Enzo® Life Sciences

Product Manual

BioArray® Low Input RNA Amplification and Biotin Labeling System

Catalog #: ENZ-42422-0010

10 Reactions

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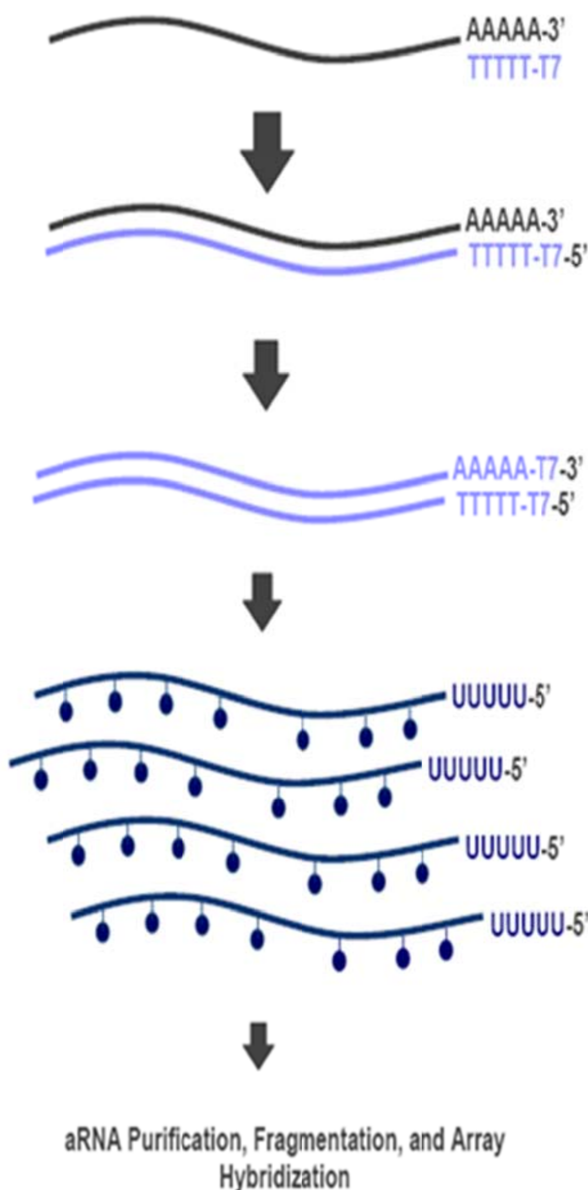
INTRODUCTION

The BioArray® Low Input RNA Amplification and Biotin Labeling System provides an optimized protocol and reagents for the production of biotin-labeled antisense RNA (*a*RNA) from total cellular RNA samples in less than 24 hours for array analysis. This kit has been improved to yield sufficient *a*RNA quantity for most microarray platforms (10 µg minimum with total RNA input as low as 20 ng and up to 500 ng). Entire amplification reaction can be performed in a single tube and the biotin-labeled *a*RNA can be purified using either magnetic beads or purification columns. The BioArray® Low Input 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 and *in vitro* transcript labeling.

PROCEDURAL OVERVIEW



First Strand cDNA Synthesis

- Bring total volume of RNA to 5 mL with Nuclease-free water
- Add 4 mL of First Strand Buffer Mix
- Add 1 mL of First Strand Enzyme Mix
- Incubate at 42˚C for 2 hours
- Chill to 4°C

Second Strand cDNA Synthesis

- Add 15 mL Nuclease-free water
- Add 3 mL Second Strand Buffer Mix
- Add 2 mL Second Strand Enzyme Mix
- Incubate at 15.5˚C for 2 hours.
- Incubate at 65°C for 10 minutes to stop the reaction.

In Vitro *Transcription and Biotin Labeling*

- Add 25 mL Transcription Buffer Mix
- Add 7.5 mL DTT
- Add 7.5 mL Enhance Cocktail
- Add 5 mL T7 RNA polymerase
- Incubate at 37˚C for up to 16 hours for input RNA 20-500 ng

SAFETY WARNINGS AND PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

Handle with care

REAGENTS PROVIDED AND STORAGE

Storage notes

Avoid freeze / thaw cycles

All reagents are shipped on dry ice. Upon receipt, the kit should be stored at ≤-20°C, protected from light. When stored properly, these reagents are stable for at least twelve months. **Avoid repeated freezing and thawing.**

Reagents provided in the kit are sufficient for approximately 100 assays using either live, permeabilized or fixed cells.

Table 1. Components

Reagent	Quantity	Vial ID
First Strand Buffer Mix	40 µL	FSB (Yellow)
First Strand Enzyme Mix	10 µL	FSE (Yellow)
Second Strand Buffer Mix	30 µL	SSB (Purple)
Second Strand Enzyme Mix	20 µL	SSE (Purple)
Transcription Buffer Mix	250 µL	TB
DTT	75 µL	DTT
Enhancer Cocktail	75 µL	EC
T7 RNA Polymerase	50 µL	T7
Nuclease-free Water	1 mL	W

Table 2. Related Product (availability separately)

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

ADDITIONAL MATERIALS REQUIRED

Equipment

- Thermal cycler with heated lid or heating blocks with heated lids and a cold block (4ºC)
- Microcentrifuge
- Orbital shaker for 96-well plates (optional)
- UV Spectrophotometer (optional)
- Bioanalyzer (Agilent, optional)

Materials

- Isolated and purified RNA
- Microcentrifuge tubes
- Sterile, aerosol barrier, nuclease-free pipette tips
- RNase decontamination solution
- Molecular biology grade ethanol (100%)
- Recommended purification kits: Qiagen RNeasy™ Mini Kit (Qiagen No. 74104) or Agencourt RNA Clean (Agencourt No. A63987)

METHODS AND PROCEDURES

Important

NOTE: *PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING. The Appendix (page 9) contains procedures to prevent RNase contamination to optimize results. It also provides details on preparation of input RNA. After thawing solutions gently hand-mix and bring reagents to the bottom of the tubes by quick centrifugation, prior to use.*

The entire process involves three major steps that are accomplished by using the BioArray® Low Input RNA Amplification and Biotin labeling System with recommended purification and analysis procedures.

With the exception of *a*RNA purification, the entire procedure may be performed in a thermal cycler (Table 3). Alternatively, heating blocks with heated lids, water baths, or hybridization ovens set at the prescribed temperatures can be used.

Table 3. Thermal Cycler Amplification Program

Process	ºC	Time
A. First Strand Synthesis	42º 4º	120 minutes Pause
B. Second Strand Synthesis	15.5º 65º 4º	120 minutes 10 minutes Pause
C. *In vitro* Transcription	37º 4º	2-16 hours Pause

To maintain 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 allow for losses in distributing components into each reaction vial.

A. First Strand Synthesis

Table 4. First Strand Master Mix		
Component	**Vial ID**	**Vol. per Reaction**
First Strand Buffer Mix (Yellow)	FSB	4.0 µL
First Strand Enzyme Mix (Yellow)	FSE	1.0 µL
Total		**5.0 µL**

Thaw

Hand mix gently

Centrifuge

Gently pipette up and down

Incubate

1. Thaw vials W (Nuclease-free Water) and FSB (First Strand Buffer Mix). If the content in vial FSB does not thaw easily, place the vial in a 37ºC – 50ºC water bath or heating block briefly to facilitate thawing. Gently mix, centrifuge (~ 5 sec) and place the tubes on ice until needed. Briefly centrifuge all tubes containing enzymes immediately prior to use.

2. Dilute the total RNA sample with Nuclease-free Water to a final concentration of 200 ng – 500 ng per 5 µL. For guidelines on preparation of RNA sample, see Appendix on page 9. Transfer 5 µL of total RNA samples into individual nuclease-free microcentrifuge reaction tubes.

3. Prepare the First Strand Master Mix by combining the reagents (Table 4) in a sterile, nuclease-free microcentrifuge tube. Gently mix by pipetting up and down several times, centrifuge and place the First Strand Master Mix on ice.

4. To each reaction tube containing 5 µL RNA sample (from step 2), add 5 µL of First Strand Master Mix.

5. Gently mix by pipetting up and down several times and close the reaction tube. Centrifuge briefly if necessary to collect the reaction mixture at the bottom of the tube. Incubate for 2 hours at 42ºC. Transfer the reactions to 4ºC in the thermo cycler or place on ice.

B. Second Strand Synthesis

Thaw

Hand mix gently

Centrifuge

Gently pipette up and down

Incubate

Table 5. Second Strand Master Mix		
Component	**Vial ID**	**Vol. per Reaction**
Nuclease-free Water	W	15.0 µL
Second Strand Buffer Mix (Purple)	SSB	3.0 µL
Second Strand Enzyme Mix (Purple)	SSE	2.0 µL
Total		**20.0 µL**

1. Thaw vials SSB (Second Strand Buffer Mix) and W (Nuclease-free Water), gently mix well, briefly centrifuge and keep on ice.
2. Shortly before the completion of the first strand synthesis reaction, prepare the Second Strand Master Mix (Table 5) in a sterile, nuclease-free microcentrifuge tube. Mix gently by pipetting up and down several times, centrifuge and place on ice.
3. Add 20 µL of the Second Strand Master Mix to each of the First Strand reaction tubes on ice. Mix gently by pipetting up and down several times. Centrifuge briefly to collect the contents.
4. Incubate at 15.5ºC for 2 hours.
5. Denature the enzymes by incubation at 65ºC for 10 minutes.
6. Transfer the reaction tubes to a thermo cycler set to 4ºC or place on ice.

C. *In Vitro* Transcription and Transcript Labeling

Table 6. *In Vitro* Transcription Master Mix		
Component	**Vial ID**	**Vol. per Reaction**
Transcription Buffer Mix	TB	25 µL
DTT	DTT	7.5 µL
Enhancer Cocktail	EC	7.5 µL
T7 RNA Polymerase	T7	5.0 µL
Total		**45.0 µL**

Centrifuge

1. Thaw vials TB (Transcription Buffer Mix) and DTT. Mix well gently, centrifuge and keep at room temperature. Briefly centrifuge vials EC (Enhancer Cocktail) and T7 (T7 RNA Polymerase) immediately prior to use and place on ice.
2. Prepare the *In Vitro* Transcription Master Mix as per Table 6, in a sterile, nuclease-free microcentrifuge tube. Gently mix well and centrifuge briefly.

3. Add 45 µL of *In Vitro* Transcription Master Mix to each of the 30 µL cDNA synthesis reaction from Step B, page 5.
4. Mix gently and centrifuge briefly to collect content to the bottom of the tubes.

Incubate

5. Immediately place the reaction tubes in a 37ºC thermo cycler (or heating block with heated lid) and incubate for 2 to 16 hours. For total RNA input ≥ 250 ng, a 2-4 hour incubation is sufficient.

Storage

6. Place the reaction tubes on ice. If using a thermo cycler, bring the reaction temperature to 4ºC and hold. *a*RNA samples should be purified immediately. However, if necessary, unpurified samples can be stored at ≤ –70ºC for up to 3 days.

D. aRNA Purification

The following procedures are recommended and may be used for *a*RNA purification:

Column Filter Purification

There are several commercially available column filter-based purification systems for the purification of the *a*RNA. 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 *a*RNA 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 (Qiagen No. 74104), we recommend the following modified protocol for maximum biotinylated *a*RNA recovery.

Important

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*.
- Pre-heat RNase-free water for elution to 55°C.

Centrifuge

Room Temp.

1. Set up the appropriate number of columns in collection tubes.
2. Briefly centrifuge samples (~5 sec) and place tubes at room temperature.
3. Carefully transfer all the contents to a 1.5 mL nuclease-free microcentrifuge tube. Add 265 µL Buffer RLT, mix and briefly centrifuge samples (~5 sec).
4. Add 320 µL of 100% ethanol (molecular biology grade), 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 Buffer RPE 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 Buffer RPE 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.

Preheat

10. Place columns in 1.5 mL microcentrifuge tubes, add 30 µL of Nuclease-free water (pre-heated to 55°C) to the center of each column, let stand for 2 minutes then centrifuge for 1 minute to elute RNA.
11. Add another 30 µL of Nuclease-free water (pre-heated to 55°C) 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.

Magnetic Beads Purification

Incubate at room temp.

1. Equilibrate the Agencourt™ RNAClean reagents (Agencourt No. A63987) to room temperature and then resuspend the magnetic beads during the last 30 minutes of the *In Vitro* Transcription Master Mix reaction.
2. Transfer the samples from the *In Vitro* Transcription Master Mix reaction into a 96-well U-bottom plate.
3. Add 108 µL of magnetic beads to each sample and incubate for 5 minutes at room temperature.
4. Separate by placing the 96-well plate on the magnetic stand for 10 minutes or until the mixture is clear.

5. Gently remove the supernatant while keeping the plate on the magnetic stand.
6. Remove the plate from the magnetic stand and wash the beads with 200 µL of 70% ethanol (freshly prepared) and incubate for 30 seconds at room temperature.
7. Separate by placing the 96-well plate on the magnetic stand for approximately 5 minutes or until the mixture is clear.
8. Aspirate and discard the supernatant and repeat steps 6 and 7. Aspirate trace amounts of ethanol without disturbing the beads.
9. Remove the plate from the magnetic stand and allow the beads to air dry for 10 minutes.

Vortex

10. Add 62 µL of Nuclease-free water (25˚C) to each well, seal and vortex or shake for 1 minute.
11. Separate the beads by applying the magnet for 10 minutes or until clear.
12. Collect and transfer 60 µL of aRNA solution to a 96-well PCR plate.

Long-term Storage

13. Analyze the RNA as required, or store at –20˚C overnight or at –80˚C for long term storage.

E. Results

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 *a*RNA 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 *a*RNA should range from 200 nt to 6000 nt a broad peak between 1 kb and 2 kb (Figure 1).

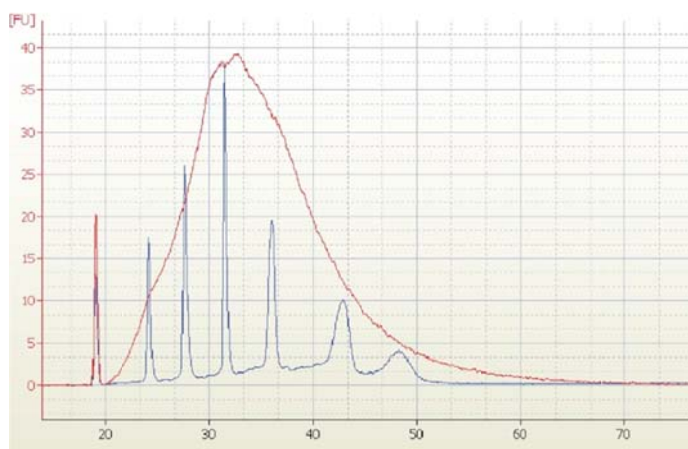


Figure 1. Analysis of biotin-labeled aRNA using the BioArray® Low Input RNA Amplification and Biotin Labeling System. After purification, 400 ng of synthesized *a*RNA was evaluated using the RNA Nano/Pico LabChip kit on an Agilent 2100 BioAnalyzer.

F. *a*RNA Fragmentation

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

APPENDIX

Recommendations for RNase-Free Technique

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

- Wear disposable powder-free gloves and change frequently throughout procedure
- 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.

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.

Important/ Warning

- Most commercially available RNA isolation reagents are compatible with the BioArray® Low Input RNA Amplification and Biotin Labeling Kit. **However, 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 accessed via a spectrophotometer with acceptable A_{260}/ A_{280} ratios between 1.8 and 2.2.
- RNA integrity can be evaluated by gel or by capillary electrophoresis.

Avoid freeze / thaw cycles

- Input RNA and amplified *a*RNA should be used immediately after purification or stored at ≤ -70°C until use. Results may vary for samples subject to multiple freeze-thaw events.

REFERENCE

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

TROUBLESHOOTING GUIDE

Problem	Potential Cause	Suggestion
Low *a*RNA yields with experimental samples	Organic, protein or salt contaminants in the input RNA	Perform parallel reaction with control RNA (ENZO 42406-CR, sold separately). Use 1 uL (100 ng) in the first strand synthesis reaction. Typical yield from this input is 25 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 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 on page 9 in the Appendix to prevent RNase contamination. Use nuclease-free plasticware and de-contaminate 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 on page 9 in the Appendix 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 tubes(s). Mix gently and move the reaction tube(s) to an incubator where condensation does not occur.

Problem	Potential Cause	Suggestion
Low yields with both experimental samples and control RNA *(continuation..)*	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.
	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 *a*RNA	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 spectro-photometer. Alternatively, use another method for quantitation of *a*RNA, such as staining with a sensitive RNA dye.
Average size of control *a*RNA is greater than 1 kb but average size of experimental *a*RNA is less than 0.5 kb.	RNA is partially degraded	Prepare fresh samples following the guidelines in the Appendix to prevent RNase contamination. Use nuclease-free plasticware and decontaminate pipettes and work surfaces with commercially available RNase decontamination solutions.

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