USER GUIDE

Ovation® Pico WTA System

PART NO. 3300-12, 3300-60, 3300-A01

NuGEN®
imagine more from less™

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I. Introduction

A. Background

The Ovation Pico WTA System provides a fast and simple method for preparing amplified cDNA for gene expression analysis. Amplification is initiated at the 3′ end as well as randomly throughout the whole transcriptome in the sample. This feature makes the Ovation Pico WTA System ideal for amplification of partially degraded and compromised RNA samples. The amplified product of the Ovation Pico WTA System is optimized for analysis on Affymetrix® GeneChip® arrays, Agilent Gene Expression microarrays and Illumina Genome-Wide Expression BeadChips utilizing the appropriate NuGEN® labeling modules and protocols. It may also be used for the detection of low-, medium- and high-abundance gene transcripts using quantitative PCR (qPCR). For details please visit our website, www.nugeninc.com.

The Ovation Pico WTA System is powered by Ribo-SPIA® technology, a rapid, simple and sensitive RNA amplification process developed by NuGEN. Using Ribo-SPIA technology and starting with 500 pg to 50 ng total RNA, microgram quantities of cDNA can be prepared in approximately five hours.

The Ovation Pico WTA System (Cat. #3300-12, 3300-60, 3300-A01) provides optimized reagent mixes and a protocol to process 12, 60 or 96 RNA samples. Control RNA is not provided with the Ovation Pico WTA System but we recommend the use of a control RNA when first using this product.

B. Ribo-SPIA Technology

Ribo-SPIA technology is a three-step process that generates amplified cDNA from as little as 500 picograms of total RNA (see Figure 1).

1. **Generation of First Strand cDNA (1 hour)**
   First strand cDNA is prepared using a unique first strand DNA/RNA chimeric primer mix and reverse transcriptase (RT). The primers have a DNA portion that hybridizes either to the 5′ portion of the poly(A) sequence or randomly across the transcript. RT extends the 3′ DNA end of each primer generating first strand cDNA. The resulting cDNA/mRNA hybrid molecule contains a unique RNA sequence at the 5′ end of the cDNA strand.

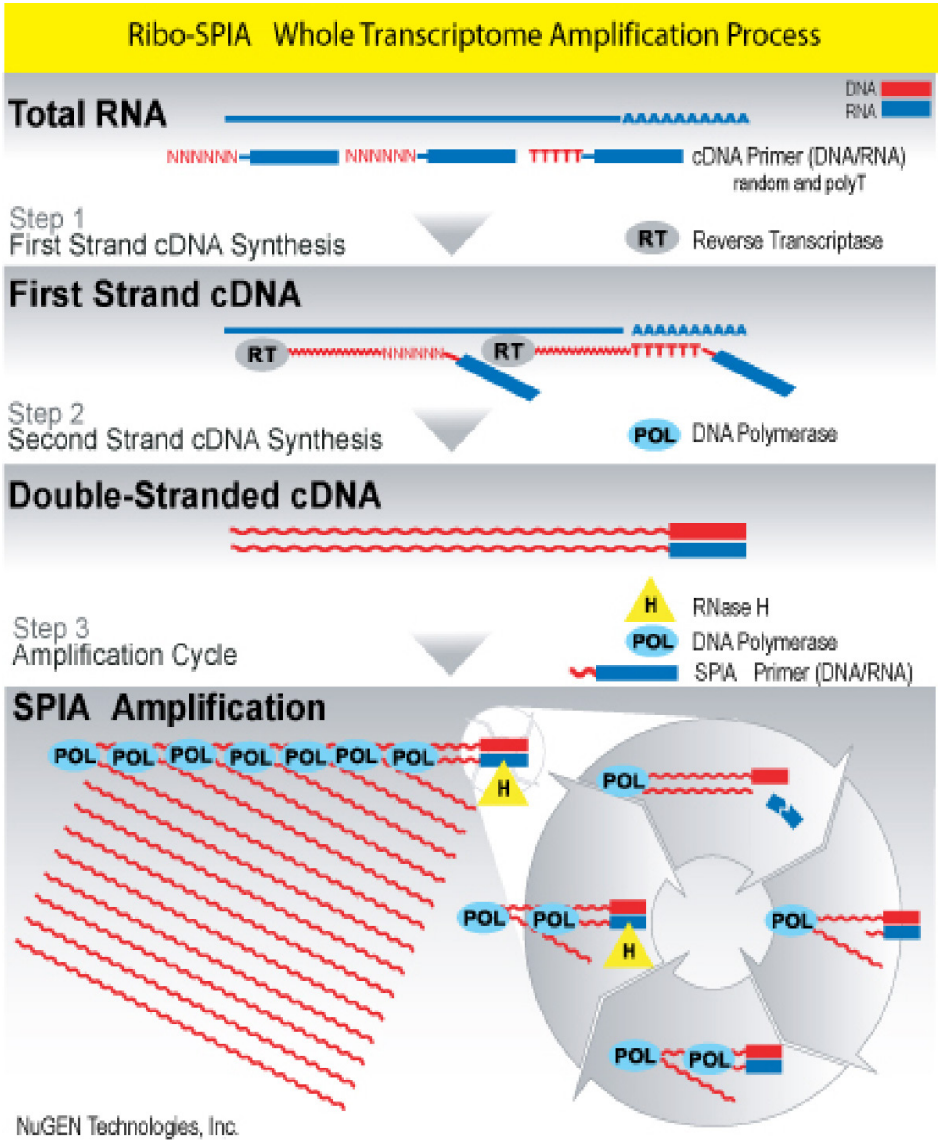
2. **Generation of a DNA/RNA Heteroduplex Double Strand cDNA (1.5 hours)**
   Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthesize a second strand, which includes DNA complementary to the 5′ unique sequence from the first strand chimeric primers. The result is a double-stranded cDNA with a unique DNA/RNA heteroduplex at one end.

3. **SPIA® Amplification (1.5 hours)**
   SPIA amplification is a linear isothermal DNA amplification process developed by NuGEN. It uses a SPIA DNA/RNA chimeric primer, DNA polymerase and RNase H in a homogeneous isothermal assay that provides highly efficient amplification of DNA sequences. RNase H is used to degrade RNA in the DNA/RNA heteroduplex at the 5′ end of the first cDNA strand. This results in the exposure of a DNA

sequence that is available for binding a second SPIA DNA/RNA chimeric primer. DNA polymerase then initiates replication at the 3′ end of the primer, displacing the existing forward strand. The RNA portion at the 5′ end of the newly synthesized strand is again removed by RNase H, exposing part of the unique priming site for initiation of the next round of cDNA synthesis. The process of SPIA DNA/RNA primer binding, DNA replication, strand displacement and RNA cleavage is repeated, resulting in rapid accumulation of cDNA with sequence complementary to the original mRNA. An average mRNA amplification of 15,000-fold is observed with 500 pg starting total RNA.

I. Introduction

Figure 1. The Ribo-SPIA RNA Amplification Process used in the Ovation Pico WTA System



I. Introduction

C. Performance Specifications

The Ovation Pico WTA System synthesizes microgram quantities of amplified cDNA starting with total cellular RNA input amounts of 500 pg to 50 ng. In approximately five hours, the Ovation Pico WTA System can produce six to 10 µg of cDNA ready for qPCR or other analytical tests. When used with intact input RNA, the size of the majority of the cDNA products produced by the Ribo-SPIA amplification process is between 50 bases and 1.5 Kb. When used with degraded input RNA the size of the amplified cDNA products will be smaller, proportional to the degree of input RNA degradation. With a 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 Pico WTA System lot is tested to meet specifications of yield, qPCR and array performance of the product.

E. Storage and Stability

The Ovation Pico WTA System is shipped on dry ice and should be unpacked immediately upon receipt.

Note: This product contains components with multiple storage temperatures.

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

The 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 separately at –80°C.

Store the RNAClean beads at 4°C

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

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

Kits handled and stored according to the above guidelines will perform to specifications for at least six months. We have not yet established long-term storage conditions for Ovation Pico WTA Systems.

F. Material Safety Data Sheet (MSDS)

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

II. Kit Components

A. Reagents and Supplies Provided

Table 1. First Strand cDNA Reagents

COMPONENT	3300-12 PART NUMBER	3300-60 PART NUMBER	3300-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
First Strand Primer Mix	S01163	S01195	S01293	Blue	A1 VER 3
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	3300-12 PART NUMBER	3300-60 PART NUMBER	3300-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	3300-12 PART NUMBER	3300-60 PART NUMBER	3300-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
SPIA Primer Mix	S01162	S01196	S01294	Red	C1 VER 4
SPIA Buffer Mix	S01164	S01194	S01291	Red	C2 VER 5
SPIA Enzyme Mix	S01165	S01166	S01292	Red	C3 VER 5

II. Kit Components

Table 4. Additional Reagents

COMPONENT	3300-12 PART NUMBER	3300-60 PART NUMBER	3300-A01 PART NUMBER	VIAL CAP	VIAL NUMBER
Nuclease-free Water	S01001	S01113	—	Green	D1
Agencourt RNAClean Beads	1200-01	1200-01	1200-01	Clear	—

Note: The reagents in the Ovation Pico WTA System product are similar to reagents in our 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 to 10 µL pipette, 2 to 20 µL pipette, 20 to 200 µL pipette, and 200 to 1000 µL pipette
  - Vortexer
  - Thermal cycler with 0.2 mL tube heat block, heated lid, and 100 µL reaction capacity
  - Appropriate spectrophotometer and cuvettes, or Nanodrop® UV-Vis Spectrophotometer
- **Reagents**
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - 0.2 mL individual thin wall PCR tubes or 8 X 0.2 mL strip PCR tubes or 0.2 mL thin wall PCR plates
  - SPRIPlate® 96R, Ring Magnet Plate (Beckman Coulter Genomics, Cat. #A29164) or 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):
  - RNAClean 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)
- Disposable gloves
- Kimwipes
- Ice bucket
- Decontamination solutions such as RNaseZap® (Ambion, Cat.#AM9780) and DNA-OFF™ (MP Biomedicals, Cat.#QD0500)

- **Optional Materials**
  - Agilent 2100 Bioanalyzer or materials and equipment for electrophoretic analysis of RNA
  - Real-time PCR system

To Order:

- Beckman Coulter Genomics, www.beckmancoulter.com
- Ambion Inc., www.ambion.com
- MP Biomedicals, www.mpbio.com
- New England BioLabs, www.neb.com/nebecomm/default.asp
- QIAGEN Inc., www1.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

It is important to assess the quality of your RNA sample prior to planning your amplification. While the Ovation Pico WTA System will allow the amplification of many RNA samples of variable quality, use of highly degraded RNA samples can lead to lower yields and shorter amplified cDNA. It is impossible to guarantee success with all degraded RNA samples. To assess RNA quality prior to using the Ovation Pico WTA System, follow the guidelines below.

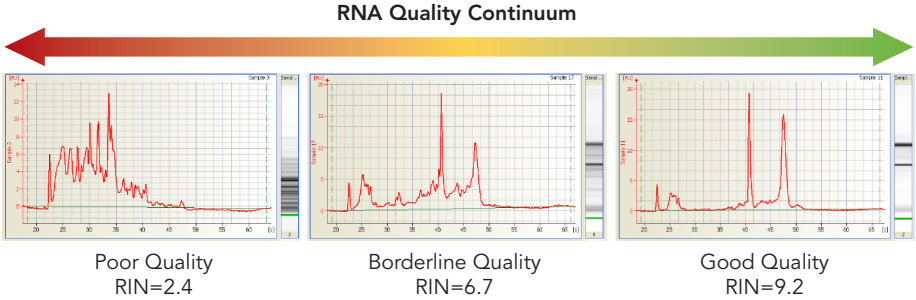
1. **RNA Quantity**
   Total RNA input must be between 500 pg and 50 ng. Inputs above 50 ng per reaction may inhibit amplification, while lower amounts of input will potentially result in insufficient yields depending on required analytical platforms.

2. **RNA Purity**
   RNA samples must be free of contaminating proteins and other cellular material, organic solvents (including phenol and ethanol) and salts used in many RNA isolation methods. Use of a commercially available system for preparing small amounts of RNA that does not require organic solvents is recommended. If a method such as Trizol is used, we recommend using good quality Trizol and column purification after isolation, if possible. One measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The A260:A280 ratio for RNA samples of acceptable purity should be in excess of 1.8. RNA samples with lower ratios may result in low amplification yield.

3. **RNA Integrity**
   RNA samples of high molecular weight with little or no evidence of degradation will amplify very well with this product. Due to the whole transcriptome amplification approach, even lower quality RNA samples and transcripts with a compromised poly(A) tail can also amplify successfully using the Ovation Pico WTA System.

   RNA integrity can be determined using the Agilent 2100 Bioanalyzer, RNA 6000 Nano LabChip® or RNA 6000 Pico LabChip®, and the RNA Integrity Number (RIN) calculation, available in the Bioanalyzer 2100 Expert Software. The instrument provides a sensitive and rapid way of confirming RNA integrity prior to amplification, both visually, with a detailed electrophoretic trace of the RNA, and computationally, by calculating a RIN score. While it is impossible to guarantee satisfactory results with all degraded samples, the Ovation Pico WTA System can work with many samples that are moderately to severely degraded. In our tests using an RNA degradation model system, RNA samples that are severely degraded showing RIN numbers of approximately 2 to 5 still amplify successfully and reproducibly. We strongly recommend quantitation of total RNA to ensure the minimum input requirement is met. On occasions when the Bioanalyzer software fails to calculate a RIN score, we recommend viewing the electrophoretic trace to determine if the sample may still be of adequate integrity for use.

Figure 2. This continuum of RNA quality shows Bioanalyzer traces of three different RNAs with varying degrees of degradation, all of which have amplified successfully using the Ovation Pico WTA System approach.



4. **User Quality Control Guidelines for RNA sample**
   The quantity and purity of RNA plays an important role in the success of amplification; however, there are instances where quantitative and qualitative data are not available or difficult to obtain for a sample set. We have developed a tool for assessment of RNA sample suitability, which includes a data set, procedures, sequence information for a set of reference qPCR assays and some assessment recommendations. This document may be obtained from our website's Technical Documents page or by contacting the NuGEN technical services team at: techserv@nugeninc.com, or in Europe: europe@nugeninc.com.

5. **DNase Treatment**
   It is generally recommended to use DNase-treated RNA for amplification using the Ovation Pico WTA System. One reason is that presence of genomic DNA in the RNA sample may potentially have adverse effects on downstream analytical platforms. Contaminating genomic DNA may also be amplified along with the RNA. Also if the total RNA sample contains a significant amount of contaminating genomic DNA, it will be difficult to accurately quantitate the true RNA concentration. The RNA input quantity may, therefore, be over-estimated based on an absorbance measurement. Since it is important that RNA input be within the stated range of 500 pg to 50 ng, we recommend using a DNase treatment that will remove contaminating genomic DNA during RNA purification.

6. **Carrier use for RNA isolation**
   We strongly recommend against the use of nucleic acid based carriers during RNA purification because many have been shown to produce cDNA product in first strand synthesis. We also advise against the use of glycogen in RNA isolation, as it inhibits reverse transcription. For the latest information regarding other carriers, contact our technical services team.

B. Using RNase-free Techniques

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

- Wear disposable gloves and change them frequently.
- Avoid touching surfaces or materials that could introduce RNases.
- Use reagents provided. Substitutions may introduce RNases.
- Prior to initiating protocol, clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination 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 Pico WTA System must be stored at –80°C. Avoid frequent freeze/thaw cycles or RNA shearing may result.

D. Amplified cDNA Storage

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

IV. Protocol

A. Overview

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

1. First strand cDNA synthesis:	1 hour
2. Second strand cDNA synthesis and purification:	1.5 hours
3. SPIA isothermal linear amplification and purification:	1.5 hours
Total time to prepare amplified cDNA	**~4 hours**

Ovation Pico 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 cDNA must be purified following amplification if you intend to use the cDNA for fragmentation and labeling using a validated NuGEN labeling system for applications such as microarray analysis.

The Ovation Pico 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 amplified cDNA, we recommend proceeding with purification of cDNA immediately after SPIA especially if you plan to mass normalize qPCR input. Spectrophotometric quantitation of unpurified amplification products will result in artificially high readings due to amplification components present in the sample.

B. Protocol Notes

- We recommend the routine use of a positive control RNA. Especially the first time you set up an amplification reaction, the use of a positive control RNA will allow the establishment of a baseline of performance and provide the opportunity to become familiar with the bead purification step. This step may be unfamiliar to many users and can be especially prone to handling variability in using the magnet plate, so a practice run with the plate is highly recommended.
- In working with very small, picogram amounts of RNA we strongly recommend the use of low retention tips and tubes for storage and dilution of the samples in order to reduce the loss of RNA samples due to adhesion to polypropylene surfaces.
- Due to the high sensitivity inherent in this amplification system we strongly recommend taking measures to minimize the potential for contamination of amplification reactions by carry-over of nucleic acids or other laboratory contaminants. The two steps to accomplish this are: 1. Designating separate workspaces for "pre-amplification" and "post-amplification" steps and materials and 2. Implementing routine cleanup protocols for workspaces as standard operating procedure. A detailed set of these recommendations is listed in the Appendix.

- Use the water provided with the kit (green: D1) or an alternate source of nuclease-free water. We do not recommend the use of DEPC-treated water with this protocol.
- 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).
- The reagent volumes recovered greatly depend on the number of batches processed with each kit. Set up no fewer than four reactions at a time with the 3300-12 kit, no fewer than 10 reactions at a time with 3300-60. This ensures sufficient reagent recoveries for 12 total amplifications from a single 12-reaction kit, and 60 from the 60-reaction kit.
- Thaw components used in each step and immediately place them on ice. It is best to 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 12 reactions in the kit. The Ovation Pico WTA System Quick Protocol has been updated to automatically calculate an appropriate overfill volume which can be used as a guideline in setting up master mixes.
- Components and reagents from other Ovation® System products should not be used with this product.
- Use only fresh ethanol stocks to make 70% ethanol used in the post-second strand bead purification (Section G), and 80% ethanol for washes in the amplified cDNA purification protocols (Section I, Appendix A). Make the ethanol mixes fresh as well, carefully measuring both the ethanol and water with pipettes. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the beads or column.

C. Beckman Coulter Genomics' Agencourt® RNAClean Purification Beads

Tips and notes Relevant to the Second Strand cDNA Cleanup, Section G:

There are significant modifications to the Beckman Coulter Genomics' Agencourt RNAClean beads standard procedure; therefore, you must follow the procedures outlined in this user guide for the use of these beads with the Ovation Pico WTA System. However,

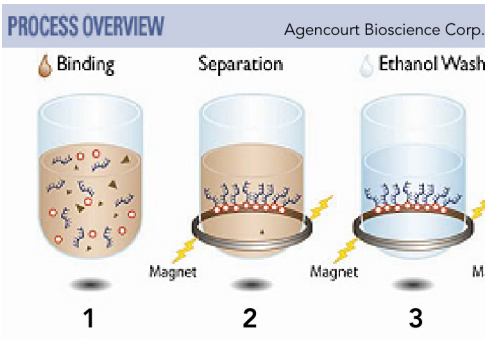
you may review the Agencourt user guide to become familiar with the manufacturer's recommendations at the following website: http://www.agencourt.com/documents/products/rnaclean/Agencourt_RNAClean_Protocol.pdf

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

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

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

Figure 3. Bead purification process overview.



Additional Tips and Notes

- Remove beads from 4°C and leave at room temperature for at least 15 minutes and before use. Ensure that they have completely reached room temperature. Cold beads will result in reduced recovery.
- Fully resuspend beads by inverting and tapping before adding to sample.
- Note that we recommend using 1.6 volumes (32 µL) of RNAClean beads. This is different from the standard Agencourt protocol.
- It is critical to let the beads separate on the magnet for a full five minutes. Removing binding buffer before the beads have completely separated will impact cDNA yields.
- After the binding step has been completed, it is important to minimize bead loss when removing the binding buffer. With the samples placed on the magnet, remove only 45 µL of the binding buffer from each sample. Some liquid will remain at the bottom of the tube but this will minimize bead loss.
- Any significant loss of beads bound to the magnet during the ethanol washes will impact cDNA yields, so make sure the beads are not lost with the wash.
- Ensure that the 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 the sample wells or tubes in a small ring.

- It is critical that all residual ethanol be removed prior to continuing with the SPIA amplification. Therefore, when removing the final ethanol wash, first remove most of the ethanol, then allow the excess to collect at the bottom of the tube before removing the remaining ethanol. This reduces the required bead air drying time.
- After drying the beads for at least 15 to 20 minutes, inspect each tube carefully and make certain that all the ethanol has evaporated before proceeding with the amplification step.
- It is strongly recommended that strip tubes or partial plates are firmly placed when used with the magnetic plate. We don't advise the use of individual tubes as they are not very stably supported on the magnetic plates.

D. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes, equipped with a heated lid, and with a capacity of 100 µL reaction volume. Prepare the programs shown in Table 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 – 1 min, 25°C – 10 min, 42°C – 10 min, 70°C – 15 min, hold at 4°C
SECOND STRAND cDNA SYNTHESIS	
Program 3 Second Strand Synthesis	4°C – 1 min, 25°C – 10 min, 50°C – 30 min, 70°C – 5 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

E. First Strand cDNA Synthesis Protocol

Important Note: Carry out steps E (First-Strand Synthesis Protocol) through H, step 8 (SPIA Amplification Protocol) in a pre-amplification workspace using dedicated pre-amplification consumables and equipment. Wipe all surfaces, equipment and instrumentation with a DNA decontaminant solution such as DNA-OFF (MP Biomedicals, Cat#Q0500) to avoid the potential introduction of previously amplified cDNA into new amplifications. For more information on our recommendations for workflow compartmentalization and routine lab cleanup please refer to Appendix E of this User Guide. If you have any questions on this important topic, please contact NuGEN Technical Services (techserv@nugeninc.com, (888) 654-6544).

1. Obtain First Strand Buffer Mix (blue: A2), First Strand Enzyme Mix (blue: A3) and the water (green: D1) from the components stored at –20°C and the First Strand Primer Mix (blue: A1) stored at –80°C.
2. Spin down contents of A3 for 2 seconds, then place on ice.
3. Thaw the other reagents at room temperature. Mix by vortexing for 2 seconds, spin for 2 seconds, then place on ice. Leave water D1 at room temperature.
4. Add 2 µL of A1 to a 0.2 mL PCR tube.
5. Add 5 µL of total RNA sample (500 pg to 50 ng) to the primer.
6. Cap and spin tube(s) for 2 seconds and return tubes to ice.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 5):

   65°C – 2 min, hold at 4°C
8. Remove tubes from the thermal cycler and place tubes on ice.
9. Once Primer Annealing (Step 7) is complete, prepare a master mix by combining A2 and A3 in a 0.5 mL capped tube, according to the volumes shown in Table 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

10. Add 3 µL of the First Strand Master Mix to each tube.

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

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

11. Mix by pipetting 3 times, spin for 2 seconds.
12. Place tubes in a pre-cooled thermal cycler programmed to run Program 2 (First Strand Synthesis; see Table 5):

    4°C – 1 min, 25°C – 10 min, 42°C – 10 min, 70°C – 15 min, hold at 4°C
13. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
14. Continue immediately with second strand cDNA synthesis.

F. Second Strand cDNA Synthesis Protocol

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 purification beads from 4°C and place on bench top to reach room temperature for use in the next step.

2. Obtain the Second Strand Buffer Mix (yellow: B1) and Second Strand Enzyme Mix (yellow: B2) from the components stored at –20°C.
3. Spin down contents of B2 for 2 seconds, then place on ice.
4. Thaw reagent B1 at room temperature, mix by vortexing for 2 seconds, spin for 2 seconds, then place on ice.

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

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)
9.75 µL	0.25 µL

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

6. Add 10 µL of the Second Strand Master Mix to each First Strand reaction tube.
7. Mix by pipetting 3 times, spin for 2 seconds, then place on ice.
8. Place tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand Synthesis; see Table 5):

    4°C – 1 min, 25°C – 10 min, 50°C – 30 min, 70°C – 5 min, hold at 4°C
9. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation. Place in a rack on the bench top.
10. Continue immediately with purification of unamplified cDNA.

G. Purification of cDNA Protocol

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

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

3. Prepare a 70% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content, which may reduce amplification yield.
4. At room temperature, add 32 µL (1.6 volumes) bead suspension to each reaction and mix by pipetting up and down 10 times.
5. Incubate at room temperature for 10 minutes.
6. Transfer tubes to magnet plate and let stand 5 minutes to completely clear the solution of beads.

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

7. Carefully remove only 45 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

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

! *Ensure that all residual ethanol is removed prior to continuing with SPIA Amplification.*

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. Proceed immediately with SPIA Amplification with the cDNA still bound to the dry beads.

H. SPIA Amplification Protocol

1. Obtain the SPIA Buffer Mix (red: C2) and SPIA Enzyme Mix (red: C3) stored at –20°C and the SPIA Primer Mix (red: C1) stored at –80°C.
2. Thaw reagents C1 and C2 at room temperature, mix by vortexing for 2 seconds, spin in a microcentrifuge for 2 seconds, then place on ice.

! *Ensure the enzyme is well mixed without introducing bubbles.*

3. Thaw C3 on ice and mix the contents by inverting gently 5 times. Ensure the enzyme is well mixed without introducing bubbles, spin in a microcentrifuge for 2 seconds, then 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. 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 5)	SPIA PRIMER MIX (RED:C1 VER 4)	SPIA ENZYME MIX (RED:C3 VER 5)
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 well by pipetting up and down 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 off the beads.
6. Transfer one half of the reaction volume (80 µL) to a second tube.
7. Place 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 tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice. Do not re-open tubes in the pre-amplification workspace.

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

Note: If using Beckman Coulter Genomics' Agencourt RNAClean bead method for final cDNA cleanup, skip steps 9–12 below and go directly to the RNAClean Bead cleanup protocol on page 22. It is not necessary to recombine the half-reactions or to remove the beads.

9. Recombine the half-reactions.
10. Transfer tubes to magnet plate 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. At this stage, the cDNA may be purified or stored at –20°C.

I. 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 labeling reactions, or other enzymatic reactions such as the WT-Ovation™ Exon Module protocol. We recommend that the amplified SPIA cDNA product be purified prior to qPCR analysis.

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.

J. Measuring cDNA Yield

1. Mix purified cDNA sample by brief vortexing and spinning prior to checking the concentration.
2. Measure the absorbance at 260, 280 and 320 nm of your amplified cDNA product. You may need to make a 1:20 dilution of the cDNA in water prior to measuring the absorbance.

3. Purity: Subtract the A320 value from both A260 and A280 values. The adjusted (A260 – A320) / (A280 – A320) ratio should be > 1.8.
4. Yield: Assume 1 absorbance unit at 260 nm of single-stranded DNA = 33 µg/mL.

   To calculate:

   (A260 – A320 of diluted sample) X (dilution factor) X 33 (concentration in µg/mL of a 1 absorbance unit solution) X 0.03 (final volume in mL) = total yield in micrograms
5. Alternatively you may measure the concentration and purity of cDNA with a Nano-drop, using 1 absorbance unit at 260 nm of single-stranded DNA = 33 µg/ mL as the constant.
6. The purified 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.

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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 Magnetic Beads, 2) the QIAGEN MinElute Reaction Cleanup Kit, 3) the QIAGEN QIAquick PCR Purification Kit and 4) the Zymo Clean & Concentrator-25.

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

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

Note: Stop after step 8. on page 18. It is not necessary to recombine the half-reactions or to remove the beads from the SPIA reactions. Begin purification as follows:

1. Obtain and vigorously shake the RNAClean bottle to resuspend the magnetic beads. Allow the bead solution to reach room temperature.

Best results can be obtained by using fresh 80% EtOH in wash step. Lower percent EtOH mixes will reduce 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 144 µL of resuspended RNAClean beads (1.8 times the sample volume) to one set of the paired 80 µL SPIA half-reactions.
4. Mix the sample and beads thoroughly by pipetting up and down 10 times.

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

9. Using a multi-channel pipette, add the sample/bead mix from the second set of half-reactions (prepared in step 7) to the appropriate tubes or wells containing the beads from the first half-reaction while it is still placed on the magnet. Add slowly as to not disturb the bead ring already in each well.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

    Note: Blot column tip prior to transferring it to a new tube to prevent any wash buffer transferring to the eluted sample.
15. Place the MinElute Column in a clean, labeled 1.5 mL microcentrifuge tube.

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Use nuclease-free water at room temperature to elute sample

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

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

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

9. Add 700 µL of 80% ethanol.

    Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.

10. Centrifuge the column for 1 minute at 13,000 rpm. Discard flow-through. Place the column back in the same collection tube.
11. Repeat steps 9 and 10 once.
12. To remove remaining liquid, centrifuge column for 1 additional minute at 13,000 rpm.
13. Remove the column from the centrifuge. Discard the collection tube with the flow-through.
14. Blot the tip of the column onto a filter paper in order to remove any residual wash buffer from the tip of the column.

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Note: Blot column tip prior to transferring it to a new tube to prevent any wash buffer transferring to the eluted sample.

15. Place the column in clean 2.0 mL collection tube, appropriately labeled.
16. Add 30 µL of nuclease-free water (green: D1) to the center of each column.

    Important: Do not use cold water!

17. Let columns stand for 5 minutes at room temperature to elute purified cDNA.
18. Centrifuge at 13,000 rpm for 1 minute to collect sample. There should be approximately 30 µL of purified cDNA.
19. Mix sample by vortexing, then spin briefly.
20. Proceed to Measuring cDNA Product Yield and Purity or store purified cDNA at –20°C.

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

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

Important Note: 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. Use of Cat. #4033/4034 will result in low yields.

1. Into a clean 1.5 mL tube add 320 µL of DNA Binding Buffer.
2. Add 160 µL of amplified SPIA cDNA product.
3. Vortex and spin down briefly.
4. Obtain one Zymo-Spin II Column and place it into a collection tube.
5. Load the entire volume of sample (480 µL) onto the Zymo-Spin II Column.
6. Centrifuge column in the collection tube for 10 seconds at >10,000 X g in a microcentrifuge.

   Note: Be sure to wait until rotor achieves desired speed before starting timer for spins less than 1 minute in this procedure. Discard flow-through.

7. Place the column back in the same collection tube.
8. Wash sample by adding 200 µL of room temperature 80% ethanol. Do not use the Wash Buffer provided with the Zymo columns.

   Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.

9. Centrifuge column in the collection tube for 10 seconds at >10,000 X g in a microcentrifuge. Discard flow-through.
10. Add 200 µL of room temperature 80% ethanol.
11. Centrifuge column in the collection tube for 90 seconds at >10,000 X g in a microcentrifuge. Discard flow-through.

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.

12. Blot the column tip onto a filter paper to remove any residual wash buffer from the tip of the column.

    Note: Blot column tip prior to transferring it to a new tube to prevent any wash buffer transferring to the eluted sample.

13. Place the column in a clean 1.5 mL microcentrifuge tube.

14. Add 30 µL of room temperature nuclease-free water (green: D1) from the kit to the center of the column.

    Important: Do not use cold water!

15. Let column stand for 1 minute at room temperature.

16. Centrifuge column and microcentrifuge tube for 30 seconds at >10,000 X g in a microcentrifuge.

17. Collect sample. There should be approximately 30 µL of purified cDNA.

18. Mix sample by vortexing, then spin briefly.

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

B. Performing Quantitative PCR on Amplified cDNA

It is recommended that the amplified cDNA generated from the Ovation Pico WTA System be purified prior to use in real time quantitative PCR reactions. Since different amplified 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 amplified cDNA produced with the kit has been successfully used as template for qPCR systems, including TaqMan® and SYBR® Green. Note that RT-PCR master mixes containing the enzyme Uracil N-Glycosylase (UNG) are not compatible with the Ovation Pico WTA System.

We can recommend the following reagents for qPCR:

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

Recommendations to Achieve Optimal Results

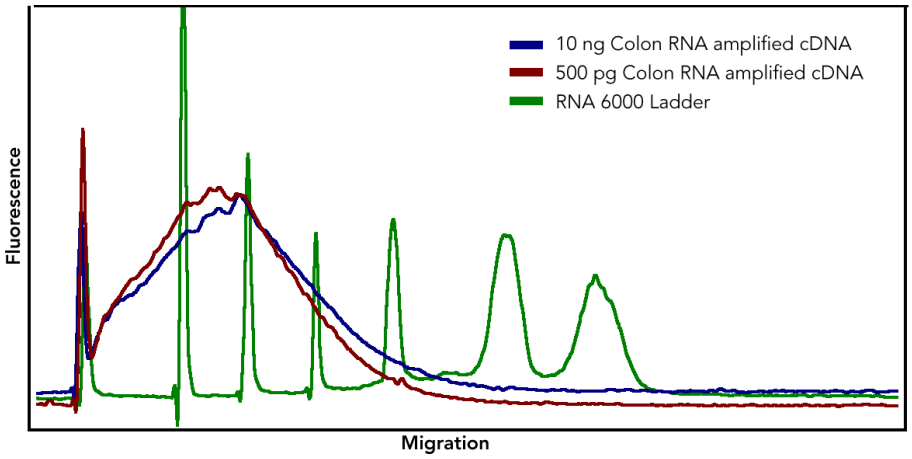
1. **Dilute the Amplified Product**
   After purification and quantitation of amplified cDNA, it can be diluted to an appropriate concentration for qPCR reaction. We recommend using 20 ng of cDNA in a 20 µL TaqMan reaction and 2 ng of cDNA for a 25 µL SYBR Green reaction. Depending on the abundance of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

2. **Primer Design**
   We recommend using primers and probes designed with amplicon sizes of less than 200 nt. Primers may be designed at any position along a transcript since the Ovation Pico WTA System amplification covers the whole transcriptome.

C. Quality Control of Amplified cDNA Product

As a quality control test you may want to analyze the size distribution of the amplified cDNA product using an Agilent Bioanalyzer. Note that the shape of this distribution trace is highly dependent on the input RNA integrity as well as RNA source. We recommend using an RNA 6000 Nano LabChip (Agilent Cat. #5065-4476) and the Eukaryotic Total RNA Nano program (Nano assay in the Expert 2100 software) and following the manufacturer's instructions. Depending on availability of amplified product you may choose to load less than 100 ng of amplified cDNA product on the Bioanalyzer chips. A typical size distribution trace may look like the one obtained from normal colon total RNA, see Figure 4 below.

Figure 4. Bioanalyzer trace of amplified cDNA product obtained from 500 pg and 10 ng of colon RNA input.



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 β-mercaptoethanol according to the type of sample as described in the RNeasy Mini Kit protocol.
2. Add 1X volume of 70% ethanol to the homogenized lysate, 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 Reagent 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 on the bench top (~ 25°C) for 15 min.
10. Add 350 µL Buffer RW1 into the RNeasy mini column, and centrifuge for 15 seconds at ≥ 8000 X g (≥10,000 rpm) to wash. Discard the flow-through.
11. Transfer the RNeasy column to a fresh 2 mL collection tube. Add 500 µL Buffer RPE (with the added ethanol) to the RNeasy column.
12. Close the tube gently, and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
13. Add another 500 µL Buffer RPE to the RNeasy column.
14. Close the tube gently and centrifuge for 2 minutes at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
15. Transfer the RNeasy column to a new 1.5 mL collection tube.
16. Pipet 30 to 50 µL RNase-free water directly onto the RNeasy membrane.

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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 RNA Clean & Concentrator™-5 Columns or the RNeasy MinElute Columns

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

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

Purification with RNA Clean & Concentrator-5 (Zymo Research, Cat. #R1015)

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

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

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

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

   Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
8. Spin for 2 minutes at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
9. Place the RNeasy MinElute Spin Column in a fresh 2 mL collection tube and place in the microcentrifuge with the cap open. Spin for 5 minutes at ≥8000 X g (≥10,000 rpm) and discard the flow-through.
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.

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

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

E. Preventing Crossover Contamination

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

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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, and the second strand product cleanup.
    b. Post-amplification includes all steps and materials related to the handling of the final amplified cDNA product, purification, array hybridization and any other analytical work. This also includes any work and materials related to other non-NuGEN protocols.
    c. Ideally pre-amplification workspace would be in a separate workroom. If this is not possible, ensure the pre-amplification area is sufficiently distant and not in the path of post-amplification work.
    d. We recommend the use of "PCR Workstation" enclosure with UV illumination dedicated for NuGEN pre-amplification protocol.
    e. Materials and consumables for pre-amplification work should be regularly exposed to UV illumination to control nucleic acid surface contamination.
2. Establish and maintain a clean work environment:
    a. Initially clean the entire lab thoroughly with DNA-OFF and RNaseZap.
    b. In the pre-amplification area, remove all small equipment, and then clean every surface that may have been exposed to amplified SPIA cDNA (drawer handles, key pads, etc.). Before reintroducing any equipment, clean every piece of equipment thoroughly. Clean wells of thermal cycler(s) and magnetic plate(s) with a Q-tip or by filling with decontamination solution.
    c. Always wear gloves and don fresh gloves upon entry into this controlled area. Frequently change gloves while working in the pre-amplification area, especially prior to handling stock reagents, reactions and RNA samples.
    d. Stock this area with clean (preferably new) equipment (pipettes, racks, consumables) that has not been exposed to post-amplification workspace.
    e. Make it a policy to carry out continual regular decontamination of all workspaces.
    f. Capture waste generated in both pre- and post-amplification areas (tips, columns, wash solutions from beads and columns, tubes, everything) in sealable plastic bags and dispose of promptly after each experiment to avoid waste spillage.
    g. Do not open amplified product reaction vessels in the pre-amplification workspace.
3. Utilize negative controls in order to detect and troubleshoot any contamination issues. The clearest indication that an amplification reaction is contaminated is the appearance of significant amounts of amplified product in a 'negative' control or No Template Control (NTC).
    a. In the absence of contamination:

i. NTC yields for WT-Ovation FFPE and Ovation Pico WTA System amplifications are typically at or below 3 micrograms.

ii. Products generated from uncontaminated NTC reactions do not yield significant array hybridization even when applied to arrays at standard input amounts.

iii. Bioanalyzer trace of this normal NTC product is very characteristic.

b. In the presence of contamination:

i. NTC yields are generally significantly higher than 3 micrograms, making NTC results the most reliable indicator of contamination.

ii. Contaminated NTC yields can be as high as or even higher than template containing reactions (i.e., your experimental samples or positive controls).

iii. The bioanalyzer traces of contaminated NTC reactions look significantly different than the typical non-contaminated NTC reaction traces.

4. When contamination is detected in reactions containing templates:

a. The amount of product generated from a template containing amplification reaction may or may not be affected, depending on the source of the contamination.

b. The bioanalyzer trace of the amplified product may or may not look altered.

c. The % present calls on arrays run with amplified product generated from a contaminated sample may be lower than expected.

VI. Appendix

F. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the Ovation Pico WTA System?
The Ovation Pico 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 magnetic beads for double-stranded cDNA purification.

Q2. Does the Ovation Pico WTA System provide any fragmentation and labeling reagents?
No. The Ovation Pico WTA System is used to generate single-stranded cDNA from small amounts of total RNA for qPCR analysis or cDNA storage; however, the cDNA output of this kit may be processed further using other validated NuGEN products such as the Encore™ Biotin Module for fragmentation and labeling cDNA for analysis on GeneChip arrays.

Q3. What equipment is required or will be useful?
Required equipment includes a microcentrifuge, pipettes, vortexer, a thermal cycler, a spectrophotometer and a magnetic plate. 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?
RNA samples of high molecular weight with little or no evidence of degradation, as expected, will amplify very well with this product. However, due to the whole transcriptome amplification approach, lower quality RNA samples and transcripts with a compromised poly(A) tail can also be amplified successfully using the Ovation Pico WTA System. The RNA should have high purity, however, and be free of contaminants.

Q6. Can I do reactions in smaller batches than four?
We recommend a minimum batch size of four reactions. Smaller batch sizes may result in difficulty with pipetting small volumes, as well as obtaining fewer than 12 reactions in total.

Q7. Is the Ovation Pico 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 the product to be analyzed on 3' expression arrays when used with an appropriate F&L module, at the same time the random primers allow the detection of the entire transcripts when used as a pre-qPCR amplification system.

Q8. Where in my target sequence can I design my qPCR primers?
The Ovation Pico WTA System does not have a 3' bias and, therefore, primers can be designed at any location within the mRNA. In order to avoid qPCR interference from possible genomic DNA contamination, we recommend treating your RNA with DNase and designing your amplicons to span an intron.

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Q9. How much total RNA do I need for amplification?
We recommend staying within the range of 500 pg to 50 ng total RNA as starting material. Input amounts outside this range may produce unsatisfactory and variable results.

Q10. How much cDNA can I expect from a single reaction?
You should expect 6 to 10 µg of cDNA from input of 500 pg to 50 ng total RNA.

Q11. Is the cDNA yield dependent upon the quantity of total RNA input?
Yes, higher RNA inputs will produce higher yields. However, at inputs of above 50 ng, the yields may become variable without increasing.

Q12. What is the amplification efficiency of the Ovation Pico WTA System?
Based on qPCR data, an average amplification efficiency of 10,000 to 15,000 fold is observed.

Q13. What size cDNA is generated by the Ovation Pico WTA System?
The amplified cDNA size distribution is somewhat dependent 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.

Q14. Can contaminating genomic DNA interfere with the amplification performance?
This system is designed to amplify RNA but large amounts of contaminating genomic DNA may amplify during the process. For this reason we recommend DNase treatment during RNA purification.

Q15. Can I use the Ovation Pico WTA System on bacterial RNA samples?
The Ovation Pico WTA System amplification process has been shown to work with some bacterial RNAs. However, currently, the kit has not been optimized for this purpose.

Q16. Are there any tissues that will not work with the Ovation Pico WTA System?
We have not encountered any specific RNA sources that will not work with the Ovation Pico WTA System. The RNA should have high purity and be free of contaminants.

Q17. Has NuGEN performed reproducibility studies on the Ovation Pico WTA System?
Yes. Sample-to-sample, lot-to-lot and operator-to-operator reproducibility tests are conducted.

Q18. Does the Ovation Pico WTA System generate product in the absence of RNA input?
In the complete absence of input RNA, non-specific product is generated with 1 to 2 µg yields. However, note that in the presence of even very small amounts of RNA, while the yields may be low, the cDNA is likely specific and an actual amplification product.

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Q19. How many rounds of amplification are performed with the Ovation Pico WTA System?
This System has a single round of amplification. It cannot be used for multiple rounds.

Q20. Can I use the Ovation Pico WTA System for archiving cDNA?
Amplified cDNA maybe stored at –20°C for at least six months.

Q21. Do I have to use your DNA/RNA primers, or can I order specific primers for the amplification?
The Ovation Pico WTA System will not work properly with other primers. There is no need to order any primers, as DNA/RNA primers provided in the Ovation Pico WTA System are universal.

Q22. Do you recommend purification of the cDNA prior to qPCR analysis?
Yes. Although this is not absolutely necessary, it is important to be able to quantitate the amplified cDNA. This allows assessment of amplification success based on the amplification yields. It also allows mass normalization of the cDNA into qPCR.

Q23. What purification methods do you recommend?

- For the Second Strand cDNA purification step (pre-amplification) we require the use of the Agencourt RNAClean magnetic beads provided with the kit.
- Several purification options are available for the final SPIA cDNA cleanup step. These are described in Appendix A of this user guide. Selection of the optimum purification option can depend on many factors. Please contact the NuGEN Technical Support team for assistance in selecting the appropriate option for your application. Refer to section II.B. for ordering information.

Q24. How do I measure my amplified cDNA product yield?
You may use a standard spectrophotometer or a Nanodrop. See section IV.J. of this user guide.

Q25. Where can I safely stop in the protocol?
The SPIA cDNA can be stored at -20°C prior to performing the purification. We do not recommend stopping at any intermediate stage of the protocol.

Q26. Do you recommend DNase treatment of my total RNA sample?
Yes. For an explanation of DNase requirements see section III.A.5. You may also find recommended procedures for DNase treatment in Appendix D.

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Q27. How many qPCR reactions will I get from one Ovation Pico 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 you will need to use more cDNA per reaction. The user will need to determine how much cDNA to use per reaction depending on the abundance of the gene being interrogated. Note that we recommend purification of the amplified cDNA prior to qPCR analysis.

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