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

Cat. No. TAN07908 – 8 Reactions

Cat. No. TAN07924 – 24 Reactions

Cat. No. TAN091096 – 96 Reactions

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1. Kit Contents

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

Component Name	Tube Label	Volume			Cap Color
		8	24	96	
TargetAmp™-Nano T7-Oligo(dT) Primer	T7-Oligo(dT) Primer	12 µl	30 µl	120 µl	Red
TargetAmp™-Nano 1st-Strand cDNA PreMix	1st-Strand cDNA PreMix	20 µl	50 µl	200 µl	
TargetAmp™-Nano 2nd-Strand cDNA PreMix	2nd-Strand cDNA PreMix	45 µl	125 µl	500 µl	
TargetAmp™-Nano 2nd-Strand DNA Polymerase	2nd-Strand DNA Polymerase	10 µl	18 µl	72 µl	
TargetAmp™-Nano T7 RNA Polymerase	T7 RNA Polymerase	25 µl	60 µl	240 µl	Blue
TargetAmp™-Nano T7 Transcription Buffer	T7 Transcription Buffer	25 µl	60 µl	240 µl	
TargetAmp™-Nano NTP PreMix	NTP PreMix	90 µl	270 µl	1,080 µl	
TargetAmp™-Nano UTP / Biotin-UTP	UTP / Biotin-UTP	30 µl	90 µl	330 µl	
RNase-Free DNase I	RNase-Free DNase I	20 µl	60 µl	240 µl	Clear
100 mM Dithiothreitol (DTT)	DTT	35 µl	100 µl	400 ul	
HeLa Total RNA Control (40 ng/µl)	HeLa Total RNA Control	10 µl	10 µl	10 ul	
RNase-Free Water	RNase-Free Water	500 µl	500 µl	2 x 1 ml	

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 Reverse Transcriptase (Life Technologies)
Thermocycler or water bath
Microcentrifuge
RNase-Free Water
RNeasy® MinElute® Cleanup Kit (Qiagen) or RNeasy Mini Kit (Qiagen)
(see "Biotin-aRNA Purification" for details)

Performance Specifications and Quality Control

The TargetAmp - Nano Labeling Kit is function-tested in a control reaction. The kit must produce at least 8 µg of Biotin-aRNA from 80 ng of HeLa Total RNA Control, corresponding to a greater than 5,000-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 1 µg of Biotin-aRNA.

2. Introduction

The TargetAmp - Nano Labeling Kit for Illumina® Expression BeadChip Process

1. **First-strand cDNA Synthesis:** The Poly(A) RNA contained in a total RNA sample is reverse transcribed into cDNA. The reaction is primed from an oligo(dT)-primer containing a phage T7 RNA Polymerase promoter sequence at its 5′ end. 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. **Second-strand cDNA Synthesis:** The cDNA produced in Step 1 is converted to double-stranded cDNA containing a T7 transcription promoter in an orientation that that will generate anti-sense RNA (aRNA; also called cRNA) during the subsequent *in vitro* transcription reaction. The cDNA produced can be used in the *in vitro* transcription reaction without the need for purification.
3. ***In Vitro* Transcription of Biotin-aRNA:** High yields of Biotin-aRNA (Biotin-cRNA) are produced in a rapid *in vitro* transcription reaction that utilizes the double-stranded cDNA produced in Step 2 as template.

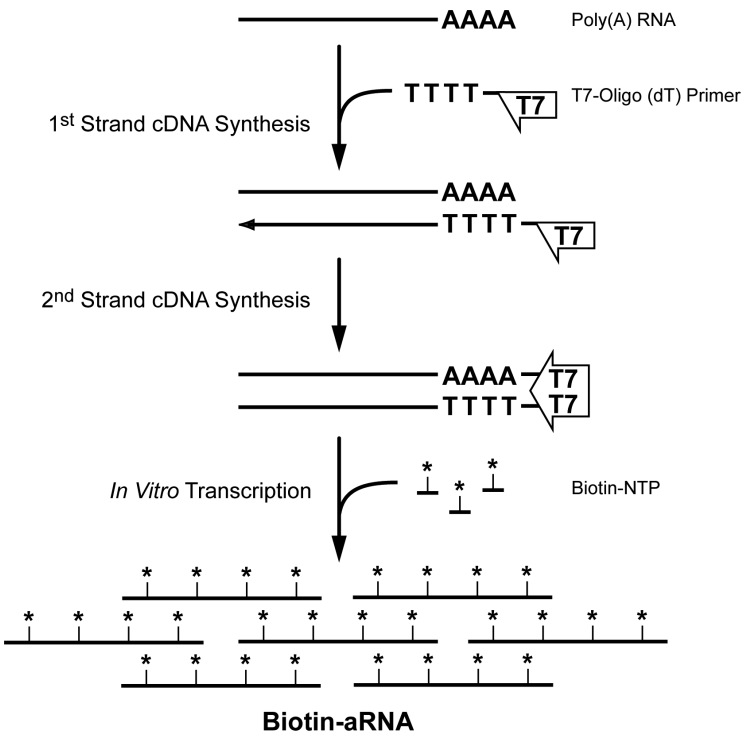

Figure 1. An Overview of the TargetAmp™-Nano Labeling Kit for Illumina® Expression BeadChip®.

3. Preparation

Maintaining an RNase-free Environment:

Ribonuclease contamination is a significant concern for those working with RNA amplification. Creating an RNase-free work environment and maintaining RNase-free solutions is critical for performing successful reactions. Therefore, we strongly recommend that the user:

1) Use RNase-free or autoclaved tubes and pipette tips
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 RNA:

The success of a TargetAmp-Nano 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 method, such as a Bioanalyzer 2100 (Agilent). Poor quality RNA is the most common cause of sub-optimal labeling results.

A TargetAmp-Nano Kit reaction utilizes 25 ng to 500 ng of RNA in a volume of 2 µl of RNase-free water.

Biotin-aRNA Yield:

The TargetAmp-Nano Labeling Kit has been evaluated for production of Biotin-aRNA from as little as 25 ng of total RNA using the HeLa Total RNA control provided in the kit. User results may vary depending on the quality of the total RNA sample and the Poly(A) content of the sample. See Table 1 (below).

Table 1. Yields of Biotin-aRNA obtained using the TargetAmp™ -Nano Labeling Kit for Illumina® Expression BeadChip®. Results summarize experiments performed using the HeLa Total RNA control provided in the kit.

HeLa Control total RNA	Biotin-aRNA Yield
25 ng	3.1 µg
100 ng	10.8 µg
500 ng	75.6 µg

Important! *Labeling >500 ng 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 it is desirable to perform labeling reactions using >500 ng of total RNA, we strongly recommend that the user perform multiple reactions, each containing up to but not exceeding 500 ng of total RNA.*

Additional Suggestions:

Familiarize Yourself with the TargetAmp-Nano Labeling Kit Procedure:

The TargetAmp-Nano Labeling Kit includes many reagents. Before starting, please read this protocol carefully and familiarize yourself with each kit component and in which step of the procedure it is used. Be sure to wear gloves when handling the kit components.

Importance of Running the Control Reaction:

We strongly recommend that those who are not experienced with the TargetAmp - Nano Labeling 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.

Performing the TargetAmp-Nano Labeling Kit Reactions:

We recommend that all reactions be performed in sterile 0.2- or 0.5-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, simultaneously.

4. RNA Labeling Procedure

Quick Protocol for RNA Labeling Procedure

Detailed procedure begins on next page.

For experienced users only!

Step	Procedure	Pages
First-strand cDNA Synthesis	1. Combine: X µl RNase-Free Water X µl RNA sample (25ng – 500 ng) 1 µl T7-Oligo(dT) Primer 3 µl total volume 2. Incubate at 65°C for 5 minutes then place on ice. 3. Add to each reaction: 1. 5 µl 1st-Strand cDNA PreMix 0.25 µl DTT 0.25 µl SuperScript III Reverse Transcriptase 4. Mix and incubate at 50°C for 30 minutes, then place on ice.	7
Second-strand cDNA Synthesis	1. Add to each reaction, on ice: 4.5 µl 2nd-Strand cDNA PreMix 0.5 µl 2nd-Strand DNA Polymerase 2. Mix and incubate at 65°C for 10 minutes 3. Incubate at 80°C for 3 minutes, then centrifuge briefly and place on ice. Optional: Store at –20°C overnight , if desired.	8
In Vitro Transcription of Biotin-aRNA	1. Warm T7 RNA Polymerase and T7 Transcription Buffer to room temperature. Mix the Transcription Buffer thoroughly. 2. Add to each reaction, at room temperature: 2 µl T7 Transcription Buffer 3 µl UTP/Biotin-UTP 10 µl NTP PreMix 3 µl DTT 2 µl T7 RNA Polymerase 3. Mix and incubate at 42°C for 4 hours 4. Add 2 µl of RNase-Free DNase to each reaction 5. Incubate at 37°C for 15 minutes	9
Biotin-aRNA Purification		10

A. First-strand cDNA Synthesis

SuperScript III Reverse Transcriptase (Life Technologies) is strongly recommended for use in this Step. The SuperScript III enzyme is provided by the user. For best results, the RNA sample should be dissolved in RNase-Free water.

Required in Step A

Component Name	Tube Label	Cap Color
TargetAmp™-Nano T7-Oligo(dT) Primer	T7-Oligo(dT) Primer	Red
TargetAmp™-Nano 1st-Strand cDNA PreMix	1st-Strand cDNA PreMix	
Dithiothreitol	DTT	Clear
RNase-Free Water	RNase-Free Water	

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

Important! *Labeling >500 ng 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 it is desirable to perform an amplification reaction using >500 ng of total RNA, we strongly recommend that the user perform multiple reactions, each containing up to but not exceeding 500 ng of total RNA.*

1. Anneal the T7-Oligo(dT) Primer 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 (25-500 ng)
1	µl	T7-Oligo(dT) Primer
3	µl	Total reaction volume

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 1st-Strand cDNA Synthesis Master Mix.

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

1.5	µl	1st-Strand cDNA PreMix
0.25	µl	DTT
0.25	µl	SuperScript III Reverse Transcriptase (200 U/µl)
2	µl	Total reaction volume

5. Gently mix the 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.

B. Second-strand cDNA Synthesis

Required in Step B

Component Name	Tube Label	Cap Color
TargetAmp™-Nano 2nd-Strand cDNA PreMix	2nd-Strand cDNA PreMix	Red
TargetAmp™-Nano 2nd-Strand DNA Polymerase	2nd-Strand DNA Polymerase	

Incubation temperatures performed in Step B: 65°C and 80°C.

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

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

4.5 µl	2nd-Strand cDNA PreMix
0.5 µl	2nd-Strand DNA Polymerase
5 µl	Total reaction volume

2. Gently mix the 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.

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

C. *In Vitro* Transcription of Biotin-aRNA

The *in vitro* transcription reaction produces Biotin-aRNA by incorporating Biotin-UTP into the RNA transcripts.

Required in Step C

Component Name	Tube Label	Cap Color
TargetAmp™-Nano T7 RNA Polymerase	T7 RNA Polymerase	Blue
TargetAmp™-Nano T7 Transcription Buffer	T7 Transcription Buffer	
TargetAmp™-Nano NTP PreMix	NTP PreMix	
TargetAmp™-Nano UTP / Biotin-UTP	UTP / Biotin-UTP	
RNase-Free DNase I	RNase-Free DNase I	
Dithiothreitol	DTT	Clear
RNase-Free Water	RNase-Free Water	

Incubation temperatures performed in Step C: 37°C and 42°C.

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

   Important! *If a precipitate is visible in the thawed 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 *In Vitro* Transcription Master Mix.

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

2	µl	T7 Transcription Buffer
3	µl	UTP / Biotin-UTP
10	µl	NTP PreMix
3	µl	DTT
2	µl	T7 RNA Polymerase
20	µl	Total reaction volume

4. Gently but thoroughly mix the *In Vitro* Transcription Master Mix and then add 20 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 50°C. If the lid temperature can not be maintained at about 50°C, then perform the incubations without heating the lid.

   Important! *Do not exceed 4 hour incubation at 42°C. Optimal yield and length of Biotin-aRNA is achieved in 4 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.

D. Biotin-aRNA Purification

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

If the expected yield of biotin-aRNA is <40 µg:

Purify the 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) with 10 µl of β-ME (β-mercaptoethanol) as described in the RNeasy kit's handbook.

2. Prepare RPE Solution as described in the purification kit's handbook.
3. To each sample add:

48	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 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 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 cRNA:
   If using the MinElute Cleanup Column, apply 20 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.
   If using the RNeasy Mini Column, apply 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.
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 1 min. 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.
   Note: *If desired, reactions can now be quick frozen (e.g., dry ice/ethanol bath) and stored at –80°C.*

E. Evaluate the Biotin-aRNA

Biotin-aRNA yield: A TargetAmp-Nano kit reaction produces sufficient Biotin-aRNA that can generally be quantified by standard UV spectroscopy. Alternative methods such as a NanoDrop spectrophotometer (Thermo Scientific) or Bioanalyzer (Agilent) can be used.

Assessing the size of the Biotin-aRNA: The size distribution of the Biotin-aRNA produced can be determined by several methods including:

a) 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.

b) 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

Appendix 1: The TargetAmp - Nano Labeling Kit Control Reaction

TargetAmp -Nano Labeling Kit provides 400 ng of human HeLa RNA total at a concentration of 40 ng/µl.

Required for the Control Reaction

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

1. Thaw the HeLa Total RNA Control on ice.
2. Anneal the T7-Oligo(dT) Primer to the HeLa Total RNA Control.

   The standard control reaction utilizes 80 ng of the HeLa Total RNA Control.

   If using less Control RNA, make up the volume of the annealing reaction with RNase-Free Water.

2 µl	HeLa Total RNA Control (80 ng)
1 µl	T7-Oligo(dT) Primer
3 µl	Total reaction volume

3. 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.
4. 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.
5. Continue the Control Reaction as described beginning in Part A, Step 4.

6. Additional TargetAmp Kits and Related Products

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

TAP120210 10 Reactions

TAP120224 24 Reactions

Produces micrograms of biotin-aRNA (biotin-cRNA) from 50 pg - 500 pg of total RNA.

ScriptSeq™ v2 RNA-Seq Library Preparation Kit

SSv21106 6 Reactions

SSv21124 24 Reactions

Kits for Isolating Total Cellular RNA and mRNA

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.

7. Reference

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

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