WHOLE TRANSCRIPTOME WT-OVATION™ SYSTEM

WT-Ovation™ FFPE RNA Amplification System V2

Catalog # 3400-12, 3400-60

USER GUIDE

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I. Introduction

A. Background

The WT-Ovation™ FFPE RNA Amplification System V2 provides a fast and simple method for preparing amplified cDNA from FFPE-derived total RNA for gene expression analysis. Amplification is initiated at the 3′ end as well as randomly throughout the whole transcriptome in the sample, making this system ideal for amplification of the severely degraded and compromised RNA typically obtained from FFPE samples. The amplified cDNA generated using the WT-Ovation FFPE System V2 can be used for analysis on Affymetrix GeneChip® arrays, Agilent Gene Expression microarrays as well as Illumina Genome-Wide Expression BeadChips utilizing the appropriate NuGEN fragmentation and labeling modules and protocols. For details please visit the NuGEN website. This cDNA also enables the detection of gene transcripts in a wide range of abundance using real-time quantitative PCR (qPCR).

The WT-Ovation FFPE System V2 is powered by Ribo-SPIA technology, a rapid, simple and sensitive RNA amplification process developed by NuGEN. Using whole transcriptome Ribo-SPIA technology and starting with 50 to 100 ng of FFPE-derived total RNA, microgram quantities of cDNA can be prepared in approximately 6 hours.

The WT-Ovation FFPE RNA Amplification System V2 (Cat. # 3400-12, 3400-60) provides optimized reagent mixes and a protocol to process 12 and 60 RNA samples, respectively. Control RNA is not provided with the WT-Ovation FFPE System V2 but we recommend routinely using a control RNA with this product.

B. Ribo-SPIA Technology

Ribo-SPIA technology is a three-step process that generates amplified cDNA from as little as 50 nanograms of total FFPE RNA (see Figure 1).

1. Generation of First Strand cDNA (65 minutes)

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

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

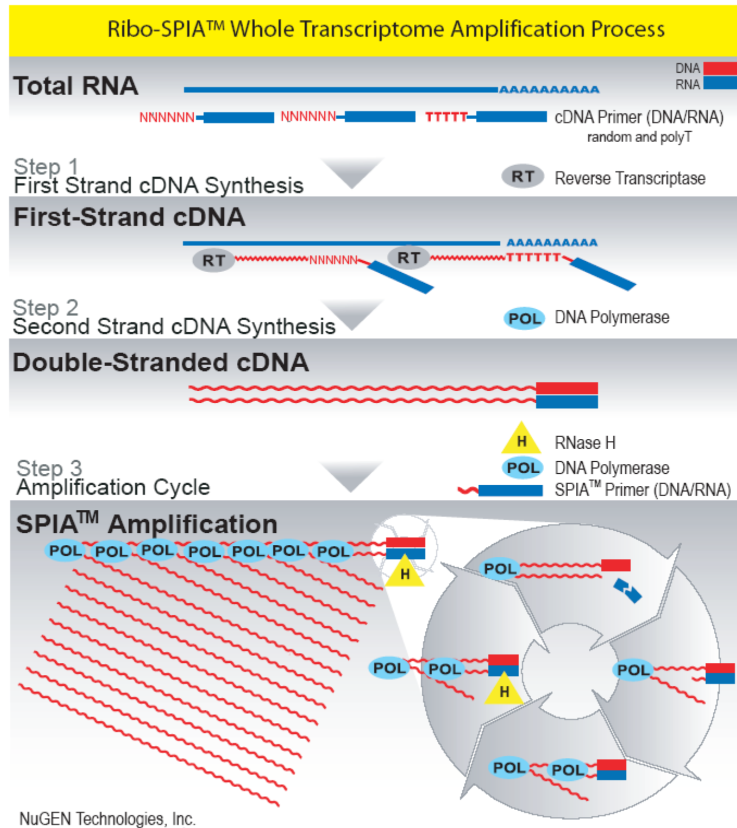
Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthesize a second strand, which includes DNA complementary to the 5′ unique sequence from the first strand chimeric primers. The result is a double stranded cDNA with a unique DNA/RNA heteroduplex at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

3. SPIA Amplification (30 minutes + 60 minutes)

SPIA amplification is a linear isothermal DNA amplification process developed by NuGEN. It uses a SPIA DNA/RNA chimeric primer, DNA polymerase and RNase H in a homogeneous isothermal assay that provides highly efficient amplification of DNA sequences.

RNase H is used to degrade RNA in the DNA/RNA heteroduplex at the 5′ end of the first cDNA strand. This results in the exposure of a DNA sequence that is available for binding a second SPIA DNA/RNA chimeric primer. DNA polymerase then initiates replication at the 3′ end of the primer, displacing the existing forward strand. The RNA portion at the 5′ end of the newly synthesized strand is again removed by RNase H, exposing part of the unique priming site for initiation of the next round of cDNA synthesis. The process of SPIA DNA/RNA primer binding, DNA replication, strand displacement and RNA cleavage is repeated, resulting in rapid accumulation of cDNA with sequence complementary to the original mRNA.

Figure 1. The Ribo-SPIA RNA Amplification Process used in the WT-Ovation™ FFPE System V2



C. Performance Specifications

The WT-Ovation FFPE RNA Amplification System V2 synthesizes microgram quantities of amplified cDNA starting with total cellular RNA extracted from FFPE samples in input amounts of 50 to 100 ng. In approximately 6 hours, the WT-Ovation FFPE System V2 can produce up to 4 to 7 µg of cDNA ready for qPCR or other analysis; high quality RNA templates can yield greater than 7 µg of cDNA. The size of the amplified cDNA products is directly proportional to the size of the degraded RNA used for the amplification reactions. With the whole transcriptome amplification approach the size distribution of the product is far less important compared to a 3' amplifications strategy, since it results in densely overlapping cDNA fragments representing the entire transcriptome. We recommend using the system for a minimum of 4 reactions at a time to prevent pipetting of very small volumes. Setting up fewer numbers of reactions in a batch may result in poor performance due to inaccurate pipetting of such small volumes, and obtaining fewer total reactions than specified.

D. Quality Control

Each WT-Ovation FFPE System V2 lot is tested to meet specifications of yield, qPCR and array performance of the product.

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

E. Storage and Stability

The WT-Ovation FFPE System V2 is shipped on dry ice and should be unpacked immediately upon receipt. The RNAClean® beads are shipped at room temperature.

Note: this product contains components with multiple storage temperatures.

The vials labeled *First Strand Primer Mix* (blue: A1), *SPIA Primer Mix1* (red: C1) and *SPIA Primer Mix2* (red: C5) should be removed from the shipping carton upon delivery and **stored separately at –80 °C**.

Store the RNAClean beads at 4 °C

The vial labeled Agencourt® RNAClean Beads (clear cap) should be removed from the top of the shipping carton upon delivery and **stored at 4 °C, do not freeze the RNAClean Beads!**

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

Kits handled and stored according to the above guidelines will perform to specifications for up to 6 months.

F. Material Safety Data Sheet (MSDS)

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

II. Kit Components

A. Reagents and Supplies Provided

Table 1. First Strand cDNA Reagents

Component	3400-12 Part Number	3400-60 Part Number	Vial Cap	Vial Number
First Strand Primer Mix	S01163	S01195	Blue	**A1 ver3**
First Strand Buffer Mix	S01174	S01191	Blue	**A2 ver3**
First Strand Enzyme Mix	S01040	S01102	Blue	**A3 ver1**

Table 2. Second Strand cDNA Reagents

Component	3400-12 Part Number	3400-60 Part Number	Vial Cap	Vial Number
Second Strand Buffer Mix	S01176	S01192	Yellow	**B1 ver3**
Second Strand Enzyme Mix	S01126	S01193	Yellow	**B2 ver2**

Table 3. SPIA Reagents

Component	3400-12 Part Number	3400-60 Part Number	Vial Cap	Vial Number
SPIA Primer Mix 1	S01218	S01215	Red	**C1 ver5**
SPIA Buffer Mix	S01216	S01194	Red	**C2 ver5**
SPIA Enzyme Mix	S01245	S01166	Red	**C3 ver5**
SPIA Primer Mix 2	S01219	S01214	Red	**C5 ver1**
SPIA Enhancer	S01246	S01247	Red	**C6 ver1**

Table 4. Additional Reagents

Component	3400-12 Part Number	3400-60 Part Number	Vial Cap	Vial Number
Nuclease-free Water	S01001	S01113	Green	**D1**
Agencourt RNAClean Beads	1200-01	1200-01	Clear	**---**

Note: The reagents in the WT-Ovation FFPE System V2 product are similar to reagents in NuGEN's other kits, however, unless the part numbers are identical, these reagents do not have exactly the same composition and therefore are not interchangeable. Do not exchange or replace one reagent named, for example, A1 with another A1, as it will adversely affect performance.

B. Additional Reagents, Supplies and Equipment

Required materials

- **Equipment**
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - 0.5 to 10 µL pipette, 2 to 20 µL pipette, 20 to 200 µL pipette, and 200 to 1000 µL pipette
  - Vortexer
  - Thermal cycler with 0.2 mL tube heat block, heated lid, and 100 µL reaction capacity
  - Appropriate spectrophotometer and cuvettes, or Nanodrop® ND-1000 UV-Vis Spectrophotometer
- **Reagents**
  - Ethanol (Sigma-Aldrich, Cat. # E7023), for purification steps
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - 0.2 mL individual thin wall PCR tubes or 8 x 0.2 mL strip PCR tubes or 0.2 mL thin wall PCR plates
  - Agencourt SPRIPlate® 96R, Ring Magnet Plate (Agencourt, Cat. # A29164) or Agencourt SPRIPlate® Ring Super Magnet Plate, (Agencourt, 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 Beads (Agencourt, Cat. #A29168)
    - MinElute® Reaction Cleanup Kit (QIAGEN, Cat. #28204)
    - QIAquick® PCR Purification Kit (QIAGEN, Cat. #28104)
    - DNA Clean & Concentrator™-25 (Zymo Research, Cat. # D4005)
  - Disposable gloves
  - Kimwipes
  - Ice bucket
  - Decontamination solutions such as RNaseZap® (Ambion, Cat.# AM9780) and DNA -OFF™ (MP Biomedicals, Cat.# QD0500)

Optional materials

- Agilent 2100 bioanalyzer or materials and equipment for electrophoretic analysis of RNA
- Real Time PCR system

To Order:

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

III. Planning the Experiment

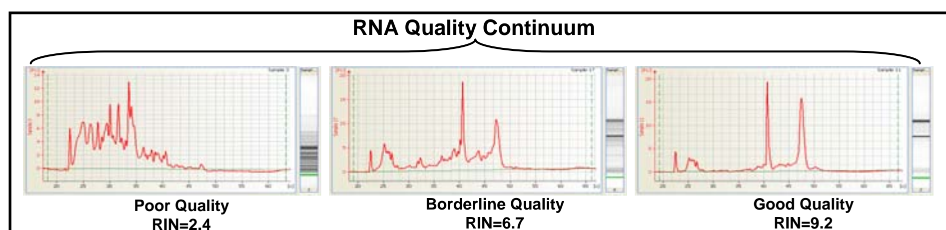
A. Input RNA Requirements

1. **RNA Quantity:** Total FFPE-derived RNA input must be between 50 to 100 ng. Inputs above 100 ng per reaction may inhibit amplification, while lower amounts of input under 50 ng may result in insufficient yields for analysis.
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 good quality Trizol and column purification after isolation, if possible. One measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The A260:A280 ratio for RNA samples of acceptable purity should be in excess of 1.8. RNA samples with lower ratios may result in low amplification yield.
3. **RNA Integrity:** The WT-Ovation FFPE System V2 was designed and optimized for use with highly degraded RNA samples. The whole transcriptome amplification approach allows low quality, highly degraded RNA samples with compromised poly-A tails to be amplified successfully.

   In our tests using degraded FFPE RNA samples showing RIN numbers as low as approximately 2.0 amplified reproducibly, given at least 50 ng of input RNA is used for amplification. We strongly recommend quantitation of total RNA to assure the minimum input requirement is met.

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

   Figure 2. This continuum of RNA quality shows Bioanalyzer traces of 3 different RNAs with varying degrees of quality, all of which have amplified successfully with the WT-Ovation™ FFPE System V2.



4. **RNA Sample Quality Assessment:** The quantity and purity of RNA plays an important role in the success of amplification, however there are instances where quantitative and qualitative data are difficult to obtain for a sample set. NuGEN has developed a tool for assessment of RNA sample suitability for WT-Ovation FFPE System V2 amplification. The WT-Ovation FFPE System Technical Report #1 describes this tool, which includes a data set, procedures, and sequence information for a set of reference qPCR assays, along with assessment recommendations.

   This document may be obtained from the NuGEN website's Technical Documents page, or by contacting the NuGEN technical services team at techserv@nugeninc.com or in Europe europe@nugeninc.com

5. **DNase Treatment**

   It is generally recommended to use DNase-treated RNA for amplification using the WT-Ovation FFPE System V2. One reason is that 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. Additionally, if the total RNA sample contains a significant amount of contaminating genomic DNA, it will be difficult to accurately quantitate the true RNA concentration. The RNA input quantity may therefore be over-estimated based on an absorbance measurement. We recommend using a DNase treatment that will remove contaminating genomic DNA during RNA purification, see section VI.D of this user guide for procedural recommendations.

   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.

6. Carrier use for RNA isolation

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

B. Using RNase-free Techniques

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

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

C. RNA Storage

RNA samples for use with the WT-Ovation FFPE System V2 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 WT-Ovation FFPE System V2 may be stored at –20 °C.

IV. Protocol

A. Overview

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

Stage	Time
1. First strand cDNA synthesis:	**1.2 hours**
2. Second strand cDNA synthesis and Purification:	**2 hours**
3. SPIA Isothermal Linear Amplification 1:	**0.5 hours**
4. SPIA Isothermal Linear Amplification 2 and Purification:	**2 hours**
Total time to prepare amplified cDNA	**~6 hours**

WT-Ovation FFPE 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 WT-Ovation FFPE System V2 may be used as a method of pre-amplification prior to qPCR. Although for qPCR applications it is not absolutely necessary to purify the amplified cDNA, if quantitation of the cDNA product is desired, purification is required. We also recommend purification of cDNA immediately after SPIA if you plan to mass normalize qPCR input. Spectrophotometric quantitation of unpurified amplification products will result in artificially high readings due to amplification components present in the sample.

The cDNA must be purified following amplification if you intend to use the cDNA for fragmentation and labeling using a validated NuGEN fragmentation and labeling system.

B. Protocol Notes

1. 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 maybe 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.
2. In working with FFPE-derived RNA we strongly recommend the use of low retention tips and tubes for storage and diluting the samples, in order to reduce the loss of RNA samples due to adhesion to polypropylene surfaces.
3. Due to the high sensitivity inherent in this amplification system we strongly recommend taking measures to minimize the potential for contamination of amplification reactions by carry-over of nucleic acids or other laboratory contaminants. The two steps to accomplish this are: 1. Designating separate workspaces for “pre-amplified” and “post-amplified” steps and materials and 2. Implementing routine cleanup protocols for workspaces as standard operating procedure. A detailed set of these recommendations is listed in the Appendix.
4. 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.
5. Setting up a minimum of 4 reactions at a time ensures that you are not pipetting very small volumes (see the second strand synthesis section).
6. The reagent volumes recovered greatly depend on the number of batches processed with each kit. Set up no fewer than four reactions at a time with the 3400-12 kit, no fewer than 10 reactions at a time with 3400-60. This ensures sufficient reagent recoveries for 12 total amplifications from a single 12-reaction kit, and 60 from the 60-reaction kit.
7. Thaw components used in each step and immediately place them on ice. Do not thaw all reagents at once.
8. Always keep thawed reagents and reaction tubes on ice unless otherwise instructed.
9. After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve it completely prior to use. You may gently warm the buffer mix for 2 minutes at room temperature followed by brief vortexing. Do not warm any enzyme or primer mixes.
10. When placing small amounts of reagents into the reaction mix, pipette up and down several times to ensure complete transfer.
11. When instructed to pipette mix, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.
12. Always allow the thermal cycler to reach the initial incubation temperature prior to placing the tubes or plates in the block.
13. When preparing master mixes, use the minimal amount of extra material to ensure recovery of the maximum number of reactions.

14. Components and reagents from other NUGEN kits should not be used with this product.
15. Use only fresh ethanol stocks to make 70% ethanol used in the post-second strand bead purification (Section H), and 80% ethanol for washes in the amplified cDNA purification protocols (Section J, Appendix A). Make the ethanol mixes fresh as well, carefully measuring both the ethanol and water with pipettes. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the beads or column.

C. Beckman Coulter's Agencourt® RNAClean® purification beads

Tips and notes relevant to the Second Strand cDNA Cleanup, section H:

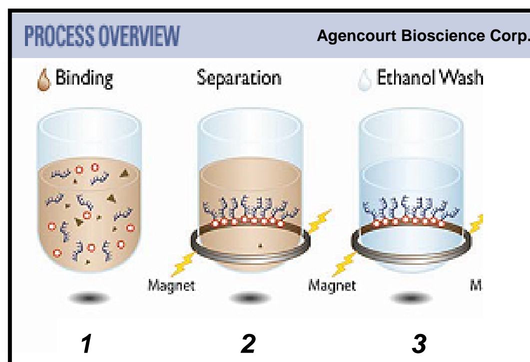
There are significant modifications to the Agencourt® RNAClean® bead's standard procedure; therefore you must follow the procedures outlined in this user guide for the use of these beads with the WT-Ovation™ FFPE System V2. It is recommended to review the Agencourt user guide to become familiar with the manufacturer's recommendations, at the following website: www.agencourt.com/documents/products/rnaclean/Agencourt_RNAClean_Protocol.pdf

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

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

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

Figure 3.



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 reduce recovery.
- Fully resuspend beads by inverting and tapping before adding to sample.
- Note that we recommend using **1.6 volumes** (32 µL) of RNAClean® beads. This is different from the standard Agencourt protocol.
- It is critical to let the beads separate on the magnet for a full five minutes. Removing binding buffer before the beads have completely separated will impact cDNA yields.
- After completing the binding step, 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 during the ethanol washes will impact cDNA yields, so make to minimize bead loss throughout the procedure.
- Ensure that the ethanol wash is freshly prepared from fresh ethanol stocks at the indicated concentration. 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 sample wells or tubes in a small ring. It is critical that all residual ethanol be removed prior to continuing with the SPIA amplification, therefore, when removing the final ethanol wash, first remove most of the ethanol, then allow the excess to collect at the bottom of the tube before removing the remaining ethanol. This reduces the required bead air drying time.
- After drying the beads for at least 15 to 20 minutes, inspect each tube carefully and make certain that all the ethanol has evaporated before proceeding.
- 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 difficult to stably position 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	65 °C for 2 minutes, then 4 °C forever
Program 2 First Strand Synthesis	4 °C for 2 minutes, 25 °C for 30 minutes, 42 °C for 15 minutes, 70 °C for 15 minutes, then 4°C forever
SECOND STRAND cDNA SYNTHESIS	
Program 3 Second Strand Synthesis	4 °C for 1 minute, 25 °C for 10 minutes, 50 °C for 30 minutes, 70 °C for 5 minutes, then 4 °C forever
SPIA AMPLIFICATION	
Program 4 SPIA Amplification 1	4 °C for 1 minute, 47 °C for 30 minutes, then 4 °C forever
Program 5 SPIA Amplification 2	4 °C for 1 minute, 47 °C for 60 minutes, 95 °C for 5 minutes, then 4 °C forever

E. Optional Demodification Protocol

Important Note: Carry out steps E (Optional Demodification Protocol) through I, step 12 (SPIA Amplification Protocol) in a ***pre-amplification workspace*** using dedicated pre-amplification consumables and equipment. Wipe all surfaces, equipment and instrumentation with a DNA decontaminant solution such as DNA-OFF (MP Biomedicals, Cat# Q0500) 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 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).

Formalin fixation of tissues results in a high degree of cross linking of the RNA. It is essential to reverse these cross links 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 de-modification is incomplete, we strongly recommend that you perform the following steps for demodification prior to using the WT-Ovation FFPE System V2.

This example assumes a 50 ng input RNA into cDNA synthesis:

1. Add 2.5 µL of 20 ng/µL RNA 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 First Strand cDNA Synthesis.

F. First Strand cDNA Synthesis Protocol

1. Obtain First Strand Buffer Mix (blue: A2), First Strand Enzyme Mix (blue: A3), and the water (green: D1) from the components stored at –20 °C and the First Strand Primer Mix (blue: A1) stored at –80 °C.
2. Flick to mix, then spin down contents of A3 for 2 seconds and place on ice.

Flick, do not vortex any enzyme mixes.

3. Thaw the other reagents at room temperature. Mix by vortexing for 2 seconds then spin for 2 seconds and place on ice. Leave water, D1 at room temperature.
4. Add 2 µL of A1 to a 0.2 mL PCR tube.
5. Add 5 µL of total RNA sample (50-100 ng) to the primer. Dilute RNA in Nuclease-free Water (D1), if necessary.
   Note: If you have performed the demodification step, add 2 µL of A1 directly to the RNA.
6. Cap and spin tube(s) for 2 seconds and return tubes to ice.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 5):
   a. Incubate at 65 °C for 2 minutes
   b. Cool to 4 °C
8. Remove tubes from the thermal cycler and place tubes on ice.

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

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

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

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 VER1)
2.5 µL	**0.5 µL**

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

10. Add 3 µL of the First Strand master mix to each tube.
11. Mix by pipetting three times, spin for 2 seconds.
12. Place tubes in a pre-cooled thermal cycler programmed to run Program 2 (First Strand Synthesis; see Table 5):
    a. Incubate at 4 °C for 2 minutes
    b. Incubate at 25 °C for 30 minutes
    c. Incubate at 42 °C for 15 minutes
    d. Heat at 70 °C for 15 minutes
    e. Cool to 4 °C
13. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
14. Continue immediately with second strand cDNA synthesis.

G. Second Strand cDNA Synthesis Protocol

1. Remove the RNAClean purification beads from 4 °C and place on bench-top to reach room temperature for use in the next step
2. Obtain the Second Strand Buffer Mix (yellow: B1) and Second Strand Enzyme Mix (yellow: B2), from the components stored at –20 °C.
3. Flick to mix, then spin down contents of B2 for 2 seconds and place on ice.
4. Thaw reagent B1 at room temperature, mix by vortexing for 2 seconds, spin for 2 seconds, and then place on ice.
5. Make a master mix by combining B1 and B2 in a 0.5 mL capped tube, according to the volumes shown in Table 7.

   In order to ensure accurate measurement of the B2 reagent, do not make this mix for fewer than 4 reactions

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

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

SECOND STRAND BUFFER MIX (YELLOW: B1 VER3)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER2)
9.75 µL	**0.25 µL**

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

6. Add 10 µL of the Second Strand master mix to each First Strand reaction tube.
7. Mix by pipetting three times, spin for 2 seconds, then place on ice.
8. Place tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand Synthesis; see Table 5):
    a. Incubate at 4 °C 1 minute
    b. Incubate at 25 °C for 10 minutes
    c. Incubate at 50 °C for 30 minutes
    d. Heat at 70 °C for 5 minutes
    e. Cool to 4 °C
9. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
10. It is highly recommended to remove 2 µL of cDNA prior to purification for qPCR assessment of overall RNA quality. Refer to WT-Ovation FFPE System Technical Report #1 for detailed instruction and recommendations on the use of this sample quality assessment tool.
11. Continue immediately with purification of unamplified cDNA.

Best results are obtained by using fresh 70% EtOH in wash step recovery.

H. Purification of cDNA Protocol

1. Ensure the Agencourt RNAClean beads have completely reached room temperature before proceeding.
2. Resuspend beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample.

3. After resuspending, do not spin the beads. A large excess of beads is provided, therefore it is not necessary to recover any trapped in the cap.
4. 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.

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

5. At room temperature, add 32 µL (**1.6 volumes**) of the bead suspension to each reaction and mix by pipetting up and down 10 times.
6. Incubate at room temperature for 10 minutes.
7. Transfer tubes or plate to magnet plate and let stand 5 minutes to completely clear the solution of beads.
8. Carefully remove only 45 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

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

Ensure that all residual ethanol is removed prior to continuing with the SPIA™ Amplification

10. Remove the 70% ethanol wash using a pipette.
11. Repeat the 70% ethanol 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.
12. Air dry the beads on the magnet for a minimum of 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 the SPIA Amplification.
13. Proceed immediately with SPIA Amplification with the cDNA still bound to the dry beads.

I. SPIA® Amplification Protocol

1. Obtain the SPIA Buffer Mix (red: C2), SPIA Enzyme Mix (red: C3) and SPIA Enhancer (red: C6) stored at –20°C and the SPIA Primer Mix 1 (red: C1) and SPIA Primer Mix 2 (red: C5) stored at –80°C.
2. Thaw reagents C1, C2 and C5 at room temperature, and mix by vortexing for 2 seconds, spin in a microcentrifuge for 2 seconds, then place on ice.
3. Thaw C3 and C6 on ice and mix the contents by pipetting gently. *Ensure the enzymes are well mixed without introducing bubbles,* spin for 2 seconds and place on ice.
4. Make a master mix by sequentially combining C2, C1, C6 and C3 in an appropriately sized capped tube according to the volumes shown in Table 8. Mix well after the addition of the C6. Add C3 and mix well at the last moment.

Use SPIA Master Mix immediately after preparation.

Table 8. SPIA® Master Mix 1 (volumes listed are for a single reaction)

SPIA® BUFFER MIX (RED:C2 VER5)	SPIA® PRIMER MIX 1 (RED:C1 VER5)	SPIA® ENHANCER (RED:C6 VER1)	SPIA® ENZYME MIX (RED:C3 VER5)
50 µL	**20 µL**	**0.7 µL**	**10 µL**

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

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 60 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 1, see Table 5):
   a. Incubate at 4 °C for 1 minute
   b. Incubate at 47 °C for 30 minutes
   c. Cool to 4 °C
7. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
8. Make a master mix by sequentially combining C2, C5, C6 and C3 in an appropriately sized capped tube according to the volumes shown in Table 8. Mix well after the addition of the C6. Add C3 and mix well at the last moment.

Table 9. SPIA® Master Mix 2 (volumes listed are for a single reaction)

SPIA® BUFFER MIX (RED:C2 VER5)	SPIA® PRIMER MIX 2 (RED:C5 VER1)	SPIA® ENHANCER (RED:C6 VER1)	SPIA® ENZYME MIX (RED:C3 VER5)
30 µL	**20 µL**	**2.3 µL**	**30 µL**

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

9. Add 80 µL of the SPIA master mix to each tube containing the SPIA reaction. Use a pipette set to 80 µL and mix well by pipetting up and down at least 3 times.
10. Transfer one half of the reaction volume (80 µL) to a second tube.
11. Place tubes in a pre-cooled thermal cycler programmed to run Program 5 (SPIA Amplification 2, see Table 5):
    a. Incubate at 4 °C for 1 minute
    b. Incubate at 47 °C for 60 minutes
    c. Heat at 95 °C for 5 minutes
    d. Cool to 4 °C
12. Remove tubes from the thermal cycler, spin for 2 seconds, then place on ice. **Do not re-open tubes or plate 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 contamination of your pre-amplification workspace with amplified cDNA present in the post-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).

13. Recombine the half-reactions.
    Note: If using the Agencourt RNAClean Bead method for final cDNA cleanup it is not necessary to recombine the half-reactions or to remove the beads.
14. Transfer tubes or plate to magnet plate and let stand 5 minutes to completely clear the solution of beads.
15. Carefully remove all of the cleared supernatant containing the eluted cDNA and transfer to a fresh tube. The beads may now be discarded.
16. At this stage, the cDNA may be purified or stored at –20 °C.

J. Purification of Amplified cDNA Protocol

Amplified SPIA cDNA product can be purified using various methods listed in Appendix A. Purification is required if the amplified cDNA is intended for use in fragmentation and labeling reactions.

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

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

K. Measuring cDNA Product Yield and Purity

1. Mix your sample by brief vortexing and spinning prior to checking the concentration.
2. Measure the absorbance at 260, 280 and 320 nm of your amplified cDNA product. You may need to make a 1:20 dilution of the cDNA in water prior to measuring the absorbance.
3. Purity: Subtract the Abs_{320} value from both Abs_{260} and Abs_{280} values. The adjusted ($Abs_{260} – Abs_{320} / Abs_{280} – Abs_{320}$) ratio should be > 1.8.
4. Yield: Assume 1 absorbance unit at 260 nm of single-stranded DNA = 33 µg/ mL.
   To calculate:
   ($Abs_{260} – Abs_{320}$ of diluted sample) x (dilution factor) x 33 (concentration in µg/mL of a 1 absorbance unit solution) x 0.03 (final volume in mL) = total yield in micrograms
5. Alternatively, you may measure the concentration and purity of cDNA with a Nanodrop, using 1 absorbance unit at 260 nm of single-stranded DNA = 33 µg/ mL as the constant.
6. The purifed cDNA may be stored at -20 °C.

V. Technical Support

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

In Europe contact NuGEN at +31(0)135780215 (Phone) or +31(0)135780216 (Fax) or email 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) Agencourt RNAClean Magnetic Beads, 2) the Qiagen MinElute Reaction Cleanup Kit, 3) the Qiagen QIAQuick PCR Purification Kit, and 4) the Zymo Clean & Concentrator-25.

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.

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

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

Note: Do not recombine 80 µL half-reactions. Stop after step 12 on page 15. It is not necessary to remove the beads from the SPIA reactions. Begin purification as follows:

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

   Note: If using a 96-well plate format with both half-reactions on the same plate, it will be necessary to transfer the sample/bead mixture to a fresh plate at this point.
5. Incubate sample/bead mixture at room temperature for 5 minutes.
6. Place the first set of samples (containing beads) on the SPRIPlate® 96R Magnet Plate for 10 minutes or until the solution appears clear.
7. After 5 minutes of the 10 minute incubation in step 6 have elapsed, add 144 µL of resuspended RNAClean beads (1.8 times the sample volume) to *second* set of half-reactions containing the remaining 80µL cDNA samples. Incubate samples at room temperature for 5 minutes.
8. Using a multi-channel pipette, remove and discard the supernatant from first set of samples (on magnet). Do not disturb the ring of magnetic beads.
9. Using a multi-channel pipette add the sample/bead mix from the second set of half-reactions (prepared in step 7) to the appropriate tubes or wells containing the beads from the first half-reaction while it is still placed on the magnet. Add slowly as to not disturb the bead ring already in each well.

   Note: Here the potential for sample cross contamination is high; take care to combine the correct half-reactions.
10. 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 pipette 10 to 15 µL up and down at the liquid surface to break the tension and allow the beads to sink to the magnet ring.
11. Using a multi-channel pipette, remove and discard the supernatant. Do not disturb the ring of magnetic beads.
12. With the samples still on the magnet plate, add 200 µL of freshly prepared 80% ethanol to each well of the reaction plate and incubate for 30 seconds or until the solution clears. Add slowly as to not disturb the separated magnetic beads.
13. Using a multi-channel pipette, remove and discard the ethanol.
14. Repeat the 80% ethanol wash once more. Ensure as much ethanol as possible is removed from the plate.
15. Remove the reaction tubes or plate from the magnet and air dry the reaction plate on bench top for no more than 2 minutes. If the beads dry too long, they are difficult to resuspend.

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

16. With the plate on bench top, add 30 µL of room temperature nuclease-free water to each well. Holding the plate firmly, very carefully vortex for 30 seconds or use a plate shaker set to medium speed. Ensure the beads are fully resuspended, vortex longer if necessary. Alternatively, the beads may be resuspended by repeated pipetting.
17. Replace reaction tubes or plate on the plate magnet; allow the beads to separate for 5 minutes or until the solution clears.
18. Using a multi-channel pipette remove the eluted sample and place into a fresh reaction tube or plate. There should be approximately 30 µL of purified cDNA.

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

Qiagen MinElute Spin Column (instructions for a single full reaction, 2 columns are required per reaction)

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

1. Ensure that 100% ethanol has been added to Buffer PE as described in the Qiagen MinElute Handbook. Failure to add ethanol to this buffer will result in low amplification yield.
2. Prepare an 80% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content which may reduce amplification yield.
3. Add 600 µL of Buffer ERC to a labeled 1.5 mL tube for each amplification reaction.
4. Transfer each full reaction (160 µL) into a tube containing the Buffer ERC.
5. Vortex for 5 seconds and spin down briefly.
6. Obtain and label two Qiagen MinElute Spin Columns for each amplification reaction and place them into collection tubes.
7. Load 380 µL (one-half) of each reaction/buffer mix onto each of the two labeled Qiagen MinElute Spin Columns.

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

8. Centrifuge columns in the collection tube for 1 minute at >10,000 x g in a microcentrifuge.
9. Discard flow-through and replace the Qiagen MinElute Spin Column in the same collection tube.
10. Wash sample by adding 500 µL of Buffer PE (prepared according to manufacturer's recommendations). Centrifuge column in the collection tube for 1 minute at >10,000 x g. Discard flow-through.
11. Add 500 µL of the room temperature 80% ethanol prepared in Step 1 above.

    Note: Use fresh 80% ethanol.
12. Centrifuge column in the collection tube for 1 minute at >10,000 x g. Discard flow-through.
13. Place the column back in the same collection tube and spin for an additional 2 minutes at >10,000 x g.

    Important: Residual ethanol from the wash buffers will not be completely removed unless the flow-through is discarded before this additional centrifugation.
14. Blot the column tip onto a filter paper to remove any residual wash buffer from the tip of the column.

    Note: Blot column tip prior to transferring it to a new tube to prevent any wash buffer transferring to the eluted sample.
15. Place the MinElute Column in a clean, labeled 1.5 mL microcentrifuge tube.
16. Add 15 µL of room temperature nuclease-free water (green: D1) from the kit to the center of each column. ***Do not use cold water!***

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

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

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

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

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

1. Into a clean 1.5 mL tube add 800 µL of PB buffer from the QIAGEN system.
2. Add the 160 µL of amplified cDNA product to the tube.
3. Vortex for five seconds and spin down for two seconds.
4. Obtain one QIAquick® spin column and insert into a collection tube.
5. Load 480 µL of sample onto the column.
6. Centrifuge column in a collection tube for one minute at 13,000 rpm (~17,900 x g).

7. Discard flow-through. Place the column back in the same collection tube.
8. Load remaining 480 µL onto the same column. Centrifuge column in collection tube for one minute at 13,000 rpm. Discard flow-through.
9. Place the column back in the same collection tube. Add 700 µL of 80% ethanol.
   Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
10. Centrifuge the column for one minute at 13,000 rpm. Discard flow-through.
11. Repeat steps 9 and 10 once.
12. To remove remaining liquid, centrifuge column for one additional minute at 13,000 rpm.
13. Remove the column from the centrifuge. Discard flow-through with the collection tube.
14. Blot the tip of the column onto a filter paper in order to remove any residual wash buffer from the tip of the column.
   Note: Blot column tip prior to transferring it to a new tube to prevent any wash buffer transferring to the eluted sample.

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

15. Place the column in clean 2.0 mL collection tube, appropriately labeled.
16. Add 30 µL of nuclease-free water (green: D1) to the center of each column.
   Do not use cold water!
17. Let columns stand for five minutes at room temperature to elute purified cDNA.
18. Centrifuge at 13,000 rpm for one minute to collect sample. There should be approximately 30 µL of purified cDNA.
19. Mix sample by vortexing, then spin briefly.
20. Proceed to Measuring cDNA Product Yield and Purity or store purified cDNA at –20 °C.

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

1. Into a clean 1.5 mL tube add 320 µL of DNA Binding Buffer.
2. Add 160 µL of amplified SPIA™cDNA product.
3. Vortex and spin down briefly.
4. Obtain one Zymo-Spin II Column and place it into a collection tube.
5. Load the entire volume of sample (480 µL) onto the Zymo-Spin II Column.

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

6. Centrifuge column in the collection tube for 10 seconds at >10,000 x g in a microcentrifuge.
   Note: Be sure to wait until rotor achieves desired speed before starting timer for spins less than 1 minute in this procedure.
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 90 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.
13. Place the Zymo-Spin II Column in a clean 1.5 mL microcentrifuge tube.

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

14. Add 30 µL of room temperature nuclease-free water (green: D1) from the kit to the center of each Zymo-Spin II column. Do not use cold water!
15. Let columns stand for 1 minute at room temperature.
16. Centrifuge column and microcentrifuge tube for 30 seconds at >10,000 x g in a microcentrifuge.
17. Collect sample. There should be approximately 30 µL of purified cDNA.
18. Mix sample by vortexing, then spin briefly.
19. Proceed to Measuring cDNA Product Yield and Purity or store purified cDNA at –20 °C.

B. Performing Quantitative PCR on Amplified cDNA

It is recommended that the amplified cDNA generated from the WT-Ovation FFPE System V2 be purified prior to use in real time quantitative PCR reactions. Since different amplified cDNA samples may be variable in concentration, the purified products can be quantitated and mass normalized to ensure the cDNA inputs to qPCR are equal for all samples. Purified amplified cDNA produced with the kit has been successfully used as templates for qPCR systems including TaqMan® and SYBR® Green. Note that RT-PCR master mixes containing the enzyme Uracil N-Glycosylase (UNG) are not compatible with the WT-Ovation FFPE System V2. NuGEN can recommend 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), or 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:

Dilution of the Amplified Product: After purification and quantitation of amplified cDNA, it can be diluted to an appropriate concentration for qPCR reaction. We recommend using 20 ng of cDNA in a 20 µL Taqman reaction and 2 ng of cDNA for a 25 µL SYBR Green reaction. Depending on the abundance of the transcripts of interest you may use more or less cDNA.

Primer Design: We strongly recommend designing multiple assays across the length of the transcript since the starting FFPE RNA is likely to be highly degraded. We also recommend using primers and probes designed with as small an amplicon size as possible due to the degraded nature of the input RNA. Primers may be designed at any position along a transcript since the WT-Ovation amplification covers the entire length of transcripts.

C. Quality Control of Amplified cDNA Product

The primary quality test for the amplified DNA is the yield of amplification. We highly recommend using the RNA quality assessment tool for at least a subset of samples being amplified, in order to determine the expected level of performance on arrays.

D. DNase Treatment of RNA

i. DNase treatment during purification: using the QIAGEN RNase-Free DNase Set and the RNeasy Mini RNA purification kit

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

   Note: Other **DNase I** enzymes we can recommend to use in this step are the Shrimp DNase (recombinant) from USB Corp. (use 10 µL) , or the DNase I (RNase-free) from New England BioLabs (use 10 µL). See the Additional Reagent section of this user guide for ordering information.
9. Pipette the DNase I incubation mix (80 µL) directly onto the membrane inside the RNeasy mini column. Incubate on the bench top (~ 25 °C) for 15 min.
10. Add 350 µL Buffer RW1 into the RNeasy mini column, and centrifuge for 15 seconds at ≥ 8000 x g (≥10,000 rpm) to wash. Discard the flow through.
11. Transfer the RNeasy column to a fresh 2 mL collection tube. Add 500 µL Buffer *RPE* (with the added ethanol) to the RNeasy column.
12. Close the tube gently, and centrifuge for 15 seconds at ≥ 8000 x g (≥10,000 rpm). Discard the flow-through.
13. Add another 500 µL Buffer *RPE* to the RNeasy column.
14. Close the tube gently, and centrifuge for 2 minutes at ≥8000 x g (≥10,000 rpm). Discard the flow-through.
15. Transfer the RNeasy column to a new 1.5 mL collection tube.
16. Pipet 30 to 50 µL RNase-free water directly onto the RNeasy membrane.
17. Close the tube gently, and centrifuge for 1 minute at ≥8000 x g (≥10,000 rpm) to elute.
18. If yields of greater than 30 µg are expected, repeat elution step and collect in the same collection tube.

ii. DNase Treatment of RNA post-purification: using RNase-free DNase and either the Zymo RNA Clean-up Kit™ - 5 columns or the RNeasy MinElute columns

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

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

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

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

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

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

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

- Add 80 µL ice-cold RNase-free water (D1, green cap) to the sample on ice.
- Add 350 µL Buffer *RLT* and mix by pipetting.
- Add 250 µL 96 to 100% ethanol and mix thoroughly by pipetting.
- Place an RNeasy MinElute Spin Column into a 2 mL collection tube (one column per sample) and apply the 700 µL sample to the column and cap the column
- Spin for 15 seconds at ≥8000 x g (≥10,000 rpm) and discard the flow-through.
- Place the RNeasy MinElute Spin Column into a fresh 2 mL collection tube. Add 500 µL Buffer *RPE* to the column and close the tube. Spin for 15 seconds at ≥8000 x g (≥10,000 rpm) and discard the flow-through keeping the same collection tube.
- Add 500 µL 80% ethanol to the RNeasy MinElute Spin Column and close the tube.
  Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
- Spin for 2 minutes at ≥8000 x g (≥10,000 rpm) and discard the flow-through.
- Place the RNeasy MinElute Spin Column in a fresh 2 mL collection tube and place in the microcentrifuge with the cap open.
- Spin for 5 minutes at ≥8000 x g (≥10,000 rpm) and discard the flow-through.
- Place the RNeasy MinElute Spin Column in a fresh 1.5 mL collection tube.
- Add 14 µL nuclease-free water (D1, green cap) directly to the center of the filter in the tube and close the cap. ***Do not use cold water!***
- Spin for 1 minute at ≥8000 x g (≥10,000 rpm) to collect the purified RNA.

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.

E. Preventing Crossover Contamination

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

1. Designate a pre-amplification workspace separate from the post-amplification workspace or general lab areas:
   a. Post-amplification include all steps and materials related to the handling of the final amplified cDNA product, purification, array hybridization, and any other analytical work. This also includes any work and materials related to other non-NuGEN protocols.
   b. Pre-amplification include all steps and materials related to RNA sample handling and dilution, NuGEN's first strand reaction, second strand reaction, and the second strand product cleanup.
   c. Ideally pre-amplification workspace would be in a separate work room. If this is not possible, ensure the pre-amplification area is sufficiently distant and not in the path of post-amplification work.
   d. We recommend the use of "PCR Workstation" enclosure with UV illumination dedicated for NuGEN pre-amplification protocol.
   e. Materials and consumables for pre-amplification work should be regularly exposed to UV illumination to control nucleic acid surface contamination.

2. Establish and maintain a clean work environment:
   a. Initially clean the entire lab thoroughly with DNA OFF™ and RNaseZap®.
      i. In the pre-amplification area, remove all small equipment, and then clean every surface that may have been handled without gloves (drawer handles, key pads, etc). Before reintroducing any equipment, clean every piece of equipment thoroughly. Especially clean wells of thermal cycler(s) and magnetic plate(s) with a Q-tip or by filling with decontamination solution.
   b. Always wear gloves and don fresh gloves upon entry into this controlled area. Frequently change gloves while working in the pre-amplification area, especially prior to handling stock reagents.
   c. Stock this area with clean (preferably new) equipment (pipettes, racks, consumables) that has not been exposed to post-amplification workspace.
   d. Make it a policy to carry out continual regular decontamination of all workspaces.
   e. Capture waste generated in both pre- and post-amplification areas (tips, columns, wash solutions from beads and columns, tubes, everything) in seal-able plastic bags and dispose of promptly after each experiment to avoid waste spillage.
   f. Do not open amplified product reaction vessels in the pre-amplification workspace.
3. Utilize negative controls in order to detect and troubleshoot a contamination issues. The clearest indication that an amplification reaction is contaminated is the appearance of significant amounts of amplified product in a 'negative' control or No Template Control (NTC).
   a. **In the absence of contamination:**
      i. NTC yields for WT-Ovation FFPE and WT-Ovation Pico amplifications are typically at or below 3 micrograms.
      ii. Products generated from uncontaminated NTC reactions do not yield significant array hybridization even when applied to arrays at standard input amounts.
      iii. Bioanalyzer trace of this normal NTC product is very characteristic.
   b. **In the presence of contamination:**
      i. NTC yields are generally significantly higher than 3 micrograms, making NTC results the most reliable indicator of contamination.
      ii. Contaminated NTC yields can be as high as or even higher than template containing reactions (i.e. your experimental samples or positive controls).
      iii. The bioanalyzer traces of contaminated NTC reactions look significantly different than the typical non-contaminated NTC reaction traces.
4. When contamination is detected in reactions containing templates:
   a. The amount of product generated from a template containing amplification reaction may or may not be affected, depending on the source of the contamination.
   b. The bioanalyzer trace of the amplified product may or may not look altered.
   c. The % present calls on arrays run with amplified product generated from a contaminated sample may be lower than expected.

F. Troubleshooting Suggestions

What can I do if my FFPE RNA samples give amplified cDNA yields consistently below specifications?
While keeping in mind that some FFPE samples are of extremely poor quality and may never produce usable data, there are a couple of ways the user can boost their amplified cDNA yield with the WT-Ovation FFPE kit. First, increase the 47 degree incubation time from 60 minutes to 90 minutes during the SPIA Amplification 2 incubation (page 12, step I.11).

Second, use the Qiagen MinElute method (page 14, Appendix A) for the final cDNA purification.

These two minor protocol enhancements can significantly increase the cDNA yield for some FFPE RNA samples without negatively impacting the expression profile generated. Contact NuGEN Technical Services if you have any questions about these recommendations.

G. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the WT-Ovation FFPE System V2?
The WT-Ovation FFPE 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 Agencourt RNAClean magnetic beads for double stranded cDNA purification.

Q2. Does the WT-Ovation FFPE System V2 provide any fragmentation and labeling reagents?
No, however the cDNA output of this kit may be processed further using validated NuGEN fragmentation and labeling products and protocols.

Q3. 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.

Q4. What additional consumables does the user need? For the SPIA cDNA purification step, purification columns or beads are required.

Q5. Do I need to use high quality total RNA?
The WT-Ovation FFPE System V2 employs the whole transcriptome amplification approach and was designed and optimized for use with highly degraded, lower quality RNA samples and transcripts with a compromised poly-A.

Q6. Can I use RNA from other sources than FFPE?
Yes. Degraded as well as intact RNA from other sources may be successfully amplified using the WT-Ovation FFPE System V2. With good quality RNA we recommend you use 2 to 20 ng starting material.

Q7. Is the WT-Ovation FFPE System V2 3 prime 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. We have tested the system with both degraded and intact RNA on 3' biased microarrays as well as arrays interrogating sequence in all regions of the transcript (i.e. "exon" arrays) with successful results.

Q8. Where in my target sequence can I design my qPCR primers?
The WT-Ovation System V2 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 since the starting FFPE RNA is likely to be highly degraded.

Q9. How much FFPE total RNA do I need for amplification?
We recommend total FFPE RNA inputs in the range of 50 ng to 100 ng. Input amounts outside this range may produce unsatisfactory variable results, especially for more degraded RNA.

Q10. How much cDNA can I expect from a single reaction?
You should expect 4 to 7 µg of cDNA from 50 to 100 ng total FFPE RNA starting material, if it is of sufficient quality. Yields using 10 ng of high quality control RNA can be significantly higher (10+ µg).

Although yield is 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 Technical Report #1.

Q11. Is the cDNA yield dependent upon the quantity of total RNA input?
Yes, the higher the RNA input into the amplification reaction, the higher the yield will be, however, at FFPE RNA inputs of above 100 ng, the yields may become variable.

Q12. What is the amplification efficiency of the WT-Ovation FFPE System V2?
Based on qPCR on a variety of genes, an average amplification efficiency of 10,000 to15,000 fold is observed.

Q13. What size cDNA is generated by the WT-Ovation FFPE System V2?
The amplified cDNA size distribution is entirely dependent on the input RNA integrity. In a whole transcriptome amplifications strategy however, the size of the resulting cDNA is not of significant consequence for use on arrays.

Q14. Can DNA be used as input for the WT-Ovation FFPE System V2?
No. The WT-Ovation FFPE System V2 is designed to amplify mRNA, not DNA.

Q15. Can contaminating genomic DNA interfere with the WT-Ovation FFPE System V2?
This system is designed to amplify RNA, but large amounts of contaminating genomic DNA may amplify during the process, so we recommend DNase treatment during RNA purification.

Q16. Can I use the WT-Ovation FFPE System V2 on bacterial RNA samples?
The WT-Ovation amplification process theoretically will work with some bacterial RNAs. However, currently, the kit has not been optimized or validated for this purpose.

Q17. Has NuGEN performed reproducibility studies on the WT-Ovation FFPE System V2?
Yes. Sample to sample, and lot-to-lot reproducibility studies are routinely conducted.

Q18. Does the WT-Ovation FFPE System V2 generate product in the absence of RNA input?
In the complete absence of input RNA non-specific product is generated with <3 µg yields. However, note that in the presence of even very small amount of RNA, while the yields may be low the cDNA is likely specific, and representing actual amplification products.

Q19. Can I use the WT-Ovation FFPE System V2 for archiving cDNA?
Amplified cDNA may be stored at –20 °C for up to 6 months. Long term tests are in progress.

Q20. Do I need to order specific primers for the amplification?
No. The DNA/RNA primers provided in the WT-Ovation FFPE System V2 are universal.

Q21. Do I have to use your DNA/RNA primers?
Yes, the system will not perform with other primers.

Q22. Do you recommend purification of the cDNA prior to qPCR analysis?
Yes. Although this is not an absolute requirement, it is an essential step prior to spectrophotometric quantitation of amplification yields. The assessment of the amplification yields allows mass normalization of the cDNA into qPCR.

Q23. What purification methods do you recommend?

- For the Second Strand cDNA purification step (pre-amplification) we require the use of the Agencourt RNAClean® magnetic beads provided with the kit.
- Several purification options are available for the final SPIA cDNA cleanup 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.

Q24. Where can I safely stop in the protocol?
We do not recommend stopping at any intermediate stage of the protocol.

Q25. Do you recommend DNase treatment of my total RNA sample?
Yes, see section **III.A.5.**

Q26. How many qPCR reactions will I get from one WT-Ovation FFPE amplification?
The number of qPCR reactions depends on the abundance level of the genes being interrogated. For medium to high copy genes, the cDNA may be diluted as much as 400-fold, enough for thousands of qPCR reactions. For very low copy genes more cDNA must be used per qPCR reaction. We recommend purification of the amplified cDNA prior to qPCR analysis.

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