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TargetAmp™ 2-Round Biotin-aRNA Amplification Kit 3.0

Cat. No. TAB2R71010 – 10 Reactions

Cat. No. TAB2R71024 – 24 Reactions

Important!

The procedure for using this TargetAmp kit has changed. RiboGuard™ RNase Inhibitor should be added to each of the two *in vitro* transcription (IVT) reactions on page 12 and page 16.

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1. Introduction

Features and Benefits of the TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0

1) Greater than 5,000,000-fold amplification of the Poly(A) RNA contained in a total RNA sample.
2) Produce microgram amounts of Biotin-aRNA from as little as 50 pg of total cellular RNA.
3) Utilizes an improved "Eberwine" linear RNA amplification process.
4) Virtually eliminates template-independent reactions.
5) Reproducible amplification results.
6) No need to purify the cDNA transcription template prior to the *in vitro* transcription reactions.
7) Fast reaction times...2-rounds of amplification can be completed in 2 days.
8) Easy to use...color-coded tubes and a simplified pipetting scheme reduce labor and the possibility of error.

The TargetAmp™ 2-Round Biotin-aRNA Amplification Kit 3.0 utilizes an improved "Eberwine" procedure[1] for amplifying Poly(A) RNA from as little as 10 pg of total cellular RNA. The kit requires both SuperScript® III and SuperScript II Reverse Transcriptases (Life Technologies™; provided by the user). See Fig. 1.

A. **Round-One, 1st-strand cDNA Synthesis:** The Poly(A) RNA component of a total RNA sample is reverse transcribed into first strand cDNA. The reaction is primed from a T7-Oligo(dT) primer...a synthetic oligo(dT) primer containing a phage T7 RNA Polymerase promoter sequence at its 5′ end. Round-one, 1st-strand cDNA synthesis is catalyzed by SuperScript III Reverse Transcriptase (provided by the user) and performed at an elevated temperature to reduce RNA secondary structure.

B. **Round-One, 2nd-strand cDNA Synthesis:** The RNA component of the cDNA:RNA hybrid produced in Step 1 is digested into small RNA fragments by RNase H. The RNA fragments then prime 2nd-strand cDNA synthesis. The resulting product is a double-stranded cDNA containing a T7 transcription promoter in an orientation that that will generate anti-sense RNA (aRNA sometimes called cRNA) during the subsequent *in vitro* transcription reaction.

C. **Round-One, *In Vitro* Transcription:** High yields of aRNA are produced in a rapid *in vitro* transcription reaction that utilizes the double-stranded cDNA produced.

D. **Round-One, RNA Purification:** The aRNA produced in the first round amplification procedure (Parts A-C) is purified by spin column chromatography (supplied by the user).

E. **Round-Two, 1st-strand cDNA Synthesis:** The aRNA produced and purified in the first round amplification process is reverse transcribed into first strand cDNA using SuperScript II Reverse Transcriptase (Life Technologies™; supplied by the user). The reaction is primed using random sequence hexamer primers.

F. **Round-Two, 2nd-strand cDNA Synthesis:** The RNA component of the cDNA:aRNA hybrid is digested into small RNA fragments by RNase H. Second-strand cDNA synthesis is then primed using a T7-Oligo(dT) Primer. The resulting product is a double-stranded cDNA containing a T7 transcription promoter in an orientation that that will generate Biotin-aRNA during the second round *in vitro* transcription reaction.

G. ***In Vitro* Transcription of Biotin-aRNA:** High yields of Biotin-aRNA are produced in a rapid *in vitro* transcription reaction that utilizes the double-stranded cDNA produced as template. During the *in vitro* transcription reaction, Biotin-UTP replaces a portion of the UTP.

2. Kit Contents

The kit components are supplied in tubes with colored caps for easier identification.

The kit has been developed for use with and will provide optimal results with SuperScript® III and SuperScript II Reverse Transcriptases (Life Technologies™; provided by the user).

Component Name	Tube Label	Amount		Cap Color
		10-rxn	24-rxn	
TargetAmp T7-Oligo(dT) Primer 1	T7-Oligo(dT) Primer 1	15 µl	30 µl	Red
TargetAmp Reverse Transcription PreMix	RT PreMix	50 µl	90 µl	
RiboGuard RNase Inhibitor	RiboGuard™ RNase Inhibitor	30 µl	60 µl	
TargetAmp DNA Polymerase PreMix 1	DNA Pol PreMix 1	60 µl	120 µl	
TargetAmp DNA Polymerase 1	DNA Polymerase 1	10 µl	18 µl	
TargetAmp cDNA Finishing Solution	cDNA Finishing Solution	15 µl	30 µl	
TargetAmp Random Primers	Random Primers	30 µl	55 µl	Blue
TargetAmp RNase H	RNase H	10 µl	18 µl	
TargetAmp T7-Oligo(dT) Primer 2	T7-Oligo(dT) Primer 2	15 µl	30 µl	
TargetAmp DNA Polymerase PreMix 2	DNA Pol PreMix 2	150 µl	350 µl	
TargetAmp DNA Polymerase 2	DNA Polymerase 2	10 µl	18 µl	
TargetAmp T7 RNA Polymerase	T7 RNA Polymerase	100 µl	225 µl	Green
TargetAmp T7 Transcription Buffer 1	Transcription Buffer 1	50 µl	125 µl	
NTP PreMix 1	NTP PreMix 1	300 µl	725 µl	
RNase-Free DNase I	DNase I	50 µl	115 µl	
TargetAmp T7 Transcription Buffer 2	Transcription Buffer 2	100 µl	220 µl	Yellow
NTP PreMix 2	NTP PreMix 2	225 µl	525 µl	
Biotin-UTP	Biotin-UTP	50 µl	120 µl	
Dithiothreitol (DTT)	DTT	100 µl	250 µl	Clear
HeLa Total RNA Control (40 ng/µl)	HeLa Total RNA Control	10 µl	10 µl	
RNase-Free Water	RNase-Free Water	2 x 1 ml	2 x 1 ml	
Poly(I)	Poly(I)	15 µl	30 µl	

Storage: Upon receipt of this kit, remove the tube containing the HeLa Total RNA Control and store it at –70°C to –80°C. Store the remainder of the kit at –20°C.

Additional Required Reagents and Equipment:

SuperScript III and SuperScript II Reverse Transcriptase (Life Technologies™)
Thermocycler
Microcentrifuge
RNase-Free Water
RNeasy® MinElute® Cleanup Kit
or
RNeasy Mini Kit (Qiagen) (see "Biotin-aRNA Purification" for details)

Performance Specifications and Quality Control

The TargetAmp 2-Round Biotin-aRNA Amplification Kit is function-tested in a control reaction. The kit must produce at least 20 µg of Biotin-aRNA (cRNA) from 200 pg of HeLa Total RNA Control, corresponding to a greater than 5,000,000-fold amplification of the Poly(A) RNA assuming that 2% of the HeLa Total RNA Control is Poly(A) RNA. A negative control reaction ("no-RNA" control) produces less than 2 µg of Biotin-aRNA.

3. Preparation

Assessing the Quality of the Total RNA:

The success of microarray experiments is strongly influenced by the quality of the RNA. RNA quality has two components…purity of the RNA (or absence of contaminants) and integrity (intactness) of the RNA. RNA quality should be assessed prior to every RNA amplification reaction. Poor quality RNA is the most common cause of sub-optimal RNA amplification results!

RNA Purification Methods and RNA Purity. Total cellular RNA, isolated by a number of methods, can be amplified successfully using the TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0. However, it is very important that the purified RNA be free of salts, metal ions, ethanol, and phenol which can inhibit the enzymatic reactions performed in the RNA amplification process. Commonly used RNA extraction and purification methods that are compatible with the TargetAmp RNA amplification process include but are not limited to:

TRIzol®/TRI Reagent®, a homogeneous solution of the powerful denaturants guanidinium isothiocyanate and phenol, is very effective at extracting the RNA from the cells. However all traces of guanidinium salts and phenol must be removed from the RNA sample prior to the RNA amplification process. If you precipitate the RNA from TRIzol-extracted cells, be sure to wash the RNA pellet at least two times with cold 70-75% ethanol to remove all traces of phenol and guanidinium salts. Air dry the RNA pellet (do not use a vacuum centrifuge) to remove residual ethanol. Then, resuspend the RNA in RNase-Free water. If you purify the RNA from TRIzol-extracted cells by column purification methods, please read the section "Spin Columns" immediately following.

Spin Columns (e.g., the RNeasy MinElute Cleanup Kit and RNeasy Mini Kit from Qiagen) are effective in purifying RNA samples that are free of the contaminants that may inhibit the RNA amplification process. Spin columns can be used with most RNA extraction procedures (e.g. TRIzol reagent). If using spin columns, follow

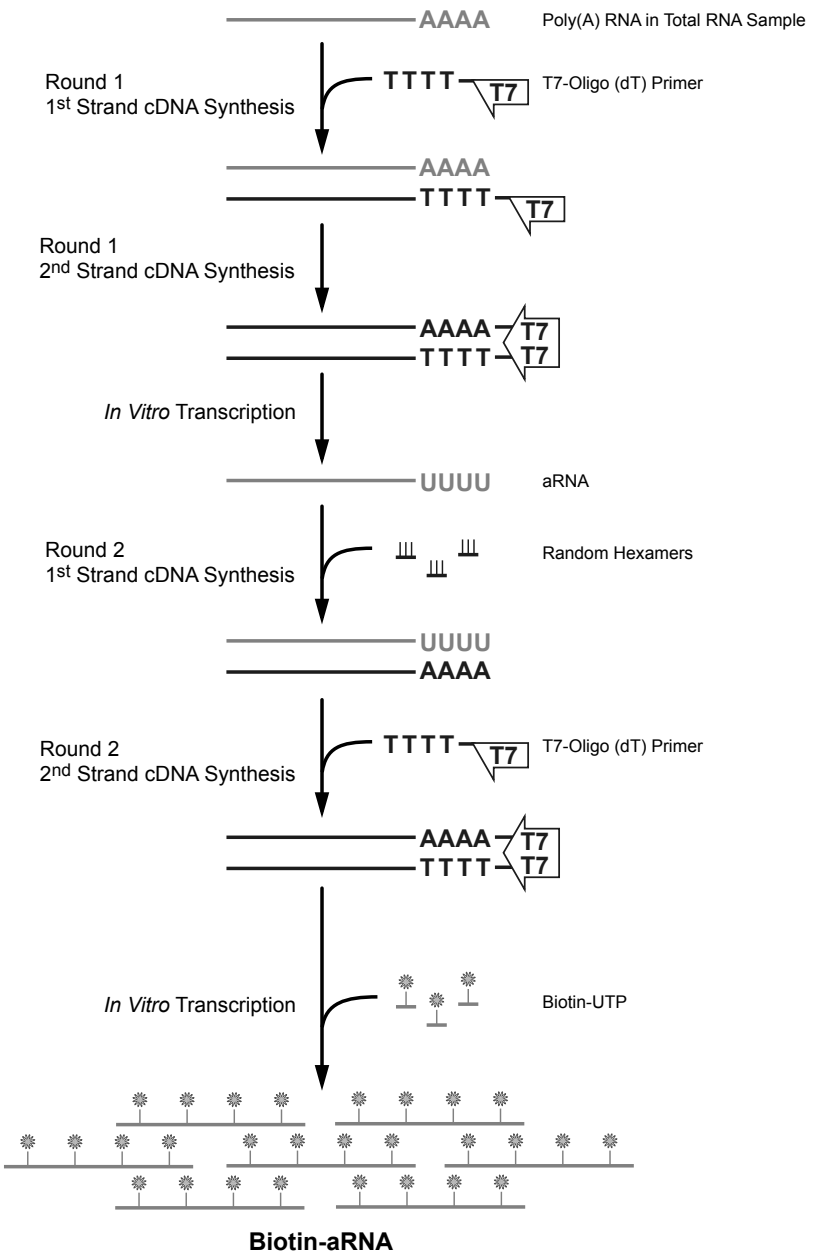

Figure 1. TargetAmp™ 2-Round Biotin-aRNA Amplification Kit 3.0 Procedure.

the manufacturer's instructions closely, especially if an ethanol wash of the RNA is performed prior to the RNA elution step. Then, elute the RNA from the column membrane using RNase-Free water. We recommend using spin columns to isolate RNA from tissue samples treated with RNA preservatives such as RNA*later*® or RNA*later*-ICE.

Salt-Fractionation: RNA purification that employs gentle salt-fractionation, such as EPICENTRE's ArrayPure™ Nano-scale RNA Purification Kit, routinely produce the highest yield of intact RNA without the use of phenol, guanidinium salts, or columns. The ArrayPure kit has been developed for total RNA purification from 1-10,000 cells obtained by laser-capture methods such as Laser Capture Microdissection (LCM), from biopsy samples, from cell culture or quick-frozen tissue. To purify RNA from >10,000 cells, EPICENTRE's MasterPure™ RNA Purification Kit is recommended. When using these kits, be sure to wash the RNA pellet at least two times with cold 70-75% ethanol to remove all traces of salts. Air dry the RNA pellet (do not use a vacuum centrifuge) to remove residual ethanol. Then, resuspend the RNA in RNase-Free water. The ArrayPure kit and MasterPure kit are not recommended for purification of RNA from tissue samples preserved with RNA*later* or RNA*later*-ICE.

RNA Integrity. Successful microarray analysis using amplified RNA is dependent on an RNA sample that contains full-length, intact Poly(A) RNA. The most commonly used methods for assaying RNA integrity are by denaturing agarose gel electrophoresis or using an Agilent 2100 Bioanalyzer.

The advantages of denaturing agarose gel electrophoresis are its low cost and ready availability of the reagents required. Denaturing gel electrophoresis separates the RNAs by size (electrophoretic mobility) under denaturing conditions. Denaturing conditions are necessary to eliminate inter- and intra-molecular secondary structure within the RNA sample which may cause degraded RNA to appear intact. Following electrophoresis, the denaturing gel is stained with, for example, ethidium bromide and the user looks for the highly stained 18S and 28S rRNAs. These bands should be sharp and discrete with an absence of smearing under either. Based on these visual observations, the user infers that the mRNA in the sample is equally intact (or degraded). If the rRNA bands appear degraded, as evidenced by smearing under each, the RNA sample should be discarded and a new sample of total RNA purified. Ideally, the ethidium bromide stained 28S rRNA band should appear to be about twice as intense as the 18S rRNA band. The main disadvantage of denaturing gels is that they require a minimum of 1 µg of total RNA be loaded per lane…a quantity of RNA that often is not expendable for those performing RNA amplification.

The Agilent 2100 Bioanalyzer is currently the preferred method for evaluating the integrity of an RNA sample. Like a denaturing gel, the bioanalyzer separates the RNAs by size (electrophoretic mobility). However, in contrast to a denaturing gel, the 2100 Bioanalyzer consumes nanograms amounts of total RNA per well when using the manufacturer's RNA 6000 Nano LabChip®. When analyzing the RNA sample using the Agilent 2100 Bioanalyzer, the 18S and 28S rRNA species should appear as distinct, sharp peaks on the electropherogram. A slightly increased baseline, indicative of the 1-5% Poly(A) RNA contained in the sample, can be seen between the two peaks.

Maintaining an RNase-free Environment:

Ribonuclease contamination is a significant concern for those performing RNA amplification. The ubiquitous RNase A is a highly stable and active ribonuclease that can contaminate any lab environment and is present on human skin. All components of the TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 have been tested to ensure the lack of contaminating ribonuclease activities. However, creating an RNase-free work environment and maintaining RNase-free solutions is critical for performing successful RNA amplification reactions. Therefore, we strongly recommend that the user:

1) Autoclave all tubes and pipette tips that will be used in the RNA amplification reactions.
2) Always wear gloves when handling samples containing RNA. Change gloves frequently especially after touching potential sources of RNase contamination such as door knobs, pens, pencils, and human skin.
3) Always wear gloves when handling kit components. Do not pick up any kit component with an ungloved hand.
4) Keep all kit components tightly sealed when not in use. Keep all tubes containing RNA tightly sealed during the incubation steps.

Total RNA Input and Biotin-aRNA Yield:

The TargetAmp 2-Round Biotin-aRNA Amplification Kit is extremely efficient at producing microgram amounts of Biotin-aRNA from picogram quantities of input total RNA. The actual Biotin-aRNA yield and fold-amplification of Poly(A) RNA from a total RNA sample is dependent on:

1) The integrity (intactness) of the total RNA sample (discussed above).
2) The amount of total RNA used in the reaction.
3) The Poly(A) RNA content of the total RNA sample.

Typically, a total RNA sample from eukaryotic cells contains 1-5% Poly(A) RNA depending on the type of cell and its metabolic state. Table 1 shows the Biotin-aRNA yield and fold-amplification obtained from high-integrity total RNA from three different cell types, each containing a different percentage of Poly(A) RNA. The information in Table 1 provides guidance to the amount of Biotin-aRNA that can be produced using exceptionally high-integrity total RNA of known Poly(A) RNA content and is not intended as a guarantee of yield. If the Poly(A) RNA content of the sample is not known, a commonly used assumption is that Poly(A) RNA constitutes 2% of a total RNA sample.

Table 1. Yields of Biotin-aRNA obtained from the HeLa Total RNA Control provided in the kit, using the TargetAmp™ 2-Round Biotin-aRNA Amplification Kit 3.0. Results are the average of multiple experiments. The fold-amplification of the Poly(A) RNA in the total RNA sample is shown in parentheses.

Amount of Total RNA Amplified	Biotin-aRNA yield from Total HeLa RNA [assume 2% of HeLa total RNA is Poly(A) RNA]
50 pg	8 µg (8×10^6)
100 pg	19 µg (9.5×10^6)
200 pg	34 µg (8.5×10^6)
500 pg	71 µg (7.1×10^6)

Important! *The TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 has been optimized for producing >5,000,000-fold amplification of the Poly(A) RNA from 50-500 pg of total cellular RNA per reaction. Amplifying >500 pg of total RNA in a single TargetAmp 2-Round Amplification reaction may result in less than 5,000,000-fold amplification of the Poly(A) RNA and may result in under-representation of some Poly(A) RNA sequences in the Biotin-aRNA produced. Therefore, if it is desirable to perform an amplification reaction using >500 pg of total RNA, we strongly recommend that the user perform multiple reactions, each containing up to but not exceeding 500 pg of total RNA.*

Biotin-aRNA Purification:

The appropriate Biotin-aRNA purification process (Part H) to use is dependent on the expected yield of Biotin-aRNA. Use Table 1 to estimate the yield of Biotin-aRNA from the amount of total RNA and the Poly(A) content of the total RNA used in each amplification reaction. If the Poly(A) RNA content of the sample is not known, assume it is 2% (comparable to the HeLa Total RNA Control). Then:

1) If the expected yield of Biotin-aRNA is <40 µg, purify the Biotin-aRNA using the Qiagen MinElute Cleanup Kit.
2) If the expected yield of Biotin-aRNA is >40 µg, purify the Biotin-aRNA using the Qiagen RNeasy Mini Kit.

Additional Suggestions:

Familiarize Yourself with the TargetAmp Kit and Procedure:

The TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 includes many reagents. Before starting, please read this protocol carefully and familiarize yourself with each kit component and in which step of the RNA amplification process it is used. Be sure to wear gloves when handling the kit components.

Importance of Running the TargetAmp 2-Round Biotin-aRNA Kit Control Reaction:

We strongly recommend that those who are not experienced with the TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 perform a control amplification reaction (Appendix 1) prior to committing a precious sample. HeLa total RNA is provided with the kit as a control.

Performing the TargetAmp 2-Round Biotin-aRNA Amplification Reactions:

We recommend that all reactions be performed in sterile 0.2 ml thin-walled tubes using sterile pipette tips and recently calibrated pipettors. Very small volumes of some kit components are required for each reaction. Therefore, we recommend the user prepare Master Mixes of reaction components when amplifying multiple samples.

Some Simple but Important Factors for Obtaining Optimal Results:

1) Familiarize yourself with the kit by running a control reaction (Appendix 1) before committing a precious sample.
2) Use up to but not more than 500 pg of total RNA per reaction.
3) Assemble the two *in vitro* transcription reactions (Part C and Part G) at room temperature. Do not exceed the *in vitro* transcription reaction times indicated in the procedure.
4) Use the appropriate RNA purification columns for purifying the Biotin-aRNA produced.
5) Optional stopping points are noted following the 1st-round and 2nd-round 2nd-strand cDNA synthesis steps (Part B and Part F) and after purification of the Biotin-aRNA (Part D and Part H).

4. RNA Amplification Procedure

Please read through the TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 procedure carefully before beginning. We strongly recommend that those who are not experienced with the TargetAmp kit perform a control amplification reaction (Appendix 1) prior to committing a precious sample. HeLa total RNA is provided with the kit as a control.

A. Round-One, 1st-strand cDNA Synthesis

SuperScript III Reverse Transcriptase (Invitrogen) is required for use in this step. The SuperScript III enzyme is provided by the user. The total RNA sample must be free of contaminating salts, metal ions, ethanol, and phenol. For best results, the RNA sample should be dissolved in RNase-Free water.

Required in Part A

Component Name	Tube Label	Cap Color
TargetAmp T7-Oligo(dT) Primer 1	T7-Oligo(dT) Primer 1	Red
TargetAmp Reverse Transcription PreMix	RT PreMix	
RiboGuard RNase Inhibitor	RiboGuard RNase Inhibitor	
Dithiothreitol	DTT	Clear
RNase-Free Water	RNase-Free Water	

Thermocycler programs: 65°C, 5 minutes and 50°C, 30 minutes.

Important! *The TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 has been optimized for producing >5,000,000-fold amplification of the Poly(A) RNA from 50-500 pg of total cellular RNA per reaction. Amplifying >500 pg of total RNA in a single TargetAmp 2-Round Amplification reaction may result in less than 5,000,000-fold amplification of the Poly(A) RNA and may result in under-representation of some Poly(A) RNA sequences in the Biotin-aRNA produced. Therefore, if it is desirable to perform an amplification reaction using >500 pg of total RNA, we strongly recommend that the user perform multiple reactions, each containing up to but not exceeding 500 pg of total RNA.*

1. Anneal the TargetAmp T7-Oligo(dT) Primer 1 to the RNA sample. If a "no template" control reaction is performed, substitute 2 µl of RNase-Free Water for the Total RNA sample.

   Important! *Be sure to use the TargetAmp T7-Oligo(dT) Primer 1 in this Step.*

x	µl	RNase-Free Water
x	µl	Total RNA sample (10-500 pg)
1	µl	TargetAmp T7-Oligo(dT) Primer 1
3	µl	Total

2. Incubate at 65°C for 5 minutes in a thermocycler.
3. Chill on ice for 1 minute. Centrifuge briefly in a microcentrifuge.
4. Prepare the Round-One, 1st-Strand cDNA Synthesis Master Mix.

   For each 1st-strand cDNA synthesis reaction, combine on ice:

1.25	µl	TargetAmp Reverse Transcription PreMix
0.25	µl	RiboGuard RNase Inhibitor
0.25	µl	DTT
0.25	µl	SuperScript III Reverse Transcriptase (200 U/µl)
2	µl	Total

Important! *Be sure to use SuperScript III Reverse Transcriptase. Do not use the SuperScript 5X Buffer or the DTT that is provided with the enzyme.*

5. Gently but thoroughly mix the Round-One, 1st-Strand cDNA Synthesis Master Mix and then add 2 µl of it to each reaction.
6. Gently but thoroughly mix the reactions and then incubate each at 50°C for 30 minutes in a thermocycler. If the thermocycler has a heated lid function, heat the lid only if the temperature of the lid can be maintained at about 50°C.

B. Round-One, 2nd-strand cDNA Synthesis

Required in Part B

Component Name	Tube Label	Cap Color
TargetAmp DNA Polymerase PreMix 1	DNA Pol PreMix 1	Red
TargetAmp DNA Polymerase 1	DNA Polymerase 1	
TargetAmp cDNA Finishing Solution	cDNA Finishing Solution	

Thermocycler programs: 65°C, 10 minutes/80°C, 3 minutes and 37°C, 10 minutes/80°C, 3 minutes.

1. Prepare the Round-One, 2nd-Strand cDNA Synthesis Master Mix.

   For each 2nd-strand cDNA synthesis reaction, combine on ice:

4.5	µl	TargetAmp DNA Polymerase PreMix 1
0.5	µl	TargetAmp DNA Polymerase 1
5	µl	Total

   Important! *Be sure to use the TargetAmp DNA Polymerase PreMix 1 and the TargetAmp DNA Polymerase 1 in this reaction.*

2. Gently but thoroughly mix the Round-One, 2nd-Strand cDNA Synthesis Master Mix and then add 5 µl of it to each reaction.
3. Gently but thoroughly mix the reactions and then incubate at 65°C for 10 minutes in a thermocycler.

   Important! *Be sure to incubate the reactions at 65°C.*

4. Incubate the reactions at 80°C for 3 minutes.
   Centrifuge briefly in a microcentrifuge then chill on ice.
5. Add 1 µl of TargetAmp cDNA Finishing Solution to each reaction.
6. Gently but thoroughly mix the reactions and then incubate at 37°C for 10 minutes in a thermocycler.
7. Incubate the reactions at 80°C for 3 minutes.
   Centrifuge briefly in a microcentrifuge then chill on ice.

   Note: *If desired, the reactions can now be frozen and stored overnight at –20°C.*

C. Round-One, *In Vitro* Transcription

Required in Part C

Component Name	Tube Label	Cap Color
NTP PreMix 1	NTP PreMix 1	Green
TargetAmp T7 RNA Polymerase	T7 RNA Polymerase	
TargetAmp T7 Transcription Buffer 1	Transcription Buffer 1	
RNase-Free DNase I	DNase I	
Dithiothreitol	DTT	Clear
RiboGuard RNase Inhibitor	RiboGuard RNase Inhibitor	Red

Thermocycler programs: 42°C, 4 hours and 37°C, 15 minutes.

1. Warm the TargetAmp T7 RNA Polymerase to room temperature. Thaw the remaining *in vitro* transcription reagents at room temperature and mix each thoroughly. If a precipitate is visible in the thawed TargetAmp T7 Transcription Buffer 1, heat the Buffer to 37°C until it dissolves. Keep the TargetAmp T7 Transcription Buffer at room temperature.

Important! *Be sure to thaw and use the NTP PreMix 1 in the Green-cap tube in this Step.*

2. Thoroughly mix the thawed TargetAmp T7 Transcription Buffer 1.

   Important! *Be sure to use the TargetAmp T7 Transcription Buffer 1 in the Green-cap tube in this step. If a precipitate is visible in the thawed TargetAmp T7 Transcription Buffer, heat the Buffer to 37°C until it dissolves. Mix the Buffer thoroughly. Keep the Buffer at room temperature.*

3. Prepare the Round-One, *In Vitro* Transcription Master Mix.

   For each *in vitro* transcription reaction, combine at room temperature:

4	µl	TargetAmp T7 Transcription Buffer 1
27	µl	NTP PreMix 1
4	µl	DTT
1	µl	RiboGuard RNase Inhibitor
4	µl	TargetAmp T7 RNA Polymerase
40	µl	Total

4. Gently but thoroughly mix the Round-One, *In Vitro* Transcription Master Mix and then add 40 µl of it to each reaction.
5. Gently but thoroughly mix the reactions and then incubate at 42°C for 4 hours in a thermocycler. If the thermocycler has a heated lid function, heat the lid only if the temperature of the lid can be maintained at about 42°C.

   Important! *Do not exceed 4 hour incubation. The 4 hour incubation gives optimal RNA yield and quality (length). Longer incubation times will result in lower quality RNA.*

6. Add 2 µl of RNase-Free DNase I to each reaction.
   Gently but thoroughly mix each and then incubate at 37°C for 15 minutes.

D. Round-One, RNA Purification

Purification of the RNA is necessary prior to the second round of RNA amplification. We recommend using the RNA Clean & Concentrator™-5 Kit (Zymo Research, Cat. No. R1015; provided by the user). These columns elute the purified RNA in a volume that is compatible with the second round amplification reaction. Other commercial RNA purification columns can be used (e.g., RNeasy MinElute Cleanup Kit; Qiagen cat. no. 74204) but their use will require the user to reduce the volume of the eluted RNA prior to the Round-Two, 1st-strand cDNA Synthesis step.

1a. RNA purification using the RNA Clean & Concentrator-5 Kit (Zymo Research). Follow the manufacturer's procedure for purifying Total RNA including small RNAs with the following suggestion: In Step 7 of the RNA Clean & Concentrator-5 column procedure, elute the RNA using 8 µl of RNase-Free Water. The eluted RNA can be used immediately in the Round-Two, 1st-strand cDNA Synthesis step (Part E). If desired, the purified RNA can be quick frozen and stored overnight at –70°C to –80°C.

1b. RNA purification using the Qiagen RNeasy MinElute Cleanup Kit (Qiagen). This procedure is slightly modified from the standard MinElute Cleanup Kit Procedure and requires that the user reduce the volume of the eluted RNA using a speed vacuum centrifugation prior to the Round-Two, 1st-strand cDNA Synthesis step.

TargetAmp Kit Component Required

Component Name	Tube Label	Cap Color
Poly(I)	Poly(I)	Clear

Note: *Use the RNase-Free Water provided in the RNeasy MinElute Cleanup Kit in this Step.*

2b. Prepare 350 µl of RLT/β-ME Solution for each sample as described in the MinElute kit handbook.

3b. Prepare 650 µl of RPE Solution for each sample as described in the MinElute kit handbook.

4b. To each sample add:

47.5	µl	RNase-Free Water
0.5	µl	Poly(I)
350	µl	RLT/β-ME Solution
250	µl	100% Ethanol

5b. Apply each sample to an RNeasy MinElute spin column in a 2 ml collection tube. Centrifuge at >8,000 x g for 15 seconds. Discard the flow-through.

6b. Apply 650 µl RPE Solution onto the column. Centrifuge at >8,000 x g for 15 seconds. Discard the flow-through.

7b. Apply 650 µl 80% ethanol onto the column. Centrifuge at >8,000 x g for 15 seconds. Discard the flow-through.

8b. Transfer the RNeasy MinElute spin column into a new collection tube. Centrifuge at full speed for 5 minutes.

9b. Transfer the spin column to a 1.5-ml collection tube. Elute the RNA by applying 14 µl of RNase-Free Water directly onto the center of the silica-gel membrane. Wait for 5 minutes. Centrifuge at full speed for 1 minute.

If desired, the RNA can now be quick frozen and stored overnight at –70°C to –80°C.

E. Round-Two, 1st-strand cDNA Synthesis

SuperScript II Reverse Transcriptase is required for use in this Step.

The SuperScript II enzyme is provided by the user.

Required in Part E

Component Name	Tube Label	Cap Color
TargetAmp Reverse Transcription PreMix	RT PreMix	Red
TargetAmp Random Primers	Random Primers	Blue
TargetAmp RNase H	RNase H	

Thermocycler programs: 65°C, 5 minutes; 37°C, 1 hour; 37°C, 20 minutes and 95°C, 2 minutes.

1. To each sample, add 2 µl of the TargetAmp Random Primers.
2. Transfer the entire volume of the purified aRNA from Part E, Step 1 into a 0.2 to 0.6-ml sterile reaction tube in which the remainder of the amplification reactions will be performed.

3a. If the RNA was purified using the Zymo Research RNA Clean & Concentrator-5 Kit in Part D, proceed immediately to Part E, Step 4.

3b. If the RNA was purified using the Qiagen RNeasy MinElute Cleanup Kit or other commercial RNA purification kit in Part D, then the user must adjust the volume of the reaction to 3 µl by speed vacuum centrifugation without heat. Important: Do not allow the RNA samples to completely dry.

Note: *Two suggestions are presented for efficiently performing the speed vacuum concentration step:*

Before concentrating the RNA sample, add 3 µl of water to a separate reaction tube. Mark the level of the 3 µl of water with a marking pen. Then, concentrate the RNA sample(s) using the speed vacuum centrifuge until it (they) is (are) at the same level in its (their) tube(s) as the 3-µl water sample.

Use water samples to determine the time necessary to reduce the RNA samples to 3 µl. For example, if there are four RNA samples to concentrate, add a volume of water equal to the volume of the RNA samples to each of four separate tubes. Speed vacuum centrifuge the water sample(s) to 3 µl final volume. Record the time needed to reduce the water samples volume to 3 µl. Then, concentrate the RNA sample(s) using the speed vacuum centrifuge for the same amount of time as needed to reduce the volume of the water sample(s) to 3 µl.

4. Incubate at 65°C for 5 minutes in a thermocycler.
5. Chill on ice for 1 minute. Centrifuge briefly in a microcentrifuge.
6. Prepare the Round-Two, 1st-Strand cDNA Synthesis Master Mix.

   For each 1st-strand cDNA synthesis reaction, combine on ice:

1.5	µl	TargetAmp Reverse Transcription PreMix
0.25	µl	DTT
0.25	µl	SuperScript II Reverse Transcriptase (200 U/µl)
2	µl	Total

Important! *Be sure to use SuperScript II Reverse Transcriptase. Do not use the SuperScript 5X Buffer or the DTT that is provided with the enzyme.*

7. Gently but thoroughly mix the Round-Two, 1st-Strand cDNA Synthesis Master Mix and then add 2 µl of it to each reaction.
8. Gently but thoroughly mix the reactions and then incubate each at room temperature for 10 minutes.
9. Transfer the reactions to 37°C and incubate each at 37°C for 1 hour in a thermocycler. If the thermocycler has a heated lid function, heat the lid only if the temperature of the lid can be maintained at about 37°C.
10. To each sample, add 0.5 µl of TargetAmp RNase H.

11. Gently but thoroughly mix each reaction and then incubate each at 37°C for 20 minutes in a thermocycler. If the thermocycler has a heated lid function, heat the lid only if the temperature of the lid can be maintained at about 37°C.
12. Transfer the reactions to 95°C.
    Incubate each at 95°C for 2 minutes in a thermocycler.
    Chill on ice for 1 minute.
    Centrifuge briefly in a microcentrifuge.

F. Round-Two, 2nd-strand cDNA Synthesis

Required in Part F

Component Name	Tube Label	Cap Color
TargetAmp T7-Oligo(dT) Primer 2	T7-Oligo(dT) Primer 2	Blue
TargetAmp DNA Polymerase PreMix 2	DNA Pol PreMix 2	
TargetAmp DNA Polymerase 2	DNA Polymerase 2	

Thermocycler programs: 70°C, 5 minutes; 42°C, 10 minutes; and 37°C, 10 minutes/80°C, 3 minutes.

1. To each reaction add 1 µl of the TargetAmp T7-Oligo(dT) Primer 2.

   Important! *Be sure to use the TargetAmp T7-Oligo(dT) Primer 2 in the blue-cap tube in this Step.*
2. Gently mix the reactions and then incubate at 70°C for 5 minutes, then incubate at 42°C for 10 minutes in a thermocycler.
   Centrifuge briefly in a microcentrifuge.
3. Prepare the Round-Two, 2nd-Strand cDNA Synthesis Master Mix.

   For each 2nd-strand cDNA synthesis reaction, combine on ice:

13	µl	TargetAmp DNA Polymerase PreMix 2
0.5	µl	TargetAmp DNA Polymerase 2
13.5	µl	Total

   Important! *Be sure to use the TargetAmp DNA Polymerase PreMix 2 in the Blue-cap tube and the TargetAmp DNA Polymerase 2 in the Blue-cap tube in this reaction.*
4. Gently but thoroughly mix the Round-Two, 2nd-Strand cDNA Synthesis Master Mix and then add 13.5 µl of it to each reaction.
5. Gently but thoroughly mix the reactions and then incubate at 37°C for 10 minutes in a thermocycler.
6. Incubate the reactions at 80°C for 3 minutes.

   Centrifuge briefly in a microcentrifuge then chill on ice.

   Note: *If desired, the reactions can now be frozen and stored overnight at –20°C.*

G. *In Vitro* Transcription of Biotin-aRNA

The *In Vitro* Transcription reaction produces Biotin-aRNA by incorporating Biotin-UTP into the RNA transcripts.

Required in Part G

Component Name	Tube Label	Cap Color
TargetAmp T7 RNA Polymerase	T7 RNA Polymerase	Green
RNase-Free DNase I	DNase I	
TargetAmp T7 Transcription Buffer 2	Transcription Buffer 2	Yellow
NTP PreMix 2	NTP PreMix 2	
Biotin-UTP	Biotin-UTP	
Dithiothreitol	DTT	Clear
RiboGuard RNase Inhibitor	RiboGuard RNase Inhibitor	Red

Thermocycler programs: 42°C, 9 hours/4°C, soak and 37°C, 15 minutes/4°C, soak.

1. Warm the TargetAmp T7 RNA Polymerase to room temperature. Thaw the remaining *in vitro* transcription reagents at room temperature and mix each thoroughly. If a precipitate is visible in the thawed TargetAmp T7 Transcription Buffer 2, heat the Buffer to 37°C until it dissolves. Keep the TargetAmp T7 Transcription Buffer at room temperature.
2. Thoroughly mix the thawed TargetAmp T7 Transcription Buffer 2.

   Important! *Use the TargetAmp T7 Transcription Buffer 2 in the Yellow-cap tube and the NTP PreMix 2 in the Yellow-cap tube in this step. If a precipitate is visible in the thawed TargetAmp T7 Transcription Buffer 2, heat the Buffer to 37°C until it dissolves. Mix the Buffer thoroughly. Keep the Buffer at room temperature.*
3. Prepare the *In Vitro* Transcription Master Mix.
   For each *in vitro* transcription reaction, combine in order at room temperature:

7.5	µl	TargetAmp T7 Transcription Buffer 2
4.5	µl	Biotin-UTP
20	µl	NTP PreMix 2
4	µl	DTT
1	µl	RiboGuard RNase Inhibitor
4	µl	TargetAmp T7 RNA Polymerase
41	µl	Total

4. Gently but thoroughly mix the *In Vitro* Transcription Master Mix and then add 41 µl of it to each reaction.
5. Gently mix the reactions and then incubate at 42°C for 9 hours in a thermocycler. If the thermocycler has a heated lid function, heat the lid only if the temperature of the lid can be maintained at about 42°C. If the lid temperature can not be maintained at about 42°C, then perform the incubations without heating the lid. If an overnight reaction is planned, program the thermocycler to hold the samples at 4°C after the 9 hour incubation is complete.

Important! *Do not exceed 9 hour incubation at 42°C. Optimal yield and quality (length) of Biotin-aRNA is achieved in 9 hours.*

6. Add 2 µl of RNase-Free DNase I to each reaction.
   Gently but thoroughly mix and then incubate each at 37°C for 15 minutes.

H. Biotin-aRNA Purification

The purification column to use is dependent upon the expected yield of aRNA. Use Table 1 to estimate the yield of aRNA expected from the amount of total RNA used in each amplification reaction. Then,

If the expected yield of AA-aRNA is <40 µg: purify the AA-aRNA using the Qiagen RNeasy MinElute Cleanup Kit. (Qiagen cat. no. 74204)

If the expected yield of AA-aRNA is >40 µg: purify the AA-aRNA using the Qiagen RNeasy Mini Kit. (Qiagen cat. no. 74014)

Use the RNase-Free Water that is provided in the MinElute Cleanup Kit or the RNeasy Mini Kit. The following procedure should be used with either the MinElute Cleanup Kit or the RNeasy Mini Kit.

1. Prepare 350 µl of RLT/β-ME Solution for each sample. Combine the RLT/β-ME in the ratio of 1 ml of Buffer RLT (provided in the RNeasy kit) with 10 µl of β-ME (β-mercaptoethanol) as described in the RNeasy kit's handbook.
2. Make sure that the RPE solution has Ethanol added to it, as described in the RNeasy kit's handbook.
3. To each sample add:
   38 µl RNase-Free Water
   350 µl RLT/β-ME Solution
   250 µl 100% Ethanol
4. Apply each sample to the purification kit's spin column in a 2 ml collection tube. Centrifuge at >8,000 x g for 15 seconds. Discard the flow-through.
5. Apply 700 µl of RPE Solution onto the column.
   Centrifuge at >8,000 x g for 15 seconds. Discard the flow-through.
6. Apply another 700 µl of RPE Solution onto the column.
   Centrifuge at >8,000 x g for 2 minutes. Discard the flow-through.
7. Transfer the spin column into a new collection tube.
   Centrifuge at full speed for 1 minute.
8. Transfer the spin column to a 1.5 ml collection tube. Elute the aRNA:

   If using the MinElute Cleanup Column, apply 20 µl of RNase-Free Water directly onto the center of the silica-gel membrane. Wait for 2 minutes. Centrifuge at full speed for 1 minute.

   If using the RNeasy Mini Column, apply 40 µl of RNase-Free Water directly onto the center of the silica-gel membrane. Wait for 2 minutes. Centrifuge at full speed for 1 minute.
9. If using the MinElute Cleanup Column, apply an additional 20 µl of RNase-Free Water directly onto the center of the silica-gel membrane. Wait for 2 minutes. Centrifuge at full speed for 1 minute.

If using the RNeasy Mini Column, apply an additional 40 µl of RNase-Free Water directly onto the center of the silica-gel membrane. Wait for 1 minute. Centrifuge at full speed for 1 minute.

This second elution step recovers 25% – 35% of the final yield of biotin-aRNA.

If desired, the RNA can now be quick frozen and stored overnight at –70°C to –80°C.

I. Quantifying the Concentration, Yield and Fold-Amplification of the Biotin-aRNA

Concentration and yield: The concentration of the Biotin-aRNA can be readily determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer available from NanoDrop Technologies. Alternately, due to the high yield of Biotin-aRNA that is produced by a TargetAmp reaction, the yield and concentration of Biotin-aRNA can be determined by standard UV spectroscopy.

1. Prepare a dilution of the Biotin-aRNA into the minimum volume of water or TE Buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) required by the spectrophotometer cuvette that will be used.
2. Zero the spectrophotometer at 260 nm using the diluents (water or TE buffer) that was used to dilute the Biotin-aRNA sample.
3. Measure and record the absorbance of the diluted Biotin-aRNA at 260 nm (A_{260}).
4. Calculate the concentration of the Biotin-aRNA. Use the conversion factor that an A_{260} reading of 1.0 is equal to an RNA concentration of 40 µg/ml.
   Biotin-aRNA concentration = (A_{260} reading) x (dilution factor) x (40 µg/ml).
   Example: Dilution for A_{260} measurement = 1:100 with an A_{260} of the 1:100 dilution = 0.15.
   Biotin-aRNA concentration = (0.15) x (100) x (40 µg/ml) = 600 µg/ml = (0.6 µg/µl) Biotin-aRNA.
5. Calculate the yield of Biotin-aRNA using the formula:
   Yield of Biotin-aRNA = (Biotin-aRNA Concentration) x (Volume of Biotin-aRNA).
   Example: 50 µl of Biotin-aRNA recovered from column, 0.6 µg/µl Biotin-aRNA determined in Part I, Step 4.
   Biotin-aRNA yield = (0.6 µg/µl) x (50 µl) = 30 µg of Biotin-aRNA.

In this example, 1 µl of 0.6 µg/µl of Biotin-aRNA was used for the spectrophotometer reading so there are now 29.4 µg of Biotin-aRNA remaining.

Fold amplification: The fold-amplification of the reaction can be calculated once the yield of the Biotin-aRNA has been determined. However, if the input RNA was total RNA, an accurate calculation of fold-amplification requires knowledge of the Poly(A) content of the original total RNA sample. If the Poly(A) content of the sample is not known, a commonly used assumption is that Poly(A) RNA constitutes 2% of the RNA in a total RNA sample.

Fold-amplification = (amount of Biotin-aRNA produced) / ([amount of total RNA input] x [Percentage of Poly(A) RNA in the total RNA sample]).

Example: Amount of input total RNA = 200 pg, Percentage of Poly(A) RNA in the sample (assumed) = 2% (0.02), Amount of Biotin-aRNA produced = 30,000,000 pg (30 µg).

Fold-amplification = 30,000,000 pg / (200 pg x 0.02) = 7,500,000.

J. Assessing the Size of the Biotin-aRNA Produced

A TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 reaction typically produces Biotin-aRNA with a size distribution between 300-3,000 bases with an average size of about 600 bases.

Sizing the Biotin-aRNA by denaturing gel electrophoresis: When assessing the size distribution of the Biotin-aRNA by denaturing agarose gel electrophoresis, load at least 1 µg into the well of a 1% formaldehyde-agarose gel. Load RNA size markers that cover the size range of approximately 100-2,000 bases.

Sizing the Biotin-aRNA using the Agilent 2100 Bioanalyzer: Dilute an aliquot of the Biotin-aRNA with water to approximately 100 ng/µl. Using the Agilent RNA 6000 Nano LabChip, load 1 µl of the diluted Biotin-aRNA per well. It is advisable to load and run duplicates of each sample tested including a no-input RNA control sample. Run the samples per instrument procedure.

5. Appendix

Appendix 1: The TargetAmp 2-Round Biotin-aRNA Amplification Kit 3.0 Control Reaction

The TargetAmp kit provides 400 ng of total human HeLa RNA at a concentration of 40 ng/µl.

Required for the TargetAmp Control Reaction

Component Name	Tube Label	Cap Color
HeLa Total RNA Control (40 ng/µl)	HeLa Total RNA	Clear
RNase-Free Water	RNase-Free Water	
TargetAmp T7-Oligo(dT) Primer 1	T7-Oligo(dT) Primer B	Red

Thermocycler programs: 60°C, 5 minutes and 50°C, 30 minutes.

1. Thaw the HeLa Total RNA Control on ice.
2. On ice, dilute the thawed HeLa Total RNA Control 1:200 with RNase-Free Water by adding 1 µl of the HeLa Total RNA Control to 199 µl of RNase-Free Water and mix thoroughly. The concentration of the diluted RNA Control is 200 pg/µl.
3. Anneal the TargetAmp T7-Oligo(dT) Primer 1 to the HeLa Total RNA Control.

   The standard control reaction utilizes 200 pg of the HeLa Total RNA Control.

   Important! *Be sure to use the TargetAmp T7-Oligo(dT) Primer 1 in the red-cap tube in this Step.*

1 µl	RNase-Free Water
1 µl	HeLa Total RNA Control (200 pg)
1 µl	TargetAmp T7-Oligo(dT) Primer 1
3 µl	Total

4. Incubate the reaction at 65°C for 5 minutes in a thermocycler. While the reaction incubates, quick-freeze the HeLa Total RNA Control (40 ng/µl; e.g., in a dry ice/ethanol bath) and return it to –70°C to –80°C storage. Discard the diluted RNA Control.
5. Cool the annealing reaction on ice for at least 1 minute. Centrifuge the tube for 5-10 seconds to bring the sample to the bottom of the tube.
6. Continue the Control Reaction as described beginning in Part A, Step 4.

6. Additional TargetAmp™ aRNA Amplification Kits & Guide

TargetAmp™ 1-Round Aminoallyl-aRNA Amplification Kit 101

TAA1R4924 — 24 Reactions

This kit will amplify Poly(A) RNA by >5,000 fold from as little as 25 ng of total cellular RNA. The kit produces aminoallyl-aRNA and is optimized for use of SuperScript III Reverse Transcriptase (provided by the user).

TargetAmp™ 1-Round aRNA Amplification Kit 103

TAU1R5110 — 10 Reactions

TAU1R5124 — 24 Reactions

This kit will amplify Poly(A) RNA by >5,000 fold producing microgram amounts of unlabeled aRNA from as little as 25 ng of total cellular RNA. The kit is optimized for use with SuperScript III Reverse Transcriptase (provided by the user).

TargetAmp™ 1-Round Biotin-aRNA Amplification Kit 105

TAB1R80524 — 24 Reactions

This kit will amplify Poly(A) RNA by >5,000 fold producing microgram amounts of Biotin-aRNA from as little as 25 ng of total cellular RNA. The kit is optimized for use with SuperScript III Reverse Transcriptase (provided by the user).

TargetAmp™ Nano Labeling Kit for Illumina® Expression BeadChip®

TAN07924 — 24 Reactions

TAN091096 — 96 Reactions

This kit will produces microgram amounts of Biotin-aRNA from as little as 25 ng of total cellular RNA for hybridization to Illumina® Expression BeadChips. The kit is optimized for use with SuperScript III Reverse Transcriptase (provided by the user).

TargetAmp™ 2-Round Aminoallyl-aRNA Amplification Kit 1.0

TAA2R4924 — 24 Reactions

This kit will amplify Poly(A) RNA by >5,000,000 fold producing microgram amounts of aminoallyl-aRNA from as little as 10 pg of total cellular RNA. The kit is optimized for use with SuperScript III and SuperScript II Reverse Transcriptases (provided by the user).

TargetAmp™ 2-Round aRNA Amplification Kit 2.0

TAU2R51224 — 24 Reactions

This kit will amplify Poly(A) RNA by >5,000,000 fold producing microgram amounts of unlabeled aRNA from as little as 10 pg of total cellular RNA. The kit is optimized for use with SuperScript III and SuperScript II Reverse Transcriptases (provided by the user).

TargetAmp™-Pico Labeling Kit for Illumina® Expression BeadChip®

This kit will produces microgram amounts of Biotin-aRNA from as little as 50 pg of total cellular RNA for hybridization to Illumina Expression BeadChips. The kit is optimized for use with SuperScript III and SuperScript II Reverse Transcriptase (provided by the user).

TAP120210 10 Reactions

TAP120224 24 Reactions

	TargetAmp™ 1-Round Aminoallyl-aRNA Amplification Kit 101	**TargetAmp™ 1-Round aRNA Amplification Kit 103**	**TargetAmp™ 1-Round Biotin-aRNA Amplification Kit 105**
Starting Total RNA	25-500 ng	25-500 ng	25-500 ng
Reverse Transcriptase(s) Used	SuperScript® III (provided by the user)	SuperScript® III (provided by the user)	SuperScript® III (provided by the user)
Fold Amplification	> 5,000	> 5,000	> 5,000
Time Required	1 Day	1 Day	1 Day
End Product	Aminoallyl-aRNA	Unlabeled-aRNA	Biotin-aRNA

	TargetAmp™ Nano Labeling Kit for Illumina® Expression BeadChip®	**TargetAmp™ 2-Round Aminoallyl-aRNA Amplification Kit 1.0**	**TargetAmp™ 2-Round aRNA Amplification Kit 2.0**
Starting Total RNA	25-500 ng	10-500 pg	10-500 pg
Reverse Transcriptase(s) Used	SuperScript® III (provided by the user)	SuperScript® III & SuperScript® II (provided by the user)	SuperScript® III & SuperScript® II (provided by the user)
Fold Amplification	> 5,000	> 5,000,000	> 5,000,000
Time Required	1 Day	2 Days	2 Days
End Product	Biotin-aRNA	Aminoallyl-aRNA	Biotin-aRNA

7. Related Products

ArrayPure™ Nano-scale RNA Purification Kit
MPS04050 50 Purifications

The ArrayPure Kit provides all reagents needed to purify total RNA from 1-10,000 eukaryotic cells without the use of organic solvents such as phenol or columns.

MasterPure™ RNA Purification Kit
MCR85102 100 Purifications

The MasterPure RNA Purification Kit provides all reagents needed to purify total RNA from >10,000 eukaryotic cells without the use of organic solvents such as phenol or columns.

ArrayPure™ Nano-scale RNA Purification Kit
MPS04050 50 Purifications

MasterPure™ RNA Purification Kit
MCR85102 100 Purifications

Ribo-Zero™ rRNA Removal Kits
Visit www.epicentre.com/ribozero.asp for a complete listing and descriptions of the Ribo-Zero Kits

ScriptSeq™ v2 RNA-Seq Library Preparation Kit
SSV21106 6 Reactions
SSV21124 24 Reactions

ScriptSeq™ Complete Kits
Visit www.epicentre.com for a complete listing and descriptions of the ScriptSeq Complete Kits

8. Reference

1. Van Gelder, R. N. *et al.*, (1990) *Proc. Natl. Acad. Sci. USA* **87** (5), 1663.

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