BD™ MTE Multiple Tissue Expression Array User Manual

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I. Introduction

The BD™ Multiple Tissue Expression (MTE) Array is a positively charged nylon membrane to which poly A^+ RNAs from different human tissues and cancer cell lines have been normalized and immobilized in separate dots, along with several controls. The MTE Array provides a fast, easy method for obtaining accurate information about the expression of a gene of interest across >70 human tissues. This method is particularly useful for determining tissue or developmental-stage specificity of gene expression, and also yields information about relative levels of mRNA abundance. Exact tissue representation may vary from lot to lot based on tissue availability.

The BD MTE Array is prepared using BD Biosciences Clontech Premium Poly A^+ RNA, which guarantees the presence of full length, rare transcripts, and virtually no genomic DNA. Although the MTE Array can be made from total RNA, we use Poly A^+ RNA to increase the sensitivity of detection approximately 50 fold and reduce nonspecific background. Detailed source information for each poly A^+ RNA sample, along with the amount of each sample applied to the MTE Array, is provided on the Product Analysis Certificate. Each array is shipped with a sample size of BD ExpressHyb™ Hybridization Solution and a ubiquitin positive control cDNA probe. If you follow the protocol provided, you can strip and reprobe each MTE Array up to four times without any significant loss of sensitivity.

Quantification of gene expression

The MTE Array allows you to determine the relative expression levels of a target mRNA in different tissues and developmental stages (Spanakis & Brouty-Boyé, 1994; Spanakis, 1993; Liew *et al.*, 1994). To this end, poly A^+ RNA samples on each MTE Array have been normalized to the mRNA expression levels of eight different "housekeeping" genes. Typically, poly A^+ RNA arrays are normalized to only one housekeeping gene; normalization to eight genes minimizes the small tissue-specific variations in expression of any single housekeeping gene. Correct normalization produces a fairly consistent hybridization signal from all samples when the array is probed with a constitutively transcribed housekeeping gene.

Figure 1 illustrates the high quality of data provided by each MTE Array. Figure 1A shows the fairly consistent hybridization signal obtained with the Human Ubiquitin cDNA Control Probe, one of the housekeeping cDNA probes used in the normalization process for the MTE Array. Panel B shows the hybridization signal from the liver-specific cDNA probe encoding tyrosine aminotransferase (TAT). TAT is expressed in only fetal and adult liver tissue. Because MTE Arrays are normalized using eight different housekeeping genes, the results seen in Figure 1 can be attributed to actual differences in target mRNA abundance, rather than differences in amounts loaded. (Our procedure for normalizing the loading of poly A^+ RNA on the MTE Array is explained in Appendix C). Our detailed normalization process ensures an accurate assessment of mRNA abundance in different tissues.

I. Introduction *continued*

Because mRNA expression can vary greatly between tissues, we recommend that you expose arrays to x-ray film or phosphorimaging screens for varying lengths of time before drawing any conclusions about the presence or absence of transcripts. Direct quantification of the hybridization signal (e.g., with a phosphorimager) provides the most accurate estimate of relative transcript abundance.

Options for Labeling and Detection

MTE Arrays are compatible with both radioactive and nonradioactive labeling and detection methods. Appendices A and B provide protocols for synthesizing radiolabeled oligo nucleotide and cDNA probes, respectively.

For nonradioactive detection using biotinylated probes, we recommend our BD SpotLight™ Chemiluminescent Labeling and Detection System. The BD SpotLight kits are specially formulated to eliminate problems with low sensitivity and high background that plague other nonradioactive methods. A key component of this system is the BD SpotHyb™ Hybridization Buffer which is optimized to minimize nonspecific hybridization. Furthermore, SpotLight employs the strong and specific affinity of the HRP-conjugated streptavidin for the biotinylated probe to further reduce background problems during detection.

The BD SpotLight™ Random Primer Labeling Kit (Cat. No. 634801) provides all the necessary reagents and protocol for synthesizing and purifying biotinylated cDNA probes. Hybridization of SpotLight-generated probes to MTE Arrays should be performed with the BD SpotLight™ Chemiluminescent Hybridization & Detection Kit (Cat. No. 634802), which contains all the necessary components for performing hybridization and chemiluminescent signal detection.

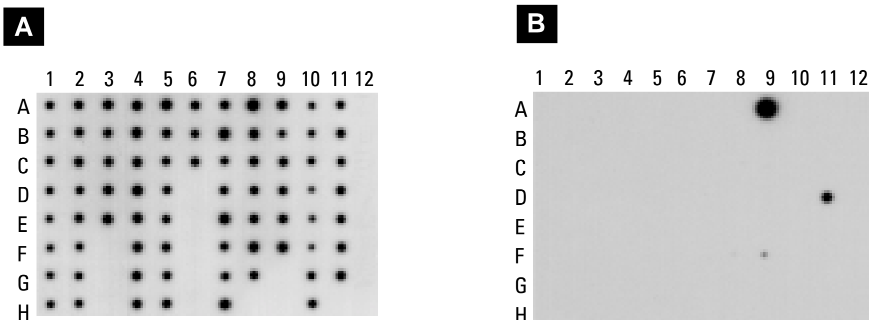

Figure 1. Sample MTE Array data. Panel A. Hybridization with the Human Ubiquitin Control cDNA Probe. Because this array was hybridized with a probe for a housekeeping gene, the hybridization signal is fairly consistent for all samples. **Panel B.** Hybridization with a cDNA probe encoding tyrosine aminotransferase (TAT). These are hybridization signals of a gene whose mRNA expression is limited to human adult and fetal liver. **All Panels.** The amount of poly A^+ RNA applied to each 1-mm dot was 53–780 ng and was normalized as described in Appendix C. Arrays were hybridized overnight with radioactively randomly primed cDNA probes using BD ExpressHyb Hybridization Solution. Arrays were washed with 2X SSC, 1% SDS at 65°C, and 0.1X SSC, 0.5% SDS at 55°C, and were exposed to x-ray film.

I. Introduction *continued*

Sensitivity of mRNA detection

In some instances, a gene is expressed at very low levels, even in the tissue from which the cDNA for the gene was originally isolated. Detection of rare transcripts on an MTE Array depends on transcript size, the specific activity of the probe, and the level of nonspecific background, but is superior to what can be achieved on a standard Northern blot.

To detect rare transcripts, it is important to minimize nonspecific background hybridization. Each MTE Array is shipped with BD™ ExpressHyb Hybridization Solution, an optimized, low-viscosity hybridization solution which significantly reduces hybridization time and background (Yang & Kain, 1995). The MTE Array and BD ExpressHyb Solution can be used with both radioactive and nonradioactive detection methods. Because the MTE Array protocol has been optimized with BD ExpressHyb Hybridization Solution, we recommend that you do not substitute your own hybridization solution, unless you are using the SpotLight system.

Positive and negative controls

Each MTE Array is shipped with the positive Human Ubiquitin Control cDNA Probe. Due to the strong signal generated with this probe (e.g., Figure 1A), we suggest that you use it *after* performing your experimental hybridization.

Several useful negative controls are applied to each MTE Array; none of these should hybridize to gene-specific probes. Hybridization of your probe to the dots containing *E. coli* rRNA or DNA, synthetic poly r(A), yeast tRNA or total RNA, or C_ot-1 DNA (representing the most abundant repetitive sequences) indicates the presence of undesirable sequences in the probe which may mask gene-specific hybridization. For more information on these controls, see Interpretation of Hybridization Results (Section VII.D).

BD MTE™ Arrays and BD MTN™ Blots

The MTE Array enables you to screen for the presence and relative abundance of a target mRNA in a broad spectrum of fetal and adult tissues; however, it does not reveal mRNA transcript size. Because poly A^+ RNA is not size fractionated on an MTE Array, hybridization of the probe to polymorphic forms of mRNA cannot be distinguished. For instance, if the target RNA belongs to a multi-gene family, the hybridization signal from the dots will reflect the pooled signal from all of these transcripts that hybridize to the probe. Multiple transcripts can also be generated from a single gene through alternative splicing and varied transcription initiation or polyadenylation sites. To distinguish between mRNAs of different sizes, we suggest that you use our BD™ MTN Multiple Tissue Northern Blots. Nevertheless, the MTE Array provides a fast way to simultaneously compare the relative abundance of a target mRNA in a wide array of tissues. Furthermore, because hybridization is confined to 1-mm dots, the MTE Array gives more accurate quantitative data (see Appendix C).

II. List of Components

Store unused arrays at –20°C, **sealed in a plastic bag** away from light. Store control probe at –20°C. Store BD ExpressHyb Solution at room temperature.

BD™ MTE Human Array 3 (Cat. No. 636903)

- 1 **BD™ MTE Human Array 3**
- 100 ng **Human Ubiquitin Control cDNA Probe** (5 ng/µl; sufficient for 5 radioactive probes)
- 25 ml **BD ExpressHyb™ Hybridization Solution**

BD™ MTE Mouse Array (Cat. No. 636827)

- 1 **BD™ MTE Mouse Array**
- 100 ng **HPRT Control cDNA Probe** (5 ng/µl; sufficient for 5 radioactive probes)
- 25 ml **BD ExpressHyb™ Hybridization Solution**

III. Additional Materials Required

The following materials are required but not supplied:

- 10 mg/ml **Sheared salmon testes DNA** (Sigma, Cat. No. D7656)
- 1 mg/ml **C_ot-1 DNA** (e.g., Boehringer Mannheim, Cat. No. 1 581 074)
- **20X SSC**

  3 M NaCl 175.3 g
  0.3 M Na_3Citrate•$2H_2O$ 88.2 g

  Adjust pH to 7.0 with 1 M HCl. Add H_2O to 1 L. Store at room temperature.
- **20% SDS**

  SDS 200 g

  Add H_2O to 1 L. Heat to 65°C to dissolve. Store at room temperature.
- **Wash solution 1**

  2X SSC
  1% SDS

  Store at room temperature.
- **Wash solution 2**

  0.1X SSC
  0.5% SDS

  Store at room temperature.

IV. General Considerations for Using the BD™ MTE Array

- **Never allow the BD™ MTE Array to dry, even slightly.** After your final wash, shake off excess solution with forceps (do not blot-dry), and immediately wrap the array completely with plastic wrap. When reprobing, unwrap the array and immediately place in the stripping solution; avoid prolonged exposure of the array to air. Always store MTE Arrays at –20°C when not in use. When unstripped arrays are stored at room temperature, degraded probe fragments hybridize to the membrane, resulting in a high level of nonspecific background that cannot be removed by stripping. Always strip the array immediately after you have developed your x-ray film or analyzed your phosphorimaging results.

- Each MTE Array may be stripped and reprobed several times if used according to these procedures; however, the strength of the hybridization signal may decrease at each reprobing.

- We recommend that you use the BD ExpressHyb Hybridization Solution shipped with each MTE Array, rather than substituting your own recipe. The recommended procedure is essential to generate a strong hybridization signal with low background.

- BD ExpressHyb Solution may be stored at room temperature. However, when the temperature drops below 25°C, a precipitate may form. (This often occurs during shipping.) To completely dissove any precipitate, warm the solution at 50–60°C and stir thoroughly. Avoid foaming by placing a stir-bar into the bottle and stirring slowly on a magnetic plate.

- The MTE Array can be used with either radioactive or nonradioactive detection methods. The procedure in this User Manual is for radioactive detection. Hybridization of biotin labeled cDNA probes to the MTE Array should be performed with the BD SpotLight™ Chemiluminescent Hybridization & Detection Kit (Cat. No. 634802), which contains all the necessary components for performing hybridization and chemiluminescent signal detection.

- Radioactively labeled probes should have a high specific activity, especially for detecting low-abundance mRNAs. For oligonucleotide probes, follow the procedure for 5'-end labeling described in Appendix A. For cDNA probes, follow the procedure described in Appendix B.

- For best results, remove unincorporated nucleotides from your hybridization probe by chromatography or gel electrophoresis. We typically use BD CHROMA SPIN™ 30 Columns (Cat. No. 636058) to purify oligonucleotides that are ≥20 bases. We recommend BD CHROMA SPIN™ 100 Columns (Cat. No. 636060) to remove unincorporated nucleotides and small probe fragments (<50 nucleotides) from cDNA probe preparations. (For additional information, please refer to Appendices A and B.)

IV. General Considerations...*continued*

- Do not add concentrated probe directly to the membrane; uneven initial concentrations may result in anomalous signals.
- Continuous agitation of the array is necessary during all prehybridization, hybridization, and washing steps. For best results and to reduce risk of radioactive contamination, we recommend the use of a hybridization incubator with rotating bottles. If using a hybridization incubator, rotate bottles continuously at 3–5 rpm for all steps that call for continuous agitation. Alternatively, prehybridization, hybridization, and washing steps may be performed in a sealed plastic bag or small plastic container with a tight-fitting lid, placed in a shaking incubator.

V. Using Oligonucleotide Probes with the BD™ MTE Array

A. Hybridization of Oligonucleotide Probes

1. Estimate the T_m of the probe using the appropriate formula (below). Synthetic oligonucleotide probes should be >22 residues with a T_m >60°C. However, for best results, we recommend using oligonucleotides 30–50 residues in length.

   (Assume that the T_m for DNA–RNA is the same as for DNA–DNA.)

   For oligonucleotides 14–30 nucleotides in length, under standard conditions of 1.0 M Na^+, the estimated T_m is:

   $$T_m = 4°C\ (G + C) + 2°C\ (A + T)$$

   For longer oligonucleotides:

   $$T_m = 81.5°C + 16.6\ (\log_{10} [Na^+]) + 0.41\ [(\frac{G + C}{n})(100)] - \frac{500}{n}$$

   where n = the number of nucleotides and the concentration of Na^+ in 6X SSC (or 5X SSPE) is 1.0 M.
2. Prepare a solution of BD ExpressHyb Hybridization Solution and sheared salmon testes DNA.
   a. Prewarm 15 ml of BD ExpressHyb Solution at 50–60°C.
   b. Heat 1.5 mg of sheared salmon testes DNA at 95–100°C for 5 min, and then chill quickly on ice. (See Appendix A for recommendations on proble labeling procedures.)
   c. Mix heat-denatured sheared salmon testes DNA with prewarmed BD ExpressHyb Solution.
3. Place the MTE Array in a hybridization container and add 10 ml of the solution prepared in Step 2.
4. Prehybridize for 30 min with continuous agitation at a temperature 30°C below the estimated T_m, but not exceeding 60°C.

   Note: Do not remove the MTE Array from the container during prehybridization, hybridization, or washing steps.
5. Denature your labeled oligonucleotide probe by heating at 95–100°C for 5 min; then chill quickly on ice.
6. Add your denatured, labeled oligonucleotide probe to the remaining 5 ml of the solution prepared in Step 2. Make sure that the probe is thoroughly mixed into the solution.

   The final concentration of your radioactively labeled oligonucleotide probe should be 5–10 x 10^6 cpm/ml. Probe concentrations >2 x 10^7 cpm/ml will reduce the time needed for hybridization, but may increase background. (For additional information on 5'-end labeling of oligonucleotide probes, please refer to Appendix A.)
7. Pour out the prehybridization solution and discard. Replace with the remaining 5 ml of solution prepared in Step 6. If hybridizing in a plastic bag, remove all air bubbles and make sure BD ExpressHyb Solution is

V. Using Oligonucleotide Probes ...*continued*

evenly distributed over the entire array.

8. Hybridize for 4 hr (or overnight) with continuous agitation at the same temperature used for prehybridization (Step 4).
9. Carefully remove the hybridization solution and discard in an appropriate waste container.
   a. Replace with 200 ml of Wash solution 1.
   b. Wash the array for 20 min with continuous agitation at room temperature.
   c. Repeat steps a and b four times.
10. Wash the array once in 200 ml of Wash solution 1 for 20 min with continuous agitation at the hybridization temperature.
11. Using forceps, remove the MTE Array from the container and shake off excess wash solution. Do not blot-dry or allow the membrane to dry. If the membrane dries even partially, subsequent removal of the probe (stripping) from the array may be difficult.
12. Immediately wrap the damp array in plastic wrap.
13. Mount plastic-wrapped array on Whatman paper (3 mm Chr). To provide orientation after autoradiography, mark mounting paper at several points with dye that has been mixed with a small amount of radiolabeled probe. Expose the MTE Array to x-ray film at –70°C with an intensifying screen; be sure to try several exposures for varying lengths of time (e.g., 3 hr, 6 hr, overnight, and 3 days). Alternatively, use a phosphorimager. **If you plan to reuse the MTE Array, strip the probe from it *immediately* and store the array at –20°C until needed. Never store arrays at room temperature.**

B. Stripping Oligonucleotide Probes from BD™ MTE Arrays

To re-use an MTE Array after hybridization, remove the oligonucleotide probe by stripping.

1. Heat 150–250 ml of Wash solution 2 to boiling; remove from heat source.
2. Remove plastic wrap from the array and immediately place the array in the heated solution. Avoid prolonged exposure of the array to air.
3. Incubate for 10 min as the solution cools, shaking frequently.
4. Remove the array from the solution and check the efficiency of stripping with a Geiger hand counter or by exposure to x-ray film. If radioactivity can still be detected, repeat the stripping procedure (Steps 1–3).
5. Place the MTE Array in a hybridization container and proceed with the next hybridization experiment. Alternatively, the array can be stored in plastic wrap at –20°C until needed.

VI. Using cDNA Probes with the BD™ MTE Array

A. Hybridization of cDNA Probes

The protocol given below is for radiolabeled probes only. Hybridization of biotin labeled cDNA probes to the MTE Array should be performed with the BD SpotLight™ Chemiluminescent Hybridization & Detection Kit (Cat. No. 634802), which contains all the necessary components for performing hybridization and chemiluminescent signal detection.

1. Prepare a solution of BD ExpressHyb Solution and sheared salmon testes DNA.
   a. Prewarm 15 ml of BD ExpressHyb Solution at 50–60°C.
   b. Heat 1.5 mg of the sheared salmon testes DNA at 95–100°C for 5 min, and then chill quickly on ice.
   c. Mix heat-denatured sheared salmon testes DNA with prewarmed BD ExpressHyb Solution.
2. Place the MTE Array in a hybridization container and add 10 ml of the solution prepared in Step 1.
3. Prehybridize for 30 min with continuous agitation at 65°C.

   Note: Do not remove the MTE Array from the container during prehybridization, hybridization, or washing steps.
4. Mix your labeled cDNA probe with 30 μg of C_ot-1 DNA, 150 μg of sheared salmon testes DNA, and 50 μl of 20X SSC, in a total volume of 200 μl. (See Appendix B for a cDNA probe labeling procedure.)

   Note: For radioactively labeled cDNA probes, use ~20 ng, 10–20 x 10^6 cpm of probe. Specific activity should be >10^9 cpm/μg (Sambrook *et al.*, 1989). Probe concentrations >10 ng/ml will reduce the time needed for hybridization, but may increase background. (For additional information on random-primer labeling of cDNA probes, please refer to Appendix B.)
5. Heat the mixture prepared in Step 4 at 95–100°C for 5 min, and then at 68°C for 30 min.
6. Add the mixture prepared in Step 4 to the remaining 5 ml of solution prepared in Step 1. Make sure that the two solutions are mixed together thoroughly.
7. Pour out the prehybridization solution and discard. Replace with the solution prepared in Step 6. If hybridizing in a plastic bag, remove all air bubbles from container, and make sure BD ExpressHyb Solution is evenly distributed over the entire array.
8. Hybridize for 6 hr (or overnight) with continuous agitation at 65°C.
9. Carefully remove the hybridization solution and discard in an appropriate waste container.
   a. Replace with 200 ml of Wash solution 1.
   b. Wash the array for 20 min with continuous agitation at 65°C.
   c. Repeat steps a and b four times.

VI. Using cDNA Probes ...*continued*

10. Perform two additional 20-min washes in 200 ml of prewarmed Wash solution 2 with continuous agitation at 55°C.
11. Using forceps, remove the MTE Array from the container and shake off excess wash solution. Do not blot-dry or allow the membrane to dry. If the membrane dries even partially, subsequent removal of the probe (stripping) from the array may be difficult.
12. **Immediately wrap the damp array in plastic wrap.**
13. Mount plastic-wrapped array on Whatman paper (3 mm Chr). To provide orientation after autoradiography, mark mounting paper at several points with dye that has been mixed with a small amount of radiolabeled probe. Expose the MTE Array to x-ray film at –70°C with an intensifying screen; be sure to try several exposures for varying lengths of time (e.g., 3 hr, 6 hr, overnight, and 3 days). Alternatively, use a phosphorimager. **If you plan to reuse the MTE Array, strip the probe from it *immediately* and store the array at –20°C until needed. Never store the array at room temperature.**

B. Stripping cDNA Probes from BD™ MTE Arrays

To re-use an MTE Array after hybridization, remove the cDNA probe by stripping.

1. Heat 150–250 ml of 0.5% SDS solution to boiling.
2. Remove plastic wrap from the array and immediately place the array in the boiling solution. Avoid prolonged exposure of the array to air.
3. Continue to boil for 5–10 min.
4. Remove the solution from heat and allow to cool for 10 min.
5. Remove the array from the solution and check the efficiency of stripping with a Geiger hand counter or by exposure to x-ray film. If radioactivity can still be detected, repeat the stripping procedure (Steps 1–4).
6. Place the MTE Array in a hybridization container and proceed with the next hybridization experiment. Alternatively, the array can be stored in plastic wrap at –20°C until needed.

VII. Interpretation of Hybridization Results

A. mRNA Polymorphisms

Although the MTE Array provides an excellent tool for determining relative levels of gene expression, it cannot be used to distinguish closely related or polymorphic mRNAs of different sizes. There are several possible situations in which probes may hybridize to multiple mRNAs. If the target gene belongs to a multi-gene family, the probe may hybridize to several related genes. Similarly, the probe may hybridize to multiple individual mRNAs that also contain homologous sequences, such as conservative structural domains or repetitive sequences. In another possible scenario, multiple-sized transcripts of a single gene may be generated by alternative splicing, transcription initiation from alternative promoters, or polyadenylation at different termination sites. BD™ MTN Multiple Tissue Northern Blots can be used to study mRNA polymorphisms.

B. Linear Range of Quantification

Genes are rarely expressed exclusively in one tissue; rather, expression levels usually vary from tissue to tissue. For example, we have found that the MHC class Ic gene is expressed at high levels in tissues such as peripheral leukocytes and lymph node, lower levels in testis and skeletal muscle, and very low levels in brain tissue. In this case, a short exposure to x-ray film might indicate that MHC is expressed only in peripheral leukocytes and lymph node. This example illustrates the need to expose the MTE Array to x-ray film for varying lengths of time before drawing conclusions about tissue-specific expression. A phosphorimager allows direct quantification of hybridization signals over a linear range of 10^4–10^5 without multiple exposure times.

C. Sensitivity of Detection

Because each poly A^+ RNA sample is confined to a 1-mm dot, the sensitivity of an MTE Array is higher than that of a Northern Blot. Using random primer-labeled cDNA probes with high specific activities (>10^9 cpm/µg), we are typically able to detect expression of mRNAs at 0.01% of the total poly A^+ RNA abundance—a level that corresponds to approximately 100 target mRNA molecules per cell. Using oligonucleotide probes that have been 5'-end labeled with ^{32}P, we can typically detect mRNA expression at 0.1–0.01% of the total poly A^+ RNA abundance.

Some mRNAs are expressed at very low levels, even in the tissues from which the cDNA probe was originally derived. For the detection of rare mRNAs, always expose x-ray film at –70°C with an intensifying screen, and use fresh solutions for developing the film. Using a phosphorimager allows you to further increase sensitivity 3–5-fold. Alternatively, some x-ray film, such as Kodak BioMax MS film (Kodak, Cat. No. 118 8077, with the corresponding BioMax intensifying screen) may be nearly as sensitive as a phosphorimager for ^{32}P detection.

VII. Interpretation of Hybridization Results *continued*

If longer exposure times fail to generate a strong signal, try increasing the specific activity of the probe and exposing the array to x-ray film for several days. A cDNA probe will give greater sensitivity than an oligonucleotide. Sensitivity can also be increased by using ^{32}P-labeled RNA probes or single-stranded gene-specific cDNA probes (Sambrook *et al.*, 1989). Sensitivity can be further increased by reducing nonspecific background (see Troubleshooting Section VIII.B). This is especially important for rare mRNAs, where the hybridization signal may be comparable with the level of nonspecific background.

D. Hybridization Controls

Reducing the level of nonspecific background is crucial for achieving high sensitivity. For more information on general methods for reducing background, consult the Troubleshooting Guide (Section VIII.B).

1. Negative controls

   Each MTE Array contains several negative controls that can be used to estimate levels of nonspecific background.

   a. Yeast total RNA, yeast tRNA, *E. coli* rRNA, and *E. coli* DNA

      Hybridization to these four controls indicates nonspecific binding of your probe. If all four controls give a clearly detectable signal, you should increase the hybridization stringency or redesign your probe.

      In some cases, you may observe a signal for *E. coli* DNA, but not the other three controls. This may be explained by the presence of *E. coli* sequences that are homologous to mammalian mRNAs. If your probe hybridizes to the dot containing *E. coli* DNA, but not to yeast total RNA, yeast tRNA, or *E. coli* rRNA, you should verify that your gene has homology to bacterial sequences.

   b. Poly r(A)

      Hybridization to poly r(A) indicates the presence of oligo(dT) sequence(s) in your hybridization probe. If this is the case, you will usually observe hybridization to many or all poly A^+ RNAs immobilized on the MTE Array. To detect your target mRNA, you must redesign your probe so that it does not contain oligo(dT) sequences.

   c. C_ot-1 DNA

      C_ot-1 DNA is enriched for repetitive DNA sequences such as the *Alu* and *Kpn* families. If you observe hybridization to the dot containing C_ot-1 DNA, your probe may contain repetitive sequences. In this case, you should redesign your probe so that it does not contain any repetitive sequences.

VII. Interpretation of Hybridization Results *continued*

d. Two genomic DNA controls (100 ng and 500 ng)

As with the C_ot-1 DNA control, if you observe hybridization to either of these dots, your probe may contain repetitive sequences. You may also observe hybridization if your gene is highly abundant or belongs to a multi-gene family.

2. Positive control

As a positive control, we have provided a cDNA probe for one of the eight housekeeping genes used for normalization (see Appendix C). This probe may be used to check the efficiency of your labeling procedure and to control for hybridization. Furthermore, you may use the probe to confirm that the normalization process was successful. The control probe should give a strong, consistent hybridization signal for all dots after only a few hours of exposure to x-ray film, and there should be little or no background.

Because the control probe provided with the MTE Array generates a strong hybridization signal, we recommend that you use it after you have completed your analysis for your target gene, or hybridize it to a separate MTE Array.

VIII. Troubleshooting Guide

A. General Considerations

- We seldom encounter problems with the MTE Array when we follow the procedures described in this manual. These procedures may seem arduous, but they should be followed closely. Not only do they improve the odds of success, but they also make troubleshooting much easier.
- For best results, use the BD ExpressHyb Hybridization Solution included with the MTE Array. Do not substitute another solution, unless using the BD SpotLight system.
- If you do not have experience with hybridization techniques and you have problems with your experiment, we suggest that you try hybridizing the MTE Array with a cDNA probe which has already been tested for Northern hybridization. The control cDNA probe is included with the MTE Array for this purpose, or you may use any well-investigated probe from your lab. Because of the strong signal generated by hybridization to the ubiquitin control probe, we recommend that you use it after trying your experiment.

B. High Background (with or without hybridization signals)

Lengthening the time recommended for washes, both low and high stringency, rarely improves high backgrounds on arrays. Strip the probe from the array as described in Section V.B (for oligonucleotide probes) or Section VI.B (for cDNA probes). For radioactively labeled probes, monitor the stripping process with a Geiger counter; cpm should be reduced to almost background levels. Then repeat the hybridization using the recommendations listed below.

Several factors may account for high levels of nonspecific background:

1. Unincorporated [^{32}P]dNTPs are not fully removed from the probe.

   Check the ratio of TCA-precipitable cpm to total cpm in the final probe preparation. Adjust chromatography conditions to achieve a ratio of >95%. We also recommend using exclusion chromatography (e.g., BD CHROMA SPIN 100 Columns) to purify your hybridization probe.
2. Probe is too old.

   Use your probe as soon as possible after preparation, since radioactive decay results in probe fragmentation. Small radioactive decay products can contribute to high background.
3. Composition of hybridization mix is incorrect.

   If you substituted another hybridization solution for BD ExpressHyb Solution, remove the probe from the array as you would for reprobing. Repeat prehybridization and hybridization as described in Section III.

VIII. Troubleshooting Guide *continued*

4. Average labeled cDNA fragment is too long.

   Optimal size is 200–800 nucleotides. If your cDNA probe is longer than 800 nucleotides, increase the ratio of random primers to template DNA.

5. Concentration of probe in hybridization solution is too high.

   For cDNA probes, do not exceed 5 x 10^6 cpm/ml. For oligonucleotide probes, do not exceed 10^7 cpm/ml.

6. Hybridization probe contains repetitive sequence(s).

   Including C_ot-1 DNA in the hybridization solution may help with this problem. If this fails, use a hybridization probe targeted to another portion of the cDNA. Alternatively, use a shorter fragment of the probe.

   If some or all of the negative controls hybridize strongly to your probe, or all poly A^+ RNAs immobilized on the MTE Array give unexpectedly strong signals, use a different hybridization probe.

C. Hybridization to Negative Controls

If you detect a signal for the negative controls immobilized on the MTE Array that is comparable to other signals, you may have a problem with nonspecific hybridization. See Interpretation of Hybridization Results (Section VII.B) for more information about the negative controls.

D. Hybridization Signals Absent or Very Weak

If signals are not generated after one week of x-ray film exposure, the hybridization probe may have a low specific activity.

1. Hybridization to an oligonucleotide probe

   Check the specific activity of your probe (see Appendix A). It should always be >2 x 10^6 cpm/pmol. For maximum sensitivity, it should be ≥5 x 10^6 cpm/pmol. If the specific activity of your oligonucleotide probe is too low, try the following:

   a. If the ^{32}P you used for labeling was older than one week, make a new probe with fresh ^{32}P. Be sure to use [γ-^{32}P]ATP with a specific activity of 6,000 Ci/mmol.
   b. Purify your oligonucleotide by acrylamide gel electrophoresis before labeling.
   c. Optimize the 5'-end labeling procedure (see Appendix A) with an oligonucleotide that has worked well in your lab.
   d. Reduce the stringency of hybridization and the final washing step (Sec. V) in Wash solution 1 by decreasing the temperature for these steps by 5–10°C.
   e. Increase the length of your oligonucleotide probe or use a cDNA probe.

VIII. Troubleshooting Guide *continued*

2. Hybridization to a cDNA probe

   Check the specific activity of your cDNA probe (see Appendix B). It should always be >5 x 10^8 cpm/µg; for maximum sensitivity, it should be >10^9 cpm/µg. If the specific activity of your cDNA probe is too low, try the following:

   a. If the ^{32}P you used for labeling was older than one week, make a new probe with fresh ^{32}P.

   b. You may not be using an optimal amount of cDNA to prepare your hybridization probe. We typically use 25–50 ng (measured from the OD_{260} of a concentrated stock). If you have a small quantity of cDNA, electrophorese the amount of DNA you have been using on an agarose gel next to a known amount of DNA markers and estimate the amount of probe cDNA. Repeat the labeling procedure using the recommended amount of cDNA.

   c. Optimize the random primer labeling procedure (Appendix B) with a known amount of control probe, such as the control cDNA probe provided with each MTE Array.

   d. If you are reprobing, try hybridizing the MTE Array to the control cDNA probe to confirm that the poly A^+ RNA on the array was not removed during stripping.

E. Probe Not Fully Homologous to Target

If you are using a probe from a different species, you may need to reduce the stringency of your final wash and/or hybridization by reducing the temperature and substituting Wash solution 1 for Wash solution 2 (use Wash solution 2 to reduce stringency). When using a synthetic oligonucleotide probe, use a sequence that is completely homologous to the target, if possible.

F. Unable to Strip and Reprobe

If you observe high background when reprobing the array, the membrane may not have been stripped completely or may have been allowed to dry. If a membrane is allowed to dry even partially, subsequent removal of the probe may be difficult. To prevent drying after your final wash, shake off excess solution with forceps (do not blot-dry) and seal in polyethylene bag or wrap the array immediately with plastic wrap. Always store MTE Arrays at –20°C when not in use. When reprobing, uncover the array, immediately place it in stripping solution, and follow the rest of the protocol provided for removing probes. If the membrane has not partially dried, consult the paragraph discussing high background in Section B, above.

IX. References

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X. Related Products

For a complete listing of all BD Biosciences Clontech products, please visit **www.bdbiosciences.com**

Product	Cat. No.
• BD™ MTN Multiple Tissue Northern Blots	many
• BD™ MTC Multiple Tissue cDNA Panels	many
• BD SpotLight™ Chemiluminescent Hybridization & Detection Kit	634802
• BD SpotLight™ Random Primer Labeling Kit	634801
• BD Atlas™ cDNA Arrays	many
• BD ExpressHyb™ Hybridization Solution	636831 636832 636833
• cDNA Probes	many
• Premium Poly A$^+$ RNA	many
• BD CHROMA SPIN™ Columns	many
• BD Marathon™ cDNA Amplification Kit	634913
• BD™ Marathon-Ready cDNAs	many
• BD QUICK-Clone™ cDNAs	many
• BD Clontech PCR-Select™ cDNA Subtraction Kit	637401
• BD SMART™ RACE cDNA Amplification Kit	634914
• BD SMART™ cDNA Library Construction Kit	634901

Appendix A: 5'-End Labeling of Oligonucleotide Probes

A. General Considerations

- Use only fresh (<one week old) ^{32}P-labeled nucleotide of the highest specific activity available (typically 6,000 Ci/mmol for [γ-^{32}P]dATP).
- It is important to remove unincorporated ^{32}P-labeled nucleotides. This can be achieved by chromatography using BD CHROMA SPIN 30 Columns or equivalent.
- Always calculate the specific activity of your probe. The specific activity lets you know if you have actually labeled a useful probe before you start the hybridization. Determining the specific activity also allows you to judge the best x-ray film exposure times. A probe with a 2X higher specific activity will require 2X less x-ray film exposure time to detect the same gene. It also allows you to calculate the amount of probe necessary to achieve the recommended concentration in the hybridization. Do not exceed the recommended concentration of probe in the hybridization or high background will result.

B. Required Reagents

- **10X Kinase buffer**

500	mM	Tris-HCl (pH 7.6)
100	mM	$MgCl_2$
50	mM	Dithiothreitol
1	mM	Spermidine
1	mM	EDTA

- **0.5 M EDTA**
- **Distilled H_2O**
- **T4 polynucleotide kinase**
- **[γ-^{32}P]ATP** (6,000 Ci/mmol; 10 µCi/µl)

C. Procedure for 5'-End Labeling Oligonucleotide Probes

1. Combine the following components:

10	pmol	Oligonucleotide probe*
2.5	µl	10X Kinase buffer
10	µl	[γ-^{32}P]ATP
5	units	T4 Polynucleotide kinase

   Add distilled H_2O to a final volume of 25 µl.

   * Use the following approximate conversion factor to determine pmol:
   1 µg of n-mer = (30/n) (100 pmol)
   where n = length of oligonucleotide probe

2. Mix gently and incubate at 37°C for 30 min.
3. Stop the reaction by adding 1 µl of 0.5 M EDTA (pH 7.5).

Appendix A: 5'-End Labeling of Oligonucleotide ... *continued*

4. Remove unincorporated nucleotides from labeled probe using a BD CHROMA SPIN™ 30 Column or equivalent. (BD CHROMA SPIN 30 Columns can remove 99% of the unincorporated ^{32}P and are recommended for oligonucleotides larger than 20 nucleotides.)
   a. Spin column for 3 min at 700 x g in a standard variable speed microcentrifuge (3,500 rpm in Eppendorf Model 5415C).
   b. Add 10–15 µg of carrier nucleic acid (e.g., yeast tRNA) to the labeled oligonucleotide probe.
   c. Load the entire labeling reaction on the center of the column.
   d. Spin for 3 min at 700 x g in a standard variable speed microcentrifuge.
5. Take 1 µl of eluant and dilute 100X.
6. Determine the specific activity of the probe by scintillation counting 1 µl of the dilution in any counting cocktail.
7. The labeled probe can be stored at –20°C for up to 3 days.

Appendix B: Random Primer Labeling of cDNA Probes

A. General Considerations

- Use only fresh (<one week old) ^{32}P-labeled nucleotide of the highest specific activity available (typically 3000 Ci/mmol for [α-^{32}P]dCTP).
- It is important to remove unincorporated ^{32}P-labeled nucleotides. This can be achieved by chromatography using BD CHROMA SPIN Columns or equivalent.
- Always calculate the specific activity of your probe. The specific activity lets you know if you have actually labeled a useful probe before you start the hybridization. Determining the specific activity also allows you to judge the best x-ray film exposure times. A probe with a 2X higher specific activity will require 2X less x-ray film exposure time to detect the same gene. It also allows you to calculate the amount of probe necessary to achieve the recommended concentration in the hybridization. Do not exceed the recommended concentration of probe in the hybridization.
- To synthesize nonradioactively-labeled cDNA probes, use the BD SpotLight Random Primer Labeling Kit. This kit provides reagents necessary to synthesize and purify biotinylated cDNA probes.

B. Required Reagents

- **5X Random hexamer primer solution**
  0.5 µg/µl $(dN)_6$ in H_2O
- **10X Reaction buffer**
  0.4 M Tris-HCl (pH 7.5)
  0.1 M $MgCl_2$
  50 mM DTT
  1 mg/ml BSA
- **0.5 mM dATP**
- **0.5 mM dTTP**
- **0.5 mM dGTP**
- **T7 DNA polymerase** (5–10 units/µl)
- **Enzyme dilution buffer**
  20 mM Tris-HCl (pH 7.5)
  5 mM DTT
  100 µg/ml BSA
  5% Glycerol
- **10X Stop solution**
  250 mM EDTA
- **[α-^{32}P]dCTP** (3,000 Ci/mmol; 10 µCi/µl)
- **Sheared salmon testes DNA** (10 mg/ml; Sigma, Cat. No. D7656)

Appendix B: Random Primer Labeling ... *continued*

C. Random Primer Labeling Procedure (Feinberg & Vogelstein, 1984)

1. Add 25 ng (up to 22 µl) of template DNA to a sterile microcentrifuge tube. Add distilled H_2O to a final volume of 22 µl.
2. Add 10 µl of 5X random hexamer primer solution and mix.
3. Heat mixture at 95–100°C for 5 min, and place on ice.
4. Spin briefly at room temperature to collect droplets formed by condensation.
5. Add the following components:

   5 µl 10X reaction buffer
   2 µl dATP
   2 µl dTTP
   2 µl dGTP
6. Add 5 µl of [α-^{32}P]dCTP.
7. Dilute 1 µl of T7 polymerase with 6.5 µl of enzyme dilution buffer. (Store enzyme on ice at all times; diluted enzyme can be stored at –20°C for up to one week without losing significant activity).
8. Add 2 µl of diluted enzyme to the reaction mixture.
9. Incubate mixture at 37°C for 20 min.
10. Add 5 µl of 10X stop solution to complete the reaction.
11. Take 1 µl of the reaction and dilute 100X in 99 µl of distilled H_2O. Reserve dilution for determination of specific activity by TCA precipitation. (For additional information on TCA precipitation, please refer to Section E.)

D. Probe Purification

We recommend purifying the labeled probe on BD CHROMA SPIN 100 Columns or BD CHROMA SPIN +TE-100 Columns (Cat. No. 636073).

1. Remove unincorporated nucleotides from labeled probe.
   a. Spin column for 3 min at 700 x g in a standard variable speed microcentrifuge (3,500 rpm in Eppendorf Model 5415C).
   b. Add 10–15 µg of carrier nucleic acid (e.g., yeast tRNA) to the labeled cDNA probe.
   c. Load the entire labeling reaction on the center of the column.
   d. Spin for 3 min at 700 x g in a standard variable speed microcentrifuge
2. Following chromatography, spot 1/50 of the eluate onto a 2.4-cm glass fiber filter (Whatman, Cat. No. 934-AH). Determine specific activity by scintillation counting. (For additional information on specific activity determination, please refer to Section F.)

Appendix B: Random Primer Labeling ... *continued*

E. TCA Precipitation

We use the following protocol for TCA precipitation, but other methods are also suitable (Sambrook *et al.*, 1989).

1. To a 1.5-ml microfuge tube, add 25 µg of sheared salmon testes DNA to serve as a carrier for TCA precipitation.
2. Add 1 µl of diluted reaction mix (prechromatography) and 50 µl of 10% TCA cooled to 4°C. The TCA/DNA solution should become cloudy immediately.
3. Place a 2.4-cm diameter glass fiber filter onto a filter holder attached to a vacuum apparatus. (We use a Hoefer filter unit for 2.4-cm filters; Hoefer, Cat. No. FH124.) Pour the contents of the test tube onto the filter under vacuum.
4. Rinse the tube with 5% TCA cooled to 4°C, and pour the rinse solution onto the filter.
5. Repeat five times with 2 ml of cooled 5% TCA and once with ethanol.
6. Remove the filter from the holder and rinse the filter holder with ethanol.

F. Determination of Specific Activity

1. Place the two filters (pre- and postchromatography) into separate scintillation vials. Add 3–4 ml of counting cocktail to each vial. Measure the levels of radioactivity in a scintillation counter.
2. Use the following formulas to calculate the specific activity of the probe (prechromatography) and the radioactivity of the purified probe fraction (postchromatography).
   a. **Specific activity of probe (cpm/µg)** = cpm (prechromatography) x 100 (the dilution factor) x 55 µl (the final reaction volume) ÷ µg of DNA used in labeling reaction

      The specific activity of the probe should be ≥10^9 cpm/µg DNA for fresh 3,000 Ci/mmol [^{32}P]dCTP (<1 week old).

   b. **Total cpm of probe fraction** = cpm (postchromatography) x volume of probe fraction (µl) x 50 (the dilution factor)

   Note: When higher specific activity is desired, two different [α-^{32}P]-labeled nucleotides can be used simultaneously during the labeling reaction. In this case, omit the corresponding unlabeled dNTP and add the [α-^{32}P]-labeled nucleotide instead. Adjust the volume of the reaction with H_2O to ensure that the final volume of the labeling reaction is 50 µl.

Appendix C: BD™ MTE Array Normalization Procedure

In this User Manual, the term "normalization" refers to the BD Biosciences Clontech method for using the gene expression levels of eight housekeeping genes to standardize loading of the poly A^+ RNAs on to our MTE Array. Because it accounts for differences in levels of transcription, normalization allows you to use the MTE Array to estimate the relative abundance of your target gene transcript in different tissues.

In some tissues, such as brain, the overall level of transcription is relatively low, producing the mRNAs necessary for basal metabolism (i.e., housekeeping gene transcripts) and a small quantity of other mRNAs. In contrast, other tissues, such as glands, are transcriptional powerhouses, producing large quantities of other mRNAs along with housekeeping transcripts. At the same time, the level of expression of housekeeping genes is rather constant for all tissues (Adams *et al.*, 1993; Adams *et al.*, 1995; Liew *et al.*, 1994). Thus, the fraction of housekeeping gene transcripts in total poly A^+ RNA is generally much lower in tissues with high overall levels of transcription than in less transcriptionally active tissues.

RNA dot blots and Northern blots are often standardized by loading equal gram amounts of poly A^+ RNA on each dot. However, this method is not ideal for quantifying gene expression, because it does not reflect differences between

TABLE I: HOUSEKEEPING GENES USED FOR NORMALIZATION

Housekeeping gene	Function	mRNA size (kb)[a]
β-actin[b]	cytoskeletal protein	1.8
Glyceraldehyde 3-phosphate dehydrogenase (G3PDH)	glycolysis	1.3
Ubiquitin[b]	protein degradation	2.3
23-kDa highly basic protein[b]	unknown	0.7
α-tubulin	cytoskeletal protein	1.6
Phospholipase A2[b]	lipid metabolism	2.8
Ribosomal protein S9 (RPS9)	protein synthesis	0.7
Hypoxanthine guanine phosphoribosyl transferase (HPRT)	nucleotide synthesis	1.3

[a] The size given here is based on sequence analysis of the mRNA and may therefore differ from the size of the corresponding band on a Northern blot. Electrophoretic mobility of an mRNA depends on the length of the poly A^+ tail, which cannot necessarily be determined from the sequence, as well as secondary structure that may persist even in a denaturing formaldehyde/agarose gel. The average length of a poly A^+ tail is 150 nucleotides.

[b] This probe is a member of a multigene family and therefore would generate multiple bands on a Northern blot. The intensity of these individual bands may vary in different tissues or cell types.

Appendix C: BD™ MTE Array Normalization ... *continued*

tissues in overall transcriptional activity. If equal gram amounts of RNA are loaded on a membrane, dots from transcriptionally active tissues will contain fewer housekeeping gene transcripts, while dots from transcriptionally less active tissues will contain disproportionately more housekeeping mRNAs because there are fewer tissue-specific transcripts. Thus, any attempt to determine the relative abundance of a target mRNA in different tissues using such a blot will be misleading. Furthermore, although the expression of housekeeping genes is relatively constant between different tissues and developmental stages, every housekeeping gene displays *some* genuine tissue- and stage-specific variation (Spanakis & Brouty-Boyé, 1994; Spanakis, 1993; Liew *et al.*, 1994; Adams *et al.*, 1993; Adams *et al.*, 1995).

To account for these differences in transcription levels, we normalize the loading of our MTE Array to *eight different housekeeping genes* (Table I): phospholipase (PL), ribosomal protein S9 (RPS9), tubulin, 23-kDa highly basic protein (23 kDa), glyceraldehyde-3-phosphate dehydrogenase (G3PDH), hypoxanthine guanine phosphoribosyl transferase (HPRT), β-actin, and ubiquitin. After extensive testing of 20 housekeeping genes, these genes were chosen because they show minimal variation and belong to different functional classes (Table 1; Liew *et al.*, 1994; Adams *et al.*, 1995). Normalization to these eight standards helps compensate for the tissue-specific variations in expression of any single housekeeping gene.

Normalization is performed prior to manufacturing each lot of MTE Array. Ideally, the intensity percentages for all tissues would be equal after normalization; however, we find that some variation persists. These dot-to-dot variations probably reflect some inconsistencies in hybridization conditions, as well as inaccuracies in quantification by phosphorimaging. As a result, two to three rounds of normalization are generally required to correctly adjust the individual amounts of poly A^+ RNA loaded on the array.

Thus, although the gram quantity of poly A^+ RNA on each dot varies from 53–780 ng (see PAC for exact quantities), the signal produced from a hypothetical gene that has *no* tissue-to-tissue variation in its expression would be consistent for all tissues.

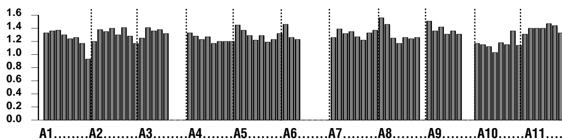

Figure 2. Average intensity percentages for all eight housekeeping genes after normalization. These data are only intended as background information for MTE Array users. Data should not be used to draw general conclusions about transcript abundance.