USER GUIDE

Ovation® RNA-Seq System V2

PART NO. 7102

NuGEN

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I. Introduction

A. Background

The Ovation® RNA-Seq System V2 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 transcriptome in the sample. This feature makes the Ovation RNA-Seq System V2 ideal for amplification prior to Next Generation Sequencing, as reads are distributed across the transcript. This, our second generation RNA-Seq System, has a streamlined protocol which takes only 4.5 hours to complete. The Ovation RNA-Seq System V2 is suitable for sequencing library construction for use with a variety of deep-sequencing platforms. It may also be suitable for use in qPCR applications.

The Ovation RNA-Seq System V2 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. Isolated poly(A)+ RNA or mRNA may be substituted for studies focused on the analysis of mature coding transcripts. Ribo-SPIA contributes minimal coverage bias which has been shown to be highly reproducible. After purification, the cDNA can be fragmented to the appropriate size and ligated into Ovation Ultralow Library Systems, Ovation Rapid Library Systems or other suitable library construction methods.

The Ovation RNA-Seq System V2 (Part No. 7102) provides optimized reagent mixes and a protocol to process total RNA samples. Control RNA is not provided with the Ovation RNA-Seq System V2, but we recommend amplifying a high-quality commercial RNA as a positive control when first using this product.

B. Ribo-SPIA® Technology

Ribo-SPIA technology is a process that generates amplified cDNA from as little as 500 pg of total RNA.

1. **Generation of First Strand cDNA (1 hour)**
   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 (2 hours)**
   Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthesize a second strand, which includes DNA complementary to the 5′ unique sequence of the first strand chimeric primers. The result is a double-stranded cDNA with a unique DNA/RNA heteroduplex at one end.

3. **SPIA® Amplification (1.5 hours)**
   SPIA is a robust isothermal strand-displacement 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 SPIA cDNA.

C. Performance Specifications

The Ovation RNA-Seq System V2 synthesizes microgram quantities of SPIA cDNA starting with total cellular RNA input amounts of 500 pg to 100 ng. In approximately 4.5 hours, the Ovation RNA-Seq System V2 produces a minimum of 2.5 µg of amplified SPIA cDNA ready for sequencing library construction using an Ovation Ultralow Library System, Ovation Rapid Library System or other suitable library construction method. Reduced amplification yield may be experienced when using RNA inputs below 500 pg or with lower quality RNA samples.

When used with intact total RNA, the size of the cDNA products produced by the Ovation RNA-Seq System V2 is typically between 200 base pairs (bp) and 1,500 bp.

D. Quality Control

Each Ovation RNA-Seq System V2 lot is tested to meet minimum performance specifications.

E. Storage and Stability

! *This product contains components with multiple storage temperatures.*

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

The vial labeled First Strand Enzyme Mix (blue: A3), should be removed from the shipping carton upon delivery and stored separately at –80°C.

The vial labeled Agencourt® Beads (clear cap) should be removed from the top of the shipping carton upon delivery and stored at 4°C.

! *Store First Strand Enzyme Mix (blue: A3) at –80°C and the Agencourt beads at 4°C*

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

This product has been tested to perform to specifications after as many as six freeze/thaw cycles. Kits handled and stored according to the above guidelines will perform to

specifications for at least six months. NuGEN has not yet established long-term storage conditions for the Ovation RNA-Seq System V2.

F. Safety Data Sheet (SDS)

If appropriate, an SDS for this product is available on the NuGEN website at http://www.nugen.com/products/ovation-rna-seq-system-v2

II. Kit Components

A. Reagents Provided

Table 1. First Strand cDNA Reagents

COMPONENT	7102-08 PART NUMBER	7102-32 PART NUMBER	7102-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
First Strand Primer Mix	S01278	S01373	S01390	Blue	A1 VER 4
First Strand Buffer Mix	S01174	S01374	S01391	Blue	A2 VER 3
First Strand Enzyme Mix	S02250	S02269	S02270	Blue	A3 VER 7

Table 2. Second Strand cDNA Reagents

COMPONENT	7102-08 PART NUMBER	7102-32 PART NUMBER	7102-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
Second Strand Buffer Mix	S01176	S01376	S01386	Yellow	B1 VER 3
Second Strand Enzyme Mix	S01126	S01377	S01290	Yellow	B2 VER 2

Table 3. SPIA Reagents

COMPONENT	7102-08 PART NUMBER	7102-32 PART NUMBER	7102-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
SPIA Primer Mix	S01279	S01378	S01393	Red	C1 VER 9
SPIA Buffer Mix	S01410	S01412	S01414	Red	C2 VER 11
SPIA Enzyme Mix	S01411	S01413	S01415	Red	C3 VER 7

Table 4. Additional Reagents

COMPONENT	7102-08 PART NUMBER	7102-32 PART NUMBER	7102-A01 PART NUMBER	VIAL LABEL	VIAL NUMBER
Nuclease-free Water	S01001	S01001	—	Green	D1
Agencourt Beads	S01307	S01307	S01307	Clear	—

Note: The reagents in the Ovation RNA-Seq System V2 product are similar to reagents in our 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, for example, A1 with another A1, as it will adversely affect performance.

Ovation RNA-Seq V2 plus Ovation Ultralow V2 Bundles (Part Nos. 0505NB-32, 0510-32, 0511-A01)

The Ovation RNA-Seq V2 System (Part No. 7102) and the Ovation Ultralow System V2 (Part No. 0344 or 0347) can now be purchased together as a bundle to provide a complete workflow from total or poly(A) selected RNA to libraries ready for sequencing. The bundled version of these products includes all the same components as the individual kits with the exception of Agencourt beads, which are not included in Part Nos. 0505NB-32 and 0511-A01. Agencourt beads can be sourced directly from Beckman Coulter, www.beckmancoulter.com

The entire workflow for 32 reactions requires a minimum of 14 mL of bead solution.

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - Covaris S-series Sonication System
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - 0.5–10 µL pipette, 2–20 µL pipette, 20–200 µL pipette, 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® UV-Vis Spectrophotometer

II. Kit Components

- **Reagents**
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps
  - 1X TE buffer (pH=8.0)
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - Low-retention microcentrifuge tubes (SafeSeal Low Binding 0.65 mL Microcentrifuge Tubes, Sorenson Biosciences, Inc., Cat. #11300)
  - 1.5 mL amber, DNase-free microcentrifuge tubes
  - 0.2 mL individual thin-wall PCR tubes, 8 X 0.2 mL strip PCR tubes or 0.2 mL thin-wall PCR plates
  - SPRIPlate® 96R, Ring Magnet Plate (Beckman Coulter, Cat. #A29164) or SPRIPlate Ring Super Magnet Plate, (Beckman Coulter, Cat. #A32782). Other magnetic stands may be used as well, although their performance has not been validated by NuGEN.
  - Purification options for final SPIA cDNA purification (select one option):
    - (Recommended) MinElute® Reaction Cleanup Kit (QIAGEN, Cat. #28204)
    - Agencourt Kit (Beckman Coulter, Cat. #A63881)
    - QIAquick® PCR Purification Kit (QIAGEN, Cat. #28104)
    - DNA Clean & Concentrator™-25 (Zymo Research, Cat. #D4005/D4006)
  - Cleaning solutions such as RNaseZap® (Thermo Fisher Scientific, Cat. #AM9780) and DNA-OFF™ (MP Biomedicals, Cat. #QD0500)
  - Disposable gloves
  - Kimwipes
  - Ice bucket
- **Optional Materials**
  - Agilent 2100 Bioanalyzer or materials and equipment for electrophoretic analysis of RNA
  - Real-time PCR system

To Order:

- Beckman Coulter, www.beckmancoulter.com
- Invitrogen Life Technologies, www.invitrogen.com
- MP Biomedicals, www.mpbio.com
- QIAGEN Inc., www.qiagen.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com
- Sorenson Biosciences, Inc., www.sorbio.com
- Thermo Fisher Scientific, www.thermofisher.com

III. Planning the Experiment

A. Input RNA Requirements

It is important to assess the quality of your RNA sample prior to planning your amplification. While the Ovation RNA-Seq System V2 is designed for use with high quality RNA samples, it may allow the amplification of many RNA samples of variable quality. Use of degraded RNA samples can lead to lower yields and shorter SPIA cDNA. It is impossible to guarantee success with all degraded RNA samples. To assess RNA quality prior to using the Ovation RNA-Seq System V2, follow the guidelines below.

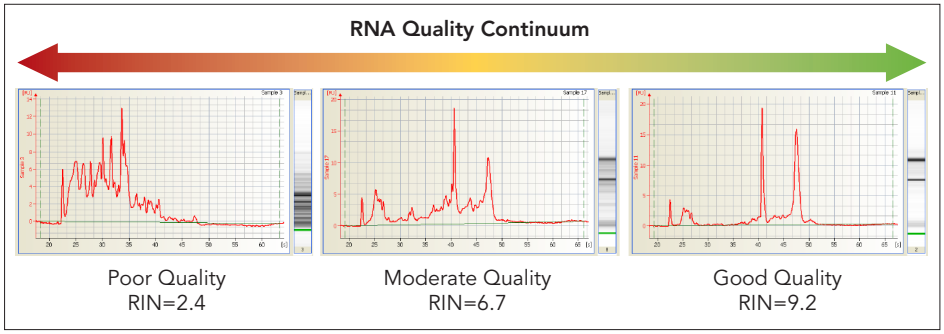
1. **RNA Quantity**
   Total RNA input must be between 500 pg and 100 ng. Total RNA inputs above 100 ng per reaction may inhibit amplification, while inputs of less than 500 pg will potentially result in insufficient yields depending on the requirements of the analytical platform. We strongly recommend quantitation of total RNA to assure the minimum input requirement is met. Isolated poly(A)+ RNA or mRNA may be substituted for studies focused on the analysis of mature coding transcripts. Please see the FAQ section for further details.

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 a column purification after isolation to remove any residual organic solvents that may be present. One measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The A260:A280 ratio for RNA samples 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. Due to the whole transcriptome amplification approach, lower quality RNA samples may also be amplified successfully using the Ovation RNA-Seq System V2.

   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 estimating RNA integrity prior to amplification, both visually, with a detailed electrophoretic trace of the RNA, and computationally, by calculating a RIN score. 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 1. This continuum of RNA quality shows Bioanalyzer traces of three different RNAs with varying levels of degradation. High-quality RNAs have been shown to amplify robustly with this kit. Use of lower quality mRNAs may result in lower amplification yields.



4. **DNase Treatment**
   It is generally recommended to use DNase-treated RNA for amplification using the Ovation RNA-Seq System V2. The presence of genomic DNA in the RNA sample may potentially have adverse effects on downstream analytical platforms. Contaminating genomic DNA may be amplified along with the RNA. Additionally, 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 nucleic acid based carriers during RNA purification because many have been shown to produce cDNA product in first strand synthesis. We also advise against the use of glycogen in RNA isolation, as it inhibits reverse transcription. For the latest information regarding other carriers, contact our technical services team.

B. 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.
- Clean work areas and instruments, including pipettes, with commercially available cleaning reagents, such as RNaseZap.
- Use only new RNase-free pipette tips and microcentrifuge tubes.
- Use a work area specifically designated for RNA work and do not use other high copy number materials in the same area.

C. RNA Storage

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

D. SPIA cDNA Storage

The amplified SPIA cDNA produced by the Ovation RNA-Seq System V2 may be stored at –20°C.

IV. Protocol

A. Overview

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

1. First strand cDNA synthesis	1 hour
2. Second strand cDNA synthesis and purification	2 hours
3. SPIA amplification and purification	1.5 hours
Total time to prepare amplified cDNA	**~4.5 hours**

Ovation RNA-Seq System V2 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 sequencing library preparation.

B. Protocol Notes

- We strongly recommend the SPIA cDNA produced by this system be fragmented to an appropriate size prior to use in sequencing library construction protocols. Failure to do so may lead to poor performance in sequencing library construction or produce poor sequencing results. Please refer to Appendix C for further details.
- We recommend the routine use of a positive control RNA. Especially the first time you set up an amplification reaction, the use of a positive control RNA will allow the establishment of a baseline of performance and provide the opportunity to become familiar with the bead purification step. This step may be unfamiliar to many users and can be especially prone to handling variability in using the magnet plate, so a practice run with the plate is highly recommended.
- The level of rRNA present may fluctuate among different sample types and may result in varying representation of ribosomal content in the sequencing data.
- When working with very small, picogram amounts of RNA we strongly recommend the use of low retention tubes for storage and dilution of the samples in order to reduce the loss of RNA samples due to adhesion to polypropylene surfaces.
- Set up no fewer than 4 reactions at a time. This ensures sufficient reagent recoveries for the full nominal number of amplifications from the kit. Making master mixes for fewer than 4 samples at a time may affect reagent recovery volumes.
- Due to the high sensitivity inherent in this amplification system, we strongly recommend taking measures to minimize the potential for the carryover of previously amplified SPIA cDNA into new amplification reactions. The two steps to accomplish this are: 1. Designating separate workspaces for "pre-amplification" and "post-amplification" steps and materials, and 2. Implementing routine

clean-up protocols for workspaces as standard operating procedure. A detailed set of these recommendations is listed in the Appendix.

- Thaw components used in each step and immediately place them on ice. It is best not to thaw all reagents at once.
- 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.
- Always keep thawed reagents and reaction tubes on ice unless otherwise instructed.
- After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve 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.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer.
- When instructed to pipet mix, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.
- Always allow the thermal cycler to reach the 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 8 reactions in the kit. The Ovation RNA-Seq System V2 Quick Protocol will automatically calculate an appropriate overfill volume which can be used as a guideline in setting up master mixes.
- Components and reagents from other Ovation System products should not be used with this product.
- 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. Agencourt Purification Beads

Tips and Notes Relevant to the Second Strand cDNA Cleanup, Section IV. Part G

There are significant modifications to the Beckman Coulter Agencourt 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 V2. However, you may review the Agencourt Kit user guide to become familiar with the manufacturer's recommendations.

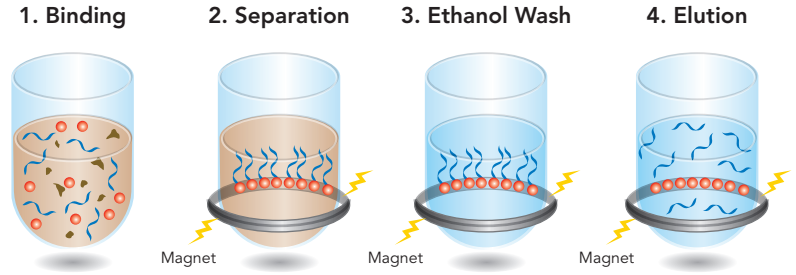
The bead purification process used for cDNA purification before amplification consists of:

1. Binding of cDNA to Agencourt beads
2. Magnetic separation of beads from supernatant
3. Ethanol wash of bound beads to remove contaminants

IV. Protocol

Elution takes place upon addition of the SPIA Master Mix. At this stage the beads are left in the cDNA tube and removed only after amplification.

Figure 2. Agencourt bead purification process overview.



Reproduced from original picture from Agencourt/Beckman Coulter Genomics

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 specific sample to bead volume ratios in our Agencourt bead protocols. In many cases our recommendations differ from the standard Beckman Coulter 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 45 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.
- 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 15 to 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 stable 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 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	(For RNA inputs ≤1 ng) 65°C – 2 min, hold at 4°C (For RNA inputs >1 ng) 65°C – 5 min, hold at 4°C
Program 2 First Strand Synthesis	4°C – 1 min, 25°C – 10 min, 42°C – 10 min, 70°C – 15 min, hold at 4°C
SECOND STRAND cDNA SYNTHESIS	
Program 3 Second Strand Synthesis	4°C – 1 min, 25°C – 10 min, 50°C – 30 min, 80°C – 20 min, hold at 4°C
SPIA AMPLIFICATION	
Program 4 SPIA Amplification	4°C – 1 min, 47°C – 60 min, 80°C – 20 min, hold at 4°C

Important Note: Carry out steps E (First-Strand cDNA Synthesis) through H, step #8 (SPIA Amplification) in a pre-amplification workspace using dedicated pre-amplification consumables and equipment. Wipe all surfaces, equipment and instrumentation with a DNA removal solution such as DNA-OFF (MP Biomedicals, Cat. #QD0500) to avoid the potential introduction of previously amplified cDNA into new amplifications. For more information on our recommendations for workflow compartmentalization and routine lab cleanup please refer to Appendix F of this user guide. If you have any questions on this important topic, please contact NuGEN Technical Services (techserv@nugen.com, (888) 654-6544).

E. First Strand cDNA Synthesis

1. Obtain the First Strand Primer Mix (blue: A1), First Strand Buffer Mix (blue: A2) and the water (green: D1) from –20°C storage and the First Strand Enzyme Mix (blue: A3) from –80°C storage.

Do not vortex any enzyme mixes.

2. Spin down the contents of A3 and place on ice.
3. Thaw the other reagents at room temperature. Mix by vortexing, spin and place on ice. Leave Nuclease-free Water at room temperature.
4. Add 2 µL of A1 to a 0.2 mL PCR tube.
5. Add 5 µL of total RNA sample (500 pg to 100 ng) to the primer.
6. Mix by pipetting 5 times, spin and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 5):

   a. For RNA inputs of 500 pg to 1 ng: 65°C – 2 min, hold at 4°C

   b. For RNA inputs >1 ng: 65°C – 5 min, hold at 4°C

8. Remove the tubes from the thermal cycler and place 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 6.

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

FIRST STRAND BUFFER MIX (BLUE: A2 VER 3)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 7)
2.5 µL	0.5 µL

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 5 times, spin and place on ice.

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

12. Place the tubes in a pre-cooled thermal cycler programmed to run Program 2 (First Strand Synthesis; see Table 5):

    4°C – 1 min, 25°C – 10 min, 42°C – 10 min, 70°C – 15 min, hold at 4°C

13. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
14. Continue immediately with the Second Strand cDNA Synthesis protocol.

F. Second Strand cDNA Synthesis

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

1. Remove the Agencourt purification beads (supplied with the Ovation RNA-Seq System V2) from 4°C storage and place on the bench top to reach room temperature for use in the next step.
2. Obtain the Second Strand Buffer Mix (yellow: B1) and the Second Strand Enzyme Mix (yellow: B2), from –20°C storage.
3. Spin down the contents of B2 and place on ice.
4. Thaw reagent B1 at room temperature, mix by vortexing, spin and 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 7.

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

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

SECOND STRAND BUFFER MIX (YELLOW: B1 VER 3)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER 2)
9.7 µL	0.3 µL

6. Add 10 µL of the Second Strand Master Mix to each First Strand reaction tube.
7. Mix by pipetting 5 times, spin and place on ice.
8. Place the tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand Synthesis; see Table 5):

   4°C – 1 min, 25°C – 10 min, 50°C – 30 min, 80°C – 20 min, hold at 4°C
9. Remove the tubes from the thermal cycler and spin to collect condensation. Place in a rack on the bench top.
10. Continue immediately with the Purification of cDNA protocol.

G. Purification of cDNA

1. Ensure the Agencourt beads have completely reached room temperature before proceeding.
2. Prepare a 70% 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. Resuspend the beads by inverting and tapping the tube. Ensure that the beads are fully resuspended before adding to the sample. 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) of the bead suspension to each reaction and mix by pipetting 10 times.
5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.

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

7. Keeping the tubes on the magnet, carefully remove only 45 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

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

8. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact cDNA yields, so ensure beads are not removed with the binding buffer or the washes.

9. Remove the 70% ethanol wash using a pipette.
10. Repeat the wash two more times.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least 2 pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

11. Air-dry the beads on the magnet for 15 to 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 SPIA amplification.
12. Continue immediately with the SPIA Amplification protocol with the cDNA still bound to the dry beads.

H. SPIA Amplification

1. Obtain the SPIA Primer Mix (red: C1), SPIA Buffer Mix (red: C2) and SPIA Enzyme Mix (red: C3) from –20°C storage.
2. Thaw C3 on ice and mix the contents by inverting gently 5 times. Ensure the enzymes are well mixed without introducing bubbles, spin and place on ice.
3. Thaw reagents C1 and C2 at room temperature, mix by vortexing, spin and place on ice.

! *Use SPIA Master Mix immediately after preparation.*

4. Make a master mix by sequentially combining C2, C1 and C3 in an appropriately sized, capped tube according to the volumes shown in Table 8.

   Note: Make sure the addition of C3 is at the last moment.

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

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

SPIA BUFFER MIX (RED: C2 VER 11)	SPIA PRIMER MIX (RED: C1 VER 9)	SPIA ENZYME MIX (RED: C3 VER 7)
20 µL	10 µL	10 µL

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 thoroughly by pipetting 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: The 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 from the beads.

6. Place the tubes in a pre-cooled thermal cycler programmed to run Program 4 (SPIA Amplification, see Table 5):

   4°C – 1 min, 47°C – 60 min, 80°C – 20 min, hold at 4°C

7. Remove the tubes from thermal cycler, spin to collect condensation and place on ice. **Do not re-open the tubes in the pre-amplification workspace.**

Important Note: At this point the tubes should be removed from the pre-amplification workspace. Carry out all remaining steps in a post-amplification workspace using dedicated post-amplification consumables and equipment. Take care to avoid the introduction of previously amplified cDNA into your pre-amplification workspace. For more information on our recommendations for workflow compartmentalization and routine lab cleanup, please refer to Appendix F of this user guide. If you have any questions on this important topic, please contact NuGEN Technical Services (techserv@nugen.com, (888) 654-6544).

8. Transfer the tubes to the magnet and let stand for 5 minutes to completely clear the solution of beads.
9. Carefully transfer 40 µL of the cleared supernatant containing the SPIA cDNA to a fresh tube.
10. Continue immediately with the Purification of SPIA cDNA protocol or store the reaction products at –20°C prior to continuing.

IV. Protocol

I. Purification of SPIA cDNA

We recommend using the QIAGEN MinElute Reaction Cleanup Kit for the purification of the amplified SPIA cDNA produced with the Ovation RNA-Seq System V2. Refer to Appendix A for additional purification options.

QIAGEN MinElute Reaction Cleanup Kit (instructions for a single full reaction)

Important Notes:

- Buffer ERC is considered hazardous according to QIAGEN, and an MSDS may be consulted.
- Add the appropriate amount of 100% ethanol to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at maximum speed in a conventional tabletop microcentrifuge at room temperature.

1. Into a clean, labeled 1.5 mL microcentrifuge tube, add 300 µL of Buffer ERC from the QIAGEN kit.
2. Add the entire volume (40 µL) of the SPIA reaction to the tube.
3. Vortex for 5 seconds, then spin briefly.
4. Obtain and label a MinElute spin column and place it into a collection tube.
5. Load the sample/buffer mixture onto the column.
6. Centrifuge for 1 minute at maximum speed in a microcentrifuge.
7. Discard the flow-through and replace the column in the same collection tube.
8. Add 750 µL of Buffer PE to the column.
9. Centrifuge for 1 minute at maximum speed.
10. Discard the flow-through and replace the column in the same collection tube.
11. Centrifuge the column for an additional 2 minutes at maximum speed to remove all residual Buffer PE.

    Note: Residual ethanol from the wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. Discard the flow-through along with the collection tube. Blot the column tip onto clean, absorbant paper to remove any residual wash buffer from the tip of the column.

    Note: Blotting the column tip prior to transferring it to a new tube is necessary to prevent any wash buffer transferring to the eluted sample.
13. Place the column into a clean, labeled 1.5 mL microcentrifuge tube.

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

Use 1X TE or Buffer EB at room temperature to elute sample

14. Add 22 µL of room temperature 1X TE or Buffer EB to the center of each column.

    Note: Ensure that the elution buffer is dispensed directly onto the membrane for complete elution of the bound cDNA.

15. Let the column stand for 1 minute at room temperature.
16. Centrifuge for 1 minute at maximum speed.
17. Discard the column and measure the volume recovered. There should be approximately 20 µL of purified SPIA cDNA.
18. Mix the sample by vortexing, then spin briefly.
19. Continue with the Measuring SPIA cDNA Yield and Purity protocol or store the purified SPIA cDNA at –20°C.

J. Measuring SPIA cDNA Yield and Purity

1. Mix the purified SPIA cDNA sample by brief vortexing and spinning prior to checking the concentration.
2. Measure the absorbance at 260, 280 and 320 nm. You may need to make a 1:20 dilution of the cDNA in water prior to measuring the absorbance.
3. Purity: Subtract the A320 value from both A260 and A280 values. The adjusted (A260–A320) / (A280–A320) ratio should be >1.8.
4. Yield: Assume 1 A260 unit = 50 µg/mL.

   To calculate: (A260–A320 of diluted sample) X (dilution factor) X 50 (concentration in µg/mL of a 1 A260 unit solution) X 0.02 (final volume in mL) = total yield in micrograms

   Note: Alternatively, you may measure the concentration and purity of cDNA with a Nanodrop by setting 1 A260 unit = 50 µg/mL as the constant.

5. The purifed cDNA may be stored at –20°C.

V. Technical Support

For help with any of our products, please contact NuGEN Technical Support at 650.590.3674 (direct) or 888.654.6544, option 2 (toll-free, U.S. only). You may also send faxes to 888.296.6544 (toll-free) or email techserv@nugen.com.

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

In all other locations, contact your NuGEN distributor for technical support.

VI. Appendix

A. Additional Purification Options for SPIA cDNA

We recommend using the QIAGEN MinElute Reaction Cleanup Kit for the purification of the amplified SPIA cDNA produced with the Ovation RNA-Seq System V2 due to its low elution volume. Alternatively, there are three other options for purification of SPIA cDNA. Listed alphabetically, they are: 1) Beckman Coulter Agencourt Kit, 2) QIAGEN QIAquick PCR Purification Kit and 3) the Zymo DNA Clean & Concentrator™-25.

Selection of the optimum purification option can depend on many factors. Generally speaking, the column based options provide for superior recovery (higher amplification yield) while the Agencourt bead method is more scalable for larger batch sizes and can be easily automated. We have found that the Agencourt bead method may result in somewhat lower amplification yields, so this option may not be appropriate for the most demanding applications where small and/or degraded RNA samples are anticipated. Please contact the NuGEN Technical Support Team for assistance in selecting the appropriate purification option for your application.

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 Kit (instructions for a single reaction)

Important Notes:

- Prepare a room temperature 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.
- The use of 96-well microplates and multi-channel pipettes is recommended for processing large batches with this procedure.

1. Obtain the Agencourt bead bottle from 4°C storage. Allow the bead solution to reach room temperature.
2. Invert the Agencourt bead bottle several times to ensure the beads are fully in suspension. It may be necessary to remix the bead stock from time to time to ensure beads remain in suspension while in use.
3. At room temperature, add 72 µL of resuspended Agencourt beads to the 40 µL SPIA reaction (1.8 times the sample volume).
4. Mix the sample and beads thoroughly by pipetting 10 times.
5. Incubate sample/bead mixture at room temperature for 5 minutes.
6. Transfer the tube to the magnet and let stand 10 minutes to completely clear the solution of beads.

! *Best results are obtained by using fresh 80% ethanol in wash steps. Lower percent ethanol mixes will reduce recovery.*

7. Keeping the tube on the magnet, carefully remove and discard the supernatant. Do not disturb the ring of beads.

   Note: Leaving several microliters of supernatant behind at this step can help minimize bead loss.

8. Keeping the tube on the magnet, add 200 µL of freshly prepared 80% ethanol to each tube and allow to stand for 30 seconds or until the solution clears. Add slowly so as to not disturb the separated magnetic beads.
9. Carefully remove and discard the ethanol.
10. Repeat the 80% ethanol wash once more. Ensure as much ethanol as possible is removed from the plate after the final wash.

    Note: With the final wash, it is important to remove as much of the ethanol as possible. Use at least 2 pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

11. Remove the tubes from the magnet and air dry on the bench top for no more than 2 minutes. If the beads dry too long, they are difficult to resuspend.

! *Use 1X TE at room temperature to elute sample.*

12. Add 33 µL of room temperature 1X TE to each tube.
13. Resuspend the beads by repeated pipetting. Alternatively, the beads may be resuspended by carefully vortexing the tubes for 30 seconds or using a plate shaker set to medium speed. Ensure the beads are fully resuspended. Vortex longer if necessary.
14. Replace the tube on the magnet and allow the beads to separate for 5 minutes or until the solution clears.
15. Carefully remove the eluted sample and place into a fresh tube.

    Note: Small amounts of bead carryover may interfere with sample quantitation. Take care to minimize bead carryover.

16. Continue with the Measuring SPIA cDNA Yield and Purity protocol or store the purified SPIA cDNA at –20°C.

VI. Appendix

QIAGEN QIAquick PCR Purification Kit (instructions for a single reaction)

Important Notes:

- 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.
- All centrifugation steps are carried out at 17,900 X g (13,000 RPM) in a conventional tabletop microcentrifuge at room temperature.
- It is not necessary to add the pH Indicator I from the QIAGEN kit to Buffer PB for this protocol.

1. Into a clean, labeled 1.5 mL microcentrifuge tube, add 250 µL of Buffer PB from the QIAGEN kit.
2. Add the entire volume (40 µL) of the SPIA reaction to the tube.
3. Vortex for 5 seconds, then spin briefly.
4. Obtain and label a QIAquick spin column and place it into a collection tube.
5. Load the sample/buffer mixture onto the column.
6. Centrifuge for 1 minute at 17,900 X g in a microcentrifuge.
7. Discard the flow-through and replace the column in the same collection tube.

! *Best results are obtained by using fresh 80% ethanol in wash steps. Lower percent ethanol mixes will reduce recovery.*

8. Add 700 µL of 80% ethanol to the column.
9. Centrifuge for 1 minute at 17,900 X g.
10. Discard the flow-through and replace the column in the same collection tube.
11. Repeat steps 8 through 10 once.
12. Centrifuge the column for an additional 2 minutes at 17,900 X g to remove all residual ethanol.

    Note: Residual ethanol from the wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation.
13. Discard the flow-through along with the collection tube. Blot the column tip onto clean, absorbant paper to remove any residual wash buffer from the tip of the column.

    Note: Blotting the column tip prior to transferring it to a new tube is necessary to prevent any wash buffer transferring to the eluted sample.
14. Place the column into a clean, labeled 1.5 mL microcentrifuge tube.

! *Use 1X TE or Buffer EB at room temperature to elute sample.*

15. Add 30 µL of room temperature 1X TE or Buffer EB to the center of each column.

    Note: Ensure that the elution buffer is dispensed directly onto the membrane for complete elution of the bound cDNA.

16. Let the column stand for 5 minutes at room temperature.
17. Centrifuge for 1 minute at 17,900 X g.
18. Discard the column and measure the volume recovered. There should be approximately 28 µL of purified SPIA cDNA.
19. Mix the sample by vortexing, then spin briefly.
20. Continue with the Measuring SPIA cDNA Yield and Purity protocol or store the purified SPIA cDNA at –20°C.

VI. Appendix

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

Important Notes:

- Zymo Research has two products sharing the DNA Clean & Concentrator-25 name, Cat. #4005/4006 and Cat. #4033/4034. Make certain to use only Cat. #4005/4006 with this protocol. Do not use Cat. #4033/4034 as this will result in low yields.
- Prepare room temperature 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 yield.
- All centrifugation steps are carried out at 10,000 X g in a conventional tabletop microcentrifuge at room temperature.
- When instructed to centrifuge for durations of less than 1 minute, allow the centrifuge to reach the target RCF before starting the timer.

1. Into a clean, labeled 1.5 mL microcentrifuge tube, add 320 µL of DNA Binding Buffer from the Zymo kit.
2. Add the entire volume (40 µL) of the SPIA reaction to the tube.
3. Vortex for 5 seconds, then spin briefly.
4. Obtain and label a Zymo Spin-II column and place it into a collection tube.
5. Load the sample/buffer mixture onto the column.
6. Centrifuge for 10 seconds at 10,000 X g in a microcentrifuge. Allow the centrifuge to reach full speed before starting the timer.
7. Discard the flow-through and replace the column in the same collection tube.
8. Add 200 µL of 80% ethanol to the column. Do not use the Wash Buffer provided in the Zymo kit.
9. Centrifuge for 10 seconds at 10,000 X g.
10. Discard the flow-through and replace the column in the same collection tube.
11. Add an additional 200 µL of 80% ethanol to the column.
12. Centrifuge for 90 seconds at 10,000 X g.

    Note: Extending the centrifugation time here helps ensure all residual ethanol is removed from the column.
13. Discard the flow-through along with the collection tube. Blot the column tip onto clean, absorbant paper to remove any residual wash buffer from the tip of the column.

    Note: Blotting the column tip prior to transferring it to a new tube is necessary to prevent any wash buffer transferring to the eluted sample.
14. Place the column into a clean, labeled 1.5 mL microcentrifuge tube.

! *Best results are obtained by using fresh 80% ethanol in wash steps. Lower percent ethanol mixes will reduce recovery.*

Use 1X TE at room temperature to elute sample.

15. Add 30 µL of room temperature 1X TE to the center of the column.

    Note: Ensure that the elution buffer is dispensed directly onto the membrane for complete elution of the bound cDNA.

16. Let the column stand for 1 minute at room temperature.
17. Centrifuge for 30 seconds at 10,000 X g.
18. Discard the column and measure the volume recovered. There should be approximately 28 µL of purified SPIA cDNA.
19. Mix the sample by vortexing, then spin briefly.
20. Continue with the Measuring SPIA cDNA Yield and Purity protocol or store the purified SPIA cDNA at –20°C.

VI. Appendix

B. Quality Control of the SPIA cDNA

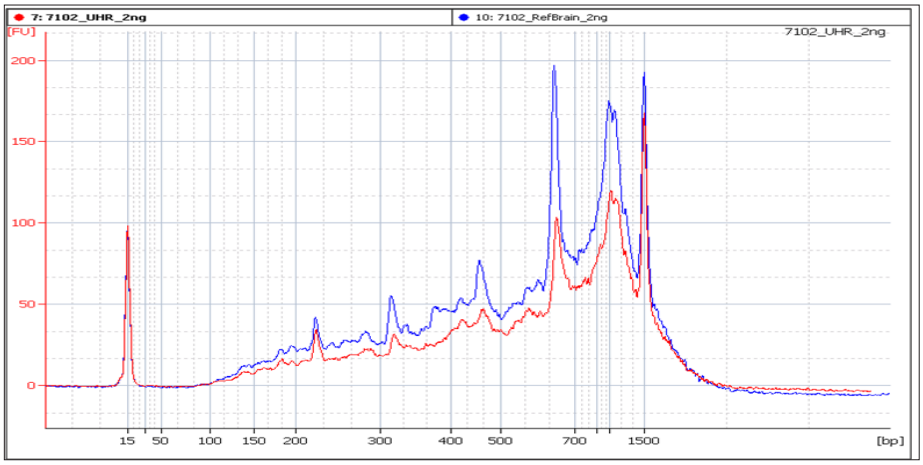
There are several methods which we can recommend for quality control analysis of the SPIA cDNA including spectrophotometric quantification of amplification yield (as described in Section IV, Protocol J on page 19) and fragment size distribution analysis using the Agilent Bioanalyzer.

When performing fragment size distribution analysis on the Bioanalyzer, we recommend using either the Agilent DNA 1000 LabChip (Agilent Cat. #5067-1504) or the RNA 6000 Nano LabChip (Agilent Cat. #5065-4476) following the manufacturer's instructions and the specific guidelines given below.

Guidelines for Using the Agilent DNA 1000 LabChip

When using the DNA 1000 LabChip (Agilent Cat. # 5065-1504) for analysis of SPIA cDNA fragment distribution, use the DNA 1000 Series II program and follow the manufacturer's instructions. A typical size distribution trace may look like the one obtained from Human Reference Brain and Universal Human Reference total RNA (Figure 3). Note that the shape of this distribution trace is highly dependent on the input RNA integrity as well as RNA source, and can vary significantly.

Figure 3. Bioanalyzer trace of SPIA cDNA product obtained from 2 ng of Human Brain and Universal Human Reference RNA using the DNA 1000 Labchip.



2 ng Human Brain 2 ng Universal Human Reference DNA Ladder

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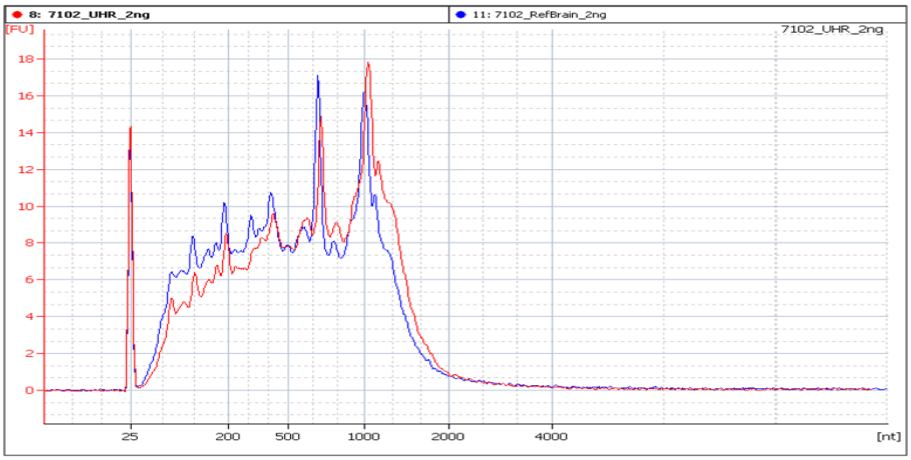
Guidelines for Using the Agilent RNA 6000 Nano LabChip

When using the RNA 6000 Nano LabChip (Agilent Cat. #5065-4476) for analysis of SPIA cDNA fragment distribution, use the Eukaryotic Total RNA Nano program and follow the manufacturer's instructions. Denaturation of the SPIA cDNA sample (as described in the Agilent RNA 6000 Nano protocol) is required for optimum resolution and reproducibility using this option. A typical size distribution trace may look like the one obtained from total Human Brain and Universal Human Reference RNA (Figure 4). Note that the shape of this distribution trace is highly dependent on the input RNA integrity as well as RNA source, and can vary significantly.

Figure 4. Bioanalyzer trace of SPIA cDNA product obtained from 2 ng of Human Brain and Universal Human Reference RNA using the RNA 6000 Nano LabChip.



2 ng Human Brain 2 ng Universal Human Reference RNA 6000 Ladder

C. RNA-Seq Library Preparation

Fragmentation of the SPIA cDNA produced by these systems is required prior to use in sequencing library construction protocols..

For the best results, SPIA cDNA must be fragmented to an appropriate size prior to preparing DNA libraries for sequencing.

We recommend fragmentation using Covaris Adaptive Focused Acoustics method, following the manufacturer's recommendations for the desired fragment length. Fragment length should be chosen with consideration for the desired sequencing read length, and whether the sequencing will be single-end or paired-end.

Fragmentation should generate a uniform distribution of library molecules for optimal library preparation. Other means of fragmentation may also be suitable, but have not been tested by NuGEN and will require validation. For alternatives to the Covaris system, please contact NuGEN Technical Support.

The double stranded cDNA produced by the Ovation RNA-Seq System V2 may be used with Ovation Ultralow Library Systems or Ovation Rapid Library Systems, or with other suitable library construction kits for use with double-stranded cDNA samples.

VI. Appendix

D. Performing Quantitative PCR on Amplified cDNA

Amplified cDNA produced with the kit has been used successfully as template for qPCR systems including TaqMan® and SYBR® Green.

We have successfully used 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. #352042)
- 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)

Recommendations to Achieve Optimal Results

1. Dilute the Amplified Product

   The unpurified amplified cDNA should be diluted 10-fold for TaqMan assays and 40-fold for SYBR Green assays.

2. 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 System V2 amplification process covers the whole transcriptome.

VI. Appendix

E. 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, pipet up and down to mix sample well. Do not centrifuge.
3. Place an RNeasy mini column in a 2 mL collection tube.
4. Apply the sample (up to 700 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.
9. Pipet 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. Pipet 30–50 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.

VI. Appendix

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) with 1 µL rDNase (10 Units Roche Cat. #04716728001).
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 & Concentrator-5 (Zymo Research, Cat. #R1015)

1. Add 4 volumes (100 µL) of RNA binding buffer to the sample.
2. Obtain one RNA Clean & Concentrator-5 Kit column and apply sample to column.
3. Spin column for 30 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
4. Add 200 µL wash buffer (with ethanol added as per vendor's specifications).
5. After closing the column spin for 30 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
6. Add 200 µL fresh 80% ethanol, close cap and spin for 30 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
7. Place the RNA Clean & Concentrator-5 Kit column in a fresh 1.5 mL collection tube.
8. Add 10 µL nuclease-free water (green: D1) directly to the center of the filter in the tube and close the cap.

   Important: Do not use cold water!
9. 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)

1. Add 80 µL ice-cold RNase-free water (D1, green cap) to the sample on ice.
2. Add 350 µL Buffer RLT and mix by pipetting.
3. Add 250 µL 96 to 100% ethanol and mix thoroughly by pipetting.
4. Place an RNeasy MinElute Spin Column into a 2 mL collection tube (one column per sample) and apply the 700 µL sample to the column.

Best results are 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.

5. After closing the column, spin for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
6. 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). Discard the flow-through, keeping the same collection tube.
7. 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.
8. Spin for 2 minutes at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
9. Place the RNeasy MinElute Spin Column in a fresh 2 mL collection tube and place in the microcentrifuge with the cap open. Spin for 5 minutes at ≥8000 X g (≥10,000 rpm) and discard the flow-through.
10. Place the RNeasy MinElute Spin Column in a fresh 1.5 mL collection tube.
11. 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!**
12. Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

VI. Appendix

F. Preventing Non-specific Amplification

Due to the high sensitivity inherent in our amplification systems, we have developed a set of recommendations designed to minimize the potential for the generation of non-specific amplification products by carryover of previously amplified SPIA cDNA. We strongly recommend implementing these procedures, especially for high-throughput and low-RNA input environments typical in today's gene expression laboratories. We have two general recommendations. First, 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 clean-up protocols for workspaces as standard operating procedure. This will prevent amplification products from accumulating in laboratory workspaces. Details regarding establishing and maintaining a suitable 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 cleanup and SPIA amplification reaction setup. After SPIA incubation the reactions are immediately removed from the pre-amplification workspace and opened only in the post-amplification area.

    b. Post-amplification includes all steps and materials related to the handling of the amplified SPIA cDNA product, including bead removal, final purification, post-SPIA modification, array hybridization and any other analytical work.

    c. Ideally, the pre-amplification workspace will be in a separate room. If this is not possible, ensure the pre-amplification area is sufficiently isolated from post-amplification work.

    d. PCR Workstation enclosures with UV illumination for use as pre-amplification workspaces can be an option in situations where conditions preclude physical separation of pre- and post-amplification activities.

2. Establish and maintain a clean work environment:

    a. Initially clean the entire lab thoroughly with DNA-OFF. Follow treatment with a thorough rinse with water to ensure no residual cleaning agents are left behind.

    b. In the pre-amplification area, remove all small equipment, and then clean every surface that may have been exposed to amplified SPIA cDNA (surfaces, drawer handles, keypad, etc.). Before reintroducing any equipment, clean every piece of equipment thoroughly. Clean wells of thermal cycler(s) and magnetic plate(s) with a cotton swab or by filling with cleaning solution.

    Clean the thermal cycler block by heating to 99°C for 15 minutes, then wipe down exposed surfaces and keypad with cleaning solution.

    Clean magnets by immersion in cleaning solution or use a cotton swab.

c. Carry out a thorough external and internal cleaning of all pipettes with DNA-OFF. Carefully follow the manufacturer's instructions for this process to avoid damaging the pipettes. It is a good idea to keep a clean set of pipettes as a backup.

d. 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, reactions and RNA samples.

e. Stock the pre-amplification workspace with clean, preferably new, equipment (pipettes, racks, etc.) that has not been exposed to the post-amplification workspace. Establish dedicated stocks of all consumables used in the pre-amplification workspace.

f. Make it a policy to carry out regular cleaning of all workspaces.

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

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

3. Avoid running negative controls (i.e., no RNA input reactions). Instead use low-template controls (inputs of 50 pg to 100 pg) in order to detect and monitor any non-specific amplification issues. The clearest indication that non-specific amplification is taking place is the appearance of higher than expected yields or irregular bioanalyzer traces in a low template control (LTC) reaction.

   a. Typical amplification performance:

      i. LTC yields for Ovation RNA-Seq System V2 amplifications should be significantly lower than yields for RNA inputs within the recommended input range of 500 pg to 50 ng.

      ii. The Bioanalyzer trace of the LTC amplification product is consistent with that seen with higher input.

   b. Atypical amplification performance:

      i. LTC yields may be similar to those obtained using higher inputs of total RNA.

      ii. The Bioanalyzer traces of amplification products may look significantly different than the typical Ovation RNA-Seq System V2 reaction traces. The LTC reaction is designed to be an especially sensitive indicator of atypical amplification performance.

      iii. Sensitivity on microarrays and the proportion of mapped reads from RNASeq runs with atypical samples may be lower than expected.

      iv. Contact NuGEN Technical Services when atypical performance is suspected.

VI. Appendix

G. Frequently Asked Questions (FAQs)

Q1. Which deep-sequencing platforms are suitable for use with the Ovation RNA-Seq System V2?
The cDNA produced by the Ovation RNA-Seq System V2 is compatible with most deep-sequencing platforms. Please contact the NuGEN Technical Services Team for information on compatibility with specific platforms.

Q2. Do I need to perform an rRNA depletion or Poly(A) enrichment step before processing with the Ovation® RNA-Seq System V2?
rRNA depletion or Poly(A) enrichment is not required. The input range of 500 pg to 100 ng refers to total RNA.

Q3. What size cDNA is generated by the Ovation RNA-Seq System V2?
Figure 3 (page 27) shows a representative fragment distribution pattern obtained with 2 ng of high quality Universal Human Reference and human brain total RNA samples.

Q4. What fragment size range should I use for sequencing library preparation?
NuGEN has had the best results using fragment size ranges consistent with or slightly larger than those recommended by the sequencing platform provider.

Q5. What materials are provided with the Ovation RNA-Seq System V2?
The Ovation RNA-Seq System V2 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 Agencourt magnetic beads for second strand reaction cDNA purification.

Q6. What equipment is required or will be useful?
Required equipment includes a microcentrifuge, pipettes, vortexer, a thermal cycler, a spectrophotometer and magnetic plate. An Agilent Bioanalyzer may also be useful for optional analytical tests.

Q7. What additional consumables does the user need?
For the SPIA cDNA purification step, we recommend using the QIAGEN MinElute Reaction Cleanup Kit. Refer to Appendix A for other purification options.

Q8. How much total RNA do I need for amplification?
The Ovation RNA-Seq System V2 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.

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Q9. Can I use poly(A)+ RNA as an alternative to total RNA?
Yes, if you are primarily interested in sequencing reads from mature coding transcripts then poly(A)+ RNA may be substituted for total RNA. NuGEN has demonstrated that poly(A)+ RNA can be isolated from 5 to 500 ng of total RNA using MPG mRNA Purification Kit (PureBiotech product number MRRK 1010) and input to the Ovation RNA-Seq System V2 protocol with good results. We recommend not using less than 500 pg of isolated poly(A)+ RNA.

Q10. Do I need to use high-quality total RNA?
The Ovation RNA-Seq System V2 is designed to work with purified total RNA. When using purified total RNA, samples should be of high molecular weight with little or no evidence of degradation. While it is impossible to guarantee the highest levels of performance when using RNA of lower quality, this system should allow the successful analysis of somewhat degraded samples. With such samples, users may experience lower amplification yields and should plan to use relatively higher RNA inputs.

Q11. Can DNA be used as input for the Ovation RNA-Seq System V2?
No. The Ovation RNA-Seq System V2 is designed to amplify mRNA, not DNA.

Q12. Can contaminating genomic DNA interfere with the Ovation RNA-Seq System V2 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.

Q13. Do you recommend DNase treatment of purified total RNA samples?
Yes. For an explanation of DNase requirements see page 8. For DNase treatment of RNA samples, refer to Appendix E for guidelines.

Q14. Can I use the Ovation RNA-Seq System V2 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 V2?
We have not encountered any specific RNA sources that will not work with the Ovation System.

Q16. How much SPIA cDNA can I expect from a single reaction?
You should expect a minimum yield of several micrograms of SPIA cDNA from a starting input of 500 pg of good quality total RNA.

Q17. How do I measure my SPIA cDNA yield?
Refer to the Measuring SPIA cDNA Yield and Purity protocol on page 19 in this User Guide for guidance.

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Q18. Is the cDNA yield dependent upon the quantity of total RNA input?
Yes, higher inputs of RNA will lead to somewhat higher amplification yields.

Q19. Can I use an Agilent Bioanalyzer to evaluate the amplification products?
Yes. Refer to Appendix B of this User Guide for guidelines.

Q20. Can I use the final purified SPIA cDNA for qPCR analysis?
Yes. qPCR can be performed on the final SPIA cDNA before or after purification. Guidelines for qPCR analysis of SPIA cDNA can be found in Appendix D.

Q21. Has NuGEN performed reproducibility studies on the Ovation RNA-Seq System V2?
Yes. Sample-to-sample, lot-to-lot, and operator-to-operator reproducibility studies are routinely conducted.

Q22. Does the Ovation RNA-Seq System V2 generate product in the absence of RNA input?
In the complete absence of input RNA, approximately 0.3 µg or less of non-specific product is generated. However, in the presence of even a very small amount of RNA the amplified cDNA has been demonstrated to be specific.

Q23. How many rounds of amplification are performed with the Ovation RNA-Seq System V2?
This System performs a single round of amplification and is not designed to support multiple rounds of amplification.

Q24. Do I need to order specific primers for the amplification?
No. The DNA/RNA primers provided in the Ovation RNA-Seq System V2 are universal. No gene-specific primers are required.

Q25. Do I have to use the supplied DNA/RNA primers?
Yes. The Ovation RNA-Seq System V2 will not work properly with other primers.

Q26. Can I perform fewer than 4 reactions at a time?
We recommend a minimum batch size of 4 reactions. Smaller batch sizes may result in difficulty pipetting small volumes and lead to poor performance.

Q27. Where can I safely stop in the protocol?
It is safe to stop after the SPIA Amplification protocol prior to final cleanup at the point specifically noted in the protocol. Store reaction products at –20°C.

Q28. What purification methods do you recommend?
For the final SPIA cDNA purification step we recommend using the QIAGEN MinElute Reaction Cleanup Kit. Alternative purification methods can be found in Appendix A.

For the double-stranded cDNA purification step prior to SPIA amplification, we require the use of the Agencourt magnetic beads provided with the Ovation RNA-Seq System V2.

VI. Appendix

H. Update History

This document, the Ovation RNA-Seq System V2 user guide (M01206 v7), has been updated from the previous version to address the following topics:

Description	Section	Page(s)
Updated the First Strand Enzyme Mix storage conditions	I.E.	2

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