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TargetAmp™ 2-Round aRNA Amplification Kit 2.0

Cat. No. TAU2R51224 – 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 11 and page 15.

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1. Introduction

The TargetAmp™ 2-Round aRNA Amplification Kit 2.0 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.

1. **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.
2. **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 will generate anti-sense RNA (aRNA) during the subsequent *in vitro* transcription reaction.
3. **Round-One, *In Vitro* Transcription:** High yields of anti-sense RNA (aRNA) are produced in a rapid *in vitro* transcription reaction that utilizes the double-stranded cDNA produced in Step 2.
4. **Round-One, RNA Purification:** The aRNA produced in the first round amplification procedure (Steps 1-3) is purified by spin column chromatography (supplied by the user).
5. **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.
6. **Round-Two, 2nd-strand cDNA Synthesis:** The RNA component of the cDNA:aRNA hybrid produced in Step 5 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 aRNA during the second round *in vitro* transcription reaction.
7. ***In Vitro* Transcription of aRNA:** High yields of aRNA (cRNA) are produced in an *in vitro* transcription reaction that utilizes the double-stranded cDNA produced in Step 6.

2. Performance Specifications and Quality Control

The TargetAmp 2-Round aRNA Amplification Kit 2.0 is function-tested in a control reaction. The kit must produce at least 20 µg of 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 aRNA.

3. 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	Volume 24-rxn	Cap Color
TargetAmp T7-Oligo(dT) Primer B	T7-Oligo(dT) Primer B	30 µl	Red
TargetAmp Reverse Transcription PreMix-SS	RT PreMix-SS	90 µl	
TargetAmp DNA Polymerase PreMix-SS 1	DNA Pol PreMix-SS 1	120 µl	
TargetAmp DNA Polymerase-SS 1	DNA Polymerase-SS 1	18 µl	
TargetAmp cDNA Finishing Solution-SS	Finishing Solution-SS	30 µl	
RiboGuard RNase Inhibitor	RiboGuard RNase Inhibitor	60 µl	
TargetAmp Random Primers-SS	Random Primers-SS	55 µl	Blue
TargetAmp RNase H-SS	RNase H-SS	18 µl	
TargetAmp T7-Oligo(dT) Primer C	T7-Oligo(dT) Primer C	30 µl	
TargetAmp DNA Polymerase PreMix-SS 2	DNA Pol PreMix-SS 2	360 µl	
TargetAmp DNA Polymerase-SS 2	DNA Polymerase-SS 2	18 µl	
TargetAmp *In Vitro* Transcription PreMix A	IVT PreMix A	725 µl	Green
TargetAmp T7 RNA Polymerase	T7 RNA Polymerase	225 µl	
TargetAmp T7 Transcription Buffer	T7 Transcription Buffer	225 µl	
100 mM ATP	ATP	110 µl	
100 mM CTP	CTP	110 µl	
100 mM GTP	GTP	110 µl	
100 mM UTP	UTP	110 µl	
RNase-Free DNase I	DNase I	115 µl	
Dithiothreitol (DTT)	DTT	250 µl	Clear
HeLa Total RNA Control (40 ng/µl)	HeLa Total RNA Control	10 µl	
RNase-Free Water	RNase-Free Water	2 x 1 ml	
Poly(I)	Poly(I)	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 or water bath
Microcentrifuge
RNase-Free Water
RNeasy® MinElute® Cleanup Kit
or
RNeasy Mini Kit (Qiagen) (see "aRNA Purification" for details)

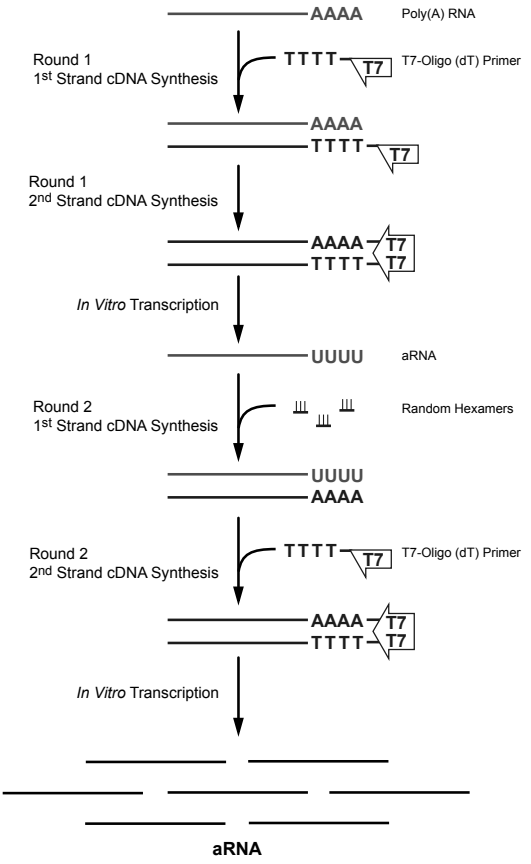

Figure 1. TargetAmp™ 2-Round aRNA Amplification Kit 2.0 Procedure.

4. 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 aRNA Amplification Kit. 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 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 as little as 5 ng 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 aRNA Amplification Kit 2.0 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.

Input Total RNA and aRNA Yield:

The TargetAmp 2-Round aRNA Amplification Kit 2.0 is extremely efficient at producing microgram amounts of aRNA from picogram quantities of total RNA. The actual 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 aRNA yield and fold-amplification obtained from high-integrity total RNA from 3 different cell types, each containing a different percentage of Poly(A) RNA. The information in Table 1 provides guidance to the amount of 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 aRNA obtained from three total RNA sources, including the HeLa Total RNA Control provided in the kit, using the TargetAmp™ 2-Round aRNA Amplification Kit 2.0. 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	aRNA yield from Total Rat Brain RNA [assume 5% of rat brain total RNA is Poly(A) RNA]	aRNA yield from Total HeLa RNA [assume 2% of HeLa total RNA is Poly(A) RNA]	aRNA yield from Total Rat Kidney RNA [assume 1.5%-2% of rat kidney total RNA is Poly(A) RNA]
0 pg	0.6 µg	0.6 µg	0.6 µg
10 pg	3 µg (6×10^6)	1.9 µg (9.5×10^6)	1.4 µg (~9×10^6)
50 pg	21 µg (8.4×10^6)	9 µg (9×10^6)	7 µg (~9×10^6)
100 pg	54 µg (10.8×10^6)	19 µg (9.5×10^6)	17 µg (~11×10^6)
200 pg	75 µg (7.5×10^6)	37 µg (9.2×10^6)	33 µg (~11×10^6)
500 pg	143 µg (5.7×10^6)	74 µg (7.4×10^6)	71 µg (~9×10^6)

Important! *The TargetAmp 2-Round aRNA Amplification Kit 2.0 has been optimized for producing >5,000,000-fold amplification of the Poly(A) RNA from 10-500 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 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.*

aRNA Purification:

The appropriate aRNA purification process (Step H) to use is dependent on the expected yield of aRNA. Use Table 1 to estimate the yield of 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 aRNA is <40 µg, purify the aRNA using the Qiagen MinElute Cleanup Kit.
2) If the expected yield of aRNA is >40 µg, purify the aRNA using the Qiagen RNeasy Mini Kit.

5. Additional Suggestions

Familiarize Yourself with the TargetAmp Kit and Procedure:

The TargetAmp 2-Round aRNA Amplification Kit 2.0 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 aRNA Kit Control Reaction:

We strongly recommend that those who are not experienced with the TargetAmp 2-Round aRNA Amplification Kit 2.0 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 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 6.C and Part 6.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 aRNA produced.
5) Optional stopping points are noted following the 1st-round and 2nd-round 2nd-strand cDNA synthesis steps (Part 6.B and Part 6.F) and after purification of the aRNA (Part 6.D and Part6. H).

6. RNA Amplification Procedure

Please read through the TargetAmp 2-Round aRNA Amplification Kit 2.0 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. A HeLa total RNA control is provided with the kit.

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

SuperScript III reverse Transcriptase (Life Technologies) is required for use in this Part. 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 6.A

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

Incubation temperatures performed in Part 5.A: 50°C and 65°C.

Important! *The TargetAmp 2-Round aRNA Amplification Kit 2.0 has been optimized for producing >5,000,000-fold amplification of the Poly(A) RNA from 10-500 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 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 B 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 B in this Step.*

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

2. Incubate at 65°C for 5 minutes in a water bath or 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:

1.5	µl	TargetAmp Reverse Transcription PreMix-SS
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 mix the Round-One, 1st-Strand cDNA Synthesis Master Mix and then add 2 µl of it to each reaction.
6. Gently mix the reactions and then incubate each at 50°C for 30 minutes in a water bath or 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.

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

Required in Part 6.B

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

Incubation temperatures performed in Part 6.B: 37°C, 65°C and 80°C.

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-SS 1
0.5 µl	TargetAmp DNA Polymerase-SS 1
5 µl	Total

   Important! *Be sure to use the TargetAmp DNA Polymerase PreMix-SS 1 and the TargetAmp DNA Polymerase-SS 1 in this reaction.*
2. Gently mix the Round-One, 2nd-Strand cDNA Synthesis Master Mix and then add 5 µl of it to each reaction.
3. Gently mix the reactions and then incubate at 65°C for 10 minutes in a water bath or thermocycler. Centrifuge briefly in a microcentrifuge.

   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-SS to each reaction.
6. Gently mix the reactions and then incubate at 37°C for 10 minutes.
7. Transfer the reactions to 80°C and then 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.*

6.C. Round-One, *In Vitro* Transcription

Required in Part 6.C

Component Name	Tube Label	Cap Color
TargetAmp *In Vitro* Transcription PreMix A	IVT PreMix A	Green
TargetAmp T7 RNA Polymerase	T7 RNA Polymerase	
TargetAmp T7 Transcription Buffer	T7 Transcription Buffer	
RNase-Free DNase I	DNase I	
Dithiothreitol	DTT	Clear
RiboGuard RNase Inhibitor	RiboGuard RNase Inhibitor	Red

Incubation temperatures performed in Part 6.C: 37°C and 42°C.

1. Warm the TargetAmp T7 RNA Polymerase to room temperature. Thaw the remaining *in vitro* transcription reagents at room temperature. If a precipitate is visible in the thawed TargetAmp T7 Transcription Buffer, 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 TargetAmp *In Vitro* Transcription PreMix A in this Step.
2. Thoroughly mix the thawed TargetAmp T7 Transcription Buffer.

   Important! *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
27	µl	TargetAmp *In Vitro* Transcription PreMix A
4	µl	DTT
4	µl	TargetAmp T7 RNA Polymerase
1	ul	RiboGuard RNase Inhibitor
40	µl	Total

4. Gently mix the Round-One, *In Vitro* Transcription Master Mix and then add 40 µl of it to each reaction.
5. Gently mix the reactions and then incubate at 42°C for 4 hours in a thermocycler or a water bath. 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.

   Mix gently and then incubate each at 37°C for 15 minutes.

6.D. Round-One, RNA Purification

This step uses the Qiagen RNeasy MinElute Cleanup Kit (Qiagen cat. no. 74204).

Use the RNase-Free Water that is provided in the MinElute Cleanup Kit.

Required in Part 6.D

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.*

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 MinElute kit) with 10 µl of β-ME (β-mercaptoethanol) as described in the MinElute kit handbook.
2. Prepare 650 µl of RPE Solution for each sample by diluting 1 volume of Buffer RPE (provided in the MinElute kit) with 4 volumes of 96-100% ethanol as described in the MinElute kit handbook.
3. 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

4. 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.
5. Apply 650 µl RPE Solution onto the column.
   Centrifuge at >8,000 x g for 15 seconds. Discard the flow-through.
6. Apply 650 µl 80% ethanol onto the column.
   Centrifuge at >8,000 x g for 15 seconds. Discard the flow-through.
7. Transfer the RNeasy MinElute spin column into a new collection tube.
   Centrifuge at full speed for 5 minutes.
8. Transfer the spin column to a 1.5 ml collection tube. Elute the aRNA 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.

   Note: *If desired, the RNA can now be quick frozen (e.g., dry ice/ethanol bath) and stored overnight at –80°C.*

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

SuperScript II Reverse Transcriptase (Life Technologies) is required for use in this Part.

The SuperScript II enzyme is provided by the user.

Required in Part 6.E

Component Name	Tube Label	Cap Color
TargetAmp Reverse Transcription PreMix-SS	RT PreMix-SS	Red
TargetAmp Random Primers-SS	Random Primers-SS	Blue
TargetAmp RNase H-SS	RNase H-SS	
Dithiothreitol	DTT	Clear

Incubation temperatures performed in Part 6.E: 37°C, 65°C and 95°C.

1. To each sample, add 2 µl of the TargetAmp Random Primers-SS.
2. Transfer the entire volume of the purified aRNA from Part 6.E.1 into a 0.2-0.6 ml sterile reaction tube in which the remainder of the amplification reactions will be performed.
3. Adjust the volume of each aRNA sample 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 using the speed vacuum centrifuge until it is at the same level in its tube 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 4 RNA samples to concentrate, add a volume of water equal to the volume of the RNA samples to each of 4 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 using the speed vacuum centrifuge for the same amount of time as needed to reduce the volume of the water sample to 3 µl.

4. Incubate at 65°C for 5 minutes in a water bath or 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-SS
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 mix the Round-Two, 1st-Strand cDNA Synthesis Master Mix and then add 2 µl of it to each reaction.
8. Gently 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 water bath or 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-SS.
11. Gently mix each reaction and then incubate each at 37°C for 20 minutes in a water bath or 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 water bath or thermocycler.

    Chill on ice for 1 minute.

    Centrifuge briefly in a microcentrifuge.

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

Required in Part 6.F

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

Incubation temperatures performed in Part 6.F: 37°C, 42°C, 70°C and 80°C.

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

   Important! *Be sure to use the TargetAmp T7-Oligo(dT) Primer C 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 water bath or 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-SS 2
0.5	µl	TargetAmp DNA Polymerase-SS 2
13.5	µl	Total

   Important! *Be sure to use the TargetAmp DNA Polymerase PreMix-SS 2 and the TargetAmp DNA Polymerase-SS 2 in this reaction.*
4. Gently mix the Round-Two, 2nd-Strand cDNA Synthesis Master Mix and then add 13.5 µl of it to each reaction.
5. Gently mix the reactions and then incubate at 37°C for 10 minutes in a water bath or thermocycler. Centrifuge briefly in a microcentrifuge.

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.*

6.G. *In Vitro* Transcription of aRNA

Required in Part 6.G

Component Name	Tube Label	Cap Color
TargetAmp T7 RNA Polymerase	T7 RNA Polymerase	Green
TargetAmp T7 Transcription Buffer	T7 Transcription Buffer	
100 mM ATP	ATP	
100 mM CTP	CTP	
100 mM GTP	GTP	
100 mM UTP	UTP	
RNase-Free DNase I	DNase I	
DTT	DTT	Clear
RNase-Free Water	RNase-Free Water	
RiboGuard RNase Inhibitor	RiboGuard RNase Inhibitor	Red

Incubation temperatures performed in Part 6.G: 37°C and 42°C.

1. Warm the TargetAmp T7 RNA Polymerase to room temperature. Thaw the remaining *in vitro* transcription reagents at room temperature. If a precipitate is visible in the thawed TargetAmp T7 Transcription Buffer, 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.

   Important! *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-Two, *In Vitro* Transcription Master Mix.

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

13.6	µl	RNase-Free Water
4	µl	TargetAmp T7 Transcription Buffer
3.6	µl	ATP
3.6	µl	CTP
3.6	µl	GTP
3.6	µl	UTP
4	µl	DTT
4	µl	TargetAmp T7 RNA Polymerase
1	µl	RiboGuard RNase Inhibitor
41	µl	Total

4. Gently mix the Round-Two, *In Vitro* Transcription Master Mix and then add 40 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 aRNA is achieved in 9 hours.*

6. Add 2 µl of RNase-Free DNase I to each reaction.

   Mix gently and then incubate each at 37°C for 15 minutes.

6.H. aRNA Purification

The purification column to use is dependent upon the expected yield of aRNA. Use Table 1 to estimate the yield of 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,

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

If the expected yield of aRNA is >40 µg: purify the 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. Prepare 1.4 ml of RPE Solution for each sample by diluting 1 volume of Buffer RPE (provided in the RNeasy kit) with 4 volumes of 96-100% ethanol 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 RNeasy spin column in a 1.5 ml collection tube. Centrifuge at >8,000 x g for 15 seconds. Discard the flow-through.
5. Apply 700 µl RPE Solution onto the column. Centrifuge at >8,000 x g for 15 seconds. Discard the flow-through. Reattach the 1.5 ml collection tube to the column.
6. Apply another 700 µl RPE Solution onto the column. Centrifuge at >8,000 x g for 2 minutes. Discard the flow-through. Reattach the 1.5 ml collection tube to the column.
7. Centrifuge at full speed for 1 minute. Discard the flow-through.

8. Transfer the RNeasy spin column to a new 1.5 ml collection tube.

   Apply at least 25 µl of RNase-Free Water directly to the column.

   Wait for 5 minutes at room temperature.

9. Centrifuge at >8,000 x g for 1 minute to collect the aRNA.

   Note: *If desired, the reactions can now be quick frozen (e.g., dry ice/ethanol bath) and stored overnight at –80°C.*

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

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

1. Prepare a dilution of the 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 aRNA sample.
3. Measure and record the absorbance of the diluted aRNA at 260 nm (A_{260}).
4. Calculate the concentration of the aRNA. Use the conversion factor that an A_{260} reading of 1.0 is equal to an RNA concentration of 40 µg/ml.

   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.

   aRNA concentration = (0.15) x (100) x (40 µg/ml) = 600 µg/ml = (0.6 µg/µl) aRNA.

5. Calculate the yield of aRNA using the formula:

   Yield of aRNA = (aRNA Concentration) x (Volume of aRNA).

   Example: 50 µl of aRNA recovered from column, 0.6 µg/µl aRNA determined in Part 6.I, Step 4.

   aRNA yield = (0.6 µg/µl) x (50 µl) = 30 µg of aRNA.

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

Fold amplification: The fold-amplification of the reaction can be calculated once the yield of the 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 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 aRNA produced = 30,000,000 pg (30 µg).

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

6.J. Assessing the Size of the aRNA Produced

A TargetAmp 2-Round aRNA Amplification reaction typically produces aRNA with a size distribution between 300-3000 bases with an average size of about 600 bases.

Sizing the aRNA by denaturing gel electrophoresis: The advantages of denaturing agarose gel electrophoresis are its relatively low cost and ready availability of the reagents required. When assessing the size distribution of the aRNA, 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 100-2000 bases.

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

7. Appendix

TargetAmp 2-Round aRNA Amplification Kit 2.0 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 B	T7-Oligo(dT) Primer B	Red

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. The concentration of the diluted RNA Control is 200 pg/µl.
3. Anneal the TargetAmp T7-Oligo(dT) Primer B 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 B in this Step.*

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

4. Incubate the reaction at 65°C for 5 minutes in a water bath or thermocycler. While the reaction incubates, quick-freeze the HeLa Total RNA Control (40 ng/µl; for example 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 6.A, Step 4.

8. Additional TargetAmp aRNA Amplification Kits & Selection Guide

	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™ 2-Round aRNA Amplification Kit 1.0	TargetAmp™ Nano Labeling Kit for Illumina® Expression BeadChip®	TargetAmp™ 2-Round Biotin-aRNA Amplification Kit 3.0
Starting Total RNA	10-500 pg	25-500 pg	50-500 pg
Reverse Transcriptase(s) Used	SuperScript® III & SuperScript® II (provided by the user)	SuperScript® III & SuperScript® II (provided by the user)	SuperScript® III & SuperScript® II (provided by the user)
Fold Amplification	>5,000,000	>5,000	>5,000,000
Time Required	2 Days	1 Day	2 Days
End Product	Unlabeled-aRNA	Biotin-aRNA	Biotin-aRNA

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

TAA2R4910 10 Reactions

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 Biotin-aRNA Amplification Kit 3.0

TAB2R71010 10 Reactions

TAB2R71024 24 Reactions

This kit will amplify Poly(A) RNA by >5,000,000 fold producing microgram amounts of Biotin-aRNA from as little as 50 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®

TAP120210 10 Reactions

TAP120224 24 Reactions

This kit will produce 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).

9. 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.

Ribo-Zero™ rRNA Removal Kits

Visit www.epicentre.com/ribozero 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.

10. Reference

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

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