

RampUP™ and RampUP Plus™

RNA Amplification and Labeling Kits

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Introduction

Genisphere's RampUP kits allow for two rounds of RNA amplification with little hands-on time required. The amplified RNA can be used for expression analysis on microarrays (see below), in qRT-PCR, cloning experiments, or other RNA-based applications. If only one round of RNA amplification is needed, please refer to Genisphere's SenseAMP products.

Characteristics of RampUP Amplification

- RampUP is a flexible amplification process that amplifies a wide range of RNA samples, including partially degraded RNA and prokaryotic RNA, by using both dT and random priming. The kit accommodates total RNA samples from 1-30ng and produces **senseRNA** after two rounds of amplification.
- RampUP utilizes a proprietary linear amplification process that makes two rounds of amplification as easy and accurate as one round. The use of tandem T7 and T3 RNA polymerase promoters allows for a quick and easy transition from round 1 to round 2 (see diagram on the next page).
- RampUP offers a high fidelity amplification process from which amplified RNA accurately represents the starting material. Amplification begins by synthesizing cDNA from the 3' end of the message. The T7 amplification reaction that follows initiates at the 3' end of the **cDNA** (originally the 5' end of the RNA). Therefore, both ends of the message are represented during the process (Goff L.A., *et al.*). In contrast, conventional Eberwine-based amplification methods prime and amplify from the 3' end of the message, typically resulting in a 3' bias (Van Gelder R.N., *et al.*).
- The RampUP protocol includes an optimized procedure for synthesis of cDNA from small quantities of total RNA (Baugh L.R., *et al.*).
- RampUP offers high-yield amplification and is designed to reduce amplification artifacts.
- RampUP has been optimized with low-quantity and degraded RNA samples such as those obtained from Laser Capture Microdissection (LCM) and Formalin Fixed Paraffin Embedded (FFPE) samples.
- For most applications, random priming of RampUP senseRNA is required. RampUP kits include random primer and dNTP mix with a lower dTTP concentration. Any labeled dUTP can be incorporated into the cDNA synthesis reaction. Biotin-dUTP is incorporated into the cDNA for hybridization to Affymetrix 3' Gene Expression Analysis Arrays, and for hybridization to Illumina arrays. Recent publications (Barker C.S., *et al.* and Eklund, A.C., *et al.*) have shown that labeled cDNA produces expression profiles equal to or better than labeled aRNA on Affymetrix GeneChips. Fluorescent- or aminoallyl-dUTP may be incorporated into the cDNA for hybridization to other arrays including those manufactured by Agilent.
- For applications that require dT priming of RampUP senseRNA, the RampUP Plus kit includes the PLUS module for producing polyadenylated RNA molecules. If necessary, the PLUS module may be ordered separately (Genisphere cat. no. P10PLUS).

RampUP RNA Amplification and Labeling

Round 1 Reverse Transcription of total RNA

Purification of cDNA

Tailing of cDNA

Annealing of T7/T3 Template Oligo

T7/T3 Promoter Synthesis

T7 In Vitro Transcription

Round 2 Reverse Transcription of senseRNA

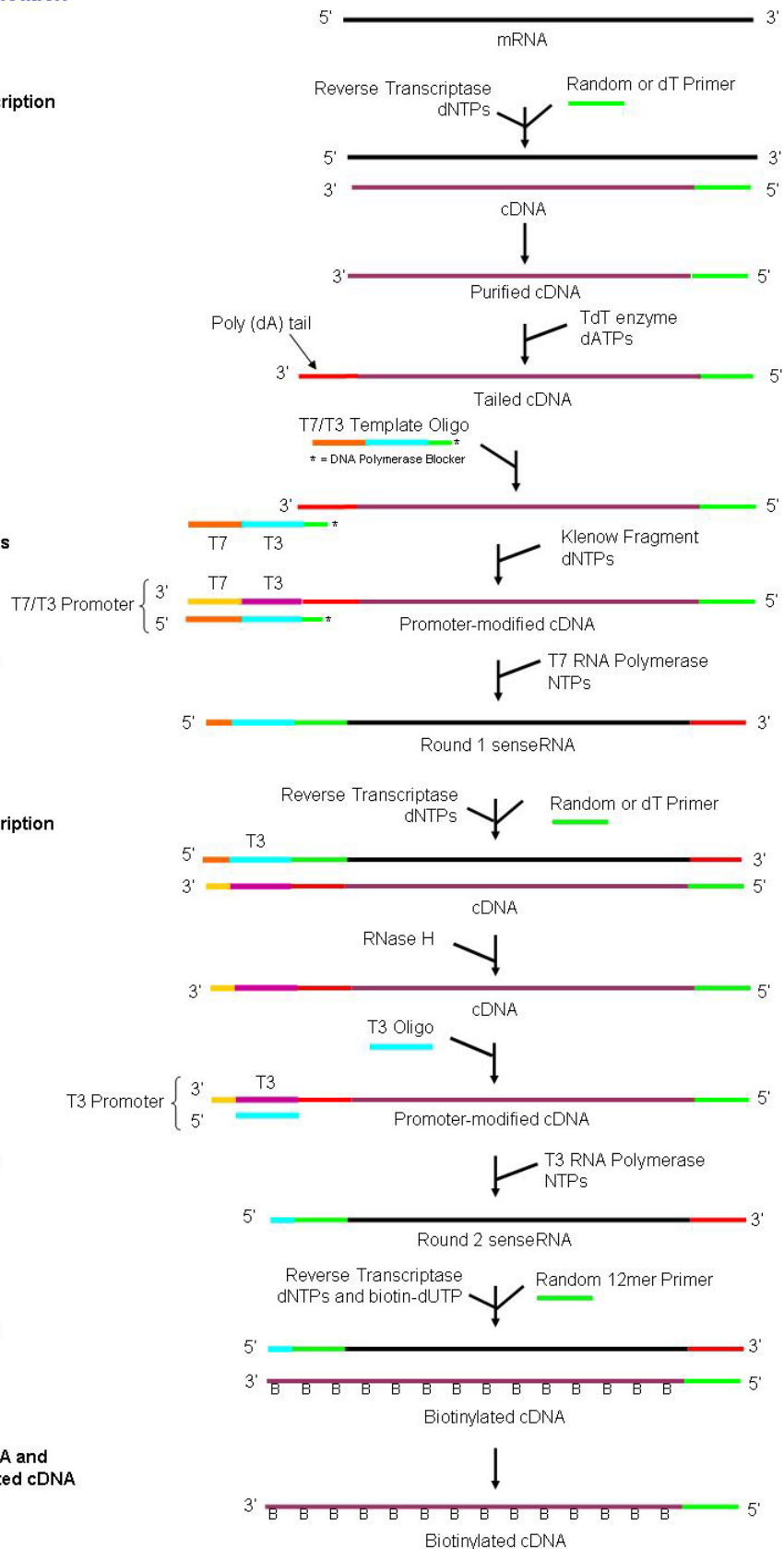
RNase H degradation of senseRNA

Annealing of T3 Oligo

T3 In Vitro Transcription

cDNA Synthesis and biotin incorporation (for most applications)

Degradation of senseRNA and Purification of Biotinylated cDNA (for most applications)



Kit Specifications

Components and Storage

RampUP Components

Vial 1	Round 1 dT RT Primer (50ng/μl)
Vial 2	Round 1 Random RT Primer (50ng/μl)
Vial 3	dNTP Mix (10mM each dATP, dCTP, dGTP, dTTP)
Vial 4	Suprase-In™ RNase Inhibitor
Vial 5	0.2mM dATP
Vial 6	10X Reaction Buffer
Vial 7	Terminal Deoxynucleotidyl Transferase
Vial 8	T7/T3 Template Oligo (10ng/μl)
Vial 9	Klenow Enzyme
Vial 10	Nuclease-Free Water
Vial 11	T7 Nucleotide Mix (ATP,CTP,GTP, and UTP)
Vial 12	10X IVT Reaction Buffer
Vial 13	T7 Enzyme Mix
Vial 14	Round 2 dT RT Primer (100ng/μl)
Vial 15	Round 2 Random RT Primer (1μg/μl)
Vial 16	RNase H
Vial 17	T3 Nucleotide Mix (ATP,CTP,GTP, and UTP)
Vial 18	T3 Oligo (50ng/μl)
Vial 19	T3 Enzyme Mix
Vial 20	Random 12mer Primer (15000ng/μl)
Vial 21	Low dTTP dNTP mix (2mM dTTP, 10mM each dATP, dCTP, dGTP)
Yellow cap	Control DNA (5ng/μl)
	Microcon® YM-50 columns (10) and collection tubes (20)

Plus Module Components (Included in RampUP Plus kits and available separately)

Vial 1P	ATP Mix
Vial 2P	5X PAP Buffer
Vial 3P	PAP Enzyme
Vial 4P	25mM MnCl ₂
Vial 5P	Control Oligo (Note: Vial 5P, Control Oligo, is not used in this protocol)

Store all vials at -20°C.

Store Microcon YM-50 columns and collection tubes at room temperature.

Instructions for Handling Kit Contents at Time of Use

Vials 1, 2, 6, 8, 10, 11, 12, 14, 15, 17, 18, 20, Yellow cap, 1P, 2P, and 4P:

Thaw at room temperature, briefly vortex and centrifuge. Keep at room temperature until use.

Vials 3, 4, 5, 7, 9, 13, 16, 19, 21 and 3P:

Thaw on ice, briefly centrifuge if necessary, and keep on ice at all times. Do not vortex.

Additional Materials/Equipment Required

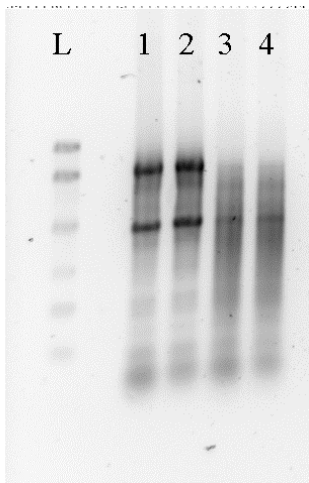
- SuperScript™ II Reverse Transcriptase (Invitrogen cat. no. 18064-014)
- 1X TE Buffer (10mM Tris-HCl, 1mM EDTA pH 8.0) (Ambion cat. no. AM9858 or equivalent)
- Nuclease-Free Water (Ambion cat. no. AM9934 or equivalent)
- RNeasy MinElute Kit (Qiagen cat. no. 74204)
 - * RNeasy MinElute columns require 80% ethanol and 95-100% ethanol
- 1mM Tris pH 8.0 (for RampUP Plus only)
- 0.5M EDTA (for RampUP Plus only)
- Heating devices for incubations at 37°C, 42°C, 65°C, and 80°C
Refer to *Procedure for Use* for specific required heating devices
- UV/Vis Spectrophotometer for cDNA quantitation (or other quantitation instrument)
 - 0.1X TE Buffer (1mM Tris-HCl, 0.1mM EDTA pH 8.0) is recommend for UV/Vis spectrophotometer quantitation
- Pipette capable of accurately measuring 0.5µl

Optional Materials/Equipment

- T4gene32 Protein, ≥10µg/µl (T4gp32) (USB cat. No. 74029Y)
- Gel electrophoresis apparatus and reagents for analyzing agarose gels (or other instrument to visualize RNA)
- Genisphere® 3DNA® labeling kit for glass microarray analysis of senseRNA
- Biotin 16-dUTP, 1mM (Roche cat. no. 11093070910) or other labeled dUTP
- 0.5M NaOH, 50mM EDTA
- 1M Tris-HCl, pH 8.0
- 10mM Tris-HCl, pH 8.0

RNA Quality and Primer Selection

- Carriers composed of nucleic acid should not be used when purifying RNA samples for amplification, since these carriers will also be amplified by RampUP. Choose a carrier that does not reverse transcribe or have a functional 3' hydroxyl, like linear acrylamide.
- DNase treatment of the RNA sample is recommended, as contaminating genomic DNA will also be amplified by RampUP. After treatment, completely inactivate the DNase by phenol-chloroform extraction, or by using the RNeasy kit from Qiagen or an equivalent method.
- If any stored RNA samples are suspected to be contaminated with RNases, treat the samples with RNase inhibitor.
- Prior to amplification, check the integrity of each RNA sample. The RampUP kits will amplify partially degraded RNA samples, but cannot amplify completely degraded RNA samples. One of the following methods should be used to determine the integrity of the sample:
 - **Arcturus Paradise™ QC Process (Beta-Actin 3'/5' value).** Intact RNA samples have a QC Metric value between 1 and 10. Partially degraded RNA samples have a QC Metric value between 11 and 164.
 - **Quantitation of ribosomal subunits.** Intact RNA samples have a subunit ratio between 1.5 and 2.4. Partially degraded RNA samples have a subunit ratio less than 1.5.
 - **Gel electrophoresis.** Intact RNA samples have two sharp, bright bands of rRNA. Partially degraded RNA samples have some visible smear of RNA, between 0.1-2Kb in length. See Figure 1 for examples of both intact and partially degraded RNA samples.



One microgram of RNA per lane

L Ambion RNA 6000 Ladder

1 Intact Mouse Brain Total RNA

2 Intact Mouse Liver Total RNA

3 Degraded Mouse Brain Total RNA

4 Degraded Mouse Liver Total RNA

The Degraded RNA samples were successfully amplified with RampUP.

Figure 1: 1% denaturing agarose gel of intact and partially degraded total RNA samples.

RNA Input and senseRNA Yield

The amplification yield of RampUP will vary from sample to sample due to differences in mRNA content and quality. For example, 20ng of a very intact RNA sample (Mouse Brain Total RNA, Ambion cat. no. AM7812) may yield 100µg of senseRNA, whereas 20ng of an FFPE sample may yield 20µg senseRNA or more. Therefore, Genisphere recommends using 1-30ng of intact RNA or 20-30ng of FFPE RNA with RampUP.

Sample Contamination

Contaminants may be introduced into an RNA sample either at the point of cell harvesting (prior to RNA purification) or after the RNA is purified. Studies requiring the amplification of RNA should be designed to avoid the inadvertent introduction of exogenous cells (technician skin cells), bacteria, or dust. These contaminants will be amplified in the assay, making data analysis difficult. While most enzymes, kits and buffers are available free of nuclease contamination, they are not always qualified for the presence of nucleic acid. Select these additional reagents appropriately.

Protocol Timelines

The RampUP protocol is designed to reduce the amount of hands-on time required for two rounds of RNA amplification. Times below reflect total times (including incubation times).

Round 1

Round 1 Reverse Transcription of Total RNA	80 minutes
Purification of cDNA	40 minutes
Tailing of First Strand cDNA	30 minutes
Annealing of T7/T3 Template oligo	15 minutes
T7/T3 Promoter Synthesis	50 minutes
T7 In Vitro Transcription	overnight

Total Time to Round 1 IVT

~3.6 hours

Round 2

Round 2 Reverse Transcription of senseRNA	90 minutes
RNAse H degradation of senseRNA	60 minutes
Annealing of T3 Oligo	10 minutes
T3 In Vitro Transcription	overnight

Total Time to Round 2 IVT

~2.7 hours

For most applications, random priming of RampUP senseRNA is required.

cDNA Synthesis of senseRNA	
cDNA Synthesis with Random 12mer Primer	210 minutes
Microcon YM-50 Purification and concentration of cDNA	30 minutes

Total Time to application

~4 hours

--OR--

For applications that require dT priming of RampUP senseRNA:

Poly (A) Tailing of senseRNA	
Poly (A) Tailing	20 minutes
Purification of poly (A) tailed senseRNA	30 minutes
cDNA Synthesis with dT Primer	60-180 minutes

Total Time to application

~2-4 hours

Procedural Note: When preparing master mixes, the enzymes should be added last and just prior to adding the master mix to the reaction. After the master mix is added, gently tap the bottom of the tube 2-4 times and briefly centrifuge. DO NOT VORTEX.

Procedure for Use

Round 1 Reverse Transcription of Total RNA

1. Concentrate or reconstitute the total RNA sample (1-30ng) in Nuclease-Free Water (Vial 10) to a volume of 3 μ l.
2. In a microtube on ice, prepare the **RNA-RT primer mix**:
 - 3 μ l Total RNA (1-30ng of intact RNA or 20-30ng of FFPE RNA)
 - 1 μ l Round 1 dT RT Primer (50ng/ μ l) (Vial 1)
 - 1 μ l Round 1 Random RT Primer (50ng/ μ l) (Vial 2)

Note: When using prokaryotic total RNA, dT primer is not required for cDNA synthesis. Use Nuclease-Free Water (Vial 10) in place of the dT Primer.
3. Gently mix the **RNA-RT primer mix** and briefly centrifuge.
4. Heat the **RNA-RT primer mix** at 80°C in a heat block for 5 minutes and immediately ice for 2-3 minutes.
5. In a separate microtube on ice, prepare a 5 μ l **reaction mix** for every RT reaction:
 - 2.0 μ l 5X First Strand Buffer (provided with SuperScript II)
 - 1.0 μ l 0.1M DTT (provided with SuperScript II)
 - 0.5 μ l dNTP Mix (Vial 3)
 - 0.5 μ l Superase-In RNase Inhibitor (Vial 4)
 - 0.5 μ l SuperScript II, 200 units/ μ l

Optional: 0.5 μ l T4gene32 Protein, \geq 10 μ g/ μ l (USB cat. No. 74029Y)

Note: If T4gene32 Protein is not included, increase SuperScript II from 0.5 μ l to 1 μ l.
6. Gently mix the **reaction mix** and briefly centrifuge. Keep on ice until used.
7. Add the 5 μ l of **reaction mix** to the 5 μ l of **RNA-RT primer mix** for a volume of 10 μ l.
8. Gently mix, briefly centrifuge, and incubate at 42°C as follows:
 - 1 hour in a thermal cycler, with heated lid, set to 42°C
 - OR
 - 5 minutes in a 42°C water bath, then 55 minutes in a 42°C dry oven.
9. Microfuge briefly. Add 90 μ l 1X TE Buffer to adjust the volume to 100 μ l.

Purification of cDNA

Purify the 100 μ l of cDNA using the Qiagen RNeasy MinElute Kit (cat. no. 74204) as indicated below.

1. Prewash the RNeasy MinElute columns prior to use as follows:
 - Apply 500 μ l of Nuclease-Free Water to the spin column. Incubate at room temperature for 1 minute. Centrifuge at $\geq 12,000$ rpm for 1 minute. Discard the flow-through.
 - Apply 20 μ l of Nuclease-Free Water to the spin column. Incubate at room temperature for 1 minute. Centrifuge at $\geq 12,000$ rpm for 1 minute.
 - Apply 20 μ l of Nuclease-Free Water to the spin column. Incubate at room temperature for 1 minute. Centrifuge at $\geq 12,000$ rpm for 1 minute. Discard the flow-through and proceed with purification of the cDNA.
2. Add 350 μ l of RLT Buffer (no BME added) to the 100 μ l cDNA sample and mix well by pipetting up and down.
3. Add 250 μ l of 95-100% ethanol and mix well by pipetting up and down. Transfer each sample into a RNeasy spin column. Centrifuge at $\geq 12,000$ rpm for 15 seconds. Discard the flow-through and collection tube. Place each column into a new collection tube supplied with the kit.
4. Add 500 μ l of RPE Buffer (prepared with ethanol as indicated by Qiagen) to each spin column. Centrifuge at $\geq 12,000$ rpm for 15 seconds. Discard the flow-through and place each spin column back in the same collection tube.
5. Add 500 μ l of 80% ethanol to each spin column. Centrifuge at $\geq 12,000$ rpm for 2 minutes. Discard the flow-through and collection tube. Place each spin column in a new collection tube supplied with the kit.
6. Centrifuge spin columns with lids open for 5 minutes at $\geq 12,000$ rpm to dry and remove any residual ethanol.
7. Place each spin column into a new 1.5mL centrifuge tube. Add 12 μ l RNase-free water to the center of the filter disk in each spin column. Do not touch the column reservoir with the pipet tip. Incubate at room temperature for 2 minutes. Centrifuge at $\geq 12,000$ rpm for 1 minute. The final volume eluted from the column should be 12 μ l. If necessary, bring the volume to 12 μ l with Nuclease-Free Water (Vial 10).

Tailing of First Strand cDNA

1. Heat the 12 μ l purified cDNA at 80°C for 10 minutes. Ice immediately for 2 minutes. Briefly centrifuge and return to ice.
2. For each reaction, prepare a **reaction mix** in a separate tube on ice:
 - 2 μ l 10X Reaction Buffer (Vial 6)
 - 4 μ l 0.2 mM dATP (Vial 5)
 - 2 μ l Terminal Deoxynucleotidyl Transferase (Vial 7)
3. Add the 8 μ l **reaction mix** to the 12 μ l cDNA for a volume of 20 μ l. Mix gently and centrifuge.
4. Incubate in a 37°C heat block for 2 minutes. **Do not exceed 2 minutes.**
5. Immediately stop the reaction by heating to 80°C for 10 minutes. Ice for 2 minutes and briefly centrifuge.

T7/T3 Promoter Synthesis

1. Add 2 μ l of T7/T3 Template Oligo (Vial 8). Mix gently and centrifuge.
2. Incubate at 37°C for 10 minutes to anneal the strands.
3. For each reaction, add the following for a volume of 25 μ l:
 - 1 μ l 10X Reaction Buffer (Vial 6)
 - 1 μ l dNTP Mix (Vial 3)
 - 1 μ l Klenow Enzyme (Vial 9)
4. Mix gently and briefly centrifuge. Incubate at room temperature (20-25°C) for 30 minutes.
5. Stop the reaction by heating to 65°C for 10 minutes. Briefly microfuge and place on ice for 2 minutes.
6. Proceed to the *T7 In Vitro Transcription* reaction using half (12.5 μ l) of the promoter-modified cDNA. The remaining modified cDNA may be stored at -20°C for future use.

T7 In Vitro Transcription

1. Incubate the 12.5 μ l of cDNA at 37°C for 10 minutes to re-anneal the strands.
2. Thaw the T7 Nucleotide Mix (Vial 11) and 10X IVT Reaction Buffer (Vial 12) at room temperature (20-25°C), and **keep at room temperature until use**. Thoroughly vortex the 10X IVT Reaction Buffer (Vial 12) to avoid precipitation of certain buffer components.
3. For each reaction, add the following components **at room temperature** (20-25°C), in the order listed, for a final volume of 25 μ l:
 - 8.0 μ l T7 Nucleotide Mix (Vial 11)
 - 2.5 μ l 10X IVT Reaction Buffer (Vial 12)
 - 2.0 μ l T7 Enzyme Mix (Vial 13)

4. Mix gently and centrifuge. Incubate at 37°C as follows:
 6-16 hours in a thermal cycler, with heated lid, set to 37°C
 OR
 5 minutes in a 37°C heat block, then 6-16 hours in a 37°C dry oven

It is essential to avoid condensation of the reaction during this step.

5. Stop the reaction by placing samples at -20°C until ready to proceed with *Round 2 Reverse Transcription of senseRNA*.

Note: for Control DNA (Yellow cap) reactions with either T7 or T3 enzyme, purify according to Appendix A and quantitate. Refer to Appendix B for the typical amounts of senseRNA recovered.

Round 2 Reverse Transcription of senseRNA

1. Dilute the Round 2 Random RT Primer (1µg/µl) (Vial 15) with Nuclease-Free Water (Vial 10) according to the Total RNA Input:

Total RNA Input Into RampUP	Round 2 Random RT Primer Required (ng)	Dilution of Round 2 Random RT Primer (Vial 15)
2ng to 30ng	500ng	No Dilution
1ng to 2ng	150ng	Dilute to 300ng/µl

2. To the 25µl senseRNA (unpurified IVT reaction), on ice, add:
 0.5µl Round 2 dT RT primer (Vial 14, 100ng/µl)
 0.5µl appropriate dilution of Round 2 Random RT Primer (Vial 15)
3. Gently mix the senseRNA-RT primer mix and briefly centrifuge.
4. Heat to 65°C for 10 minutes. Ice immediately for 2 minutes. Briefly centrifuge and return to ice.
5. For each reaction, add the following for a volume of 28µl:
 1µl dNTP Mix (Vial 3)
 1µl SuperScript II RT enzyme
6. Gently mix, briefly centrifuge, and incubate at 42°C for 1 hour.
7. Incubate at 65°C for 10 minutes. Ice for 2 minutes, and briefly centrifuge.
8. Add 1µl RNaseH (Vial 16). Gently mix, briefly centrifuge, and incubate at 37°C for 30 minutes.
9. Incubate at 80°C for 20 minutes to stop enzyme activity.
10. Allow samples to cool to room temperature for 2 minutes. Briefly centrifuge.

T3 In Vitro Transcription

1. Add 2 μ l of the T3 Oligo (Vial 18) to each reaction. Mix gently and centrifuge. Incubate the 31 μ l of cDNA at 37°C for 10 minutes to anneal the strands.
2. Thaw the T3 Nucleotide Mix (Vial 17) and 10X IVT Reaction Buffer (Vial 12) at room temperature (20-25 °C), and **keep at room temperature until use**. Thoroughly vortex the 10X IVT Reaction Buffer (Vial 12) to avoid precipitation of certain buffer components.
3. For each reaction, add the following components **at room temperature** (20-25 °C), in the order listed, for a final volume of 50 μ l:

13.2 μ l T3 Nucleotide Mix (Vial 17)
2.5 μ l 10X IVT Reaction Buffer (Vial 12)
3.3 μ l T3 Enzyme Mix (Vial 19)

Note: A precipitate may form in the reaction when adding the T3 IVT components. This is normal. Continue with the experiment.

4. Mix gently and centrifuge. Incubate at 37°C as follows:
6-16 hours in a thermal cycler, with heated lid, set to 37°C
OR
5 minutes in a 37°C heat block, then 6-16 hours in a 37°C dry oven
It is essential to avoid condensation of the reaction during this step.
5. Stop the reaction by placing samples at -20°C until ready to purify the senseRNA.

Purification of Round 2 senseRNA

Purify senseRNA using the RNeasy MinElute Kit (Qiagen cat. no. 74204) following the protocol in Appendix A.

Quantitation of Round 2 senseRNA

Quantitate the senseRNA by using a spectrophotometer or other instrument. Calculate the A260/280 ratio to determine RNA purity. A ratio of 2.0-2.5 is most desirable.

Optional: Gel electrophoresis under denaturing conditions may be used to visualize senseRNA size distribution and sample purity.

Applications of senseRNA

For most applications, random priming of senseRNA is required. Follow pages 13-17 for most microarray applications; alternatively, proceed to Genisphere's Array 900MPX protocol using 1-3 μ g senseRNA. SenseRNA may also be used directly in RT-PCR protocols.

For applications that require dT priming of senseRNA, continue to page 18.

cDNA Synthesis of senseRNA

cDNA Synthesis from 20 μ g of senseRNA

This procedure is recommended for most microarray applications to ensure maximum yield of labeled cDNA for hybridization. If 20 μ g of senseRNA is not available, please refer to *cDNA Synthesis from 10 μ g of senseRNA* on page 14.

Note: Incorporate biotin-16-dUTP (Roche cat. no. 11093070910) into the cDNA for hybridization to Affymetrix 3' Gene Expression Analysis Arrays and Illumina arrays. Incorporate a fluorescent dUTP into the cDNA for hybridization to other glass arrays.

1. Bring the volume of 20 μ g of senseRNA (minimum concentration of 1.25 μ g/ μ l) to 16 μ l with Nuclease-Free Water. If necessary concentrate the senseRNA sample to a minimum concentration of 1.25 μ g/ μ l using one of the following methods:
 - SpeedVac or similar vacuum concentrator
 - Lyophilization
 - Evaporation in a 60°C heat block
 - Microcon YM-3 concentrator (Millipore™ cat. no. 42404)
2. Add 2 μ l Random 12-mer Primer (Vial 20, 15000ng/ μ l).
3. Incubate the senseRNA/primer mix at 80°C for 5 minutes.
4. During the 5 minute senseRNA/primer mix incubation at 80°C, prepare a 22 μ l reverse transcription reaction mix on ice for each reaction:
 - 8.0 μ l 5X First Strand Buffer, included with SuperScript II
 - 4.0 μ l 0.1M DTT, included with SuperScript II
 - 1.0 μ l Superase-In RNase Inhibitor (Vial 4)
 - 2.0 μ l low dTTP dNTP mix (Vial 21)
 - 5.0 μ l Biotin-16-dUTP, 1mM (or other labeled-dUTP)
 - 2.0 μ l SuperScript II Reverse Transcriptase
5. Immediately ice the senseRNA/primer mix for 2 minutes. Briefly centrifuge.
6. Incubate the senseRNA/primer mix at room temperature for 3 minutes.
7. Add the 22 μ l reverse transcription reaction mix to each reaction for a final volume of 40 μ l.
8. Incubate the samples at 42°C for 2.5 hours.
9. Add 7 μ l of 0.5M NaOH, 50mM EDTA.
10. Incubate the samples at 65°C for 30 minutes.
11. Add 10 μ l of 1M Tris-HCl, pH 8.0.
12. Add 43 μ l of 1XTE Buffer to adjust the volume of each reaction to 100 μ l.
13. Proceed to *Microcon YM-50 Purification and Concentration* on page 15.

cDNA Synthesis from 10µg of senseRNA

Note: Incorporate biotin-16-dUTP (Roche cat. no. 11093070910) into the cDNA for hybridization to Affymetrix 3' Gene Expression Analysis Arrays and Illumina arrays. Incorporate a fluorescent dUTP into the cDNA for hybridization to other glass arrays.

1. Bring the volume of 10µg of senseRNA (minimum concentration of 1.54µg/µl) to 6.5µl with Nuclease-Free Water. If necessary concentrate the senseRNA sample to a minimum concentration of 1.54µg/µl using one of the following methods:
 - SpeedVac or similar vacuum concentrator
 - Lyophilization
 - Evaporation in a 60°C heat block
 - Microcon YM-3 concentrator (Millipore™ cat. no. 42404)
2. Add 1µl Random 12-mer Primer (Vial 20, 15000ng/µl).
3. Incubate the senseRNA/primer mix at 80°C for 5 minutes.
4. During the 5 minute senseRNA/primer mix incubation at 80°C, prepare a 12.5µl reverse transcription reaction mix on ice for each reaction:
 - 4.0µl 5X First Strand Buffer, included with SuperScript II
 - 2.0µl 0.1M DTT, included with SuperScript II
 - 1.0µl Superase-In RNase Inhibitor (Vial 4)
 - 1.0µl low dTTP dNTP mix (Vial 21)
 - 2.5µl Biotin 16-dUTP, 1mM (or other labeled-dUTP)
 - 2.0µl SuperScript II Reverse Transcriptase
5. Immediately ice the senseRNA/primer mix for 2 minutes. Briefly centrifuge.
6. Incubate the senseRNA/primer mix at room temperature for 3 minutes.
7. Add the 12.5µl reverse transcription reaction mix to each reaction for a final volume of 20µl.
8. Incubate the samples at 42°C for 2.5 hours.
9. Add 3.5µl of 0.5M NaOH, 50mM EDTA.
10. Incubate the samples at 65°C for 30 minutes.
11. Add 5µl of 1M Tris-HCl, pH 8.0.
12. Add 71.5µl of 1XTE Buffer to adjust the volume of each reaction to 100µl.
13. Proceed to *Microcon YM-50 Purification and Concentration* on page 15.

Microcon YM-50 Purification and Concentration of Labeled cDNA

Purify the 100 μ l of cDNA using a Microcon YM-50 column (provided) as described below.

Note: Do not use alternative columns for this step.

1. Place a Microcon YM-50 column into a collection tube provided.
2. Add the 100 μ l cDNA to the sample reservoir. Do not touch the membrane with the pipet tip. Secure the tube cap and centrifuge for 6 minutes at 13,000g.
3. Add 200 μ l of 1X TE buffer to the sample reservoir without touching the membrane. Gently mix by pipetting up and down 5 times. Secure the tube cap and centrifuge for 6 minutes at 13,000g.
4. Carefully separate the sample reservoir from the collection tube. Discard the flow-through. Place the YM-50 column into the same collection tube.
5. Add 200 μ l of 1X TE buffer to the sample reservoir without touching the membrane. Gently mix by pipetting up and down 5 times. Secure the tube cap and centrifuge for 6 minutes at 13,000g.

Note: If using fluorescent labeled deoxynucleotide to generate labeled cDNA, repeat steps 3 and 4 two additional times to remove excess fluorescent label.

6. Carefully separate the sample reservoir from the collection tube. Discard the collection tube.
7. Add 10-20 μ l 10mM Tris-HCl, pH 8.0 to the sample reservoir without touching the membrane. Gently tap the side of the reservoir to mix.
8. Carefully place the sample reservoir **upside-down** in a **new collection tube** provided. Centrifuge for 3 minutes at 10,000-13,000g.
9. Measure the volume of cDNA collected in the bottom of the tube (10-20 μ l).

Quantitation and Visualization of cDNA

Quantitate the cDNA by using a spectrophotometer or other instrument. From the OD, determine the nucleic acid concentration of each labeled sample:

$$A_{260\text{nm}} \times 33 \text{ (single-stranded DNA extinction coefficient)} \times \text{dilution factor} = \text{concentration of cDNA in ng}/\mu\text{l}$$

The cDNA should range from 50 to 600 bases. The average fragment size should be 50-250 bases, which is ideal for microarray hybridization. If cDNA fragments are too long, contact Genisphere Technical Support for recommendations for cDNA fragmentation.

Array Hybridization Recommendations

Affymetrix GeneChip

Hybridize approximately 3-6 μ g of biotinylated cDNA to the Affymetrix GeneChip.

Note: Do not apply less than 2.5 μ g of biotinylated cDNA to an Affymetrix GeneChip.

For example:

Prepare labeled cDNA to 40 μ l: Combine 3-6 μ g of biotinylated cDNA sample with Nuclease-Free Water to give a volume of 32 μ l. Add 8 μ l Affymetrix 5X Fragmentation Buffer. Vortex to mix and briefly centrifuge. Incubate at 94 $^{\circ}$ C for 35 minutes. (Note: the cDNA is not being fragmented during this incubation because the 5X Fragmentation Buffer is designed to fragment RNA. However, this step is important to reduce array background.) Immediately place tubes on ice for 2 minutes and briefly centrifuge.

Allow GeneChips to equilibrate to room temperature (20-25 $^{\circ}$ C).

Prepare cDNA Hybridization Buffer for Midi Array:

100.0 μ l	2X Hybridization Buffer
22.7 μ l	Nuclease-Free Water
20.0 μ l	DMSO
3.3 μ l	Control Oligonucleotide B2
10.0 μ l	20X Eukaryotic Hybridization Controls
2.0 μ l	10mg/ml Herring Sperm DNA
2.0 μ l	50mg/ml Acetylated BSA
<hr/>	
160 μ l	(sufficient hybridization buffer for single reaction)

Add 160 μ l of cDNA hybridization buffer to each 40 μ l labeled cDNA sample for a final hybridization volume of 200 μ l. Incubate samples at 99 $^{\circ}$ C for 5 minutes. Transfer tubes to 45 $^{\circ}$ C for 5 minutes. Briefly centrifuge samples at 13,000g for 5 minutes.

Pre-Hybridization of GeneChip: Load 140 μ l of pre-hybridization buffer into each Midi GeneChip and rotate in an Affymetrix oven at 60rpm for 10 minutes at 45 $^{\circ}$ C.

cDNA Hybridization: Remove chips from oven, remove pre-hybridization buffer, and load 140 μ l of each 200 μ l fragmented cDNA sample into the GeneChip. In an Affymetrix oven, hybridize with rotation at 60rpm for 16 hours at **47 $^{\circ}$ C**.

Washing and Staining: Prepare staining vials as recommended in the standard Affymetrix protocol. For U133Av2.0 midi chip, use fluidics protocol Midi_euk2v3.

Scan GeneChips using Affymetrix scanner.

Illumina Array

Follow the manufacturer's suggestions for array hybridization, washing, and signal development. Substitute biotin-cDNA from page 15 for biotin-aRNA.

Glass Array

Hybridize approximately 5-20 μ g of direct fluorescent or aminoallyl labeled cDNA to the glass array. **Note:** Due to the presence of ribosomal RNA in the amplified senseRNA labeled, it may be necessary to increase the stringency of the post cDNA hybridization wash procedure to preserve differential data.

For example:

cDNA Hybridization

Perform cDNA hybridization according to standard protocol for the array. Depending on array type, the effective hybridization temperature should be 55-65°C.

Post cDNA Hybridization Wash and Scan

Prewarm 2X SSC, 0.2% SDS wash buffer to:
55°C for oligo arrays
65°C for cDNA arrays

Remove coverslip and wash the array in the prewarmed buffer for 15 minutes. Transfer the array to 2X SSC wash buffer at room temperature and wash for 10 minutes. Transfer the array to 0.2X SSC wash buffer at room temperature and wash for 10 minutes.

Dry and scan the array following standard protocol.

Poly (A) Tailing of senseRNA (For RampUP Plus kits only)

This procedure is for RampUP Plus kits. If necessary, the PLUS module may be ordered separately (Genisphere cat. no. P10PLUS).

1. Some, or all, of the purified senseRNA may be used in the Poly (A) tailing reaction. Bring the desired volume of senseRNA to 15.5µl with Nuclease-Free Water (Vial 10).
2. For each tailing reaction, dilute the ATP Mix (Vial 1P) in **1mM Tris pH 8.0** according to the following formula or table below:

(µg of senseRNA) divided by 50 = Dilution factor of ATP Mix

<u>Ex. amount of senseRNA</u>	<u>Dilution of ATP Mix</u>
10µg	1:5
5µg	1:10
2µg	1:25
1µg	1:50
500ng	1:100

3. Make a 1:5 dilution of the PAP Enzyme (Vial 3P) in 1X PAP Buffer, as follows:
 - To prepare 1X PAP Buffer, add 2µl of 5X PAP Buffer (Vial 2P) to 8µl Nuclease-Free Water (Vial 10). Briefly vortex and microfuge.
 - Add 1µl of PAP Enzyme (Vial 3P) to 4µl of the 1X PAP Buffer. Tap gently to mix, briefly microfuge, and place on ice until use.
4. For each reaction, add the following components for a volume of 25µl:
 - 5.0µl 5X PAP Buffer (Vial 2P)
 - 2.5µl MnCl₂ (Vial 4P)
 - 1.0µl ATP Mix (Vial 1P dilution from step 2)
 - 1.0µl PAP Enzyme (Diluted 1:5 from step 3)
5. Mix gently and microfuge. Incubate at 37°C for 15 minutes.
(Note: Discard any unused 1X PAP buffer, diluted PAP enzyme, or diluted ATP mix.)
6. Stop the reaction by adding 3µl of 0.5M EDTA. Tap to mix, and briefly microfuge.
7. Purify the poly (A) tailed senseRNA using the RNeasy MinElute Kit following the protocol in Appendix A.

Poly (A) tailed senseRNA from RampUP Plus:

After the poly (A) tailed senseRNA has been purified and quantitated, it is ready for any reverse transcription reaction using a dT primer. Generally, 1µg of poly (A) tailed senseRNA requires 7.5-10 picomoles of oligo(dT) primer for efficient reverse transcription.

Recommended amounts of input poly (A) tailed senseRNA for glass microarray detection:

Genisphere's 3DNA Array 900: 1-3µg senseRNA
Genisphere's 3DNA Array 350: 3-5µg senseRNA
Genisphere's 3DNA Array 50: 5-10µg senseRNA
Direct incorporation or amino allyl methods: 5-20µg senseRNA

Recommended amounts of input poly (A) tailed senseRNA for Agilent arrays:

T7 amplification-labeling methods (Agilent): 500ng senseRNA

Troubleshooting

Low senseRNA Yield

Poor RNA sample quality

- If possible, analyze the RNA sample on an agarose gel or other equipment (see page 6). The RampUP kits will amplify partially degraded RNA samples, but cannot amplify completely degraded RNA samples. If the RNA sample is excessively degraded, the overall yield of senseRNA will be reduced.

Poor RNA sample purity

- Contaminating proteins and other RNA extraction components such as ethanol, phenol, or salts, can inhibit reverse transcription and thus reduce overall amplification yield. Be certain to use an RNA extraction method that purifies away these products.
- All reagents and materials that come in contact with RNA samples should be nuclease and protease-free.

Incorrect RNA sample concentration

- Accurate quantitation of RNA samples is critical to calculating amplification fold. When possible, quantitate the RNA samples before beginning the RampUP procedure.
- RNA samples contain varying amounts of mRNA, depending on sample source. Typically, mRNA constitutes 1-5% of a total RNA population. Take this into consideration when calculating fold amplification.

Failed cDNA synthesis

- The initial reverse transcription reaction is the most critical step in the RampUP process. Failure of any one of the components of this reaction will result in a poor or failed synthesis reaction. Store and handle all components properly. DO NOT use expired reagents. Ensure that the recommended reaction temperatures and times are followed. Be certain that the sample, reagents, pipette tips and tubes are nuclease and protease-free.
- When starting with less than 10ng of total RNA, be sure to include the optional T4gene32 Protein (T4gp32) (USB cat. No. 74029Y, $\geq 10\mu\text{g}/\mu\text{l}$) in the reverse transcription reactions.

Failed Tailing or Promoter Synthesis reactions

- Be sure to purify the cDNA following the RNeasy MinElute procedure on page 9. This procedure has been studied thoroughly, and is required for subsequent Tailing and Promoter Synthesis Reactions.
- All of the enzymatic reactions are vital to the RampUP process. Failure of any one of the reaction components will result in a poor or failed amplification. Properly store and handle all components. DO NOT use expired reagents. Ensure that the recommended reaction temperatures and times are followed. Be certain that the sample, reagents, pipette tips and tubes are nuclease and protease-free. Use of the supplied control DNA can help identify a failed enzymatic reaction. Refer to Appendix B: Use of Control DNA (Yellow cap).

Precipitation of In Vitro Transcription reagents

- Be sure to follow the protocol when preparing the T7 and T3 IVT reactions. Thaw the NTPs and 10X IVT Reaction Buffer at room temperature. Thoroughly vortex the 10X IVT Reaction Buffer. Add the reagents to the cDNA, in the order listed in the protocol, at room temperature.

Incorrect incubation conditions

- Confirm the actual incubation temperatures of all heating devices, using a calibrated thermometer. Use heating devices that will prevent condensation from forming inside the reaction tube during the IVT reaction (i.e. thermal cycler or hybridization oven).

Inaccurate pipetting

- Some of the procedures outlined in the RampUP protocol require pipetting extremely small volumes. Be sure to use pipettors that can accurately dispense a volume of 0.5µl.

Inaccurate senseRNA absorbance readings

- Before quantitating senseRNA, calibrate the spectrophotometer to ensure accurate measurements.
- Be sure that the spectrophotometer is being used within the linear range of the instrument (typically absorbance units between 0.1 and 1). Prepare higher dilutions of senseRNA if necessary.
- Measure an RNA sample with known concentration for comparison purposes.
- Confirm concentration of senseRNA with a second method, for example, gel electrophoresis or an equivalent instrument.

Poor Data Quality when Analyzing senseRNA

High artifact content due to incorrect procedure for cDNA purification

- Be sure to purify the cDNA following the RNeasy MinElute procedure on page 9. Failure to prewash the column can result in amplification of nucleic acid artifacts present on the column that can negatively impact data quality or labeling efficiency.

High artifact content due to RNA sample contamination

- Contaminating DNA will be amplified by RampUP. These amplified products will interfere with the analysis of the senseRNA. Treat RNA samples with DNase I, to remove contaminating DNA. **Be sure the DNase is fully removed or inactivated before proceeding with RampUP.**
- Nucleic acid carriers for RNA purification, such as tRNA, will be amplified by RampUP. These amplified products will interfere with the analysis of the senseRNA. When purifying RNA samples, use non-nucleic acid carriers such as linear acrylamide.

Excessive poly (T) or poly (A) in senseRNA samples

- In the first round of RampUP amplification, a poly (A) tail is added to 3' end of the first-strand cDNA. If the reaction time is too long, the poly (A) tails will be too long, resulting in a large amount of amplified poly (T) in the senseRNA. Be sure to limit the tailing reaction time to 2 minutes, as outlined in the protocol.
- For RampUP Plus kits, a poly (A) tail is added to 3' ends of senseRNAs. The Poly (A) tailing procedure on page 18 is designed to produce functional, yet short, Poly (A) tails. Be sure to follow the dilutions of tailing reagents, as outlined in the protocol. In addition, remember that 1µg of poly (A) tailed senseRNA requires 7.5-10 picomoles of oligo(dT) primer for efficient reverse transcription. Be sure to use sufficient amounts of dT primer.

Residual ethanol in senseRNA

- Residual ethanol from the senseRNA purification procedure will inhibit reverse transcriptase. Be sure to follow step 7 of the RNeasy MinElute procedure (Appendix A) to fully remove ethanol from the samples.

For further assistance, please contact Genisphere Technical Support.

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Patent pending (published application no. US20060281153).

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Appendix A: Use of RNeasy MinElute Kit (Qiagen cat. no. 74204)

1. If necessary, bring each sample to be purified to 100 μ l by adding the appropriate volume of RNase-free water supplied with the kit.
2. Warm additional RNase-free water to 50°C. The warmed water will be used in step 8, to elute the sample.
3. Add 350 μ l of RLT Buffer (no BME added) and mix well by pipetting up and down.
4. Add 250 μ l of 95-100% ethanol and mix well by pipetting up and down. Transfer each sample into an RNeasy spin column. Centrifuge at $\geq 12,000$ rpm for 15 seconds. Discard the flow-through and collection tube. Place each column into a new collection tube supplied with the kit.
5. Add 500 μ l of RPE Buffer (prepared with ethanol as indicated by Qiagen) to each spin column. Centrifuge at $\geq 12,000$ rpm for 15 seconds. Discard the flow-through and place each spin column back in the same collection tube.
6. Add 500 μ l of 80% ethanol to each spin column. Centrifuge at $\geq 12,000$ rpm for 2 minutes. Discard the flow-through and collection tube. Place each spin column in a new collection tube supplied with the kit.
7. Centrifuge spin columns with lids open for 5 minutes at $\geq 12,000$ rpm to dry and remove any residual ethanol.
8. Place each spin column into a 1.5mL collection tube supplied with the kit. Add 12 μ l of 50°C water (from step 2) to each spin column, taking care to not touch the column reservoir with the pipette tip. Incubate at room temperature for 2 minutes. Centrifuge at $\geq 12,000$ rpm for 1 minute.

Appendix B: Use of Control DNA (Yellow cap)

In the RampUP kit, the most critical step is the initial reverse transcription. The Control DNA (Yellow cap) can be used to test reactions subsequent to the initial reverse transcription. This procedure can be followed to ensure that the following kit components are working appropriately: Vials 3, 5-9, 11-13 and 17. Vial 19, T3 Enzyme Mix, may also be tested by using it in place of the T7 Enzyme Mix (Vial 13) in the first round IVT reaction (see step 7, on the next page).

Tailing of Control DNA (Yellow cap)

1. Prepare 50ng of the Control DNA for tailing:
 - 10 μ l Control DNA (Yellow cap)
 - 2 μ l Nuclease-Free Water (Vial 10)
2. Heat the 12 μ l Control DNA to 80°C for 10 minutes. Ice immediately for 2 minutes. Briefly centrifuge and return to ice.
3. For each control reaction, prepare a Master Mix in a separate tube on ice:
 - 2 μ l 10X Reaction Buffer (Vial 6)
 - 4 μ l 0.2 mM dATP (Vial 5)
 - 2 μ l TdT Enzyme (Vial 7)
4. Combine the Master Mix and the Control DNA for a volume of 20 μ l. Mix gently and centrifuge.
5. Incubate in a 37°C heat block for 2 minutes. **Do not exceed 2 minutes.**
6. Stop the reaction by heating to 80°C for 10 minutes. Ice immediately for 2 minutes. Briefly centrifuge.
7. Add 4 μ l (10ng) of the dA tailed Control DNA to 16 μ l of Nuclease-Free Water (Vial 10) for a volume of 20 μ l. Proceed with T7/T3 promoter synthesis, below.

Note: The remaining dA tailed Control DNA maybe stored at –20°C to be used for future control reactions, or a portion may be run on a gel (see next page).

T7/T3 Promoter Synthesis

1. Add 2 μ l of T7/T3 Template Oligo (Vial 8) for a volume of 22 μ l. Mix gently and centrifuge.
2. Incubate at 37°C for 10 minutes to anneal the strands.
3. For each reaction, add the following for a volume of 25 μ l:
 - 1 μ l 10X Reaction Buffer (Vial 6)
 - 1 μ l dNTP mix (Vial 3)
 - 1 μ l Klenow enzyme (Vial 9)
4. Mix gently and briefly centrifuge.
5. Incubate at room temperature for 30 minutes.
6. Stop the reaction by heating to 65°C for at least 10 minutes. Place on ice.

7. Proceed to the *T7 In Vitro Transcription* reaction on page 10 using half (5ng in 12.5 μ l) of the promoter-modified Control DNA. Save the remaining modified Control DNA at -20°C for future use as a control amplification reaction, or run a parallel T3 In Vitro Transcription by using the T3 Enzyme Mix (Vial 22) in place of the T7 Enzyme Mix (Vial 13) in the In Vitro Transcription reaction described on page 10. Vial 17 may also be tested in a parallel In Vitro Transcription reaction by using 8 μ l T3 Nucleotide Mix in place of the T7 Nucleotide Mix (Vial 11) in the reaction.

Gel Analysis Demonstrating the Use of Control DNA (Yellow Cap):

After taking 5ng of promoter-modified control DNA into each overnight In Vitro Transcription reaction, and purifying, the following amounts of senseRNA are typically recovered:

- T7 IVT: 30 μ g or more
- T3 IVT: 10 μ g or more

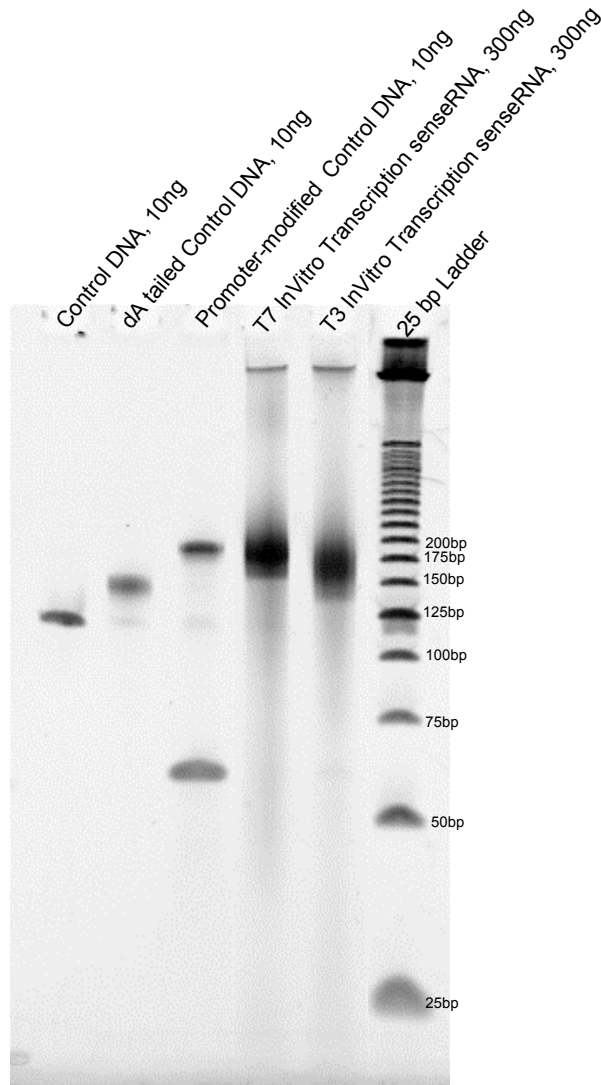


Figure 2. 10% TBE-Urea gel showing the Control DNA (Yellow cap) after each reaction. The gel was run at 150 volts for 45 minutes and stained with 1X SYBR[®] Gold solution.