USER GUIDE

Ovation® Whole Blood Solution

PART NOS. 3100, 1300, 4200

NuGEN®

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I. Introduction

A. Background

The Ovation® Whole Blood Solution is comprised of two kits for amplification of whole blood total RNA and one kit for the preparation of labeled target for Applied Biosystems (formerly Affymetrix) GeneChip™ array analysis. The amplification protocol requires as little as 5 ng of total RNA and yields up to 12 µg of cDNA in 4 hours, without the need for globin transcript reduction. Following a two-hour fragmentation and labeling process, the target cDNA can be hybridized directly to GeneChip arrays without further purification.

Note: With whole blood RNA samples, we advise using a minimum of 20 ng of input total RNA due to the high levels of globin present in such samples.

The Ovation Whole Blood Solution includes:

- Ovation RNA Amplification System V2 (Part No. 3100), providing a fast and simple method for preparing microgram quantities of amplified, single-stranded cDNA product from 5–100 ng of whole blood total RNA for gene expression analysis by microarray or qPCR. The amplified cDNA can also be archived for future analysis. The Ovation RNA Amplification System V2 is powered by Ribo-SPIA® technology, a rapid, simple and sensitive RNA amplification process developed by NuGEN.
- Ovation WB Reagent (Part No. 1300) is a companion reagent for use with the Ovation RNA Amplification System V2. The incorporation of this reagent into the SPIA® amplification procedure, as described in this user guide, significantly increases amplified cDNA yields. This reagent is particularly important for challenging samples such as whole blood total RNA and other sources of RNA that do not yield sufficient amplified cDNA through standard SPIA amplifications. This reagent and protocol may also be employed in cases requiring large amounts of amplified cDNA.
- Encore® Biotin Module (Part No. 4200) is used for fragmentation and labeling of the amplified cDNA for analysis on Applied Biosystems GeneChip arrays.

B. Ribo-SPIA® Technology

Ribo-SPIA technology is a four-step process that generates amplified cDNA from as little as 5 ng of total RNA.

1. **Generation of First Strand cDNA (1.5 hours)**
   First strand cDNA is prepared from total RNA using a unique first strand DNA/RNA chimeric primer and reverse transcriptase. The primer has a DNA portion that hybridizes to the 5′ portion of the poly(A) sequence. 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 (0.75 hours)**
   Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthesize a second strand, including DNA comple-

mentary to the 5′ unique sequence from the first strand chimeric primer. The result is double stranded cDNA with a unique DNA/RNA heteroduplex at one end.

3. **SPIA Amplification (1.7 hours, including purification)**
SPIA amplification is an 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 process exposes a DNA sequence that is available for binding a 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. Up to 10,000-fold amplification of all mRNA species is observed when starting with 5 ng of total RNA.

C. Performance Specifications

When used with the Ovation WB Reagent, the Ovation RNA Amplification System V2 produces 5–12 µg of cDNA starting with input amounts of 20–50 ng total RNA from whole blood or 5–100 ng total cellular RNA. In approximately four hours, amplified cDNA is generated and is ready for fragmentation and biotin attachment. The size of the majority of the products produced by the Ribo-SPIA amplification process is between 200 bases and 2.0 Kb.

The Encore Biotin Module enables the fragmentation and biotin labeling of up to 5 µg of Ribo-SPIA amplified cDNA per reaction in approximately 1.5 hours. On average, these fragments are 50–100 bases long.

D. Quality Control

Each of the products used in the Ovation Whole Blood Solution is tested to meet performance specifications.

E. Storage and Stability

The Ovation RNA Amplification System V2 is shipped on dry ice and should be unpacked immediately upon receipt. The small package of vials labeled First Strand Primer Mix (blue: A1) and SPIA Primer Mix (red: C1) should be removed from the shipping carton upon delivery and stored at –80°C. All remaining components should be stored at –20°C on internal shelves of a freezer without a defrost cycle.

The Ovation WB Reagent product is shipped on dry ice and should be stored at –80°C.

The Encore Biotin Module product is shipped on dry ice and should be stored at –20°C.

Products handled and stored according to the above guidelines should perform to specifications for approximately six months. NuGEN does not recommend long-term storage of Ovation RNA Amplification System V2, the Ovation WB Reagent or the Encore Biotin Module.

F. Safety Data Sheet (SDS)

If appropriate, an SDS for this product is available on the NuGEN website at www.nugen.com/products/ovation-whole-blood-solution

II. Kit Components

A. Reagents and Supplies Provided

Table 1. Ovation RNA Amplification System V2 (Part Nos. 3100-12, 3100-60, 3100-A01)

COMPONENT	3100-12 PART NUMBER	3100-60 PART NUMBER	3100-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
First Strand Primer Mix	S01038	S01100	S01212	Blue	A1 VER 1
First Strand Buffer Mix	S01150	S01154	S01206	Blue	A2 VER 4
First Strand Enzyme Mix	S01040	S01102	S01207	Blue	A3 VER 1
Second Strand Buffer Mix	S01151	S01155	S01208	Yellow	B1 VER 4
Second Strand Enzyme Mix	S01042	S01104	S01209	Yellow	B2 VER 1
SPIA Primer Mix	S01089	S01105	S01141	Red	C1 VER 1
SPIA Buffer Mix	S01152	S01156	S01210	Red	C2 VER 6
SPIA Enzyme Mix	S01165	S01166	S01211	Red	C3 VER 5
Nuclease-free Water	S01001	S01113	S01001	Green	D1

Note: The reagents in the Ovation RNA Amplification System V2 product may be 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 are not interchangeable.

Table 2. Ovation WB Reagent (Part Nos. 1300-60, 1300-A01)

COMPONENT	1300-60 PART NUMBER	1300-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
Ovation WB Reagent	S01190	S01213	Red	C4 VER 1

II. Kit Components

Table 3. Encore Biotin Module (Part No. 4200)

COMPONENT	4200-12 PART NUMBER	4200-60 PART NUMBER	4200-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
Fragmentation Buffer Mix	S01182	S01182	S01177	Orange	FL1
Fragmentation Enzyme Mix	S01175	S01183	S01178	Orange	FL2
Labeling Buffer Mix	S01184	S01184	S01179	Orange	FL3
Biotin Reagent	S01172	S01185	S01180	Orange	FL4
Labeling Enzyme Mix	S01173	S01186	S01181	Orange	FL5

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - Microcentrifuge for 0.2 mL individual and 8 x 0.2 mL strip PCR tubes (e.g. PGC Cat. #16-7009-70/72 or similar)
  - 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 and Supplies**
  - 100% ethanol, to make 80% ethanol for the cDNA column purification step. (Sigma-Aldrich, Inc., Cat. # E7023)
  - 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
  - Appropriate spectrophotometer cuvettes
  - Disposable gloves
  - Lab wipes and ice bucket

II. Kit Components

- **Optional Equipment and Reagents**
  - Agilent 2100 Bioanalyzer or materials and equipment for electrophoretic analysis of RNA
  - Real-time PCR system
  - Cleaning solutions such as RNaseZap® (Ambion, Cat. #AM9780) and DNA-OFF™ (MP Biomedicals, Cat. #QD0500)
  - Purification options for final SPIA cDNA purification (select one option):
    - Agencourt® RNAClean® XP Beads (Beckman Coulter Genomics, Cat. #A29168)
    - MinElute® Reaction Cleanup Kit (QIAGEN, Cat. #28204)
    - QIAquick® PCR Purification Kit (QIAGEN, Cat. #28104)
    - DNA Clean & Concentrator™-25 (Zymo Research, Cat. #D4005)
  - SPRIPlate® 96R, Ring Magnet Plate (Beckman Coulter Genomics, Cat. #A29164)

To Order

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

III. Planning the Experiment

A. Input RNA Requirements

The most important requirement for achieving successful results with the Ovation RNA Amplification System V2 is to use total RNA of high purity and molecular weight. Use of low purity or degraded RNA may lead to low yield and spurious microarray results. To assess total RNA quality prior to using the Ovation RNA Amplification System V2, follow the guidelines below.

1. **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. We recommend using a commercially available system that does not require organic solvents to prepare small amounts of RNA. 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. Using RNA samples with lower ratios may result in low amplification yield.

2. **RNA Integrity**
   RNA samples used with the Ovation RNA Amplification System V2 must be of high molecular weight and show little or no evidence of degradation. Amplification of degraded RNA may have a significant effect on microarray results.

   Very small samples limit the number of available methods that can be used to determine integrity. One system NuGEN uses is the Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip®. This instrument provides a sensitive and rapid way of confirming RNA integrity prior to amplification.

3. **DNase Treatment**
   When using the Ovation RNA Amplification System V2, it is generally recommended that you use DNase-treated RNA for amplification. Presence of genomic DNA in the RNA sample may have adverse effects on downstream analytical platforms. Contaminating genomic DNA may be amplified along with the RNA. Additionally, if the total RNA sample contains a significant amount of contaminating genomic DNA, it will be difficult to accurately quantitate the RNA concentration. The RNA input quantity may therefore be overestimated based on an absorbance measurement. Since it is important that RNA input be within the stated range of 5–100 ng, we recommend using a DNase treatment that will remove contaminating genomic DNA during RNA purification.

   Note: With whole blood RNA samples, we advise using a minimum of 20 ng of input total RNA due to the high levels of globin present in such samples.

B. Using RNase-free Techniques

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

- Wear disposable gloves and change them frequently
- Avoid touching surfaces or materials that could introduce RNases

- Use the reagents provided; substitutions may introduce RNases
- Prior to initiating the protocol, clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents, such as RNaseZap® and DNA-OFF™
- Use only new RNase-free pipette tips and microcentrifuge tubes
- Use a work area specifically designated for RNA work and do not use other high copy number materials in the same area

C. RNA Storage

RNA samples for use with the Ovation RNA Amplification System V2 must be stored at –80°C. To minimize degradation of your RNA samples, avoid frequent freeze/thaw cycles.

D. Amplified cDNA Storage

Ideally, the unlabeled cDNA product produced by the Ovation RNA Amplification System V2 is used immediately after preparation. If necessary, the cDNA product may be stored at –20°C after purification and prior to fragmentation and labeling. The cDNA will be stable for up to six months.

IV. Ovation RNA Amplification System V2 Protocol

A. Overview

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

1. First strand cDNA synthesis	1.5 hours
2. Second strand cDNA synthesis	0.75 hours
3. SPIA amplification	1.25 hours
Total time to prepare amplified cDNA	**~3.5 hours**
cDNA purification	**+0.5 hours**

cDNA fragmentation and biotin labeling using the Encore Biotin Module is performed in two stages:

1. cDNA fragmentation	0.5 hours
2. Biotin labeling	1.25 hours
Total time to fragment and label amplified cDNA	**1.75 hours**
Total time	**5.75 hours**

The entire amplification, fragmentation and labeling process can be completed in one working day, and the amplified, fragmented and labeled cDNA can either be hybridized to arrays or stored at –20°C.

Ovation RNA Amplification System V2 components are color coded, with each reagent vial linked to a specific stage of the process. Performing each stage requires the creation of a master mix and addition of other reagents, followed by incubation. Master mixes are prepared by mixing components provided for that stage.

B. Protocol Notes

- Thaw those components used in each step and immediately place them on ice.
- 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 2 minutes at room temperature followed by brief vortexing. Do not warm any enzyme or primer mixes.
- Use the water provided with the kit (green: D1) or an alternate source of nuclease-free water. We do not recommend using DEPC treated water with this protocol.
- The reagent volumes recovered depend greatly upon the number of batches processed with each kit. Set up no fewer than 10 reactions at a time with 1300-60 and no fewer than 48 reactions at a time with 1300-A01. The A01 kit has been designed for use with an automation protocol requiring large batch sizes. For more information on automation solutions contact our technical support team.

- When placing small amounts of reagents into the reaction mix, pipette up and down several times to ensure complete transfer.
- When instructed to pipette mix, gently aspirate and dispense a volume that is at least half of total volume of the reaction mix. Repeat a minimum of five times for complete mixing.
- Always allow thermal cycler to reach initial temperature prior to placing tubes in the block.
- When working with more than one sample, excess master mix may be needed.
- Components of the Ovation RNA Amplification System V2 cannot be used interchangeably with other Ovation System kits and vice versa.
- Use only fresh ethanol stocks to make 80% ethanol for washes in the amplified cDNA purification protocols (Appendix A). Make the ethanol mixes the same day, 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. Preparing RNA Samples

We highly recommend isolating total RNA using a commercial system containing no organic solvents or removing any trace organic solvents by column purification. Phenol or ethanol contamination in the RNA sample will lower cDNA yields. The PAXgene Blood RNA Kit (QIAGEN, Cat. # 762164) for sample stabilization and whole blood RNA isolation has been tested and validated for use with the Ovation Whole Blood Solution.

Use high quality RNA for optimal performance. Verify O.D. 260/280 and 28S:18S ribosomal ratios of samples before using. A control amplification step should be performed with each batch of RNA samples until you become familiar with the protocol. Adjust the total RNA sample to be amplified using Nuclease-free Water (green: D1) so that 5–100 ng is present in 5 µL.

Note: With whole blood RNA samples, we advise using a minimum of 20 ng of input total RNA due to the high levels of globin present in such samples.

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 five programs shown in Table 4, 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 100–105°C).

Table 4. Thermal Cycler Programming

PRIMER ANNEALING AND FIRST STRAND SYNTHESIS	
Program 1 Primer Annealing	65°C – 5 min, hold at 4°C
Program 2 First Strand Synthesis	48°C – 60 min, 70°C – 15 min, hold at 4°C
SECOND STRAND SYNTHESIS	
Program 3 Second Strand Synthesis	37°C – 30 min, 75°C – 15 min, hold at 4°C
SPIA AMPLIFICATION	
Program 4 SPIA Amplification 1	48°C – 30 min, hold at 4°C
Program 5 SPIA Amplification 2	48°C – 30 min, 95°C – 5 min, hold at 4°C
cDNA FRAGMENTATION AND BIOTIN LABELING (ENCORE BIOTIN MODULE)	
Program 6 cDNA Fragmentation	37°C – 30 min, 95°C – 2 min, hold at 4°C
Program 7 Biotin Labeling	37°C – 60 min, 70°C – 10 min, hold at 4°C

E. First Strand cDNA Synthesis

1. Obtain First Strand Buffer Mix (blue: A2) and First Strand Enzyme Mix (blue: A3) from the components stored at –20°C and the First Strand Primer Mix (blue:A1) stored at –80°C.
2. Place A3 on ice and thaw A1 and A2 at room temperature. Once thawed, place reagents on ice.
3. Add 5 µL of total RNA sample (5–100 ng RNA from whole blood or total cellular RNA) to a 0.2 mL PCR tube.
4. Mix contents of A1 by vortexing for 2 seconds, and then spin in a microcentrifuge for 2 seconds.
5. Add 2 µL of A1 to the RNA aliquot.
6. Cap and flick tubes 6–8 times, spin for 2 seconds and place on ice.

! *Flick, do not vortex any enzyme mixes.*

7. Place the tube(s) in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 4):

   65°C – 5 min, hold at 4°C

8. Remove the tubes from the thermal cycler and snap cool by placing tubes on ice.
9. Mix contents of A2 by vortexing for 2 seconds. Spin for 2 seconds and place on ice.
10. Once the 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 5.

Table 5. First Strand Master Mix (volumes listed are for a single reaction)

FIRST STRAND BUFFER MIX (BLUE: A2 VER 4)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 1)
12 µL	1 µL

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

11. Spin tubes for 2 seconds and add 13 µL of the First Strand Master Mix to each tube.
12. Cap and flick tubes 6–8 times, spin for 2 seconds and place on ice.

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

13. Place the tubes in a pre-warmed thermal cycler programmed to run Program 2 (First Strand cDNA Synthesis; see Table 4):

    48°C – 60 min, 70°C – 15 min, hold at 4°C

14. Remove the tubes from the thermal cycler, flick the tubes 6–8 times, spin for 2 seconds to collect condensation and place on ice.
15. Continue immediately with the Second Strand cDNA Synthesis protocol.

F. Second Strand cDNA Synthesis

1. Remove the Second Strand Buffer (yellow: B1) and Second Strand Enzyme Mix (yellow: B2) from –20°C storage.
2. Mix the contents of B2 by flicking tube 6–8 times. Spin in a microcentrifuge for 2 seconds and place on ice.
3. Thaw reagent B1 at room temperature. Mix contents of B1 by vortexing for 2 seconds. Spin for 2 seconds and place on ice.
4. Make a master mix by combining B1 and B2 in a 0.5 mL capped tube, according to the volumes shown in Table 6.

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

The SPIA reagents may be thawed and put on ice 10 minutes before the completion of Second Strand Synthesis.

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

SECOND STRAND BUFFER MIX (YELLOW: B1 VER 4)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER 1)
18 µL	2 µL

5. Add 20 µL of the Second Strand Master Mix to each First Strand reaction tube.
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 3 (Second Strand cDNA Synthesis; see Table 4):

   37°C – 30 min, 75°C – 15 min, hold at 4°C
8. Remove the tubes from the thermal cycler, flick 6–8 times, spin for 2 seconds to collect condensation and place on ice.
9. Continue immediately with the SPIA Amplification protocol.

G. SPIA Amplification

1. Remove the SPIA Buffer Mix (red: C2), SPIA Enzyme Mix (red: C3), and water (green:D1) from –20°C storage. Also remove the SPIA Primer Mix (red: C1) and the Ovation WB Reagent (red: C4) stored at –80°C.

Ensure the enzymes are well mixed without introducing bubbles.

2. Thaw reagents at room temperature.
3. Mix contents of C1 by vortexing for 2 seconds, then spin in a microcentrifuge for 2 seconds and place on ice. Repeat for C2.
4. Mix the contents of C3 by inverting gently 5 times. Make sure the enzyme is well mixed without introducing bubbles. Spin for 2 seconds and place on ice.
5. Place new 0.2 mL PCR tubes in a rack on ice.

Use SPIA Master Mix immediately after preparation.

6. Make a master mix by sequentially combining C2, C1, D1, and C3 in a 1.5 mL capped tube according to the volumes shown in Table 7.

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

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

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

SPIA BUFFER MIX (RED: C2 VER 6)	SPIA PRIMER MIX (RED: C1 VER 1)	WATER (GREEN: D1)	SPIA ENZYME MIX (RED: C3 VER 5)
72 µL	2 µL	4 µL	40 µL

7. Add 118 µL of the SPIA master mix to the entire volume (40 µL) of the Second Strand reaction. Mix well by pipetting up and down 6–8 times.
8. Cap and spin tubes in a microcentrifuge for 2 seconds and return tubes to ice.
9. Split the 158 µL reaction volume into two 79 µL volumes in new, chilled 0.2 mL PCR tubes.
10. Spin tubes in a microcentrifuge for 2 seconds and place on ice.
11. Place tubes in a pre-warmed thermal cycler programmed to run Program 4 (SPIA Amplification 1, see Table 4):

    48°C – 30 min, hold at 4°C
12. Remove tubes and put on ice.
13. Add 3 µL C4 VER 1 to each 79 µL SPIA reaction.
14. Pipette up and down to mix with the pipette set at a large volume (>40 µL).
15. Place tubes in a pre-warmed thermal cycler programmed to run Program 5 (SPIA Amplification 2, see Table 4):

    48°C – 30 min, 95°C – 5 min, hold at 4°C
16. Remove tubes from the thermal cycler, flick 6–8 times and spin for 2 seconds to collect condensation. Place on ice.
17. Recombine the 2 x 82 µL volumes of each reaction into a single tube.

    Note: If using the Agencourt® RNAClean® XP Bead method for final cDNA cleanup, it is not necessary to recombine the half-reactions.
18. Vortex and spin for 2 seconds. Return samples to ice before proceeding with cDNA purification.
19. You may remove a 3 µL aliquot of pooled SPIA cDNA for analytical purposes, if desired.

H. 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.

Many factors may contribute to selecting the optimum purification method. Please contact the NuGEN Technical Support team for assistance in selecting the appropriate purification option for your purposes.

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

IV. Ovation RNA Amplification System V2 Protocol

I. Measuring cDNA Product Yield and Purity

1. Mix the sample by brief vortexing and spinning prior to checking the concentration.
2. Measure the absorbance of your cDNA product 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 of ssDNA = 33 µg/mL.

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

   The expected yield is 5–12 µg of cDNA.
5. Alternatively, you may measure the concentration and purity of cDNA with a Nanodrop, using 1 absorbance unit at 260 nm of ssDNA = 33 µg/mL as the constant.
6. The purified cDNA product may be stored at –20°C.

J. Fragmentation and Biotin Labeling

The Encore Biotin Module (Part No. 4200) has been validated for use with the cDNA generated using the Ovation RNA Amplification System V2 (Part No. 3100). You may proceed immediately with the fragmentation and labeling of the amplified cDNA following the procedure outlined in Encore Biotin Module user guide.

The only exception from standard fragmentation and labeling procedure is that the cDNA input into fragmentation and labeling reactions is 4.4 µg (per reaction) for whole blood samples.

For GeneChip array hybridization recommendations see Appendix C of this user guide.

V. Encore Biotin Module Protocol

A. Overview

The cDNA labeling reaction is performed in two steps:

1. cDNA fragmentation	0.5 hours
2. Biotin labeling	1.25 hours
Total time to label amplified cDNA	**1.75 hours**

B. Protocol Notes

- This protocol should be carried out in a post-amplification workspace designated for handling SPIA cDNA amplification products using dedicated post-amplification equipment and consumables. Exercise care to avoid the introduction of SPIA cDNA into workspaces used to set up SPIA amplification reactions.
- Thaw only components used in each step and immediately place them on ice.
- 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 mixes.
- The FL3 labeling buffer may appear to have pink coloration. This is normal.
- Spin down labeling master mix briefly at low speed. High speed spins for long periods can cause formation of a precipitate.
- The reagent volumes recovered greatly depend on the number of batches processed with each kit. Set up no fewer than three reactions at a time with the 4200-12 kit, no fewer than 10 reactions at a time with 4200-60, and no fewer than 48 reactions at a time with 4200-A01. The 4200-A01 kit has been designed for use with automation protocols requiring large batch sizes. For information on automation solutions contact NuGEN Technical Services.
- When placing small amounts of reagents into reaction mix, gently pipet up and down several times to ensure complete transfer.
- When instructed to pipet mix, gently aspirate and dispense at least half of the total reaction mix volume. Repeat a minimum of five times to ensure complete mixing.
- Allow the thermal cycler to reach incubation temperature before placing samples in the block.
- When working with more than one sample, excess master mix may be needed.
- Components of this NuGEN product should not be used or combined with any other Ovation Systems or Applause products or vice versa.

V. Encore Biotin Module Protocol

C. Preparing cDNA Samples

The amount of amplified cDNA required for each fragmentation and labeling reaction depends on the method of cDNA generation. When using cDNA generated from the Ovation Whole Blood Solution, you should use 4.4 µg input cDNA for a final concentration of 20 ng/µL. The concentration of cDNA should be assessed using 33 µg/mL/A260 as the constant.

D. Programming the Thermal Cycler

Table 8. Thermal Cycler Programming for the Encore Biotin Module

cDNA FRAGMENTATION AND BIOTIN LABELING	
Program 6 cDNA Fragmentation	37°C – 30 min, 95°C – 2 min, hold at 4°C
Program 7 Biotin Labeling	37°C – 60 min, 70°C – 10 min, hold at 4°C

E. cDNA Fragmentation

1. Obtain the Fragmentation Buffer Mix (Orange: FL1) and Fragmentation Enzyme Mix (Orange: FL2) from –20°C storage.

   Note: You may thaw all reagents at once. Refer to the Biotin Labeling protocol for thawing and mixing instructions for the labeling reagents.

2. Thaw FL1 at room temperature, mix by vortexing, spin and place on ice.
3. Mix FL2 by inverting the tube 3 times, spin and place on ice.
4. Add 4.4 µg of the purified SPIA cDNA into a PCR tube on ice. Add water, if necessary, to bring up the volume of the cDNA sample to 25 µL.
5. Make the Fragmentation Master Mix by combining FL1 and FL2 in a 0.5 mL capped tube, according to the volumes shown in Table 9.

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

FRAGMENTATION BUFFER MIX (ORANGE: FL1)	FRAGMENTATION ENZYME MIX (ORANGE: FL2)
5 µL	2 µL

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

6. Add 7 µL of the Fragmentation Master Mix to each sample.
7. Mix well by pipetting 8–10 times.
8. Vortex briefly to ensure thorough mixing, spin and place on ice.
9. Place the tubes in a pre-warmed thermal cycler programmed to run Program 6 (cDNA Fragmentation, see Table 8):

   37°C – 30 min, 95°C – 2 min, hold at 4°C
10. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice. Continue immediately with the Biotin Labeling protocol.

F. Biotin Labeling

1. Obtain the Labeling Buffer Mix (Orange: FL3), Biotin Reagent (Orange: FL4) and the Labeling Enzyme Mix (Orange: FL5) from the product box stored at –20°C.
2. Place all reagents on ice.
3. Thaw FL3 and FL4 at room temperature, mix by vortexing, spin and place on ice.
4. Mix FL5 by inverting the tube 3 times, spin and place on ice.
5. Make the Labeling Master Mix by combining FL3, FL4 and FL2 in a 0.5 mL capped tube, according to the volumes shown in Table 10.

Table 10. Labeling Master Mix (volumes listed are for a single reaction)

LABELING BUFFER MIX (ORANGE: FL3)	BIOTIN REAGENT (ORANGE: FL4)	LABELING ENZYME MIX (ORANGE: FL5)
15 µL	1.5 µL	1.5 µL

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

6. Add 18 µL of the Labeling Master Mix to each fragmented cDNA sample tube.
7. Mix well by pipetting 8–10 times.
8. Vortex briefly to ensure thorough mixing, spin and place on ice.
9. Place the tubes in a pre-warmed thermal cycler programmed to run Program 7 (Biotin Labeling, see Table 8):

   37°C – 60 min, 70°C – 10 min, hold at 4°C
10. After completion, remove tubes from thermal cycler, spin to collect condensation and place on ice.

V. Encore Biotin Module Protocol

The fragmented and labeled cDNA may be processed immediately for array hybridization or stored at –20°C. See Appendix B for information on quality control of the amplified, fragmented and labeled cDNA product. For recommendations on array hybridization, see Appendix C.

VI. Technical Support

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

In Europe contact NuGEN at +31.13.5780215 (Phone) or +31.13.5780216 (Fax) or email europe-gn@tecan.com.

In all other locations, contact your NuGEN distributor's Technical Support team.

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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® XP 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.

Agencourt® RNAClean® XP Beads (instructions for a single reaction)

Important notes:

- We recommend using a the SPRIPlate 96R Magnet Plate or similar 96-well ring magnet plate designed for 0.2 mL PCR tubes.
- Do not recombine the 82 µL half-reactions. Stop after step 16 on page 15 and begin purification as detailed below.

1. Ensure the Agencourt RNAClean XP Beads have completely reached room temperature before proceeding. Using cold beads may reduce the expected recovery.
2. Resuspend the beads by inverting and tapping the tube. Ensure that the beads are fully resuspended before adding to the sample.
3. 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 expected recovery.
4. Add 144 µL of resuspended beads (1.8 times the sample volume) to one set of the paired 80 µL SPIA half-reactions.
5. Mix the sample and beads thoroughly by pipetting up and down 10 times.

   Note: If using a 96-well plate with both half-reactions on the same plate, you must transfer the sample/bead mixture to a fresh plate at this point.

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

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

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

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

13. 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.
14. Using a multi-channel pipette, remove and discard the ethanol.
15. Repeat the 80% ethanol wash once more. Ensure as much ethanol as possible is removed from the plate.
16. Remove the reaction tubes or plate from the magnet and air dry the reaction plate on a 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. With the plate on a bench top, add 30 µL of room temperature Nuclease-free Water (green: D1) from the kit to each well. Holding the plate firmly, very carefully vortex for 30 seconds or use a plate shaker set to medium speed. To ensure the beads are fully resuspended, vortex longer if necessary. Alternatively, the beads may be resuspended by repeated pipetting.
18. Replace reaction tubes or plate on the plate magnet; allow the beads to separate for 5 minutes or until the solution clears.

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19. 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: It is important to minimize bead carry-over as small amounts of magnetic bead carry-over may interfere with sample quantitation.

20. 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.

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

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 recovery.
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.
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.

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

11. Add 500 µL of the room temperature 80% ethanol prepared in Step 1 above.
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.

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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 filter paper to remove any residual wash buffer from the tip of the column, preventing any wash buffer from 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.
17. Let the columns stand for 1 minute at room temperature.
18. Centrifuge the columns and microcentrifuge tubes for 1 minute at >10,000 X g.
19. Pool eluates from each half-reaction and measure the volume recovered. There should be approximately 25–30 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.

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

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.

1. Into a clean, labeled 1.5 mL microcentrifuge tube, add 800 µL of Buffer PB from the QIAGEN kit.
2. Add the the entire volume (160 µL) of amplified cDNA product to the tube.
3. Vortex for 5 seconds and spin down for 2 seconds.
4. Obtain and label a QIAquick spin column and place it into a collection tube.
5. Load 480 µL of sample onto the column.
6. Centrifuge column in a collection tube for 1 minute at 17,900 X g.
7. Discard flow-through. Place the column back in the same collection tube.

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

8. Load remaining 480 µL onto the same column. Centrifuge column in collection tube for 1 minute at 17,900 X g. Discard flow-through.
9. Place the column back in the same collection tube. Add 700 µL of 80% ethanol.
10. Centrifuge the column for 1 minute at 17,900 X g. Discard flow-through.
11. Repeat steps 9 and 10 once.
12. To remove remaining liquid, centrifuge column for one additional minute at 17,900 X g.
13. Remove the column from the centrifuge. Discard flow-through with the collection tube.
14. Blot the column tip onto filter paper to remove any residual wash buffer from the tip of the column, preventing any wash buffer from transferring to the eluted sample.
15. Place the column in a clean 2.0 mL collection tube, appropriately labeled.

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

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 5 minutes at room temperature to elute purified cDNA.
18. Centrifuge at 17,900 X g for 1 minute to collect sample. There should be approximately 30 µL of purified cDNA.
19. Mix the 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)

Important notes:

- Prepare a room temperature 80% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content, which may reduce 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. Add 320 µL of DNA Binding Buffer from the Zymo kit to a clean, labeled 1.5 mL microcentrifuge tube.
2. Add 160 µL of amplified SPIA cDNA product.
3. Vortex for 5 seconds, then spin briefly.

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

Be sure to wait until rotor achieves desired speed before starting timer for spins less than 1 minute in this procedure.

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

4. Obtain and label a Zymo Spin-II column and place it in a collection tube.
5. Load the entire volume of sample (480 µL) 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. Wash sample by adding 200 µL of room temperature 80% ethanol. Do not use the Wash Buffer provided with the Zymo columns.
9. Centrifuge for 10 seconds at >10,000 X g.
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 filter paper to remove any residual wash buffer from the tip of the column, preventing any wash buffer from transferring to the eluted sample.
13. Place the column into a clean, labeled 1.5 mL microcentrifuge tube.
14. Add 30 µL of room temperature Nuclease-free Water (green: D1) from the kit to the center of each 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.
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. Quality Control of Amplified, Fragmented and Labeled cDNA Product

We recommend viewing the size of the final fragmented and biotinylated product on an Agilent Bioanalyzer. Insufficiently fragmented product may yield poor results on Applied Biosystems GeneChips. We recommend that 100 ng of each fragmented/biotinylated cDNA sample be analyzed on an RNA 6000 Nano LabChip® (Agilent cat #5065-4476) using the Eukaryotic Total RNA Nano program (Nano assay in the Expert 2100 software) following the manufacturer's instructions. To obtain the best results from the GeneChip, 80% or greater of the fragmented cDNA product should be smaller than 200 bases in length. Figure 1, below, provides examples of Bioanalyzer traces for unfragmented and fragmented cDNA product.

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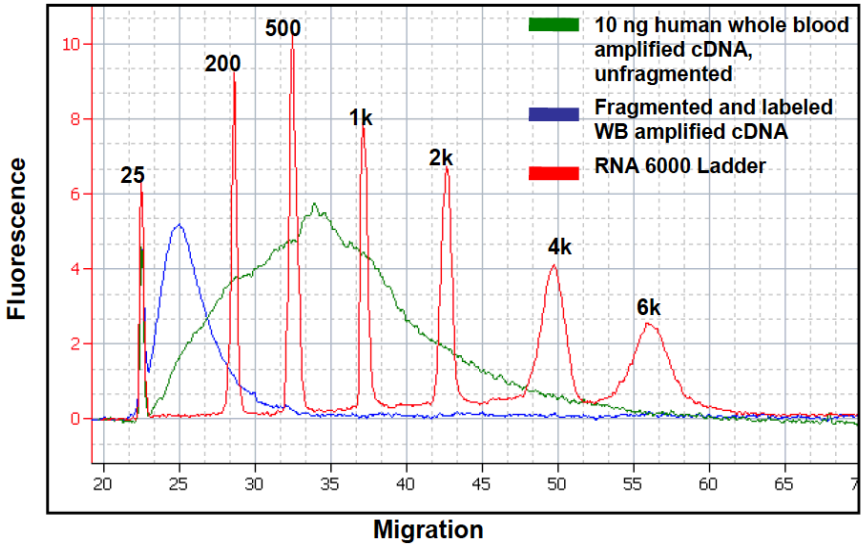
Figure 1. Bioanalyzer trace of human whole blood amplified, (un-fragmented and fragmented) cDNA product. The size of the RNA 6000 ladder (Ambion Inc., Cat. #7152) is indicated above each peak.



C. Target Preparation for Applied Biosystems GeneChip Eukaryotic Array Analysis

In general, cDNA targets labeled using the Encore Biotin Module are prepared for analysis on GeneChip arrays according to the manufacturer's guidelines. Some specific exceptions are noted below.

Using the Applied Biosystems GeneChip Hybridization Wash and Stain Kit with GeneChip 3′ Expression Arrays

Guidelines for processing GeneChip 3′ Expression Arrays (e.g., HG-U133 arrays) can be found in the Applied Biosystems GeneChip Expression Analysis Technical Manual (Thermo Fisher Scientific Part No. 702232) unless otherwise noted below.

- Refer to Table 11 for guidelines on hybridization cocktail formulation and hybridization cocktail loading volume with NuGEN prepared samples.
- Heat denature the hybridization cocktail at 99°C for 2 minutes (not 5 minutes as specified by Applied Biosystems), then follow the Applied Biosystems protocol (45°C in a heat block for 5 minutes then centrifuge at maximum speed for 1 minute just prior to loading).
- We recommend a hybridization time of 18 hours ± 2 hours. Hybridization for 16–20 hours yields comparable results.

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- Refer to Table 11 for guidelines on selection of the appropriate fluidics scripts. Note that in some cases the optimal fluidics script will be different for NuGEN targets than for Applied Biosystems labeled targets.

Table 11. Hybridization, Cocktail Assembly and Fluidics Protocols for Single GeneChip Arrays using Applied Biosystems GeneChip HWS kit (Thermo Fisher Scientific P/N 900720)

COMPONENT	STANDARD ARRAY (49 or 64 FORMAT)	MIDI ARRAY (100 FORMAT)	MINI ARRAY (169 FORMAT)	FINAL CONCENTRATION
Fragmented, biotin-labeled amplified cDNA	50 µL	34 µL	25 µL	Depends on sample type and amplification method
Control oligo-nucleotide B2 (2 nM)	3.7 µL	2.5 µL	1.9 µL	50 pM
20X eukaryotic hybridization controls (bioB, bioC, bioD, cre)	11 µL	7.5 µL	5.5 µL	1.5, 5, 25 and 100 pM, respectively
2X Hybridization buffer	110 µL	75 µL	55 µL	1X
100% DMSO	22 µL	15 µL	11 µL	10%
Water	23.3 µL	16 µL	11.6 µL	N/A
Total Volume	220 µL	150 µL	110 µL	
Array Loading Volume	200 µL	130 µL	90 µL	
FLUIDICS PROTOCOLS				
For 3' arrays	FS450_0004	FS450_0002		

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D. Performing Quantitative PCR on Amplified cDNA

Prior to purification of the cDNA, a small aliquot of cDNA may be saved for qPCR validation of array results. This unpurified cDNA may be diluted and used directly as template for qPCR systems including TaqMan® and SYBR® Green. If you would like to include mass normalization, the qPCR reaction should be run on the purified and quantitated cDNA samples.

Note: RT-PCR master mixes containing the enzyme Uracil N-Glycosylase (UNG) are not compatible with cDNA from the Ovation Whole Blood Solution.

NuGEN recommends the following reagents for qPCR:

- TaqMan: FastStart TaqMan Probe Master (Rox) (Roche, Cat. # 04 673 476 001), ABsolute qPCR Mix plus ROX (ABgene, Cat. #AB-1136/B), and Fast Universal PCR Master Mix 2x (Invitrogen, Cat. #4352042)
- SYBR: QuantiTect™ SYBR Green PCR Kit (QIAGEN, Cat. #204143), iQ SYBR Green Supermix (BioRad, Cat. # 170-8880) and FastStart SYBR Green Master (ROX) (Roche, Cat. # 04 673 514 001)

Recommendations to Achieve Optimal Results

1. **Dilute the Amplified Product**
   The concentration of your purified cDNA should be 160–400 ng/µL. We recommend that the cDNA be diluted before performing qPCR because inhibition has been observed with undiluted product. Typically, the amplified product is diluted 1:10 in nuclease-free water or in a buffer specified by the qPCR system manufacturer. After dilution, add 2 µL to a 25 µL qPCR reaction. Depending on the abundance of the transcripts you are measuring you may wish to dilute the cDNA further than 1:10.

2. **Primer Design**
   Amplified cDNA produced using the Ribo-SPIA process is generated from the 3' end of the mRNA. For best results, primer sets for qPCR procedures using this cDNA should be designed within 1,500 bases from the poly(A) tail.

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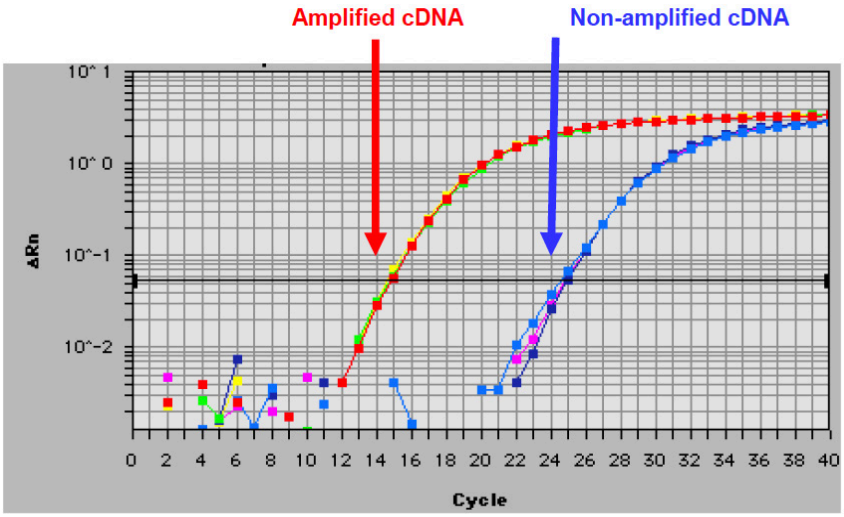
Figure 2. TaqMan Analysis of GAPDH Using Amplified cDNA.



Ovation RNA Amplification System V2, amplification of 20 ng UHR total RNA input. TaqMan® data indicated amplification efficiency of greater than 10^3. TaqMan reactions were conducted on duplicate samples of each dilution with the ABsolute QPCR ROX mix (ABgene Cat. #AB-1136/B), using a probe/primer set for GAPDH; located approximately 1kb from the poly(A) site. Fluorescence measurements were made with an ABI 7700 system.

E. DNase Treatment of RNA

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

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

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

DNase Treatment of RNA Post-purification: Using RNase-free DNase and either the RNA Clean-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) with 1 µL rDNase (10 Units Roche Cat. #04716728001).
2. Add up to 500 ng of the RNA sample and Nuclease-free Water (D1, green cap) to bring the final volume to 25 µL.
3. Incubate at 25°C for 15 minutes followed by 37°C for 15 minutes and return to ice.
4. After the DNase treatment, the sample must be purified. We recommend either of the two purification procedures below:

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

1. Add 4 volumes (100 µL) of RNA binding buffer to the sample.
2. Obtain one RNA Clean-up Kit-5 column and apply sample to column.
3. Spin column for 30 seconds at ≥8000 X g (≥10,000 rpm) and 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) and 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) and discard the flow-through.
7. Place the RNA Clean-up Kit-5 column in a fresh 1.5 mL collection tube.
8. Add 10 µL Nuclease-free Water (green: D1) directly to the center of the filter in the tube and close the cap.

   Important: Do not use cold water!
9. Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

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

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

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

1. Add 80 µL ice-cold Nuclease-free Water (D1, green cap) to the sample on ice.
2. Add 350 µL Buffer RLT and mix by pipetting.
3. Add 250 µL 96 to 100% ethanol and mix thoroughly by pipetting.
4. Place an RNeasy MinElute Spin Column into a 2 mL collection tube (one column per sample) and apply the 700 µL sample to the column.
5. After closing the column, spin for 15 seconds at ≥8000 X g (≥10,000 rpm) and discard the flow-through.
6. Place the RNeasy MinElute Spin Column into a fresh 2 mL collection tube. Add 500 µL Buffer RPE to the column and close the tube. Spin for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through, keeping the same collection tube.
7. Add 500 µL 80% ethanol to the RNeasy MinElute Spin Column and close the tube.

   Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
8. Spin for 2 minutes at ≥8000 X g (≥10,000 rpm). Discard the flow-through.

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

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

9. Place the RNeasy MinElute Spin Column in a fresh 2 mL collection tube and place in the microcentrifuge with the cap open. Spin for 5 minutes at ≥8000 X g (≥10,000 rpm) and discard the flow-through.
10. Place the RNeasy MinElute Spin Column in a fresh 1.5 mL collection tube.
11. Add 14 µL Nuclease-free Water (D1, green cap) directly to the center of the filter in the tube and close the cap. **Do not use cold water!**
12. Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

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F. Frequently Asked Questions (FAQs)

Q1. Do I need to use high quality total RNA?
Yes. Use of lower quality RNA may result in poor performance. One approach to determining RNA quality is the Agilent Bioanalyzer's RNA Integrity Number (RIN). Clean RNA with a RIN score greater than 7 should amplify well.

Q2. How much total RNA input do I need for amplification?
We recommend RNA input of 5–100ng RNA from whole blood or total cellular RNA; however, due to the high levels of globin present in whole blood RNA samples, we advise using inputs of 20 ng or higher when feasible.

Q3. Can I use mRNA instead of total RNA as starting material?
Purified poly(A) RNA has been successfully used as input to the Ovation System. It is important to use much lower amounts of input mRNA, comparable to mRNA present in 5–100 ng of a total RNA sample from the same tissue.

Q4. Is the Ovation RNA Amplification System V2 amplification 3′ biased?
Yes. Since the Ovation RNA Amplification System V2 primes the poly(A) tail of transcripts, it is 3′ biased, resulting in coverage up to a range of 1500 bases from the 3′ poly(A) tail.

Q5. What is the expected cDNA yield from one reaction of the Ovation RNA Amplification System V2 used with the Ovation WB Reagent?
For a standard reaction the expected yield is 5–12 µg of amplified cDNA.

Q6. Is the cDNA yield dependent upon the quantity of input total RNA?
The total yield of cDNA is not directly dependent upon input RNA amount due to upper limit constraints on cDNA production in the reaction.

Q7. What is the amplification efficiency of the Ovation RNA Amplification System V2?
Based on qPCR results of a collection of housekeeping genes tested on HeLa RNA, amplification efficiency ranges from 1,000–10,000 fold or higher depending on the input amount.

Q8. What is the size range of cDNA generated by the Ovation RNA Amplification System V2?
As measured with an Agilent Bioanalyzer, the majority of amplified SPIA cDNA is 200–2000 bases long. After fragmentation, 80% of the cDNA falls below 200 bases with an average peak at 85 bases.

Q9. Has NuGEN performed reproducibility studies on the Ovation RNA Amplification System V2?
Yes. Our studies have included sample-to-sample, lot-to-lot, and operator-to-operator reproducibility.

Q10. Can DNA be used as input for the Ovation RNA Amplification System V2?
No. The Ovation RNA Amplification System V2 is designed to amplify mRNA.

VII. Appendix

Q11. Are there any tissues that will not work with the Ovation RNA Amplification System V2?
We have not encountered any good-quality, clean RNA samples containing poly(A) RNA that will not work with the Ovation RNA Amplification System V2.

Q12. Does the Ovation RNA Amplification System V2 generate product in a no-RNA reaction?
As with most amplification systems, non-specific product is generated using the Ovation RNA Amplification System V2 in the absence of input template. Array and qPCR analysis show these amplification products to be non-specific.

Q13. How many rounds of amplification are performed with the Ovation RNA Amplification System V2?
The Ovation Amplification System V2 performs a single round of amplification in fewer than 4 hours. Our products are designed to provide high sensitivity through robust amplification without necessitating a second round of amplification.

Q14. Do I need to order specific primers for the amplification?
The chimeric DNA/RNA primers provided with the Ovation RNA Amplification System V2 kits are universal. There is no need for additional primers.

Q15. Do I have to use the DNA/RNA primers from the kit?
Yes. The Ovation RNA Amplification System V2 was designed and optimized to work with the primers provided. NuGEN does not support the use of other primers with the Ovation RNA Amplification System V2.

Q16. Do I need to change the sense of the cDNA for use on oligo arrays?
The Ovation RNA Amplification System V2 generates antisense cDNA. There is no need to change the sense.

Q17. Can I use the Ovation RNA Amplification System V2 for archiving cDNA, and what are the recommend storage conditions?
Yes. Resulting cDNA may be stored at –20°C following purification for at least six months. Ensure the vials are well sealed and avoid multiple freeze/thaw cycles.

Q18. For quantitative real time PCR applications, what is the optimal distance from the 3′ poly(A) tail for design of primer probe sets?
Due to the amplification mechanism of the Ovation RNA Amplification System V2, we recommend primer/probe sets be designed within the first 1,500 bases from the poly(A) tail.

Q19. Where can I safely stop in the Ovation RNA Amplification System V2 protocol?
You may safely stop after SPIA amplification or after purifying the amplified cDNA and store the cDNA at –20°C. After processing with the Encore Biotin Module, the fragmented and labeled cDNA may also be stored at –20°C for short term storage or at –80°C for longer term storage.

VII. Appendix

Q20. How can I ensure good yields at the cDNA purification step?
In order to maximize yields, we recommend the following:

a. Do NOT use cold water for the elution step. Use the D1 Nuclease-free Water included in the Ovation RNA Amplification System V2 kit at room temperature.

b. Do NOT spin the columns at an incorrect speed. Strictly adhere to the guidelines in the user guide.

c. Use a fresh dilution of ethanol from a fresh stock for any washing steps.

d. Vortex the eluted cDNA sample prior to measuring the absorbance.

Q21. Should I purify the cDNA before determining the concentration?
Yes. The primers and reagents present in the amplified cDNA will interfere with accurate quantitation. Details on measuring the concentration of cDNA are included in this user guide.

Q22. Do you recommend any specific whole blood RNA isolation methods?
We do not specifically require one method of RNA preparation, however, for whole blood samples the QIAGEN PAXgene products for stabilization and RNA isolation have been validated and are compatible with the Ovation Whole Blood Solution. In general, any method that yields high quality, non-degraded RNA that is free of organic solvents and contaminants should work well.

Fragmentation and Labeling

Q23. What materials are provided with the Encore Biotin Module?
The Encore Biotin Module provides all necessary buffers and enzymes for fragmentation and labeling of cDNA generated by the Ovation Whole Blood Solution.

Q24. How much fragmented and labeled cDNA does this kit yield?
Since the Encore Biotin Module does not require any purification, the final yield is equal to the amount of cDNA input.

Q25. What is the size range of cDNA fragments generated by the Encore Biotin Module?
As measured with an Agilent Bioanalyzer, 80% of product falls below 200 bases with an average peak at 85 bases.

Q26. Has NuGEN performed reproducibility studies on the Encore Biotin Module?
Yes. Our studies have included sample-to-sample, lot-to-lot and operator-to-operator reproducibility. Refer to the NuGEN Technical Report on Encore Biotin Module Performance for a summary of our performance data.

Q27. Can the Encore Biotin Module be used for fragmentation and labeling of RNA?
No.

Q28. Should I purify the labeled cDNA before hybridization?
No. Purification of the labeled cDNA is not necessary.

Q29. What are the recommended storage conditions for the labeled cDNA?
The labeled cDNA should be stored at –20°C. Ensure the vials are well sealed and avoid multiple freeze/thaw cycles.

Q30. Are the array hybridization reagents included in the Encore Biotin Module?
No. Refer to Appendix C for information on recommended array hybridization reagents.

Q31. What hybridization and wash protocols do you recommend for Applied Biosystems GeneChip applications?
Refer to Appendix C for information on array hybridization protocols.

Q32. Where can I safely stop in the Encore Biotin Module protocol?
We do not recommend stopping at any step of the protocol.

Q33. How do I determine fragmentation success?
You may use an Agilent Bioanalyzer to inspect the size distribution of samples before and after fragmentation as described in Appendix B.

G. Update History

This document, the Ovation Whole Blood Solution User Guide (M01110 v6.1) is an update to address the following topics:

Description	Section	Page(s)
Updated legal information, emails, address, trademarks, and logo.	Throughout	Throughout
Updated Affymetrix GeneChip array info to Applied Biosystems GeneChip.	Throughout	Throughout

VII. Appendix

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