

SenseAMP™ and SenseAMP Plus™

RNA Amplification Kits

Table of Contents	Page
Introduction	
Characteristics of SenseAMP Amplification	2
SenseAMP Procedure Overview	3
Kit Specifications	
Components and Storage	4
Instructions for Handling Kit Contents at Time of Use	4
Additional Materials/Equipment Required	5
RNA Quality and Primer Selection	6
RNA Input Recommendations	7
Protocol Timelines	7
Procedural Notes	8
Procedure for Use	
First Strand cDNA Synthesis	8
Purification of cDNA	9
Tailing of First Strand cDNA	10
T7 Promoter Synthesis	10
In Vitro Transcription	11
Purification of senseRNA	11
Quantitation of senseRNA	11
Optional: Analysis of senseRNA using Gel Electrophoresis	11
Poly (A) Tailing of senseRNA (for SenseAMP Plus kits only)	12
Analysis of senseRNA	13
Troubleshooting	14
References	16
Appendices	
Appendix A: Use of RNeasy® MinElute™ Kit	17
Appendix B: Use of RNeasy® Mini Kit	18
Appendix C: Use of MinElute™ PCR Purification Kit	19
Appendix D: Use of Control DNA (Yellow Cap)	20

Introduction

Genisphere's SenseAMP kits use a unique RNA amplification method that produces thousands of nearly identical copies of each original RNA molecule. Based on the use of T7 RNA polymerase enzyme, SenseAMP is simple to use and generates high quality amplified RNA for further use in expression analysis experiments such as microarrays and/or quantitative RT-PCR, cDNA cloning, or other relevant RNA-based applications. For total RNA samples of 25ng or less, please refer to Genisphere's RampUP kit for two rounds of RNA amplification.

Characteristics of SenseAMP Amplification

- SenseAMP synthesizes **sense-strand** RNA, referred to in this protocol as **senseRNA**, which is nearly identical to the original message RNA. Due to its unique design, the SenseAMP kit produces amplified RNA that can be labeled for further expression analysis using conventional reverse transcription methods (on both oligo and cDNA arrays). In contrast, conventional T7-based RNA amplification methods ("Eberwine" amplification, Van Gelder R.N. *et al.* 1990) produce anti-sense RNA and may require incorporation of modified ribonucleotides, an often-inefficient method of labeling RNA.
- SenseAMP amplification begins by synthesizing cDNA from the 3' end of the message (dT priming). 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. In contrast, conventional Eberwine-based amplification methods prime and amplify from the 3' end of the message, typically resulting in a 3' bias.
- SenseAMP amplification provides unprecedented data quality due to its high fidelity RNA amplification products. When compared to unamplified RNA by qRT-PCR analysis, RNA produced by the SenseAMP kit demonstrates a significantly higher correlation ($R^2 = 0.93$) than does RNA produced by conventional Eberwine amplification ($R^2 = 0.86$) (Goff L.A. *et al.* 2004).
- SenseAMP 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 primers in the first reaction (cDNA synthesis).
- Since the SenseAMP Plus kit includes the PLUS module for producing polyadenylated RNA molecules, it can be used to generate high-quality input material for downstream amplification or labeling procedures which require dT primed reverse transcription. If necessary, the PLUS module may be ordered separately (Genisphere cat. no. P10PLUS).
- SenseAMP Plus can also be used to amplify Low Molecular Weight RNA samples like snRNA, hnRNA, piRNA, miRNA, etc. An optimized protocol for the amplification of Low Molecular Weight RNA is available on Genisphere's website, www.genisphere.com.
- SenseAMP senseRNA can easily be prepared for analysis on Affymetrix® GeneChips®. Genisphere's cDNA Synthesis Kit (cat. no. CDNAMOD) is required to generate biotinylated cDNA for hybridization. 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.

SenseAmp Procedure Overview

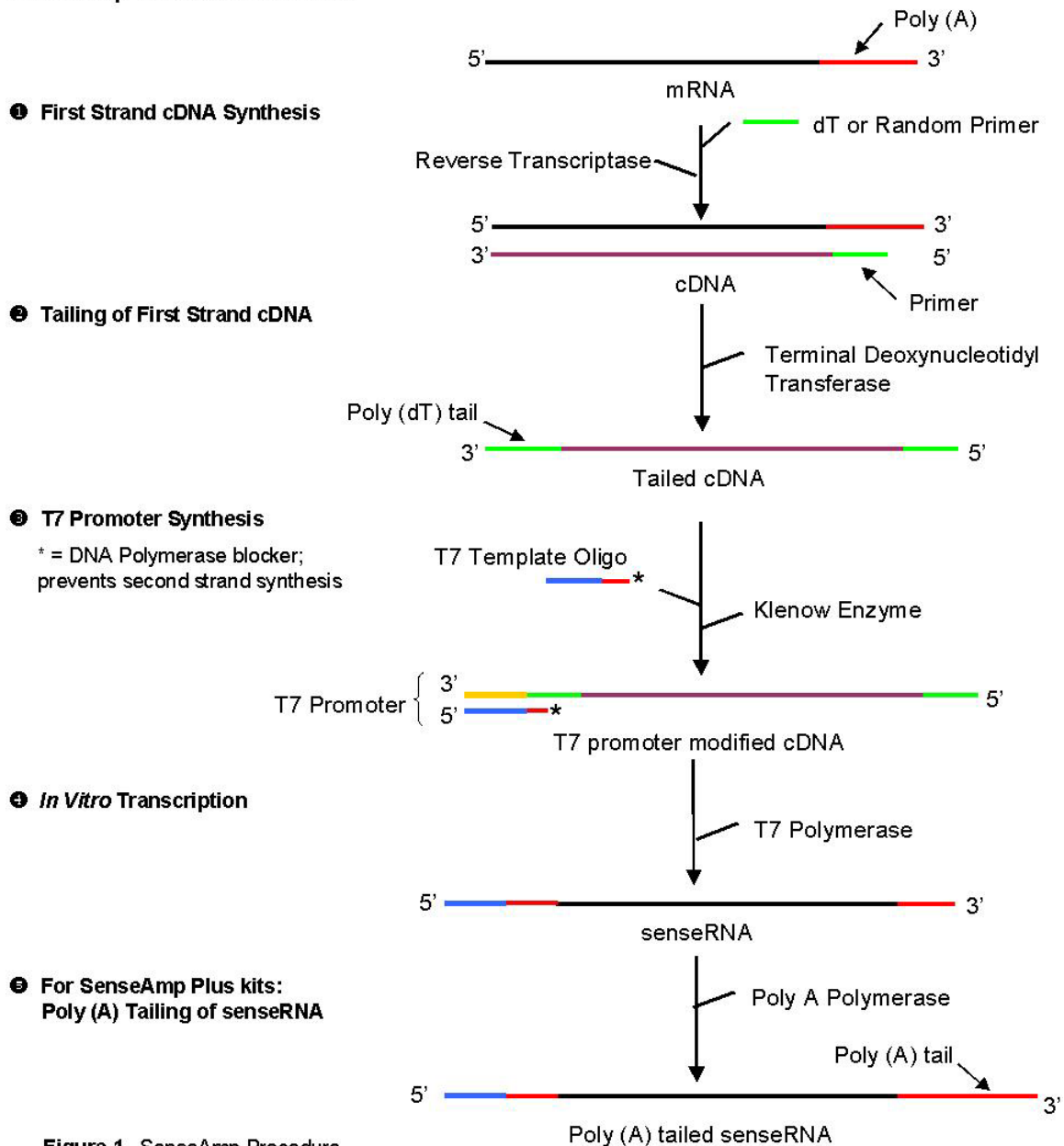


Figure 1. SenseAmp Procedure.

- 1 First Strand cDNA Synthesis.** RNA is primed using an Oligo (dT) and/or random primer to produce single-stranded cDNA.
- 2 Tailing of First Strand cDNA.** First strand cDNA is purified then tailed with dTTP using Terminal Deoxynucleotidyl Transferase.
- 3 T7 Promoter Synthesis.** The T7 Template is annealed to the 3' end of the cDNA. Klenow enzyme fills in the 3' end of first strand cDNA to produce a double-stranded T7 promoter. The T7 Template contains a blocker to prevent second strand synthesis.
- 4 In Vitro Transcription.** senseRNA copies of the original starting material are generated.
- 5 For SenseAmp Plus kits: Poly (A) Tailing of senseRNA.** Poly (A) tails are regenerated on all senseRNA molecules.

Kit Specifications

Components and Storage

SenseAMP Components

Vial 1	SenseAMP dT24 RT Primer (50ng/μl)
Vial 2	SenseAMP Random 9mer RT Primer (250ng/μl)
Vial 3	dNTP Mix (10mM each dATP, dCTP, dGTP, dTTP)
Vial 4	Suprase-In™ RNase Inhibitor
Vial 5	10mM dTTP
Vial 6	10X Reaction Buffer
Vial 7	Terminal Deoxynucleotidyl Transferase
Vial 8	SenseAMP T7 Template Oligo
Vial 9	Klenow Enzyme
Vial 10	Nuclease-Free Water
Vial 11	T7 Nucleotide Mix (ATP,GTP,CTP, and UTP)
Vial 12	10X T7 Reaction Buffer
Vial 13	T7 Enzyme Mix
Yellow Cap	Control DNA (5ng/μl)

Plus Module Components

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.

Instructions for Handling Kit Contents at Time of Use

Vials 1, 2, 6, 8, 10, 11, 12, Yellow Cap, 1P, 2P, and 4P:
Thaw at room temperature, vortex, and briefly microfuge. Keep at room temperature until use.

Vials 3, 4, 5, 7, 9, 13, and 3P:
Thaw on ice, briefly microfuge if necessary, and keep on ice at all times. Do not vortex.

Additional Materials/Equipment Required

- SuperScript™ II (Invitrogen) or other Reverse Transcriptase enzyme
- 10mM Tris-HCl, 1mM EDTA pH 8.0 (1X TE Buffer) (Ambion cat. no. 9858 or equivalent)
- RNeasy MinElute Kit (Qiagen cat. no. 74204)
- 80% Ethanol
- 100% Ethanol
- 0.5M EDTA (for PLUS poly(A) tailing only)
- 1mM Tris pH 8.0 (for PLUS poly(A) tailing only)
- Microcentrifuge
- Heating devices for incubations at 37°C, 42°C, 65°C, 80°C
- UV/Vis Spectrophotometer for senseRNA quantitation (or other RNA quantitation instrument)
Note: 1mM Tris-HCl, 0.1mM EDTA pH 8.0 (0.1X TE Buffer) is recommend for UV/Vis spectrophotometer quantitation

Optional Materials/Equipment

- RNeasy Mini Kit (Qiagen cat. no. 74106) (for senseRNA >50µg; see page 11)
- MinElute™ PCR Purification Kit (Qiagen cat. no. 28006) (for alternate cDNA purification; see Appendix C)
- MegaScript T7 Kit (Applied Biosystems cat. no. AM1334) (for amplifying the other half of T7-promoter-modified cDNA; see page 10)
- Gel electrophoresis apparatus and reagents for analyzing agarose gels (or other instrument to visualize RNA)
- Genisphere® 3DNA® labeling kit for microarray analysis of senseRNA
- Genisphere cDNA Synthesis Kit (cat. no. CDNAMOD) for Affymetrix GeneChip or glass microarray analysis of senseRNA

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 SenseAMP. 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 SenseAMP. 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. If the RNA is intact, Random Primer (Vial 2) may be omitted from the first reverse transcription step (see page 8). If the RNA is partially degraded, use both random and dT primers in the first reverse transcription step, as described on page 8. The SenseAMP 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 2 for examples of both intact and partially degraded RNA samples.

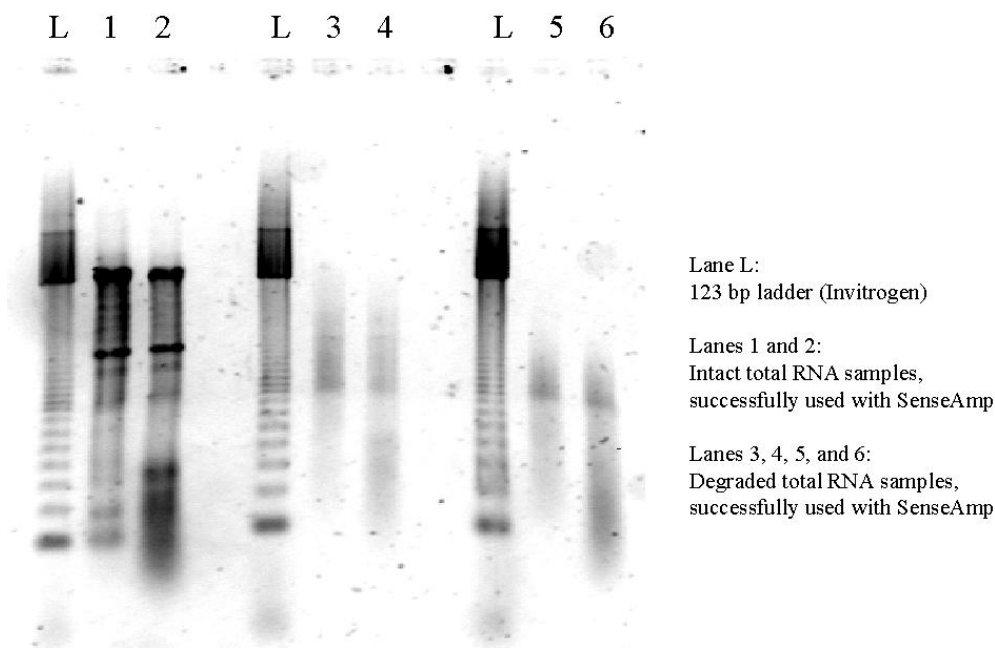


Figure 2: 1% denaturing agarose gel of intact and partially degraded RNA samples. One microgram of each RNA sample was loaded onto the gel.

RNA Input Recommendations

The SenseAMP kit is designed for one round of amplification using 25-250ng of total RNA. For high-quality RNA which can be reverse transcribed without random primers (see page 6), 25-2000ng of total RNA can be used.

For smaller amounts of total RNA (25ng or less), please refer to Genisphere's RampUP RNA amplification kit, for two rounds of RNA amplification.

Note: This protocol is not written for the amplification of Low Molecular Weight RNA samples like snRNA, hnRNA, piRNA, miRNA, etc. An optimized protocol for the amplification of Low Molecular Weight RNA is available on Genisphere's website, www.genisphere.com.

Protocol Timelines

The SenseAmp protocol is designed to reduce the amount of hands-on time required for one round of RNA amplification.

First Strand cDNA Synthesis	140 minutes
Purification of cDNA	30 minutes
Tailing of First Strand cDNA	30 minutes
T7 Promoter Synthesis	65 minutes
T7 In Vitro Transcription	overnight
Total Time to Round 1 IVT	~4.5 hours

Procedural Notes

- When preparing master mixes, the enzyme 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 microfuge. DO NOT VORTEX.
- All incubations longer than 30 minutes should be transferred from a heat block to a hybridization oven, or performed in a thermal cycler. This prevents the reaction from concentrating due to evaporation and condensation of the sample on the unheated sides and top of the tube.

Procedure for Use

First Strand cDNA Synthesis

1. Dilute Random Primer, 250ng/μl (Vial 2) using Nuclease-Free Water (Vial 10) to 1X by mass of the RNA sample. For example, when reverse transcribing 100ng of total RNA, dilute the Random Primer to 100ng/μl.
2. Adjust the volume of total RNA to 7μl with Nuclease-Free Water (Vial 10).
3. Add the following components to the 7μl total RNA to make the **RNA-Primer Mix**:
 - 2μl SenseAMP dT24 RT primer (50ng/μl) (Vial 1)
 - 2μl Random Primer (Vial 2 dilution from step 1)

Notes:

- When using intact total RNA, the Random Primer may not be required for cDNA synthesis (see page 6). Use Nuclease-Free Water (Vial 10) in place of the Random Primer.
 - 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. Heat the **RNA-Primer Mix** to 80°C for 10 minutes. Ice immediately for 2 minutes. Briefly microfuge and return to ice.
 4. For each reaction, prepare a **Master Mix** in a separate tube on ice:
 - 4μl 5X First Strand Buffer (or equivalent 5X buffer)
 - 2μl 0.1M DTT (if supplied with enzyme, otherwise use Nuclease-Free water)
 - 1μl Superase-In (Vial 4)
 - 1μl dNTP Mix (Vial 3)
 - 1μl Superscript II (or equivalent reverse transcriptase enzyme)
 - 9μl
 5. Add the **Master Mix** to the **RNA-Primer Mix** for a volume of 20μl. Mix gently and microfuge.
 6. Incubate at 42°C for 2 hours.
 7. Microfuge briefly. Add 80μl of 1X TE Buffer to adjust the volume to 100μl.

Purification of cDNA

Currently, Genisphere recommends using the RNeasy MinElute kit (Qiagen cat. no. 74204) to purify cDNA (see procedure below). In previous versions of the SenseAMP protocol, Genisphere recommended the MinElute PCR Purification Kit (Qiagen cat. no. 28006) to purify cDNA. If it is necessary to purify the cDNA with the MinElute PCR Purification kit, follow the procedure in Appendix C.

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.
8. 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 purified cDNA (12 μ l) to 80°C for 10 minutes. Ice immediately for 2 minutes. Briefly microfuge and return to ice.
2. For each reaction, prepare a Master Mix in a separate tube on ice:
2 μ l 10X Reaction Buffer (Vial 6)
4 μ l 10mM dTTP (Vial 5)
2 μ l TdT Enzyme (Vial 7)
8 μ l
3. Combine the Master Mix and the cDNA for a volume of 20 μ l. Mix gently and microfuge.
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. Briefly microfuge and cool to room temperature (20-25°C) for 1-2 minutes.

T7 Promoter Synthesis

1. Add 2 μ l of T7 Template Oligo (Vial 8) to the tailed cDNA for a volume of 22 μ l. Briefly vortex and microfuge.
2. Incubate at 37°C for 10 minutes to anneal the strands.
3. For each reaction, add the following components 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 microfuge.
5. Incubate at room temperature (20-25 °C) for 30 minutes.
6. Stop the reaction by heating to 65°C for 10 minutes. Briefly microfuge and place on ice for 2 minutes.
7. Proceed to the *In Vitro Transcription* reaction using half (12.5 μ l) of the promoter-modified cDNA. Save the remaining modified cDNA at -20°C for future use (PCR, gel analysis, or a parallel amplification reaction using the MegaScript T7 Kit (Applied Biosystems cat. no. AM1334) and the In Vitro Transcription procedure on page 11).

In Vitro Transcription

1. Incubate the 12.5µl of cDNA at 37°C for 10 minutes to re-anneal the strands.
2. Thaw the T7 Nucleotide Mix (Vial 11) and 10X T7 Reaction Buffer (Vial 12) at room temperature, and **keep at room temperature until use**. Thoroughly vortex the 10X T7 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 25µl:
 - 8.0µl T7 Nucleotide Mix (Vial 11)
 - 2.5µl 10X T7 Reaction Buffer (Vial 12)
 - 2.0µl T7 Enzyme Mix (Vial 13)
4. Mix gently and microfuge. 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 air hybridization oven.**It is essential to avoid evaporation and condensation of the reaction during this step.**
Note: An incubation of 14-16 hours (overnight) is recommended for maximum senseRNA yield.
5. Stop the reaction by placing samples at -20°C until ready to proceed with the next step.

Purification of senseRNA

Purify the senseRNA using the appropriate Qiagen RNeasy column:

- Recommended: For anticipated senseRNA amounts less than 50µg, follow Appendix A, Use of RNeasy MinElute Kit
- For anticipated senseRNA amounts more than 50µg, follow Appendix B, Use of RNeasy Mini Kit

Quantitation of senseRNA

Quantitate the senseRNA using a spectrophotometer or other instrument. Calculate the A260/280 ratio to determine RNA purity. A ratio of 2.0-2.3 is most desirable. Higher ratios may indicate that an excessive poly (A) tail was generated during the amplification reaction.

Optional: Gel electrophoresis under denaturing conditions may be used to visualize senseRNA size distribution and sample purity. If random primers were used for first strand cDNA synthesis, the resulting senseRNAs will have shorter fragments than if the dT primer alone was used for first strand cDNA synthesis.

Procedural Note:

For SenseAMP Plus, continue to page 12. For SenseAMP, continue to page 13.

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

This procedure is for SenseAMP 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 tailed senseRNA following Appendix A, Use of RNeasy MinElute Kit.

Analysis of senseRNA

SenseRNA from SenseAMP:

After the senseRNA has been purified and quantitated, it is ready for any reverse transcription reaction using a random primer or gene-specific primer. Random primers should be used at 1.5-2X by mass to the senseRNA for efficient reverse transcription.

Recommended amounts of input senseRNA for glass microarray detection:

Array 900MPX: 1-3 μ g senseRNA

Direct incorporation or amino allyl methods: 5-20 μ g senseRNA

Recommended amounts of input senseRNA for Affymetrix Gene Expression Analysis Arrays:

10-20 μ g senseRNA

Note: Genisphere's cDNA Synthesis Kit (cat. no. CDNAMOD) is required to generate biotinylated cDNA for hybridization on Affymetrix GeneChips.

Poly (A) tailed senseRNA from SenseAMP 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, 1 μ g 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:

Array 900: 1-3 μ g senseRNA

Array 350: 3-5 μ g senseRNA

Array 50: 5-10 μ g senseRNA

Direct incorporation or amino allyl methods: 5-20 μ g senseRNA

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

T7 amplification-labeling methods (Agilent): 500ng senseRNA
(labeled cRNA is hybridized to the array)

Troubleshooting

For troubleshooting purposes, we recommend following Appendix D, Use of Control DNA (Yellow cap), to ensure that certain kit components are working appropriately. The information below is also useful for troubleshooting.

Low senseRNA Yield

Poor RNA sample purity

- Contaminating proteins, and other extraction components such as ethanol, phenol, or salts, can inhibit reverse transcription and thus reduce overall amplification yield. We recommend Qiagen's RNeasy kits for purifying extracted RNA samples before use. Alternatively, ethanol precipitation may be used to remove protein contaminants.
- **Contaminating DNA will be amplified by this protocol**, as it will be tailed by Terminal Deoxynucleotidyl Transferase along with synthesized cDNA. This will affect absorbance readings and significantly alter calculated concentration of amplified senseRNA. We recommend treating DNA contaminated RNA samples with DNase I. Please refer to *RNA Quality and Primer Selection* for information on the handling of DNase I treated samples.

Incorrect RNA sample concentration

- When possible, quantitate the RNA sample before beginning this procedure. If the concentration of RNA is too low to quantitate, consider using Genisphere's RampUP kit for multiple rounds of RNA amplification.
- RNA samples can 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

- All of the components in the reverse transcription reaction (RT enzyme, dNTPs, reaction buffer, etc.) are vital to efficient cDNA synthesis. Failure of any one of these components will result in a failed synthesis reaction. Store and handle all components properly. DO NOT use expired reagents.
- Check peer-reviewed articles to determine which enzyme is commonly used for a species of interest. RT enzymes have different attributes and variable performance characteristics, particularly among various species.

Incorrect conditions for Tailing of cDNA

- Do not exceed the amount of equivalent RNA recommended for the tailing reaction. Using too much cDNA in the tailing reaction will reduce the length of the dTTP tails, and the T7 promoter template will not be able to hybridize.
- Do not exceed the 2 minute incubation time, as described in the protocol.

Incorrect purification of cDNA

- Follow the special procedure for the use of RNeasy MinElute columns. Genisphere's protocol includes a pre-wash of the column for ideal purification, allowing the cDNA to properly function in the subsequent enzymatic reactions.

Incorrect use of T7 Template Oligo

- Failure to add T7 template oligo prior to T7 promoter synthesis will prevent the double stranded promoter complex from being produced, thus preventing *in vitro* transcription.

Incorrect incubation conditions

- Confirm actual incubation temperature of the heating device using a thermometer.
- Use a heating device that will prevent condensation from forming inside the reaction tube during the IVT reaction (i.e. thermal cycler or oven incubator). Condensation can alter overall concentration of reaction components.

SenseRNA absorbance readings are inaccurate

- Calibrate the spectrophotometer to ensure accurate measurements.
- Measure an RNA sample with known concentration for comparison purposes.
- Be sure that A260 and A280 measurements are within detection limits of spectrophotometer. Prepare additional higher concentration dilutions of your senseRNA for analysis if necessary.
- Confirm concentration of senseRNA samples by analyzing samples on an agarose gel in comparison to samples of known concentration.
- The SenseAMP procedure can generate poly (A) sequences on the 5' end of senseRNA because TdT enzyme is used to tail cDNA with dTTP. A A260/280 ratio of greater than 2.4 may indicate that a large amount poly (A) was produced during the amplification procedure. Repeat the SenseAMP procedure using less Terminal Deoxynucleotidyl Transferase in the cDNA Tailing procedure. Dilute the enzyme (1:4) in 1X Reaction Buffer (Vial 6 diluted 1:10). Use 2µl of the diluted enzyme in the tailing reaction.

Nuclease contamination

- Be sure to use disposable microfuge tubes and pipet tips that are free of contaminating nucleases.

Inefficient senseRNA Labeling

Residual ethanol

- Residual ethanol from senseRNA purification procedure can inhibit reverse transcriptase. Be sure to follow the protocols in Appendix A and Appendix B when using Qiagen RNeasy columns to purify senseRNA.

Failed or omitted Poly (A) Polymerase Reaction

- SenseRNA that is not efficiently tailed with poly (A) will not efficiently reverse transcribe using a dT primer, resulting in reduced sensitivity in array experiments. Repeat the tailing and labeling procedure using the purified senseRNA.

Insufficient senseRNA used in labeling reactions

- In all RNA amplification methods, considerable quantities of amplified RNA are labeled for hybridization to microarrays. Genisphere has compared sensitivity on microarrays using both amplified and unamplified RNA. Based on these experiments, we have made recommendations in our protocols for how much senseRNA to use in your labeling reactions. Be sure to follow these recommendations for input senseRNA to be used in labeling reactions.

Insufficient quantity of primers used in labeling reactions

- For labeling senseRNA from the SenseAMP kit, use random primers at 1.5-2X by mass to the senseRNA.
- For labeling poly (A) tailed senseRNA from the SenseAMP Plus kit, use 7.5-10 picomoles of dT primer for every microgram of poly (A) tailed senseRNA.

References

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Patents pending.

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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 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.
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 RNeasy Mini Kit (Qiagen cat. no. 74106)

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 a RNeasy spin column. Centrifuge at \geq 12,000rpm 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 by adding ethanol as indicated by Qiagen) to each spin column. Centrifuge at \geq 12,000rpm for 15 seconds. Discard flow-through and place each spin column back in the same collection tube.
6. Add another 500 μ l of RPE Buffer to each spin column. Centrifuge for 2 minutes at \geq 12,000rpm. Discard the flow-through and collection tube. Place each spin column into a new 1.5mL centrifuge tube (not supplied with the kit).
7. Centrifuge for 1 minute at \geq 12,000rpm to remove any residual ethanol.
8. Place each spin column into a 1.5mL collection tube supplied with the kit. Add 30-50 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 12,000rpm for 1 minute.
9. Re-apply the 30-50 μ l volume eluted back onto the same column. Incubate at room temperature for 2 minutes. Centrifuge \geq 12,000rpm for 1 minute.

Appendix C: Use of MinElute PCR Purification Kit (Qiagen cat. no. 28006)

Currently, Genisphere recommends using the RNeasy MinElute kit (Qiagen cat. no. 74204) to purify cDNA (see page 9). In previous versions of the SenseAMP protocol, Genisphere recommended the MinElute PCR Purification Kit (Qiagen cat. no. 28006) to purify cDNA. If it is necessary to purify the cDNA with the MinElute PCR Purification kit, follow the procedure below.

Purify the 100 μ l of cDNA using the MinElute PCR Purification Kit (Qiagen cat. no. 28006) as follows:

1. Add 500 μ l Buffer PB to the 100 μ l cDNA sample and mix.
2. Apply the cDNA mixture to the MinElute column and centrifuge for 1 minute at 10-14,000 x g (~13,000 rpm) in a conventional tabletop microcentrifuge.
3. Discard the flow-through. Place the MinElute column into the same collection tube.
4. Add 750 μ l Buffer PE to the MinElute column and centrifuge for 1 minute.
5. Discard the flow-through. Place the MinElute column back into the same collection tube.
6. Add 500 μ l 80% ethanol to the MinElute column and centrifuge for 2 minutes.
7. Discard the flow-through. Place the MinElute column back into the same collection tube.
8. Open the column caps and place in a microfuge with the cap opposite the direction of rotation of the rotor to avoid breaking the cap off. Centrifuge for 5 minutes.
9. Place the MinElute column into a clean, labeled, 1.5mL microfuge tube.
10. To elute cDNA, add 10 μ l Buffer EB to the center of the column membrane. Incubate at room temperature for 2 minutes. Centrifuge for 2 minutes. Discard column and save the eluted cDNA.
11. **Bring the volume of cDNA to 12 μ l with Nuclease-Free Water (Vial 10).**
12. Proceed to Tailing of First Strand cDNA on page 10.

Appendix D: Use of Control DNA (Yellow cap)

In the SenseAMP 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 the following kit components are working appropriately: Vials 3, 5-9, and 11-13. After following this procedure, at least 15ug of senseRNA should be obtained.

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 10mM dTTP (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 tailed Control DNA to 16 μ l of Nuclease-Free Water (Vial 10) for a volume of 20 μ l. Proceed with T7 promoter synthesis, below.

Note: The remaining tailed Control DNA maybe stored at –20°C to be used for future control reactions.

T7 Promoter Synthesis

1. Add 2 μ l of T7 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 11 of SenseAMP, 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 control reactions.
8. After the overnight In Vitro Transcription, purify with RNeasy columns and quantitate. At least 15ug of senseRNA should be obtained.