USER GUIDE

Ovation® RNA-Seq FFPE System

PART NO. 7150-08

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imagine more from less™

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A. Background

The Ovation® RNA-Seq FFPE System provides a fast and simple method for preparing amplified cDNA from FFPE-derived 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, making the Ovation RNA-Seq FFPE System ideal for amplification of the severely degraded and chemically modified RNA typically obtained from FFPE samples. This approach is ideal for processing samples for RNA-Seq on Next Generation Sequencing (NGS) systems, as reads are distributed across the transcript. The Ovation RNA-Seq FFPE System is optimized for the generation of double-stranded cDNA suitable for sequencing library construction for use with a variety of NGS platforms. The amplified cDNA generated with the Ovation RNA-Seq FFPE System is suitable for qPCR or microarray analysis on some array platforms.

The Ovation RNA-Seq FFPE 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 100 ng FFPE RNA, microgram quantities of cDNA can be prepared in approximately six hours.

The Ovation RNA-Seq FFPE System (Part No. 7150) provides optimized reagent mixes and a protocol to process eight RNA samples.

I. Introduction

B. Ribo-SPIA® Technology

Ribo-SPIA technology is a three-step process that generates amplified cDNA from as little as 100 nanograms of FFPE RNA (see Figure 1).

1. **Generation of First Strand cDNA (1.2 hours)**
   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.

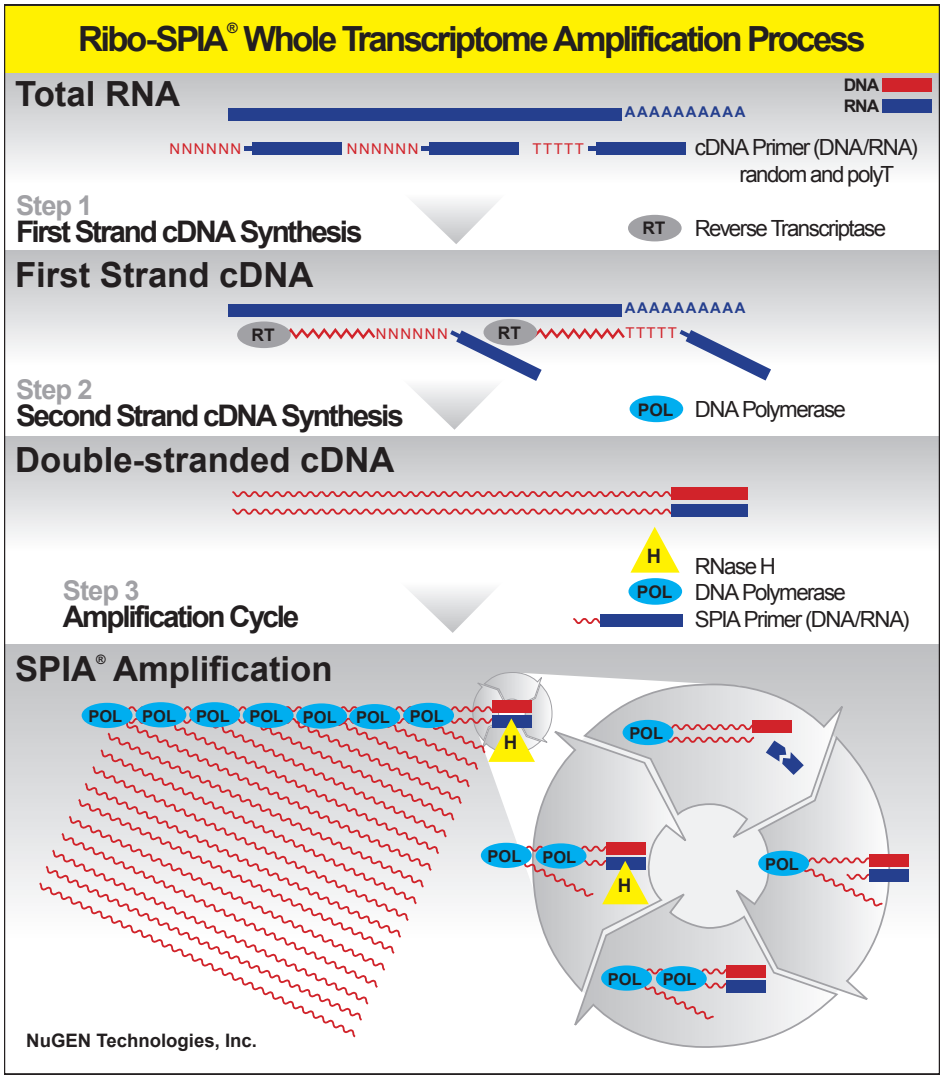
2. **Generation of a DNA/RNA Heteroduplex Double-stranded cDNA (2 hours)**
   Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthesize a second strand, which includes DNA complementary to the 5′ unique sequence of the first strand chimeric primers.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

3. **Amplification (1.5 hours)**
   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 cDNA strand. This results in the exposure of a DNA sequence that is available for binding the 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.

4. **Post-SPIA Modification (1.5 hours)**
   The Post-SPIA Modification process completes the amplification process. The first step allows 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 sequencing library construction (not shown on Figure 1).

I. Introduction

Figure 1. The Ribo-SPIA RNA Amplification process used in the Ovation RNA-Seq FFPE System



I. Introduction

C. Performance Specifications

The Ovation RNA-Seq FFPE System synthesizes microgram quantities of amplified cDNA starting with FFPE RNA input amounts of as little as 100 ng. In approximately six hours, the Ovation RNA-Seq FFPE System can produce sufficient double-stranded cDNA for sequencing library construction using the Encore™ NGS Library System I (Part No. 300), Encore NGS Multiplex System I (Part No. 301) or other suitable library construction methods for leading NGS platforms. The size of the amplified cDNA products is directly proportional to the size of the degraded RNA used for the amplification reactions.

D. Quality Control

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

E. Storage and Stability

This product contains components with multiple storage temperatures.

The Ovation RNA-Seq FFPE 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' Agencourt® RNAClean® XP Beads (clear cap), should be stored at –20°C in a freezer without a defrost cycle.

Store the RNAClean XP Beads at 4°C

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

The Ovation RNA-Seq FFPE System 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 FFPE System.

F. Material Safety Data Sheet (MSDS)

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

II. Kit Components

A. Reagents Provided

Table 1. First Strand cDNA Reagents

COMPONENT	7150-08 PART NUMBER	VIAL CAP	VIAL NUMBER
First Strand Primer Mix	S01278	Blue	A1 VER 4
First Strand Buffer Mix	S01174	Blue	A2 VER 3
First Strand Enzyme Mix	S01040	Blue	A3 VER 1

Table 2. Second Strand cDNA Reagents

COMPONENT	7150-08 PART NUMBER	VIAL CAP	VIAL NUMBER
Second Strand Buffer Mix	S01176	Yellow	B1 VER 3
Second Strand Enzyme Mix	S01126	Yellow	B2 VER 2

Table 3. SPIA Reagents

COMPONENT	7150-08 PART NUMBER	VIAL CAP	VIAL NUMBER
SPIA Primer Mix	S01331	Red	C1 VER 9
SPIA Buffer Mix	S01332	Red	C2 VER 9
SPIA Enzyme Mix	S01199	Red	C3 VER 5

II. Kit Components

Table 4. Post-SPIA Modification Reagents

COMPONENT	7150-08 PART NUMBER	VIAL CAP	VIAL NUMBER
Primer Mix	S01334	Violet	E1 VER 3
Buffer Mix	S01335	Violet	E2 VER 3
Enzyme Mix	S01267	Violet	E3 VER 1

Table 5. Additional Reagents

COMPONENT	7150-08 PART NUMBER	VIAL CAP	VIAL NUMBER
Nuclease-free Water	S01001	Green	D1
Agencourt RNAClean XP Beads	S01307	Clear	—

B. Additional Equipment, Reagents and Labware

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, 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
  - Fluorescent spectrophotometer
- **Reagents**
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps
  - 1X TE buffer
  - QuantIT PicoGreen dsDNA Assay Kit (Invitrogen, Cat. #P11496)
- **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

II. Kit Components

- 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. #A29164) or Beckman Coulter Genomics SPRIPlate Ring Super Magnet Plate, (Beckman Coulter Genomics, 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):
  - RNAClean XP Kit (Beckman Coulter Genomics, Cat. #A63987)
  - QIAquick® PCR Purification Kit (QIAGEN, Cat. #28104)
  - MinElute® Reaction Cleanup Kit (QIAGEN, Cat. #28204)
  - DNA Clean & Concentrator™-25 (Zymo Research, Cat. #D4005)
- Disposable gloves
- Kimwipes
- Ice bucket

- **Optional Materials**
  - Agilent 2100 Bioanalyzer or materials and equipment for electrophoretic analysis of RNA
  - Real-time PCR system
  - Cleaning solutions such as RNaseZap® (Ambion, Cat. #AM9780) and DNA-OFF™ (MP Biomedicals, Cat. #QD0500)

To Order:

- Ambion Inc., www.ambion.com
- Beckman Coulter Genomics, www.beckmangenomics.com
- Invitrogen Life Technologies, www.invitrogen.com
- MP Biomedicals, www.mpbio.com
- New England BioLabs, www.neb.com
- QIAGEN Inc., www.qiagen.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com
- USB Corporation, www.usbweb.com
- Zymo Research, www.zymoresearch.com

III. Planning the Experiment

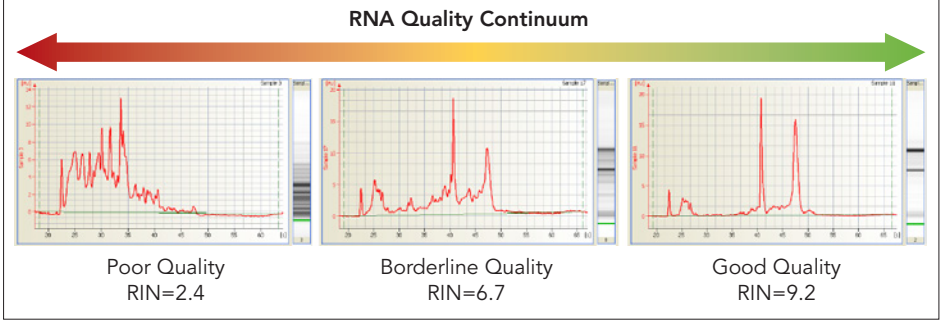
A. Input RNA Requirements

1. **RNA Quantity**
   Total RNA input must be between 100–200 ng. Inputs above 200 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. 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**
   The Ovation RNA-Seq FFPE System was designed and optimized for use with degraded RNA samples. The whole transcriptome amplification approach allows low-quality, degraded RNA samples with compromised poly(A) tails to be amplified successfully. Tests with FFPE RNA samples that were highly degraded (RIN approx 2), showed reproducible amplification when the recommended quantity of input total RNA was used. We strongly recommend quantitation of total RNA to ensure the minimum input requirement is met. RNA samples of high molecular weight with little or no evidence of degradation, will also amplify very well with this product. Some FFPE-derived RNA samples may be too severely compromised to work with the Ovation RNA-Seq FFPE System.

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



4. **DNase Treatment**
   It is highly recommended to use DNase-treated RNA for amplification using the Ovation RNA-Seq FFPE System. 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. Also, if the total RNA sample contains a significant amount of contami-

! *It is highly recommended to perform DNase treatment of FFPE samples prior to amplification.*

nating genomic DNA, it may 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 100–200 ng, we recommend using a DNase treatment that will remove contaminating genomic DNA during RNA purification.

To ensure that RNA is free of significant genomic DNA contamination following DNase treatment, the RNA can be assayed directly by qPCR using the primers described in WT-Ovation FFPE System Technical Report #1. Ct values generated from 100 ng of sample RNA are compared to a standard curve generated by running qPCR on a 5-fold dilution series of a control cell line DNA into qPCR. A control cell line such as K562 DNA High Molecular weight may be used (Cat. #DD2011, Promega). The cell line DNA equivalent mass of contaminating DNA is calculated from the standard curve. For very-high-quality samples such as Ambion FirstChoice® total RNA we typically see <0.003% of the nucleic acid as genomic DNA. In FFPE samples that have not been treated with DNase, genomic DNA contamination of 0.5 to 4.2% is not uncommon.

5. **Carrier Use for RNA Isolation**
   We strongly discourage 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 FFPE System must be stored at –80°C. Avoid frequent freeze/thaw cycles, or RNA degradation may result.

D. Amplified cDNA Storage

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

IV. Protocol

A. Overview

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

1. First strand cDNA synthesis	1.2 hours
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	**~6.2 hours**

Ovation RNA-Seq FFPE 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 Post-SPIA modification if you intend to use the cDNA for sequencing library preparation.

B. Protocol Notes

- We recommend the routine use of a high-quality commercial 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 magnet is highly recommended.
- Set up no fewer than 4 reactions at a time with the 7150 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.
- Due to the high sensitivity inherent in this amplification system, we strongly recommend taking measures to minimize the introduction of exogenous nucleic acids and the potential for the carryover of previously amplified cDNA into new amplification reactions. The two steps to accomplish this are: 1. Designate separate workspaces for "pre-amplification" and "post-amplification" steps and materials, and 2. Implement 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 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 FFPE System 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® RNAClean® XP Purification Beads

Tips and Notes:

There are significant modifications to the Beckman Coulter Genomics' Agencourt RNAClean XP 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 FFPE System. However, you may review the Beckman Coulter Genomics user guide to become familiar with the manufacturer's recommendations.

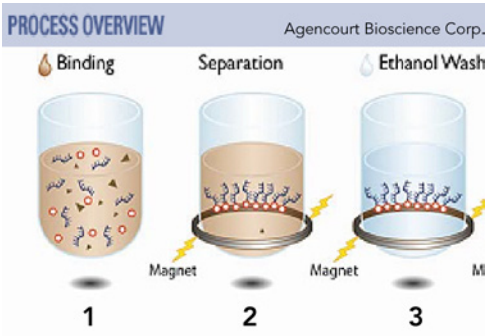
The bead purification process used for cDNA purification before amplification consists of:

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

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

IV. Protocol

Figure 3. Bead purification process overview.



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 1.2 volumes (24 µL) of RNAClean XP 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 5 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 35 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 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	65°C – 2 min, hold at 4°C
Program 2 First Strand Synthesis	4°C – 2 min, 25°C – 30 min, 42°C – 15 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, 95°C – 5 min, hold at 4°C
POST-SPIA MODIFICATION	
Program 5 Post-SPIA Modification I	98°C – 3 min, hold at 4°C
Program 6 Post-SPIA Modification II	4°C – 1 min, 30°C – 10 min, 42°C – 15 min, 75°C – 10 min, hold at 4°C

IV. Protocol

Important Note: Carry out steps E (Optional Demodification) through I (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. #Q0500) to avoid the potential introduction of exogenous nucleic acid or previously amplified cDNA into new amplifications. For more information on our recommendations for workflow compartmentalization and routine lab cleanup please refer to Appendix E of this User Guide. If you have any questions on this important topic, please contact NuGEN Technical Services (techserv@nugeninc.com, (888) 654-6544).

E. Optional Demodification

Formalin fixation of tissues results in a high degree of cross linking of the RNA. It is essential to reverse as much of this cross-linking as possible for successful cDNA synthesis and amplification. Many commercial RNA isolation kits designed for the extraction of RNA from FFPE tissues include a demodification step. If the RNA isolation kit you have used does not include this step or if you suspect the demodification is incomplete, we strongly recommend that you perform the following steps for demodification prior to using the Ovation RNA-Seq FFPE System.

1. Add 2.5 µL of RNA sample (100–200 ng) to a 0.2 mL PCR tube.
2. Add 2.5 µL of 100 mM Tris-HCl pH 8.0.
3. Incubate sample at 70°C for 15 minutes, then cool sample to 4°C.
4. Proceed to the First Strand cDNA Synthesis protocol.

F. First Strand cDNA Synthesis

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.

Do not vortex any enzyme mixes.

2. Spin down contents of A3 and place on ice.
3. Thaw the other reagents at room temperature. Mix by vortexing, then spin and place on ice.
4. Add 2 µL of A1 to a 0.2 mL PCR tube.
5. Add 5 µL of total RNA sample (100–200 ng) to the primer.
6. Mix by pipetting 3 times, spin and place on ice.

7. Place tube(s) in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 6):

   65°C – 2 min, hold at 4°C

8. Remove tubes from the thermal cycler and place tubes on ice.

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

Table 7. 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 1)
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 3 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 tubes in a pre-cooled thermal cycler programmed to run Program 2 (First Strand Synthesis; see Table 6):

    4°C – 2 min, 25°C – 30 min, 42°C – 15 min, 70°C – 15 min, hold at 4°C

13. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.

14. Continue immediately with the Second Strand cDNA Synthesis protocol.

G. Second Strand cDNA Synthesis

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

1. Remove the RNAClean XP 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. Spin down 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 8.

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

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

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 3 times, spin and place on ice.
8. Place tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand Synthesis; see Table 6):

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

H. Purification of cDNA

1. Ensure the RNAClean XP beads have completely reached room temperature before proceeding. Resuspend beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample.
2. 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.
3. 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.
4. Ensuring the beads are fully resuspended, add 24 µL (1.2 volumes) bead suspension at room temperature to each reaction and mix by pipetting up and down 10 times.
5. Incubate at room temperature for 10 minutes.
6. Transfer tubes or plate to 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. Carefully remove only 35 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. Significant loss of beads at this stage will impact cDNA yields, so ensure beads are not removed with the binding buffer or the wash.

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

8. With the plate still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
9. Remove the 70% ethanol wash using a pipette.
10. 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 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 – 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.
12. Proceed immediately with the SPIA Amplification protocol with the cDNA still bound to the dry beads.

I. SPIA® Amplification

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, spin and 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, 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 9. Make sure the addition of C3 is at the last moment.

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

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

SPIA BUFFER MIX (RED: C2 VER 9)	SPIA PRIMER MIX (RED: C1 VER 9)	SPIA ENZYME MIX (RED: C3 VER 5)
40 µL	20 µL	20 µL

5. Add 80 µL of the SPIA Master Mix to each tube containing the double-stranded cDNA bound to the dried beads. Use a pipette set to 50 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):

   4°C – 1 min, 47°C – 60 min, 95°C – 5 min, hold at 4°C

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

Important Note: At this point the tubes or plate 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 E of this User Guide. If you have any questions on this important topic, please contact NuGEN Technical Services (techserv@nugeninc.com, (888) 654-6544).

8. Transfer tubes or plate to magnet plate and let stand 5 minutes to completely clear the solution of beads.
9. Carefully transfer 70 µL of the cleared supernatant containing the amplified cDNA to fresh tubes. The beads may now be discarded.

   Note: At this point, a small aliquot of the amplified cDNA can be removed for qPCR analysis, if desired.

10. Proceed immediately with the Post-SPIA Modification I protocol or store reaction products at –20°C overnight prior to continuing.

J. Post-SPIA Modification I

1. Obtain the Primer Mix (Violet: E1) stored at –20°C. Thaw E1 at room temperature, mix by vortexing, spin and place on ice.
2. Add 10 µL of the Primer Mix E1 to 70 µL of the SPIA reaction.
3. Mix thoroughly by pipetting 6–8 times at the 50 µL setting, spin and place on ice.
4. Place tubes in a pre-warmed thermal cycler programmed to run Program 5 (Post-SPIA Modification I, see Table 6):

   98°C – 3 min, hold at 4°C

5. Remove tubes from the thermal cycler, spin and place on ice.
6. Proceed immediately with the Post-SPIA Modification II protocol.

K. Post-SPIA Modification 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, spin and place on ice.
3. Spin down E3 and 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 VER 3)	E3 ENZYME MIX (VIOLET: E3 VER 1)
10 µL	10 µL

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

5. Add 20 µL of the Post-SPIA Modification II Master Mix to the entire volume of the Post-SPIA Modification I reaction. Final reaction volume is 100 µL.
6. Mix thoroughly by pipetting 6–8 times at the 70 µL pipettor setting, spin and place on ice.
7. Place tubes in a pre-cooled thermal cycler programmed to run Program 6 (Post-SPIA Modification II, see Table 6):

   4°C – 1 min, 30°C – 10 min, 42°C – 15 min, 75°C – 10 min, hold at 4°C
8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Proceed to Purification of Amplified cDNA protocol, or store cDNA at –20°C.

L. Purification of Amplified cDNA

The amplified cDNA should be purified prior to subsequent processing for RNA-Seq library construction. Selection of the optimum purification option can depend on many factors. Suitable purification methods are listed in the Appendix. Please contact the NuGEN Technical Support team for assistance in selecting the appropriate purification option for your application.

M. 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 cDNA 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 purified cDNA 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-squared value 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 X 1000)/1000 = 200 ng/µL).

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 at europe@nugeninc.com.

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

VI. Appendix

A. Purification Protocols for Amplified cDNA

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

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

Beckman Coulter Genomics' Agencourt RNAClean XP Purification Magnetic Beads
(instructions for a single reaction)

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

1. Obtain and vigorously shake the RNAClean XP bottle to resuspend the magnetic beads. Allow the bead solution to reach room temperature.
2. Prepare an 80% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content which may reduce amplification yield.
3. Split the 100 µL SPIA cDNA sample into two 50 µL volumes in clean 0.2 mL strip tubes, PCR plate, or round bottom 96-well plate.
4. Add 90 µL of resuspended RNAClean XP beads (1.8 times the sample volume) to one set of the paired 50 µL SPIA half-reactions.
5. Mix the sample and beads thoroughly by pipetting up and down 10 times.

   Note: If using a 96-well plate format with both half-reactions on the same plate, it will be necessary to transfer the sample/bead mixture to a fresh plate at this point.
6. Incubate sample/bead mixture at room temperature for 5 minutes.
7. Place the first set of samples (containing beads) on the magnet for 10 minutes or until the solution appears clear.
8. After 5 minutes of the 10 minute incubation in step 7 have elapsed, add 90 µL of resuspended RNAClean XP beads (1.8 times the sample volume) to second set of half-reactions containing the remaining 50 µL cDNA samples. Mix the sample and beads thoroughly by pipetting up and down 10 times. Incubate samples at room temperature for 5 minutes.
9. Using a multi-channel pipette, remove and discard the supernatant from first set of samples (on magnet). Do not disturb the ring of magnetic beads.

10. Using a multi-channel pipette, add the sample/bead mix from the second set of half-reactions (prepared in step 8) to the appropriate tubes or wells containing the beads from the first half-reaction while it is still placed on the magnet. Add slowly as to not disturb the bead ring already in each well.

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

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

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

12. Using a multi-channel pipette, remove and discard the supernatant. Do not disturb the ring of magnetic beads.

13. With the samples still on the magnet plate, add 200 µL of freshly prepared 80% ethanol to each sample and incubate for 30 seconds or until the solution clears. Add slowly as to not disturb the separated magnetic beads.

14. Using a multi-channel pipette, remove and discard the ethanol.

15. Repeat the 80% ethanol wash once more. Ensure as much ethanol as possible is removed from the samples.

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

17. With the tubes on the bench top, add 30 µL of room temperature DNase-free 1X TE to each tube. Holding the tubes firmly, very carefully vortex for 30 seconds or use a plate shaker set to medium speed. Ensure the beads are fully resuspended, vortex longer if necessary. Alternatively, the beads may be resuspended by repeated pipetting.

18. Replace reaction tubes on the magnet. Allow the beads to separate for 5 minutes or until the solution clears.

19. Using a multi-channel pipette, remove the eluted sample and place into a fresh reaction tube. There should be approximately 30 µL of purified cDNA.

    Note: Small amounts of magnetic bead carry-over may interfere with sample quantitation; take care to minimize bead carry-over.

20. Proceed to Measuring cDNA Product Yield and Purity or store purified cDNA at –20°C.

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QIAGEN MinElute Reaction Cleanup Kit (QIAGEN, Cat. #28204)
(instructions for a single reaction)

1. Ensure that 100% ethanol has been added to Buffer PE as described in the QIAGEN MinElute Handbook. Failure to add ethanol to this buffer will result in low amplification yield.
2. Into a clean 1.5 mL tube, add 300 µL of ERC buffer from the QIAGEN kit.
3. Add the 100 µL of amplified cDNA product to the tube. Mix sample by vortexing, then spin briefly.
4. Obtain 1 MinElute spin column and insert into a collection tube.
5. Load the entire solution of sample and buffer onto column.
6. Centrifuge for 1 minute at maximum speed. Discard flow-through.
7. Place column back in the same collection tube. Add 750 µL of Buffer PE with ethanol.
8. Centrifuge for 1 minute at maximum speed. Discard flow-through.
9. Centrifuge again for 1 minute at maximum speed to remove all residual Buffer PE with ethanol.
10. Remove the MinElute spin column from the centrifuge. Discard flow-through along with the collection tube.
11. Blot the column tip onto a filter paper to remove any residual wash buffer from the tip of the column.

    Note: Blotting of the column tip MUST be done prior to transferring the column to a clean 1.5 mL microcentrifuge tube. Failure to do so may result in a small quantity of wash buffer in your final eluted sample.
12. Place the MinElute spin column in clean 1.5 mL collection tube.
13. Add 20–25 µL of 1X TE or buffer EB to the center of each MinElute spin column.
14. Let columns stand for 1 minute at room temperature.
15. Centrifuge at maximum speed for 1 minute.
16. Collect sample. There should be approximately 18–23 µL of purified cDNA.
17. Mix sample by vortexing, then spin briefly.
18. Proceed to Measuring Double-stranded cDNA Product Yield protocol or store cDNA at –20°C.

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

1. Into a clean 1.5 mL tube add 500 µL of PB buffer from the QIAGEN system.
2. Add the 100 µL of amplified cDNA product to the tube.
3. Mix sample by vortexing, then spin briefly.
4. Obtain 1 QIAquick spin column and insert into a collection tube.
5. Load 600 µL of sample onto the column.
6. Centrifuge column in a collection tube for 1 minute at 13,000 rpm (~17,900 X g). Discard flow-through.
7. 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 8 and 9 once.
11. To remove remaining liquid, centrifuge column for 1 additional minute at 13,000 rpm.
12. Remove the column from the centrifuge. Discard flow-through with the collection tube.
13. Blot the tip of the column onto a filter paper in order to remove any residual wash buffer from the tip of the column.

    Note: Blot column tip prior to transferring it to a new tube to prevent any wash buffer transferring to the eluted sample.
14. Place the column in a clean 2.0 mL collection tube, appropriately labeled.
15. Add 30 µL of 1X TE or buffer EB 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 Double-stranded cDNA Product Yield protocol or store cDNA at –20°C.

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

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

1. Into a clean 1.5 mL tube add 200 µL of DNA Binding Buffer.
2. Add 100 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 (420 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 TE at room temperature to elute sample.

13. Place the Zymo-Spin II Column in a clean 1.5 mL microcentrifuge tube. Add 25 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 25 µL of purified cDNA.
16. Mix sample by vortexing, then spin briefly.
17. Proceed to Measuring Double-stranded cDNA Product Yield protocol or store cDNA at –20°C.

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B. 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 successfully used as a template for qPCR systems including TaqMan® and SYBR® Green as well as others. NuGEN has successfully used the following reagents for qPCR:

- TaqMan: ABsolute qPCR Mix plus ROX (ABgene, Cat. #AB-1136/B) or 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) or FastStart SYBR Green Master (ROX) (Roche, Cat. #04 673 514 001)

Recommendations to Achieve Optimal Results

1. **Dilute the Amplified Product**
   The unpurified SPIA-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 amplification process covers the whole transcriptome.

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 FFPE RNA and Fresh Frozen RNA (see Figure 4, below).

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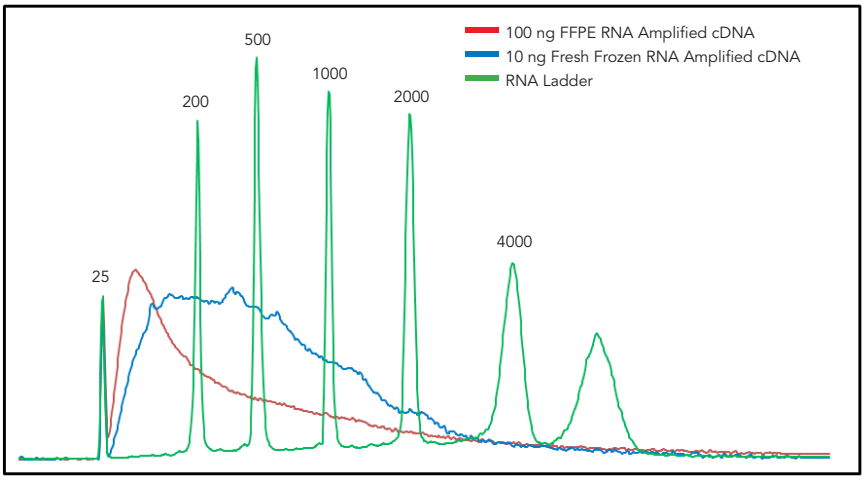
Figure 4. Bioanalyzer Trace of Amplified cDNA Product obtained from 100 ng of FFPE RNA and 10 ng of Fresh Frozen total RNA.



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, 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.

   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 Reagents section of this user guide for ordering information.

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.

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 Cat. #78316) with 1 µL rDNase (10 Units Roche Cat. #04716728001 or 2 Units USB Cat. #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:

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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.
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.

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

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

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.

E. 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 spreading through 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 final amplified 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 workroom. If this is not possible, ensure the pre-amplification area is sufficiently isolated from post-amplification work.
    d. We recommend the use of "PCR Workstation" enclosures with UV illumination as pre-amplification workspaces 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 and RNaseZap.
    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, keypads, etc.). Before reintroducing any equipment, clean every piece

of equipment thoroughly. It is important to clean wells of thermal cycler(s) and magnetic plate(s) with a cotton swab or by filling with cleaning solution.

c. 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.

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

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

f. 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.

g. 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 FFPE System amplifications (following the SPIA step) should be significantly lower than yields for RNA inputs within the recommended input range of 100 to 200 ng.

   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 FFPE System reaction traces. The LTC reaction may be an especially sensitive indicator of atypical amplification performance.

      iii. Performance of libraries produced from samples with atypical amplified product may be lower than expected.

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

VI. Appendix

F. RNA-Seq Library Preparation

Illumina Hi-Seq 1000, Hi-Seq 2000, Genome Analyzer, or HiScan SQ

For the best results, we recommend using NuGEN's Encore NGS Library Systems to prepare DNA libraries for sequencing. For use on Illumina platforms, we offer the Encore NGS Library System I (Part No. 300) for non-multiplex sequencing, and Encore NGS Multiplex System I (Part No. 301) to enable multiplexing of up to eight samples per lane.

There are two recommendations for using the NuGEN Encore NGS Library System I or Encore NGS Multiplex System I to create libraries from material generated using the NuGEN RNA-Seq FFPE System:

1. **Fragmentation:** Due to the smaller size of amplicons expected from FFPE-derived material, most samples of FFPE RNA origin that are amplified using the Ovation RNA-Seq FFPE System do not require fragmentation prior to library preparation, unless the amplified product appears to be considerably larger than 200 bp. It is recommended to perform analysis using the Agilent Bioanalyzer to confirm the size distribution of amplified material.
2. **Input quantity:** We recommend using 400 ng double-stranded cDNA as starting input. Because the amplified product will contain populations of both double-stranded and single-stranded cDNA, it is important to measure the quantity of double-stranded cDNA to avoid underestimation of the quantity of viable material for library preparation. Please see III.M: Measuring Double-stranded cDNA Product Yield for details on performing this measurement.

Alternatively, 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 FFPE 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.

Other NGS Platforms

The double-stranded cDNA produced by the Ovation RNA-Seq FFPE System may also be used with other leading NGS platform providers such as Life Technologies and Roche 454. Please contact the NuGEN Technical Support team for suitable library construction recommendations.

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G. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the Ovation RNA-Seq FFPE System?
The Ovation RNA-Seq FFPE 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 Agencourt RNAClean XP magnetic beads for cDNA purification.

Q2. What equipment is required or will be useful?
A microcentrifuge, pipettes, vortexer, a thermal cycler, a spectrophotometer and a magnetic plate are required. An Agilent Bioanalyzer may also be useful.

Q3. What additional consumables does the user need?
For the SPIA cDNA purification step, purification columns or beads are required.

Q4. Can I use RNA from sources other than FFPE?
While it may be possible to use RNA from other sources with the Ovation RNA-Seq FFPE System, it was developed specifically for use with FFPE total RNA.

Q5. Is the Ovation RNA-Seq FFPE System 3′- biased?
In this system, oligo dT primers are mixed with random primers for the first strand synthesis of cDNA products. This enables the amplification of highly degraded RNA in which much of the amplifiable sequence has become separated from the poly(A) sequence.

Q6. Where in my target sequence can I design my qPCR primers?
The Ovation RNA-Seq FFPE System includes random priming and, therefore, primers can be designed at any location within the mRNA. In order to avoid qPCR interference from possible genomic DNA contamination, we recommend treating your RNA with DNase and designing your amplicons to span an intron. We strongly recommend designing your assays for multiple locations across the transcript with the smallest amplicon size possible, since the starting FFPE RNA is likely to be highly degraded.

Q7. How much FFPE total RNA do I need for amplification?
We recommend total FFPE RNA input of as little as 100 ng. If more sample is available, this amount may be increased up to 200 ng. Input amounts outside this range may produce unsatisfactory variable results, especially for more degraded RNA.

Q8. How much cDNA can I expect from a single reaction?
You should expect a sufficient amount of cDNA from 100–200 ng total FFPE RNA starting material for NGS library preparation. Although yield can be a critical sample quality indicator, success of a given FFPE sample set in array analysis may be predicted using the RNA Sample Quality Assessment Tool described in WT-Ovation FFPE System V2 Technical Report #1.

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Q9. Is the cDNA yield dependent upon the quantity of total RNA input?
Yes. Generally, higher RNA inputs will result in slightly higher amplification yields; however, at FFPE RNA inputs above 200 ng the yields may become variable.

Q10. What size cDNA is generated by the Ovation RNA-Seq FFPE System?
The amplified cDNA size distribution is highly dependent on the input RNA integrity.

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

Q12. Can genomic DNA interfere with the Ovation RNA-Seq FFPE System?
Yes. This system is designed to amplify RNA, but genomic DNA may amplify during the process, so we recommend DNase treatment during RNA purification.

Q13. Can I use the Ovation RNA-Seq FFPE System on bacterial RNA samples?
No, the use of this system on bacterial samples is not recommended.

Q14. Has NuGEN performed reproducibility studies on the Ovation RNA-Seq FFPE System?
Yes. Sample-to-sample, lot-to-lot, and operator-to-operator reproducibility studies are routinely conducted.

Q15. Does the Ovation RNA-Seq FFPE System generate product in the absence of RNA input?
In the complete absence of input RNA non-specific product is generated. However, note that in the presence of even a very small amount of RNA, while the yields may be low the cDNA has been demonstrated to be specific. Please see the Appendix of this User Guide for our recommendations regarding the use of low-template controls.

Q16. Can I use the Ovation RNA-Seq FFPE System for archiving cDNA?
Yes. Amplified cDNA may be stored at –20°C for as long as six months.

Q17. Do I need to order specific primers for the amplification?
No. The DNA/RNA primers provided in the Ovation RNA-Seq FFPE System are universal.

Q18. Do I have to use your DNA/RNA primers?
Yes. The system will not perform with other primers.

Q19. Do you recommend purification of the cDNA prior to qPCR analysis?
No. We recommend using the material before Post-SPIA Modification. A small aliquot can be removed immediately following SPIA, diluted and used directly for qPCR. Refer to Appendix B of the User Guide for recommendations on achieving optimal results.

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Q20. What purification methods do you recommend?
For the Second Strand cDNA purification step (pre-amplification) we require the use of the Agencourt RNAClean XP magnetic beads provided with the kit.

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

Q21. Where can I safely stop in the protocol?
You may stop immediately following the SPIA Amplification or after Post-SPIA Modification II prior to final cleanup at the points specifically noted in the protocol. Store reaction products at –20°C.

Q22. Do you recommend DNase treatment of my total RNA sample?
Yes. Refer to section III.A.4 for more information about DNase treatment of RNA samples.

NuGEN Technologies, Inc.

Headquarters USA

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

Europe

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

For our international distributors contact information, visit our website

www.nugeninc.com

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