

USER GUIDE

Ovation[®] RNA Amplification System V2

PART NO. 3100-12, 3100-60, 3100-A01

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I. Introduction

A. Background

The Ovation® RNA Amplification System V2 (Cat.#3100-12, 3100-60, 3100-A01) provides a fast, simple and sensitive method for preparing microgram quantities of amplified cDNA from 5 to 100 nanograms of total RNA for gene expression analysis. The Ovation RNA Amplification System V2 is powered by Ribo-SPIA® technology, an amplification process developed by NuGEN®.

The Ovation RNA Amplification System V2 (Cat. #3100-A01) is intended for automation solutions, and includes software scripts for various automation platforms: <http://www.nugeninc.com/nugen/index.cfm/products/automation-solutions/>

The amplified cDNA can be used for quantitative PCR (qPCR) analysis or for cDNA storage. It may also be used for analysis on Affymetrix® GeneChip® arrays, Agilent Gene Expression microarrays and Illumina Genome-Wide Expression BeadChips utilizing the appropriate NuGEN fragmentation and labeling modules and protocols. For details, please visit the NuGEN website.

The Ovation RNA Amplification System V2 will generally produce sufficient yield for multiple analytical applications. Prior to fragmentation and labeling, an aliquot of the amplified cDNA may be reserved for performing gene specific analyses using methods such as qPCR for validation of array findings.

B. Ribo-SPIA Technology

Ribo-SPIA technology is a three-step process that generates micrograms of cDNA from nanograms of total RNA (see Figure 1).

1. Generation of First Strand cDNA (1.5 hours)

First strand cDNA is prepared from total RNA using a unique first strand DNA/RNA chimeric primer and a reverse transcriptase. The primer has a DNA portion that hybridizes to the 5' portion of the poly(A) sequence. The resulting cDNA/mRNA hybrid molecule contains a unique RNA sequence at the 5' end of the cDNA strand.

2. Generation of a DNA/RNA Heteroduplex Double-stranded cDNA (0.75 hours)

Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthesize a second strand, which includes DNA complementary to the 5' unique sequence from the first strand chimeric primer. The result is a double-stranded cDNA with a unique DNA/RNA heteroduplex at one end.

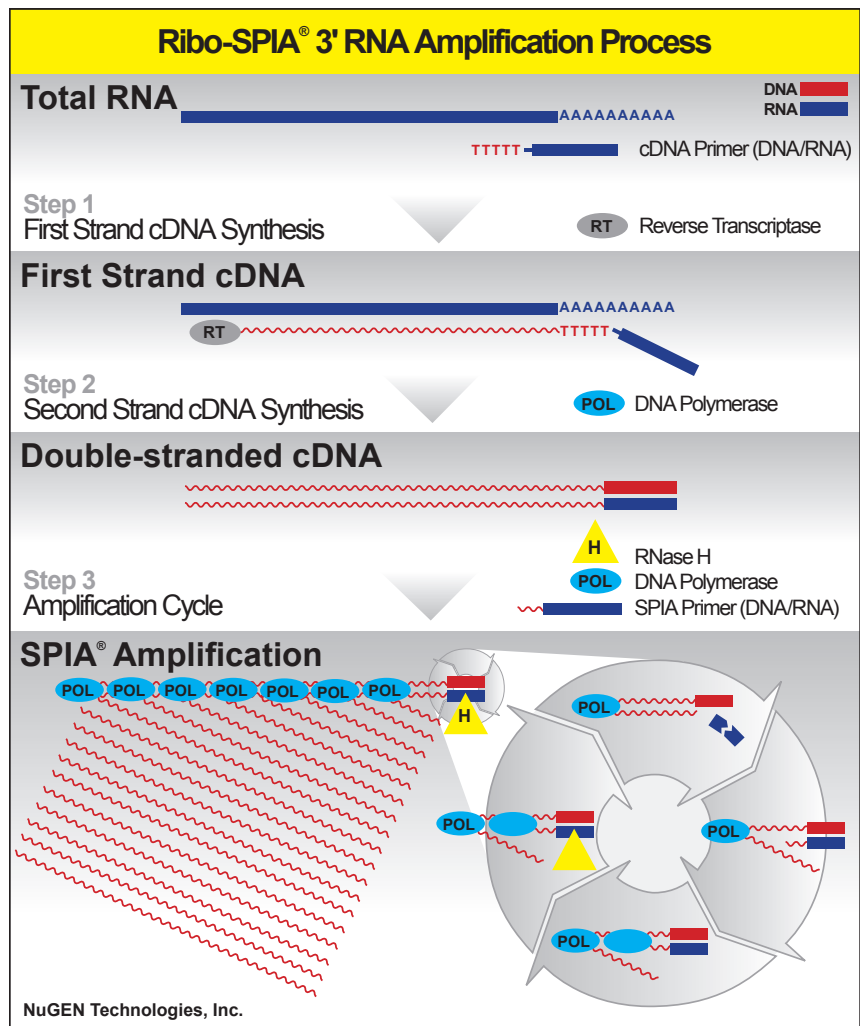
3. SPIA® Amplification (1.75 hours, including purification required for microarray applications)

SPIA amplification is a linear isothermal DNA amplification process developed by NuGEN Technologies, Inc. It uses a SPIA DNA/RNA chimeric primer, DNA polymerase and RNase H in a homogeneous isothermal assay that provides highly efficient amplification of DNA sequences. RNase H is used to degrade RNA in the DNA/RNA heteroduplex at the 5' end of the first cDNA strand. This results in the exposure of a DNA sequence that is available for hybridizing a second SPIA DNA/RNA chimeric primer. DNA polymerase then initiates replication at the 3'

I. Introduction

end of the primer, displacing the existing strand. The RNA portion at the 5' end of the newly synthesized strand is again removed by RNase H, exposing part of the unique priming site for initiation of the next round of cDNA synthesis. The process of SPIA DNA/RNA primer hybridization, DNA replication, strand displacement and RNA cleavage is repeated, resulting in rapid accumulation of cDNA with sequence complementary to the original mRNA. Up to ten thousand-fold amplification of all mRNA species is observed with 5 ng starting total RNA.

Figure 1. The Ribo-SPIA Amplification Process Used in the Ovation RNA Amplification System V2



The Ovation System family of products and methods is covered by U.S. Patent Nos. 6,692,918, 6,251,639, 6,946,251 and 7,354,717, and other issued and pending patents in the U.S. and other countries.

I. Introduction

C. Performance Specifications

The Ovation RNA Amplification System V2 produces 4 to 7 µg of cDNA starting with input amounts of total cellular RNA of 5 to 100 ng, in approximately four hours. The size of the majority of the products produced by the Ribo-SPIA amplification process is between 200 bases and 2.0 Kb.

D. Quality Control

Each Ovation RNA Amplification System V2 lot is tested to meet performance specifications.

E. Storage and Stability

The Ovation RNA Amplification System V2 is shipped on dry ice and should be unpacked immediately upon receipt.

Note: This product contains components with multiple storage temperatures.

The vials labeled First Strand Primer Mix (blue: A1) and SPIA Primer Mix (red: C1) should be removed from the shipping carton upon delivery and stored separately at -80°C.

All remaining components should be stored at -20°C in a freezer without a defrost cycle.

Kits handled and stored according to the above guidelines will perform to specifications for at least six months. NuGEN has not yet established long-term storage conditions for the Ovation RNA Amplification System V2.

F. Material Safety Data Sheet (MSDS)

An MSDS for this product is available from NuGEN Technical Service by calling 888-654-6544 or by sending an email to: techserv@nugeninc.com.



Store First Strand and SPIA
Primer Mixes at -80°C

II. Kit Components

A. Reagents and Supplies Provided

Table 1. **First Strand cDNA Reagents**

COMPONENT	3100-12 PART NUMBER	3100-60 PART NUMBER	3100-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
First Strand Primer Mix	S01138	S01100	S01212	Blue	A1 VER 2
First Strand Buffer Mix	S01150	S01154	S01206	Blue	A2 VER 4
First Strand Enzyme Mix	S01040	S01102	S01207	Blue	A3 VER 1

Table 2. **Second Strand cDNA Reagents**

COMPONENT	3100-12 PART NUMBER	3100-60 PART NUMBER	3100-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
Second Strand Buffer Mix	S01151	S01155	S01208	Yellow	B1 VER 4
Second Strand Enzyme Mix	S01042	S01104	S01209	Yellow	B2 VER 1

Table 3. **SPIA Reagents**

COMPONENT	3100-12 PART NUMBER	3100-60 PART NUMBER	3100-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
SPIA Primer Mix	S01089	S01105	S01141	Red	C1 VER 1
SPIA Buffer Mix	S01152	S01156	S01210	Red	C2 VER 6
SPIA Enzyme Mix	S01165	S01166	S01211	Red	C3 VER 5

II. Kit Components

Table 4. Additional Reagents

COMPONENT	3100-12 PART NUMBER	3100-60 PART NUMBER	3100-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
Nuclease-free Water	S01001	S01113	S01001	Green	D1

Note: The reagents in many of the Ovation and WT-Ovation™ System products are similar. However, unless the part numbers are identical, these reagents do not have exactly the same composition and, therefore, are not interchangeable. Do not exchange or replace one reagent named, for example, A1 with another A1, as it will adversely affect performance.

B. Additional Equipment, Reagents and Labware

Required Equipment

- Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
- Microcentrifuge for 0.2 mL individual and 8 X 0.2 mL strip PCR tubes (e.g., PGC #16-7009-70/72 or similar)
- 0.5 to 10 µL pipette, 2 to 20 µL pipette, 20 to 200 µL pipette, 200 to 1000 µL pipette
- Vortexer
- Thermal cycler with 0.2 mL tube heat block, heated lid and 100 µL reaction capacity
- Appropriate spectrophotometer or Nanodrop® ND-1000 UV-Vis Spectrophotometer

Required Reagents and Supplies

- Nuclease-free pipette tips
- 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
- 0.2 mL individual thin wall PCR tubes or 8 X 0.2 mL strip PCR tubes
- Appropriate spectrophotometer cuvettes
- Disposable gloves
- Kimwipes
- Ice bucket

II. Kit Components

Optional Reagents and Supplies and Equipment

- Purification options for final SPIA cDNA purification (select one option):
 - RNAClean® Beads (Beckman Coulter Genomics, Cat. #A29168)
 - MinElute® Reaction Cleanup Kit (QIAGEN, Cat. #28204)
 - QIAquick® PCR Purification Kit (QIAGEN, Cat. #28104)
 - DNA Clean & Concentrator™-25 (Zymo Research, Cat. #D4005)
- (Optional) SPRIPlate® 96R, Ring Magnet Plate (Beckman Coulter Genomics, Cat. #A29164)

Note: Necessary only when using the RNAClean Bead option for final SPIA cDNA cleanup.

- 100% ethanol, to make 80% ethanol for the cDNA column purification step (Sigma-Aldrich, Inc., Cat. #E7023)
- Agilent 2100 Bioanalyzer or other equipment for electrophoretic analysis of small RNA and cDNA samples
- Real-time PCR system
- Decontamination solutions such as RNaseZap® and DNA-OFF™

To Order:

- Beckman Coulter Genomics, www.beckmancoultergenomics.com
- Ambion Inc., www.ambion.com
- MP Biomedicals, www.mpbio.com
- New England BioLabs, www.neb.com/nebecomm/default.asp
- QIAGEN Inc., www1.qiagen.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com
- USB Corporation, www.usbweb.com
- Zymo Research, www.zymoresearch.com

III. Planning the Experiment

A. Input RNA Requirements

The most important requirement for achieving successful results with the Ovation RNA Amplification System V2 is to use total RNA of high purity and molecular weight. Use of low purity or degraded RNA may lead to low yield and spurious microarray results. To assess total RNA quality prior to using the Ovation RNA Amplification System V2, follow the guidelines below.

1. RNA Purity

RNA samples must be free of contaminating proteins and other cellular material, organic solvents (including phenol and ethanol) and salts used in many RNA isolation methods. Use of a commercially available system for preparing small amounts of RNA that does not require organic solvents is recommended. One measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The A260:A280 ratio for RNA samples of acceptable purity should be in excess of 1.8. RNA samples with lower ratios may result in low amplification yield.

2. RNA Integrity

RNA samples used with the Ovation RNA Amplification System V2 must be of high molecular weight and show little or no evidence of degradation. Amplification of degraded RNA may have a significant effect on microarray results.

Determining the integrity of RNA with very small samples limits the methods that can be used. One system NuGEN uses is the Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip®. The instrument provides a sensitive and rapid way of confirming RNA integrity prior to amplification.

3. DNase Treatment

It is generally recommended to use DNase-treated RNA for amplification with the Ovation RNA Amplification System V2. Presence of genomic DNA in the RNA sample may have adverse effects on downstream analytical platforms, such as microarrays. Significant amounts of contaminating genomic DNA also make it difficult to accurately quantitate the true RNA concentration. The RNA input quantity may, therefore, be over-estimated based on an absorbance measurement. Since it is important that RNA input be within the specified range, we recommend using DNase treatment during RNA purification.

4. Carrier Use for RNA Isolation

We strongly recommend against the use of yeast tRNA during RNA purification because it has been shown to produce cDNA product in first strand synthesis. We also advise against the use of glycogen in RNA isolation, as it inhibits reverse transcription. For the latest information regarding other carriers, contact our technical services team.

III. Planning the Experiment

B. Using RNase-free Techniques

RNase contamination by reagents and work environment will lead to experimental failure. Follow these guidelines to minimize contamination:

- Wear disposable gloves and change them frequently.
- Avoid touching surfaces or materials that could introduce RNases.
- Use reagents provided. Substitutions may introduce RNases.
- Prior to initiating protocol, clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents, such as RNaseZap and DNA-OFF.
- Use only new RNase-free pipette tips and microcentrifuge tubes.
- Use a work area specifically designated for RNA work and do not use other high copy number materials in the same area.

C. RNA Storage

RNA samples for use with the Ovation RNA Amplification System V2 must be stored at -80°C . Avoid frequent freeze/thaw cycles or RNA degradation may result.

D. Amplified cDNA Storage

The unlabeled, cDNA product produced by the Ovation RNA Amplification System V2 is preferably used immediately after preparation. If necessary, the cDNA product may be stored at -20°C , after purification and prior to fragmentation and labeling. The cDNA will be stable for a minimum of six months.

IV. Protocol

A. Overview

The Ribo-SPIA amplification process used in the Ovation RNA Amplification System V2 is performed in three stages:

1. First strand cDNA synthesis:	1.5 hour
2. Second strand cDNA synthesis:	0.75 hours
3. SPIA amplification and final cDNA purification:	1.75 hours
Total time to prepare amplified cDNA	~4 hours

The entire amplification process can easily be completed in one day. Amplified cDNA may be stored at -20°C . The purified cDNA can be prepared for analysis on Affymetrix GeneChip arrays using our Encore™ Biotin Module with a simple procedure that is completed in less than two hours.

Ovation RNA Amplification System V2 components are color coded, with each reagent vial's cap and label color linked to a specific step in the process. Performing each step requires the simple preparation and addition of a master mix to the reaction, followed by incubation.

B. Protocol Notes

- Thaw the components used in each step and immediately place them on ice.
- Always keep thawed reagents and reaction tubes on ice unless otherwise instructed.
- After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve it completely prior to use. You may gently warm the buffer mix for two minutes at room temperature followed by brief vortexing. Do not warm any enzyme or primer mixes.
- Use the water provided with the kit (green: D1) or an alternate source of nuclease-free water. We do not recommend the use of DEPC-treated water with this protocol.
- The reagent volumes recovered greatly depend on the number of batched process with each kit. Set up no fewer than three reactions at a time with the 3100-12 kit, no fewer than 10 reactions at a time with 3100-60, and no fewer than 48 reactions at a time with 3100-A01. The A01 kit has been designed for use with an automation protocol requiring large batch sizes. For more information about our automation solutions contact our technical support team.
- When placing small amounts of reagents into reaction mix, pipet up and down several times to ensure complete transfer.
- When instructed to pipet mix, gently aspirate and dispense a volume that is at least half of total volume of the reaction mix. Repeat a minimum of five times to ensure complete mixing.
- Always allow thermal cycler to reach initial incubation temperature prior to placing PCR tubes in the block.
- When working with more than one sample, excess master mix may be needed.

IV. Protocol

- Components of the Ovation RNA Amplification System V2 should not be used in place of similar components in other Ovation Amplification kits and vice versa.
- Use only fresh ethanol stocks to make 80% ethanol for washes in the amplified cDNA purification protocols (Section H and Appendix A). Make the ethanol mixes fresh as well, carefully measuring both the ethanol and water with pipettes. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the beads or column.

C. Preparing RNA Samples

Isolate total RNA with a commercial system containing no organic solvents or remove any trace organic solvents by column purification. Phenol or ethanol contamination in the RNA sample will lower cDNA yields. Use high-quality RNA for optimal performance. Verify A260:A280 ratio and 28S:18S ribosomal ratios of samples before using. Initially, a control amplification step should be performed with each batch of RNA samples until the user becomes familiar with the protocol. Adjust the total RNA sample to be amplified using nuclease-free water (green: D1) so that 5 to 100 ng of total RNA is present in 5 μ L.

D. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes, equipped with a heated lid and with a capacity of 100 μ L reaction volume. Prepare the five programs shown in Table 5, following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature at 100°C. For thermal cyclers with a fixed temperature heated lid (e.g., ABI GeneAmp® PCR 9600 and 9700 models) use the default settings (typically 100 to 105°C).



Never vortex RNA; flick tubes containing RNA to mix components

IV. Protocol

Table 5. Thermal Cycler Programming

FIRST STRAND cDNA SYNTHESIS	
Program 1 Primer Annealing	65°C – 5 min, hold at 4°C
Program 2 First Strand Synthesis	48°C – 60 min, 70°C – 15 min, hold at 4°C
SECOND STRAND cDNA SYNTHESIS	
Program 3 Second Strand Synthesis	37°C – 30 min, 75°C – 15 min, hold at 4°C
SPIA AMPLIFICATION	
Program 4 SPIA Amplification	48°C – 60 min, 95°C – 5 min, hold at 4°C

E. First Strand cDNA Synthesis Protocol

1. Obtain First Strand Buffer Mix (blue: A2) and First Strand Enzyme Mix (blue: A3) from the components stored at –20°C and the First Strand Primer Mix (blue: A1) stored at –80°C.
2. Place A3 on ice and thaw A1 and A2 at room temperature. Once thawed, place reagents on ice.
3. Add 5 µL of total RNA sample (5 to 100 ng) to a 0.2 mL PCR tube.
4. Mix contents of A1 by vortexing for 2 seconds to collect condensation, then spin in a microcentrifuge for 2 seconds.
5. Add 2 µL of A1 to the RNA aliquot.
6. Cap and flick tubes 6 to 8 times, spin tube(s) in a microcentrifuge for 2 seconds, then return to ice.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 5):
65°C – 5 min, hold at 4°C
Remove tubes from the thermal cycler and snap cool by placing tubes on ice.
8. Mix the contents of A3 by flicking tube 6 to 8 times, spin in a microcentrifuge for 2 seconds, then place on ice.
9. Mix contents of A2 by vortexing for 2 seconds, spin in a microcentrifuge for 2 seconds, then place on ice.



Never vortex RNA; flick tubes containing RNA to mix components

IV. Protocol

- Once Primer Annealing (Step 7) is complete, prepare a master mix by combining A2 and A3 in a 0.5 mL capped tube, according to the volumes shown in Table 6.

Table 6. First Strand Master Mix (volumes listed are for a single reaction)

FIRST STRAND BUFFER MIX (BLUE:A2 VER 4)	FIRST STRAND ENZYME MIX (BLUE:A3 VER 1)
12 μ L	1 μ L

! Mix by pipetting and spin down the master mix briefly. Immediately place on ice.

! The second strand reagents may be thawed and put on ice 10 minutes before the completion of First Strand Synthesis.

- Spin reaction tube(s) in a microcentrifuge for 2 seconds and add 13 μ L of the First Strand Master Mix to each tube.
- Cap and flick tubes 6 to 8 times, spin in a microcentrifuge for 2 seconds, then place on ice.
- Place tubes in a pre-warmed thermal cycler programmed to run Program 2 (First Strand Synthesis; see Table 5):
48°C – 60 min, 70°C – 15 min, hold at 4°C.
- Once the temperature has reached 4°C, remove tubes from the thermal cycler, flick the tubes 6 to 8 times, spin for 2 seconds to collect condensation, then place on ice.
- Continue immediately with Second Strand cDNA Synthesis.

F. Second Strand cDNA Synthesis Protocol

- Obtain the Second Strand Buffer (yellow: B1) and Second Strand Enzyme Mix (yellow: B2) from the components stored at –20°C.
- Mix the contents of B2 by flicking tube 6 to 8 times, spin in a microcentrifuge for 2 seconds, then place on ice.
- Thaw reagent B1 at room temperature, mix contents of B1 by vortexing for 2 seconds, spin for 2 seconds in a microcentrifuge, then place on ice.
- Make a master mix by combining B1 and B2 in a 0.5 mL capped tube, according to the volumes shown in Table 7.

Table 7. Second Strand Master Mix (volumes listed are for a single reaction)

SECOND STRAND BUFFER MIX (YELLOW:B1 VER 4)	SECOND STRAND ENZYME MIX (YELLOW:B2 VER 1)
18 μ L	2 μ L

! Mix by pipetting and spin down the master mix briefly. Immediately place on ice.

IV. Protocol



The SPIA reagents may be put on ice 15 minutes before the completion of Second Strand Synthesis.

5. Add 20 μ L of Second Strand Master Mix to each First Strand reaction tube.
6. Cap and flick tubes 6-8 times, spin in a microcentrifuge for 2 seconds, then place on ice.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 3 (Second Strand Synthesis; see Table 5):
37°C – 30 min, 75°C – 15 min, hold at 4°C.
8. Remove tubes from the thermal cycler, flick 6 to 8 times, spin in a microcentrifuge for 2 seconds to collect condensation, then place on ice.
9. Continue immediately with SPIA amplification.

G. SPIA Amplification Protocol

1. Obtain the SPIA Buffer Mix (red:C2), SPIA Enzyme Mix (red:C3) and water (green:D1) from the components stored at -20°C and the SPIA Primer Mix (red:C1) stored at -80°C .
2. Thaw reagents at room temperature.
3. Mix contents of C1 and C2 by vortexing for 2 seconds, spin in a microcentrifuge for 2 seconds, then place on ice.
4. Mix the contents of C3 by inverting gently 5 times. Make sure the enzyme is well mixed without introducing bubbles. Spin in a microcentrifuge for 2 seconds, then place on ice.
5. Place new 0.2 mL PCR tubes in a rack on ice.
6. Make a master mix by combining sequentially C2, C1, water and C3 in a 1.5 mL capped tube according to the volumes shown in Table 8.

IV. Protocol



Mix by pipetting and spin down the master mix briefly. Immediately place on ice. Use master mix immediately.

Table 8. SPIA Master Mix (volumes listed are for a single reaction)

SPIA BUFFER MIX (RED:C2 VER 6)	SPIA PRIMER MIX (RED:C1 VER 1)	WATER (GREEN:D1)	SPIA ENZYME MIX (RED:C3 VER 5)
72 μ L	4 μ L	4 μ L	40 μ L

7. Add 120 μ L of the SPIA Master Mix to the entire volume (40 μ L) of the Second Strand reaction. Mix well by pipetting up and down 6 to 8 times.
8. Cap and spin tube(s) in a microcentrifuge for 2 seconds and return tubes to ice.
9. Split the 160 μ L reaction volume into two 80 μ L volumes in new, chilled 0.2 mL PCR tubes.
10. Spin tube(s) in a microcentrifuge for 2 seconds and place on ice.
11. Place tubes in a pre-warmed thermal cycler programmed to run Program 4 (SPIA Amplification, see Table 5):
48°C – 60 min, 95°C – 5 min, hold at 4°C.
12. Remove tubes from the thermal cycler, flick the tubes 6 to 8 times, spin for 2 seconds to collect condensation, and then place on ice.
Note: If using the RNAClean Bead method for final cDNA cleanup, it is not necessary to recombine the half-reactions.
13. Recombine the 2 X 80 μ L volumes of each reaction into a single tube.
14. Vortex and spin for 2 seconds. Return samples to ice before proceeding with step H.
Note: Remove a 3 μ L aliquot of pooled SPIA cDNA for analytical purposes, if desired.

H. Purification of Amplified cDNA (Required for microarray applications)

Amplified SPIA cDNA product can be purified using various methods listed in Appendix A. Purification is required if the amplified cDNA is intended for use in subsequent labeling protocols such as the Encore Biotin Module, NuGEN Agilent Solution, or Illumina Protocol.

Selection of the optimum purification option can depend on many factors. Please contact the NuGEN Technical Support team for assistance in selecting the appropriate purification option for your application.

We recommend that the amplified SPIA cDNA product be purified prior to qPCR analysis.

I. Measuring cDNA Product Yield and Purity

1. Mix your sample by briefly vortexing and spinning prior to checking the concentration.

IV. Protocol

2. Measure the absorbance at 260, 280 and 320 nm of your amplified cDNA product. You may need to make a 1:20 dilution of the cDNA in water prior to measuring the absorbance.
3. Purity: Subtract the A320 value from both A260 and A280 values. The adjusted $(A_{260} - A_{320} / A_{280} - A_{320})$ ratio should be > 1.8 .
4. Yield: Assume 1 absorbance unit at 260 nm of single-stranded DNA = 33 $\mu\text{g/mL}$.
5. To calculate:
 $(A_{260} - A_{320} \text{ of diluted sample}) \times (\text{dilution factor}) \times 33 \text{ (concentration in } \mu\text{g/mL of a 1 absorbance unit solution)} \times 0.03 \text{ (final volume in mL)} = \text{total yield in micrograms}$
6. Alternatively, you may measure the concentration and purity of cDNA with a Nano-drop, using 1 absorbance unit at 260 nm of single-stranded DNA = 33 $\mu\text{g/mL}$ as the constant.
7. The purified cDNA may be stored at -20°C .

V. Technical Support

For Technical Support, please contact NuGEN at (U.S. only) 888.654.6544 (Toll-Free Phone) or 888.296.6544 (Toll-Free Fax) or email techserv@nugeninc.com.

In Europe contact NuGEN at +31(0)135780215 (Phone) or +31(0)135780216(Fax) or email at europe@nugeninc.com.

In all other locations, contact your NuGEN distributors Technical Support team.

VI. Appendix

A. Purification Protocols for Amplified cDNA

There are four currently supported alternatives for carrying out the final purification of SPIA cDNA. Listed alphabetically, they are: 1) Beckman Coulter Genomics RNAClean Magnetic Beads, 2) the QIAGEN MinElute Reaction Cleanup Kit, 3) the QIAGEN QIAquick PCR Purification Kit and 4) the Zymo Clean & Concentrator-25.

The procedures given below are specifically adapted for use with NuGEN products and may differ significantly from the protocols published by the manufacturers. Failure to follow the purification procedures as given below may negatively impact your results.

Beckman Coulter Genomics RNAClean Magnetic Beads

(instructions for a single reaction)

Note: Do not recombine 80 μ L half-reactions. Stop after step 12 in the SPIA Amplification Protocol on page 14 (IV. Protocol, G. SPIA Amplification Protocol) and proceed with the purification as follows:

1. Obtain and vigorously shake the RNAClean bottle to resuspend the magnetic beads.
2. Prepare an 80% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content which may reduce amplification yield.
3. Add 144 μ L of resuspended RNAClean beads (1.8 times the sample volume) to one set of the paired 80 μ L SPIA half-reactions.
4. Mix the sample and beads thoroughly by pipetting up and down 10 times.

Note: If using a 96-well plate format with both half-reactions on the same plate, it will be necessary to transfer the sample/bead mixture to a fresh plate at this point.

5. Incubate sample/bead mixture at room temperature for 5 minutes.
6. Place the first set of samples (containing beads) on the SPRIPlate 96R Magnet Plate for 10 minutes or until the solution appears clear.
7. After 5 minutes of the 10 minute incubation in step 6 have elapsed, add 144 μ L of resuspended RNAClean beads (1.8 times the sample volume) to second set of half-reactions containing the remaining 80 μ L cDNA samples. Incubate samples at room temperature for 5 minutes.
8. Using a multi-channel pipette, remove and discard the supernatant from first set of samples (on magnet). Do not disturb the ring of magnetic beads.



Best results can be obtained by using fresh 80% ethanol in the wash step. Lower percent ethanol mixes will reduce recovery.

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- Using a multi-channel pipette, add the sample/bead mix from the second set of half-reactions (prepared in step 7) to the appropriate tubes or wells containing the beads from the first half-reaction while it is still placed on the magnet. Add slowly as to not disturb the bead ring already in each well.

Note: Here the potential for sample cross-contamination is high; take care to combine the correct half-reactions.

- Wait for an additional 10 minutes or until the solution appears clear.

Note: If the surface tension traps a small halo of beads suspended at the liquid surface, use a multi-channel pipette and gently pipet 10-15 μL up and down at the liquid surface to break the tension and allow the beads to sink to the magnet ring.

- Using a multi-channel pipette, remove and discard the supernatant. Do not disturb the ring of magnetic beads.
- With the samples still on the magnet plate, add 200 μL of freshly prepared 80% ethanol to each well of the reaction plate and incubate for 30 seconds or until the solution clears. Add slowly as to not disturb the separated magnetic beads.
- Using a multi-channel pipette, remove and discard the ethanol.
- Repeat the 80% ethanol wash once more. Ensure as much ethanol as possible is removed from the plate.
- Remove the reaction tubes or plate from the magnet and air dry the reaction plate on bench top for no more than 2 minutes. If the beads dry too long, they are difficult to resuspend.
- With the plate on bench top, add 30 μL of room temperature nuclease-free water to each well. Holding the plate firmly, very carefully vortex for 30 seconds or use a plate shaker set to medium speed. Ensure the beads are fully resuspended, vortex longer if necessary. Alternatively, the beads may be resuspended by repeated pipetting.
- Replace reaction tubes or plate on the plate magnet. Allow the beads to separate for 5 minutes or until the solution clears.
- Using a multi-channel pipette, remove the eluted sample and place into a fresh reaction tube or plate. There should be approximately 30 μL of purified cDNA.

Note: Small amounts of magnetic bead carry-over may interfere with sample quantitation. Take care to minimize bead carry-over.
- Proceed to Measuring cDNA Product Yield and Purity or store purified cDNA at -20°C .



Use nuclease-free water at room temperature to elute sample.

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100% ethanol must be added to the QIAGEN Buffer PE upon first use. Failure to do so will result in low amplification.



Best results can be obtained by using fresh 80% ethanol in the wash step. Lower percent ethanol mixes will reduce recovery.



Residual ethanol from the wash buffers will not be completely removed unless the flow-through is discarded before this additional centrifugation.



Use nuclease-free water at room temperature to elute sample.

Ensure that the water is dispensed directly onto the membrane for complete elution of bound cDNA.

QIAGEN MinElute Spin Column

(instructions for a single full reaction, 2 columns are required per reaction)

1. Ensure that 100% ethanol has been added to Buffer PE as described in the QIAGEN MinElute Handbook. Failure to add ethanol to this buffer will result in low amplification yield.
2. Prepare an 80% ethanol wash solution. It is critical that this solution be prepared fresh on the same day as the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content which may reduce amplification yield.
3. Add 600 μL of Buffer ERC to a labeled 1.5 mL tube for each amplification reaction.
4. Transfer each full reaction (160 μL) into a tube containing the Buffer ERC.
5. Vortex for 5 seconds and spin down briefly.
6. Obtain and label two QIAGEN MinElute Spin Columns for each amplification reaction and place them into collection tubes.
7. Load 380 μL (one half) of each reaction/buffer mix onto each of the two labeled QIAGEN MinElute Spin Columns.
8. Centrifuge columns in the collection tube for 1 minute at $>10,000 \times g$ in a microcentrifuge. Discard flow-through and replace the QIAGEN MinElute Spin Column in the same collection tube.
9. Wash sample by adding 500 μL of Buffer PE (prepared according to manufacturer's recommendations). Centrifuge column in the collection tube for 1 minute at $>10,000 \times g$. Discard flow-through.
10. Add 500 μL of the room temperature 80% ethanol prepared in Step 1 above.
Note: Use fresh 80% ethanol.
11. Centrifuge column in the collection tube for 1 minute at $>10,000 \times g$. Discard flow-through.
12. Place the column back in the same collection tube and spin for an additional 2 minutes at $>10,000 \times g$.
13. Blot the column tip onto a filter paper to remove any residual wash buffer from the tip of the column.
Note: Blot column tip prior to transferring it to a new tube to prevent any wash buffer transferring to the eluted sample.
14. Place the MinElute Column in a clean, labeled 1.5 mL microcentrifuge tube.
15. Add 15 μL of room temperature nuclease-free water (green: D1) from the kit to the center of each column. **Do not use cold water!**
16. Let columns stand for 1 minute at room temperature.

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17. Centrifuge column and microcentrifuge tube for 1 minute at $>10,000 \times g$.
18. Pool eluates from each half-reaction and measure the volume recovered. There should be approximately 25 to 30 μL of purified cDNA.
19. Mix sample by vortexing, then spin briefly.
20. Proceed to Measuring cDNA Product Yield and Purity or store purified cDNA at -20°C .

QIAGEN QIAquick PCR Purification Kit, Cat. #28104

(instructions for a single reaction)

1. Into a clean 1.5 mL tube, add 800 μL of PB buffer from the QIAGEN system.
2. Add the 160 μL of amplified cDNA product to the tube.
3. Vortex for 5 seconds and spin down for 2 seconds.
4. Obtain one QIAquick spin column and insert into a collection tube.
5. Load 480 μL of sample onto the column.
6. Centrifuge column in a collection tube for 1 minute at 13,000 rpm ($\sim 17,900 \times g$). Discard flow-through. Place the column back in the same collection tube.
7. Load remaining 480 μL onto the same column. Centrifuge column in collection tube for 1 minute at 13,000 rpm. Discard flow-through.
8. Place the column back in the same collection tube. Add 700 μL of 80% ethanol.
Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
9. Centrifuge the column for 1 minute at 13,000 rpm. Discard flow-through.
10. Repeat steps 9 and 10 once.
11. To remove remaining liquid, centrifuge column for 1 additional minute at 13,000 rpm.
12. Remove the column from the centrifuge. Discard flow-through with the collection tube.
13. Blot the tip of the column onto a filter paper in order to remove any residual wash buffer from the tip of the column.
Note: Blot column tip prior to transferring it to a new tube to prevent any wash buffer transferring to the eluted sample.
14. Place the column in clean 2.0 mL collection tube, appropriately labeled.
15. Add 30 μL of nuclease-free water (green: D1) to the center of each column. **Do not use cold water!**
16. Let columns stand for 5 minutes at room temperature to elute purified cDNA.



Best results can be obtained by using fresh 80% ethanol in the wash step. Lower percent ethanol mixes will reduce recovery.



Use nuclease-free water at room temperature to elute sample.

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17. Centrifuge at 13,000 rpm for 1 minute to collect sample. There should be approximately 30 μL of purified cDNA.
18. Mix sample by vortexing, then spin briefly.
19. Proceed to Measuring cDNA Product Yield and Purity or store purified cDNA at -20°C .

Zymo Research DNA Clean & Concentrator™-25

(instructions for a single reaction)

1. Into a clean 1.5 mL tube add 320 μL of DNA Binding Buffer.
2. Add 160 μL of amplified SPIA cDNA product.
3. Vortex and spin down briefly.
4. Obtain one Zymo-Spin™ II Column and place it into a collection tube.
5. Load the entire volume of sample (480 μL) onto the Zymo-Spin II Column.
6. Centrifuge column in the collection tube for 10 seconds at $>10,000 \times g$ in a microcentrifuge.

Note: Be sure to wait until rotor achieves desired speed before starting timer for spins less than 1 minute in this procedure.

7. Discard flow-through. Place the Zymo-Spin II Column back in the same collection tube.
8. Wash sample by adding 200 μL of room temperature 80% ethanol. Do not use the Wash Buffer provided with the Zymo columns.

Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.

9. Centrifuge column in the collection tube for 10 seconds at $>10,000 \times g$ in a microcentrifuge. Discard flow-through.
10. Add 200 μL of room temperature 80% ethanol.
11. Centrifuge column in the collection tube for 90 seconds at $>10,000 \times g$ in a microcentrifuge. Discard flow-through.
12. Blot the column tip onto a filter paper to remove any residual wash buffer from the tip of the column.

Note: Blot column tip prior to transferring it to a new tube to prevent any wash buffer transferring to the eluted sample.

13. Place the Zymo-Spin II Column in a clean 1.5 mL microcentrifuge tube.
14. Add 30 μL of room temperature nuclease-free water (green: D1) from the kit to the center of each Zymo-Spin II column. **Do not use cold water!**
15. Let columns stand for 1 minute at room temperature.



Best results can be obtained by using fresh 80% ethanol in the wash step. Lower percent ethanol mixes will reduce recovery.



Use nuclease-free water at room temperature to elute sample.

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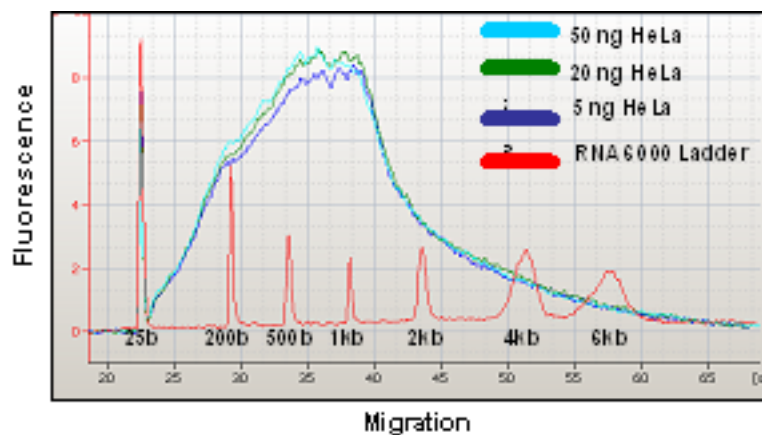
16. Centrifuge column and microcentrifuge tube for 30 seconds at $>10,000 \times g$ in a microcentrifuge.
17. Collect sample. There should be approximately 30 μL of purified cDNA.
18. Mix sample by vortexing, then spin briefly.
19. Proceed to Measuring cDNA Product Yield and Purity or store purified cDNA at -20°C .

B. Quality Control of Amplified cDNA Product

An Agilent Bioanalyzer may be used to assess the amplified cDNA size distribution. The size distribution of the resulting amplified SPIA cDNA is between 200 bases and 2 Kb in length. A shift in size to a much smaller size may be indicative of significantly degraded input RNA or failure of amplification. The shape of the Bioanalyzer curve will vary depending on the tissue origin of the RNA sample. We recommend 100 ng of each cDNA sample be analyzed on an RNA 6000 Nano LabChip (Agilent Cat.#5065-4476) using the Eukaryotic Total RNA Nano program following the manufacturer's instructions. If the amplified cDNA is used with the Encore Biotin Module, the fragmented and labeled cDNA may also be run on the Bioanalyzer to assess success of fragmentation. Note that we do not recommend determining the concentration of amplified cDNA using the Bioanalyzer.

Examples of Bioanalyzer traces from unfragmented SPIA cDNA products are shown in Figure 2 below.

Figure 2. Bioanalyzer trace of amplified cDNA product from three different input amounts of HeLa total RNA: The size of the RNA 6000 ladder (Ambion Inc., Cat. #7152) is indicated above each peak.



C. Performing Quantitative PCR on Amplified cDNA

The amplified cDNA generated from the Ovation RNA Amplification System V2 may be used directly in real time quantitative PCR reactions or first purified in order to quantitate and allow for mass normalization of input for qPCR reactions. Amplified cDNA produced with the kit has been successfully used as template for qPCR systems including TaqMan® and SYBR® Green. Note that RT-PCR master mixes containing the enzyme Uracil N-Glycosylase (UNG) are not compatible with the Ovation System. We can recommend the following reagents for qPCR:

- TaqMan: FastStart TaqMan Probe Master (Rox) (Roche, Cat. #04 673 476 001)
- TaqMan: ABsolute qPCR Mix plus ROX (ABgene, Cat. #AB-1136/B)
- TaqMan: Fast Universal PCR Master Mix 2x (Applied Biosystems, Cat. #4352042)
- SYBR: QuantiTect™ SYBR Green PCR Kit (QIAGEN, Cat. #204143)
- SYBR: iQ SYBR Green Supermix (BioRad, Cat. #170-8880)
- SYBR: FastStart SYBR Green Master (ROX) (Roche, Cat. #04 673 514 001)

Recommendations to Achieve Optimal Results

Using unpurified SPIA cDNA as input into qPCR reactions: It is recommended that the unpurified cDNA be diluted before performing qPCR since inhibition has been observed with undiluted product. Typically the amplified product is diluted 1:10 in nuclease-free water or in a buffer specified by the qPCR system manufacturer. 2 µL is then added to a 25 µL qPCR reaction. Depending on the abundance of the transcripts you are measuring you may wish to dilute the cDNA further than 1:10.

Using purified SPIA cDNA as input: Generally, purified cDNA is obtained at a concentration of 150 to 250 ng/µL. It will be necessary to dilute the cDNA to an appropriate level for use in qPCR reactions. Typically, inputs in the range from 5 to 10ng in a 25 µL qPCR reaction will be appropriate, however it may be necessary to empirically optimize the input for low or high copy-number transcripts.

Primer design: The amplified cDNA produced using the Ovation RNA Amplification System V2 is generated from the 3' end of the mRNA. For best results, primer sets used in qPCR procedures using amplified cDNA should be within 1.5 Kb from the poly(A) tail.

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Figure 3. TaqMan analysis of GAPDH using amplified and non-amplified cDNA

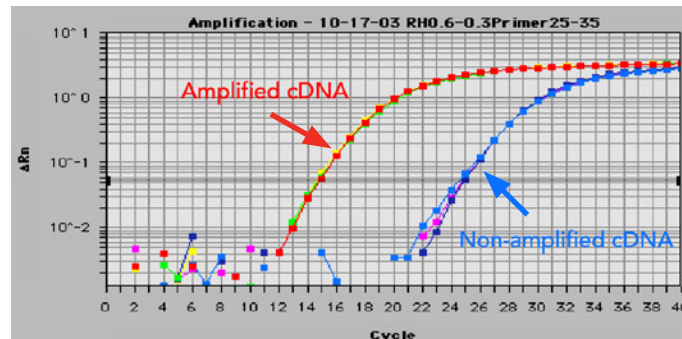


Figure 3 shows the results of GAPDH quantitative RT-PCR (qPCR) comparing amplified SPIA cDNA (red) to the second strand cDNA prior to SPIA amplification (blue) from a 20 ng sample of Universal Human Reference RNA processed with the Ovation System. The qPCR data indicate an amplification efficiency of greater than 10^3 . The qPCR (TaqMan) reactions were carried out in triplicate on an ABI 7700 system using the Absolute QPCR ROX Mix (ABgene Cat. #AB-1136/b), and a probe/primer set for GAPDH located approximately 1 Kb from the poly(A) site.

D. DNase Treatment of RNA

DNase Treatment During Purification: Using the QIAGEN RNase-Free DNase Set and the RNeasy Mini RNA Purification Kit

1. Homogenize sample in RLT buffer including β -mercaptoethanol according to the type of sample as described in the RNeasy Mini Kit protocol.
2. Add 1X volume of 70% ethanol to the homogenized lysate, pipet up and down to mix sample well. Do not centrifuge.
3. Place an RNeasy mini column in a 2 mL collection tube.
4. Apply the sample (up to 700 μ L), including any precipitate that may have formed, to the column.
5. Close the tube gently and centrifuge for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
6. For volumes greater than 700 μ L, load aliquots onto the RNeasy column successively and centrifuge as before.
7. Add 350 μ L Buffer RW1 into the RNeasy mini column to wash and centrifuge for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
8. Add 10 μ L DNase I to 70 μ L Buffer RDD. Gently invert the tube to mix.

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Note: Other DNase I enzymes we can recommend to use in this step are the Shrimp DNase (recombinant) from USB Corp. (use 10 μ L), or the DNase I (RNase-free) from New England BioLabs (use 10 μ L). See the Additional Reagents section of this user guide for ordering information.

9. Pipet the DNase I incubation mix (80 μ L) directly onto the membrane inside the RNeasy mini column. Incubate on the bench top (~ 25°C) for 15 min.
10. Add 350 μ L Buffer RW1 into the RNeasy mini column and centrifuge for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash. Discard the flow-through.
11. Transfer the RNeasy column to a fresh 2 mL collection tube. Add 500 μ L Buffer RPE (with the added ethanol) to the RNeasy column.
12. Close the tube gently and centrifuge for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
13. Add another 500 μ L Buffer RPE to the RNeasy column.
14. Close the tube gently and centrifuge for 2 minutes at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
15. Transfer the RNeasy column to a new 1.5 mL collection tube.
16. Pipet 30–50 μ L RNase-free water directly onto the RNeasy membrane.
17. Close the tube gently and centrifuge for 1 minute at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute.
18. If yields of greater than 30 μ g are expected, repeat elution step and collect in the same collection tube.

DNase Treatment of RNA Post-Purification: Using RNase-free DNase and either the RNA Clean & Concentrator™-5 Columns or the RNeasy MinElute Columns

Note: If you are unable to quantify your RNA because the sample is contaminated with DNA, we recommend DNase treatment followed by purification.

1. On ice, mix together 2.5 μ L 10X DNase I Reaction buffer (Roche Cat. #04716728001 or USB PN 78316) with 1 μ L rDNase (10 Units Roche Cat. #04716728001 or 2 Units USB PN 78311).
2. Add RNA sample (up to 500 ng) and add RNase-free water (D1, green cap) to bring the final volume to 25 μ L.
3. Incubate at 25°C for 15 minutes followed by 37°C for 15 minutes and return to ice.
4. After the DNase treatment, the sample must be purified. We recommend either of the two purification procedures below:



Best results can be obtained by using fresh 80% ethanol in the wash step. Lower percent ethanol mixes will reduce recovery.

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Use nuclease-free water at room temperature to elute sample.

Purification with RNA Clean & Concentrator-5 (Zymo Research, Cat. #R1015)

1. Add 4 volumes (100 μ L) of RNA binding buffer to the sample.
2. Obtain one RNA Clean & Concentrator-5 column and apply sample to column.
3. Spin column for 30 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
4. Add 200 μ L wash buffer (with ethanol added as per vendor's specifications).
5. After closing the column spin for 30 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
6. Add 200 μ L fresh 80% ethanol, close cap, spin for 30 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
7. Place the RNA Clean & Concentrator-5 column in a fresh 1.5 mL collection tube.
8. Add 10 μ L nuclease-free water (green: D1) directly to the center of the filter in the tube and close the cap. **Do not use cold water!**
9. Spin for 1 minute at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to collect the purified RNA.

Purification with QIAGEN RNeasy MinElute Cleanup Columns (QIAGEN, Cat. #74204)

1. Add 80 μ L ice-cold RNase-free water (D1, green cap) to the sample on ice.
2. Add 350 μ L Buffer RLT and mix by pipetting.
3. Add 250 μ L 96 to 100% ethanol and mix thoroughly by pipetting.
4. Place an RNeasy MinElute Spin Column into a 2 mL collection tube (one column per sample) and apply the 700 μ L sample to the column.
5. After closing the column, spin for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
6. Place the RNeasy MinElute Spin Column into a fresh 2 mL collection tube. Add 500 μ L Buffer RPE to the column and close the tube. Spin for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through, keeping the same collection tube.
7. Add 500 μ L 80% ethanol to the RNeasy MinElute Spin Column and close the tube.
8. Spin for 2 minutes at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
9. Place the RNeasy MinElute Spin Column in a fresh 2 mL collection tube and place in the microcentrifuge with the cap open. Spin for 5 minutes at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
10. Place the RNeasy MinElute Spin Column in a fresh 1.5 mL collection tube.



Best results can be obtained by using fresh 80% ethanol in the wash step. Lower percent ethanol mixes will reduce recovery.

11. Add 14 μ L nuclease-free water (D1, green cap) directly to the center of the filter in the tube and close the cap. **Do not use cold water!**
12. Spin for 1 minute at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to collect the purified RNA.

E. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the Ovation RNA Amplification System V2?

The Ovation RNA Amplification System V2 provides all necessary buffers, primers and enzymes for first strand synthesis, second strand synthesis and SPIA amplification.

Q2. What equipment is required or will be useful?

Required equipment includes a microcentrifuge, pipettes, vortexer and a thermal cycler. A UV/Vis spectrophotometer and an Agilent Bioanalyzer will be useful and a real time PCR system may be used for quality control.

Q3. What additional consumables are required or useful for the Ovation RNA Amplification System V2?

For the SPIA cDNA purification step, purification columns or beads are required. Please refer to page 17 for purification options, as well as page 5 for other consumables.

Q4. Do I need to use high-quality total RNA?

Use of lower quality RNA may result in poor performance. One approach to determining RNA quality is the Agilent Bioanalyzer's RNA Integrity Number (RIN). Clean RNA with a RIN score of greater than 7 should amplify well.

Q5. How much total RNA input do I need for amplification?

We recommend staying within the specified range of 5 to 100 ng of total RNA starting material. We often suggest 20 ng input as an appropriate starting point. Input greater than 100 ng may adversely affect amplification.

Q6. What is the dynamic range of input mRNA copies that are linearly amplified?

Our studies demonstrate linear amplification of transcripts present at 100 to 100,000,000 copies in a 5 ng sample of HeLa RNA. Different input amounts of RNA from different tissues may affect the lower limit of detection.

Q7. Can I use a total RNA input of less than 5 ng?

The Ovation RNA Amplification System V2 has been validated for total RNA input amounts of 5 to 100 ng. Using input quantities outside the recommended range will affect quality and quantity of resulting cDNA.

Q8. Can I omit quantitation of input RNA?

We do not recommend omitting quantitation of input RNA. However, if sample size constraints absolutely prohibit quantitation, you may save a small aliquot (2 μ L) following the second strand cDNA synthesis in order to retrospectively estimate the starting input RNA concentration by quantitative RT-PCR. (Refer to Technical Note 1 for more detail on this procedure.)

Q9. Can I use mRNA instead of total RNA as starting material?

Purified poly(A)⁺ RNA has been successfully used as input to the Ovation RNA Amplification System V2. It may be necessary to reduce the input of mRNA to a level comparable to the mRNA present in 5 ng to 100 ng of total RNA.

Q10. Is the Ovation RNA Amplification System V2 3' biased?

Since the Ovation RNA Amplification System V2 primes the poly(A) tail of transcripts, it is 3' biased, resulting in coverage to a range of 1.5 Kb from the 3' poly(A) tail.

Q11. How much cDNA yield can I expect from one reaction of Ovation amplification?

For a standard reaction the expected yield is 4–7 µg of amplified cDNA.

Q12. Is the cDNA yield dependent upon the quantity of input total RNA?

The total yield of cDNA is not directly dependent upon input RNA amount due to upper limit constraints on cDNA production in the reaction.

Q13. What is the amplification efficiency of the Ovation RNA Amplification System V2?

Based on qPCR results of a collection of housekeeping genes, amplification efficiency ranges from 1,000- to 10,000-fold or higher depending on the input amount.

Q14. What is the size range of cDNA generated by the Ovation RNA Amplification System V2?

As measured with an Agilent Bioanalyzer, the majority of amplified SPIA cDNA is between 200 bases and 2 Kb in length. After fragmentation, 80% of product falls below 200 bases with an average peak at 85 bases.

Q15. Has NuGEN performed reproducibility studies on the Ovation RNA Amplification System V2?

Our studies have included sample-to-sample, lot-to-lot, and operator-to-operator reproducibility.

Q16. Can the Ovation RNA Amplification System V2 kits be used for amplification of DNA?

The Ovation RNA Amplification System V2 is designed to amplify mRNA, not DNA.

Q17. Can I use the Ovation RNA Amplification System V2 on prokaryotic RNA samples?

The Ovation RNA Amplification System V2 relies on the presence of a poly(A) tail for priming. Therefore, it will not amplify most prokaryotic RNA.

Q18. Are there any tissues that will not work with the Ovation RNA Amplification System V2?

We have not encountered any good-quality, clean RNA samples containing poly(A) + RNA that will not work with the Ovation RNA Amplification System V2.

Q19. Does the Ovation RNA Amplification System V2 generate product in a no-RNA reaction?

As with most amplification systems, non-specific product is generated using the Ovation RNA Amplification System V2 in the absence of input template. Array and qPCR analysis show these amplification products to be non-specific.

Q20. How many rounds of amplification are performed with the Ovation RNA Amplification System V2?

The Ovation RNA Amplification System V2 performs a single round of amplification in less than four hours. Our products are designed to provide high sensitivity through robust amplification without necessitating a second round of amplification.

Q21. Do I need to order specific primers for the amplification?

The chimeric DNA/RNA primers provided with the Ovation RNA Amplification System V2 kits are universal, and there is no need for additional primers.

Q22. Do I have to use your DNA/RNA primers?

The Ovation RNA Amplification System V2 was designed and optimized to work with the primers provided. The use of other primers with the Ovation RNA Amplification System V2 is not supported.

Q23. Why is the purification of cDNA step optional?

cDNA purification is required for microarray applications. cDNA for fragmentation and labeling reactions and array analysis must be purified to allow for accurate quantitation of cDNA as well as ensure proper performance of the Encore Biotin Module.

If the amplified cDNA is to be used for qPCR analysis alone, then purification is optional. Purification, however, will enable mass normalization of cDNA input into the qPCR reaction, potentially reducing variability.

Q24. Can I fragment the cDNA?

Yes, cDNA fragmentation and labeling can be achieved using the NuGEN Encore Biotin Module to prepare target for analysis on Affymetrix GeneChip arrays.

Q25. Can I use the Ovation RNA Amplification System V2 for archiving cDNA?

cDNA may be stored following purification for at least six months. Longer term stability tests are in progress.

Q26. What are the recommended storage conditions for amplified cDNA?

The amplified cDNA may be stored at -20°C . Ensure the vials are well sealed and avoid multiple freeze thaw cycles.

Q27. What types of arrays work with the cDNA generated with the Ovation RNA Amplification System V2?

The cDNA generated with the Ovation RNA Amplification System V2 has been validated on Affymetrix GeneChip arrays, Agilent Dual-Mode Gene Expression Arrays and Illumina Whole Genome Expression BeadChips when used in conjunction with the appropriate labeling Module or protocol. Contact NuGEN Technical Services for more information.

Note: Encore Biotin Module is used solely with Affymetrix arrays.

Q28. For quantitative real time PCR applications, what is the optimal distance from the 3' poly(A) tail for design of primer probe sets?

Due to the amplification mechanism of the Ovation RNA Amplification System V2, we recommend primer/probe sets to be designed within the first 1.5 Kb from the poly(A) tail.

Q29. What are the incubation temperatures for each step?

First strand primer annealing = 65°C

First strand synthesis = 48°C

Second strand synthesis = 37°C

SPIA amplification = 48°C

Q30. Where can I safely stop in the protocol?

You may safely stop after the SPIA Amplification step or final SPIA cDNA purification and store the cDNA at -20°C.

Q31. How can I ensure good yields at the cDNA purification step?

In order to maximize yields, we recommend the following:

- Do NOT use cold water for the elution step. Use the D1 nuclease-free water included in the Ovation RNA Amplification System V2 kit at room temperature.
- Do NOT spin the columns at an incorrect speed. Strictly adhere to the guidelines in the User Guide.
- Use a fresh dilution of Ethanol from a fresh stock for any washing steps.
- Vortex the eluted cDNA sample prior to measuring the O.D.

Q32. Should I purify the cDNA before determining the concentration?

Yes, the primers and reagents present in the amplified cDNA will interfere with accurate quantitation. Other details on measuring the concentration of cDNA are included in the user guide.

Q33. Do you recommend any RNA preparation methods?

We do not specifically require one method of RNA preparation, as long as the method yields high-quality, non-degraded RNA that is free of organic solvents and contaminants.

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Q34. What carriers do you recommend?

In general, we discourage the use of carriers as they can interfere with amplification, and have not been shown to be necessary at the RNA concentrations used in this application.



NuGEN Technologies, Inc.

Headquarters USA

201 Industrial Road, Suite 310
San Carlos, CA 94070 USA
Toll Free Tel: 888.654.6544
Toll Free Fax: 888.296.6544
custserv@nugeninc.com
techserv@nugeninc.com

Europe

P.O. Box 149,
6680 AC Bommel,
The Netherlands
Tel: +31-13-5780215
Fax: +31-13-5780216
europe@nugeninc.com

For our international distributors contact information, visit our website

www.nugeninc.com