

SenseAMP Plus™

Procedure for Amplification of Low Molecular Weight RNA

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Introduction

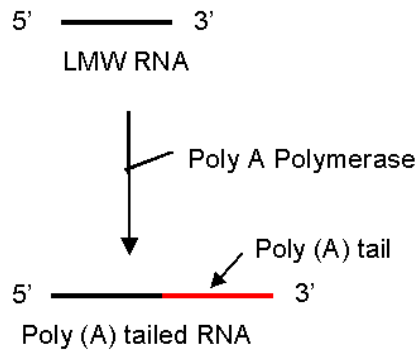
Background Information

Genisphere's SenseAmp kits use a unique RNA amplification method that produces thousands of nearly identical copies of each original RNA molecule (1). Based on the use of T7 RNA polymerase enzyme, SenseAmp is simple to use and generates high quality amplified RNA for further analysis by microarrays (2), quantitative RT-PCR (2), ELOSA (3), or other relevant RNA-based applications.

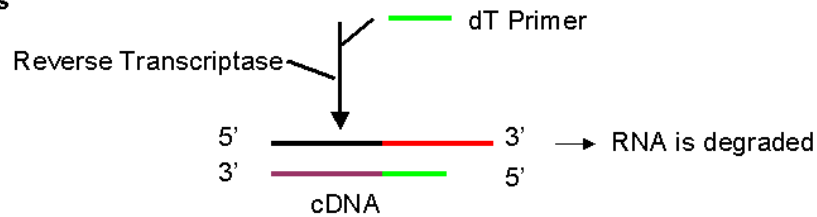
The following protocol is an adaptation of the SenseAmp Plus procedure, optimized for Low Molecular Weight (LMW) RNA samples. A number of roles have been described for LMW RNA molecules (snRNA, hnRNA, piRNA, miRNA, etc.) including remodeling of chromosomal architecture and regulation of mRNA translation. Up to 30ng of enriched LMW RNA sample can be used in this amplification procedure.

Amplification of LMW RNA with SenseAmp Plus: Procedure Overview

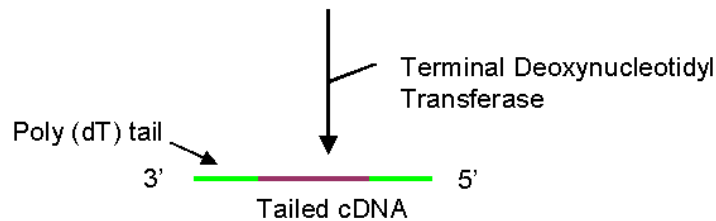
1 Poly (A) Tailing



2 First Strand cDNA Synthesis

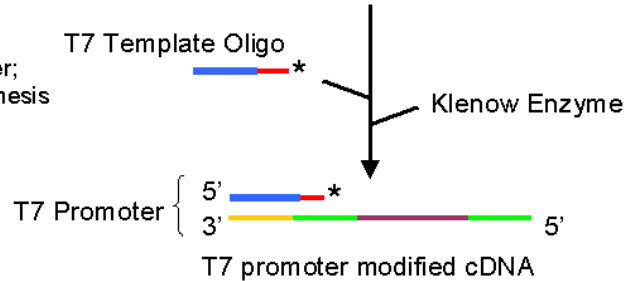


3 Tailing of First Strand cDNA

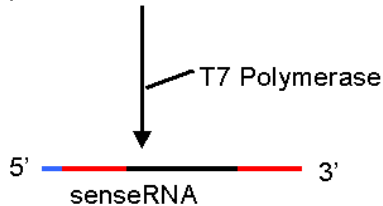


4 T7 Promoter Synthesis

* = DNA Polymerase blocker; prevents second strand synthesis



5 In Vitro Transcription



- 1 **Poly (A) Tailing:** Poly (A) tails are generated on all LMW RNA molecules.
- 2 **First Strand cDNA Synthesis:** RNA is primed using an Oligo (dT) and/or random primer to produce single-stranded cDNA.
- 3 **Tailing of First Strand cDNA:** First strand cDNA is purified then tailed with dTTP using Terminal Deoxynucleotidyl Transferase.
- 4 **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.
- 5 **In Vitro Transcription:** senseRNA copies of the original RNA molecules are generated.

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 Superase-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

Plus Module Components

- Vial 1P ATP Mix
- Vial 2P 5X PAP Buffer **Note: Vial 2P, 5X PAP Buffer, is NOT used in this protocol**
- Vial 3P PAP Enzyme
- Vial 4P 25mM MnCl₂
- Vial 5P Control Oligo (40ng/μl)

Store all vials at -20°C.

Instructions for Handling Kit Contents at Time of Use

Vials 1, 2, 6, 8, 10, 11, 12, 1P, 4P and 5P:
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.

Other Materials Required

- SenseAmp Plus Kit (Genisphere® cat. no. RAMP110MIR or RAMP120MIR)
- 1M Tris pH 8.0
- 10mM Tris pH 8.0
- 1mM Tris pH 8.0
- 0.5M NaOH/50mM EDTA
- 10mM Tris-HCl, 1mM EDTA pH 8.0 (1X TE Buffer)
- 1mM Tris-HCl, 0.1mM EDTA pH 8.0 (0.1X TE Buffer)
- SuperScript™II (Invitrogen™) or other reverse transcriptase enzyme
- Microcon® YM-100 Centrifugal Filter Devices (Millipore™ cat. no. 42413)
- RNeasy® MinElute™ Kit (Qiagen cat. no. 74204)
- RiboGreen® RNA Quantitation Kit (Molecular Probes™ cat. no. R-11490)

Procedure

Enrichment of LMW RNA

Note: When using a severely fragmented or degraded RNA sample, this enrichment procedure may not be necessary. In this case, proceed to the Poly (A) Tailing procedure below.

Enrich the total RNA sample using a Microcon YM-100 column and a conventional tabletop microfuge.

1. Dilute the total RNA sample to 100 μ L with 10mM Tris pH 8.
2. Heat to 80°C for 3 minutes, then cool on ice for 3 minutes.
3. While the sample is cooling on ice, add 50 μ l of 10mM Tris pH 8 to the Microcon column, and spin for 3 minutes at top speed.
4. Discard the flow-through and the collection tube. Place the column into a new collection tube.
5. Add the 100 μ l of RNA to the Microcon column, and centrifuge for 7 minutes at top speed.
6. Save the eluate in the collection tube. This is the enriched LMW RNA. 10-20 μ l of enriched LMW RNA may be quantitated with the RiboGreen RNA Quantitation Kit.

Poly (A) Tailing

Note: Vial 2P, 5X PAP Buffer, is NOT used in this protocol

1. In this protocol, up to 30 nanograms of Low Molecular Weight RNA can be used. Adjust the volume to 18 μ l with Nuclease-Free Water (Vial 10).
2. For LMW RNA samples between 1 and 30 nanograms: dilute the ATP (Vial 1P) in 1mM Tris pH 8.0 according to the following formula:

$$\text{ATP dilution factor} = \frac{5000}{\text{____ng of LMW RNA}}$$

For example, if starting with 2ng RNA, the ATP dilution factor = 5000 \div 2ng = 2500.
Dilute the ATP 1:2500 by adding 1 μ l of ATP (Vial 1P) to 2499 μ l of 1mM Tris pH 8.0.

For LMW RNA samples less than 1ng: dilute the ATP (Vial 1P) 1:5000 in 1mM Tris pH 8.0.

3. Add the following components to the 18 μ l LMW RNA, for a volume of 25 μ l:
 - 2.5 μ l 10X Reaction Buffer (Vial 6)
 - 2.5 μ l 25mM MnCl₂ (Vial 4P)
 - 1 μ l diluted ATP (Vial 1P dilution from step 2)
 - 1 μ l PAP Enzyme (Vial 3P)
4. Mix gently and microfuge.
5. Incubate in a 37°C heat block for 15 minutes.

First Strand cDNA Synthesis

1. Briefly microfuge the 25 μ l of tailed RNA and place on ice.
2. Prepare a 1:10 dilution of SenseAmp dT primer (Vial 1) by adding 1 μ l SenseAmp dT primer to 9 μ l 0.1X TE. Vortex and briefly microfuge.
3. On ice, add 2 μ l of 1:10 diluted SenseAmp dT primer (Vial 1 dilution from step 2).
4. Mix gently and microfuge. Incubate at 65°C for 10 minutes and immediately transfer to ice for 2 minutes.
5. Add the following components on ice, for a volume of 50 μ l:
 - 10 μ l 5X First Strand Buffer (or equivalent buffer supplied with the reverse transcriptase)
 - 5 μ l 0.1M DTT (If supplied with the reverse transcriptase; otherwise use nuclease free water)
 - 2.5 μ l dNTP mix (Vial 3)
 - 1 μ l Superase-in™ RNase inhibitor (Vial 4)
 - 2 μ l SuperScript II reverse transcriptase, 200 units (or equivalent reverse transcriptase)
 - 2.5 μ l Nuclease-Free Water (Vial 10)
6. Gently mix (do not vortex) and incubate at 42°C for one hour.
7. Stop the reaction by adding 8.75 μ l of 0.5M NaOH/50mM EDTA. Briefly vortex and microfuge.
Note: the reaction may turn to a brown color; this is normal.
9. Incubate at 65°C for 30 minutes to degrade the RNA.
Note: the reaction may turn from brown to clear; this is normal.
10. Neutralize the reaction by adding 12.5 μ l of 1M Tris pH 8.0. Bring the sample to 100 μ l by adding 28.75 μ l 1XTE Buffer. Briefly vortex and microfuge. This is the cDNA.

Purification and Concentration of cDNA

Purify the cDNA using a Microcon YM-100 column and a conventional tabletop microfuge.

1. Add the 100 μ l cDNA to the sample reservoir. Do not touch the membrane with the pipette tip. Secure the tube cap and centrifuge for 6 minutes at 13,000g.
2. 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.
3. Carefully separate the sample reservoir from the collection tube. Discard the flow-through. Place the YM-100 column into the same collection tube.
4. 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.
5. Carefully separate the sample reservoir from the collection tube. Discard the collection tube.
6. Add 5 μ l 1mM Tris pH 8.0 to the sample reservoir without touching the membrane. Gently tap the side of the reservoir to mix.
7. Carefully place the sample reservoir upside down in a **new collection tube**. Centrifuge for 3 minutes at 13,000g.
8. Note the volume of cDNA collected in the bottom of the tube (5-10 μ l). Bring the volume of cDNA to 10 μ l with Nuclease Free Water (Vial 10).

Tailing of First Strand cDNA

1. Heat purified cDNA (10 μ l) to 80°C for 10 minutes. Ice immediately for 1-2 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)
 - 2 μ l Nuclease Free Water (Vial 10)
 - 4 μ l 10mM dTTP (Vial 5)
 - 2 μ l TdT Enzyme (Vial 7)
 - 10 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 3 minutes. **Do not exceed 3 minutes.**
5. Stop the reaction by heating to 80°C for 10 minutes. Briefly microfuge and cool to room temperature for 1-2 minutes.

T7 Promoter Synthesis

1. Add 2 μ l of SenseAmp 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. To 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. Incubate at room temperature for 30 minutes.
5. Stop the reaction by heating to 65°C for 10 minutes. Place on ice.
6. 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 or for use in a parallel amplification reaction.

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 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 to the 12.5 μ l of cDNA **at room temperature**, for a final volume of 25 μ l:
 - 8.0 μ l T7 Nucleotide Mix (Vial 11)
 - 2.5 μ l 10X T7 Reaction Buffer (Vial 12)
 - 2.0 μ l T7 Enzyme Mix (Vial 13)
4. Mix gently and microfuge. Incubate in a thermalcycler (with heated lid) at 37°C for 4-16 hours. Or, place the reaction in a 37°C heat block for 5 minutes and then transfer to a 37°C air hybridization oven for 4-16 hours. **It is essential to avoid evaporation and condensation of the reaction during this step.**

Purification of senseRNA

Purify the senseRNA using the RNeasy MinElute Kit (Qiagen cat. no. 74204) following Qiagen's protocol for RNA Cleanup. To elute, add 14 μ l Nuclease Free Water, incubate for two minutes, and then spin. The recovered volume should be approximately 12 μ l.

Quantitation of senseRNA

Determine the concentration of the senseRNA using the RiboGreen RNA Quantitation Kit (Molecular Probes cat. no. R-11490). Use the Ribosomal RNA standard provided with the kit to prepare a standard curve. Use 1 μ l of the purified senseRNA to quantitate.

Analysis of senseRNA

Since the senseRNA is in the original orientation as the unamplified RNA, and has a 3' poly(A) tail, several standard methods can be used to analyze the senseRNA.

For microarray analysis: FlashTag™ RNA Labeling Kit (Genisphere).

Proceed directly to *FlashTag Ligation*, using 125-750ng of the purified senseRNA. The senseRNA does not need to be tailed with Poly (A) Polymerase.

For qRT-PCR analysis, ELOSA methods, or other applications:

Contact Genisphere's Technical Support group for recommendations.

References

1. Goff et al. Evaluation of sense-strand mRNA amplification by comparative quantitative PCR. BMC Genomics 2004, 5:76.
2. Mattie et al. Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies. Molecular Cancer 2006, 5:24.
3. Mora, JR, and Getts, RC. Enzymatic microRNA detection in microtiter plates with DNA dendrimers. BioTechniques October 2006, Volume 41, Number 4: pp 420-424

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Appendix A: Use of Control Oligo (Vial 5P)

A control LMW RNA oligo is provided to test the reagents in the SenseAmp Plus kit. The use of 10ng of Vial 5P Control Oligo in the SenseAmp Plus procedure should result in 5-10 μ g of senseRNA recovered.

Note: Vial 2P, 5X PAP Buffer, is NOT used in this protocol

1. Dilute the Control Oligo (Vial 5P, 40ng/ μ l) to 10ng/ μ l by adding 1 μ l Control Oligo to 3 μ l Nuclease-Free Water (Vial 10). Then, add 1 μ l of diluted Control Oligo (10ng) to 17 μ l Nuclease-Free Water (Vial 10), to adjust the volume to 18 μ l.
2. Dilute the ATP (Vial 1P) 1:500 by adding 1 μ l ATP to 499 μ l 1mM Tris pH 8.0. Mix gently and microfuge.
3. Add the following components to the 18 μ l Control Oligo, for a volume of 25 μ l:
 - 2.5 μ l 10X Reaction Buffer (Vial 6)
 - 2.5 μ l 25mM MnCl₂ (Vial 4P)
 - 1 μ l diluted ATP (Vial 1P dilution from step 2)
 - 1 μ l PAP Enzyme (Vial 3P)
4. Mix gently and microfuge.
5. Incubate in a 37°C heat block for 15 minutes.

Continue to **First Strand cDNA Synthesis** on page 7.