USER GUIDE

Ovation® FFPE WTA System

PART NO. 3403

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I. Introduction

A. Background

The Ovation® FFPE WTA System 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 chemically modified RNA typically obtained from FFPE samples. The amplified cDNA generated using the Ovation FFPE WTA System can be used for analysis on microarrays (Affymetrix GeneChip® Arrays, Agilent Gene Expression microarrays, Illumina Genome-Wide Expression BeadChips and others) utilizing the appropriate Encore® labeling module or validated labeling protocol. The amplified cDNA can also be used as input for qPCR reactions. For details please visit the NuGEN website.

The Ovation FFPE WTA System 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 4.7 hours.

The Ovation FFPE WTA System (Part No. 3403) provides optimized reagent mixes and a protocol to process total RNA samples. Control RNA is not provided with the Ovation FFPE WTA System but we recommend routinely using a control RNA with this product.

I. Introduction

B. Ribo-SPIA® Technology

Ribo-SPIA technology is a three-step process that generates amplified cDNA from as little as 50 nanograms of FFPE derived total 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 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 (1.5 hours)**
   SPIA is a robust isothermal strand displacement amplification process developed by NuGEN. It uses a DNA/RNA chimeric SPIA primer, DNA polymerase and RNase H in a homogeneous isothermal assay that provides highly efficient amplification of DNA sequences. RNase H is used to degrade RNA in the DNA/RNA heteroduplex at the 5′ end of the first cDNA strand. This results in the exposure of a DNA sequence that is available for binding the first SPIA primer. DNA polymerase then initiates replication at the 3′ end of the primer, displacing the existing forward strand. The RNA portion at the 5′ end of the newly synthesized strand is again removed by RNase H, exposing part of the unique priming site for initiation of the next round of cDNA synthesis. The process of SPIA DNA/RNA primer binding, DNA replication, strand displacement and RNA cleavage is repeated, resulting in rapid accumulation of SPIA cDNA.

I. Introduction

C. Performance Specifications

The Ovation FFPE WTA System synthesizes microgram quantities of SPIA cDNA starting with total cellular RNA extracted from FFPE samples in input amounts of 50 to 100 ng. In approximately 4.7 hours, the Ovation FFPE WTA System can produce at least 4 µg of cDNA ready for labeling for microarray hybridization, qPCR or other analysis. High quality, non-FFPE RNA samples may produce higher yields. The size of the amplified cDNA is proportional to the size of the input RNA used. With our whole transcriptome amplification approach the size distribution of the product is far less important compared to a 3´ amplification strategy, since it results in densely overlapping cDNA fragments representing the entire transcriptome.

D. Quality Control

Each Ovation FFPE WTA System lot is tested to meet specifications of yield, qPCR and array performance.

E. Storage and Stability

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

All kit components, except the Agencourt RNAClean XP Beads (clear cap), should be stored at –20°C in a freezer without a defrost cycle.

! *Store 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.

This product has been tested to perform to specifications after as many as six freeze/thaw cycles. Kits handled and stored according to the above guidelines will perform to specifications for at least six months. NuGEN has not yet established long-term storage conditions for the Ovation FFPE WTA System.

F. Material Safety Data Sheet (MSDS)

An MSDS for this product is available on the NuGEN website at http://www.nugeninc.com/nugen/index.cfm/support/user-guides/.

II. Kit Components

A. Reagents Provided

Table 1. First Strand cDNA Reagents

COMPONENT	3403-12 PART NUMBER	3403-60 PART NUMBER	3403-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
First Strand Primer Mix	S01399	S01403	S01406	Blue	A1 VER 8
First Strand Buffer Mix	S01174	S01191	S01287	Blue	A2 VER 3
First Strand Enzyme Mix	S01040	S01102	S01288	Blue	A3 VER 1

Table 2. Second Strand cDNA Reagents

COMPONENT	3403-12 PART NUMBER	3403-60 PART NUMBER	3403-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
Second Strand Buffer Mix	S01176	S01192	S01289	Yellow	B1 VER 3
Second Strand Enzyme Mix	S01126	S01193	S01290	Yellow	B2 VER 2

Table 3. SPIA Reagents

COMPONENT	3403-12 PART NUMBER	3403-60 PART NUMBER	3403-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
SPIA Primer Mix	S01400	S01416	S01407	Red	C1 VER 9
SPIA Buffer Mix	S01401	S01404	S01408	Red	C2 VER 10
SPIA Enzyme Mix	S01402	S01405	S01409	Red	C3 VER 7

II. Kit Components

Table 4. Additional Reagents

COMPONENT	3403-12 PART NUMBER	3403-60 PART NUMBER	3403-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
Nuclease-free Water	S01001	S01113	—	Green	D1
Agencourt® RNAClean® XP Beads	S01307	S01307	S01307	Clear	—

Note: The reagents in the Ovation FFPE WTA System 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 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
- **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, 8 X 0.2 mL strip PCR tubes or 0.2 mL thin-wall PCR plates
  - Agencourt SPRIPlate® 96R, Ring Magnet Plate (Beckman Coulter Genomics, Cat. #A29164) or Agencourt 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.

II. Kit Components

- Purification options for final SPIA cDNA purification (select one option):
  - Agencourt RNAClean XP Kit (Beckman Coulter Genomics, Cat. #A63987)
  - MinElute® Reaction Cleanup Kit (QIAGEN, Cat. #28204)
  - QIAquick® PCR Purification Kit (QIAGEN, Cat. #28104)
  - DNA Clean & Concentrator™-25 (Zymo Research, Cat. #D4005/D4006)
- Disposable gloves
- Kimwipes
- Ice bucket
- Cleaning 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:

- Beckman Coulter Genomics, www.beckmangenomics.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

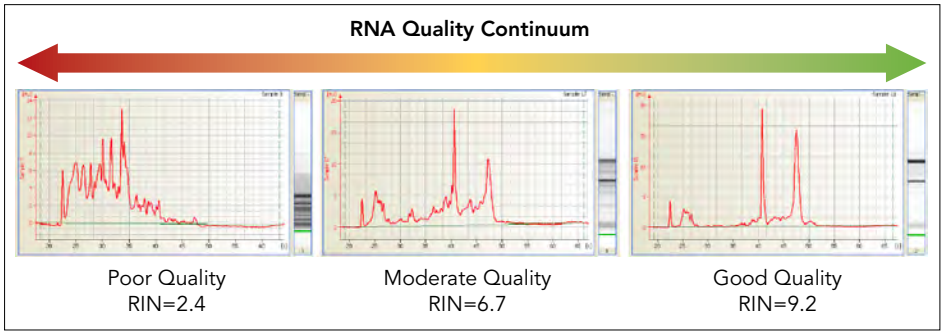
1. **RNA Quantity**
   Total FFPE-derived RNA input must be in the range of 50 to 100 ng. Inputs above 100 ng per reaction may inhibit amplification, while an input under 50 ng may result in insufficient yield for analysis. We strongly recommend quantitation of total RNA to assure the minimum and maximum input requirements are met.

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 RNA from FFPE samples that does not require organic solvents is recommended. One measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The A260:A280 ratio for RNA samples should be in excess of 1.8. RNA samples with lower ratios may result in low amplification yield.

3. **RNA Integrity**
   The Ovation FFPE WTA System was designed and optimized for use with highly degraded RNA samples. The whole transcriptome amplification approach allows highly degraded RNA samples with compromised poly(A) tails to be amplified successfully.

   The Agilent 2100 Bioanalyzer provides a common method for assessing RNA integrity, specifically the RNA Integrity Number (RIN) calculation available in the Bioanalyzer 2100 Expert software. In our tests using FFPE RNA, RIN scores were observed to be uniformly low and highly variable between samples. RIN scores have not proven to be predictive of sample performance on the microarray. Some FFPE-derived RNA samples may be too severely compromised to work with the Ovation RNA-Seq FFPE System.

Figure 1. This continuum of RNA quality shows Bioanalyzer traces of 3 different RNAs with varying levels of degradation. RNA from FFPE samples typically falls in the lowest range of the quality spectrum; however, we have demonstrated successful amplification and analysis of a wide range of FFPE RNA samples using the Ovation FFPE WTA System.



4. **RNA Sample Quality Assessment**
   Such metrics as RIN score, age of FFPE blocks, amplification yield, etc. have been shown to correlate only weakly with sample performance on array. We have developed a predictive tool for assessment of the suitability of FFPE RNA samples for array analysis. The WT-Ovation® FFPE System RNA Sample Quality Assessment Test technical report describes this tool, including a data set, procedures and sequence information for a set of reference qPCR assays, along with assessment recommendations. This document can be downloaded at http://www.nugeninc.com/tasks/sites/nugen/assets/File/technical_documents/techdoc_wt_ov_ffpe_rep_01.pdf.

5. **DNase Treatment**
   It is generally recommended to use DNase-treated RNA for amplification using the Ovation FFPE WTA 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 contaminating genomic DNA, it will be difficult to accurately quantify 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. Refer to Appendix D of this user guide for procedural recommendations.

6. **Carrier Use for RNA Isolation**
   Generally speaking, the addition of carrier to RNA samples is not required for successful RNA isolation. We strongly recommend against the use of nucleic acid based carriers during RNA purification because many have been shown to produce cDNA product in first strand synthesis. We also advise against the use of glycogen in RNA isolation, as it inhibits reverse transcription. For the latest information regarding other carriers, contact the NuGEN 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 RNase 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 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 FFPE WTA System must be stored at –80°C. Avoid frequent freeze/thaw cycles or RNA degradation may result.

D. Amplified cDNA Storage

The SPIA cDNA produced by the Ovation FFPE WTA System may be stored at –20°C.

IV. Protocol

A. Overview

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

1.	First strand cDNA synthesis	1.2 hours
2.	Second strand cDNA synthesis and purification	2 hours
3.	SPIA isothermal amplification and purification	1.5 hours
	Total time to prepare amplified cDNA	**~4.7 hours**

Ovation FFPE WTA 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 SPIA cDNA must be purified following amplification if you intend to use the cDNA for labeling using a NuGEN Encore labeling module or other validated labeling protocol.

The Ovation FFPE WTA System may also be used as a method of pre-amplification prior to qPCR. Although for qPCR applications it is not absolutely necessary to purify the SPIA cDNA, we recommend purifying the cDNA after SPIA. If quantitation of the cDNA product is desired, purification is required. Spectrophotometric quantitation of unpurified amplification products will result in artificially high readings due to amplification components present in the sample.

B. Protocol Notes

- We recommend the routine use of a high-quality 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, so a practice run with the magnet is highly recommended.
- Due to the high sensitivity inherent in this amplification system we strongly recommend taking measures to minimize the potential for the carryover of previously amplified cDNA into new amplification reactions. The two steps to accomplish this are: 1. Designating separate workspaces for "pre-amplification" and "post-amplification" steps and materials and 2. Implementing routine clean-up protocols for workspaces as standard operating procedure. A detailed set of these recommendations is listed in Appendix E.
- Use the Nuclease-free Water (green: D1) provided with the kit or an alternate source of nuclease-free water. We do not recommend the use of DEPC-treated water with this protocol.
- Setting up a minimum of four reactions at a time ensures that you are not pipetting very small volumes (see the second strand synthesis section).

IV. Protocol

- 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 a 12-reaction kit, no fewer than 10 reactions at a time with a 60-reaction kit and no fewer than 48 reactions with an automation (A01) kit. This ensures there will be sufficient reagent volumes to perform the full number of reactions specified for each kit size.
- Thaw components used in each step and immediately place them on ice. Do not thaw all reagents at once.
- Always keep thawed reagents and reaction tubes on ice unless otherwise instructed.
- 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 two minutes at room temperature followed by brief vortexing. Do not warm any enzyme or primer mixes.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer.
- When instructed to pipet mix, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.
- Always allow the thermal cycler to reach the initial incubation temperature prior to placing the tubes in the block.
- When preparing master mixes, use the minimal amount of extra material to ensure recovery of the maximum number of reactions. The Ovation FFPE WTA Quick Protocol has been designed to automatically calculate an appropriate master mix volume, based on the desired number of reactions, which can be used as a guideline in setting up master mixes.
- Components and reagents from other NuGEN products should not be interchanged with the components supplied with this product.
- Use only fresh ethanol stocks to make 70% ethanol used in the post-second strand bead purification (Protocol H), and 80% ethanol for washes in the amplified cDNA purification protocols (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. Agencourt® RNAClean® XP Purification Beads

Tips and Notes Relevant to the Second Strand cDNA Cleanup, Protocol H:

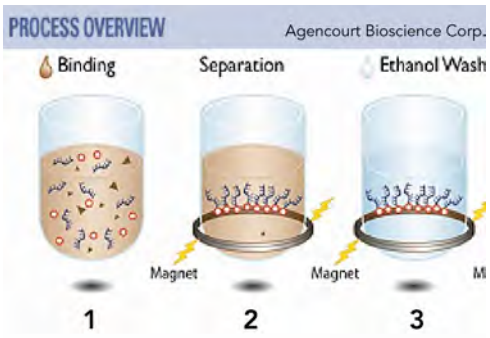
There are significant modifications to the Beckman Coulter Genomics Agencourt RNAClean XP Kit standard procedure; therefore, you must follow the procedures outlined in this user guide for the use of these beads with the Ovation FFPE WTA System. However, you may review the Agencourt RNAClean XP Kit user guide to become familiar with the manufacturer's recommendations.

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

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

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

Figure 2. 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 the beads by inverting and tapping before adding to the sample.
- Note that we recommend using 1.6 volumes (32 µL) of RNAClean XP 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 the binding buffer before the beads have completely separated will impact cDNA yields.
- After the binding step has been completed, it is important to minimize bead loss when removing the binding buffer. With the samples placed on the magnet,

remove only 45 µL of the binding buffer from each sample. Some liquid will remain at the bottom of the tube but this will minimize bead loss.

- Any significant loss of beads during the ethanol washes will impact cDNA yields, so make certain 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 tubes.
- It is critical that all residual ethanol be removed prior to continuing with the SPIA amplification. Therefore, when removing the final ethanol wash, first remove most of the ethanol, then allow the excess to collect at the bottom of the tube before removing the remaining ethanol. This reduces the required bead air drying time.
- After drying the beads for 15 to 20 minutes, inspect each tube carefully and make certain that all the ethanol has evaporated before proceeding with the amplification step.
- It is strongly recommended that strip tubes or partial plates are firmly placed when used with the magnet. We do not advise the use of individual tubes as they are difficult to position stably on the magnet.

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 – 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

IV. Protocol

Important Note: Carry out Protocol E (RNA Demodification) through Protocol I, step 8 (SPIA Amplification) in a pre-amplification workspace using dedicated pre-amplification consumables and equipment. Wipe all surfaces, equipment and instrumentation with a DNA removal solution such as DNA-OFF (MP Biomedicals, Cat#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 the NuGEN Technical Services Team (techserv@nugeninc.com, (888) 654-6544).

E. RNA Demodification (Optional)

Note: Formalin fixation of tissues can result in the cross linking of RNA. Many commercial RNA isolation kits designed for the extraction of RNA from FFPE tissues include a demodification step which is designed to reverse the cross linking process to the extent possible. If the RNA isolation kit you have used does not include this step or if you suspect the demodification is incomplete, we recommend that you perform the following steps for demodification prior to using the Ovation FFPE WTA System. While this step is not necessary for many FFPE RNA samples, we have found that it can be helpful in some cases where poor RNA sample quality has rendered amplification difficult.

1. Add 2.5 µL of total RNA (50 to 100 ng) to a 0.2 mL PCR tube.
2. Add 2.5 µL of 100 mM Tris-HCl pH 8.0.
3. Incubate the sample at 70°C for 15 minutes, then cool to 4°C.
4. Spin briefly and place on ice.
5. Continue immediately with the First Strand cDNA Synthesis protocol.

F. First Strand cDNA Synthesis

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

! *Do not vortex any enzyme mixes.*

2. Spin down the contents of A3 and place on ice.
3. Thaw the other reagents at room temperature. Mix by vortexing, spin and place on ice. Leave the Nuclease-free Water at room temperature.
4. Add 2 µL of A1 to a 0.2 mL PCR tube.

5. Add 5 µL of total RNA sample (50 to 100 ng) to the primer.

   Note: If you have performed the RNA Demodification protocol, add 2 µL of A1 directly to the 5 µL RNA Demodification product.

6. Mix by pipetting 5 times, spin and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 5):

   65°C – 2 min, hold at 4°C

8. Remove the tubes from the thermal cycler and place on ice.
9. Once Primer Annealing (Step 7) is complete, prepare a master mix by combining A2 and A3 in a 0.5 mL capped tube, according to the volumes shown in Table 6.

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

FIRST STRAND BUFFER MIX (BLUE: A2 VER 3)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 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 5 times, spin and place on ice.

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

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

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

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

G. Second Strand cDNA Synthesis

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

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

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

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

5. Make a master mix by combining B1 and B2 in a 0.5 mL capped tube, according to the volumes shown in Table 7.

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

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

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

   4°C – 1 min, 25°C – 10 min, 50°C – 30 min, 80°C – 20 min, hold at 4°C
9. Remove the tubes from the thermal cycler and spin to collect condensation. Place in a rack on the bench top.
10. It is highly recommended to remove 2 µL of cDNA prior to purification for the RNA Sample Quality Assessment. Refer to WT-Ovation FFPE System Technical Report #1 for detailed instructions on the use of the Sample Quality Assessment Tool.
11. Continue immediately with the Purification of cDNA protocol.

H. Purification of cDNA

1. Ensure the Agencourt RNAClean XP beads have completely reached room temperature before proceeding.
2. Prepare a 70% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content which may reduce amplification yield.
3. Resuspend the beads by inverting and tapping the tube. Ensure that the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads. A large excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.
4. At room temperature, add 32 µL (1.6 volumes) of the bead suspension to each reaction and mix by pipetting 10 times.
5. Incubate at room temperature for 10 minutes.

IV. Protocol

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

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

6. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
7. Keeping the tubes on the magnet, carefully remove only 45 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.
8. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.

   Note: The beads should not disperse; instead they will stay on the walls of the tubes. Significant loss of beads at this stage will impact cDNA yields, so ensure beads are not removed with the binding buffer or the wash.
9. Remove the 70% ethanol wash using a pipette.
10. Repeat the wash 2 more times.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least 2 pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.
11. Air dry the beads on the magnet for 15 to 20 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing with SPIA Amplification.
12. Continue immediately with the SPIA Amplification protocol with the cDNA still bound to the dry beads.

I. SPIA® Amplification

Ensure the enzyme is well mixed without introducing bubbles.

1. Obtain the SPIA Buffer Mix (red: C2), SPIA Primer Mix (red: C1) and SPIA Enzyme Mix (red: C3) from –20°C storage.
2. Thaw C3 on ice and mix the contents by inverting gently 5 times, spin and place on ice. Ensure the enzyme is well mixed without introducing bubbles.
3. Thaw reagents C1 and C2 at room temperature, mix by vortexing, spin and place on ice.
4. Make a master mix by sequentially combining C2, C1, and C3 in an appropriately sized capped tube, according to the volumes shown in Table 8.

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

Use SPIA Master Mix immediately after preparation.

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

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

SPIA BUFFER MIX (RED: C2 VER 10)	SPIA PRIMER MIX (RED: C1 VER 9)	SPIA ENZYME MIX (RED: C3 VER 7)
80 µL	40 µL	40 µL

5. Add 160 µL of the SPIA Master Mix to each tube containing the double-stranded cDNA bound to the dried beads. Use a pipette set to 80 µL and mix thoroughly by pipetting at least 8 to 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 from the beads.

6. Transfer one half of the reaction volume (80 µL) to a second tube.

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

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

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

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

Note: If using the Agencourt RNAClean XP method for final SPIA cDNA cleanup, skip steps 9–12 below and go directly to the Agencourt RNAClean XP Kit protocol on 22. It is not necessary to recombine the half-reactions or to remove the beads at this point.

9. Recombine the half-reactions.

10. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.

11. Carefully remove all of the cleared supernatant containing the eluted cDNA and transfer to a fresh tube. The beads may now be discarded.
12. Continue immediately with the Purification of SPIA cDNA protocol, or store the SPIA cDNA at –20°C.

J. Purification of SPIA cDNA

The 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 an Encore labeling module or other supported labeling protocol.

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 SPIA cDNA be purified prior to qPCR analysis.

K. Measuring SPIA cDNA Yield and Purity

1. Mix the purified SPIA cDNA sample by brief vortexing and spinning prior to checking the concentration.
2. Measure the absorbance at 260, 280 and 320 nm. You may need to make a 1:20 dilution of the cDNA in water prior to measuring the absorbance.
3. Purity: Subtract the A320 value from both A260 and A280 values. The adjusted (A260–A320) / (A280–A320) ratio should be >1.8.
4. Yield: Assume 1 A260 unit = 33 µg/mL for single stranded cDNA.

   To calculate: (A260–A320 of diluted sample) X (dilution factor) X 33 (concentration in µg/mL of a 1 A260 unit solution) X 0.03 (final volume in mL) = total yield in micrograms

   Note: Alternatively, you may measure the concentration and purity of cDNA with a Nanodrop using the sscDNA setting or by manually setting 1 A260 unit = 33 µg/mL as the constant.
5. The purifed cDNA may be stored at –20°C.

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 SPIA cDNA

There are four currently supported alternatives for carrying out the final purification of SPIA cDNA. Listed alphabetically, they are: 1) the Agencourt RNAClean XP Kit, 2) the QIAGEN MinElute Reaction Cleanup Kit, 3) the QIAGEN QIAquick PCR Purification Kit and 4) the Zymo DNA 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.

Agencourt RNAClean XP Kit (instructions for a single reaction)

Important notes:

- Stop after Protocol I, step 8 on 19. It is not necessary to recombine the half-reactions or to remove the beads from the SPIA reactions at this point.
- Prepare a room temperature 80% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content which may reduce amplification yield.
- The use of 96-well microplates and multi-channel pipettes is recommended for processing large batches with this procedure.

1. Obtain the RNAClean XP bottle from 4°C storage. Allow the bead solution to reach room temperature.
2. Invert the RNAClean XP bottle several times to ensure the beads are fully in suspension. It may be necessary to remix the bead stock from time to time to ensure beads remain in suspension while in use.
3. At room temperature, add 144 µL of resuspended RNAClean XP 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 10 times.

   Note: If using a 96-well plate or 8-strip tube format with both half-reactions on the same plate or strip, it will be necessary to transfer the sample/bead mixture to a fresh plate or strip at this point.
5. Incubate the sample/bead mixture at room temperature for 5 minutes.
6. Place the sample/bead mixture on the magnet for 10 minutes to completely clear the solution of beads.
7. After 5 minutes of the 10 minute incubation in step 6 have elapsed, add 144 µL of resuspended RNAClean XP beads to the second half-reaction containing the remaining 80 µL of SPIA cDNA.
8. Incubate the second sample/bead mixture at room temperature for 5 minutes.

9. Keeping the first tube on the magnet, carefully remove and discard the supernatant from first set of samples. Do not disturb the ring of beads.
10. With the first tube still on the magnet, add the sample/bead mix from the second half-reaction (prepared in step 7) to the tube containing the beads from the first half-reaction (on the magnet). Add slowly as to not disturb the bead ring already in the tube.

    Note: Here the potential for inadvertent sample mixing is high when processing multiple samples. Take care to combine the correct half-reactions. Using a multi-channel pipette can help minimize the risk of combining the half-reactions incorrectly.
11. Wait for an additional 10 minutes to completely clear the solution of beads.

    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 to15 µL up and down at the liquid surface to break the tension and allow the beads to sink to the magnet.
12. Carefully remove and discard the supernatant. Do not disturb the ring of beads.

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

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

13. Keeping the tube on the magnet, add 200 µL of freshly prepared 80% ethanol to each sample and incubate for 30 seconds or until the solution clears. Add slowly so as to not disturb the separated beads.
14. Carefully remove and discard the ethanol.
15. Repeat the 80% ethanol wash once more. Ensure as much ethanol as possible is removed from the tube.

    Note: With the final wash, it is important to remove as much of the ethanol as possible. Use at least 2 pipetting steps and allow excess ethanol to collect at the bottom of the tube after removing most of the ethanol in the first pipetting step.
16. Remove the tube from the magnet and air dry on the bench top for no more than 2 minutes. If the beads dry too long, they are difficult to resuspend.

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

17. Add 30 µL of room temperature, Nuclease-free Water (green: D1) to the tube.
18. Resuspend the beads by repeated pipetting. Alternatively, the beads may be resuspended by carefully vortexing the tube for 30 seconds or using a plate shaker set to medium speed. Ensure the beads are fully resuspended. Vortex longer if necessary.
19. Replace the tube on the magnet and allow the beads to separate for 5 minutes or until the solution clears.

20. Carefully remove the eluted sample and transfer to a fresh tube.

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

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

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

Important notes:

- Buffer ERC is considered hazardous according to QIAGEN, and an MSDS may be consulted.
- 2 columns are required per reaction if expected yield is above 8 µg.
- Add the appropriate amount of 100% ethanol to Buffer PE before use (see bottle label for volume).
- All centrifuge steps are carried out at maximum speed in a conventional table-top microcentrifuge at room temperature.

1. Into a clean, labeled 1.5 mL microcentrifuge tube, add 600 µL of Buffer ERC from the QIAGEN kit.
2. Add the entire volume (160 µL) of the SPIA reaction to the tube.
3. Vortex for 5 seconds, then spin briefly.
4. Obtain and label a MinElute spin column(s) and place it into a collection tube(s).
5. Load the sample/buffer mixture onto the column.

   Note: If using two columns per sample, load one-half of the sample/buffer mixture onto each of the two columns. Process each column as described below.
6. Centrifuge for 1 minute at maximum speed in a microcentrifuge.
7. Discard the flow-through and replace the column in the same collection tube.

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

8. Add 750 µL of Buffer PE the column.
9. Centrifuge for 1 minute at maximum speed.
10. Discard the flow-through and replace the column in the same collection tube.
11. Centrifuge the column for an additional 2 minutes at maximum speed to remove all residual Buffer PE.

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

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

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

14. Add 15 µL of room temperature, Nuclease-free Water (green: D1) from the NuGEN kit to the center of each column.

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

15. Let the column stand for 1 minute at room temperature.
16. Centrifuge for 1 minute at maximum speed.
17. If two columns were used per sample, pool the eluates.
18. Discard the column(s) and measure the volume recovered. There should be approximately 12 to 15 µL of purified SPIA cDNA (24 to 30 µL if two columns were used).
19. Mix the sample by vortexing, then spin briefly.
20. Continue with the Measuring SPIA cDNA Yield and Purity protocol or store purified SPIA cDNA at –20°C.

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

Important notes:

- Prepare an 80% ethanol wash solution and keep at room temperature. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content, which may reduce yield.
- All centrifugation steps are carried out at 17,900 X g (13,000 RPM) in a conventional tabletop microcentrifuge at room temperature.
- Do not add the pH Indicator I from the QIAGEN kit to Buffer PB for this protocol.

1. Into a clean, labeled 1.5 mL microcentrifuge tube, add 800 µL of Buffer PB from the QIAGEN kit.
2. Add the entire volume (160 µL) of the SPIA reaction to the tube.
3. Vortex for 5 seconds, then spin briefly.
4. Obtain and label a QIAquick spin column and place it into a collection tube.
5. Load 480 µL (one-half) of the sample/buffer mixture onto the column.
6. Centrifuge for 1 minute at 17,900 X g in a microcentrifuge.
7. Discard the flow-through and replace the column in the same collection tube.
8. Load the remaining 480 µL of the sample/buffer mixture onto the same column.
9. Centrifuge for 1 minute at 17,900 X g.
10. Discard the flow-through and replace the column in the same collection tube.
11. Add 700 µL of 80% ethanol to the column.
12. Centrifuge for 1 minute at 17,900 X g.
13. Discard the flow-through and replace the column in the same collection tube.
14. Repeat step 11 through step 13 once.
15. Centrifuge for an additional 2 minute at 17,900 X g.

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

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

! *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.*

18. Add 30 µL of room temperature, Nuclease-free Water (green: D1) from the NuGEN kit to the center of each column.

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

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

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

Important notes:

- Zymo Research has two products sharing the DNA Clean & Concentrator-25 name, Cat. #4005/4006 and Cat. #4033/4034. Make certain to use only Cat. #4005/4006 with this protocol. Do not use Cat. #4033/4034 as this will result in low yields.
- Prepare a room temperature 80% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content, which may reduce yield.
- All centrifugation steps are carried out at 10,000 X g in a conventional tabletop microcentrifuge at room temperature.
- When instructed to centrifuge for durations of less than 1 minute, allow the centrifuge to reach the target RCF before starting the timer.

1. Into a clean, labeled 1.5 mL microcentrifuge tube, add 320 µL of DNA Binding Buffer from the Zymo kit.
2. Add the entire volume (160 µL) of the SPIA reaction to the tube.
3. Vortex for 5 seconds, then spin briefly.
4. Obtain and label a Zymo Spin-II column and place it into a collection tube.
5. Load the sample/buffer mixture onto the column.
6. Centrifuge for 10 seconds at 10,000 X g in a microcentrifuge. Allow the centrifuge to reach full speed before starting the timer.
7. Discard the flow-through and replace the column in the same collection tube.
8. Add 200 µL of 80% ethanol to the column. Do not use the wash buffer provided in the Zymo kit.
9. Centrifuge for 10 seconds at 10,000 X g.
10. Discard the flow-through and replace the column in the same collection tube.
11. Add an additional 200 µL of 80% ethanol to each column.
12. Centrifuge for 90 seconds at 10,000 X g.

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

    Note: Blotting the column tip prior to transferring it to a clean tube is necessary to prevent any wash buffer transferring to the eluted sample.

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

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

14. Place the column into a clean, labeled 1.5 mL microcentrifuge tube.
15. Add 30 µL of room temperature, Nuclease-free Water (green: D1) from the NuGEN kit to the center of the column.

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

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B. Guidelines for Use in Microarray Experiments

The SPIA cDNA produced by the Ovation FFPE WTA System may be labeled for use in microarray analysis. NuGEN has developed specific labeling products and protocols for use with various microarray platforms. Workflows suitable for use with specific microarray platforms are described below.

Affymetrix GeneChip Arrays

The Encore Biotin Module (Part No. 4200) is designed to prepare the SPIA cDNA produced with the Ovation FFPE WTA System for hybridization to Affymetrix GeneChip Arrays. The Encore Biotin Module user guide provides an optimized protocol and specific recommendations for use with the Ovation FFPE WTA System. 4-5 µg of SPIA cDNA is required for standard (49 format) GeneChip Arrays with this method and 2-2.5 µg of cDNA is required for mini (169 format) GeneChip Arrays as well as GeneChip Array Strips and Array Plates.

Illumina Genome-Wide Expression BeadChips

The Encore BiotinIL Module (Part No. 4210) is designed to prepare the SPIA cDNA produced with the Ovation FFPE WTA System for hybridization to Illumina Genome-Wide Expression BeadChips. The Encore BiotinIL Module user guide provides an optimized protocol for use with the Ovation FFPE WTA System. 0.75-1.5 µg of SPIA cDNA is required for labeling with this method, depending on the specific array format.

Agilent Dual-mode Gene Expression Arrays

We have developed a protocol for the fluorescent labeling of SPIA cDNA for use on Agilent Dual-mode Gene Expression Arrays, which is described in the Agilent Solution technical report. This document may be found at http://www.nugeninc.com/tasks/sites/nugen/assets/File/technical_documents/techdoc_agilent_appnote_01.pdf. 2 µg of SPIA cDNA is required for labeling with this method.

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C. Performing Quantitative PCR on Amplified cDNA

It is recommended that the amplified cDNA (SPIA cDNA) generated from the Ovation FFPE WTA System be purified prior to use in real-time, quantitative PCR reactions (qPCR). Since SPIA cDNA samples will vary in concentration, the purified products may be quantitated and mass normalized to ensure the cDNA inputs are equal for all samples. Purified SPIA cDNA produced with this kit has been successfully used as a template for qPCR systems including TaqMan® and SYBR® Green. Note that qPCR master mixes containing the enzyme Uracil N-Glycosylase (UNG) are not compatible with the Ovation FFPE WTA System.

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 SPIA cDNA

   After purification and quantitation of the SPIA cDNA, it can be diluted to an appropriate concentration for the 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 wish to use more or less cDNA.

2. Primer Design

   We recommend designing multiple assays across the length of the transcript since the starting FFPE RNA is likely to be highly degraded and some amplicons may not perform robustly. 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 Ovation FFPE WTA System amplification covers the entier length of the transcript.

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D. DNase Treatment of RNA

DNase Treatment During Purification: Using the QIAGEN RNase-Free DNase Set and the RNeasy Mini RNA Purification Kit

1. Homogenize sample in RLT buffer including ß-mercaptoethanol according to the type of sample as described in the RNeasy Mini Kit protocol.
2. Add 1X volume of 70% ethanol to the homogenized lysate, 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 minutes.
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.

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17. Close the tube gently and centrifuge for 1 minute at ≥8000 X g (≥10,000 rpm) to elute.
18. If yields of greater than 30 µg are expected, repeat elution step and collect in the same collection tube.

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

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 (green: D1) to bring the final volume to 25 µL.
3. Incubate at 25°C for 15 minutes followed by 37°C for 15 minutes and return to ice.
4. After the DNase treatment, the sample must be purified. We recommend either of the two purification procedures below:

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

1. Add 4 volumes (100 µL) of RNA binding buffer to the sample.
2. Obtain 1 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 column in a fresh 1.5 mL collection tube.
8. 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.

   Important: Do not use cold water!
9. 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.*

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

1. Add 80 µL ice-cold RNase-free water (green: D1) to the sample on ice.
2. Add 350 µL Buffer RLT and mix by pipetting.
3. Add 250 µL 96–100% 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.

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

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.
10. Spin for 5 minutes at ≥8000 X g (≥10,000 rpm) and discard the flow-through.
11. Place the RNeasy MinElute Spin Column in a fresh 1.5 mL collection tube.

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

12. Add 14 µL nuclease-free water (green: D1) directly to the center of the filter in the tube and close the cap.
    Do not use cold water!
13. Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

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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 through the carry-over of previously amplified SPIA cDNA. We strongly recommend implementing these procedures, especially for the 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 non-specific amplification products from spreading through the laboratory. 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 room. If this is not possible, ensure the pre-amplification area is sufficiently isolated from post-amplification work.

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

2. Establish and maintain a clean work environment:

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

    b. In the pre-amplification area, remove all small equipment and then clean every surface that may have been exposed to amplified SPIA cDNA (surfaces, drawer handles, key pads, etc.). Before reintroducing any equipment, clean every piece of equipment thoroughly.

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

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

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

d. Always wear gloves and don fresh gloves upon entry into this controlled area. Frequently change gloves while working in the pre-amplification area, especially prior to handling stock reagents, reactions and RNA samples.

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

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

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

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

3. Avoid running negative controls (i.e., no RNA input reactions). Instead use high-quality control RNA to run both low-template controls (inputs of 50 pg to 100 pg) and positive controls (2 ng to 5 ng) 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 when compared to the positive control reaction.

   a. Typical amplification performance:

      i. LTC yields for the Ovation FFPE WTA System amplifications should be significantly lower than yields for positive control reactions.

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

   b. Atypical amplification performance:

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

      ii. The bioanalyzer traces of amplification products may look significantly different than positive controls or typical Ovation FFPE WTA System traces. The LTC reaction is designed to be an especially sensitive indicator of atypical amplification performance.

      iii. Sensitivity on arrays run with atypical samples (i.e., non-specific amplification products present at significant concentration) may be lower than expected.

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

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F. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the Ovation FFPE WTA System?
The Ovation FFPE WTA 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 double-stranded cDNA purification.

Q2. Does the Ovation FFPE WTA System provide any labeling reagents?
No. This system is used to generate SPIA cDNA from FFPE RNA samples. The SPIA cDNA may be processed further using a NuGEN Encore labeling module or other supported labeling protocol.

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

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

Q5. Do I need to use high-quality total RNA?
No. The Ovation FFPE WTA System employs a whole transcriptome amplification approach and was designed specifically for use with highly degraded, lower quality RNA samples.

Q6. Can I use RNA from sources other than FFPE?
Yes. While the Ovation FFPE WTA System was developed specifically for use with total RNA from FFPE samples, it is possible to use intact, non-FFPE RNA samples as well. Performance with intact total RNA samples below 2 ng may vary.

Q7. Is the Ovation FFPE WTA System 3´ biased?
In this system, oligo dT primers are mixed with random primers for the first strand synthesis of cDNA products. This allows amplification coverage of the whole transcript. We have tested the system with both degraded and intact RNA on 3´ microarray designs as well as arrays interrogating sequence in all regions of the transcript (i.e., Affymetrix GeneChip Gene 1.0 ST and Exon 1.0 ST Arrays) with successful results.

Q8. Where in my target sequence can I design my qPCR primers?
The Ovation FFPE WTA System allows amplification of the entire transcript; 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 highly degraded.

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Q9. How much FFPE derived 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 may be higher using intact, non-FFPE RNA samples.

Although yield is an important sample quality indicator, success of a given FFPE sample set in array analysis is best predicted using the RNA Sample Quality Assessment Tool described in the WT-Ovation FFPE System RNA Sample Quality Assessment technical report.

Q11. Is the cDNA yield dependent upon the quantity of total RNA input?
Using a higher RNA input will typically allow for higher amplification yields; however, at FFPE RNA inputs of above 100 ng, the yields may become variable.

Q12. What size cDNA is generated by the Ovation FFPE WTA System?
The amplified cDNA size distribution can vary based on the input RNA integrity. In a whole transcriptome amplification strategy, however, the size of the resulting cDNA is not of significant consequence for use on arrays.

Q13. Can DNA be used as input for the Ovation FFPE WTA System?
No. The Ovation FFPE WTA System is designed to amplify mRNA, not DNA.

Q14. Can contaminating genomic DNA interfere with the Ovation FFPE WTA System?
Yes. This system is designed to amplify RNA, but contaminating genomic DNA may amplify during the process, so we recommend DNase treatment during RNA purification.

Q15. Can I use the Ovation FFPE WTA System on bacterial RNA samples?
The Ribo-SPIA amplification process theoretically will work with some bacterial RNAs. However, the kit has not been optimized or validated for this purpose.

Q16. Has NuGEN performed reproducibility studies on the Ovation FFPE WTA System?
Yes. Sample-to-sample, and lot-to-lot reproducibility studies are conducted.

Q17. Does the Ovation FFPE WTA System generate product in the absence of RNA input?
In the complete absence of input RNA less than 3 µg of 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.

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Q18. Can I use the Ovation FFPE WTA System for archiving cDNA?
Yes. Amplified cDNA may be stored at –20°C for at least six months. Long term tests are in progress.

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

Q20. Do I have to use your DNA/RNA primers?
Yes. The system will not perform with other primers.

Q21. 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.

Q22. 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 Services Team for assistance in selecting the appropriate option for your application. Refer to section II.B. for ordering information.

Q23. Where can I safely stop in the protocol?
The SPIA cDNA may be stored prior to final purification at the point indicated in the protocol. We do not recommend stopping at any other intermediate stage of the protocol.

Q24. Do you recommend DNase treatment of my total RNA sample?
Yes. For an explanation of the DNase requirements, please refer to section III.A.5 of this user guide.

Q25. How many qPCR reactions will I get from one Ovation FFPE WTA System 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.

G. Update History

This document, the Ovation Pico WTA System V2 user guide (M01224 v2), is an update to address the following topics.

Description	Section	Page(s)
Update NuGEN logo.	Throughout	
Update copyright and IP information.	Throughout	
Revise contact information for requesting MSDS documents.	I.F.	2
Add Appendix B, Guidelines for Use on Microarrays	Appendix B	31
Add Appendix G, table of user guide updates.	Appendix G	40

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