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

Cat. No. TAP120210 – 10 Reactions

Cat. No. TAP120224 – 24 Reactions

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1. Kit Contents

The kit components are supplied in tubes with colored caps for easier identification. The kit has been developed for use, and will provide optimal results, with SuperScript® III and SuperScript II Reverse Transcriptases (Life Technologies™; provided by the user).

Component Name	Tube Label	10 Reactions	24 Reactions	Cap Color
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

RNA Clean & Concentrator™-5 columns (Zymo Research)

RNase-Free Water

RNeasy® MinElute® Cleanup Kit or RNeasy Mini Kit (Qiagen)

Performance Specifications and Quality Control

The TargetAmp-Pico Labeling Kit is function-tested in a control reaction. The kit must produce at least 20 µg of Biotin-aRNA from 200 pg of HeLa Total RNA Control. A negative control reaction ("no-RNA" control) produces less than 2 µg of Biotin-aRNA.

2. Overview of the Process

The TargetAmp-Pico Labeling Kit utilizes an improved "Eberwine" procedure[1] for amplifying poly(A) RNA from as little as 50 pg of total cellular RNA. The kit requires both SuperScript III and SuperScript II Reverse Transcriptases (provided by the user). See Fig. 1.

1. **Round-one, first-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 containing a phage T7 RNA Polymerase promoter sequence at its 5′ end. Round-one, first-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, second-strand cDNA synthesis:** Second strand of cDNA is synthesized. The resulting product is a double-stranded (ds) cDNA containing a T7 transcription promoter in an orientation that that will generate antisense RNA (aRNA; sometimes called cRNA) during the subsequent *in vitro* transcription reaction.
3. **Round-one *in vitro* transcription:** High yields of aRNA are produced in a rapid *in vitro* transcription reaction that uses the ds cDNA produced in Part 2.
4. **Round-one RNA purification:** The aRNA produced in the first-round amplification procedure (Parts 1-3) is purified by spin-column chromatography (supplied by the user).
5. **Round-two, first-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 (supplied by the user). The reaction is primed using random-sequence hexamer primers.
6. **Round-two, second-strand cDNA synthesis:** Second-strand cDNA synthesis is then primed using a T7-Oligo(dT) Primer. The resulting product is a ds cDNA containing a T7 transcription promoter in an orientation that that will generate biotin-aRNA during the second-round *in vitro* transcription reaction.
7. ***In vitro* transcription of biotin-aRNA:** High yields of biotin-aRNA are produced in a rapid *in vitro* transcription reaction that uses the ds cDNA as template.

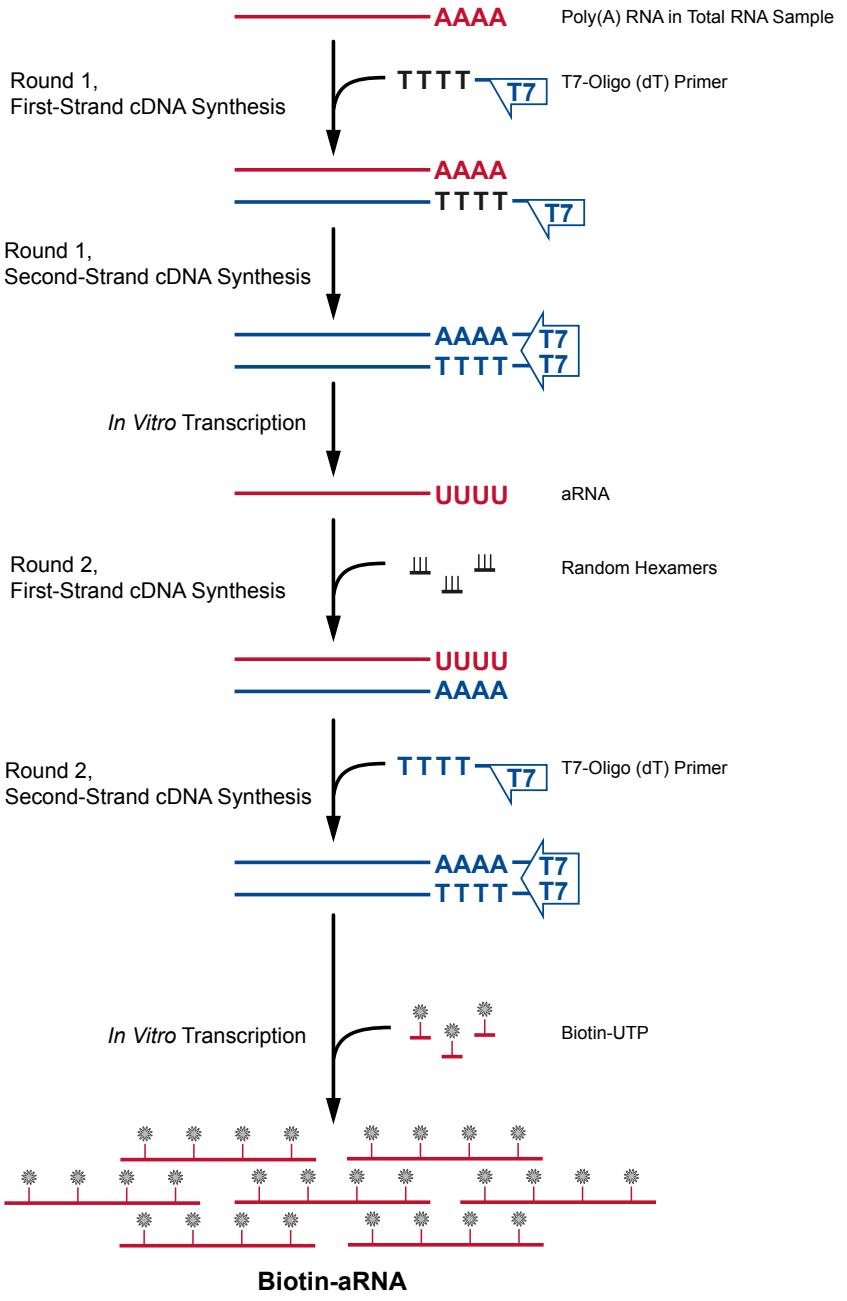

Figure 1. An overview of the procedure for the TargetAmp™-Pico Labeling Kit for Illumina® Expression BeadChip®.

3. Preparation

Maintaining an RNase-Free Environment:

Ribonuclease contamination is a significant concern for those performing RNA amplification. All components of the TargetAmp-Pico Kit 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. Therefore, we strongly recommend that you:

- Autoclave all tubes and pipette tips that will be used in the RNA amplification reactions.
- 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.
- Always wear gloves when handling kit components. Do not pick up any kit component with an ungloved hand.
- Keep all kit components tightly sealed when not in use. Keep all tubes containing RNA tightly sealed during the incubation steps.

Input RNA:

The success of a TargetAmp-Pico Kit reaction is strongly influenced by the quality of the input RNA. The RNA should dissolved in RNase-free water and be free of contaminants such as salt, ethanol, denaturants (e.g., TRIzol® reagent), and metal ions (e.g., Mg^{+2}). The RNA should also be "intact" as judged by the presence of the high-molecular-weight rRNAs by denaturing agarose gel electrophoresis or other methods, such as a Bioanlyzer 2100 (Agilent). Poor-quality RNA is the most common cause of suboptimal labeling results. A TargetAmp-Pico Kit reaction uses 50-500 pg of RNA in a volume of 2 µl of RNase-Free Water.

Biotin-aRNA Yield:

The TargetAmp-Pico Kit is extremely efficient at producing microgram amounts of biotin-aRNA from picogram quantities of input total RNA. The actual biotin-aRNA yield from a total RNA sample is dependent on:

i) the integrity (intactness) of the total RNA sample;

ii) the amount of total RNA used in the reaction; and

iii) the poly(A) RNA content of the RNA sample.

Table 1 provides guidance for the amount of biotin-aRNA that can be produced using the HeLa Control RNA provided in the kit.

Table 1. Yields of biotin-aRNA obtained from the HeLa Total RNA Control provided in the kit. Results are the average of multiple experiments.

Amount of HeLa Control	Biotin-aRNA Yield
50 pg	8 µg
100 pg	19 µg
200 pg	34 µg
500 pg	71 µg

⚠ ***Important!*** *The TargetAmp-Pico Kit has been optimized for producing biotin-aRNA from 50 pg to 500 pg of total cellular RNA per reaction. Amplifying >500 pg of total RNA in a single reaction may result in under-representation of some poly(A) RNA sequences in the biotin-aRNA produced. Therefore, if you wish to use >500 pg of input total RNA, we strongly recommend that you perform multiple reactions, each containing ≤500 pg of total RNA.*

Additional Suggestions:

The TargetAmp-Pico Kit includes many reagents. Before starting the procedure, 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.

- Familiarize yourself with the kit by running a control reaction (Appendix 1) before committing a precious sample.
- Use ≤500 pg of total RNA per reaction.
- Assemble the two *in vitro* transcription reactions (Parts 4.C and 4.G) at room temperature. Do not exceed the reaction times indicated in the procedure.
- Use the appropriate RNA purification columns for purifying the biotin-aRNA as described in Part 4.H.
- Optional stopping points are noted after the first-round and second-round, second-strand cDNA synthesis steps (Parts 4.B and 4.F) and after purification of the biotin-aRNA (Parts 4.D and 4.H).

4. Kit Procedure

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

Detailed procedure follows the Quick Protocol
For experienced users only!

Step	Procedure	Pages
Round-one, first-strand cDNA synthesis	1. Combine: X µl RNase-Free Water X µl RNA sample (50-500 pg) 1 µl T7-Oligo(dT) Primer 1 3 µl total volume 2. Incubate 5 min @ 65°C, then place on ice. 3. Add: 1.25 µl Reverse Transcription PreMix 0.25 µl RiboGuard™ RNase Inhibitor 0.25 µl DTT 0.25 µl SuperScript® III Reverse Transcriptase 4. Mix and incubate 30 min @ 50°C, then place on ice.	9
Round-one, second-strand cDNA synthesis	1. Add on ice: 4.5 µl DNA Polymerase PreMix 1 0.5 µl DNA Polymerase 1 2. Mix and incubate 10 min @ 65°C. 3. Incubate 3 min @ 80°C, then centrifuge briefly and place on ice. 4. Add 1 µl cDNA Finishing Solution 5. Mix and incubate for 10 min @ 37°C. 6. Incubate 3 min @ 80°C, then centrifuge briefly and place on ice. Optional: Store @ -20°C overnight, if desired.	10
Round-one *in vitro* transcription	1. Warm T7 RNA Polymerase and T7 Transcription Buffer 1 to RT. Mix the Transcription Buffer thoroughly. 2. Add @ RT: 4 µl T7 Transcription Buffer 27 µl NTP PreMix 1 4 µl DTT 1 µl RiboGuard RNase Inhibitor 4 µl T7 RNA Polymerase 3. Mix and incubate 4 h @ 42°C. 4. Add 2 µl RNase-Free DNase. 5. Incubate 15 min @ 37°C.	10
Round-one RNA purification	Purify using RNA Clean & Concentrator™-5 column (Zymo Research) following the Total RNA Purification protocol, except elute with 8 µl RNase-Free Water.	11

Quick Protocol (continued)

Step	Procedure	Pages
Round-two, first-strand cDNA Synthesis	1. Add 2 µl Random Primers. 2. Transfer to 0.2- or 0.6-ml tube. 3. Incubate 5 min @ 65°C, then place on ice. 4. Add: 1.5 µl Reverse Transcription PreMix 0.25 µl DTT 0.25 µl SuperScript II Reverse Transcriptase 5. Mix and incubate 10 min @ RT. 6. Incubate 1 h @ 37°C. 7. Add 0.5 µl RNase H Mix and incubate 20 min @ 37°C. 8. Incubate 2 min @ 95°C. Place on ice 1 min, then centrifuge briefly.	12
Round-two, second-strand cDNA synthesis	1. Add 1 µl T7-Oligo(dT) Primer 2. 2. Incubate 5 min @ 70°C. 3. Incubate 10 min @ 42°C, then centrifuge briefly and place on ice. 4. Add on ice: 13 µl DNA Polymerase PreMix 2 0.5 µl DNA Polymerase 2 5. Mix and incubate 10 min @ 37°C. 6. Incubate 3 min @ 80°C, then centrifuge briefly and place on ice.	14
In vitro transcription of biotin-aRNA	1. Warm T7 RNA Polymerase and T7 Transcription Buffer 2 to RT. Mix the Transcription Buffer thoroughly. 2. Add @ RT: 7.5 µl T7 Transcription Buffer 2 4.5 µl Biotin-UTP 20 µl NTP PreMix 2 4 µl DTT 1 µl RiboGuard RNase Inhibitor 4 µl T7 RNA Polymerase 3. Mix and incubate 9 h @ 42°C. 4. Add 2 µl RNase-Free DNase. 5. Mix and incubate 15 min @ 37°C.	14
Biotin-aRNA purification	Purify the biotin-aRNA using a suitable column.	15

Please read the entire TargetAmp-Pico Labeling Kit procedure before beginning. If you are not experienced with the protocol, we strongly recommend that you perform a control amplification reaction (Appendix 1) prior to committing a precious sample.

4.A. Round-One, First-Strand cDNA Synthesis

Required in Part 4.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	

SuperScript III Reverse Transcriptase (user-provided) is required in Part 4.A.

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

⚠ ***Important!*** *Use only ≤500 pg of input total RNA per reaction.*

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.

x µl	RNase-Free Water
x µl	Total RNA sample (50-500 pg)
1 µl	TargetAmp T7-Oligo(dT) Primer 1
3 µl	Total volume

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, First-Strand cDNA Synthesis Master Mix as follows:

   ⚠ ***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.*

   For each first-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 volume

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

4.B. Round-One, Second-Strand cDNA Synthesis

Required in Part 4.B

Component Name	Tube Label	Cap Color
TargetAmp-Pico DNA Polymerase PreMix 1	DNA Pol PreMix 1	Red
TargetAmp-Pico DNA Polymerase 1	DNA Polymerase 1	
TargetAmp-Pico 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, Second-Strand cDNA Synthesis Master Mix as follows:

   For each second-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 volume

2. Gently but thoroughly mix the Master Mix and then add 5 µl of it to each reaction.
3. Gently but thoroughly mix the reactions and then incubate them at 65°C for 10 minutes in a thermocycler.
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 them 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.*

4.C. Round-One *In Vitro* Transcription

Required in Part 4.C

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

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 the precipitate dissolves. Keep the TargetAmp T7 Transcription Buffer at room temperature.

   ⚠ ***Important!*** *Be sure to thaw and use the TargetAmp T7 Transcription Buffer 1 and the NTP PreMix 1 in the Green-cap tubes in this step.*

2. Thoroughly mix the thawed TargetAmp T7 Transcription Buffer 1.
3. Prepare the Round-One *In Vitro* Transcription Master Mix as follows:

   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 volume

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

   ⚠ ***Important!*** *Do not exceed a 4-hour incubation. The 4-hour incubation gives optimal RNA yield and length.*

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

4.D. Round-One RNA Purification

Option 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 Part 4.E. If desired, the purified RNA can be quick-frozen and stored overnight at –70°C to –80°C.

Option 1b.

RNA purification using the Qiagen RNeasy MinElute Cleanup Kit (Qiagen):
Using the MinElute Cleanup Kit procedure requires reducing the volume of the eluted RNA using a speed-vacuum centrifugation prior to Part 4.E. Use the following procedure (steps 2-9) if using the MinElute Kit:

TargetAmp-Pico 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.*

2. Prepare 350 µl of RLT/β-ME Solution for each sample as described in the MinElute kit handbook.
3. Prepare 650 µl of RPE Solution for each sample as described in the MinElute kit handbook.
4. 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
5. 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.
6. Apply 650 µl of RPE Solution onto the column. Centrifuge at >8,000 x g for 15 seconds. Discard the flow-through.
7. Apply 650 µl of 80% ethanol onto the column. Centrifuge at >8,000 x g for 15 seconds. Discard the flow-through.
8. Transfer the RNeasy MinElute spin column into a new collection tube. Centrifuge at full speed for 5 minutes.
9. 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.

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

4.E. Round-Two, First-Strand cDNA Synthesis

Required in Part 4.E

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

SuperScript II Reverse Transcriptase (user-provided) is required in Part 4.E.

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-Pico Random Primers.
2. Transfer the entire volume of the purified aRNA from Part 4.D 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 column in Part 4.D, proceed immediately to Part 4.E, Step 4.

3b. If the RNA was purified using the Qiagen RNeasy MinElute Cleanup Kit or other commercial RNA purification kit in Part 4.D, then 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.*

Hint: *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 water level with a marking pen. Then, concentrate the RNA samples using the speed-vacuum centrifuge until they are at the same level in their tubes 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. Record the time needed to reduce the water samples volume to 3 µl by speed-vacuum centrifugation. Then concentrate the RNA samples using the same amount of time as that determined to reduce the volume of the water samples 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, First-Strand cDNA Synthesis Master Mix as follows:

⚠ ***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.*

For each first-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 volume

7. Gently but thoroughly mix the Master Mix and then add 2 µl of it to each reaction.

8. Gently but thoroughly mix the reactions and then incubate them at room temperature for 10 minutes.

9. Transfer the reactions to a thermocycler and incubate them at 37°C for 1 hour. If the thermocycler has a heated-lid function, heat the lid only if the lid temperature 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 them at 37°C for 20 minutes in a thermocycler. If the thermocycler has a heated-lid function, heat the lid only if the lid temperature can be maintained at about 37°C.

12. Incubate the reactions at 95°C for 2 minutes. Remove the reactions from the thermocycler, chill on ice for 1 minute., and then centrifuge briefly in a microcentrifuge. Proceed to Part 4.F.

4.F. Round-Two, Second-Strand cDNA Synthesis

Required in Part 4.F

Component Name	Tube Label	Cap Color
TargetAmp-Pico T7-Oligo(dT) Primer 2	T7-Oligo(dT) Primer 2	Blue
TargetAmp-Pico DNA Polymerase PreMix 2	DNA Pol PreMix 2	
TargetAmp-Pico 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-Pico 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 incubate them at 70°C for 5 minutes, followed by 42°C for 10 minutes in a thermocycler. Centrifuge briefly in a microcentrifuge.
3. Prepare the Round-Two, Second-Strand cDNA Synthesis Master Mix as follows:

   For each second-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 volume

   Important! *Be sure to use the TargetAmp DNA Polymerase PreMix 2 and the TargetAmp DNA Polymerase 2 in the Blue-cap tubes in this reaction.*

4. Gently but thoroughly mix the Master Mix and then add 13.5 µl of it to each reaction.
5. Gently but thoroughly mix the reactions and then incubate them 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.*

4.G. *In Vitro* Transcription of Biotin-aRNA

Required in Part 4.G

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

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 the precipitate 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 and the NTP PreMix 2 in the Yellow-cap tubes in this step.*
3. Prepare the *In Vitro* Transcription Master Mix as follows.

   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 volume

4. Gently but thoroughly mix the 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 lid temperature can be maintained at about 42°C; otherwise, 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 a 9-hour incubation at 42°C. Optimal yield and 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 the reactions at 37°C for 15 minutes.

4.H. Biotin-aRNA Purification

The purification column to use is dependent upon the expected yield of biotin-aRNA. Use Table 1 in Part 3 to estimate the yield of biotin-aRNA expected from the amount of total RNA used in each amplification reaction.

If the expected yield of biotin-aRNA is <40 µg: Purify the biotin-aRNA using the Qiagen RNeasy MinElute Cleanup Kit. (Qiagen)

If the expected yield of biotin-aRNA is >40 µg: Purify the biotin-aRNA using the Qiagen RNeasy Mini Kit. (Qiagen)

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) to 10 µl of β-ME (β-mercaptoethanol) as described in the RNeasy kit handbook.
2. Make sure that the RPE Solution has ethanol added to it, as described in the RNeasy kit 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 as follows:

   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.

4.I. Evaluate the Biotin-aRNA

A TargetAmp-Pico kit reaction produces sufficient biotin-aRNA to be quantified by standard UV spectroscopy. Alternative methods, such as a NanoDrop spectrophotometer (Thermo Scientific) or Bioanalyzer (Agilent), can be used.

The size distribution of the biotin-aRNA can be determined by several methods, including:

- Denaturing 1% formaldehyde-agarose gel electrophoresis. Load at least 200 ng of biotin-aRNA for SYBR® Gold detection or 1 µg of biotin-aRNA for ethidium bromide detection.
- Bioanalyzer 2100 (Agilent). Use 100 ng of Biotin-aRNA with the RNA Nano chip or 1-5 ng of biotin-aRNA with the RNA Pico chip.

5. Appendix 1: Performing a Control Reaction

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

Required for Appendix 1

Component Name	Tube Label	Cap Color
HeLa Total RNA Control (40 ng/µl)	HeLa Total RNA	Clear
RNase-Free Water	RNase-Free Water	
TargetAmp-Pico 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.

   ⚠ ***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 volume

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 storage at –70°C to –80°C. 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 with the procedure, beginning in Part 4.A, Step 4.

6. Related Products

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

TAN07924	24 Reactions
TAN091096	96 Reactions

ScriptSeq™ v2 RNA-Seq Library Preparation Kit

SSV21106	6 Reactions
SSV21124	24 Reactions

7. Reference

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

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