instructions

product code

25-8005-67

Hybond Atlas RNA Purification and Labelling Kit

for synthesis of high sensitivity probes directly from total RNA

Warning

For research use only.

Not recommended or intended for diagnosis of disease in humans or animals.

Do not use internally or externally in humans or animals.

25800567PL Rev-A, 2001

Amersham Biosciences

Handling

Storage

The kit components should be stored as follows

- Store control total RNA at -70 °C
- Store DNase I enzyme, DNase I buffer, biotinylated oligo(dT), 10× termination mix and reverse transcriptase at -15 °C to -30 °C
- Store all other reagents at 2–8 °C

Expiry

For expiry details see outer packaging

Components

Each kit is sent out in two boxes which contain the following components:

Box 1

Denaturing solution: 50 ml
Saturation buffer: for Phenol, 65 ml
RNase-free water: 20 ml
2 M NaOAc: pH 4.5, 1 ml
10× termination mix: 1.2 ml
Streptavidin magnetic

Page finder

beads: 180 µl
1× Binding buffer: 24 ml
2× Binding buffer: 600 µl
1× Reaction buffer: 700 µl
1× Wash buffer: 1.2 ml

Box 2
DNase I (1 unit/µl): 500 µl
10× DNase I buffer:, 1 ml
Biotinylated oligo(dT): 12 µl
Control total RNA: (1:1:1 mixture of human, mouse and rat total RNA), 30 µg
Reverse transcriptase: 15 µl

Other materials required

When preparing reagents for RNA work, be sure to take the necessary precautions to prevent RNA degradation (see section ❶, page 8

RNA Isolation

- Saturated Phenol (store at 2–8 °C)
  For 160 ml:
  100 g Phenol (Sigma #P1037 or Boehringer Mannheim #100728). In a fume hood, heat the

Safety warnings and precautions

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Caution: For use with radioactive material

We recommend that this product and components are handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. As all chemicals should be considered as potentially hazardous, it is advisable when handling chemical reagents to wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In case of contact with skin or eyes, wash immediately with water.

Please note that the protocol may require the use of the following chemicals: Phenol (Toxic, causes burns); Chloroform (Harmful, irritant); Isopropanol (Highly flammable); Liquid Nitrogen, Cardice (Cause burns); Formamide (Harmful, irritant); Formaldehyde (Toxic, causes

jar of Phenol in a 70 °C water bath for 30 min or until the Phenol is completely melted. Add 95 ml of Phenol directly to the saturation buffer, and mix well. Hydroxyquinoline may be added if desired. Freeze in suitable aliquots at -15 °C to -30 °C for long-term storage.
Note: This preparation of saturated Phenol will only have one phase.

- Chloroform (Sigma #C2432 or #C0549)
- Isopropanol (Sigma #19516)
- Liquid Nitrogen or dry ice
- Tissue homogenizer (e.g., Polytron or equivalent)
  For < 200mg tissue, use 6 mm probe.
  For > 200 mg tissue, use 10 mm probe.
- Refrigerated centrifuge capable of 12 000 rpm (15 000 × g)
  (e.g. Eppendorf 5417R refrigerated centrifuge;

burns); MOPS (Irritant); Ethidium Bromide (Irritant, possible genetic damage); Ethanol (Highly flammable). Please follow the manufacturers safety data sheets relating to the handling and safe use of these materials.

rotor #F45-30-11)

Analysis of RNA quality

- Formamide (Sigma #F9037)
- 12.3 M Formaldehyde (Sigma #F8775)
- 10× MOPS buffer (Autoclave to sterilize; solution may turn yellow)
  0.4 M MOPS, pH 7.0
  0.1 M NaOAc, pH 7.0
  10 mM EDTA, pH 7.0
- Ethidium Bromide, 10 mg/ml
- Buffer for measuring O.D. (store at -15 °C to -30 °C)
  10 mM Tris, pH 7.5
  0.1 mM EDTA, pH 7.5

Probe synthesis

- Reagents included with the Hybond Atlas Array: 10× dNTP mix (for dATP label; 5 mM each dCTP, dGTP, dTTP), CDS primer mix, 5× Reaction buffer, Reverse transcriptase (to supplement the volume provided in this kit), DTT (100 mM)
- [α-^{32}P]dATP (10 µCi/µl;

Description

Hybond™ Atlas™ cDNA Expression Arrays are valuable tools for high throughput, gene expression profiling. Each array is a positively charged nylon membrane on which hundreds of cDNA fragments have been immobilized. To analyse the expression patterns of these genes, probes are generated from the RNA samples of interest using a mixture of gene-specific primers (patent pending). Probes generated using gene-specific primers are significantly less complex than probes generated using oligo(dT) or random primers, so the sensitivity of detection is increased by about 10-fold, with a concomitant reduction in the level of non-specific background and cross-hybridization.

The Hybond Atlas RNA Purification and Labelling System, when used in conjunction with reagents supplied with Hybond Atlas Arrays, allows you to synthesize highly sensitive probes directly from total RNA. The key components in this System are Streptavidin-coated magnetic beads and biotinylated oligo(dT) which allow you to carry out both poly A$^+$ RNA enrichment and probe synthesis in a single procedure. This simplicity is possible

3000 Ci/mmol; Amersham Biosciences #PB10204) or Redivue™ version AA0004
OR

- [α-^{33}P]dATP (10 µCi/µl; > 2500 Ci/mmol; Amersham Biosciences #BF1001) or Redivue version AH9904

General materials

- Ethanol
- Deionized water (Milli-Q™-filtered or equivalent; **do not** use DEPC-treated water)
- Magnetic particle separator (Promega #Z5331)
  Note: It is important that you use a separator designed for 0.5 ml tubes.
- Polypropylene centrifuge tubes
  1.5 ml (Sarstedt #72-690-051)
  2 ml (PGC Scientifics #16-8105-75)
  15 ml (Fisher: tubes #05-562-10D; caps #05-562-11E)
  50 ml (Fisher: tubes

because probes are synthesized while poly A$^+$ RNA remains bound to the magnetic beads, eliminating potentially troublesome intermediate poly A$^+$ RNA purification steps.

Using this procedure, probes made from total RNA produce results that are just as good as those from pure poly A$^+$ RNA. This is a clear advantage when only small amounts of tissues or cells are available. This flexibility is especially important for clinical researchers who wish to investigate gene expression in biopsy materials, tumours, or pathological specimens.

For best results in Hybond Atlas experiments, it is essential that you follow the instructions in this booklet to isolate total RNA from your tissue source. The quality of total RNA used is **the most important factor** for generating high-sensitivity hybridization probes. Most commercially available kits for RNA isolation are not compatible with Hybond Atlas Arrays because they result in non-specific background.

These instructions provide a method for total RNA isolation and cDNA probe synthesis. All subsequent steps in the procedure, including probe purification, are described in the Hybond Atlas cDNA Expression Arrays instructions (RPN7850PL). After completing

w/caps #05-529-1D)
Note: 15 ml and 50 ml tubes should be sterilized with 1% SDS and Ethanol before use.

Critical parameters

- Ensure that each component is stored under its recommended storage conditions
- The quality of total RNA used is the most important factor in generating high-sensitivity hybridization probes

probe synthesis (Section ❹C, see pages 22–23 of this booklet), continue with Section ❸C in the Hybond Atlas Arrays instructions. We recommend that you thoroughly read **both sets of instructions** before beginning the procedure.

Protocol

❶ General considerations

The quality of the RNA used to make probes is the most important factor influencing the sensitivity and reproducibility of the hybridization pattern in Hybond Atlas experiments. A poor quality RNA preparation leads to high background on the membrane and/or a misleading hybridization pattern.

Contamination by genomic DNA is particularly troublesome. Therefore, all total RNA samples must be treated with RNase-free DNase I as described in Section ❷C, pages 15–17 prior to use in probe synthesis.

Buffer-saturated Phenol must be prepared from solid Phenol as described on pages 3–4

- Always wear eye protection and gloves while handling reagents.
- Homogenize samples in a fume hood or other well-ventilated area.
- Prepare denaturing Agarose/Formaldehyde gels in a fume hood or other well-ventilated area.
- Always use Phenol-resistant polypropylene centrifuge tubes that can withstand centrifuge speeds of up to 12 000 rpm (15 000 × g). See page 6, for recommendations.

Guidelines for working with RNA

The following precautions should be taken to prevent RNA degradation. For a more complete discussion of these and other guidelines, please refer to Sambrook *et al*., 1991.

- Sterile, disposable plasticware is essentially free of RNases and can be used for the preparation and storage of RNA without pretreatment. However, general laboratory glassware and plasticware may be

contaminated with RNases and should be treated appropriately. To remove RNases from glassware, bake at 300 °C for 4 h (or 180 °C for 8 h). To remove RNases from polypropylene plasticware, rinse with Chloroform. Do not use autoclaved glass or plasticware without first washing it with SDS and Ethanol.

- Prepare all solutions using deionized water (Milli-Q-filtered or equivalent) that has not been treated with DEPC.
- Use only ultrapure reagents that are reserved for RNA work only; use single use plastic or baked metal spatulas when handling these reserved reagents.
- Wear disposable gloves during preparation of materials and solutions that will be used for isolation and analysis of RNA, and during manipulations involving RNA. Change gloves frequently.
- Starting material should be as fresh as possible. For best results, use tissue or cells immediately after harvesting. If this is not possible, flash freeze samples in liquid Nitrogen and store them at -70 °C until required for use.

❷ Total DNA isolation procedure

Please read through the entire protocol before starting.
You should also thoroughly read the Hybond Atlas cDNA Expression Arrays instructions (RPN7850PL), especially ❸C.

Yields will vary according to cell or tissue type (see table 3 on page 18).

A. RNA isolation from tissues

Table 1. Volumes of reagents for tissues

Weight of tissue	10–100 mg	100–300 mg	300–600 mg	0.6–1.0 g
Recommended tube size	2 × 2 ml	15 ml*	50 ml*	50 ml*
Denaturing solution	1.0 ml	3.0 ml	6.0 ml	10.0 ml
Saturated Phenol	2.0 ml	6.0 ml	12.0 ml	20.0 ml
Chloroform	0.6 ml	1.8 ml	3.6 ml	6.0 ml
Saturated Phenol (2nd round)	1.6 ml	4.8 ml	9.6 ml	16.0 ml
Chloroform (2nd round)	0.6 ml	1.8 ml	3.6 ml	6.0 ml
Isopropanol	2.0 ml	6.0 ml	12.0 ml	20.0 ml
80% EtOH wash	1.0 ml	3.0 ml	6.0 ml	10.0 ml

* WARNING! Conical 50 ml tubes can break under forces greater than 8000 rpm (10 000 × g). We recommend using sterile 15 ml and 50 ml round-bottomed polypropylene centrifuge tubes at all times. See page 6 for recommendations.

1. Harvest tissue; use immediately or flash freeze in liquid Nitrogen and store at -70 °C.

IMPORTANT: When working with frozen tissue, ensure that it remains frozen until adding the denaturing solution. Even partial thawing can result in RNA degradation. Perform all necessary manipulations on dry ice or liquid Nitrogen.

2. Cut or crush tissue into small pieces (< 1 cm^3). When working with frozen tissue, pre-chill a mortar and pestle with liquid Nitrogen, fill the mortar with liquid Nitrogen, and break frozen tissue into smaller pieces.

3. Weigh out tissue in a pre-chilled, sterile tube. See Table 1 for the appropriate tube size.

4. Add the appropriate volume of denaturing solution (see Table 1).

Note: Always make sure you add at least 1 ml per 100 mg tissue.

5. Grind the sample at 0–4 °C using a tissue homogenizer (e.g., Polytron or equivalent) at the maximum setting for 1–2 min or until completely homogenized.

6. Incubate on ice for 5–10 min.

7. Vortex well. Centrifuge homogenate at 12 000 rpm (15 000 × g) for 5 min at 4 °C to remove cellular debris.

8. Transfer the entire supernatant to new centrifuge tube(s).
Note: Avoid pipetting the insoluble upper layer, if present.

9. Add the appropriate volume of saturated Phenol (see Table 1). Cap the tubes securely and vortex for 1 min. Incubate on ice for 5 min.

10. Add the appropriate volume of Chloroform (see Table 1). Shake sample and vortex vigorously for 1–2 min. Incubate on ice for 5 min.

11. Centrifuge the homogenate at 12 000 rpm (15 000 × g) for 10 min at 4 °C.

12. Transfer the upper aqueous phase containing the RNA to a new tube. Take care not to pipette any material from the white interphase or lower organic phase.

13. Perform a second round of Phenol: Chloroform extraction, using the amounts shown in Table 1 for '2nd round'. Repeat Steps 9–12. For very RNase-rich samples (e.g., pancreas, liver and spleen), we recommend that you perform a third or fourth round of Phenol:Chloroform extraction.

14. Add the appropriate volume of isopropanol (see Table 1). Add slowly, mixing occasionally as you add it. Mix the solution well and incubate on ice for 10 min.

15. Centrifuge the samples at 12 000 rpm (15 000 × g) for 15 min at 4 °C.

16. Quickly remove the supernatant without disturbing the RNA pellet.

17. Add the appropriate volume of 80% Ethanol (see Table 1).

18. Centrifuge at 12 000 rpm (15 000 × g) for 5 min at 4 °C. Quickly and carefully discard the supernatant.

19. Air dry the pellet.

20. Resuspend the pellet in enough RNase-free water to ensure an RNA concentration of 1–2 µg/µl. Refer to Table 3 for approximate yields.

21. Allow the pellet to soak, then resuspend thoroughly by tapping the tube and pipetting gently up and down.

22. Remove a 2 µl aliquot to compare with your RNA sample following DNase treatment (Section ❷C). Store RNA samples at -70 °C until ready to proceed with DNase treatment.

B. RNA isolation from cultured cells

Table 2. Volumes of reagents for cultured cells

Cell number	10^6–10^7	1–3 × 10^7	3–6 × 10^7	6–10 × 10^7
Tube size	2 × 2 ml	15 ml*	50 ml*	50 ml*
Denaturing solution	1.0 ml	3.0 ml	6.0 ml	10.0 ml
Saturated Phenol	2.0 ml	6.0 ml	12.0 ml	20.0 ml
Chloroform	0.6 ml	1.8 ml	3.6 ml	6.0 ml
Saturated Phenol (2nd round)	1.6 ml	4.8 ml	9.6 ml	16.0 ml
Chloroform (2nd round)	0.6 ml	1.8 ml	3.6 ml	6.0 ml
Isopropanol	2.0 ml	6.0 ml	12.0 ml	20.0 ml
80% EtOH wash	1.0 ml	3.0 ml	6.0 ml	10.0 ml

* WARNING! Conical 50 ml tubes can break under forces greater than 8000 rpm (10 000 × g). We recommend using sterile 15 ml and 50 ml round bottomed polypropylene centrifuge tubes at all times. See page 6 for recommendations.

1. Transfer cultured cells from culture vessel to a sterile tube. See Table 2 for the appropriate tube size.

2. Centrifuge at 2000 rpm (500 × g) for 5 min at 4 °C. Discard the supernatant.

3. Use cells immediately, or flash freeze in liquid Nitrogen and store at -70 °C.

IMPORTANT: When working with frozen cells, ensure that they remain frozen until adding the denaturing solution. Even partial thawing can result in RNA degradation. Perform all necessary manipulations on dry ice or liquid Nitrogen.

4. Add the appropriate volume of denaturing solution (Table 2). Pipette the solution up and down vigorously and vortex well until cell pellet is completely resuspended. Incubate on ice for 5–10 min.

5. Vortex well. Centrifuge the homogenate at 12 000 rpm (15 000 × g) for 5 min at 4 °C to remove cellular debris.

6. Transfer the entire supernatant to new centrifuge tube(s). Avoid pipetting the insoluble upper layer, if present.

7. Add the appropriate volume of saturated Phenol (see Table 2). Cap the tubes securely and vortex for 1 min. Incubate on ice for 5 min.

8. Add the appropriate volume of Chloroform (see Table 2). Shake sample and vortex vigorously for 1–2 min. Incubate on ice for 5 min.

9. Centrifuge the homogenate at 12 000 rpm (15 000 × g) for 10 min at 4 °C.

10. Transfer the upper aqueous phase containing the RNA to a new tube. Take care not to pipette any material from the white interphase or lower organic phase.

11.Perform a second round of Phenol:Chloroform extraction, using the amounts shown in Table 2 for '2nd round'. Repeat Steps 7-10. For very RNase-rich cells (e.g. leukocytes), we recommend that you perform a third round of Phenol:Chloroform extraction.

12. Add the appropriate volume of Isopropanol (see Table 2). Add slowly, mixing occasionally as you add it. Mix the solution well and incubate on ice for 10 min.

13. Centrifuge the samples at 12 000 rpm (15 000 × g) for 15 min at 4 °C.

14. Quickly remove the supernatant without disturbing the RNA pellet.

15. Add the appropriate volume (see Table 2) of 80% Ethanol.

16. Centrifuge at 12 000 rpm (15 000 × g) for 5 min at 4 °C. Quickly and carefully discard the supernatant.

17. Air dry the pellet.

18. Resuspend the pellet in enough RNase-free water to ensure an RNA concentration of 1–2 µg/µl. Refer to Table 3 for approximate yields.

19. Allow pellet to soak, then resuspend thoroughly by tapping the tube and pipetting.

20. Remove a 2 µl aliquot to compare with your RNA sample following DNase treatment. Store RNA samples at -70 °C until ready to proceed with DNase treatment.

C. DNase treatment of total RNA

The following protocol describes DNase I treatment of 0.5 mg of total RNA prior to purification of poly A^+ RNA. If you are starting with more or less than 0.5 mg, adjust all volumes proportionally.

We recommend that you divide your total RNA samples prior to DNase treatment and only perform the DNase treatment on a portion. This is a safeguard, since significant degradation can occur during the 30 min incubation with DNase I if samples still contain impurities at this point. In our experience, prior to performing this procedure, some RNase-rich human tissues (e.g., pancreas, liver, leukocytes, and cerebellum) must be Phenol: Chloroform extracted twice, then precipitated and resuspended in RNase-free water in order to completely remove RNases. If you still see degradation, we recommend using Ambion's ANTI-RNase (#2692) at a concentration of 1 unit/µl with this protocol.

1. Combine the following reagents in a 1.5 ml microcentrifuge tube for each sample (you may scale up or down accordingly):

500 µl	Total RNA (1 mg/ml)
100 µl	10× DNase I buffer
50 µl	DNase I (1 unit/µl)
350 µl	Deionized water
1.0 ml	Total volume

Mix well by pipetting.

2. Incubate the reactions at 37 °C for 30 min in an air incubator.

3. Add 100 µl of 10× termination mix. Mix well by pipetting.

4. Split each reaction into two 1.5 ml microcentrifuge tubes (550 µl per tube).

5. Add 500 µl of saturated Phenol and 300 µl of Chloroform to each tube and vortex thoroughly.

6. Centrifuge at 14 000 rpm for 10 min at 4 °C to separate phases.

7. Carefully transfer the top aqueous layer to a fresh 1.5 ml microcentrifuge tube. Avoid pipetting any material from the interface or lower phase.

8. Add 550 µl of Chloroform to the aqueous layer and vortex thoroughly.

9. Centrifuge at 14 000 rpm for 10 min at 4 °C to separate phases.

10. Carefully remove the top aqueous layer and place in a 2.0 ml microcentrifuge tube.

11. Add 1/10 volume (50 µl) of 2 M NaOAc and 2.5 volumes (1.5 ml) of 95% Ethanol. If treating less than 20 µg total RNA, add 20 µg of Glycogen.

12. Vortex the mixture thoroughly and incubate on ice for 10 min.

13. Spin in a microcentrifuge at 14 000 rpm for 15 min at 4 °C.

14. Carefully remove the supernatant and any traces of Ethanol.

15. Gently overlay the pellet with 500 µl of 80% Ethanol.

16. Centrifuge at 14 000 rpm for 5 min at 4 °C.

17. Carefully remove the supernatant.

18. Air dry the pellet for approximately 10 min or until the pellet is dry.

19. Dissolve the precipitate in 250 µl of RNase-free water and assess the yield and purity of your RNA as described in section ❸ below Alternatively, store RNA at -70 °C.

❸ Assessing yield and purity of total RNA

The yield of total RNA will vary depending on the tissue or cells from which it is obtained. Perform the following analysis on both the aliquot of RNA saved before DNase treatment (see page 10 or 13, section ❷A or ❷B) and on the DNase-treated RNA.

Table 3. Representative total RNA yields

Tissue/cell source	Amount of starting material	Yield of total RNA	Yield after DNase (70% recovery)
Rat liver	100 mg	600 µg	420 µg
Rat skeletal muscle	100 mg	90 µg	60 µg
Mouse brain	100 mg	125 µg	90 µg
Mouse spleen	100 mg	245 µg	170 µg
Mouse testes	100 mg	240 µg	170 µg
Mouse thymus	100 mg	85 µg	60 µg
Human cerebellum	100 mg	85 µg	60 µg
Human prostate tumour	100 mg	100 µg	70 µg
MCF-7 cell line	1×10^7 cells	70 µg	50 µg
Mouse fibroblasts	1×10^7 cells	800 µg	560 µg
U251 cell line	1×10^7 cells	95 µg	65 µg

A. Determining A_{260}

1. Thoroughly mix your RNA. Measure the total RNA sample volume.

2. Transfer 2–5 µl of your total RNA sample to a 1.5 ml tube.

3. Bring volume up to 400 µl with O.D. buffer (see page 5) and mix by pipetting.

4. Transfer the contents to a 1 ml glass cuvette with a 1 cm path length.

5. Measure A_{260} and A_{280} using O.D. buffer as a reference blank.

6. Calculate RNA yield as follows:

RNA constant for 1 cm path length: One A_{260} unit of RNA = 40 µg/ml

- Total A_{260} = (A_{260} of dilute sample) × (dilution factor)

- Concentration (µg/ml) = (total A_{260}) × (40 µg/ml)
- Yield (µg) = (total sample volume) × (concentration)

7. Calculate the A_{260} / A_{280} ratio. Pure RNA exhibits a ratio of 1.9–2.1.

Example: The RNA sample volume was 0.5 ml. A 2 µl sample aliquot was diluted to 400 µl in O.D. buffer. The following spectrophotometric readings were taken: A_{260} = 0.231; A_{280} = 0.115

Calculations:

- Total A_{260} = (0.231) × (200) = 46.2
- Concentration = (46.2) × (40) = 1848 µg/ml
- RNA yield = (0.5 ml) × (1848 µg/ml) = 924 µg
- Purity = 0.231/0.115 = 2.01

B. Preparing a 1% denaturing Agarose gel

1. Wash a mini-gel box, a gel tray (7 × 10 cm), and combs with deionized water. Equipment should be reserved for RNA work only.

2. Add 1 g of Agarose to a 250 ml beaker containing a magnetic stir-bar.

3. Add 82.5 ml of water.

4. Microwave for 2 min or until boiling.

Perform the following steps in a fume hood:

5. Place the bottle on a magnetic stir-plate and stir slowly for 2 min to cool.

6. While stirring, add 10 ml of 10× MOPS buffer and 7.5 ml of 12.3 M Formaldehyde.

7. Continue stirring for 1 min; then pour the solution onto the gel tray.

8. Allow at least 1 h for the gel to solidify at room temperature. Do not use Formaldehyde gels that have been stored longer than 24 h.

9. Remove the gel comb and submerge the gel in a gel box with 1 × MOPS buffer.

C. RNA sample preparation

1. Prepare RNA loading solution immediately before running the gel by mixing in a sterile tube (for 6–10 samples):

Formaldehyde	45 µl
Formamide	45 µl
10× MOPS buffer	10 µl
EtBr (10 mg/ml)	3.5 µl
0.1 M EDTA (pH 7.5)	1.5 µl
Bromophenol Blue dye (in 50% Glycerol)	8 µl

2. Add 10–15 µl of RNA loading solution to 1–2 µg of total RNA; mix well.

3. Heat at 70 °C for 10–15 min.

4. Cool on ice 1 min, then load onto the gel.

D. Gel electrophoresis guidelines

1. Run gel at 4–5 V/cm (equivalent to 50–60 V on a mini-gel box).

2. Examine gel when dye has migrated 3–4 cm from the wells.

E. Expected results

Total RNA from mammalian sources should appear as two bright bands (28S and 18S ribosomal RNA) at approximately 4.5 and 1.9 kb. The ratio of intensities of the 28S and 18S rRNA bands should be 1.5–2.5:1. Lower ratios are indicative of degradation. You may also see additional

bands or a smear lower than the 18S rRNA band, including very small bands corresponding to 5S rRNA and tRNA.

❹ Poly A$^+$ RNA enrichment and probe synthesis

After completing this section proceed with column chromatography in Section ❸C of the Hybond Atlas cDNA Expression Array instructions (RPN7850PL). It is important to read the Hybond Atlas Arrays User Manual before starting. Optional: Use the entire aliquot of control total RNA in a parallel labeling reaction to help troubleshoot any problems you may encounter in incorporating label into your probe. The control total RNA contains a 1:1:1 mixture of human, mouse, and rat total RNA. As a result, it should not be used as a control for array hybridization.

A. Streptavidin magnetic bead preparation

1. Resuspend magnetic beads by inverting and gently tapping tube.

2. Pipette 15 µl of beads per probe synthesis reaction into one 0.5 ml tube.

3. Separate the beads on a magnetic particle separator.

4. Remove and discard supernatant using a pipette.

5. Wash beads with 150 µl of 1× binding buffer; pipette up and down.

6. Separate beads on a magnetic particle separator.

7. Remove and discard supernatant using a pipette.

8. Repeat Steps 5–7 three times.

9. Resuspend the beads in 15 µl of 1× binding buffer per reaction.

B. Poly A+ RNA enrichment

Perform the following steps for each total RNA sample.

Important: Do not pause between any of these steps.

1. Preheat a PCR thermal cycler to 70 °C.

2. Pipette 10–50 µg total RNA into a 0.5 ml tube. For synthesizing probes with the highest sensitivity, we recommend using as much RNA as possible, up to the 50 µg limit.

3. Add deionized water to adjust total volume to 45 µl.

4. Add 1 µl of biotinylated oligo(dT), and thoroughly mix by pipetting.

5. Incubate at 70 °C for 2 min in the preheated thermal cycler.

6. Remove from heat and cool at room temperature for 10 min.

7. Add 45 µl of 2× binding buffer, mix well by pipetting.

8. Resuspend the washed beads by pipetting up and down, and add 15 µl of this mixture to each RNA sample.

9. Mix on a vortex mixer or shaker at 1500 rpm for 25–30 min at room temperature. Ensure that beads remain suspended. Do not exceed 30 min.

10. Separate beads using the magnetic separator. Carefully pipette off and discard supernatant.

11. Gently resuspend beads in 50 µl of 1× wash buffer.

12. Being careful not to lose particles, separate beads and then pipette off and discard supernatant.

13. Repeat steps 11 and 12.

14. Gently resuspend beads in 50 µl of 1× reaction buffer.

15. Separate beads, then remove and discard supernatant using a pipette.

16. Resuspend beads in 3 µl distilled water

C. cDNA probe synthesis

Proceed with the following steps, using reagents included with your Hybond Atlas Arrays.

1. Prepare a master mix for all labelling reactions plus one extra reaction (to ensure that you have sufficient volume). Combine the following in a 0.5 ml microcentrifuge tube **at room temperature:**

	per rxn	4 rxns
5× Reaction buffer	4 µl	16 µl
10× dNTP mix (for dATP label)	2 µl	8 µl
[α-^{32}P]dATP (3000 Ci/mmol, 10 µCi/µl)		
or		
[α-^{33}P]dATP (> 2500 Ci/mmol, 10 µCi/µl)	5 µl	20 µl
DTT (100 mM)	0.5 µl	2 µl
Total volume	11.5 µl	46 µl

2. Preheat a PCR thermal cycler to 65 °C.

3. Add 4 µl primer mix from Hybond Atlas Array to the resuspended beads. Mix well by pipetting.

4. Incubate the beads and primer mix in the preheated thermal cycler at 65 °C for 2 min.

5. Reduce the temperature of the thermal cycler to 50 °C (or 48 °C if using an unregulated heating block or water bath); incubate tubes for 2 min. During this incubation, add 2 µl reverse transcriptase per reaction to the master mix (add 8 µl reverse transcriptase for the

4-reaction master mix). Mix by pipetting, and keep the master mix at room temperature.

6. After completion of the 2-min incubation at 50 °C, add 13.5 µl of the master mix to each reaction tube. Mix the contents of the tubes thoroughly by pipetting and immediately return them to the thermal cycler.

7. Incubate tubes at 50 °C (or 48 °C) for 25 min.

8. Add 2 µl 10× termination mix. Mix well.

Now proceed with column chromatography to purify your labelled probe as described in the Hybond Atlas cDNA Expression Arrays instructions (see page 31 Section ❸C).

Additional information

Troubleshooting guide

Problems	Possible causes
❶ **Lower than anticipated yield of purified total RNA**	Different types of tissues will yield different amounts of total RNA. For example, 100 mg of rat liver generally yields 600 µg of total RNA, whereas 100 mg of rat skeletal muscle generally yields only 90 µg. Table 2 lists approximate RNA yields for several types of tissues and cells. If the yield of purified total RNA is significantly lower than anticipated, consider the following: 1. **Possible cause:** Sample was not adequately homogenized. **Solution:** Use a tissue homogenizer (e.g., Polytron or equivalent), and/or homogenize for longer. 2. **Possible cause:** RNA pellet was not completely dissolved. **Solutions:** ● Let pellet soak in water longer before resuspending. ● Try pipetting the RNA solution up and down vigorously for 1 min. ● Do not vacuum-dry the pellet. ● Heat the sample at 70 °C for an extra 5 min. If sample is very viscous, add more deionized water and continue pipetting to dissolve. ● Low yields are often the result of degraded RNA. Check the RNA on a Formaldehyde gel. See page 19 Section ❸B.
❷ **Degraded RNA**	1. **Possible cause:** Tissue or cell culture sample may not have been quickly and completely homogenized in the denaturing solution. **Solution:** Ensure that you move the shaft of the

homogenizer around the sides of the tube and up and down while homogenizing for best results. Check periodically for darker clumps of tissue or cells that have not been homogenized. Do not stop until you are sure the sample is completely homogenized. Additionally, doubling the volume of the denaturing solution may help.

2. **Possible cause:** Tissues may have been old, may not have been frozen immediately after harvesting, or may have thawed before the denaturing solution was added. Fresh human tissue is often difficult to obtain so degradation is likely to occur.
   Solution: Next time, use tissue samples immediately after harvesting or flash freeze in liquid nitrogen and store at -70 °C. When using frozen samples, do not allow samples to thaw before adding the denaturing solution. Avoid thawing the samples by using liquid Nitrogen if necessary when manipulating the samples.
3. **Possible cause:** Equipment and materials used in the preparation may have not been RNase-free.
   Solution: Use new, sterile, disposable centrifuge tubes and supplies. Always wear gloves and change them frequently. Make solutions with deionized water. Bake glassware at 300 °C for 4 h, or 180 °C for 8 h, before use. If you still see degradation, we recommend using Ambion's ANTIRNase (#2692) at a concentration of 1 unit/µl throughout the protocol.

❸

RNA is contaminated by protein and/or DNA

Protein contamination is characterized by a low A_{260}/A_{280} ratio. Genomic DNA contamination causes background signal when hybridizing the cDNA probe to the Hybond Atlas Array.

1. **Possible cause:** Not enough rounds of Phenol:Chloroform extraction were performed. This is especially a problem for RNase-rich tissues.

Solution: Perform a total of 3–4 rounds of Phenol:Chloroform extraction.

2. **Possible cause:** The lower phenol phase or the interphase may have been disturbed during extraction of the aqueous phase.
   Solution: Repeat Phenol:Chloroform extraction, and then precipitate as in Step ❷C.11, page 16.
3. **Possible cause:** Sample was too viscous to efficiently separate RNA from DNA. If the tissue homogenate is too viscous, RNA will not be efficiently resolved from DNA and proteins during Phenol: Chloroform extraction.
   Solutions:

- Double the volume of denaturing solution.
- Pass homogenate through a 26–30 gauge needle to shear DNA.

4

Homogenate and/or aqueous phase darkens or turns yellow after exposure to denaturing solution.

This often occurs when a tissue sample (e.g., liver, heart) is rich in blood. This discoloration will not affect RNA quality, so there is no need for concern. No corrective measures need be taken.

Reference

Sambrook, J. *et al*., Molecular Cloning: A Laboratory Manual, 2nd Edition, (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (1991).

Legal

Product information

Product name	code
Hybond Atlas RNA Purification and Labelling System	25-8005-67

Related products

Hybond Atlas cDNA Expression Arrays	
Human 1.2	RPN7850
Human Cancer 1.2	RPN7851
Human 1.2 II	RPN7852
Mouse 1.2	RPN7853
Rat 1.2	RPN7854
Human 1.2 III	RPN7855
Rat 1.2 II	RPN7856
Mouse 1.2 II	RPN7857
Mouse Cancer 1.2 II	RPN7858
Hybond AtlasT Human Array Trial Kit	RPN7882
Hybond Northern Blots	
Human d 12 poly A^+ RNAs	RPN4800
Human a 8 poly A^+ RNAs	RPN4801
Human b 7 poly A^+ RNAs	RPN4802
Human c 8 poly A^+ RNAs	RPN4803
Mouse a 8 poly A^+ RNAs	RPN4804
[α-^{32}P] dATP, 10 μCi/μl, 3000 Ci/mmol	AA0004
[α-^{33}P] dATP, 10 μCi/μl,> 2500 Ci/mmol	AH9904
Digital Imaging	
Typhon 8600 variable mode imager	

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