WHOLE TRANSCRIPTOME WT-OVATION™ SYSTEM

WT-Ovation™ Pico RNA Amplification System

VERSION 1.0

Catalog # 3300-12

USER GUIDE

NuGEN™ *NuGEN Technologies, Inc.* www.nugeninc.com

imagine more from less™

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I. Introduction

A. Background

The WT-Ovation™ Pico RNA Amplification System provides a fast and simple method for preparing amplified cDNA from total RNA 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 WT-Ovation™ Pico System ideal for amplification of partially degraded and compromised RNA samples. The amplified product of the WT-Ovation™ Pico System is optimized for the detection of low, medium and high abundance gene transcripts using real-time quantitative PCR (qPCR). The amplified cDNA generated with the WT-Ovation™ Pico System may also be used as input in the FL-Ovation™ cDNA Biotin Module V2 and fragmented and labeled for hybridization to Affymetrix GeneChip® arrays.

The WT-Ovation™ Pico 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 5 hours. The amplified product is single-stranded cDNA in the antisense (opposite sense) direction of the mRNA starting material.

The WT-Ovation™ Pico RNA Amplification System (Cat. # 3300-12) provides optimized reagent mixes and a protocol to process 12 RNA samples. Control RNA is not provided with the WT-Ovation™ Pico System but we recommend a 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 (65 minutes)

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

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

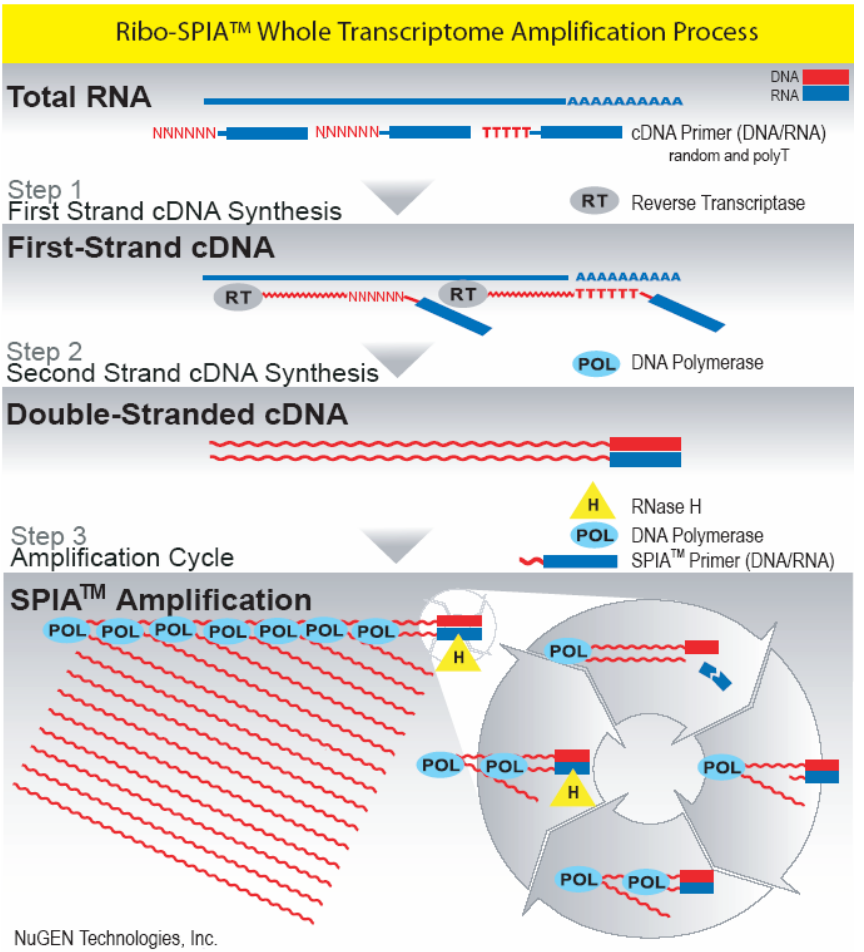
Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthesize a second strand, which includes DNA complementary to the 5′ unique sequence from the first strand chimeric primers. The result is a double stranded cDNA with a unique DNA/RNA heteroduplex at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

3. SPIA™ Amplification (80 minutes)

SPIA™ amplification is a linear isothermal DNA amplification process developed by NuGEN™. It uses a SPIA™ DNA/RNA chimeric primer, DNA polymerase and RNase H in a homogeneous isothermal assay that provides highly efficient amplification of DNA sequences. RNase H is used to degrade RNA in the DNA/RNA heteroduplex at the 5′ end

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

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



C. Performance Specifications

The WT-Ovation™ Pico RNA Amplification System synthesizes microgram quantities of amplified cDNA starting with total cellular RNA input amounts of 500 pg to 50 ng. In approximately 5 hours, the WT-Ovation™ Pico System can produce 6 to 10 µg of cDNA in a 160 µl volume ready for qPCR or other analytical tests. The size of the majority of the cDNA products produced by the Ribo-SPIA™ amplification process is between 50 bases and 1.5 Kb. We recommend using the system with a minimum of 4 reactions at a time to prevent pipetting of very small volumes. Setting up fewer numbers of reactions at a time may result in obtaining less number of reactions.

D. Quality Control

Each WT-Ovation™ Pico System lot is tested to meet specifications of yield, size and performance of the product.

E. Storage and Stability

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

The WT-Ovation™ Pico System is shipped on dry ice and should be unpacked immediately upon receipt. **Note**: this product contains components with multiple storage temperatures.

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**.

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.**

Store the RNAClean® beads 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 6 months. NuGEN has not yet established long-term storage conditions for WT-Ovation™ Pico Systems.

F. Material Safety Data Sheets (MSDS)

MSDS for this product are 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	PART NUMBER	VIAL CAP	VIAL NUMBER
First Strand Primer Mix	S01163	Blue	**A1 ver3**
First Strand Buffer Mix	S01174	Blue	**A2 ver3**
First Strand Enzyme Mix	S01040	Blue	**A3 ver1**

Table 2. Second Strand cDNA Reagents

COMPONENT	PART NUMBER	VIAL CAP	VIAL NUMBER
Second Strand Buffer Mix	S01176	Yellow	**B1 ver3**
Second Strand Enzyme Mix	S01126	Yellow	**B2 ver2**

Table 3. SPIA™ Reagents

COMPONENT	PART NUMBER	VIAL CAP	VIAL NUMBER
SPIA™ Primer Mix	S01162	Red	**C1 ver4**
SPIA™ Buffer Mix	S01164	Red	**C2 ver5**
SPIA™ Enzyme Mix	S01165	Red	**C3 ver5**

Table 4. Additional Reagents

COMPONENT	PART NUMBER	VIAL CAP	VIAL NUMBER
Nuclease-free Water	S01001	Green	**D1**
Agencourt ® RNAClean® Beads	1200-01	Clear	**---**

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

B. Additional Reagents, Supplies and Equipment

Required materials

- **Equipment**
  - Microcentrifuge for individual 1.5 ml and 0.5 ml tubes
  - 0.5 - 10 µl pipette, 2 - 20 µl pipette, 20 - 200 µl pipette, and 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® ND-1000 UV-Vis Spectrophotometer
- **Reagents**
  - Yeast tRNA (Ambion, Cat.# 7119), for bead purification step
  - Ethanol (Sigma-Aldrich, Cat. # E7023), for purification steps
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 ml and 0.5 ml RNase-free microcentrifuge tubes
  - 0.2 ml individual thin wall PCR tubes or 8 x 0.2 ml strip PCR tubes or 0.2 ml thin wall PCR plates
  - Agencourt SPRIPlate™ 96R, Ring Magnet Plate (Agencourt, Cat. # 000219) or the SPRIStand™ Magnetic 6-tube Stand (Agencourt, Cat. # 001139)
  - DNA Clean & Concentrator™-25 (Zymo Research, Cat. # D4005)
  - Disposable gloves
  - Kimwipes
  - Ice bucket

Optional materials

- Agilent 2100 bioanalyzer or materials and equipment for electrophoretic analysis of RNA
- Real Time PCR system
- Decontamination solutions such as RNase*Zap*® and DNA-OFF™

To Order:

- Agencourt Bioscience Corporation, (800) 361-7780, websales@agencourt.com, www.agencourt.com
- Ambion Inc., (800)888-8804, custom@ambion.com , www.ambion.com
- BD Clontech, (877) 232-8995, orders@clontech.com, www.clontech.com/clontech
  * The Nucleospin® Extract I kit is an unlisted item in the BD Clontech catalogue and must be requested by number. Inform the customer service representative that you are using the Extract I kit for working with NuGEN's Ovation™ Amplification System.
- New England BioLabs, 800-632-5227, orderinfo@neb.com, www.neb.com/nebecomm/default.asp
- QIAGEN Inc., (800) 426-8157, customercare-us@qiagen.com, www1.qiagen.com
- Sigma-Aldrich, Inc., (800) 325-3010, www.sigmaaldrich.com
- USB Corporation, 800.321.9322, customerserv@usbweb.com, www.usbweb.com
- Zymo Research, (888) 882-9682, order@zymoresearch.com

C. Input RNA Requirements

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

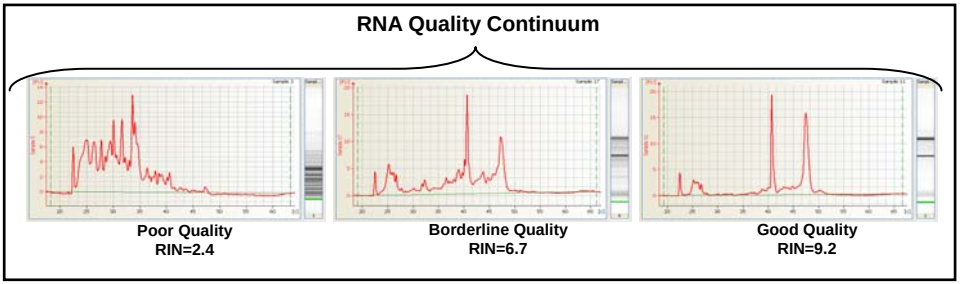
RNA samples must be free of contaminating proteins and other cellular material, organic solvents (including phenol and ethanol) and salts used in many RNA isolation methods. Use of a commercially available system for preparing small amounts of RNA that does not require organic solvents is recommended. 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 WT-Ovation™ Pico RNA Amplification System.

The extent of 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. In our tests using an RNA degradation model system, RNA samples that are severely degraded showing RIN numbers of approximately 2.0 still amplify successfully and reproducibly, given at least 500 pg of input RNA is used for amplification. NuGEN strongly recommends quantitation of total RNA to assure 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 3 different RNAs with varying degrees of quality, all of which have amplified successfully with the WT-Ovation™ Pico RNA Amplification System.



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. NuGEN has developed a tool for assessment of RNA sample suitability for WT-Ovation™ Pico System amplification. The WT-Ovation™ Pico System Technical Report #1 describes this tool 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 the NuGEN's website's Technical Documents page, listed below, or by contacting the NuGEN technical services team at techserv@nugeninc.com or in Europe europe@nugeninc.com
   http://www.nugeninc.com/html/04_technical_resources2.html

5. **DNase Treatment**

   It is generally recommended to use DNase-treated RNA for amplification using the WT-Ovation™ Pico System. One reason is that presence of genomic DNA in the RNA sample may potentially have adverse affects on downstream analytical platforms. Contaminating genomic DNA may also be amplified along with the RNA. Another reason is 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 yeast tRNA during RNA purification, because it has been shown to produce cDNA product in first strand synthesis. We also advise against the use of glycogen in RNA isolation as it inhibits reverse transcription. For the latest information regarding other carriers, contact NuGEN's technical services team.

D. Using RNase-free Techniques

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

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

E. RNA Storage

RNA samples for use with the WT-Ovation™ Pico System must be stored at –80°C. Avoid frequent freeze/thaw cycles or RNA shearing may result.

F. Amplified cDNA Storage

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

III. Protocol

A. Overview

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

1. First strand cDNA synthesis:	**1 hour**
2. Second strand cDNA synthesis and Purification:	**2 hours**
3. SPIA™ Isothermal Linear Amplification and Purification:	**2 hours**
Total time to prepare amplified cDNA	**~5 hours**

WT-Ovation™ Pico 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 WT-Ovation™ Pico System may be used as a method of pre-amplification prior to qPCR. Although for qPCR applications it is not absolutely necessary to purify the amplified cDNA, we recommend proceeding with purification of cDNA immediately after SPIA™ especially if you plan to mass normalize qPCR input. The cDNA must be purified following amplification if you or intend to use the cDNA for fragmentation and labeling using a NuGEN's FL-Ovation™ Module.

B. Protocol Notes

1. The first time you set up an amplification reaction, use a control RNA at an input level well above the minimum recommended range. This allows you to establish a baseline of performance and provides the opportunity to become familiar with the bead purification step. This step is especially prone to handling variability in using the magnet plate so a practice run with the plate is also highly recommended.
2. In working with very small, picogram amounts of RNA we strongly recommend the use of low retention tips and tubes for storage and diluting the samples, in order to reduce the loss of RNA samples due to adhesion to polypropylene surfaces.
3. Set up no fewer than four amplification reactions at a time. This assures sufficient reagent recoveries for 12 total amplifications from a single kit. In addition, setting up a minimum of 4 reactions at a time assures that you are not pipetting very small volumes (see the second strand synthesis section). Making master mixes for fewer than 4 samples at a time may affect reagent recovery volumes and pipetting errors.
4. Thaw components used in each step and immediately place them on ice. Do not thaw all reagents at once!
5. Always keep thawed reagents and reaction tubes on ice unless otherwise instructed.
6. 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.
7. When placing small amounts of reagents into the reaction mix, pipette up and down several times to ensure complete transfer.
8. When instructed to pipette mix, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.

9. Always allow the thermal cycler to reach the initial incubation temperature prior to placing the tubes or plates in the block.
10. When preparing master mixes, use the minimal amount of extra material to ensure 12 reactions in the kit.
11. Components and reagents from other Ovation™ System products should not be used with this product.
12. Use only fresh ethanol stocks to make 70% and 80% ethanol for washes in the cDNA purification protocols. Make the ethanol mixes fresh as well. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the beads or column.

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

Tips and notes:

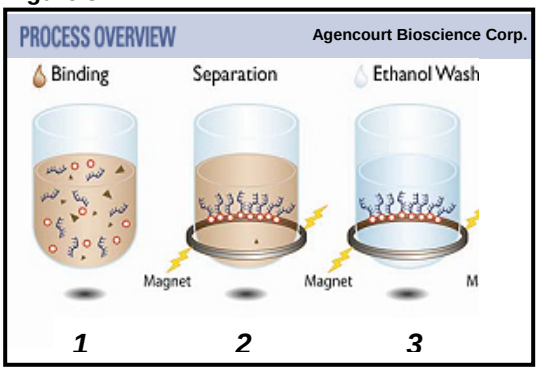
There are significant modifications to the Agencourt® RNAClean® bead's standard procedure; therefore you must follow the procedures outlined in this user guide for the use of these beads with the WT-Ovation™ Pico System. However you may review the Agencourt user guide to become familiar with the manufacturer's 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.



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 than 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 70% ethanol wash is freshly prepared from fresh ethanol stocks. Lower percent ethanol mixes will reduce recovery.
- During the ethanol washes, keep the samples on the magnet. The beads should not be allowed to disperse; the magnet will keep the beads on the walls of the sample wells or tubes in a small ring.
- It is critical that all residual ethanol be removed prior to continuing with the SPIA™ amplification. Therefore, when removing the final ethanol wash, first remove most of the ethanol, then allow the excess to collect at the bottom of the tube before removing the remaining ethanol. This reduces the required bead air drying time.
- After drying the beads for at least 15-20 minutes, inspect each tube carefully and make certain that all the ethanol has evaporated before proceeding with the amplifications step.

D. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 ml tubes, equipped with a heated lid, and with a capacity of 100 µl reaction volume. Prepare the programs shown in Table 5, following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature at 100°C. For thermal cyclers with a fixed temperature heated lid (e.g. ABI GeneAmp® PCR 9600 and 9700 models) use the default settings (typically 100 to 105°C).

Table 5. Thermal Cycler Programming

FIRST STRAND cDNA SYNTHESIS	
Program 1 Primer Annealing	65°C for 2 minutes, then 4°C forever
Program 2 First Strand Synthesis	4°C for 1 minute, 25°C for 10 minutes, 42°C for 10 minutes, 70°C for 15 minutes, then 4°C forever
SECOND STRAND cDNA SYNTHESIS	
Program 3 Second Strand Synthesis	4°C for 1 minute, 25°C for 10 minutes, 50°C for 30 minutes, 70°C for 5 minutes, then 4°C forever
SPIA™ AMPLIFICATION	
Program 4 SPIA™ Amplification	4°C for 1 minute, 47°C for 60 minutes, 95°C for 5 minutes, then 4°C forever

E. First Strand cDNA Synthesis Protocol

1. Obtain First Strand Buffer Mix (blue: A2), First Strand Enzyme Mix (blue: A3), and the water (green: D1) from the components stored at –20°C and the First Strand Primer Mix (blue: A1) stored at –80°C.
2. Flick to mix, then spin down contents of A3 for 2 seconds and place on ice.
3. Thaw the other reagents at room temperature. Mix by vortexing for 2 seconds then spin for 2 seconds and place on ice. Leave water, D1 at room temperature.
4. Add 2 µl of A1 to a 0.2 ml PCR tube.
5. Add 5 µl of total RNA sample (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):
   a. Incubate at 65°C for 2 minutes
   b. Cool to 4°C
8. Remove tubes from the thermal cycler and place tubes on ice.
9. Once Primer Annealing (Step 7) is complete, prepare a master mix by combining A2 and A3 in a 0.5 ml capped tube, according to the volumes shown in Table 6.
   Note: If you intend to run a negative Reverse Transcriptase control, set it up in this step with the addition of A2 and water (D1) and exclude A3.

Flick, do not vortex any enzyme mixes.

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

FIRST STRAND BUFFER MIX (BLUE: A2 VER 3)	FIRST STRAND ENZYME MIX (BLUE: A3 VER1)
2.5 µl	**0.5 µl**

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

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

10. Add 3 µl of the First Strand master mix to each tube.
11. Mix by pipetting three times, spin for 2 seconds.
12. Place tubes in a pre-cooled thermal cycler programmed to run Program 2 (First Strand Synthesis; see Table 5):
    a. Incubate at 4°C for 1 minute
    b. Incubate at 25°C for 10 minutes
    c. Incubate at 42°C for 10 minutes
    d. Heat at 70°C for 15 minutes
    e. Cool to 4°C
13. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
14. Continue immediately with second strand cDNA synthesis.

F. Second Strand cDNA Synthesis Protocol

1. Remove the RNAClean® purification beads from 4°C and place on bench-top to reach room temperature for use in the next step
2. Obtain the Second Strand Buffer Mix (yellow: B1) and Second Strand Enzyme Mix (yellow: B2), from the components stored at –20° C.

3. Flick to mix, then spin down contents of B2 for 2 seconds and place on ice.
4. Thaw reagent B1 at room temperature, and mix by vortexing for 2 seconds, spin for 2 seconds, and then place on ice.
5. Make a master mix by combining B1 and B2 in a 0.5 ml capped tube, according to the volumes shown in Table 7.

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

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

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

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

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

6. Add 10 µl of the Second Strand master mix to each First Strand reaction tube.
7. Mix by pipetting three times, spin for 2 seconds, then place on ice.
8. Place tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand Synthesis; see Table 5):
   a. Incubate at 4°C 1 minute
   b. Incubate at 25°C for 10 minutes
   c. Incubate at 50°C for 30 minutes
   d. Heat at 70°C for 5 minutes
   e. Cool to 4°C
9. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.
10. Continue immediately with purification of unamplified cDNA.

G. Purification of cDNA Protocol

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

1. Ensure the Agencourt® RNAClean® beads have completely reached room temperature before proceeding.
2. Resuspend beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample.
3. After resuspending do not spin the beads. A large excess of beads is provided, therefore it is not neceassay to recover any trapped in the cap.
4. Add 1 µl of 50 ng/µl yeast tRNA carrier to each reaction before adding the beads.
5. At room temperature, add 32 µl, (**1.6 volumes**), bead suspension to each reaction and mix by pipetting up and down 10 times. Incubate at room temperature for 10 minutes.
6. Transfer tubes or plate to magnet plate and let stand 5 minutes to completely clear the solution of beads.
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

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

beads are not removed with the binding buffer or the wash.

8. With the plate still on the magnet, add 200 µl of *freshly prepared* 70% ethanol and allow to stand for 30 seconds. Prepare the 70% ethanol fresh on the day of your experiment.
9. Remove the 70% ethanol wash using a pipette.
10. Repeat the 70% ethanol wash two more times.
    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least 2 pipetting steps to allow excess ethanol to collect at the bottom of the tubes.
11. Air dry the beads on the magnet for a minimum of 15 – 20 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing with the SPIA™ Amplification.
12. Proceed immediately with SPIA™ Amplification with the cDNA still bound to the dry beads.

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

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 reagent C1 and C2 at room temperature, and mix by vortexing for 2 seconds, spin in a microcentrifuge for 2 seconds, then place on ice.
3. Thaw C3 on ice and mix the contents by inverting gently 5 times. *Ensure the enzyme is well mixed without introducing bubbles,* then spin in a microcentrifuge for 2 seconds and place on ice.
4. Make a master mix by sequentially combining C2, C1, and C3 in an appropriately sized capped tube according to the volumes shown in Table 8. Make sure the addition of C3 is at the last moment.

Use SPIA™ Master Mix immediately after preparation.

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

SPIA™ BUFFER MIX (RED:C2 VER5)	SPIA™ PRIMER MIX (RED:C1 VER4)	SPIA™ ENZYME MIX (RED:C3 VER5)
80 µl	**40 µl**	**40 µl**

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

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 8-10 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):
   a. Incubate at 4°C for 1 minute
   b. Incubate at 47°C for 60 minutes
   c. Heat samples to 95°C for 5 minutes
   d. Cool to 4°C
8. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice.

9. Recombine the half-reactions.
10. Transfer tubes or plate 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

We recommend that the amplified SPIA™cDNA product be purified prior to qPCR analysis, especially if the amplified cDNA is intended for use in fragmentation and labeling reactions or needs to be mass normalized for qPCR. The recommended method is using Zymo Research Clean and Concentrator™-25, described below. For alternative purification methods see Appendix B.

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

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

1. 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.
7. Discard flow-through. Place the Zymo-Spin II Column back in the same collection tube.
8. Wash sample by adding 200 µl of room temperature 80% ethanol. Do not use the Wash Buffer provided with the Zymo columns. **Note**: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
9. Centrifuge column in the collection tube for 10 seconds at >10,000 x g in a microcentrifuge. Discard flow-through.
10. Add 200 µl of room temperature 80% ethanol.
11. Centrifuge column in the collection tube for 30 seconds at >10,000 x g in a microcentrifuge. Discard flow-through.
12. Blot the column tip onto a filter paper to remove any residual wash buffer from the tip of the column.

    Note: Blot column tip prior to transferring it to a new tube to prevent any wash buffer transferring to the eluted sample.
13. Place the Zymo-Spin II Column in a clean 1.5 ml microcentrifuge tube.

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

14. Add 35 µl of room temperature nuclease-free water (green: D1) from the kit to the center of each Zymo-Spin II column. Do not use cold water!

    Note: For small RNA input amplifications in the range of 500 pg, it is recommended to use 30 µl of water to keep concentrations higher.
15. Let columns stand for 1 minute at room temperature.
16. Centrifuge column and microcentrifuge tube for 30 seconds at >10,000 x g in a microcentrifuge.
17. Collect sample. There should be approximately 30-35 µl of purified cDNA.
18. Mix sample by vortexing, then spin briefly.
19. Proceed to Measuring cDNA Product Yield and Purity step.

J. Measuring cDNA Product Yield and Purity

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

IV. Technical Support

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

In Europe contact NuGEN at +31(0)135780215 (Phone) or +31(0)135780216 (Fax) or email europe@nugeninc.com.

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

V. Appendix

A. Performing Quantitative PCR on Amplified cDNA

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

NuGEN can recommend the following reagents for qPCR:

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

1. Recommendations to Achieve Optimal Results

a. 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.

b. 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 WT-Ovation ™ amplification covers the whole transcriptome.

B. Alternative Purification Protocols for Amplified cDNA

Although the recommended SPIA™cDNA purification method is using the Zymo Research Clean and Concentrator™-25, a number of other alternative methods may also be used and are listed below.

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

NucleoSpin® Extract I Kit (instructions for a single reaction)

1. Pipette 40 µl 1 x TE (pH 7-7.5) into a clean 1.5 ml tube, then pipette in 800 µl of Buffer NT2 into the tube.
2. Add the 160 µl of amplified cDNA product to the tube.
3. Vortex for five seconds and spin down for two seconds.
4. Insert the NucleoSpin® Extract Spin Column into a 2 ml collection tube. One column is sufficient for each sample.
5. Load 500 µl of sample onto column. Centrifuge at 11,000 x g for 1 minute at room temperature. Discard flow-through.

6. Reinsert the column in the 2 ml collection tube. Load the remaining 500 µl of sample onto the same column. Centrifuge at 11,000 x g for 1 minute at room temperature. Discard flow-through.
7. Reinsert the NucleoSpin® Extract I column back in the 2 ml collection tube and add 600 µl room temperature 80% ethanol. **Note**: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
8. Centrifuge at 11,000 x g for 2 minutes to remove 80% ethanol from the membrane filter. Discard flow-through.
9. Put the column back in the 2 ml collection tube and add 200 µl room temperature 80% ethanol. Centrifuge at 11,000 x g for 2 minutes.
10. Remove the NucleoSpin® Extract I column from the centrifuge. Discard flow-through along with the collection tube.
11. Blot the column tip onto a filter paper to remove any residual wash buffer from the tip of the column.
    Note: Blotting of the column tip **MUST** be done prior to transferring the column to a clean 1.5 ml microcentrifuge tube. Failure to do so may result in a small quantity of wash buffer in your final eluted sample.

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

12. Place the NucleoSpin® Extract I column in a clean 1.5 ml microcentrifuge tube.
13. Add 30 µl of nuclease-free water (green: D1) to the center of each column and incubate at room temperature for 1 minute to elute purified cDNA SPIA™ product. ***Do not use cold water!***
14. Centrifuge at 11,000 x g for 1 minute to collect sample. There should be approximately 30 µl of purified cDNA.
15. Mix sample by vortexing, then spin briefly.
16. Proceed to Measuring cDNA Product Yield and Purity step.

QIAquick® Purification Kit (instructions for a single reaction)

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

1. Into a clean 1.5 ml tube add 800 µl of PB buffer from the QIAGEN system.
2. Add the 160 µl of amplified cDNA product to the tube.
3. Vortex for five seconds and spin down for two seconds.
4. Obtain one QIAquick® spin column and insert into a collection tube.
5. Load 480 µl of sample onto the column.
6. Centrifuge column in a collection tube for one minute at 13,000 rpm.
7. Discard flow-through. Place the column back in the same collection tube.
8. Load remaining 480 µl onto the same column. Centrifuge column in collection tube for one minute at 13,000 rpm. Discard flow-through.
9. Place the column back in the same collection tube. Add 700 µl of 80% ethanol.
   Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
10. Centrifuge the column for one minute at 13,000 rpm. Discard flow-through.
11. Repeat steps 9 and 10 once.
12. To remove remaining liquid, centrifuge column for one additional minute at 13,000 rpm.
13. Remove the column from the centrifuge. Discard flow-through along with the collection tube.
14. Blot the tip of the column onto a filter paper in order to remove any residual wash buffer from the tip of the column.
    Note: Blot column tip prior to transferring it to a new tube to prevent any wash buffer transferring to the eluted sample.
15. Place the column in 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 five minutes at room temperature to elute purified cDNA.
18. Centrifuge at 13,000 rpm for one minute to collect sample. There should be approximately 30 µl of purified cDNA.
19. Mix sample by vortexing, then spin briefly.
20. Proceed to Measuring cDNA Product Yield and Purity step.

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

- When using individual PCR tubes for processing cDNA, use the SPRIStand™ magnet and follow the procedure as outlined below.
  1. Obtain and vigorously shake the AMPure® bottle to resuspend the magnetic beads.
  2. Place the 160 µl cDNA into a clean 1.5 ml tube.
  3. Add 288 µl of resuspended AMPure® beads to the 160 µl cDNA sample.
  4. Mix the sample and beads thoroughly by pipetting up and down 10 times.
  5. Incubate samples at room temperature for 5 minutes.
  6. Place the 1.5 ml tube on the SPRIStand magnet for 10 minutes or until the solution appears clear.
  7. Proceed with step 11 as outlined below while using the SPRIStand magnet instead of the SPRIPlate 96R magnet.

- When using strip tubes or PCR plates for processing cDNA, use the SPRIPlate™ 96R magnet and follow the procedure as outlined below.
  1. Obtain and vigorously shake the AMPure® bottle to resuspend the magnetic beads.
  2. Stop at step 9 of section IV.H, and do not combine the SPIA™cDNA 80-µl half reactions.
  3. To each of the 80-µl SPIA™cDNA reactions add 144 µl of resuspended AMPure® beads (1.8 times the sample volume).
  4. Mix the sample and beads thoroughly by pipetting up and down 10 times.
  5. Incubate samples at room temperature for 5 minutes.
  6. Place the sample plate (or strip) on the SPRIPlate 96R magnet for 10 minutes or until the solution appears clear.
     Note: If the surface tension traps a small halo of beads suspended at the liquid surface, use a multi-channel pipette and gently pipette 10-15 µl up and down at the liquid surface to break the tension and allow the beads to sink to the magnet ring.
  7. Using a multi-channel pipette, remove and discard the supernatant. Do not disturb the ring of magnetic beads.
  8. Add the sample-bead mix from the second plate to the appropriate columns of the first plate while it is still placed on the SPRIPlate® 96R magnet. Add slowly as to not disturb the bead ring already in each well.
     Note: During this step the potential for sample cross contamination is high; take care in pipetting the correct columns.
  9. Allow the sample plate (or strip) to stand on the magnet for 10 minutes or until the solution appears clear.
  10. Using a multi-channel pipette, remove and discard the supernatant. Do not disturb the ring of magnetic beads.
  11. With the reaction plate (or strip) still on the magnet, add 200 µl of freshly prepared 70% 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.

Best results can be obtained by using fresh 70% Ethanol in the wash step. Lower percent Ethanol mixes will reduce recovery.

12. Using a multi-channel pipette remove and discard the ethanol.
13. Repeat the 70% ethanol wash. Ensure all ethanol is removed from the bottom of the plate. **Note**: if the surface tension traps a small halo of beads suspended at the liquid surface, use a multi-channel pipette and gently pipette 10-15 µl up and down at the liquid surface to break the tension and allow the beads to sink to the magnet ring.
14. Air dry the samples on bench top off the SPRIPlate 96R magnet for 10-20 minutes, ensure the plate is completely dry before proceeding. Remove any remaining ethanol carefully using a pipette.

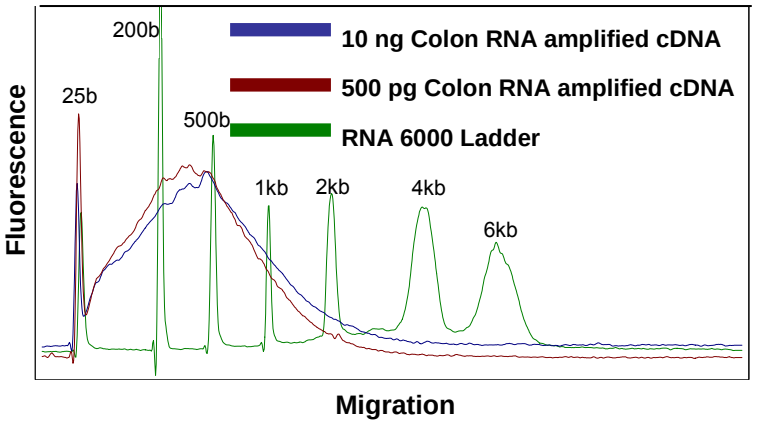
Use nuclease-free water at room temperature to elute sample.

15. With the samples still on bench top, add 30 µl of the RNase-free Water (D1, provided with the Ovation kit) to each well. Holding the plate firmly, vortex for 30 seconds or use a plate shaker set to medium speed. Ensure the beads are fully wetted, vortex longer if necessary. ***Do not use cold water!***
16. Replace reaction plate (or strip) back on the SPRIPlate 96R magnet; allow the beads to separate for 5 minutes or until the solution clears.
17. Using a multi-channel pipette remove the eluted sample and place into a fresh plate. **Note**: Small amounts of magnetic bead carry-over may interfere with sample quantitation take care to minimize bead carry-over.
18. 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 step.

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 chose to load lower than 100 ng of amplified cDNA product on the Bioanalyzer chips. A typical size distribution trace may look like the one obtained from Colon Normal Adjacent (NAT) Total RNA, see Figure 4 below.

Figure 4. Bioanalyzer Trace of Amplified cDNA Product obtained from 500 pg and 10 ng of total NAT RNA input.



D. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the WT-Ovation™ Pico RNA Amplification System?

The WT-Ovation™ Pico System provides all necessary buffers, primers and enzymes for first strand synthesis, second strand synthesis and amplification, yielding single stranded amplified cDNA. The kit also provides nuclease-free water and Agencourt RNAClean® magnetic beads for double stranded cDNA purification.

Q2. Does the WT-Ovation™ Pico RNA Amplification System provide any fragmentation and labeling reagents?

No. The WT-Ovation™ Pico 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 FL-Ovation™ cDNA Biotin Module V2 for fragmentation and labeling cDNA and analyze it on GeneChip® arrays.

Q3. What equipment is required or will be useful?

Required equipment includes a microcentrifuge, pipettes, vortexer, a thermal cycler, and a spectrophotometer, and a magnetic tube stand or 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 are required. See user guide for validated purification products and procedures.

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 can also be amplified successfully using the WT-Ovation™ Pico RNA Amplification System. The RNA should have high purity however and be free of contaminants.

Q6. Can I do reactions in smaller batches than 4?

We recommend 3 batches of 4 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 WT-Ovation™ Pico System 3 prime 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 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 WT-Ovation™ system does not have a 3 prime 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.

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 starting material.

Q11. Is the cDNA yield dependent upon the quantity of total RNA input?
Yes, the more RNA is put into the amplification reaction, the more yield is recovered. However at inputs of above 50 ng, the yields may become variable without increasing.

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

Q13. What size cDNA is generated by the WT-Ovation™ Pico System?
On a Bioanalyzer, using the RNA 6000 size markers, the median length of the amplified cDNA is approximately 340 bases. More than 50% of the product is greater than 320 bases in length.

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

Q15. Can contaminating genomic DNA interfere with the WT-Ovation™ Pico System performance?
This system is designed to amplify RNA but large amount of contaminating genomic DNA may amplify during the process. For this reason we recommend DNase treatment during RNA purification.

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

Q17. Are there any tissues that will not work with the WT-Ovation™ Pico System?
We have not encountered any specific RNA sources that will not work with the Ovation™ System. The RNA should have high purity and be free of contaminants.

Q18. Has NuGEN performed reproducibility studies on the WT-Ovation™ Pico System?
Yes. Sample to sample, lot to lot, and operator to operator reproducibility studies are routinely conducted.

Q19. Does the Ovation™ System generate product in the absence of RNA input?
In the complete absence of input RNA non-specific product is generated with 1-2 µg yields. However, note that in the presence of even very small amount of RNA, while the yields may be low, the cDNA is likely specific and an actual amplification product.

Q20. How many rounds of amplification are performed with the WT-Ovation™ Pico System?
This System performs a single round of amplification and can not be used for multiple rounds of amplification.

Q21. Can I use the WT-Ovation™ Pico System for archiving cDNA?
Amplified cDNA maybe safely stored at -20°C for 6 months. Long term stability tests are in progress.

Q22. Do I need to order specific primers for the amplification?
No. The DNA/RNA primers provided in the WT-Ovation™ Pico System are universal.

Q23. Do I have to use your DNA/RNA primers?
The WT-Ovation™ Pico System will not work properly with other primers.

Q24. What are the incubation temperatures for each step?
First strand primer annealing = 65°C
First strand synthesis = 4°C, 25°C, 42°C, and 70°C
Second strand synthesis = 4°C, 25°C, 50°C, and 70°C
SPIA™ amplification = 4°C, 47°C, and 95°C

Q25. Do you recommend purification of the cDNA prior to qPCR analysis?
Yes. Although this is not typically necessary, it is important to be able to quantitate the amplified cDNA. This allows assessment of the amplification success based on the yields obtained, it also allows mass normalization of the cDNA into qPCR.

Q26. What purification methods do you recommend?
- For the double stranded cDNA purification step (pre-amplification) we require the use of the Agencourt RNAClean® magnetic beads provided with the kit.
- For the amplified cDNA purification step we highly recommend using the Zymo Research DNA Clean & Concentrator™-25. Alternative methods have also been validated and described in Appendix B.
- See the Additional, Reagents, Supplies, and Equipment section of this user guide for order information and Appendix C for procedures.

Q27. How do I measure my amplified cDNA product?
You may use a standard spectrophotometer or a Nanodrop. See section **IV. J.** of this user guide for procedural details.

Q28. Where can I safely stop in the protocol?
We do not recommend stopping at any intermediate stage of the protocol.

Q29. Do you recommend DNase treatment of my total RNA sample?
Yes. For an explanation of DNase requirements see section **III.A.4.**.

Q30. How many qPCR reactions will I get from one WT-Ovation™ Pico 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.

NuGEN Technologies, Inc.
Headquarters USA
821 Industrial Road Unit A, San Carlos, CA 94070 USA, Toll Free Tel: 888.654.6544, Toll Free Fax: 888.296.6544
www.nugeninc.com custserv@nugeninc.com techserv@nugeninc.com

Canada
MJS BioLynx Inc.
P.O Bag 1150, 300 Laurier Blvd.
Brockville, ON K6V 5W1
Canada
Toll Free: 1-888-593-5969
Tel: +1-613-498-2126
Fax: +1-613-342-1341
sales@biolynx.ca or tech@biolynx.ca
www.biolynx.ca/contact-biolynx.html

Europe
NuGEN Technologies, Inc.
P.O. Box 149,
6680 AC Bemmel
The Netherlands
Tel: +31-13-5780215
Fax: +31-13-5780216
europe@nugeninc.com
www.nugeninc.com

Asia
MediBIC.
Daido Seimei Kasumigaseki
Building 8F, 1-4-2
Kasumigaseki, Chiyoda-ku,
Tokyo 100-0013,
Japan
Tel: +81-3-5510-2313
Fax: +81-3-5510-2312
info@medibic.com
www.medibic.com

Australia
Integrated Sciences Pty. Ltd.
2 McCabe Place
PO Box 731
Willoughby NSW 2068
Australia
Tel: +61-02-9417-7866 or
1 800-252-204 (Australia only)
Fax: +61-02-9417-5066
tech@integratedsci.com.au
www.integratedsci.com.au/contactus.asp

Israel
ZOTAL Biologicals
& Instrumentation
4 Habarzel Street
Tel Aviv 69710,
Israel
Tel: +972-3-6492444
Fax: +972-3-6496664
sales@zotal.co.il
www.zotal.co.il

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