USER GUIDE

Ovation® RNA-Seq System

CATALOG NO. 7100-08

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imagine more from less™

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I. Introduction

A. Background

The Ovation® RNA-Seq System provides a fast and simple method for preparing amplified cDNA from total RNA for RNA-Seq applications (transcriptome sequencing). Amplification is initiated at the 3′ end as well as randomly throughout the whole transcriptome in the sample. This feature makes the Ovation RNA-Seq System ideal for amplification prior to Next Generation Sequencing, as reads are distributed across transcript types. The amplified product of the Ovation RNA-Seq System is optimized for the generation of sequencing libraries for the Illumina Genome Analyzer II platform. The amplified cDNA generated with the Ovation RNA-Seq System may be suitable for qPCR or microarray analysis as well as other commercial NGS platforms.

The Ovation RNA-Seq 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 as little as 500 pg total RNA, microgram quantities of cDNA can be prepared in approximately 6 hours.

The Ovation RNA-Seq System (Cat. # 7100-08) provides optimized reagent mixes and a protocol to process 8 RNA samples. Control RNA is not provided with the Ovation RNA-Seq System, but we recommend amplifying a high-quality commercial RNA as a positive control when first using this product.

I. Introduction

B. Ribo-SPIA Technology

Ribo-SPIA technology is a 3-step process that generates amplified cDNA from as little as 500 pg (range up to 100 ng) of total RNA (see Figure 1).

1. **Generation of First Strand cDNA (40 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.

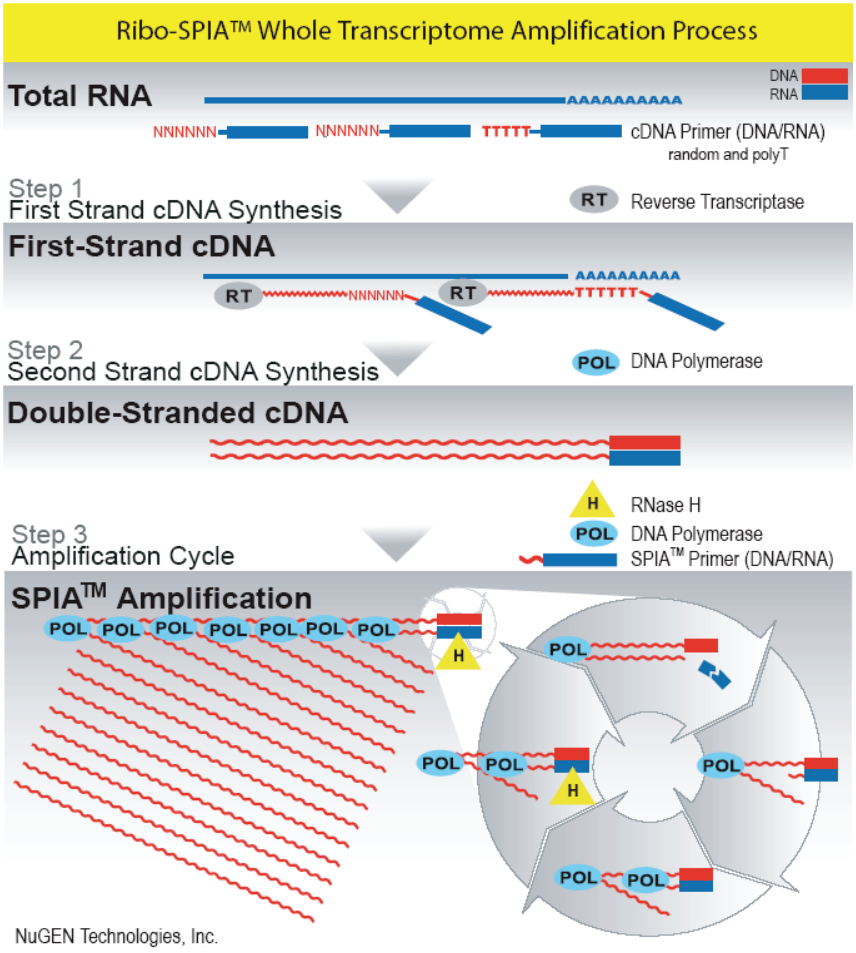
2. **Generation of a DNA/RNA Heteroduplex Double-Stranded cDNA (90 minutes)**
   Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthesize a second strand, which includes DNA complementary to the 5′ unique sequence of the first strand chimeric primers. The result is a double-stranded cDNA with a unique DNA/RNA heteroduplex at one end.

3. **Amplification (80 minutes)**
   SPIA® is a linear isothermal DNA amplification process developed by NuGEN. It uses a DNA/RNA chimeric SPIA 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 the first SPIA 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 a sequence complementary to the original mRNA. An average amplification of 800-fold is observed with 500 pg starting total RNA.

4. **Post-SPIA Modification (45 minutes)**
   The Post-SPIA Modification process completes the amplification process. The first step allows the random primers to anneal to the single-stranded, anti-sense cDNA target. The second step utilizes DNA polymerase to extend from the annealed primers on the newly synthesized strand, producing targets appropriate for Illumina library preparation (not shown on Figure 1).

I. Introduction

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



I. Introduction

C. Performance Specifications

The Ovation RNA-Seq System synthesizes microgram quantities of amplified cDNA starting with total cellular RNA input amounts of 500 pg to 100 ng. In approximately 6 hours, the Ovation RNA-Seq System can produce an average of 3 µg of double-stranded cDNA in a 30 µL volume ready for Illumina library preparation. When used with intact input RNA, the size of the majority of the cDNA products produced by the Ribo-SPIA amplification process is between 50 bases and 1.0 Kb.

D. Quality Control

Each Ovation RNA-Seq System lot is tested to meet specifications of yield and cDNA size distribution by BioAnalyzer.

E. Storage and Stability

This product contains components with multiple storage temperatures.

Store the RNAClean Beads at 4°C

The Ovation RNA-Seq System is shipped on dry ice and should be unpacked immediately upon receipt. **Note:** This product contains components with multiple storage temperatures.

All kit components, except the Beckman Coulter Genomics RNAClean® Beads (clear cap), should be stored at **–20°C** in a freezer without a defrost cycle.

The vial labeled Beckman Coulter Genomics RNAClean Beads (clear cap) should be removed from the top of the shipping carton upon delivery and **stored at 4°C.**

Kits handled and stored according to the above guidelines will perform to specifications for at least 6 months. NuGEN has not yet established long-term storage conditions for the Ovation RNA-Seq System.

F. Material Safety Data Sheets (MSDS)

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

II. Kit Components

A. Reagents Provided

Table 1. First Strand cDNA Reagents

COMPONENT	7100-08 PART NO.	VIAL CAP	VIAL NUMBER
First Strand Primer Mix	S01278	Blue	A1 ver4
First Strand Buffer Mix	S01174	Blue	A2 ver3
First Strand Enzyme Mix	S01040	Blue	A3 ver1

Table 2. Second Strand cDNA Reagents

COMPONENT	7100-08 PART NO.	VIAL CAP	VIAL NUMBER
Second Strand Buffer Mix	S01176	Yellow	B1 ver3
Second Strand Enzyme Mix	S01126	Yellow	B2 ver2

Table 3. SPIA Reagents

COMPONENT	7100-08 PART NO.	VIAL CAP	VIAL NUMBER
SPIA Primer Mix	S01279	Red	C1 ver9
SPIA Buffer Mix	S01280	Red	C2 ver9
SPIA Enzyme Mix	S01261	Red	C3 ver5

Table 4. Post-SPIA Modification Reagents

COMPONENT	7100-08 PART NO.	VIAL CAP	VIAL NUMBER
Primer Mix	S01281	Violet	E1 ver1
Buffer Mix	S01282	Violet	E2 ver3
Enzyme Mix	S01283	Violet	E3 ver1

Table 5. Additional Reagents

COMPONENT	7100-08 PART NO.	VIAL CAP	VIAL NUMBER
Nuclease-free water	S01001	Green	D1
Beckman Coulter Genomics RNAClean Beads	1200-01	Clear	—

Note: The reagents in the Ovation RNA-Seq System product are similar to reagents in NuGEN's other kits. However, unless the component 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 A1, for example, with another A1 unless the component part numbers are identical, as it will adversely affect performance.

B. Additional Reagents, Supplies and Equipment

Required materials

- **Equipment**
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - 0.5 - 10 µL pipette, 2 - 20 µL pipette, 20 - 200 µL pipette, and 200 - 1000 µL pipette
  - Vortexer
  - Thermal cycler with 0.2 mL tube heat block, heated lid, and 100 µL reaction capacity
  - Appropriate spectrophotometer and cuvettes, or Nanodrop® ND-1000 UV-Vis Spectrophotometer
  - Fluorescent Spectrophotometer
- **Reagents**
  - Ethanol (Sigma-Aldrich, Cat. # E7023), for purification steps
  - 1x TE buffer
  - QuantIT PicoGreen dsDNA Assay Kit (Invitrogen, Cat. # P11496)

II. Kit Components

- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - 1.5 mL amber, DNase-free microcentrifuge tubes
  - 0.2 mL individual thin wall PCR tubes or 8 x 0.2 mL strip PCR tubes or 0.2 mL thin wall PCR plates
  - Beckman Coulter Genomics SPRIPlate® 96R, Ring Magnet Plate (Beckman Coulter Genomics, Cat. # 000219) or Beckman Coulter Genomics SPRIPlate Ring Super Magnet Plate, (Beckman Coulter Genomics, Cat. # 000322). Other magnetic stands may be used as well, although their performance has not been validated by NuGEN.
  - DNA Clean & Concentrator™-25 (Zymo Research, Cat. # D4006)
  - Disposable gloves
  - Kimwipes
  - Ice bucket

Optional materials

- Agilent 2100 bioanalyzer or materials and equipment for electrophoretic analysis of RNA
- Real Time PCR system
- Decontamination solutions such as RNaseZap® (Ambion, Cat. # AM9780) and DNA-OFF™ (MP Biomedicals, Cat. # QD0500)

To Order:

- Ambion Inc., www.ambion.com
- Beckman Coulter Genomics Bioscience Corporation, www.Beckman-Coulter Genomics.com
- Invitrogen Life Technologies, www.invitrogen.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

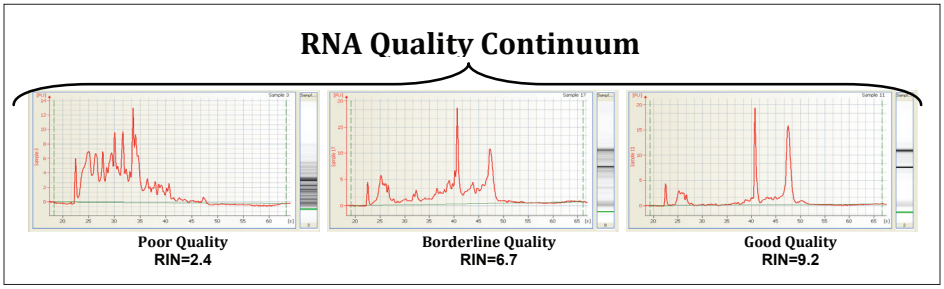
1. **RNA Quantity**
   Total RNA input must be between 500 pg and 100 ng. Inputs above 100 ng per reaction may inhibit amplification, while lower amounts of input will potentially result in insufficient yields depending on required analytical platforms.

2. **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 high-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.

3. **RNA Integrity**
   RNA samples of high molecular weight with little or no evidence of degradation will amplify very well with this product.

   The extent of RNA integrity can be determined using 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, with a detailed electrophoretic trace of the RNA, and computationally, by calculating a RIN score. We strongly recommends quantitation of total RNA to assure the minimum input requirement is met. On occasions when the Bioanalyzer software fails to calculate a RIN score, we recommend viewing the electrophoretic trace to determine if the sample is of adequate integrity for use.

Figure 2. This continuum of RNA quality shows Bioanalyzer traces of 3 different RNAs with varying degrees of quality. Only high-quality RNAs have been validated to amplify robustly with this kit.



4. **DNase Treatment**
   It is generally recommended to use DNase-treated RNA for amplification using the Ovation RNA-Seq System. One reason is that the presence of genomic DNA in the RNA sample may potentially have adverse effects on downstream analytical platforms. Contaminating genomic DNA may also be amplified along with the RNA. Another reason is that if the total RNA sample contains a significant amount of contaminating genomic DNA, it will be difficult to accurately quantify the true RNA concentration. The RNA input quantity may therefore be overestimated based on an absorbance measurement. Since it is important that RNA input be within the stated range of 500 pg to 100 ng, we recommend using a DNase treatment that will remove contaminating genomic DNA during RNA purification.

5. **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.

B. Using RNase-Free Techniques

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

1. Wear disposable gloves and change them frequently.
2. Avoid touching surfaces or materials that could introduce RNases.
3. Use reagents provided. Substitutions may introduce RNases.
4. Clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents, such as RNaseZap.
5. Use only new RNase-free pipette tips and microcentrifuge tubes.
6. 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-Seq System must be stored at –80°C. Avoid frequent freeze/thaw cycles, or RNA shearing may result.

D. Amplified cDNA Storage

The amplified cDNA produced by the Ovation RNA-Seq System may be stored at –20°C.

IV. Protocol

A. Overview

The Ribo-SPIA amplification process used in the Ovation RNA-Seq is performed in 4 stages:

1. First strand cDNA synthesis:	**1 hour**
2. Second strand cDNA synthesis and purification:	**2 hours**
3. SPIA isothermal linear amplification and purification:	**1.5 hours**
4. Post-SPIA modification and purification:	**1.5 hours**
Total time to prepare amplified cDNA	**~6 hours**

Ovation RNA-Seq System components are color-coded, with each color linked to a specific stage of the process. Performing each stage requires making a master mix, then adding it to the reaction, followed by incubation. Master mixes are prepared by mixing components provided for that stage.

The cDNA must be purified following amplification if you intend to use the cDNA for Illumina library preparation.

B. Protocol Notes

1. The first time you set up an amplification reaction, use a control RNA at an input level well above the minimum recommended input of 500 pg. This allows you to establish a baseline of performance and provides the opportunity to become familiar with the bead purification step. This step is especially prone to handling variability in using the magnet plate, so a practice run with the plate is also highly recommended.
2. Set up no fewer than 4 reactions at a time with the 7100-08 kit. This ensures sufficient reagent recoveries for 8 total amplifications from a single kit. Making master mixes for fewer than 4 samples at a time may affect reagent recovery volumes.
3. Thaw components used in each step and immediately place them on ice. It is best not to thaw all reagents at once.
4. Always keep thawed reagents and reaction tubes on ice unless otherwise instructed.
5. After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve the precipitate 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.
6. When placing small amounts of reagents into the reaction mix, pipette up and down several times to ensure complete transfer.
7. When instructed to pipet mix, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.

8. Always allow the thermal cycler to reach the initial incubation temperature prior to placing the tubes or plates in the block.
9. When preparing master mixes, use the minimal amount of extra material to ensure 8 reactions in the kit.
10. Components and reagents from other Ovation System products should not be used with this product.
11. Use only fresh ethanol stocks to make ethanol for washes in the cDNA purification protocols. Make the ethanol mixes fresh as well. 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. Beckman Coulter Genomics RNAClean Purification Beads

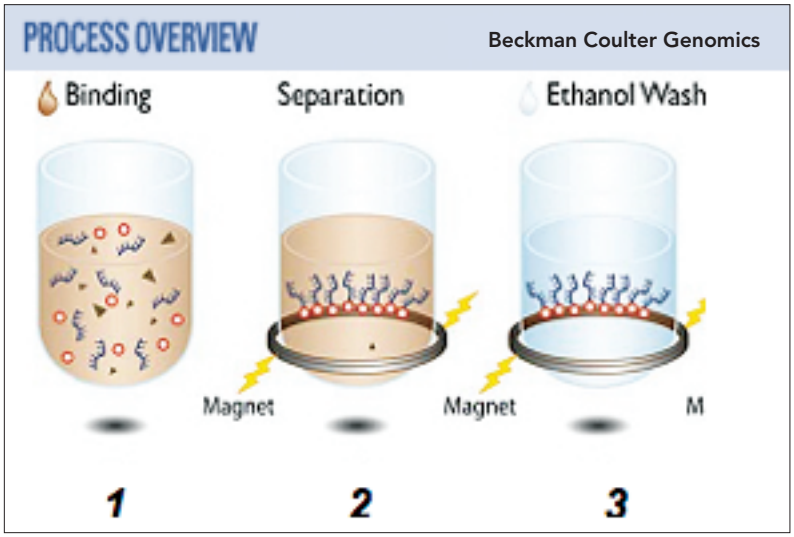
Tips and notes:
There are significant modifications to the Beckman Coulter Genomics RNAClean beads' standard procedure; therefore, you must follow the procedures outlined in this user guide for the use of these beads with the Ovation RNA-Seq System. However, you may review the Beckman Coulter Genomics user guide at the following website to become familiar with the manufacturer's recommendations: http://www.Beckman-CoulterGenomics.com/documents/products/rnaclean/Beckman-Coulter Genomics_RNAClean_Protocol.pdf.

The bead purification process used for cDNA purification before amplification consists of:

1. Binding of cDNA to magnetic beads
2. Separation of total cDNA bound to magnetic beads from contaminants, removal and discarding of supernatant
3. Washing of cDNA with ethanol

At this stage the beads are left in the cDNA tube and removed only after amplification.

Figure 3.



IV. Protocol

Additional tips and notes:

- Remove beads from 4°C and leave at room temperature for at least 15 minutes. Before use, ensure that they have completely reached room temperature. Cold beads will result in reduced recovery.
- Fully resuspend beads by inverting and tapping before adding to the sample.
- Note that we recommend using **1.6 volumes** (32 µL) of RNAClean beads. This is different than the standard Beckman Coulter Genomics protocol.
- It is critical to let the beads separate on the magnet for a full 10 minutes. Removing the binding buffer before the beads have completely separated will impact cDNA yields.
- After the binding step has been completed, it is important to minimize bead loss when removing the binding buffer. With the samples placed on the magnet, remove only 42 µL of the binding buffer from each sample. Some liquid will remain at the bottom of the tube, but this will minimize bead loss.
- Any significant loss of beads bound to the magnet during the ethanol washes will impact cDNA yields, so make sure the beads are not lost with the wash.
- Ensure that the 70% ethanol wash is freshly prepared from fresh ethanol stocks. Lower percent ethanol mixes will reduce recovery.
- During the ethanol washes, keep the samples on the magnet. The beads should not be allowed to disperse; the magnet will keep the beads on the walls of the sample wells or tubes in a small ring.
- It is critical that all residual ethanol be removed prior to continuing with the SPIA amplification. Therefore, when removing the final ethanol wash, first remove most of the ethanol, then allow the excess to collect at the bottom of the tube before removing the remaining ethanol. This reduces the required bead air-drying time.
- After drying the beads for at least 15-20 minutes, inspect each tube carefully and make certain that all the ethanol has evaporated before proceeding with the amplification step.
- It is strongly recommended that strip tubes or partial plates are firmly placed when used with the magnetic plate. We don't advise the use of individual tubes, as they are not very stably supported on the magnetic plates.

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 µL reaction volume. Prepare the programs shown in Table 6, 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 6. Thermal Cycler Programming

First Strand cDNA Synthesis	
Program 1: Primer Annealing	(≤ =1 ng) 65°C for 2 minutes, then 4°C forever (> =1 ng) 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, 80°C for 20 minutes, then 4°C forever
SPIA Amplification	
Program 4: SPIA Amplification	4°C for 1 minute, 47°C for 60 minutes, 95°C for 5 minutes, then 4°C forever
Post-SPIA Modification	
Program 5: Post-SPIA Modification I	98°C for 3 minutes, then 4°C forever
Program 6: Post-SPIA Modification II	4°C for 1 minute, 30°C for 10 minutes, 42°C for 15 minutes, 75°C for 10 minutes, then 4°C forever

E. First Strand cDNA Synthesis Protocol

1. Obtain First Strand Primer Mix (blue: A1), First Strand Buffer Mix (blue: A2), First Strand Enzyme Mix (blue: A3), and the water (green: D1) from the components stored at –20°C.
2. Flick to mix, then spin down contents of A3 for 2 seconds and place on ice.
3. Thaw the other reagents at room temperature. Mix by vortexing for 2 seconds, spin for 2 seconds, and then place on ice.
4. Add 2 µL of A1 to a 0.2 mL PCR tube.
5. Add 5 µL of total RNA sample (≥ 500 pg) to the primer.
6. Cap and spin tube(s) for 2 seconds and return tube(s) to ice.
7. Place tube(s) in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 6):

   a. Incubate at 65°C for 2 or 5 minutes (as shown in Table 6).

   b. Cool to 4°C
8. Remove tube(s) from the thermal cycler and place tube(s) on ice.
9. 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 7.

   Note: If you intend to run a negative Reverse Transcriptase control, set it up in this step with the addition of A2 and water (D1) and exclude A3.

Flick, do not vortex, any enzyme mixes.

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

FIRST STRAND BUFFER MIX (BLUE: A2 ver3)	FIRST STRAND ENZYME MIX (BLUE: A3 ver1)
2.5 µL	0.5 µL

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

10. Add 3 µL of the First Strand master mix to each tube.
11. Mix by pipetting 6 - 8 times, spin for 2 seconds.
12. Place tubes in a pre-cooled thermal cycler programmed to run Program 2 (First Strand Synthesis; see Table 6):
    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. Incubate 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.

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

F. Second Strand cDNA Synthesis Protocol

The purification beads should be removed from 4°C and left at bench top to reach room temperature well before the start of purification.

1. Remove the RNAClean purification beads from 4°C and place at bench top to reach room temperature for use in the next step
2. Obtain the Second Strand Buffer Mix (yellow: B1) and Second Strand Enzyme Mix (yellow: B2), from the components stored at –20°C.
3. Flick to mix, then spin down contents of B2 for 2 seconds and place on ice.
4. Thaw reagent B1 at room temperature, mix by vortexing for 2 seconds, spin for 2 seconds, then place on ice.
5. Make a master mix by combining B1 and B2 in a 0.5 mL capped tube, according to the volumes shown in Table 8.

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

SECOND STRAND BUFFER MIX (YELLOW: B1 ver3)	SECOND STRAND ENZYME MIX (YELLOW: B2 ver2)
9.7 µL	0.3 µL

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

6. Add 10 µL of the Second Strand master mix to each First Strand reaction tube.
7. Mix by pipetting 6 - 8 times, spin for 2 seconds, then place on ice.
8. Place tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand Synthesis; see Table 6):

   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. Incubate at 80°C for 20 minutes

   e. Cool to 4°C
9. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
10. Continue immediately with purification of unamplified cDNA.

Best results can be obtained by using fresh 70% ethanol in wash step.

Minimize bead loss by leaving a residual volume of binding buffer after completion of the binding step.

Ensure that all residual ethanol is removed prior to continuing with the SPIA amplification.

G. Purification of cDNA Protocol

1. Ensure the Beckman Coulter Genomics RNAClean beads have completely reached room temperature before proceeding.
2. Resuspend beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample.
3. After resuspending, do not spin the beads. A large excess of beads is provided, therefore it is not necessary to recover any trapped in the cap.
4. At room temperature, add 32 µL **(1.6 volumes)** bead suspension to each reaction and mix by pipetting up and down 10 times. Incubate at room temperature for 10 minutes.
5. Transfer tubes or plate to magnet plate and let stand 5 minutes to completely clear the solution of beads.
6. Carefully remove only 42 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

   Note: The beads should not disperse; instead, they will stay on the walls of the wells as a small ring. Significant loss of beads at this stage will impact cDNA yields, so ensure beads are not removed with the binding buffer or the wash.
7. With the plate still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds. Prepare the 70% ethanol fresh on the day of your experiment.

8. Remove the 70% ethanol wash using a pipette.
9. Repeat the 70% ethanol wash two more times, for a total of three washes.

   Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least 2 pipetting steps to allow excess ethanol to collect at the bottom of the tubes before removing the last of the ethanol.
10. Air-dry the beads on the magnet for a minimum of 15 – 20 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing with the SPIA amplification.
11. Proceed immediately with SPIA amplification with the cDNA still bound to the dry beads.

H. SPIA Protocol

1. Obtain the SPIA Primer Mix (red: C1), SPIA Buffer Mix (red: C2) and SPIA Enzyme Mix (red: C3), stored at –20°C.
2. Thaw reagent C1 and C2 at room temperature, mix by vortexing for 2 seconds, spin in a microcentrifuge for 2 seconds, then place on ice.
3. Thaw C3 on ice and mix the contents 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. Make sure the addition of C3 is 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:C2 ver9)	SPIA PRIMER MIX (RED:C1 ver9)	SPIA ENZYME MIX (RED:C3 ver5)
20 µL	10 µL	10 µL

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

5. Add 40 µL of the SPIA master mix to each tube containing the double-stranded cDNA bound to the dried beads. Use a pipette set to 30 µL and mix well by pipetting up and down at least 8-10 times. Attempt to get the majority of the beads in suspension and remove most of the beads from the tube walls.

   Note: Beads may not form a perfectly uniform suspension, but this will not affect the reaction. The addition of SPIA master mix will elute the cDNA off the beads.

6. Place tubes in a pre-cooled thermal cycler programmed to run Program 4 (SPIA Amplification, see Table 6):
   a. Incubate at 4°C for 1 minute
   b. Incubate at 47°C for 60 minutes
   c. Incubate at 95°C for 5 minutes
   d. Cool to 4°C
7. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
8. **Move to the post-amplification workspace** and continue immediately with Post-SPIA Modification I.
9. Transfer tubes or plate to magnet plate and let stand 5 minutes to completely clear the solution of beads.
10. Continue immediately with Post-SPIA Modification I.

Move post-amplification workspace to a new area and continue immediately with Post-SPIA Modification I.

I. Post-SPIA Modification Protocol I

The remainder of the protocol should be carried out in a workspace designated for post-amplification processes, well separated from the pre-amplification workspace (see Appendix E for further information).

The remainder of the protocol should be carried out in a workspace designated for post-amplification processes, well separated from the pre-amplification workspace (see Appendix E for further information).

1. Carefully transfer 35 µL of the cleared supernatant containing the amplified cDNA to fresh tubes. The beads may now be discarded.
2. Obtain the Primer Mix (Violet: E1) stored at –20°C. Thaw E1 at room temperature, mix by vortexing for 2 seconds, spin for 2 seconds, then place on ice.
3. Add 5 µL of the Primer Mix E1 to 35 µL of the SPIA reaction.
4. Mix thoroughly by pipetting 6 - 8 times at the 30 µL setting, then spin for 2 seconds and place on ice.
5. Place tubes in a pre-warmed thermal cycler programmed to run Program 5 (Post-SPIA Modification I, see Table 6):
   a. Incubate at 98°C for 3 minutes
   b. Cool to 4°C
6. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
7. Continue immediately to Post-SPIA Modification II.

J. Post-SPIA Modification Protocol II

1. Obtain the Buffer Mix (Violet: E2) and Enzyme Mix (Violet: E3) stored at –20°C.
2. Thaw E2 at room temperature, mix by vortexing for 2 seconds, spin for 2 seconds, then place on ice.
3. Spin down E3 in a microcentrifuge for 2 seconds, then place on ice.
4. Make a master mix by sequentially combining E2 and E3 in a 0.5 mL capped tube, according to the volumes shown in Table 10.

Table 10. Post-SPIA Modification II Master Mix (volumes listed are for a single reaction)

BUFFER MIX (VIOLET: E2 ver3)	E3 ENZYME MIX (VIOLET: E3 ver1)
5 µL	5 µL

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

5. Add 10 µL of the Post-SPIA Modification II master mix to the entire volume of the Post-SPIA Modification I reaction. Final reaction volume is 50 µL.
6. Mix thoroughly by pipetting 6 - 8 times at the 40 µL pipettor setting, spin for 2 seconds, then place on ice.
7. Place tubes in a pre-cooled thermal cycler programmed to run Program 6 (Post-SPIA Modification II, see Table 6):
   a) Incubate at 4°C for 1 minute
   b) Incubate at 30°C for 10 minutes
   c) Incubate at 42°C for 15 minutes
   d) Incubate at 75°C for 10 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. Amplified cDNA can be stored at –20°C.

K. Purification of Amplified cDNA Protocol

The amplified cDNA should be purified prior to subsequent processing for RNA-Seq library construction. Suitable purification methods are listed in the Appendix.

L. Measuring Double-Stranded cDNA Product Yield

1. For all dilutions, use 1x TE prepared from the 20x TE stock provided in the PicoGreen kit (Invitrogen, Cat. # P11496).
2. Prepare double-stranded standard curve reagents according to Table 11, using the reagents supplied in the PicoGreen kit.
3. Dilute amplified 1000-fold by performing 3 serial 10-fold dilutions with 1x TE.
4. Prepare 2x PicoGreen solution by diluting the stock 1:200 with 1x TE. Make PicoGreen solution in an amber tube and protect the stock solution from light.
5. Mix 10 µL of standards with 10 µL of 2x PicoGreen.
6. Incubate at room temperature for 5 minutes, protected from light.
7. Mix 10 µL of diluted samples with 10 µL of 2x PicoGreen. Incubate at room temperature, protected from light, while the standard curve measurements are made.
8. Blank the ND3300 with 1x TE.
9. In the Standards Tab, make 3 measurements each of the reference (TE+PicoGreen) and the standards 1 through 4, beginning with the lowest concentration standard.
10. After all standards measurements are made, view the standard curve.
11. The R-square for the linear fit reported by the software should be ≥ 0.98.
12. Measure each of the diluted samples of interest, using a dilution factor of '2' (for the dilution into the PicoGreen).
13. The ND3300 reports the concentration in ng/mL. Because the starting sample was diluted 1000-fold, this value corresponds to the ng/µL concentration of the sample (e.g., (200 ng/mL * 1000)/1000 = 200 ng/µL).
14. Proceed to Illumina library preparation, beginning with the End Repair protocol, using ≥300 ng of double-stranded cDNA product. Please see the Appendix for options on reagents for the Illumina library preparation protocol.

Table 11. Double-Stranded DNA Standard Dilutions

SAMPLE ID	CONCENTRATION TO ENTER	DILUTIONS	
Std5	1000	2000 ng/mL	2 µL stock + 98 µL 1x TE
Std4	500	1000 ng/mL	10 µL 2000 ng/mL + 10 µL 1x TE
Std3	100	200 ng/mL	10 µL 2000 ng/mL + 90 µL 1x TE
Std2	10	20 ng/mL	10 µL 200 ng/mL + 90 µL 1x TE
Std1	1	2 ng/mL	10 µL 20 ng/mL + 90 µL 1x TE
REF	0	0 ng/mL	90 µL 1x TE

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 europe@nugeninc.com.

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

VI. Appendix

A. Performing Quantitative PCR on Amplified cDNA

If qPCR is desired, it is recommended that the assay be performed using amplified cDNA prior to the Post-SPIA Modification steps. Amplified cDNA produced with the kit has been used successfully as templates for qPCR systems including TaqMan® and SYBR® Green.

We can recommend the following reagents for qPCR:

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

1. Recommendations to Achieve Optimal Results

a. Dilute the Amplified Product
The unpurified amplified cDNA should be diluted 10-fold for TaqMan assays and 40-fold for SYBR Green assays.

b. Primer Design
We recommend using primers and probes designed with amplicon sizes of less than 200 nt. Primers may be designed at any position along a transcript since the Ovation RNA-Seq amplification process covers the whole transcriptome.

B. Purification Protocols for Amplified cDNA

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

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

1. Into a clean 1.5 mL tube add 250 µL of PB buffer from the Qiagen system.
2. Add the 50 µL of amplified cDNA product to the tube.
3. Vortex for 5 seconds and spin down for 2 seconds.
4. Obtain 1 QIAquick spin column and insert into a collection tube.
5. Load 300 µL of sample onto the column.
6. Centrifuge column in a collection tube for 1 minute at 13,000 rpm (~17,900 x g).
7. Discard flow-through. Place the column back in the same collection tube.
8. 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.

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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.

! *Use nuclease-free TE at room temperature to elute sample.*

14. Place the column in a clean 2.0 mL collection tube, appropriately labeled.
15. Add 30 µL of 1x TE to the center of each column.
16. Let columns stand for 5 minutes at room temperature to elute purified cDNA.
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 step.

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

Beckman Coulter Genomics RNAClean purification magnetic beads
(instructions for a single reaction)

1. Obtain and vigorously shake the RNAClean bottle to resuspend the magnetic beads.
2. Transfer the 50 µL cDNA into a clean 1.5 mL tube, 0.2 mL strip tubes or PCR plate.
3. Add 90 µL of resuspended RNAClean beads to the 50 µL cDNA sample.
4. Mix the sample and beads thoroughly by pipetting up and down 10 times.
5. Incubate samples at room temperature for 5 minutes.
6. Place the tubes or plate on the plate magnet for 10 minutes or until solution clears.
7. Using a multi-channel pipette, remove and discard the supernatant. Do not disturb the ring of magnetic beads.

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

8. With the reaction plate still on the plate magnet, 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 2 more times. Ensure all the ethanol is removed from the plate.
11. Air-dry the tubes or plate at bench top away from the plate magnet for 10-20 minutes, ensuring the plate is completely dry before proceeding. Remove any remaining ethanol using a pipette.

12. With the reaction vessel at bench top, add 30 µL of DNase-free 1x TE to each tube or well. Vortex for 30 seconds. Ensure the beads are fully resuspended. Vortex longer if necessary.
13. Replace reaction vessel back on the plate magnet. Allow the beads to separate for 5 minutes or until the solution clears.
14. Using a pipette, remove the eluted sample and place into a fresh tube.

    Note: Small amounts of magnetic bead carryover may interfere with sample quantitation. Take care to minimize bead carryover.
15. Collect sample. There should be approximately 30 µL of purified cDNA.
16. Mix sample by vortexing, spin briefly, and proceed to measuring cDNA yield.

Zymo Clean and Concentrator Kit, Cat. # D4006
(instructions for a single reaction)

1. Into a clean 1.5 mL tube add 320 µL of DNA Binding Buffer.
2. Add 50 µL of amplified SPIA cDNA product.
3. Vortex and spin down briefly.
4. Obtain 1 Zymo-Spin II Column and place it into a collection tube.
5. Load the entire volume of sample (370 µL) onto the Zymo-Spin II Column.
6. Centrifuge column in the collection tube for 10 seconds at >10,000 x g in a microcentrifuge.
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 x g in a microcentrifuge. Discard flow-through.
10. Add 200 µL of room-temperature 80% ethanol.
11. Centrifuge column in the collection tube for 30 seconds at ≥ 10,000 x 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.

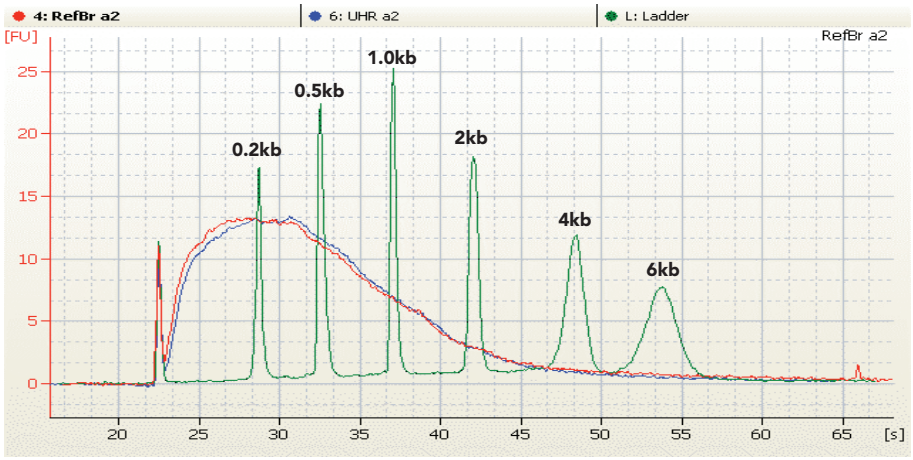
Use nuclease-free 1x TE at room temperature to elute sample.

13. Place the Zymo-Spin II Column in a clean 1.5 mL microcentrifuge tube. Add 30 µL of room-temperature nuclease-free 1x TE to the center of each Zymo-Spin II Column. Let columns stand for 1 minute at room temperature.
14. Centrifuge column and microcentrifuge tube for 30 seconds at maximum speed in a microcentrifuge.
15. Collect sample. There should be approximately 30 µL of purified cDNA.
16. Mix sample by vortexing, then spin briefly.
17. The purified cDNA may be stored at –20°C.

C. 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 Eukaryotic Total RNA Nano program (Nano assay in the Expert 2100 software) and following the manufacturer's instructions. Depending on availability of amplified product, you may choose to load less than 100 ng of amplified cDNA product on the Bioanalyzer chips. A typical size distribution trace may look like the one obtained from Human Reference Brain and Universal Human Reference total RNA (see Figure 4, below).

Figure 4. Bioanalyzer Trace of Amplified cDNA Product obtained from 10 ng of total Human Reference Brain and Universal Human Reference RNA



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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 ß-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 at 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. Pipette 30-50 µL RNase-free water directly onto the RNeasy membrane.

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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.

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

DNase Treatment of RNA post-purification: using RNase-free DNase and either the RNA Clean and 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 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 and Concentrator 5
   (Zymo Research, Cat. # R1015)

   - Add 4 volumes (100 µL) of RNA binding buffer to the sample.
   - Obtain one RNA Clean and Concentrator 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 vendor's 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 and Concentrator 5 column in a fresh 1.5 ml collection tube.
   - Add 10 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.

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

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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 96-100% ethanol and mix thoroughly by pipetting.
- Place an RNeasy MinElute Spin Column into a 2 ml collection tube (1 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. **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.

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E. Preventing Crossover Contamination

Due to the high sensitivity inherent in our amplification systems, we have a set of recommendations designed to minimize the potential for contamination of amplification reactions by carryover of nucleic acids or other laboratory contaminants. We strongly recommend implementing these procedures especially for high throughput and low RNA input environments typical in today's gene expression laboratories. Our 2 general recommendations are first to **designate separate workspaces for "pre-amplification" and "post-amplification" steps and materials.** This provides the best work environment for processing RNA using our highly sensitive amplification protocols. Our second recommendation is to **implement routine cleanup protocols for workspaces as standard operating procedure.** This will prevent contamination by amplification products, intermediaries, and exogenous nucleic acids from spreading through laboratory workspaces. Details regarding establishing and maintaining a 'clean' work environment are listed below:

1. Designate a pre-amplification workspace separate from the post-amplification workspace or general lab areas:
    a. Pre-amplification includes all steps and materials related to RNA sample handling and dilution, NuGEN's first strand reaction, second strand reaction, second strand product cleanup, and setup of the SPIA reactions.
    b. Post-amplification includes all steps and materials related to the handling of the amplified cDNA product, including the Post-SPIA Modification protocols, purification, array hybridization, and any other analytical work. This also includes any work and materials related to other non-NuGEN protocols.
    c. Ideally, pre-amplification workspace would be in a separate work room. If this is not possible, ensure the pre-amplification area is sufficiently distant and not in the path of post-amplification work.
    d. We recommend the use of "PCR Workstation" enclosure with UV illumination dedicated for NuGEN pre-amplification protocol.
    e. Materials and consumables for pre-amplification work should be regularly exposed to UV illumination to control nucleic acid surface contamination.
2. Establish and maintain a clean work environment:
    a. Initially clean the entire lab thoroughly with DNA-OFF and RNaseZap.
        i. In the pre-amplification area, remove all small equipment, and then clean every surface that may have been handled without gloves (drawer handles, keypads, etc.). Before reintroducing any equipment, clean every piece thoroughly. It is important to clean wells of thermal cycler(s) and magnetic plate(s) with a Q-tip or by filling with decontamination solution.
    b. Always wear gloves, and don fresh gloves upon entry into this controlled area. Frequently change gloves while working in the pre-amplification area, especially prior to handling stock reagents.

c. Stock this area with clean (preferably new) equipment (pipettes, racks, consumables) that has not been exposed to post-amplification workspace.

d. Make it a policy to carry out continual regular decontamination of all workspaces.

e. Capture waste generated in both pre- and post-amplification areas (tips, columns, wash solutions from beads and columns, tubes, everything) in sealable plastic bags and dispose of them promptly after each experiment to avoid waste spillage.

f. Do not open amplified product reaction vessels in the pre-amplification workspace.

3. Utilize negative controls in order to detect and troubleshoot a contamination issues. The clearest indication that an amplification reaction is contaminated is the appearance of significant amounts of amplified product in a 'negative' control or No Template Control (NTC). If negative controls are run, it is recommended that these reactions NOT be carried through the Post-SPIA Modification steps.

   a. **In the absence of contamination:**

      i. NTC yields for Ovation RNA-Seq System amplifications (following the SPIA step) are typically at or below 0.5 µg.

   b. **In the presence of contamination:**

      i. NTC yields are generally significantly higher than 0.5 µg, making NTC results the most reliable indicator of contamination.

4. When contamination is detected in reactions containing templates:

   a. The amount of product generated from a template containing amplification reaction may or may not be affected, depending on the source of the contamination.

   b. The bioanalyzer trace of the amplified product may or may not look altered.

F. Illumina RNA-Seq Library Preparation

The Ovation RNA-Seq System yields double-stranded cDNA ready for the end repair protocol and subsequent library construction. Reagents for performing these steps may be obtained from Illumina. Suitable options for completing library construction are the mRNA-Seq 8 Sample Prep Kit (Cat. # RS-100-0801) in which case only reagents for the second day of the protocol are required when used in conjunction with the Ovation RNA-Seq System, or the Paired End DNA Sample Prep Oligo Only Kit (Cat. # PE-102-1003), which provides kitted oligos and adapters used in the second day protocol.

VII. FAQs

Q1. Do I need to perform a rRNA depletion or Poly(A) enrichment step before processing with Ovation® RNA-Seq System?
rRNA depletion or Poly(A) enrichment is not necessary. The input range of 500 pg to100 ng refers to total RNA.

Q2. What materials are provided with the Ovation RNA-Seq System?
The Ovation RNA-Seq System provides all necessary buffers, primers and enzymes for first strand synthesis, second strand synthesis and amplification. The kit also provides nuclease-free water and Beckman Coulter Genomics RNAClean® magnetic beads for second strand reaction cDNA purification.

Q3. What equipment is required or will be useful?
Required equipment includes a microcentrifuge, pipettes, vortexer, a thermal cycler, a spectrophotometer, a fluorescence spectrophotometer, and magnetic plate. An Agilent Bioanalyzer may also be useful for optional analytical tests.

Q4. What additional consumables does the user need?
For the SPIA cDNA purification step, purification columns or magnetic beads are required. See user guide for validated purification products and procedures.

Q5. Do I need to use high quality total RNA?
The Ovation RNA-Seq System is designed to work with 500 pg to 100 ng of purified total RNA samples of high molecular weight with little or no evidence of degradation.

Q6. Can I do reactions in smaller batches than 4?
We recommend a minimum batch size of 4 reactions. Smaller batch sizes may result in difficulty pipetting small volumes and lead to poor performance.

Q7. How much total RNA do I need for amplification?
The Ovation RNA-Seq System can be used with purified total RNA in the range of 500 pg to 100 ng. Input amounts outside this range may produce unsatisfactory and variable results.

Q8. How much cDNA can I expect from a single reaction?
You should expect approximately 3 µg of cDNA in 30 µL volume ready for Illumina library preparation.

Q9. Is the cDNA yield dependent upon the quantity of total RNA input?
Yes, higher inputs of will lead to higher amplification yields.

Q10. What size cDNA is generated by the Ovation RNA-Seq System?
On a Bioanalyzer, using the RNA 6000 size markers, the median length of the amplified cDNA is approximately 200 bases, with the longest fragments up to 1,500 bases.

Q11. What size range should I excise from the gel for Illumina library preparation?
Best results are obtained when material corresponding to the peak of the bioanalyzer trace is excised for library preparation. In most cases, this corresponds to ~ 250 – 350 bp in length.

VII. FAQs

Q12. Can DNA be used as input for the Ovation RNA-Seq System?
No. The Ovation RNA-Seq System is designed to amplify mRNA, not DNA.

Q13. Can contaminating genomic DNA interfere with the Ovation RNA-Seq System performance?
When using purified total RNA samples, large amounts of contaminating genomic DNA may amplify during the process. For this reason we recommend DNase treatment during RNA purification.

Q14. Can I use the Ovation RNA-Seq System on bacterial RNA samples?
The Ovation amplification process theoretically will work with many bacterial RNAs; however, the kit has not been optimized for this purpose.

Q15. Are there any tissues that will not work with the Ovation RNA-Seq System?
We have not encountered any specific RNA sources that will not work with the Ovation System.

Q16. Has NuGEN performed reproducibility studies on the Ovation RNA-Seq System?
Yes. Sample-to-sample, lot-to-lot, and operator-to-operator reproducibility studies are routinely conducted.

Q17. Does the Ovation RNA-Seq System generate product in the absence of RNA input?
In the complete absence of input RNA, approximately 0.5 µg or less of non-specific product is generated prior to Post-SPIA modification. However, in the presence of even a very small amount of RNA the amplified cDNA has been demonstrated to be specific.

Q18. How many rounds of amplification are performed with the Ovation RNA-Seq System?
This System performs a single round of amplification and is not designed to support multiple rounds of amplification.

Q19. Do I need to order specific primers for the amplification?
No. The DNA/RNA primers provided in the Ovation RNA-Seq System are universal. No gene-specific primers are required.

Q20. Do I have to use the supplied DNA/RNA primers?
The Ovation RNA-Seq System will not work properly with other primers.

Q21. Do you recommend purification of the amplified cDNA prior to qPCR analysis?
No. It is recommended that qPCR be performed using the amplified cDNA prior to the Post-SPIA Modification steps. A small aliquot can be removed immediately following SPIA, diluted and used directly for qPCR. Refer to Appendix A of the User Guide for recommendations on achieving optimal results.

VII. FAQs

Q22. What purification methods do you recommend?
For the double-stranded cDNA purification step (pre-amplification) we require the use of the RNAClean magnetic beads provided with the Ovation RNA-Seq kit. For the amplified cDNA purification step we suggest using one of the methods described in Appendix B of this User Guide.

Q23. How do I measure my amplified cDNA product?
See Protocol L of this document.

Q24. Can I use an Agilent Bioanalyzer to evaluate the amplification products?
Yes. Refer to Appendix C for guidelines.

Q25. Where can I safely stop in the protocol?
It is safe to stop after the SPIA amplification step prior to the Post-SPIA Modification or after Post-SPIA Modification II prior to final cleanup. Store reaction products at -20°C.

Q26. Do you recommend DNase treatment of purified total RNA samples?
Yes. For an explanation of DNase requirements see section III.A.4. For DNase treatment of RNA samples, refer to Appendix D for guidelines.

Q27. Can I use this product on Life Technologies SOLiD or Roche 454 sequencing systems?
Validation on these platforms is ongoing. Contact technical services for the most recent information on the use of Ovation RNA-Seq System for sample preparation on these, and other sequencing platforms.

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