As a final check, make sure the gel edges overlap the Window Gasket by at least 5 mm.

11. Place the Sealing Frame on top of the Vacuum Stage. Lock the Sealing Frame onto the four latch posts. The spring latch handle of the Sealing Frame has a precut region in the middle. Push down on this exposed area of the Sealing Frame with your thumb until the latches snap onto all four latch posts.

12. Unscrew (counterclockwise) the Vacuum Regulator bleed valve several turns to prevent strong initial vacuum.

13. Start the vacuum source and slowly turn the bleed valve clockwise until the gauge reads 5 inches of Hg. If Bio-Rad's Vacuum Pump is used, prewarm the pump for 10 minutes before blotting. Without prewarming, the vacuum pump will slowly increase the pressure and then stabilize after 10 minutes. Adjust the pressure to 5 inches of Hg.

14. With a finger, apply gently pressure on top of the gel along the window border. This pressure helps to form a tight vacuum seal between the gel and the Window Gasket.

15. Gently pour 1,000–1,500 ml of the appropriate transfer solution (refer to step 5) into the upper reservoir. Check to see if the gel is displaced. The gel should be stuck to the Window Gasket. If the gel floats, disassemble the Sealing Frame to drain the transfer solution and repeat step 10 to 15 again.

16. Place the lid on the Vacuum Blotter. Occasionally, check the buffer level. It should be higher then the gel. Check the vacuum pressure and adjust the to 5 inches of Hg as needed.

17. Transfer the gel for 90 minutes at 5 inches of Hg.

18. After transfer, disassemble the Vacuum Blotter and remove the Zeta-Probe GT membrane. Rinse the membrane briefly in 2x SSC, and air dry the membrane. Dry the blotted Zeta-Probe GT membrane at 80 °C for 30 minutes or crosslink the DNA to the membrane in a GS Gene Linker. The dried membranes are stable at room temperature. The membranes can be stored dry between two filter papers in plastic bags at 23–25 °C.

2.6 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 the 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 minutes prior to electrophoretic transfer.

    b. Electrophoresis Under Non-Denaturing Conditions

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

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

After base treatment, neutralize the gel by washing in 5x transfer buffer two times, 10 minutes each. Then wash the gel once in 0.5x transfer buffer for 10 minutes.

3. While gels are being equilibrated, soak the Zeta-Probe GT membrane at least 1 minute in 0.5x transfer buffer.

4. Fill the electrophoretic transfer cell half full of 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 stirring bar. Circulate buffer in the cell by stirring to maintain temperature during the run.

5. Prepare Transfer Assembly.

Soak one fiber pad by squeezing it while it is submerged in 0.5x transfer buffer. Lay the soaked pad on the opened 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 lay it on the fiber pad. Place the gel on the filter paper. Hold the presoaked membrane with both hands so that the middle of the Zeta-Probe GT 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 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 2x SSC, and air dry the membrane. Dry the blotted Zeta-Probe GT membrane at 80 °C for 30 minutes or crosslink the DNA to the membrane in a GS Gene Linker. The dried membranes are stable at room temperature. The membrane can be stored dry between two filter papers in plastic bags at 23–25 °C.

2.7 DNA Dot/Slot Blotting

When Zeta-Probe GT 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 (covalent circles, dsDNA, ssDNA), whole blood, tissue, or cultured cells, it can simply be heated in alkali then filtered directly onto Zeta-Probe GT 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 minutes.[17] The sample may be purified or crude DNA (≤ 5 mg), whole soft tissue, *e.g.*, liver (≤ 0.5 mg), whole blood (≤ 10 ml), or cultured cells (≤ 10^5 cells).

2. Wet a sheet of Zeta-Probe GT membrane by immersing it in distilled water.

3. Assemble the microfiltration apparatus with a pre-wetted Zeta-Probe GT membrane. Make sure that all the screws or clamps have been tightened under vacuum to insure no cross well contamination.

4. Rinse wells with 0.5 ml TE or H_2O. Apply vacuum until wells are empty but not dry.

5. Apply 0.5 ml DNA samples into appropriate wells without vacuum. Apply 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. Without disconnecting the vacuum, disassemble the apparatus and remove the membrane. Then rinse the membrane briefly in 2x SSC and allow it to air dry. Dry the blotted Zeta-Probe GT membrane at 80 °C for 30 minutes or crosslink the DNA to the membrane in a GS Gene Linker. The dried membranes are stable at room temperature. The membranes can be stored dry between two filter papers in plastic bags at 23–25 °C.

2.8 RNA Dot/Slot Blotting

Both native and denatured RNA are retained quantitatively by Zeta-Probe GT membrane. However to insure optimal hybridization, RNA samples must be totally denatured before fixing onto the Zeta-Probe GT membrane. Two protocols are presented for denaturing RNA samples. The first protocol uses a mild alkali for denaturing and fixing the RNA sample. The second protocol uses glyoxal for denaturation.

Alkaline RNA Denaturation and Fixation

1. Immediately before use, dissolve the RNA samples in 0.5 ml total volume of ice-cold 10 mM NaOH, 1 mM EDTA final concentrations.

2. Assemble microfiltration apparatus with a sheet of Zeta-Probe GT membrane pre-wetted in distilled water. Make sure that all the screws or clamps have been tightened under vacuum to insure no cross well contamination.

3. Rinse wells with water or TE, apply vacuum until wells are just dry.

4. Apply 0.5 ml samples to wells without vacuum.

5. Apply vacuum until wells are just dry, then release the vacuum.

6. Add 0.5 ml of cold 10 mM NaOH, 1 mM EDTA to each well and then apply vacuum until the wells are dry.

7. With vacuum on, disassemble and remove the membrane.

8. Immediately rinse the membrane in 2x SSC, 0.1% SDS and blot lightly. Dry the blotted Zeta-Probe GT membrane at 80 °C for 30 minutes or crosslink the DNA to the membrane in a GS Gene Linker. The dried membranes are stable at room temperature. The membranes can be stored dry between two filter papers in plastic bags at 23–25 °C.

Glyoxal RNA Denaturation and Fixation

1. Add RNA sample to the following final concentrations:
   50% dimethyl sulfoxide (DMSO)
   10 mM sodium phosphate, pH 7.2
   1 M glyoxal
2. Incubate sample for 1 hour at 50 °C. Then cool the RNA sample on ice.
3. Wet a sheet of Zeta-Probe GT membrane by immersing it in distilled water.
4. Assemble the microfiltration apparatus with prewetted Zeta-Probe GT membrane. Make sure that all the screws or clamps have been tightened under vacuum to insure no cross well contamination.
5. Rinse wells with TE or water, apply vacuum until just dry.
6. Apply 0.5 ml RNA samples to appropriate wells without vacuum.

7. Apply vacuum until wells are just dry, then release vacuum.
8. Rinse all wells with 0.5 ml TE, applying vacuum until completely dry.
9. Disconnect the vacuum. Remove blotted Zeta-Probe GT membrane.
10. Rinse the membrane briefly in 2x SSC, and air dry the membrane. Dry the blotted Zeta-Probe GT membrane at 80 °C for 30 minutes or crosslink the DNA to the membrane in a GS Gene Linker. The dried membranes are stable at room temperature. The membranes can be stored dry between two filter papers in plastic bags at 23–25 °C.

Alternatively, place the membrane in a vacuum oven at 80 °C for 1 hour (omit the above step 9).

2.9 DNA Alkaline Fixation

After transfer, place the Zeta-Probe GT membrane (DNA surface uppermost) on a pad of filter paper saturated with 0.4 M NaOH for 10 minutes. Rinse in 2x SSC and air dry. The dried membranes are stable at room temperature. Crosslinking is not necessary when nucleic acids are applied under alkaline conditions.

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 GT blotting membranes the following is recommended:

Probe specific activity	10^8 cpm/µg probe
Probe concentration in the hybridization mixture	10^6 cpm/ml (10–15 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 hybridization. DNA probes prepared by nick translation are generally long. Probe fragments longer than 1 kb decrease hybridization specifically.

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 clean-up 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 bead volume using Bio-Gel® P-30 gel (catalog number 150-1340) or with Bio-Spin® 30 columns (catalog number 732-6004).

After clean-up, denature double stranded probes by increasing temperature to 95–100 °C for 5 minutes. Then cool rapidly in ice. Use the probe as soon as possible after preparation.

Section 4
Hybridization Protocols

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 GT membrane. The most important blocking reagent in the hybridization solution is sodium dodecyl sulfate (SDS). SDS is the most effective blocking reagent when used at concentration ≥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. Oligonucleotide probes can be used with the Standard Protocol (Section 4.1). Just cal-

culate the proper temperature with the following formulas. The Alternative Protocol (Section 4.3) should be used only when extreme sensitivity is needed.

The final volume of hybridization solution is also important in reducing background. During hybridization, use 70–150 µl solution/cm^2 Zeta-Probe GT membrane and use at least 2 ml solution /cm^2 Zeta-Probe GT membrane for washes.

One of the most significant advantages offered by Zeta-Probe GT 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.

Hybridization 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 - 820/L. [12]

T_m(DNA/RNA) = approximate 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 (T_H) appropriate to synthetic oligomeric DNA probes below 21 bases 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

This hybridization protocol can be used with DNA/DNA, DNA/RNA, and RNA/RNA hybrids. The Standard Protocol can also be used with oligonucleotide probes by adjusting the T_H from the previous section.

Prehybridization:

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

   0.25 M sodium phosphate, pH 7.2

   7% SDS

3. Seal the bag and incubate briefly at 65 °C for 5–30 minutes. The goal is simply to coat the membrane evenly and completely with this solution.

Hybridization:

1. Cut one corner of the plastic bag, remove the prehybridization solution, and replace it with same buffer.
2. Add denatured probe and remove all air 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 the hybridized Zeta-Probe GT membrane from the plastic bag.

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

Washes:

1. Wash membrane at 65 °C, 2 times, for 30–60 minutes each, in the following:

   20 mM sodium phosphate, pH 7.2

   5% SDS

2. Wash membrane at 65 °C, 2 times, for 30-60 minutes each, in the following:

   20 mM sodium phosphate, pH 7.2

   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, then 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 GT membrane will stick to the film.

4.2 Formamide Protocol

Prehybridization:

1. Seal blotted Zeta-Probe GT membrane inside a heat sealable plastic bag. Combine these reagents in the order given. For 100 ml:

   50 ml formamide

   25 ml 0.5 M sodium phosphate, pH 7.2

   15 ml H_2O

   1.46 g NaCl

   7.0 g SDS

   Bring to 100 ml with H_2O. If precipitation occurs, heat the solution as necessary.

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

3. Incubate at 43 °C for 5 minutes. The goal is simply to coat the membrane evenly and completely with this solution.

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 hours with agitation.

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 into 2x SSC. Rinse briefly, then wash them successively by vigorous agitation at room temperature for 15 minutes 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 hybridization. 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 GT membrane will stick to the film.

4.3 Alternative Protocol

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

Polyethylene glycol (PEG)[15,16]—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. For example, 1 g of PEG 8,000 plus hybridization solution to 10 mls. Conduct post hybridization washes the same as in Section 4.1 or 4.2, without PEG.

Section 5
Probe Stripping And Rehybridization

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

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

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

Check membrane by overnight exposure.

DNA Blots Only

Alternatively, soak a pad of filter papers in 0.4 N NaOH. Place the membrane DNA side up onto the soaked filter papers for 5 minutes. Rinse in 2x SSC. Repeat.

Check membrane by overnight exposure.

Section 6
Zeta-Probe GT Membrane Troubleshooting Guide

Problem	Solution
1. Fragments greater than ~1,000 bp can not 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 those not denatured prior to and during electrophoresis will transfer completely up to at least 2,000 bp. Solve this problem in any of three ways: 1. Omit pretreatment, and transfer dsDNA. Alkaline fix (Section 2.8) 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 hour at 50 °C. Then transfer directly.

Problem	Solution
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 minutes.
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. Proper blocking of the membrane is very important, use ≥1% SDS. We recommend 7% SDS. Refer to hybridization protocols. 1. Use a desalting gel column to remove unincorporated label, or the Bio-Spin 30 columns. 2. Use the probe as soon as possible after preparation, since decay results in fragmentation. 3. Use a different heterologous nucleic acid in the prehybridization and hybridization mixtures. Sonicate it thoroughly and denature it before use.

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 plastic bag prior to hybridization. 3. Be sure no bubbles exist in hybridization bag.
5. Lane background or extra bands.	1. Contaminated template. Make sure the probe is synthesized with the pure template of choice. Sequence the probe; if the probe contains a repeat sequence add carrier DNA/RNA.

Problem	Solution
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% PEG in the hybridization mixture. This polymer effectively reduces the solvent volume, thereby increasing the concentration of the solutes and enhancing hybridization. 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.
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. 2. Be sure probe is denatured by boiling or heating to 65 °C for 5 minutes in 50% formamide prior to hybridization.

Section 7
Appendix

20x TAE	MW	g/l
800 mM Tris Base	121.1	96.9
400 mM Na 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
0.3 M trisodium citrate	294.1	88.2

20x SSPE	MW	g/l
3.6 M NaCl	58.44	210
0.2 M Na_2HPO_4 7 H_2O	268.07	53.6
0.02 M EDTA	372.2	7.44

TE

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

20% SDS	MW	g/l
20% Sodium dodecyl sulfate	288.38	200
Heat to 65 °C to get into solution		

0.5 M sodium phosphate, pH 7.2	FW	g/l
Stock A:		
0.5 M $NaH_2PO_4 \cdot H_2O$	138.0	69
Stock B:		
0.5 M $Na_2HPO_4 \cdot 7H_2O$	268.1	134
Combine 316 ml of Stock A with 684 ml Stock B to make 1 liter of 0.5 M sodium phosphate pH 7.2. Refer to reference 18.		

50% Formamide	g/100 ml
50% formamide	50

Store at 4 °C. Immediately before use, deionize the required volume by stirring gently for 1 hour with 1 g mixed-bed ion exchange resin (AG® 501-X8 (D) resin, catalog number 142-6425)/ 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 resin, catalog number 142-6425). Store at -20 °C in small aliquots. If the aliquot is exposed to the air, it cannot be reused.

Ordering information

Catalog Number	Product Description
162-0190	**Zeta-Probe GT Membrane,** 9 x 12 cm, 15 sheets
162-0191	**Zeta-Probe GT Membrane,** 10 x 15 cm, 15 sheets
162-0192	**Zeta-Probe GT Membrane,** 15 x 15 cm, 15 sheets
162-0193	**Zeta-Probe GT Membrane,** 15 x 20 cm, 15 sheets
162-0194	**Zeta-Probe GT Membrane,** 20 x 20 cm, 15 sheets
162-0195	**Zeta-Probe GT Membrane,** 20 x 25 cm, 15 sheets
162-0196	**Zeta-Probe GT Membrane,** 30 cm x 3.3 m roll
162-0197	**Zeta-Probe GT Membrane,** 20 cm x 3.3 m roll
165-5000	**Model 785 Vacuum Blotter with Regulator**
170-3910	**Trans-Blot Electrophoretic Transfer Cell**
165-5052	**PowerPac 200 Power Supply,** 100/120 V
170-6545	**Bio-Dot® Microfiltration Apparatus**
170-6542	**Bio-Dot SF Microfiltration Apparatus**

Catalog Number	Product Description
142-6425	**AG 501-X8 (D) Mixed Bed Ion Exchange Resin**
150-1340	**Bio-Gel P-30 Fine Mesh Filtration Gel**
732-6004	**Bio-Spin 30 Columns**

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