WHOLE TRANSCRIPTOME WT-OVATION™ SYSTEM

WT-Ovation™
RNA Amplification System

VERSION 1.0

Catalog # 2210-24

USER GUIDE

NuGEN™ *NuGEN Technologies, Inc.* www.nugeninc.com

imagine more from less™

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I. Introduction

A. Background

The WT-Ovation™ RNA Amplification System provides a fast and simple method for preparing amplified cDNA from total RNA for gene expression analysis. Amplification is initiated at the 3′ end as well as randomly throughout the whole transcriptome in the sample. The amplified product of the WT-Ovation System is optimized for the detection of low, medium and high abundance gene transcripts using real-time quantitative PCR (qPCR).

The WT-Ovation System is powered by Ribo-SPIA® technology, a rapid, simple and sensitive RNA amplification process developed by NuGEN®. Using Ribo-SPIA technology and starting with 5 to 50 ng total RNA, microgram quantities of cDNA can be prepared in approximately 4 hours. The amplified product is single-stranded cDNA in the antisense (opposite sense) direction of the mRNA starting material. Amplified cDNA product is ready for qPCR and does not require purification.

The WT-Ovation System provides optimized reagent mixes and a protocol to process 24 (cat. # 2210-24) RNA samples. Control RNA is not provided with the WT-Ovation System but is recommended when using the system.

B. Ribo-SPIA® Technology

Ribo-SPIA technology is a three-step process that generates amplified cDNA from as little as 5 nanograms of total RNA (see Figure 1).

1. **Generation of First Strand cDNA** (65 minutes)

   First strand cDNA is prepared from total RNA using a unique first strand DNA/RNA chimeric primer mix and reverse transcriptase (RT). The primers have a DNA portion that hybridizes either to the 5′ portion of the poly (A) sequence or randomly across the transcript. RT extends the 3’ DNA end of each primer generating first strand cDNA. The resulting cDNA/mRNA hybrid molecule contains a unique RNA sequence at the 5′ end of the cDNA strand.

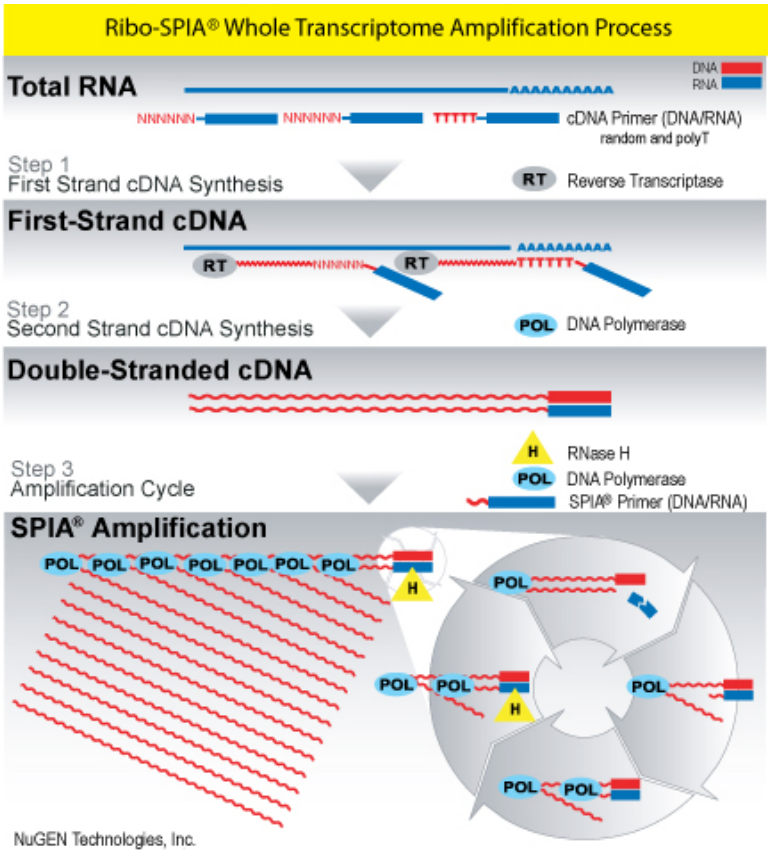
2. **Generation of a DNA/RNA Heteroduplex Double Strand cDNA** (80 minutes)

   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 primers. The result is a double stranded cDNA with a unique DNA/RNA heteroduplex at one end.

3. **SPIA® Amplification** (80 minutes)

   SPIA amplification is a linear isothermal DNA amplification process developed by NuGEN. 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 binding a second SPIA DNA/RNA chimeric primer. DNA polymerase then initiates replication at the 3′ end of the primer, displacing the existing forward 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 binding, DNA replication, strand displacement and RNA cleavage is repeated, resulting in rapid accumulation of cDNA with sequence complementary to the original mRNA. An average amplification of 1,500-fold is observed with 5 ng starting total RNA.

Figure 1. The WT- Ribo-SPIA® RNA Amplification Process used in the WT-Ovation System



C. Performance Specifications

The WT-Ovation RNA Amplification System synthesizes microgram quantities of amplified cDNA starting with total cellular RNA input amounts of 5 to 50 ng. In approximately 4 hours, the WT-Ovation System can produce 1.5 to 4 µg of cDNA in a 40 µL volume ready for qPCR or other analytical tests. The size of the majority of the cDNA products produced by the Ribo-SPIA amplification process is between 100 bases and 1.5 Kb. We recommend using the system with a minimum of 8 reactions at a time. Using the system with smaller numbers of reactions per batch may result in fewer total reactions (< 24).

D. Quality Control

Each WT-Ovation System lot is tested to meet performance specifications.

E. Storage and Stability

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

The WT-Ovation System is shipped on dry ice and should be unpacked immediately upon receipt. 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 established long-term storage conditions for WT-Ovation Systems.

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.

II. Kit Components

G. Reagents and Supplies Provided

Table 1. First Strand cDNA Reagents

Component	Part Number	Vial Cap	Vial Number
First Strand Primer Mix	S01130	Blue	A1 ver2
First Strand Buffer Mix	S01131	Blue	A2 ver2
First Strand Enzyme Mix	S01140	Blue	A3 ver1

Table 2. Second Strand cDNA Reagents

Component	Part Number	Vial Cap	Vial Number
Second Strand Buffer Mix	S01132	Yellow	**B1 ver3**
Second Strand Enzyme Mix	S01126	Yellow	**B2 ver2**
Reaction Enhancement Enzyme Mix	S01119	Yellow	**B3 ver1**

Table 3. SPIA® Reagents

Component	Part Number	Vial Cap	Vial Number
SPIA Primer Mix	S01133	Red	C1 ver3
SPIA Buffer Mix	S01134	Red	C2 ver4
SPIA Enzyme Mix	S01199	Red	C3 ver5

Table 4. Additional Reagents

Component	Part Number	Vial Cap	Vial Number
Nuclease-free Water	S01001	Green	D1

H. Additional Equipment, Reagents and Labware

1. 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, and 200 to 1000 µL pipette
- Vortexer
- Thermal cycler with 0.2 mL tube heat block, heated lid, and 40 µL reaction capacity

2. Optional Reagents and Supplies and Equipment

- Agilent 2100 bioanalyzer or materials and equipment for electrophoretic analysis of RNA
- Real Time PCR system
- Appropriate spectrophotometer and cuvettes, or Nanodrop® ND-1000 UV-Vis Spectrophotometer
- Decontamination solutions such as RNaseZap® (Ambion, Cat.# AM9780) and DNA-OFF™ (MP Biomedicals, Cat.# QD0500)
- 100% Ethanol (Sigma-Aldrich, cat. # E7023)
- Purification options for final SPIA cDNA purification (select one option):
  - RNAClean Beads (Agencourt, 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 (Agencourt, Cat. # A29164)

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

To Order:

- Agencourt Bioscience Corporation, (800) 361-7780, www.agencourt.com
- Ambion Inc., (800)888-8804, www.ambion.com
- MP Biomedicals, (800) 854-0530, www.mpbio.com
- New England BioLabs, (800) 632-5227, www.neb.com/nebecomm/default.asp
- QIAGEN Inc., (800) 426-8157, www1.qiagen.com
- Sigma-Aldrich, Inc., (800) 325-3010, www.sigmaaldrich.com
- USB Corporation, (800) 321-9322, www.usbweb.com
- Zymo Research, (888) 882-9682, www.zymoresearch.com

3. Labware

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

III. Planning the Experiment

I. Input RNA Requirements: 5 to 50 ng

An important requirement for achieving successful results with the WT-Ovation RNA Amplification System is to use RNA of high quality. Use of low quality RNA may lead to low yield and poor qPCR results. To assess RNA quality prior to using the WT-Ovation System, 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. If a method such as Trizol is used, we recommend using good quality Trizol and column purification after isolation, if possible. 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 WT-Ovation System must be of high molecular weight and show little or no evidence of degradation for best results. Determining the integrity of RNA with very small samples limits the methods that can be used. NuGEN recommends the use of the Agilent 2100 Bioanalyzer, RNA 6000 Nano LabChip® or RNA 6000 Pico LabChip®, and the RNA Integrity Number (RIN) calculation available in the Bioanalyzer 2100 Expert Software. The instrument provides a sensitive and rapid way of confirming RNA integrity prior to amplification, both visually by providing a detailed electrophoretic trace of the RNA and also computationally by calculating a RIN score. NuGEN recommends the use of uncontaminated RNA (see above) with a RIN score of 7 to 10 (see Figure 2). RNA samples with scores lower than 7 may show reduced amplification yield. It should be noted, however, that occasionally the Bioanalyzer software will fail to calculate a RIN score. In those cases, the sample may be of adequate integrity for use, but you will need to evaluate the integrity by viewing the electrophoretic trace.

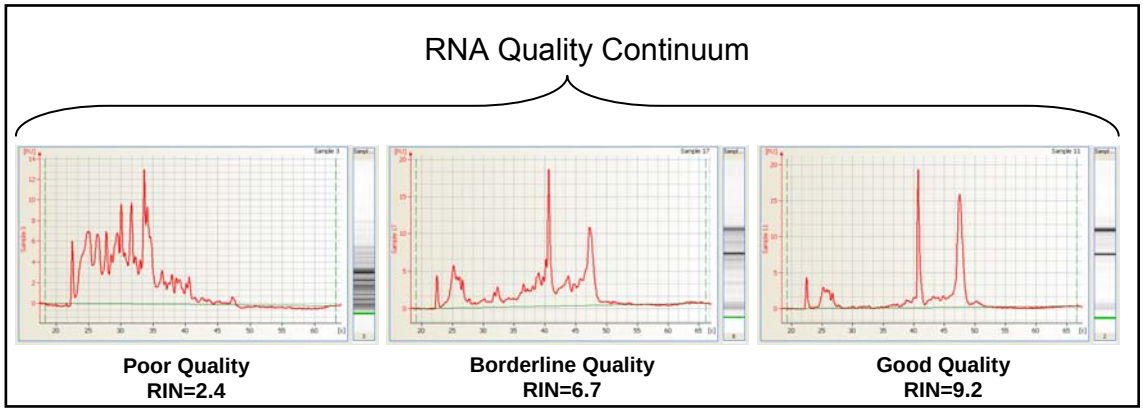
3. DNase Treatment

The presence of some genomic DNA in the RNA sample has been shown to have no adverse affects on amplification using the WT-Ovation System. However if the total RNA sample contains a significant amount of contaminating genomic DNA, it will be difficult to accurately quantitate the true RNA concentration. The RNA input quantity may therefore be over-estimated based on an O.D. measurement. Since it is important that RNA input be within the stated range of 5 to 50 ng, we recommend using a DNase treatment that will remove contaminating genomic DNA. We have validated procedures for DNase treatment of RNA samples both pre- and post- purification and these procedures are listed in Section E in the Appendix of this user guide.

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 NuGEN's technical services team.

Figure 2. This continuum of RNA quality shows Bioanalyzer traces of 3 different RNAs with varying degrees of quality.



J. Using RNase-free Techniques

RNase contamination through 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.
- **Caution**: The Reaction Enhancement Reagent, B3 (patent pending) contains a heat-labile RNase I enzyme. When using this reagent take care not to splash or contaminate gloves, bench or pipettes. Preferably use a dedicated pipette to measure out B3.

K. RNA Storage

RNA samples for use with the Ovation Biotin System must be stored at –80 °C. Avoid frequent freeze/thaw cycles or RNA shearing may result.

L. Amplified cDNA Storage

The amplified cDNA produced by the WT-Ovation System may be stored at –20 °C for several months.

IV. Protocol

M. Overview

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

1. First strand cDNA synthesis:	**1 hour**
2. Second strand cDNA synthesis and Enhancement:	**1.5 hours**
3. SPIA Isothermal Linear Amplification:	**1.5 hours**
Total time to prepare amplified cDNA	**~4 hours**

WT-Ovation System components are color coded, with each reagent vial linked to a specific process stage. Performing each stage requires the simple addition of a master mix and other reagents, followed by incubation. Master mixes are prepared by mixing components provided for that stage.

N. Protocol Notes

- Set up no fewer than eight amplification reactions at a time. This assures sufficient reagent recoveries for 24 total amplifications from a single kit. Making master mixes for fewer than 8 samples at a time may affect reagent recovery volumes.
- 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.
- Thaw components used in each step and immediately place them on ice. Do not thaw all reagents at once!
- Always keep thawed reagents and reaction tubes on ice unless otherwise noted.
- 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 2 minutes at room temperature followed by brief vortexing. Do not warm any enzyme or primer mixes.
- When placing small amounts of reagents into the reaction mix, pipette up and down several times to ensure complete transfer.
- When instructed to pipette mix, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix. Repeat a minimum of five times to ensure complete mixing.
- Always allow the thermal cycler to reach the pre-chilled initial incubation temperature prior to placing the tubes or plates in the block.
- When preparing master mixes, use the minimal amount of extra material to ensure 24 reactions in the kit.
- Components and reagents from other Ovation System products should not be used with this product.
- **Caution**: The Reaction Enhancement Reagent, B3 (patent pending) contains a heat-labile RNase I enzyme. When using this reagent, take care not to splash or contaminate gloves, bench or pipettes. Preferably use a dedicated pipette to measure out B3.
- Use only fresh ethanol stocks to make 80% ethanol for washes in the cDNA purification protocols. Make the 80% ethanol mixes fresh as well. Lower concentrations of ethanol in wash solutions will results in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the column.

O. 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 40 µL reaction volume. Prepare the 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).

Table 5. Thermal Cycler Programming

FIRST STRAND cDNA SYNTHESIS	
Program 1 Primer Annealing	65 °C for 5 minutes, then 4 °C forever
Program 2 First Strand Synthesis	4 °C for 1 minute, 25 °C for 10 minutes, 42 °C for 10 minutes, 70 °C for 15 minutes, then 4 °C forever
SECOND STRAND cDNA SYNTHESIS	
Program 3 Second Strand Synthesis	4 °C for 1 minute, 25 °C for 10 minutes, 50 °C for 30 minutes, 70 °C for 5 minutes, then 4 °C forever
POST SECOND STRAND ENHANCEMENT	
Program 4 Post Second Strand Enhancement	4 °C for 1 minute, 37 °C for 15 minutes, 80 °C for 20 minutes, then 4 °C forever
SPIA AMPLIFICATION	
Program 5 SPIA Amplification	4 °C for 1 minute, 47 °C for 60 minutes, 95 °C for 5 minutes, then 4 °C forever

P. First Strand cDNA Synthesis Protocol

Flick, do not vortex any enzyme mixes.

1. Obtain First Strand Buffer Mix (blue: A2), First Strand Enzyme Mix (blue: A3), and Nuclease-free Water (green: D1) from the components stored at –20 °C and the First Strand Primer Mix (blue: A1) stored at –80 °C.
2. Spin down contents of A3 for 2 seconds and place on ice.
3. Thaw reagent A1, A2, and D1 at room temperature. Mix A1, and A2 by vortexing for 2 seconds then spin for 2 seconds and place on ice. Leave D1 at room temperature.
4. Add 2 µL of total RNA sample (5 to 50 ng) to a 0.2 mL PCR tube.
5. Add 2 µL of A1 to the RNA aliquot.
6. Cap and spin tube(s) for 2 seconds and return tubes to ice.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 5):
   a. Incubate at 65 °C for 5 minutes
   b. Cool to 4 °C
8. Remove tubes from the thermal cycler and place tubes on ice.
9. Once Primer Annealing (Step 7) is complete, prepare a master mix by combining A2, D1 and A3 in a 0.5 mL capped tube, according to the volumes shown in Table 6.
   Note: If you intend to run a negative Reverse Transcriptase control, set it up in this step with the addition of A2 and water and exclude A3.

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

FIRST STRAND BUFFER MIX (BLUE: A2VER2)	NUCLEASE-FREE WATER (GREEN: D1)	FIRST STRAND ENZYME MIX (BLUE: A3VER1)
5 µL	0.5 µL	0.5 µL

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

10. Add 6 µL of the First Strand master mix to each tube.
11. Mix by pipetting three times, spin for 2 seconds.

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

12. Place tubes in a chilled thermal cycler programmed to run Program 2 (First Strand Synthesis; see Table 5):
    a. Incubate at 4 °C for 1 minute
    b. Incubate at 25 °C for 10 minutes
    c. Incubate at 42 °C for 10 minutes
    d. Heat at 70 °C for 15 minutes
    e. Cool to 4 °C
13. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
14. Continue immediately with second strand cDNA synthesis.

Q. Second Strand cDNA Synthesis Protocol

1. Obtain the Second Strand Buffer Mix (yellow: B1), Second Strand Enzyme Mix (yellow: B2), and Reaction Enhancement Enzyme Mix (yellow: B3) from the components stored at –20 °C.
2. Spin down contents of B2 and B3 for 2 seconds and place on ice.
3. Thaw reagent B1 at room temperature. Mix B1 by vortexing for 2 seconds, spin for 2 seconds, then place on ice.
4. 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: B1VER3)	SECOND STRAND ENZYME MIX (YELLOW: B2VER2)
9.75 µL	0.25 µL

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

5. Add 10 µL of the Second Strand master mix to each First Strand reaction tube.
6. Mix by pipetting three times, spin for 2 seconds, then place on ice.
7. Place tubes in a chilled thermal cycler programmed to run Program 3 (Second Strand Synthesis; see Table 5):
    a. Incubate at 4 °C for 1 minute
    b. Incubate at 25 °C for 10 minutes
    c. Incubate at 50 °C for 30 minutes
    d. Heat at 70 °C for 5 minutes
    e. Cool to 4 °C
8. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
9. Continue immediately with Post Second Strand Enhancement Step.

R. Post Second Strand Enhancement Protocol

Prepare master mix for at least 10 reactions for accurate pipetting of the B3. Enough reagent is included in the kit for preparing 6 X 20 uL mixes.

1. Make a master mix by combining B1 and B3 in a 0.5 mL capped tube, according to the volumes shown in Table 8.

   In order to ensure accurate measurement of the B3 reagent, do not make this mix for less than 10 reactions.

Table 8. Post Second Strand Enhancement Master Mix (volumes listed are for a single reaction)

SECOND STRAND BUFFER MIX (YELLOW: B1VER3)	REACTION ENHANCEMENT ENZYME MIX (YELLOW: B3VER1)
1.9 µL	0.1 µL

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

2. Add 2 µL of the Post Second Strand Enhancement master mix to each Second Strand reaction tube.
3. Mix by pipetting three times then spin for 2 seconds and place on ice.
4. Place tubes in chilled thermal cycler programmed to run Program 4 (Post Second Strand Enhancement; see Table 5):
    a. Incubate at 4 °C for 1 minute
    b. Incubate at 37 °C for 15 minutes
    c. Heat at 80 °C for 20 minutes
    d. Cool to 4 °C
5. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
6. Continue immediately with SPIA Amplification.

The SPIA Amplification reagents may be thawed and put on ice 10 minutes before the completion of Post Second Strand Enhancement.

S. SPIA® Amplification Protocol

1. Obtain the SPIA Buffer Mix (red: C2), and SPIA Enzyme Mix (red: C3), stored at –20 °C and the SPIA Primer Mix (red: C1) stored at –80 °C.
2. Thaw reagent C1 and C2 at room temperature. Mix C1 and C2 by vortexing for 2 seconds, spin in a microcentrifuge for 2 seconds, then place on ice.
3. Thaw C3 on ice. Mix the contents of C3 by inverting gently 5 times. Ensure the enzyme is well mixed without introducing bubbles, then spin in a microcentrifuge for 2 seconds and place on ice.
4. Make a master mix by sequentially combining C2, C1, and C3 in a 0.5 mL capped tube according to the volumes shown in Table 9. *Ensure the addition of C3 at the last moment.*

Use SPIA Master Mix immediately after preparation.

Table 9. SPIA® Master Mix (volumes listed are for a single reaction)

SPIA BUFFER MIX (RED:C2VER4)	SPIA PRIMER MIX (RED:C1VER3)	SPIA ENZYME MIX (RED:C3VER5)
5 µL	5 µL	10 µL

Note: Mix by pipetting and spin down the master mix briefly. Place on ice. Use immediately.

5. Add 20 µL of the SPIA master mix to the entire volume (20 µL) of the Enhanced Second Strand reaction.
6. Mix by pipetting 6 to 8 times, then spin for 2 seconds and place on ice.
7. Place tubes in a chilled thermal cycler programmed to run Program 5 (SPIA Amplification, see Table 5):
    a. Incubate at 4 °C for 1 minute
    b. Incubate at 47 °C for 60 minutes.
    c. Heat samples to 95 °C for 5 minutes
8. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
9. SPIA cDNA can be used unpurified for analytical purposes or stored at –20 °C. Options for purification of the SPIA cDNA are described in Appendix B.

V. Technical Support

For Technical Support, please contact NuGEN at (US 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 distributor's Technical Support team.

VI. Appendix

A. Performing Quantitative PCR on Amplified cDNA

The amplified cDNA generated from the WT-Ovation RNA Amplification System can be used directly in real time quantitative PCR reactions; however, in many cases it can be advantageous to purify the SPIA cDNA prior to beginning qPCR. Amplified cDNA produced with the kit has been successfully used as template for qPCR systems including TaqMan® and SYBR® Green. NuGEN has successfully used the following reagents for qPCR:

- 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)

1. Recommendations to Achieve Optimal Results

a. Dilute the Amplified Product

To obtain amplified product sufficient for 200 reactions, cDNA product can be diluted 1:10 (minimum of 1:4) in 1 x TE or a buffer specified by the qPCR system manufacturer. A 2 µL aliquot (or approximately 20 ng if using purified SPIA cDNA) of diluted product is used per 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 or use lower inputs of purified SPIA cDNA.

b. Test Amplified Product for SYBR Green assay

Some SYBR Green assays may not be compatible with the unpurified SPIA cDNA due to the presence of residual primer. For best results using SYBR Green detection, first check that unpurified SPIA cDNA does not interfere with detection. In cases of such interference, we have noted unusual Ct curves as well as anomalies in the dissociation profiles. If this is the case, the amplified cDNA must be purified prior to use with these assays. (Refer to Section B of Appendix).

c. Primer Design

We recommend using primers and probes designed with amplicon sizes of less than 200 nt. Primers may be designed at any length from the 3' end since the WT-Ovation amplification is not 3' biased.

Figure 3. TaqMan® Analysis of Human carcinoembryonic antigen-related cell adhesion molecule 7 (CEACAM7) cDNA Amplification.

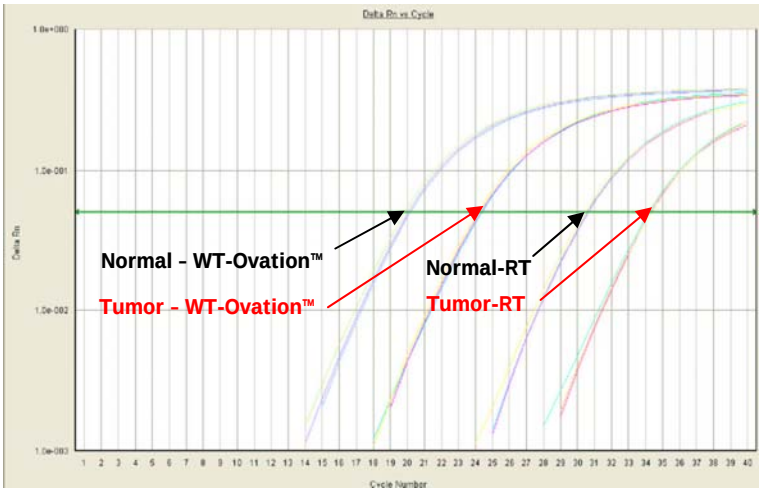


Figure 3. Comparison of Ct curves between amplified and non-amplified cDNA product. For all, the RT cDNA conversion steps were performed on triplicate samples followed by qPCR reactions. Total RNA input of 20 ng from Human Colon Tumor or Human Colon Normal Adjacent Tissue (Ambion, Austin TX), underwent 1st and 2nd strand cDNA synthesis steps of WT-Ovation RNA Amplification System. 2 µL of this non-amplified cDNA product was saved for qPCR, and the remaining 18 µL underwent SPIA Isothermal Linear Amplification. All cDNA products were diluted 1:10 in TE. 2 µL of each were added in a 25 µL qPCR reaction using the ABsolute qPCR ROX mix (ABgene cat. #AB-1136/b) and Exiqon ProbeLibrary assay (#Human64 probe for Human carcinoembryonic antigen-related cell adhesion molecule 7, CEACAM7). qPCR was performed on the ABI 7500 (Applied Biosystems). Upon amplification the Cts shift lower by approximately 10 cycles, while maintaining the magnitude of relative expression.

B. Purification of Amplified cDNA Protocol

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

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

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.

Agencourt RNAClean® magnetic beads (instructions for a single reaction)

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 76 µL of resuspended RNAClean beads (1.8 times the sample volume) each 42 µL SPIA cDNA sample.
4. Mix the sample and beads thoroughly by pipetting up and down 10 times.
5. Incubate sample/bead mixture at room temperature for 5 minutes.

6. Place the samples (containing beads) on the SPRIPlate® 96R Magnet Plate for 10 minutes or until the solution appears clear.
7. Using a multi-channel pipette, remove and discard the supernatant from the samples (on magnet). Do not disturb the ring of magnetic beads.
8. 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.
9. Using a multi-channel pipette remove and discard the ethanol.
10. Repeat the 80% ethanol wash once more. Ensure as much ethanol as possible is removed from the plate.
11. 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.
12. 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.
13. Replace reaction tubes or plate on the plate magnet; allow the beads to separate for 5 minutes or until the solution clears.
14. 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.
15. Proceed to Measuring cDNA Product Yield and Purity (Appendix C) or store purified cDNA at –20 °C.

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

Qiagen MinElute Spin Column (instructions for a single full reaction, 1 column is required per reaction)

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

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 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 300 µL of Buffer ERC to a labeled 1.5 mL tube for each amplification reaction.
4. Transfer each full reaction (42 µL) into the tube containing the Buffer ERC.
5. Vortex for 5 seconds and spin down briefly.
6. Obtain and label a Qiagen MinElute Spin Column for each amplification reaction and place it into a collection tube.
7. Load the 192 µL reaction/buffer mix onto the labeled Qiagen MinElute Spin Column.
8. Centrifuge column in the collection tube for 1 minute at >10,000 x g in a microcentrifuge.
9. Discard flow-through and replace the Qiagen MinElute Spin Column in the same collection tube.
10. 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 x g. Discard flow-through.
11. Add 500 µL of the room temperature 80% ethanol prepared in Step 1 above.
    Note: Use fresh 80% ethanol.
12. Centrifuge column in the collection tube for 1 minute at >10,000 x g. Discard flow-through.
13. Place the column back in the same collection tube and spin for an additional 2 minutes at >10,000 x g.
    Important: Residual ethanol from the wash buffers will not be completely removed unless the flow-through is discarded before this additional centrifugation.
14. Blot the column tip onto a filter paper to remove any residual wash buffer from the tip of the column.

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.

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

15. Place the MinElute Column in a clean, labeled 1.5 mL microcentrifuge tube.
16. 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!

    Important: Ensure that the water is dispensed directly onto the membrane for complete elution of bound cDNA.
17. Let columns stand for 1 minute at room temperature.
18. Centrifuge column and microcentrifuge tube for 1 minute at >10,000 x g.
19. Measure the volume recovered. There should be approximately 10 to 15 µL of purified cDNA.
20. Mix sample by vortexing, then spin briefly.
21. Proceed to Measuring cDNA Product Yield and Purity (Appendix C) or store purified cDNA at –20 °C.

Qiagen QIAquick® PCR Purification Kit, Cat. #28104 (instructions for a single reaction)

1. 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.
2. Into a clean 1.5 mL tube, add 200 µL of PB buffer from the QIAGEN system.
3. Add the 42 µL of amplified cDNA product to the tube.
4. Vortex for five seconds and spin down for two seconds.
5. Obtain one QIAquick® spin column and insert into a collection tube.
6. Load the entire reaction/buffer mix onto the column.
7. Centrifuge column in a collection tube for one minute at 13,000 rpm (~17,900 x g).
8. Discard flow-through. Place the column back in the same collection tube.
9. Add 700 µL of the room temperature 80% ethanol prepared in step 1 above.

   Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
10. Centrifuge the column for one minute at 13,000 rpm. Discard flow-through.
11. Repeat steps 9 and 10 once.
12. To remove remaining liquid, centrifuge column for one additional minute at 13,000 rpm after discarding the flow through from the final 80% ethanol wash.
13. Remove the column from the centrifuge. Discard the collection tube along with any additional flow-through.
14. 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.
15. Place the column in clean 2.0 mL collection tube, appropriately labeled.
16. Add 30 µL of nuclease-free water (green: D1) to the center of each column.
17. Do not use cold water!
18. Let columns stand for five minutes at room temperature to elute purified cDNA.
19. Centrifuge at 13,000 rpm for one minute to collect sample. There should be approximately 30 µL of purified cDNA.
20. Mix sample by vortexing, then spin briefly.
21. Proceed to Measuring cDNA Product Yield and Purity (Appendix C) or store purified cDNA at –20 °C.

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.

Zymo Research DNA Clean & Concentrator™-25 (instructions for a single reaction)

1. 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.

2. Into a clean 1.5 mL tube add 320 µL of DNA Binding Buffer.
3. Add 42 µL of amplified SPIA™cDNA product.
4. Vortex and spin down briefly.
5. Obtain one Zymo-Spin II Column and place it into a collection tube.
6. Load the entire volume of sample (362 µL) onto the Zymo-Spin II Column.
7. Centrifuge column in the collection tube for 10 seconds at >10,000 x 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.
8. Discard flow-through. Place the Zymo-Spin II Column back in the same collection tube.
9. Wash sample by adding 200 µL of the room temperature 80% ethanol prepared in step 1 above. Do not use the Wash Buffer provided with the Zymo columns. Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
10. Centrifuge column in the collection tube for 10 seconds at >10,000 x g in a microcentrifuge. Discard flow-through.
11. Add 200 µL of room temperature 80% ethanol.
12. Centrifuge column in the collection tube for 90 seconds at >10,000 x g in a microcentrifuge. Discard flow-through.
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 Zymo-Spin II Column in a clean 1.5 mL microcentrifuge tube.
15. 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!
16. Let columns stand for 1 minute at room temperature.
17. Centrifuge column and microcentrifuge tube for 30 seconds at >10,000 x g in a microcentrifuge.
18. Collect sample. There should be approximately 30 µL of purified cDNA.
19. Mix sample by vortexing, then spin briefly.
20. Proceed to Measuring cDNA Product Yield and Purity (Appendix C) or store purified cDNA at –20 °C.

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.

C. Measuring cDNA Product Yield and Purity

Note: *You must purify your amplified cDNA before measuring yield and purity*.

1. Mix your sample by brief vortexing and spinning prior to checking the concentration.
2. Measure the O.D.260, O.D.280 and O.D.320 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 O.D.320 value from both O.D.260 and O.D.280 values. The adjusted O.D.260–320/O.D.280–320 ratio should be > 1.8.
4. Yield: Assume 1 O.D.260 of single stranded DNA = 33 µg/ mL.
   To calculate:
   (O.D.260–320 of diluted sample) x (dilution factor) x 33 (concentration in µg/mL of 1 O.D. solution) x 0.03 (final volume in mL) = total yield in micrograms
5. The expected yield is between **1.5 and 4 µg** of cDNA depending on input.
6. Alternatively you may measure concentration and purity of the cDNA with a Nanodrop.
7. The purified cDNA product may be stored at –20 °C.

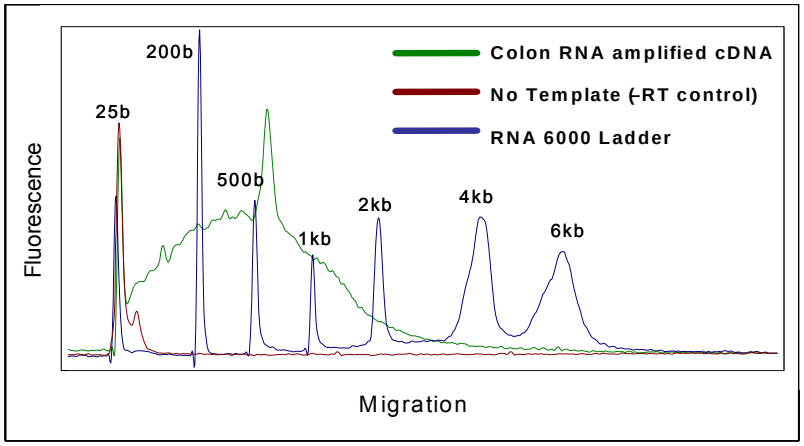
You must purify your amplified cDNA before measuring yield and purity.

D. Quality Control of Amplified cDNA Product

As a quality control test you may want to analyze the size distribution of the amplified cDNA product using an Agilent Bioanalyzer. Note that the shape of this distribution trace is highly dependent on the input RNA integrity as well as RNA source. We recommend using an RNA 6000 Nano LabChip® (Agilent cat #5065-4476) and the mRNA Smear Nano program (mRNA

Nano assay in the Expert 2100 software) and following the manufacturer's instructions. Depending on availability of amplified product you may chose to load lower than 100 ng of amplified cDNA product on the Bioanalyzer chips. A typical size distribution trace may look like the one obtained from Colon Tumor RNA. See Figure 4 below.

Figure 4. Bioanalyzer Trace of Amplified cDNA Product: The size of the RNA 6000 ladder (Ambion Inc., cat. #7152) is indicated below each peak.



E. DNase Treatment of RNA

i. 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, pipette 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 µL), including any precipitate that may have formed, to the column.
5. Close the tube gently, and centrifuge for 15 seconds at ≥ 8000 x g (≥10,000 rpm). Discard the flow-through.
6. For volumes greater than 700 µL, load aliquots onto the RNeasy column successively and centrifuge as before.
7. Add 350 µL Buffer *RW1* into the RNeasy mini column to wash, and centrifuge for 15 seconds at ≥8000 x g (≥10,000 rpm). Discard the flow through.
8. Add 10 µL **DNase I** to 70 µL Buffer *RDD*. Gently invert the tube to mix.
   Note: Other **DNase I** enzymes we can recommend to use in this step are the Shrimp DNase (recombinant) from USB Corp. (use 10 µL) , or the DNase I (RNase-free) from New England BioLabs (use 10 µL). See the Additional Reagent section of this user guide for ordering information.
9. Pipette the DNase I incubation mix (80 µL) directly onto the membrane inside the RNeasy mini column. Incubate on the bench top (~ 25 °C) for 15 min.
10. Add 350 µL Buffer RW1 into the RNeasy mini column, and centrifuge for 15 seconds at ≥ 8000 x g (≥10,000 rpm) to wash. Discard the flow through.
11. Transfer the RNeasy column to a fresh 2 mL collection tube. Add 500 µL Buffer RPE (with the added ethanol) to the RNeasy column.
12. Close the tube gently, and centrifuge for 15 seconds at ≥ 8000 x g (≥10,000 rpm). Discard the flow-through.
13. Add another 500 µL Buffer *RPE* to the RNeasy column.

14. Close the tube gently, and centrifuge for 2 minutes at ≥8000 x g (≥10,000 rpm). Discard the flow-through.
15. Transfer the RNeasy column to a new 1.5 mL collection tube.
16. Pipet 30 to 50 µL RNase-free water directly onto the RNeasy membrane.
17. Close the tube gently, and centrifuge for 1 minute at ≥8000 x g (≥10,000 rpm) to elute.
18. If yields of greater than 30 µg are expected, repeat elution step and collect in the same collection tube.

ii. DNase Treatment of RNA post-purification: using RNase-free DNase and either the RNA Clean-up Kit™- 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 µL 10X DNase I Reaction buffer (Roche cat # 04716728001 or USB PN 78316) with 1 µL 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 µL.
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:

Purification with RNA Clean-up Kit™ - 5 (Zymo Research, Cat. # R1015)

- Add 4 volumes (100 µL) of RNA binding buffer to the sample.
- Obtain one RNA Clean-up Kit™ - 5 column and apply sample to column.
- Spin column for 30 seconds at ≥8000 x g (≥10,000 rpm) and discard the flow-through.
- Add 200 µL wash buffer (with ethanol added as per vendors specifications).
- After closing the column spin for 30 seconds at ≥8000 x g (≥10,000 rpm) and discard the flow-through.
- Add 200 µL fresh 80% ethanol, close cap, spin for 30 seconds at ≥8000 x g (≥10,000 rpm) and discard the flow-through.
- Place the RNA Clean-up Kit - 5 column in a fresh 1.5 mL collection tube.
- Add 10 µL nuclease-free water (green: D1) directly to the center of the filter in the tube and close the cap. ***Do not use cold water!***
- Spin for 1 minute at ≥8000 x g (≥10,000 rpm) to collect the purified RNA.

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

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

- Add 80 µL ice-cold RNase-free water (D1, green cap) to the sample on ice.
- Add 350 µL Buffer RLT and mix by pipetting.
- Add 250 µL 96 to 100% ethanol and mix thoroughly by pipetting.
- Place an RNeasy MinElute Spin Column into a 2 mL collection tube (one column per sample) and apply the 700 µL sample to the column.
- After closing the column, spin for 15 seconds at ≥8000 x g (≥10,000 rpm) and discard the flow-through.
- Place the RNeasy MinElute Spin Column into a fresh 2 mL collection tube. Add 500 µL Buffer RPE to the column and close the tube. Spin for 15 seconds at ≥8000 x g (≥10,000 rpm) and discard the flow-through keeping the same collection tube.
- Add 500 µL 80% ethanol to the RNeasy MinElute Spin Column and close the tube.

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

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

- Spin for 2 minutes at ≥8000 x g (≥10,000 rpm) and discard the flow-through.
- 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 ≥8000 x g (≥10,000 rpm) and discard the flow-through.
- Place the RNeasy MinElute Spin Column in a fresh 1.5 mL collection tube.
- Add 14 µL 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!**
- Spin for 1 minute at ≥8000 x g (≥10,000 rpm) to collect the purified RNA.

F. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the WT-Ovation RNA Amplification System?
The WT-Ovation System provides all necessary buffers, primers and enzymes for first strand synthesis, second strand synthesis and amplification, yielding cDNA. The kit also provides nuclease-free water.

Q2. What equipment is required or will be useful?
Required equipment includes a microcentrifuge, pipettes, vortexer and a thermal cycler. An O.D. spectrophotometer and an Agilent Bioanalyzer will be useful.

Q3. What additional consumables does the user need?
None. For the optional purification of amplified cDNA, see user guide for validated purification products and procedures.

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. Can I do reactions in smaller batches than 8?
We recommend 3 batches of 8 reactions. Smaller batch sizes may result in fewer than 24 reactions in total.

Q6. Where in my target sequence can I design my qPCR primers?
The WT-Ovation system does not have a 3 prime bias and therefore primers can be designed at any location within the mRNA. In order to avoid qPCR interference from possible genomic DNA contamination, we recommend treating your RNA with DNase and designing your amplicons to span an intron.

Q7. Is the WT-Ovation System 3 prime biased?
No, this product is different from the Ovation® RNA Amplification System in that the first strand cDNA is primed with random hexamers as well as a 3 prime primer.

Q8. How much total RNA do I need for amplification?
We recommend staying within the range of 5 to 50 ng total RNA starting material. Amounts greater than 50 ng may produce variable results.

Q9. How much cDNA can I expect from a single reaction?
You should expect 1.5 to 4 µg of cDNA from 5 to 50 ng total RNA starting material.

Q10. Is the cDNA yield dependent upon the quantity of input total RNA?
Yes, the more RNA into the assay, the more yield recovered. However at inputs of above 50 ng, the yields become variable without increasing.

Q11. What is the amplification efficiency of the WT-Ovation System?
Based on qPCR on a variety of genes, an average amplification efficiency of 1500-fold is observed.

Q12. What is the dynamic range of input mRNA that is linearly amplified with the WT-Ovation System?
Our studies demonstrate linear amplification over 6 orders of magnitude starting with transcripts present as low as 20 copies in a sample.

Q13. What size cDNA is generated by the WT-Ovation System?

On a Bioanalyzer, using the RNA 6000 size markers, the average size of the amplified cDNA is 375 bases. More than 50% of the product is greater than 320 bases in length.

Q14. Can the WT-Ovation System amplify DNA?

The Ovation system is designed to amplify mRNA, not DNA.

Q15. Can I use the WT-Ovation System on bacterial RNA samples?

The WT-Ovation theoretically will work with some bacterial RNAs. However, the kit has not been optimized for this purpose.

Q16. Are there any tissues that will not work with the WT-Ovation System?

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

Q17. Has NuGEN performed reproducibility studies on the WT-Ovation System?

Yes. Sample to sample, lot to lot, and operator to operator reproducibility studies are routinely conducted.

Q18. Does the Ovation System generate product in the absence of RNA input?

No significant product is generated in the absence of input RNA.

Q19. How many rounds of amplification are performed with the WT-Ovation System?

The WT-Ovation System performs a single round of amplification.

Q20. Can I use the WT-Ovation System for archiving cDNA?

Amplified cDNA maybe stored at -20°C for at least several months. Long term stability tests are in progress.

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

No. The DNA/RNA primers provided in the WT-Ovation System are universal.

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

The WT-Ovation System will not work properly with other primers.

Q23. What are the incubation temperatures for each step?

First strand primer annealing = 65 °C
First strand synthesis = 25 °C and 42 °C
Second strand synthesis = 25 °C and 50 °C
Post second strand Enhancement = 37 °C
SPIA amplification = 47 °C

Q24. If I choose to purify my product, what method do you recommend?

Several purification options are available for the final SPIA cDNA cleanup step. These are described in Appendix B of this user guide. Selection of the optimum purification option can depend on many factors. Please contact the NuGEN Technical Support team for assistance in selecting the appropriate option for your application. Refer to section **II.B** for ordering information.

Q25. Should I purify the cDNA if I chose to determine 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.

Q26. How do I measure my cDNA product?

Measure the amplified product as suggested in Appendix C.

Q27. Where can I safely stop in the protocol?

We do not recommend stopping at any stage of the protocol.

Q28. How many qPCR reactions will I get from one WT-Ovation amplification?

The number of qPCR reactions depends on the abundance level of the genes being interrogated. For medium to high copy genes, the cDNA may be diluted as much as 400-fold, enough for hundreds of qPCR reactions. For very low copy genes you will need to use more cDNA per reaction. The user will need to determine how much cDNA to use per reaction depending on the abundance of the gene being interrogated.

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