Single-Round RNA Amplification and Biotin Labeling System

Instruction Manual

Cat. No. ENZ-42420-10 10 Reactions

For research use only.

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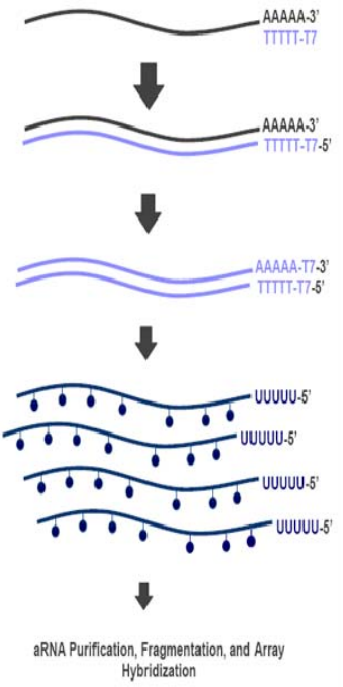
I. Introduction

The **Single-Round RNA Amplification and Biotin Labeling System** provides an optimized protocol and reagents for the production of biotin-labeled antisense RNA (aRNA) from total cellular RNA samples in less than 24 hours for array analysis. This kit has been improved to yield sufficient aRNA quantity for most microarray platforms (10 µg minimum) with total RNA input of as low as 250 ng and up to 5000 ng. The higher yield results from an improved cDNA purification system now provided with the kit. The Single-Round RNA Amplification and Biotin Labeling System has been optimized for superior performance with reduced variability and improved reproducibility and data quality.

The complete system is composed of reagents for cDNA synthesis, purification and *in vitro* transcript labeling.

II. Procedural Overview



First Strand cDNA Synthesis

- Add 1 µL T7 Oligo(dT) Primer to 0.25 - 5 µg total RNA.
- Bring total volume to 13 µL with nuclease-free water.
- Incubate at 70˚C for 10 minutes and move to 42°C.
- Add 7 µL of First Strand Master Mix.
- Incubate at 42˚C for 2 hours.

Second Strand cDNA Synthesis

- Add 130 µL Second Strand Master Mix.
- Incubate at 16˚C for 2 hours.

cDNA Purification

***In Vitro* Transcription and Biotin Labeling**

- Add 27 µL IVT Master Mix.
- Incubate at 37˚C for 6 hours for input RNA $\geq$ 2 µg.
- Incubate at 37˚C for 16 hours for input RNA 0.25-2 µg.

III. Reagents Provided and Storage

Store all components of Box A at –20°C in a non-frost-free freezer. Water can be left at room temperature after first thawing.

Table 1. Box A Components

Reagents	Min. Vol. Supplied	Vial ID
dNTP Mix	40 µL	**dN**
Promoter Primer	10 µL	**P**
First Strand Buffer	20 µL	**FB**
DTT	80 µL	**D**
Reverse Transcriptase	10 µL	**RT**
RNase Inhibitor	10 µL	**I**
DNA Polymerase	50 µL	**DP**
RNase H	10 µL	**RH**
Second Strand Buffer	150 µL	**SB**
IVT Reaction Buffer	60 µL	**RB**
10X Biotin-Labeled Ribonucleotide	60 µL	**B**
Enhancer Cocktail	60 µL	**EC**
T7 RNA Polymerase	30 µL	**T7**
Nuclease-free Water	2 mL	**W**

Store all components of Box B at room temperature.

Table 2. Box B Components

Reagents	Min. Vol. Supplied	Vial ID
cDNA Binding Buffer	4.5 mL	**BB**
5X Wash Solution	1.2 mL	**WS**
cDNA Purification Columns	10	n/a
cDNA Elution Tubes	10	n/a
Collection Tubes	10	n/a

Table 3. Related Product (Available Separately)

Reagent	Supplied	Cat. No.
Control RNA (100 µg/mL)	10 µL	**ENZ-42406-CR**

IV. Additional Materials Required

Equipment

- Thermal cycler with heated lid or heating blocks with heated lid
- Cold block (4°C).
- Microcentrifuge
- Orbital shaker for 96-well plates (optional)
- UV Spectrophotometer (optional)
- Bioanalyzer (Agilent, optional)

Materials

- Isolated and purified RNA
- 0.2 ml, 0.6 ml and 1.5 ml microcentrifuge tubes
- Sterile, aerosol barrier, nuclease-free pipette tips
- RNase decontamination solution
- Molecular Biology Grade Ethanol (100%)

V. Methods and Procedures

NOTE: *PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING. Upon thawing of solutions, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution.*

The entire process involves four major steps that are accomplished by using the Single-Round Amplification and Biotin Labeling System with recommended purification and analysis procedures.

With the exception of all purifications, the entire procedure may be performed in a thermal cycler (Table 4). Alternatively, heating blocks with heated lids, water baths, or hybridization ovens set at the prescribed temperatures can be used.

Table 4. Thermal Cycler Amplification Program

Process	°C	Time
Denaturation	70° 42° 42°	10 minutes 3 minutes Pause
Reverse Transcription	42° 4°	120 minutes Pause
Secnond Strand Synthesis	16° 4°	120 minutes Pause
In Vitro Transcription	37° 4°	16 hours (see step D-5) Pause

For the sake of consistency of common reaction components, we recommend making master mixes for each step of the procedure when performing more than one reaction. The reaction tables show exact reactant volumes per reaction. While making a master mix, Include 10% extra volume of each common reaction component in the master mix to make up for losses in distributing into each reaction vial.

A. First-Stand Synthesis

Table 5. First Strand Master Mix

Component	Vial ID	Vol. per Reaction
First Strand Buffer	**FB**	2.0 µL
DTT	**D**	2.0 µL
dNTP Mix	**dN**	1.0 µL
Reverse Transcriptase	**RT**	1.0 µL
RNase Inhibitor	**I**	1.0 µL
Total		**7.0 µL**

1. Thaw vials **dN**, **P**, **FB**, **D** and **W.** Gently mix, centrifuge (~5 sec) and place on ice until needed. Briefly centrifuge all tubes containing enzymes immediately prior to use.
2. Mix total RNA sample (250 ng - 5000 ng per reaction) with 1 µL **Promoter Primer** (**P**).
3. Add an appropriate volume of **Nuclease-free Water** (**W**) to the RNA/Primer mix for a total volume of **13 µL**.
4. Incubate for 10 minutes at 70°C to denature. **Do not place** the RNA/Primer mix on ice. Move immediately to 42°C.
5. While denaturing, prepare the First Strand Master Mix by combining the reagents (Table 5) in a sterile, nuclease-free microcentrifuge tube.
6. Gently mix by pipetting up and down several times, centrifuge and place the First Strand Master Mix on ice.
7. Incubate separately both the RNA/Primer mix (from step 4) and the First Strand Master Mix (from step 5) for 3 minutes at 42°C.
8. After 3 minutes at 42°C, add **7 µ**L of First Strand Master Mix to the RNA/Primer mix and mix by pipetting up and down several times.
9. Incubate for 2 hours at 42°C.
10. Place the tubes on ice.

B. Second Strand cDNA Synthesis

Table 6. Second Strand Master Mix		
Component	**Vial ID**	**Vol. per Reaction**
Nuclease-free Water	**W**	106 µL
dNTP Mix	**dN**	3 µL
Second Strand Buffer	**SB**	15 µL
DNA Polymerase	**DP**	5 µL
RNaseH	**RH**	1 µL
Total		**130 µL**

1. Thaw vials **dN**, **SB** and **W**, mix, briefly centrifuge and keep on ice.
2. Shortly before the completion of the first strand synthesis reaction, prepare the Second Strand Master Mix (Table 6) in a sterile, nuclease-free microcentrifuge tube. Mix gently by pipetting up and down several times, centrifuge and place on ice.
3. Add **130 µL** of the Second Strand Master Mix to the first strand reaction on ice, and mix gently by pipetting up and down several times. Centrifuge briefly to collect the contents.
4. Incubate at 16°C for 2 hours.
5. Place the tubes on ice.
6. Immediately proceed to purification of the double-stranded cDNA template.

C. cDNA Purification

NOTE: *All centrifugation steps are at 10,000 x* g.

1. Perform the following steps prior to begining cDNA purification.
   a. Make a **1X Wash Solution** by adding 4.8 mL 100% Ethanol to the 1.2 mL of **5X Wash Solution (WS)** .
   b. Pre-equilibrate each cDNA purification column with 30 µL **DNA Binding Buffer** (**BB**) for 5 minutes. **Do not centrifuge**.
   c. Place **Nuclease-free Water (W)** at 70°C for at least 10 min.
2. Add 375 µL of **DNA Binding Buffer** (**BB**) to each double-stranded cDNA sample and mix gently.
3. Add cDNA samples in binding buffer to the pre-equilibrated cDNA purification columns and centrifuge for 1 minute. Discard the flow-through.

4. Add 500 µL of **1X Wash Solution**, centrifuge for 1 minute, discard the flow-through and centrifuge for an additional minute to remove trace liquid and transfer to an elution tube.
5. Add 17.5 µL of pre-warmed (70°C) **Nuclease-free Water** (**W**), incubate for 1 minute at room temperature and centrifuge for an additional minute to elute cDNA. Repeat this entire step for a total sample volume of 35 µL.
6. If immediately proceeding to *in vitro* transcription, keep the purified cDNA at room temperature.
7. Purified cDNA can be stored at -20˚C for up to 3 days prior to use in the *in vitro* transcription reaction. Bring it to room temperature just prior to setting up *in vitro* transcription reaction.

D. RNA TRANSCRIPT LABELING

Table 7. *In Vitro* Transcription (IVT) Master Mix

Component	Vial ID	Vol. per Reaction
IVT Reaction Buffer	**RB**	6 µL
Biotin-Labeled Ribonucleotide	**B**	6 µL
DTT	**D**	6 µL
Enhancer Cocktail	**EC**	6 µL
T7 RNA Polymerase	**T7**	3 µL
Total		**27 µL**

1. Thaw vials **RB**, **B** and **D**. Mix well gently, centrifuge and keep at room temperature. Briefly centrifuge the vials containing enzymes immediately prior to use and place on ice.
2. Prepare the *in vitro* transcription master mix (Table 7) in a sterile, nuclease-free microcentrifuge tube at **room temperature**. Mix well gently and centrifuge briefly
3. Add the purified cDNA (~33 µl from step C-6) to 27 µl of IVT reaction mix.
4. Mix gently and centrifuge briefly to collect content at the bottom of the tubes.
5. Immediately place the tubes in a 37°C thermocycler (or heating block with heated lid) and incubate for 16 hours. For total RNA input ≥ 2 µg, a 6 hour incubation is sufficient.
6. Place the reaction tubes on ice. If using a thermocycler, bring the reaction temperature to 4°C and hold. aRNA samples should be purified immediately. However, if necessary, unpurified samples can be stored at ≤ -70°C for up to 3 days.

E. aRNA PURIFICATION

A suggested protocol for the aRNA Purification is provided in Appendix C.

VI. Appendices

A. Recommendations for RNase-Free Technique

Avoid all sources of RNase contamination as generation of high quality labeled aRNA product requires that full length RNA be maintained from sample preparation to cDNA synthesis and transcription.

- Wear disposable powder-free gloves and change frequently throughout.
- Avoid RNase containing surfaces.
- Use only the reagents supplied in this kit. Other reagents may not be compatible.
- Use only sterile, DNase and RNase-free pipet tips, microcentrifuge tubes and other plasticware.
- Clean pipettes and work surfaces with commercially available RNase decontaminating solutions or wipes.

B. RNA Preparation Guidelines

- RNA purity and integrity affect both the quantity and quality of the resulting cDNA .
- Starting with total cellular RNA minimizes loss of low abundance transcripts during mRNA isolation.
- Most commercially available RNA isolation reagents are compatible with the cDNA Synthesis Kit. However, the Qiagen poly-A carrier RNA is not recommended.
- Total RNA samples should be in water or buffer, free of protein, DNA, cellular material, organic solvents, salts and other RNA isolation reagents.
- RNA purity can be assessed via a spectrophotometer with acceptable A_{260}/ A_{280} ratios between 1.8 and 2.1.
- RNA integrity can be evaluated by gel or by capillary electrophoresis.
- Both the input RNA and amplified aRNA should be used immediately after purification or stored at ≤-70°C until use. Results may vary for samples subject to multiple freeze-thaw events.

C. aRNA Purification

General Guidelines for Column Filter Purification

There are several commercially available column filter-based purification systems for the purification of the aRNA. To avoid precipitation of reagents or sample, ensure that all solutions are at room temperature and thoroughly mixed before use. All excess wash solution must be removed from the column filter prior to aRNA elution. Visually monitor the addition of the elution solution to the filter to ensure saturation. Unsaturated filters will result in reduced yields.

If you are using the Qiagen RNeasy™ Mini Kit, we recommend the following modified protocol for maximum biotinylated aRNA recovery.

NOTE

- Make sure Buffer RLT, Buffer RPE and RNase-free water are at room temperature.
- Make certain that ethanol has been added to Buffer RPE.
- DO NOT add β-mercaptoethanol to Buffer RTL.
- DO NOT perform on-column DNase I digestion.
- All centrifugation steps are at 10,000 x *g*.

1. Set up the appropriate number of columns in collection tubes.
2. Briefly centrifuge samples (~ 5 sec) and place tubes at room temperature.
3. Add 210 µL RNA Binding Buffer RLT, mix and briefly centrifuge samples (~ 5 sec).
4. Add 255 µL of 100% ethanol, mix by pipetting up and down. Then transfer the mixture to the center of the column. Centrifuge for 0.5 minutes.
5. Transfer columns to fresh collection tubes and discard tubes containing flow-through.
6. Add 500 µL of RPE Wash Buffer to each column, let stand for 2 minutes, and then centrifuge for 0.5 minutes.
7. Discard the flow-through and place columns in same collection tubes.
8. Add another 500 µL of RPE Wash Buffer to each column, let stand for 2 minutes, and then centrifuge for 0.5 minutes.
9. Transfer columns to fresh collection tubes and discard tubes containing flow-through. Centrifuge for 2 minutes.
10. Place columns in 1.5 mL microcentrifuge tubes, add 30 µL of RNase-free water to the center of each column, let stand for 2 min then centrifuge for 1 min to elute RNA.

11. Add another 30 μL of RNase-free water to the center of each column, let stand for 2 minutes, and then centrifuge for 1 minute to finish the RNA elution.

12. Analyze the RNA as required, proceed with the indicated step, or store at ≤ -70˚C.

D. Analysis of Biotin-Labeled Transcript

The RNA yield can be estimated by absorbance detection at 260 nm and 280 nm using a UV/Vis spectrophotometer.

The quality of amplified aRNA can be assessed by gel electrophoresis and should result in a smear ranging from 200 nt to 5000 nt with and average size of approximately 1200 nt.

If analyzing by Agilent 2100 Bioanalyzer or similar method, the distribution of biotin-labeled aRNA should range from 200 nt to 6000 nt a broad peak between 1 kb and 2 kb (Figure 1).

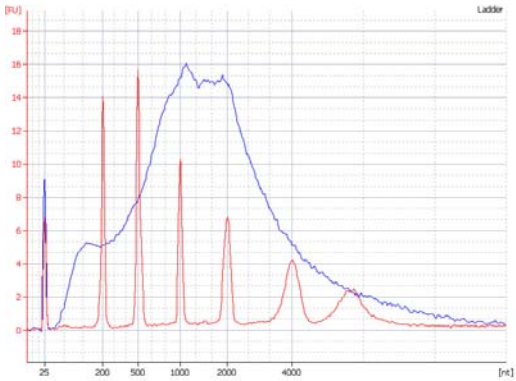


Figure 1. Analysis of biotin-labeled aRNA using the Single-Round RNA Amplification and Labeling System. After purification, 400 ng of synthesized aRNA was evaluated using the RNA Nano/Pico LabChip kit on an Agilent 2100 BioAnalyzer.

E. aRNA Fragmentation

Before hybridization of biotinylated aRNA to Affymetrix GeneChip, the labeled aRNA should be reduced in size by chemical fragmentation. We recommend that you follow the guidelines of your microarray manufacturer regarding aRNA fragmentation.

VII. References

1. Van Gelder *et al*. PNAS **87**(1990)1663-7. Van Gelder *et al*. PNAS **87**(1990)

VIII. Troubleshooting Guide

Problem	Potential Cause	Suggestion
Low aRNA yields with experimental samples	Organic, protein or salt contaminants in the input RNA	Perform parallel reaction with control RNA (ENZ-42406-CR, sold separately). Use 5 uL (500 ng) in the first strand synthesis reaction. Typical yield from this input is 30 ug or more. If control RNA amplifies to this level but not your experimental samples, repurify your starting material. Use extra caution not to carry over organic organic solvents or denaturants during purification.
	Low mRNA content of experimental sample	Increase the total RNA input if the experimental RNA sample is expected to have low mRNA content.
	Input RNA does not contain poly(A) tracts	RNA amplification with this kit requires the use of oligo dT primers. Do not substitute random or gene-specific primers with this kit.
	Degraded input RNA	Prepare fresh sample following the guidelines in Appendix A to prevent RNase contamination. Use nuclease-free plasticware and decontaminate pipettes and work surfaces with commercially available RNase decontamination solutions.
Low yields with both experimental samples and control RNA	Inadequate thawing and mixing of reagents	Ensure reagents are completely thawed with no solids remaining. Gently mix and centrifuge reagents briefly.
	Nuclease contamination	Prepare fresh samples following the guidelines in Appendix A to prevent RNase contamination. Use nuclease-free plasticware and decontaminate pipettes and work surfaces with commercially available RNase decontamination solutions.
	Incorrect incubation temperatures	Check the accuracy of the temperatures of your incubators and thermal cycler.
	Reaction tube condensation	Briefly centrifuge the reaction tube(s). Mix gently and move the reaction tube (s) to an incubator where condensation does not occur.

Continued…….

Problem	Potential Cause	Suggestion
Low yields with both experimental samples and control RNA (*continuation..)*	RNA sample vacuum centrifuged to dryness	Monitor sample volume during vacuum centrifugation to avoid drying to completion. RNA is difficult to resuspend if dried completely.
	Inadequate elution of cDNA	Heat elution buffer to 70°C. Proper maintenance of temperature at this step is critical for maximal elution of cDNA from the column filter.
	Inadequate elution of aRNA	Elute nucleic acids from column filter purification systems with enough elution solution to completely saturate the filter. Dispense the elution buffer in the center of the column bed.
	Incorrect spectrophotometer readings	Check the accuracy of your spectrophotometer. Alternatively, use another method for quantitation of aRNA such as staining with a sensitive RNA dye.
Average size of control aRNA is greater than 1 kb but average size of experimental aRNA is less than 0.5 kb.	Amount of input RNA is outside of recommended range (0.25 – 5 ug)	If input RNA is below 250 ng, increase the amount of input RNA. If input RNA is above 5 ug, decrease the amount of input RNA.

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