

Atlas[®] SMART[™] Probe Amplification Kit User Manual

Supplement for the Super SMART[™]
PCR cDNA Synthesis Kit
(Cat. No. 635000)

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I. Introduction

The advent of array technology has allowed researchers to perform high-volume screening of many genes in a single expression analysis experiment. Our Atlas® Arrays have become a leading choice among researchers for performing such expression profiling analyses. Atlas Arrays have a wide range of applications, including investigating normal biological and disease processes as well as discovering potential therapeutic drug targets and diagnostic markers. However, the application of array technology is often limited because substantial amounts of RNA are required for standard labeling techniques. The **Atlas SMART™ Probe Amplification Kit** solves this problem by employing our PCR-based SMART technology to amplify limiting amounts of starting RNA, while maintaining the relative expression profile of transcripts between samples.

When used in conjunction with our Super SMART PCR cDNA Synthesis Kit, the Atlas SMART Probe Amplification Kit allows researchers to synthesize highly sensitive array probes from minimal starting material (Gonzalez et al., 1999; Livesey et al., 2000). Using this method, probes made from small amounts of total RNA produce results that are comparable to those from pure poly A⁺ RNA—a clear advantage when only limited amounts of tissues or cells are available. In fact, the Atlas SMART Probe Amplification Kit allows you to generate array probes starting with RNA from as little as 100 cells or 0.01 mg tissue.

Super SMART™ cDNA synthesis technology

Super SMART cDNA synthesis starts with nanogram quantities of total RNA and a modified oligo(dT) primer (the 3' SMART CDS Primer II A) to initiate the first-strand synthesis reaction (Figure 1). When reverse transcriptase (RT) reaches the 5' end of the mRNA, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3' end of the cDNA. The SMART Oligonucleotide II A, which has an oligo(G) sequence at its 3' end, base-pairs with the deoxycytidine stretch, creating an extended template. RT then switches templates and continues replicating to the end of the oligonucleotide (Chenchik et al, 1998). The resulting full-length, single-stranded (ss) cDNA contains sequences that are complementary to the SMART Oligonucleotide. The SMART anchor sequence and the poly A sequence are then used as universal priming sites for end-to-end cDNA amplification by PCR.

Advantage® 2 PCR Kit for long-distance PCR

We strongly recommend using the Advantage 2 PCR Kit (Cat. Nos. 639206 & 63907) with the Super SMART PCR cDNA Synthesis Kit. The Advantage 2 PCR Kit includes the Advantage 2 Polymerase Mix, formulated for efficient, accurate, and convenient amplification of cDNA templates by long-distance PCR (LD PCR; Barnes, 1994). The mix contains TITANIUM™ Taq DNA Polymerase—a nuclease-deficient N-terminal deletion of Taq DNA polymerase plus TaqStart® Antibody to provide automatic hot-start PCR (Kellogg et al., 1994)—and a minor amount

I. Introduction continued

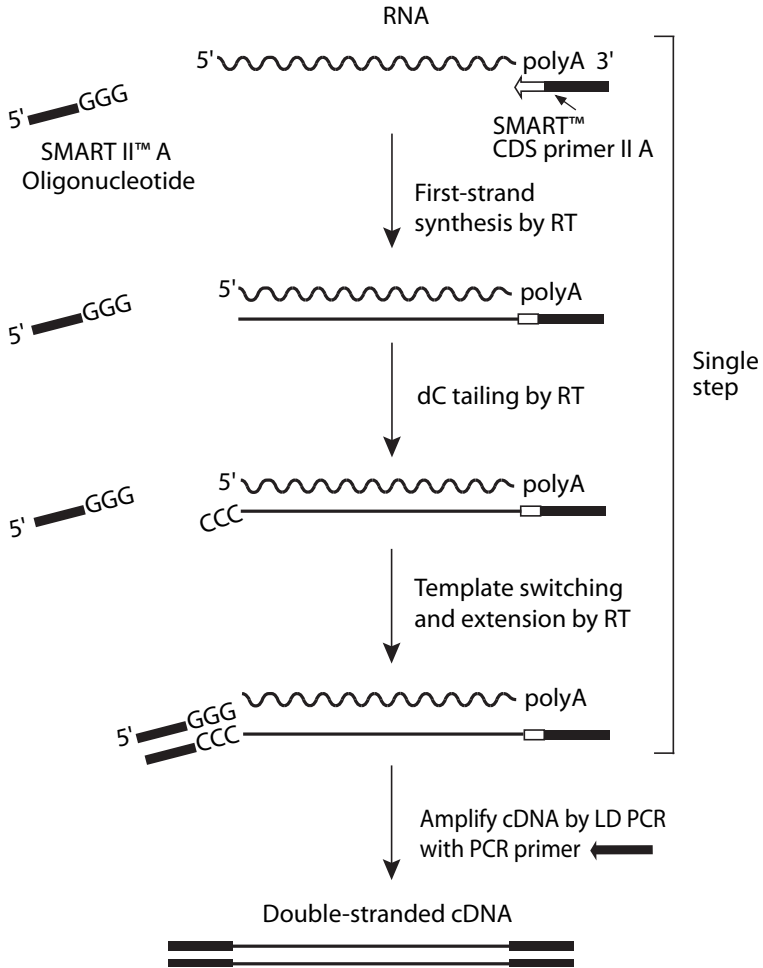


Figure 1. Flow chart of the SMART cDNA synthesis and amplification method. The SMART II Oligonucleotide, CDS primer, and PCR primer all contain a stretch of identical sequence (see the Super SMART PCR cDNA Synthesis Kit User Manual for complete sequence information).

I. Introduction continued

of a proofreading polymerase. This combination allows you to efficiently amplify full-length cDNAs with a significantly lower error rate than that of conventional PCR (Barnes, 1994).

Use of PCR amplification with array technology

Amplification of cDNA by the method described in the Super SMART User Manual has been shown to maintain the relative representation of each transcript in the original sample (Spirin et al., 1999; Wang et al., 2000). Use of the optimization method, described in Section V.C, also ensures that messages will not be amplified to saturation. Also, since array analysis compares the expression levels of individual genes between two samples, minor differences in amplification efficiency for different transcripts will be equivalent across samples and will have a negligible effect on the overall analysis.

Generation of probes for Atlas™ Arrays and for other types of arrays

Note: If you wish to generate SMART array probes for glass microarrays, use our Atlas SMART Fluorescent Probe Amplification Kit (Cat. No. 634714).

For nylon-based arrays, the Atlas SMART Probe Amplification Kit includes a Random Primer Mix (N9), which allows you to generate a probe representing **all** the transcripts in your sample. However, if you are synthesizing probes for use with our Atlas Nylon cDNA Expression Arrays, it is important that you use the CDS Primer Mix that is included with the array. Priming the probe labeling reaction with the CDS Primer Mix generates a specialized probe for the genes on the Atlas Nylon Array. Reducing probe complexity in this manner results in an approximately 10-fold increase in sensitivity, with a concomitant reduction in nonspecific background.

To use the Atlas SMART Probe Amplification Kit with Atlas Plastic Microarrays, it is essential that you use the SMART Random Primer Mix (N15) w/Synth. Control included with this kit. Using this random primer mix ensures optimal labeling of your probe with this kit. **Do not** use the Random Primer Mix that comes with your plastic microarray if you plan to generate a probe using this kit.

Important: When generating array probes for two samples it is essential to generate both probes using the same technique. Do not use the Atlas SMART procedure to generate one probe and the standard Atlas procedure to generate the other.

Comparing arrays screened with amplified probe to arrays screened with non-amplified probe will yield inaccurate results.

II. List of Components

Note: This kit is a supplement to the Super SMART PCR cDNA Synthesis Kit. **Components contained in the Super SMART™ PCR cDNA Synthesis Kit are required for the protocols in this User Manual.** See Additional Materials Required for more information.

Store NucleoSpin® Extraction Kits at room temperature.
Store all other reagents at -20°C .

Box 1:

- 30 μl **Klenow Enzyme** (2 units/ μl)
- 75 μl **10X dNTP Mix** (for dATP label)
- 75 μl **10X dNTP Mix** (for dCTP label)
- 75 μl **10X Labeling Buffer**
- 15 μl **Random Primer Mix** (N9, 1 $\mu\text{g}/\mu\text{l}$)
- 15 μl **SMART Random Primer Mix (N15) w/Synth. Control**
- 2 x 750 μl **SMART Blocking Solution for Nylon Arrays**
- 45 μl **SMART Blocking Solution for Plastic Arrays**

Box 2:

- 2 **Atlas NucleoSpin® Extraction Kits**

III. Additional Materials Required

The following reagents are required but not supplied:

Analysis of RNA Quality

- **Formamide** (Sigma Cat. No. F9037)
- **12.3 M formaldehyde** (Sigma Cat. No. F8775)
- **10X 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 Optical Density** (store at room temperature)
 - 10 mM Tris (pH 7.5)
 - 0.1 mM EDTA (pH 7.5)

First-Strand cDNA Synthesis and Super SMART PCR Amplification

- **Reagents included with the Super SMART PCR cDNA Synthesis Kit (Cat. No. 635000):**
 - SMART II A Oligonucleotide
 - 3' SMART CDS Primer II A
 - PowerScript™ Reverse Transcriptase
 - 5' PCR Primer II A
 - dNTP Mix
 - Dithiothreitol (DTT)
 - Control Human Placenta Total RNA
 - **Advantage® 2 PCR Kit (Cat. Nos. 639206 & 639207)**
 - [Optional] **Mineral oil** (Sigma Cat. No. M3516)
 - **TE buffer** (10 mM Tris [pH 7.6], 1 mM EDTA)
 - **DNA size markers** (1-kb DNA ladder; LTI Cat. No. 15615-016)
 - **50X TAE electrophoresis buffer**
 - 242.0 g Tris base
 - 57.1 ml glacial acetic acid
 - 37.2 g Na₂EDTA•2H₂O
- Add H₂O to 1 L.

III. Additional Materials Required continued

Probe Synthesis

- **For Atlas Nylon Arrays:**
 - CDS Primer Mix (included with the array)
- [α -³²P]dATP (10 μ Ci/ μ l; 3,000 Ci/mmol; GE Healthcare Cat. No. PB10204)
OR
- [α -³³P]dATP (10 μ Ci/ μ l; >2,500 Ci/mmol; GE Healthcare Cat. No. BF1001)
OR
- [α -³²P]dCTP (10 μ Ci/ μ l; 3,000 Ci/mmol; GE Healthcare Cat. No. PB10205)

Note: Do not use GE Healthcare Redivue or any other dye-containing isotope.

Hybridization

For Atlas Nylon cDNA Expression Arrays, we recommend that you use ExpressHyb Hybridization Solution (included with each Atlas Nylon Array), a low-viscosity hybridization solution that significantly reduces hybridization time and background (Yang & Kain, 1995). If you are using a Atlas Plastic Microarray, use the PlasticHyb Hybridization Solution included with your array. Otherwise, use the hybridization solution recommended by your array's manufacturer, or the General Hybridization Solution described below.

- **ExpressHyb™ Hybridization Solution** (included with Atlas cDNA Expression Arrays; also available separately under Cat. Nos. 636831, 636832, and 636833)
- **PlasticHyb Hybridization Solution** (included with Atlas Plastic Microarrays; also available separately under Cat. No. 634807)
- **General Hybridization Solution**

5X	SSPE
10X	Denhardt's solution
100 μ g/ml	Denatured sheared salmon testes DNA
2% (w/v)	SDS (Sigma Cat. No. L5409)

For short term storage (\leq 2 weeks), keep solution at 4°C; for long term storage (>2 weeks), keep at -20°C.

- **Atlas Plastic Array Hybridization Box** (Cat. No. 634810)
This hybridization box was designed to precisely fit our plastic microarrays and keep them flat during hybridization and washing. We strongly recommend using this box instead of roller bottles, which may curl the plastic film and lead to difficulties in imaging your array. Also, due to the plastic material, decontamination after experiments is easier than for roller bottles.

III. Additional Materials Required continued

- **Washes for Nylon Arrays**

Wash Solution N1

2X	SSC
1%	SDS

Store at room temperature.

Wash Solution N2

0.1X	SSC
0.5%	SDS

Store at room temperature.

- **Washes for Plastic Microarrays**

Wash Solution P1

2X	SSC
0.1%	SDS

Store at room temperature.

Wash Solution P2

0.1X	SSC
0.1%	SDS

Store at room temperature.

Wash Solution P3

0.1X	SSC
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Store at room temperature.

IV. General Considerations

- This kit is a supplement to the Super SMART PCR cDNA Synthesis Kit. **You must have the Super SMART PCR cDNA Synthesis Kit to perform the protocols in this User Manual.**
- This User Manual provides complete protocols for probe synthesis and purification, and membrane hybridization. Protocols for SMART cDNA synthesis and purification are described in the Super SMART PCR cDNA Synthesis Kit User Manual (PT3656-1). The Atlas cDNA Expression Arrays User Manual (PT3140-1) and the Atlas Plastic Microarrays User Manual (PT3591-1) provide important background information, tips for array analysis and normalization as well as additional troubleshooting suggestions. We strongly recommend that you thoroughly read all relevant User Manuals before beginning the procedure.
- When generating array probes for two samples it is essential to generate both probes using the same technique. Do not use the Atlas SMART procedure to generate one probe and the standard Atlas procedure to generate the other. **Comparing arrays screened with amplified probe to arrays screened with non-amplified probe will yield inaccurate results.**
- Proper template switching, which is essential to the SMART technology, requires the use of an MMLV RNase H⁻ point mutant (not a deletion mutant) reverse transcriptase, such as our PowerScript Reverse Transcriptase. **Do not use the reverse transcriptase included with Atlas Arrays unless it is the PowerScript™ enzyme** (refer to your array's Product Analysis Certificate). See Section III for details.
- The SMART Blocking Solutions included with this kit contain components that are essential for reducing background when using array probes generated with SMART technology. Be sure to add SMART Blocking Solution to your hybridization solution.
- The success of your experiment depends on the quality of your total RNA. There are several procedures available for RNA isolation (Chomczynski & Sacchi, 1987; Farrell, 1993; Sambrook et al., 1989).
- Before you begin first-strand synthesis, we strongly recommend that you check the integrity of your RNA. For determining the integrity of human total RNA samples, we recommend that you use the Clontech RNA/cDNA Quality Assay (Cat. No. 636841). Alternatively, you can check RNA quality by electrophoresing a sample on a formaldehyde/agarose/EtBr gel (see Appendix). For mammalian total RNA, you should observe two bright bands at approximately 4.5 and 1.9 kb; these bands represent 28S and 18S ribosomal RNA. The ratio of intensities of these bands should be 1.5–2.5:1. For more information, see Sambrook et al. (1989).

IV. General Considerations continued

- Wear gloves throughout the procedure to protect your RNA and cDNA samples from degradation by nucleases.
- The first time you use this kit, you should use the Control Human Placenta Total RNA provided with the Super SMART PCR cDNA Synthesis Kit, to synthesize cDNA in parallel with your experimental samples. Generating cDNA with this control will verify that all components are working properly, including the reverse transcriptase, and will also help you troubleshoot any problems that may arise.
- The cycling parameters in this protocol have been optimized using an authorized hot-lid thermal cycler. Optimal parameters may vary with different thermal cyclers and templates.
- To resuspend pellets and mix reaction components, gently pipet up and down, then centrifuge briefly to recover contents to the bottom of the tube.
- Add enzymes to reaction mixtures last, and thoroughly incorporate the enzyme by gently pipetting the reaction mixture up and down.
- Do not change the amount of enzyme or DNA used in the reactions. The amounts and concentrations have been carefully optimized.
- If you are using a Atlas Nylon Array, use the CDS Primer Mix provided with your array. Use the Random Primer Mix (N9; included in this kit) if you are using a nylon array from another manufacturer.
- When using a Atlas Plastic Microarray, use the SMART Random Primer Mix (N15) w/Synth. Control included in this kit.
- If you are not using Atlas Arrays, please follow the hybridization instructions provided by your array's manufacturer.

V. Array Probe Synthesis and Purification

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

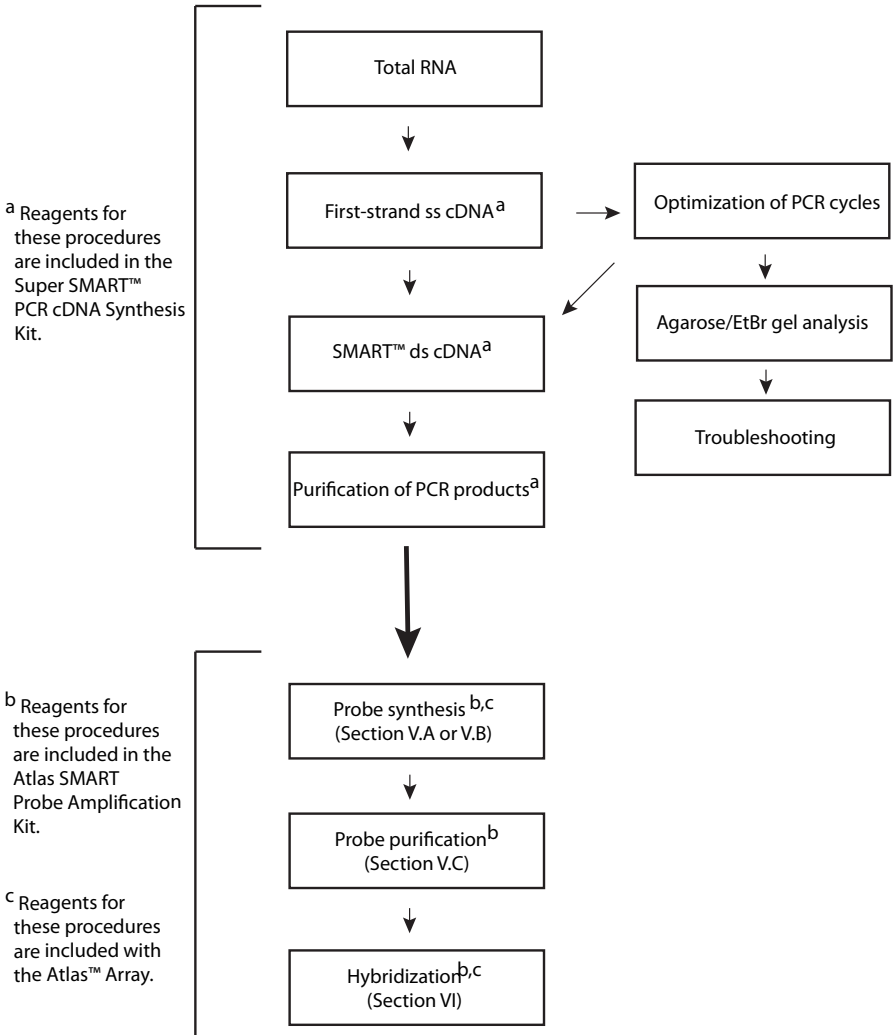


Figure 2. Overview of the Atlas SMART Probe Amplification procedure.

V. Array Probe Synthesis and Purification continued

Important: Be sure to follow the protocol designed for your particular array.

A. SMART cDNA Probe Synthesis for Nylon Arrays

The 50- μ l reaction described below converts 500 ng of purified SMART cDNA into 32 P- or 33 P-labeled cDNA. To generate SMART cDNA, follow the protocols for First-Strand cDNA Synthesis, Column Chromatography, cDNA Amplification, and Spin Column Purification as described in the Super SMART PCR cDNA Synthesis Kit User Manual (PT3656-1).

1. Preheat a PCR thermal cycler to 50°C.
2. In separate 0.5-ml microcentrifuge tubes, mix 1 μ l CDS Primer Mix (included with your Atlas Nylon Array) or 1 μ l Random Primer Mix (N9; included with this kit for other array users) with 500 ng of purified SMART cDNA. To each tube, add deionized H₂O to a final volume of 33 μ l.
3. Denature samples by heating at 95–100°C for 5 min, then chill on ice for 2 min.
4. While samples are being denatured, prepare a Master Labeling Mix for each labeling reaction plus one extra. Combine the following reagents in a 0.5-ml microcentrifuge tube **at room temperature** (do not add Klenow enzyme until just before adding labeling mix to samples):

	per rxn	3 rxns
10X Labeling Buffer	5 μ l	15 μ l
10X dNTP Mix (for dATP label)*	5 μ l	15 μ l
$[\alpha\text{-}^{32}\text{P}]\text{dATP}$ (3,000 Ci/mmol, 10 μ Ci/ μ l)	5 μ l	15 μ l
-or-		
$[\alpha\text{-}^{33}\text{P}]\text{dATP}$ (>2,500 Ci/mmol, 10 μ Ci/ μ l)		
Total volume Master Labeling Mix	15 μl	45 μl

*Substitute 10X dNTP Mix (for dCTP label) if labeling with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$.

5. After denaturation is complete, spin tubes briefly in a microcentrifuge to collect the sample contents at the bottom.
6. Incubate tubes in a preheated PCR thermal cycler at 50°C for 2–3 min.
7. Add 2 μ l Klenow enzyme per reaction to the Master Labeling Mix (i.e., 6 μ l for a 3-reaction Master Mix). Mix well.
8. After completion of the incubation at 50°C, add 17 μ l of Master Labeling Mix to each reaction tube.

Note: Do not remove the cDNA samples from the thermal cycler for longer than is necessary to add the Master Mix.
9. Mix the contents of the tubes by pipetting and immediately return them to the thermal cycler.

V. Array Probe Synthesis and Purification continued

10. Incubate tubes in the PCR thermal cycler at 50°C for 30–45 min.
11. Stop the reaction by adding 2 µl of 0.5 M EDTA.
12. Proceed with the probe purification in Part C. If necessary, you can store your probe on ice or at 4°C for a few hours.

B. SMART cDNA Probe Synthesis for Plastic Microarrays

The 50-µl reaction described below converts 500 ng of purified SMART cDNA into ³³P-labeled cDNA. To generate SMART cDNA, follow the protocols for First-Strand cDNA Synthesis, Column Chromatography, cDNA Amplification, and Spin Column Purification as described in the Super SMART PCR cDNA Synthesis Kit User Manual (PT3656-1).

1. Preheat a PCR thermal cycler to 37°C.
2. In separate 0.5-ml microcentrifuge tubes, mix 1 µl SMART Random Primer Mix (N15) w/Synth. Control (included in this kit) with 500 ng of purified SMART cDNA. To each tube, add deionized H₂O to a final volume of 33 µl.
3. Denature samples by heating at 95–100°C for 5 min, then chill on ice for 2 min.
4. While samples are being denatured, prepare a Master Labeling Mix for each labeling reaction plus one extra. Combine the following reagents in a 0.5-ml microcentrifuge tube **at room temperature** (do not add Klenow enzyme until just before adding labeling mix to samples):

	per rxn	3 rxns
10X Labeling Buffer	5 µl	15 µl
10X dNTP Mix (for dATP label)	5 µl	15 µl
[α- ³³ P]dATP (>2,500 Ci/mmol, 10 µCi/µl)	5 µl	15 µl
Total volume Master Labeling Mix	15 µl	45 µl

5. After denaturation is complete, spin tubes briefly in a microcentrifuge to collect the sample contents at the bottom.
 6. Incubate tubes in a preheated PCR thermal cycler at 37°C for 2–3 min.
 7. Add 2 µl Klenow enzyme per reaction to the Master Labeling Mix (i.e., 6 µl for a 3-reaction Master Mix). Mix well.
 8. After completion of the incubation at 37°C, add 17 µl of Master Labeling Mix to each reaction tube.
- Note:** Do not remove the cDNA samples from the thermal cycler for longer than is necessary to add the Master Mix.
9. Mix the contents of the tubes by pipetting and immediately return them to the thermal cycler.
 10. Incubate tubes in the PCR thermal cycler at 37°C for 30–45 min.
 11. Stop the reaction by adding 2 µl of 0.5 M EDTA.

V. Array Probe Synthesis and Purification continued

12. Proceed with the probe purification in Part C. If necessary, you can store your probe on ice or at 4°C for a few hours.

C. Array Probe Purification

To purify the labeled cDNA from unincorporated ³²P- or ³³P-labeled nucleotides and small (<0.1 kb) cDNA fragments, use the provided NucleoSpin Extraction Kit and follow the procedure described below. Before beginning, ensure that 95% ethanol has been added to Buffer NT3 according to the instructions on the bottle.

1. Dilute probe synthesis reactions to 400 µl total volume with Buffer NT2; mix well by pipetting.
2. Place a NucleoSpin Extraction Spin Column into a 2-ml Collection Tube, and pipet the sample into the column. Centrifuge at 14,000 rpm for 1 min. Discard Collection Tube and flowthrough into the appropriate container for radioactive waste.
3. Insert the NucleoSpin column into a fresh 2-ml Collection Tube. Add 500 µl Buffer NT3 to the column. Centrifuge at 14,000 rpm for 1 min. Discard Collection Tube and flowthrough.
4. Repeat Step 3 two more times.
5. Transfer the NucleoSpin column to a clean 1.5-ml microcentrifuge tube. Pipette 100 µl Buffer NE directly onto the filter, being careful not to touch the surface of the filter with the pipette tip. Allow filter to soak for 2 min.
6. Centrifuge at 14,000 rpm for 1 min to elute purified probe. Discard filter.
7. Check the radioactivity of the probe by scintillation counting:
 - a. Add 2 µl of each purified probe to 5 ml of scintillation fluid in separate scintillation-counter vials.
 - b. Count ³²P- or ³³P-labeled samples on the ³²P channel, and calculate the total number of counts in each sample. (Multiply counts by a dilution factor of 50.)

Probes synthesized using the procedure in this User Manual should have a total of **>5 x 10⁶ cpm** for probes made with the Atlas CDS Primer Mix (included with Atlas Nylon Arrays), **>10³ cpm** for probes made with the SMART Random Primer Mix (N15) w/Synth. Control (included in this kit), or **>10⁷ cpm** for probes made with the Random Primer Mix (N9; included with this kit). Store probes at -20°C.

Discard flowthrough fractions, columns, and elution tubes in the appropriate container for radioactive waste.

VI. Array Hybridization

If you are using Atlas Nylon Arrays (or other nylon-based array), follow the procedure outlined in Section VI.A. For Atlas Plastic Microarrays, a separate array hybridization procedure is described in Section VI.B .

For best results with nylon arrays, we recommend that you use ExpressHyb Hybridization Solution, a low-viscosity hybridization solution that significantly reduces hybridization time and background (Yang & Kain, 1995). ExpressHyb solution is included with each Atlas Nylon Array. For Atlas Plastic Microarrays, we recommend that you use the PlasticHyb Hybridization Solution included with the array. You may also purchase ExpressHyb solution separately or use the hybridization solution recommended by your array's manufacturer. A recipe for a General Hybridization Solution is also provided in Section III.

The SMART Blocking Solutions included with this kit contain components that are essential for reducing background when using array probes generated with SMART technology. Be sure to add the array-specific SMART Blocking Solution to your hybridization solution in the manner described below.

Note: Because probes made from the Control RNA generate strong hybridization signals, we suggest that you do not hybridize control probes to Atlas Arrays unless you have problems with your experiment. See the Troubleshooting Guide in the Atlas Arrays User Manual for more information.

A. Nylon Array Hybridization

1. Prepare a mixture of ExpressHyb Hybridization Solution and SMART Blocking Solution for Nylon Arrays:
 - a. Prewarm 5 ml of Hybridization Solution at 68°C.
 - b. Heat 50 µl of Blocking Solution for Nylon Arrays at 95–100°C for 5 min, then chill quickly on ice.
 - c. Mix the heated Blocking Solution with prewarmed Hybridization Solution. Keep at 68°C until use.
2. Fill a hybridization bottle with deionized H₂O. Wet the array by placing it in a dish of deionized H₂O, remove, shake off excess, and then place the membrane into the bottle. Pour off any remaining water from the bottle; the membrane should adhere to the inside walls of the container without creating air pockets. Add 5 ml of the solution prepared in Step 1. Ensure that the solution is evenly distributed over the membrane. Perform this step quickly to prevent the array membrane from drying.
3. Prehybridize for 30 min with continuous agitation at 68°C.

Notes:

 - Do not remove the array from the container during the prehybridization, hybridization, or washing steps.
 - If performing the hybridization in roller bottles, rotate at 5–7 rpm during prehybridization and hybridization steps.
4. Add 50 µl of Blocking Solution for Nylon Arrays to your purified probe; mix well.

VI. Array Hybridization continued

5. Boil probe mix for 5 min, then chill on ice for 2 min.
6. Add the mixture prepared in Step 5 directly to your array and prehybridization solution. Be sure to avoid pouring the concentrated probe directly on the surface of the membrane. Make sure that the two solutions are mixed together.
7. Hybridize overnight with continuous agitation at 68°C. Be sure that all regions of the membrane are in contact with the hybridization solution at all times. If necessary, add an extra 2–3 ml of prewarmed ExpressHyb solution.
8. The next day, prewarm Wash Solution N1 (2X SSC, 1% SDS) and Wash Solution N2 (0.1X SSC, 0.5% SDS) at 68°C.
9. Carefully remove the hybridization solution and discard in an appropriate radioactive waste container. Replace with 200 ml of prewarmed Wash Solution N1. Wash the array for 30 min with continuous agitation at 68°C. Repeat this step three more times.
Note: If using roller bottles, fill to 80% capacity and rotate at 12–15 rpm during all wash steps.
10. Perform one 30-min wash in 200 ml of prewarmed Wash Solution N2 with continuous agitation at 68°C.
11. Perform one final 5-min wash in 200 ml of 2X SSC with agitation at room temperature.
12. Using forceps, remove the 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 will be difficult.**
13. Immediately wrap the damp membrane in plastic wrap.
14. Mount the plastic-wrapped array on Whatman paper (3 MM Chr). Expose the array to x-ray film at –70°C with an intensifying screen. When setting up your exposure, make sure you are aware of the orientation of the array by noting the position of the orientation notch at the upper right-hand corner of the membrane. Also, be sure to try several exposures for varying lengths of time (e.g., 3–6 hr, overnight, and 3 days). Alternatively, use a phosphorimager. When exposing the array to a phosphorimaging screen at room temperature, be sure to seal the array membrane in plastic to prevent drying.

VI. Array Hybridization continued

B. Plastic Microarray Hybridization

The hybridization procedure described below is optimized for use with the Atlas Plastic Array Hybridization Box. Use of this hybridization box, or an equivalent container, is strongly recommended to ensure that the array is kept flat during the experiment. If you do not have a rocking platform, which allows you to use our hybridization box (we recommend the Rocker Platform [Cat. No. HS9355] from Continental Lab Products, which conveniently fits in hybridization ovens), you may use the roller bottle hybridization procedure in the Appendix of the Atlas Plastic Microarrays User Manual (PT3591-1).

- As a general rule for any hybridization vessel, ensure that there is sufficient PlasticHyb Hybridization Solution to completely bathe the microarray.
- Ensure that the printed surface of the microarray is facing up. See Section IV of the Plastic Microarrays User Manual (PT3591-1) for tips on orienting your microarray.
- Ensure that the box is well sealed and continuously agitated on a rocking platform during all steps.
 1. Prerinsing the Plastic Microarray [Optional]
 - a. Fill a Atlas Hybridization Box ~80% full with H₂O and warm to 55–60°C. Also prewarm 30 ml of PlasticHyb Hybridization Solution at 60°C in a separate container.
 - b. Carefully place the microarray into the hybridization box containing the prewarmed H₂O, with the printed surface **facing up**.
 - c. Pour off the H₂O and replace with 15 ml of prewarmed Hybridization Solution. Firmly attach the lid.
 - d. Rock the microarray for 10–30 min at 60°C.
 2. Add 3 µl of Blocking Solution for Plastic Arrays with your purified probe; mix well.
 3. Denature probe mix for 5 min at 95°C, then chill on ice for 2 min.
 4. Combine the denatured probe mix with 15 ml of prewarmed hybridization solution in a disposable 50-ml or 15-ml plastic tube. Make sure that the two solutions are thoroughly mixed together.
 5. Carefully pour off the pre-rinsing solution from the microarray and replace with the mixture prepared in Step 4.
 6. Hybridize overnight with continuous agitation at 60°C and ≥6 rpm. Ensure that all regions of the plastic are in contact with the hybridization solution at all times. Full coverage is critical because the plastic is nonporous and does not absorb liquid. If necessary, add an extra 2–3 ml of prewarmed hybridization solution.

VI. Array Hybridization continued

7. The next day, prewarm 300 ml of Wash Solution P1 (2X SSC, 0.1% SDS) and 300 ml of Wash Solution P2 (0.1X SSC, 0.1% SDS) to 58–60°C. Fill a 500 ml beaker with room-temperature Wash Solution P3.
8. Open the box containing your hybridizing microarray. Leaving the microarray inside the box, carefully pour off the hybridization solution into an appropriate container for radioactive waste. Immediately fill the hybridization box with 40–50 ml prewarmed Wash Solution P1. Do not allow the microarray to dry. Do not allow the box or the Wash Solution to cool. Reattach the lid and rock for 5 min at 58°C to remove residual radioactive probe. Discard the Wash Solution.
9. Repeat the wash in Step 8 one time.
10. After pouring off the second wash with Wash Solution P1, fill the hybridization box with 40–50 ml prewarmed Wash Solution P2 (from Step 7). Wash the microarray in a 58°C incubator for 5 min.
11. Reduce the temperature of the hybridization oven to 25–30°C. Pour off the Wash Solution P2, and fill the box to approximately 80% capacity (40–50 ml) with **room-temperature** Wash Solution P3 (0.1X SSC). Rock the microarray for 5 min.
12. Remove the microarray from the hybridization box using forceps. Immediately transfer the microarray to the beaker of room-temperature Wash Solution P3 (0.1X SSC). Rinse the microarray by dipping it several times into the Wash Solution.
13. Remove the microarray from the beaker of Wash Solution P3 very slowly, allowing the Wash Solution to drain off the surface. Usually, only small droplets of Wash Solution will remain on the microarray after this step. If large droplets are present, dip the microarray into the Wash Solution and slowly remove it again. If large droplets still remain, they can be removed by absorption with a dust-free tissue. Failure to remove these large droplets can cause “plaques” in the microarray image.
14. Allow the microarray to air-dry completely (about 5–10 min). **Do not** use a heating device to rapidly dry the microarray.
15. Expose the printed surface of the microarray to a phosphorimaging screen suitable for ³³P detection. If for any reason the plastic is warped, affix the microarray to the phosphorimaging cassette with adhesive tape along the entire length of the microarray edges. Ensure complete contact with the phosphorimager screen. **Do not** cover the microarray with plastic wrap. Typical exposure times range from 12–72 hr, but longer exposures can be performed, as necessary. When the exposure is complete, scan the phosphorimager screen at a resolution of 50 µm or higher.

VI. Array Hybridization continued

C. Stripping cDNA Probes from Atlas Arrays

To re-use Atlas Nylon or Plastic Arrays after phosphorimaging, you may remove the cDNA probe by stripping. For successful stripping, please refer to the Atlas cDNA Expression Arrays User Manual (PT3140-1) or the Atlas Plastic Microarrays User Manual (PT3591-1).

VII. Troubleshooting Guide

A. Problems with First-Strand cDNA Synthesis and SMART PCR

For analysis of cDNA amplification results and general troubleshooting of SMART cDNA synthesis, please refer to the Super SMART PCR cDNA Synthesis Kit User Manual.

B. Hybridization Probe has Low Specific Activity

1. If the [α -³²P]dATP or [α -³²P]dCTP you used for labeling was older than one week or your [α -³³P]dATP was older than two weeks, make a new probe with fresh nucleotide. Some lots of radioactive nucleotide may inhibit incorporation. If you obtain $<0.5 \times 10^6$ cpm incorporation for the Control Human Placenta Total RNA, procure a new lot of radioactive nucleotide and repeat the labeling procedure.
2. Your experimental cDNA sample contains impurities that inhibit probe synthesis and labeling.

Purification of the amplified cDNA is required for proper probe synthesis. Be sure to closely follow the protocol in the Super SMART PCR cDNA Synthesis Kit User Manual. The efficiency of purification will be diminished if the protocol is not followed exactly. In particular, using too much or too little column buffer in the washing and elution steps, or omitting the final spin before elution, is likely to cause contamination of cDNA with ethanol or high salt. These impurities will inhibit the labeling reaction.

3. Loss of Klenow activity

It is important to work quickly after adding Klenow to the Master Mix. If more than 8 minutes are allowed to elapse between adding the enzyme to the Master Mix and the addition of the Master Mix to the cDNA samples, labeling will be significantly impaired. Perform a control reaction by labeling the cDNA generated from the Control Human Placenta Total RNA to monitor the efficiency of the labeling reaction. Be sure to store Klenow at -20°C . Keep on ice while setting up reactions.

C. High Nonspecific Background (with or without hybridization signals)

Several factors may account for high levels of nonspecific background:

1. RNA used to make the hybridization probe is of poor quality
Check the quality of your RNA before performing first-strand synthesis. See the Appendix of this User Manual for specific protocols.
2. SMART Blocking Solution omitted from hybridization mix
The SMART Blocking Solution contains components that are essential for reducing background when using array probes generated with SMART technology. Be sure to add SMART Blocking solution to your hybridization solution.

VII. Troubleshooting Guide continued

3. Unincorporated radioactive nucleotide is not fully removed from the probe

If for some reason you did not purify your cDNA probe, use the NucleoSpin Extraction Spin Columns provided with the Atlas SMART Kit and follow the procedure described in Section V.C of this User Manual.

4. Probe is too old

Use your cDNA probe as soon as possible after preparation because radioactive decay results in probe fragmentation. Small radioactive decay products can contribute to high background.

5. Composition of hybridization mix is incorrect

When using Atlas Arrays, it is important to use the hybridization solution provided and follow the procedure described in Section VI of this User Manual. Do **not** substitute another hybridization solution.

Note: For additional tips on troubleshooting your hybridization, please refer to the Atlas cDNA Expression Arrays User Manual (PT3140-1) or the Atlas Plastic Microarrays User Manual (PT3591-1).

VIII. References

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IX. Related Products

For a complete listing of all Clontech products,
please visit www.clontech.com

<u>Product</u>	<u>Cat. No.</u>
• Super SMART™ PCR cDNA Synthesis Kit	635000
• Atlas® cDNA Expression Arrays	many
• Atlas® Plastic Microarrays	many
• AtlasImage™ 2.7 Software	634650
• ExpressHyb™ Hybridization Solution	636831 636832
• PlasticHyb Hybridization Solution	634807
• Atlas® Plastic Array Hybridization Box	634810
• Premium Total and Poly A ⁺ RNAs	many
• Multiple Tissue Northern (MTN®) Blots	many
• SMART™ cDNA Library Construction Kit	634901
• SMART™ PCR cDNA Synthesis Kit	634902
• SMART™ RACE cDNA Amplification Kit	634914
• Clontech RNA/cDNA Quality Assay	636841

Appendix: 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. Table I shows the representative RNA yield from a variety of tissues.

TABLE I: REPRESENTATIVE TOTAL RNA YIELDS

Tissue/Cell Source	Amount of Starting Material	Yield of Total RNA
Rat liver	100 mg	600 µg
Rat skeletal muscle	100 mg	90 µg
Mouse brain	100 mg	125 µg
Mouse spleen	100 mg	245 µg
Mouse testes	100 mg	240 µg
Mouse thymus	100 mg	85 µg
Human cerebellum	100 mg	85 µg
Human prostate tumor	100 mg	100 µg
MCF-7 cell line	1 x 10 ⁷ cells	70 µg
Mouse fibroblasts	1 x 10 ⁷ cells	800 µg
U251 cell line	1 x 10 ⁷ cells	95 µg

A. Determining A₂₆₀

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 Section III: Additional Materials Required) and mix by pipetting.
4. Transfer contents to a 1-ml glass cuvette with a 1-cm path length.
5. Measure A₂₆₀ and A₂₈₀ using O.D. Buffer as a reference blank.
6. Calculate RNA yield as follows:

RNA constant for 1-cm path length: One A₂₆₀ unit of RNA = 40 µg/ml

- Total A₂₆₀ = (A₂₆₀ of dilute sample) x (dilution factor)
- Concentration (µg/ml) = (total A₂₆₀) x (40 µg/ml)
- Yield (µg) = (total sample volume) x (concentration)

7. Calculate the A₂₆₀/A₂₈₀. Pure RNA has an A₂₆₀/A₂₈₀ 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: A₂₆₀ = 0.231; A₂₈₀ = 0.115

Appendix: Assessing Yield and Purity of Total RNA cont.

Calculations:

- Total $A_{260} = (0.231) \times (200) = 46.2$
- Concentration = $(46.2) \times (40) = 1,848 \mu\text{g/ml}$
- RNA yield = $(0.5 \text{ ml}) \times (1,848 \mu\text{g/ml}) = 924 \mu\text{g}$
- Purity = $0.231/0.115 = 2.01$

B. Preparing a 1% Denaturing Agarose Gel

1. Wash mini-gel box, gel tray (7 x 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 bottle on a magnetic stir-plate and stir slowly for 2 min to cool.
6. While stirring, add 10 ml of 10X MOPS buffer and 7.5 ml of 12.3 M formaldehyde.
7. Continue stirring for 1 min; then pour onto gel tray.
8. Allow at least 1 hr for the gel to solidify at room temperature.
Note: Do not use formaldehyde gels that have been stored longer than 24 hr.
9. Remove the gel comb and submerge the gel in gel box with 1X MOPS buffer.

C. RNA Sample Preparation

1. Prepare RNA loading solution immediately before running the gel (for 6–10 samples):

formaldehyde	45 μl
formamide	45 μl
10X 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 on 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.

Appendix: Assessing Yield and Purity of Total RNA cont.

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. 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.

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