USER GUIDE

Crescendo cDNA™ Synthesis for qPCR

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SYMBOL	MEANING
	Catalog Number
	Consult instructions for use
	Contains sufficient for <n> tests
	Warning
	Important
	Expiration Date
	Temperature limitation
	Optional stopping point
	Note
	Manufacturer

Table of Contents

I. Introduction

A. Overview

Intended Use

The Crescendo cDNA Synthesis for qPCR kit provides a fast and simple method of generating amplified cDNA from total RNA for use in qPCR applications. Crescendo cDNA Synthesis is intended for Research Use Only and not for use in diagnostic procedures.

Features

The Crescendo cDNA Synthesis kit is powered by Ribo-SPIA® technology. Ribo-SPIA is a robust isothermal strand-displacement amplification process that uses a DNA/RNA chimeric SPIA primer, DNA polymerase and RNase H in a homogeneous isothermal assay that provides highly efficient amplification of DNA sequences. Using Ribo-SPIA technology, significant quantities of cDNA can be prepared starting with as little as 500 pg total RNA. Reduced amplification yield may be experienced when using RNA inputs below 500 pg or with lower quality RNA samples.

Specifications

Input type:	Total RNA
Input amount:	500 pg – 50 ng
# Reactions available:	16, 64

B. Storage and Stability

The Crescendo cDNA Synthesis kit is shipped on dry ice and should be unpacked immediately upon receipt.

This product contains components with multiple storage temperature requirements. All shipments should be unpacked immediately upon receipt and stored as directed below.

- The vial labeled First Strand Enzyme Mix (blue: A3), should be removed from the kit box upon delivery and stored separately at –80 °C.

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

This product has been tested to perform to specifications after as many as six freeze/thaw cycles. Kits handled and stored according to the above guidelines are warranted to perform to specifications for 6 months from the date of shipment. Expiration dates listed on the kit label may be longer than the warranty period. Do not use kits that have passed the expiration date.

C. Warnings and Precautions

1. Unpack and inspect the kits immediately upon receiving. In case of severe kit package damage, no dry ice left in the package or ice pack melted, and/or missing components, please contact Tecan NGS Technical Support immediately. Please provide the kit(s) and/or component(s) part number, and lot number information. Do not use damaged components.
2. Follow your institution's safety procedures for working with chemicals and handling of biological samples. Follow good laboratory practices and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary. Changing gloves between handling samples is recommended to avoid contamination of sample or reagents.

3. Consult your institution's environmental waste personnel on proper disposal of unused reagents. Check state and local regulations as they may differ from federal disposal regulations. This material may exhibit characteristics of hazardous waste requiring specific disposal requirements. Institutions should check their country hazardous waste disposal requirements.
4. If appropriate, an SDS for this product is available on the Tecan Genomics website at https://www.nugen.com/products/crescendo-cdna-synthesis-qpcr

D. Before You Start

Please review this User Guide before using this kit for the first time, including the "Components", "Planning the Experiment", "Protocol" and "FAQ" sections. For more information, visit the Crescendo cDNA Synthesis page at https://www.nugen.com/products/crescendo-cdna-synthesis-qpcr

New to NGS? Contact Tecan NGS Technical Support at techserv-gn@tecan.com for tips and tricks on getting started.

A. Reagents Provided

This kit is provided in a 16 (-16) or 64 (-64) reaction format.

Table 1. Crescendo cDNA Synthesis (Part No. 30183903, 30183905)

COMPONENT	30183903-16 PART NUMBER	30183905-64 PART NUMBER	VIAL LABEL	VIAL NUMBER
First Strand Primer Mix	S01278	S01373	Blue	A1 VER 4
First Strand Buffer Mix	S01174	S01374	Blue	A2 VER 3
First Strand Enzyme Mix	S02250	S02269	Blue	A3 VER 7
Second Strand Buffer Mix	S01176	S01376	Yellow	B1 VER 3
Second Strand Enzyme Mix	S01126	S01377	Yellow	B2 VER 2
SPIA Primer Mix	S01279	S01378	Red	C1 VER 9
SPIA Buffer Mix	S01410	S01412	Red	C2 VER 11
SPIA Enzyme Mix	S01411	S01413	Red	C3 VER 7
Nuclease-free Water	S01001	S01001	Green	D1

Note: The reagents in the Crescendo cDNA Synthesis kit are similar to reagents in our other kits; however, unless the component part numbers are identical, these reagents do not have exactly the same composition and, therefore, are not interchangeable. Do not exchange or replace one reagent named, for example, A1 with another A1, as it will adversely affect performance.

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - 0.5–10 µL pipette, 2–20 µL pipette, 20–200 µL pipette, 200–1000 µL pipette
  - Vortexer
  - Thermal cycler with 0.2 mL tube heat block, heated lid, and 100 µL reaction capacity
  - Appropriate spectrophotometer and cuvettes, or Nanodrop® UV-Vis spectrophotometer
- **Reagents**
  - Ethanol, Absolute (200 Proof), Molecular Biology Grade (Fisher Scientific Cat. #BP2818), for purification steps
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Fisher Scientific, Cat. #75793), for diluting nucleic acids
  - Agencourt® AMPure XP Beads (Beckman Coulter, Cat. # A63880 or A63881)

- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - Low-retention microcentrifuge tubes (DNA LoBind Tubes, Eppendorf Cat.# 022431005 or 022431021)
  - 0.2 mL PCR strip tubes or 0.2 mL thin-wall PCR plates
  - Magnetic stand for 0.2 mL strip tubes or plates. (Beckman Coulter Cat. #A29164 or A32782; Thermo Fisher Scientific Cat. #12331D, 12027, or 12332D; Promega Cat. #V8351). Other magnetic stands may be used as well, although their performance has not been validated by Tecan.
  - Cleaning solutions such as RNaseZap® (Thermo Fisher Scientific, Cat. #AM9780) and DNA-OFF™ (MP Biomedicals, Cat. #QD0500)
  - Disposable gloves
  - Kimwipes
  - Ice bucket

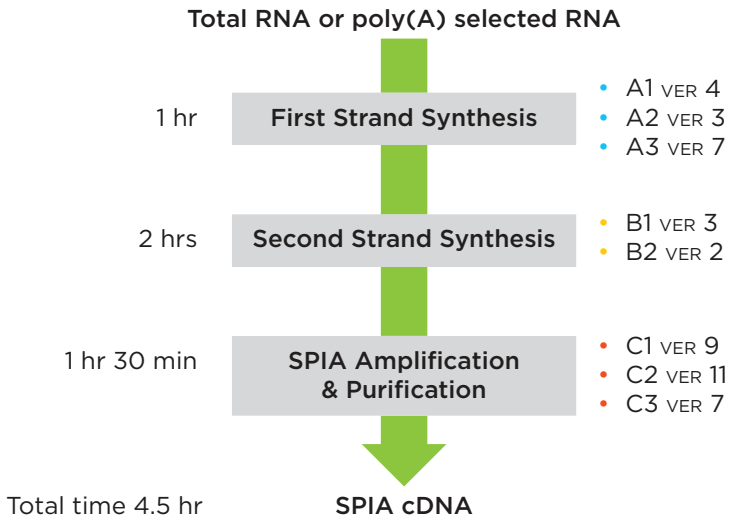
To Order:

- Beckman Coulter, www.beckmancoulter.com
- Eppendorf, www.eppendorf.com
- MP Biomedicals, www.mpbio.com
- QIAGEN Inc., www.qiagen.com
- Thermo Fisher Scientific, www.thermofisher.com

A. Workflow and Time Required

The Crescendo cDNA Synthesis workflow can be completed in approximately 4.5 hours. Double-stranded cDNA is generated followed by SPIA amplification to produce microgram quantities of cDNA when used with intact total RNA. The size of the cDNA products produced by the Crescendo cDNA Synthesis kit is typically between 200 base pairs (bp) and 1,500 bp.

Figure 1. Crescendo cDNA Synthesis Time and Workflow.



Important: For this kit, we recommend protocol sections **A (Sample Preparation) through E, step #6 (SPIA Amplification)** be performed in a pre-amplification workspace using dedicated pre-amplification consumables and equipment. 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 Tecan NGS Technical Support (techserv-gn@tecan.com).

B. Input RNA Requirements

RNA Quantity

Total RNA input must be between 500 pg and 50 ng. We strongly recommend quantitation of total RNA to assure the minimum input requirement is met. Please see the FAQ section for further details.

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. If a method such as TRIzol is used, we recommend using a column purification after isolation.

One measure of RNA purity is the ratio of absorbance readings. The A260:A280 ratio for RNA samples should be greater than 1.8 and A260:A230 should be greater than 2.0.

RNA Integrity

RNA samples of high molecular weight with little or no evidence of degradation will amplify very well with this product. Lower quality RNA samples may also be amplified successfully. Use of degraded RNA samples can lead to lower yields and shorter SPIA cDNA. It is impossible to guarantee success with all degraded RNA samples.

RNA integrity can be determined using the Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip® or RNA 6000 Pico LabChip.

DNase Treatment

It is generally recommended to use DNase-treated RNA for amplification using the Crescendo cDNA Synthesis kit. Contaminating genomic DNA may be amplified along with the RNA and may potentially have adverse effects on downstream analytical platforms. Additionally, if the total RNA sample contains a significant amount of contaminating genomic DNA, it may be difficult to accurately quantify the true RNA concentration. The RNA input quantity may be overestimated based on an absorbance measurement resulting in inappropriate input into the kit.

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. Contact Tecan NGS technical support at techserv-gn@tecan.com for more information.

C. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes, equipped with a heated lid and a capacity of 100 µL reaction volume. Prepare the programs shown in Table 2, 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 use the default settings (typically 100 to 105 °C).

Table 2. Thermal Cycler Programming

FIRST STRAND cDNA SYNTHESIS		VOLUME
Program 1 Primer Annealing	For RNA inputs ≤1 ng: 65 °C – 2 min, hold at 4 °C For RNA inputs >1 ng: 65 °C – 5 min, hold at 4 °C	3.5 µL
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	5 µL
SECOND STRAND cDNA SYNTHESIS		
Program 3 Second Strand Synthesis	4 °C – 1 min, 25 °C – 10 min, 50 °C – 30 min, 80 °C – 20 min, hold at 4 °C	10 µL
SPIA AMPLIFICATION		
Program 4 SPIA Amplification	4 °C – 1 min, 47 °C – 60 min, 80 °C – 20 min, hold at 4 °C	20 µL

D. Bead Purification

Agencourt Beads

Ampure XP or RNA Clean XP Beads (Agencourt beads) are suitable for use with this kit. There are modifications to the Agencourt beads' standard procedure; therefore, you must follow the protocols outlined in this user guide for the use of these beads.

- Remove beads from 4 °C and leave at room temperature for at least 30 minutes prior to use. Cold beads and buffer will result in reduced recovery.
- Prior to use, ensure beads are fully resuspended by vortexing or inverting and tapping the tube.
- Note that we recommend specific sample to bead volume ratios in our Agencourt bead protocols. In many cases our recommendations differ from the standard Beckman Coulter protocol.
- If using strip tubes or partial plates, ensure they are firmly placed on the magnetic plate. The use of individual tubes is not advised as they are not very stable on the magnetic plates.
- It is critical to let the beads separate on the magnet for the full time indicated at each step. Removing the binding buffer before the beads have completely separated will impact yields.
- After the binding step has been completed, take care to minimize bead loss when removing the binding buffer. Loss of beads at this step may impact yields. With the samples placed on the magnetic plate, carefully remove the specified quantity of binding buffer from each sample to avoid disturbing the beads.
- Ensure that the 70% ethanol wash is freshly prepared from fresh ethanol stocks. Lower percent ethanol mixes will reduce recovery.
- It is critical that all residual ethanol be removed prior to elution. 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 also reduces the required bead air-drying time.

- After drying the beads, inspect each tube carefully and make certain that all the ethanol has evaporated before proceeding with the amplification step.

Preparation of EtOH Wash Solution

Prepare a 70% or 80% EtOH wash solution by combining 100% EtOH and nuclease-free water. Make the ethanol mixes fresh, carefully measuring both the ethanol and water with pipettes. This protocol requires 0.6 mL of 70% EtOH for section **D. cDNA Purification** and 0.4 mL of 80% EtOH for **F. Purification of SPIA cDNA** per sample.

Important:
- It is critical that the ethanol solution in the purification steps 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 acticipated aqueous content, which may reduce amplification yield.

EtOH SOLUTION	1X REACTION VOLUME*	100% EtOH	NUCLEASE-FREE WATER
70% EtOH	0.6 mL	0.42 mL	0.18 mL
80% EtOH	0.4 mL	0.32 mL	0.08 mL

*A minimum of 10% extra volume should be prepared for each sample.

E. SPIA cDNA Storage

The amplified SPIA cDNA produced by the Crescendo cDNA Synthesis kit may be stored at –20 °C.

Protocol Notes

Controls

- We recommend the routine use of a positive control RNA, especially the first time an amplification reaction is set up. 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.
- Routine use of a low-template control (LTC) is recommended to monitor the work environment for non-specific amplification issues. Avoid running negative controls (i.e., no RNA input reactions). The clearest indication that non-specific amplification is taking place is the appearance of higher than expected yields or irregular bioanalyzer traces in a low template control (LTC) reaction.

General Workflow

- When working with very small, picogram amounts of RNA we strongly recommend the use of low retention tubes for storage and dilution of the samples in order to reduce the loss of RNA samples due to adhesion to polypropylene surfaces.
- Set up no fewer than 8 reactions at a time for the 16-reaction kit and no fewer than 16 reactions at a time for larger kit to ensure that you are not pipetting very small volumes and to ensure sufficient reagent recoveries for the full nominal number of samples from the kit. Making master mixes for fewer than 8 samples at a time may affect reagent recovery volumes.
- Due to the high sensitivity inherent in this amplification system, we strongly recommend taking measures to minimize the potential for the carryover of previously amplified SPIA cDNA into new amplification reactions. The two steps to accomplish this are: 1. Designating separate workspaces for “pre-amplification” and “post-amplification” steps and materials, and 2. Implementing routine clean-up protocols for workspaces as standard operating procedure. A detailed set of these recommendations is listed in **Appendix E**.
- Thaw components used in each step and immediately place them on ice. Always keep thawed reagents and reaction tubes on ice unless otherwise instructed. Do not thaw all reagents at once.
- After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve the precipitate completely prior to use. You may gently warm the buffer mix for 2 minutes at room temperature followed by brief vortexing.
- Keep enzyme mixes on ice after briefly spinning to collect the contents. Do not vortex enzyme mixes nor warm any enzyme, adaptor or primer mixes.
- When preparing master mixes, use the minimal amount of extra material to ensure you are able to run the maximum number of reactions using the components provided in the kit.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer from the pipet tip to the reaction mix.
- When instructed to mix via pipetting, 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 or plates in the block.

Reagents

- 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.
- Components and reagents from other Tecan products should not be used with this product.
- Use only fresh ethanol stocks to make ethanol for washes in the purification protocols.
- Make the ethanol mixes fresh, 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.

For each section of the protocol, remove reagents from recommended storage conditions listed on page 1. Thaw and place reagents at room temperature or on ice as instructed. After each section, continue immediately to the next section of the protocol unless otherwise directed.

Return all reagents to their appropriate storage conditions promptly after use unless otherwise instructed.

Note: A minimum batch size of 8 reactions is recommended to ensure that you are not pipetting very small volumes and to ensure sufficient reagent recoveries for the full nominal number of samples from the kit.

A. Sample Preparation

1. Remove Nuclease-free water (Green: D1) from –20 °C and place at room temperature.
2. Aliquot each total RNA input sample (500 pg – 50 ng) into a 0.2 mL strip tube or 96-well plate.
3. Bring final volume of RNA up to 2.5 µL with D1 if necessary and place on ice.

Note: Keep D1 at room temperature for use in subsequent sections.

B. First Strand cDNA Synthesis

1. Obtain the First Strand Primer Mix (blue: A1) from –20 °C storage.

Table 3. First Strand Master Mix

REAGENT	FIRST STRAND BUFFER MIX (BLUE: A2 VER 3)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 7)
1X REACTION VOLUME	1.25 µL	0.25 µL

2. Spin down the contents of A3 and place on ice.
3. Thaw A1 and A2 at room temperature. Mix by vortexing, spin down and place on ice.
4. Add 1 µL of A1 to each 2.5 µL of input RNA for a total of 3.5 µL. Mix by pipetting 5 times, spin down and place on ice.
5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 2):

   For RNA inputs of 500 pg to 1 ng: 65 °C – 2 min, hold at 4 °C

   For RNA inputs > 1 ng: 65 °C – 5 min, hold at 4 °C
6. Remove the tubes from the thermal cycler, spin and place on ice.
7. Once Primer Annealing (Step 5) 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 3.

8. Add 1.5 µL of First Strand Master Mix to each sample tube for a total of 5 µL. Mix by pipetting 5 times, spin and place on ice.
9. Place the tubes in a pre-cooled thermal cycler programmed to run Program 2 (First Strand Synthesis; see Table 2):

   4 °C – 1 min, 25 °C – 10 min, 42 °C – 10 min, 70 °C – 15 min, hold at 4 °C
10. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

C. Second Strand cDNA Synthesis

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

Table 4. Second Strand Master Mix

REAGENT	SECOND STRAND BUFFER MIX (YELLOW: B1 VER 3)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER 2)
1X REACTION VOLUME	4.85 µL	0.15 µL

2. Spin down the contents of B2 and place on ice.
3. Thaw reagent B1 at room temperature, mix by vortexing, spin down and place on ice.
4. Prepare a master mix by combining B1 and B2 in a 0.5 mL capped tube according to the volumes shown in Table 4. Mix well by pipetting, spin down and place on ice.
5. Add 5 µL of Second Strand Master Mix to each sample tube for a total of 10 µL. Mix by pipetting 5 times, spin down and place on ice.
6. Place the tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand Synthesis; see Table 2):

   4 °C – 1 min, 25 °C – 10 min, 50 °C – 30 min, 80 °C – 20 min, hold at 4 °C
7. Remove the tubes from the thermal cycler and spin to collect condensation. Place in a rack on the bench top.

D. cDNA Purification

1. Ensure the Agencourt beads have completely reached room temperature before proceeding.
2. Prepare a 70% ethanol wash solution.

Important:
- It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container.
- Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content, which may reduce amplification yield.

3. Resuspend the beads by vortexing. Ensure that the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.

4. At room temperature, add 16 µL (1.6 volumes) of Agencourt beads to each reaction and mix by pipetting 10 times.
5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
7. Keeping the tubes on the magnet, carefully remove the binding buffer and discard it.
8. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.

Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact cDNA yields, so ensure beads are not removed with the binding buffer or the washes.

9. Remove the 70% ethanol wash using a pipette.
10. Repeat the wash two more times.

Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least 2 pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

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.

Important:
- It is critical that all residual ethanol be removed prior to continuing with SPIA amplification.

12. Continue immediately to the SPIA Amplification protocol with the cDNA still bound to the dry beads.

E. SPIA Amplification

Table 5. SPIA Master Mix

REAGENT	SPIA BUFFER MIX (RED: C2 VER 11)	SPIA PRIMER MIX (RED: C1 VER 9)	SPIA ENZYME MIX (RED: C3 VER 7)
1X REACTION VOLUME	10 µL	5 µL	5 µL

1. Thaw C3 on ice and mix the contents by inverting gently 5 times without introducing bubbles. Spin down and place on ice.
2. Thaw reagents C1 and C2 at room temperature. Mix by vortexing, spin down and place on ice.
3. Make a master mix by sequentially combining C2, C1 and C3 in an appropriately sized, capped tube according to the volumes shown in Table 5.

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

4. Add 20 µL of the SPIA Master Mix to each tube containing the double-stranded cDNA bound to the dried beads. Use a pipette set to 15 µL and mix thoroughly by pipetting 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: The beads may not form a perfectly uniform suspension, but this will not affect the reaction. The addition of SPIA master mix will elute the cDNA from the beads.

5. Place the tubes in a pre-cooled thermal cycler programmed to run Program 4 (SPIA Amplification, see Table 2):

   4 °C – 1 min, 47 °C – 60 min, 80 °C – 20 min, hold at 4 °C

6. Remove the tubes from thermal cycler, spin to collect condensation and place on ice.

   Do not re-open the tubes in the pre-amplification workspace.

   Important:
   At this point the tubes should be removed from the pre-amplification workspace. Carry out all remaining steps in a post-amplification workspace using dedicated post-amplification consumables and equipment. Take care to avoid the introduction of previously amplified cDNA into your pre-amplification workspace. For more information, please refer to **Appendix E** of this user guide. If you have any questions on this important topic, please contact Tecan NGS Tech Support (techsupport-gn@tecan.com).

7. Transfer the tubes to the magnet and let stand for 5 minutes to completely clear the solution of beads.
8. Carefully transfer 20 µL of the cleared supernatant containing the SPIA cDNA to a fresh tube.
9. Continue immediately with the Purification of SPIA cDNA protocol or store the reaction products at –20 °C.

   Note: The amplified cDNA may be used immediately in qPCR reactions, without purification, if consistent input concentration in the qPCR reaction is not required. The unpurified amplified cDNA should be diluted at least 15-fold in TaqMan assays and 40-fold in SYBR Green assays.

Optional stopping point: Store samples at –20 °C.

F. Purification of SPIA cDNA

We recommend purification of the amplified SPIA cDNA produced with the Crescendo cDNA Synthesis using Agencourt beads before qPCR studies or long term storage.

Agencourt Beads (instructions are for a single reaction)

Important:
- Prepare a room temperature 80% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content which may reduce amplification yield.
- The use of 96-well microplates and multi-channel pipettes is recommended for processing large batches with this procedure.

1. Obtain the AMPure XP or RNAClean XP bead bottle from 4 °C storage. Allow the bead solution to reach room temperature.
2. Invert the bead bottle several times to ensure the beads are fully in suspension. It may be necessary to remix the bead stock from time to time to ensure beads remain in suspension while in use.
3. At room temperature, add 36 µL of resuspended beads to the 20 µL SPIA reaction (1.8x volume).
4. Mix the sample and beads thoroughly by pipetting 10 times.
5. Incubate sample/bead mixture at room temperature for 5 minutes.
6. Transfer the tube to the magnet and let stand 10 minutes to completely clear the solution of beads.
7. Keeping the tube on the magnet, carefully remove and discard the supernatant. Do not disturb the beads.
8. Keeping the tube on the magnet, add 200 µL of freshly prepared 80% ethanol to each tube and allow to stand for 30 seconds or until the solution clears. Add slowly so as to not disturb the separated magnetic beads.
9. Carefully remove and discard the ethanol.
10. Repeat the 80% ethanol wash once more. Ensure as much ethanol as possible is removed from the plate after the final wash.

    Note: With the final wash, it is important to remove as much of the ethanol as possible. Use at least 2 pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

11. Remove the tubes from the magnet and air dry on the bench top for no more than 2 minutes. Ensure all ethanol has evaporated from the walls of the tube, but do not overdry.
12. Add 33 µL of room temperature 1X TE to each tube.
13. Resuspend the beads by repeated pipetting. Alternatively, the beads may be resuspended by carefully vortexing the tubes for 30 seconds or using a plate shaker set to medium speed. Ensure the beads are fully resuspended. Vortex longer if necessary.
14. Replace the tube on the magnet and allow the beads to separate for 5 minutes or until the solution clears.

15. Carefully remove the eluted sample and place into a fresh tube.

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

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

Optional stopping point: Store samples at –20 °C.

G. SPIA cDNA Yield and Purity

1. Mix the purified SPIA cDNA sample by brief vortexing. Spin down contents.
2. Measure the absorbance at 230, 260, 280 and 320 nm on a spectophotometer. 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 the A230, A260 and A280 values. The adjusted (A260–A320) / (A280–A320) ratio should be greater than 1.8. The adjusted (A260–A320) / (A230–A320) ratio should be greater than 2.
4. Yield: Assume 1 A260 unit = 50 µg/mL.

   To calculate: (A260–A320 of diluted sample) X (dilution factor) X 50 (concentration in µg/mL of a 1 A260 unit solution) X 0.033 (final volume in mL) = total yield in micrograms
5. The purified cDNA may be stored at –20°C.

Optional stopping point: Store samples at –20 °C.

H. Performing Quantitative PCR on Amplified cDNA

It is recommended that the amplified cDNA generated from the Crescendo cDNA Synthesis for qPCR kit 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. Amplified cDNA produced with this kit can be used successfully as template for qPCR systems including TaqMan® and SYBR® Green.

We have successfully used the following reagents for qPCR:

- TaqMan: TaqPath qPCR Master Mix, CG (Thermo Fisher Scientific, Cat. #A16245)
- SYBR: QuantiTect™ SYBR Green PCR Kit (QIAGEN, Cat. #204143), iQ SYBR Green Supermix (BioRad, Cat. #170-8880)

Other qPCR master mixes are likely compatible with the amplifed cDNA produced with this kit but have not been thoroughly evaluated by Tecan.

Recommendations to achieve optimal results

1. Dilute the Amplified Product
   - After cDNA purification and quantification, dilute the cDNA to the appropriate concentration for the qPCR reaction. It may be necessary to empirically determine the appropriate input of amplified cDNA for use in qPCR reaction.
   - Higher inputs of cDNA may be required for qPCR when starting with limited or degraded total RNA.
   - Higher inputs of cDNA may be required for qPCR when detecting low-expressed transcripts.
2. Primer Design
   - Primers may be designed at any position along a transcript since the Crescendo cDNA Synthesis for qPCR amplification process covers the whole transcriptome.
   - When starting with high quality RNA, we recommend using primers and probes designed with amplicon sizes of less than 200 nt.
   - When starting with degraded RNA, we recommend using primers and probes designed with amplicon sizes of less than 100 nt to compensate for the smaller cDNA fragments.

V. Technical Support

For help with any of our products, please contact Tecan NGS Technical Support at 650.590.3674 (direct) or 888.654.6544, option 2 (toll-free, U.S. only) or email techserv-gn@tecan.com

In Europe contact Tecan NGS Technical Support at +31.13.5780215 (phone) or email europe-gn@tecan.com.

In all other locations, contact your Tecan NGS reagent distributor for technical support.

A. Quality Control of the SPIA cDNA

There are several methods which we can recommend for quality control analysis of the SPIA cDNA including spectrophotometric quantification of amplification yield (as described in **Section IV, Protocol G** on page 16 and fragment size distribution analysis using the Agilent Bioanalyzer.

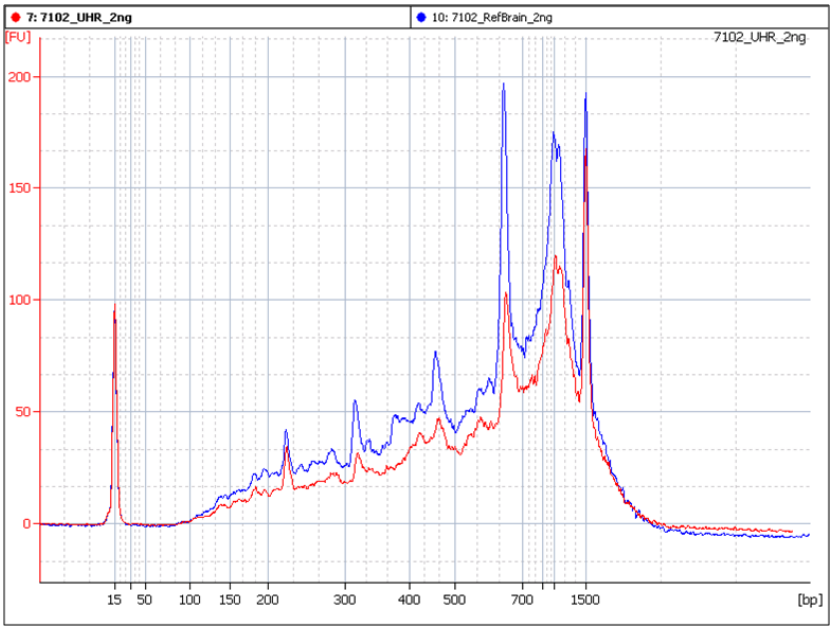
When performing fragment size distribution analysis on the Bioanalyzer, we recommend using either the Agilent DNA 1000 LabChip (Agilent Cat. #5067-1504) or the RNA 6000 Nano LabChip (Agilent Cat. #5065-4476) following the manufacturer's instructions and the specific guidelines given below.

Guidelines for Using the Agilent DNA 1000 LabChip

When using the DNA 1000 LabChip (Agilent Cat. # 5065-1504) for analysis of SPIA cDNA fragment distribution, use the DNA 1000 Series II program and follow the manufacturer's instructions. A typical size distribution trace may look like the one obtained from Human Reference Brain and Universal Human Reference total RNA (Figure 2).

Note that the shape of this distribution trace is highly dependent on the input RNA integrity as well as RNA source, and can vary significantly.

Figure 2. Bioanalyzer trace of SPIA cDNA product obtained from 2 ng of Human Brain and Universal Human Reference RNA using the DNA 1000 Labchip.

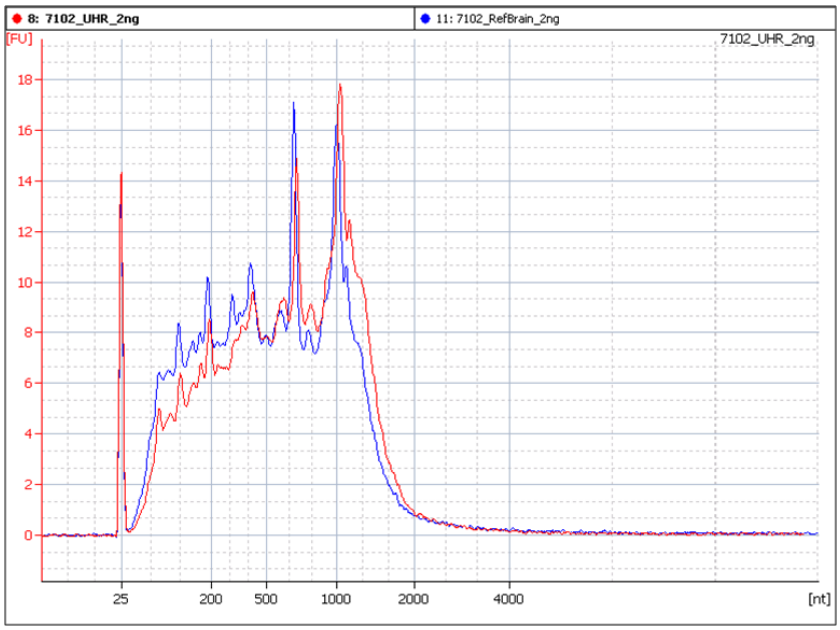


Guidelines for Using the Agilent RNA 6000 Nano LabChip

When using the RNA 6000 Nano LabChip (Agilent Cat. #5065-4476) for analysis of SPIA cDNA fragment distribution, use the Eukaryotic Total RNA Nano program and follow the manufacturer's instructions. Denaturation of the SPIA cDNA sample (as described in the Agilent RNA 6000 Nano protocol) is required for optimum resolution and reproducibility using this option. A typical size distribution trace may look like the one obtained from total Human Brain and Universal Human Reference RNA (Figure 3). Note that the shape of this distribution

trace is highly dependent on the input RNA integrity as well as RNA source, and can vary significantly.

Figure 3. Bioanalyzer trace of SPIA cDNA product obtained from 2 ng of Human Brain and Universal Human Reference RNA using the RNA 6000 Nano LabChip.



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.
- Clean work areas and instruments, including pipettes, with commercially available cleaning 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. DNase Treatment of RNA

We recommend DNase treatment of total RNA used as input for Crescendo cDNA Synthesis. Three options are provided below: DNase Treatment Integrated with First Strand Synthesis (recommended), DNase Treatment During RNA Purification, and DNase Treatment Post RNA Purification.

First Strand cDNA Synthesis with Integrated DNase Treatment

Additional reagents required:

- 20 mM Nuclease-free $MgCl_2$
- HL-dsDNase buffer (recipe in Table 7)
- HL-dsDNase (ArcticZymes Cat. #70800-201)
- 1 M Tris-HCl (Invitrogen Cat. #15567-027)
- 0.1 M DTT (dithiothreitol) (Affymetrix Cat. #70726 150 UL)

Table 6. Thermal cycler programs:

PROGRAM	
Program 1a Primer Annealing	(For RNA inputs ≤1 ng) 65 °C – 2 min, hold at 4 °C (For RNA inputs >1 ng) 65 °C – 5 min, hold at 4 °C
Program 1b DNase Treatment and Inactivation	37 °C – 10 min, 55 °C – 10 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

1. Obtain the First Strand Primer Mix (blue: A1), First Strand Buffer Mix (blue: A2), First Strand Enzyme Mix (blue: A3), water (green: D1), $MgCl_2$, 0.1 M DTT, and DNase from –20 °C storage.
2. Spin down the contents of A3 and HL-dsDNase and place on ice.
3. Thaw the other reagents at room temperature. Mix by vortexing, spin down and place on ice. Leave Nuclease-free Water at room temperature.
4. Add 1 µL of A1 to a 0.2 mL PCR tube.
5. Add 1 µL of total RNA sample (500 pg to 50 ng) to the primer.
6. Mix by pipetting 5 times, spin down and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1a:

   (For RNA inputs ≤1 ng) 65 °C – 2 min, hold at 4 °C

   (For RNA inputs >1 ng) 65 °C – 5 min, hold at 4 °C
8. Remove the tubes from the thermal cycler and place on ice.
9. Prepare HL-DNase buffer according to the volumes shown in Table 7. Mix by pipetting and spin down the master mix briefly. Place on ice. Use immediately in the DNase Master Mix (Table 8).

Table 7. HL-dsDNase Buffer

REAGENT	1 M TRIS-HCL	0.1 M DTT	NUCLEASE-FREE WATER (GREEN: D1)
1X REACTION VOLUME	7 µL	7 µL	86 µL

10. Prepare a master mix by combining $MgCl_2$, HL-dsDNase buffer, and HL-dsDNase in a 0.5 mL capped tube, according to the volumes shown in Table 8. Mix by pipetting, spin, and place on ice. (DO NOT VORTEX. DNase is sensitive to physical denaturing.)

Table 8. DNase Master Mix

REAGENT	$MgCl_2$	HL-dsDNase BUFFER	HL-dsDNase
1X REACTION VOLUME	0.5 µL	0.5 µL	0.5 µL

11. Add 1.5 µL of DNase master mix to the RNA sample/primer mix.
12. Mix by pipetting 5 times, spin, and place on ice.
13. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1b:

    37 °C – 10 min, 55 °C – 10 min, hold at 4 °C
14. Remove the tubes from the thermal cycler and place on ice.
15. Prepare a master mix by combining A2 and A3 in a 0.5 mL capped tube, according to the volumes shown in Table 9.

Table 9. First Strand Master Mix

REAGENT	FIRST STRAND BUFFER MIX (BLUE: A2 VER 3)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 1)
1X REACTION VOLUME	1.25 µL	0.25 µL

16. Add 1.5 µL of the First Strand Master Mix to each tube.
17. Mix by pipetting 5 times, spin, and place on ice.
18. Place the tubes in a pre-cooled thermal cycler programmed to run Program 2:

    4 °C – 1 min, 25 °C – 10 min, 42 °C – 10 min, 70 °C – 15 min, hold at 4 °C
19. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
20. Continue immediately with the Second Strand cDNA Synthesis protocol. (page 12; **IV. Protocol; C. Second Strand cDNA Synthesis**)

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

1. Homogenize sample in RLT buffer including ß-mercaptoethanol according to the type of sample as described in the RNeasy Mini Kit protocol.
2. Add 1X volume of 70% ethanol to the homogenized lysate, pipet up and down to mix sample well. Do not centrifuge.
3. Place an RNeasy mini column in a 2 mL collection tube.
4. Apply the sample (up to 700 µL), including any precipitate that may have formed, to the column.
5. Close the tube gently, and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
6. For volumes greater than 700 µL, load aliquots onto the RNeasy column successively and centrifuge as before.
7. Add 350 µL Buffer RW1 into the RNeasy mini column to wash, and centrifuge for 15seconds 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.
9. Pipet the DNase I incubation mix (80 µL) directly onto the membrane inside the RNeasy mini column. Incubate at the bench top (~25 °C) for 15 min.
10. Add 350 µL Buffer RW1 into the RNeasy mini column, and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm) to wash. Discard the flow-through.
11. Transfer the RNeasy column to a fresh 2 mL collection tube. Add 500 µL Buffer RPE (with the added ethanol) to the RNeasy column.
12. Close the tube gently, and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
13. Add another 500 µL Buffer RPE to the RNeasy column.
14. Close the tube gently, and centrifuge for 2 minutes at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
15. Transfer the RNeasy column to a new 1.5 mL collection tube.
16. Pipet 30–50 µL RNase-free water directly onto the RNeasy membrane.
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 and 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 10X DNase I Reaction buffer (Roche Cat. #04716728001) with 1 µL rDNase (10 Units Roche Cat. #04716728001).

2. Add RNA sample (up to 500 ng) and add Nuclease-free water (D1, green cap) to bring the final volume to 25 µL.
3. Incubate at 25 °C for 15 minutes followed by 37 °C for 15 minutes and return to ice.

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-5 Kit 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-5 Kit 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: Use room temperature water. Do not use cold water!

9. Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

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

1. Add 80 µL ice-cold Nuclease-free water (D1, green cap) to the sample on ice.
2. Add 350 µL Buffer RLT and mix by pipetting.
3. Add 250 µL 96 to 100% ethanol and mix thoroughly by pipetting.
4. Place an RNeasy MinElute Spin Column into a 2 mL collection tube (one column per sample) and apply the 700 µL sample to the column.
5. After closing the column, spin for 15 seconds at ≥8000 X g (≥10,000 rpm). 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. **Use room temperature water. Do not use cold water!**
12. Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

D. Ribo-SPIA Technology

The Crescendo cDNA Synthesis is powered by Ribo-SPIA technology, a rapid, simple and sensitive RNA amplification process. Using Ribo-SPIA technology and starting with as little as 500 pg total RNA, microgram quantities of cDNA can be prepared. Isolated poly(A) RNA or mRNA may be substituted for studies focused on the analysis of mature coding transcripts. Ribo-SPIA contributes minimal coverage bias which has been shown to be highly reproducible.

Ribo-SPIA technology is a process that generates amplified cDNA from as little as 500 pg of total RNA.

a. Generation of First Strand cDNA (1 hour)

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.

b. Generation of a DNA/RNA Heteroduplex Double-stranded cDNA (2 hours)

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

c. SPIA Amplification (1.5 hours)

SPIA is a robust isothermal strand-displacement amplification process. It uses a DNA/RNA chimeric SPIA primer, DNA polymerase and RNase H in a homogeneous isothermal assay that provides highly efficient amplification of DNA sequences. RNase H is used to degrade RNA in the DNA/RNA heteroduplex at the 5' end of the first cDNA strand. This results in the exposure of a DNA sequence that is available for binding the first SPIA primer. DNA polymerase then initiates replication at the 3' end of the primer, displacing the existing forward strand. The RNA portion at the 5' end of the newly synthesized strand is again removed by RNase H, exposing part of the unique priming site for initiation of the next round of cDNA synthesis. The process of SPIA DNA/RNA primer binding, DNA replication, strand displacement and RNA cleavage is repeated, resulting in rapid accumulation of SPIA cDNA.

E. Preventing Non-specific Amplification

Due to the high sensitivity inherent in our amplification systems, we have developed a set of recommendations designed to minimize the potential for the generation of non-specific amplification products by carryover of previously amplified SPIA cDNA. We strongly recommend implementing these procedures, especially for high-throughput and low-RNA input environments typical in today's gene expression laboratories. We have two general

recommendations. First, designate separate workspaces for “pre-amplification” and “post-amplification” steps and materials. This provides the best work environment for processing RNA using our highly sensitive amplification protocols. Our second recommendation is to implement routine clean-up protocols for workspaces as standard operating procedure. This will prevent amplification products from accumulating in laboratory workspaces. Details regarding establishing and maintaining a suitable work environment are listed below:

1. Designate a pre-amplification workspace separate from the post-amplification workspace or general lab areas:
    a. Pre-amplification includes all steps and materials related to RNA sample handling and dilution, first strand reaction, second strand reaction, second strand cleanup and SPIA amplification reaction setup. After SPIA incubation the reactions are immediately removed from the pre-amplification workspace and opened only in the post-amplification area.
    b. Post-amplification includes all steps and materials related to the handling of the amplified SPIA cDNA product, including bead removal, final purification, post-SPIA modification, array hybridization and any other analytical work.
    c. Ideally, the pre-amplification workspace will be in a separate room. If this is not possible, ensure the pre-amplification area is sufficiently isolated from post-amplification work.
    d. PCR Workstation enclosures with UV illumination for use as pre-amplification workspaces can be an option in situations where conditions preclude physical separation of pre- and post-amplification activities.
2. Establish and maintain a clean work environment:
    a. Initially clean the entire lab thoroughly with DNA-OFF. Follow treatment with a thorough rinse with water to ensure no residual cleaning agents are left behind.
    b. In the pre-amplification area, remove all small equipment, and then clean every surface that may have been exposed to amplified SPIA cDNA (surfaces, drawer handles, keypad, etc.). Before reintroducing any equipment, clean every piece of equipment thoroughly.
        i. Clean wells of thermal cycler(s) and magnetic plate(s) with a cotton swab or by filling with cleaning solution.
        ii. Clean the thermal cycler block by heating to 99 °C for 15 minutes, then wipe down exposed surfaces and keypad with cleaning solution.
        iii. Clean magnets by immersion in cleaning solution or use a cotton swab.
    c. Carry out a thorough external and internal cleaning of all pipettes with DNA-OFF. Carefully follow the manufacturer’s instructions for this process to avoid damaging the pipettes. It is a good idea to keep a clean set of pipettes as a backup.
    d. Always wear gloves, and don fresh gloves upon entry into this controlled area. Frequently change gloves while working in the pre-amplification area, especially prior to handling stock reagents, reactions and RNA samples.
    e. Stock the pre-amplification workspace with clean, preferably new, equipment (pipettes, racks, etc.) that has not been exposed to the post-amplification workspace. Establish dedicated stocks of all consumables used in the pre-amplification workspace.
    f. Make it a policy to carry out regular cleaning of all workspaces.
    g. Capture waste generated in both pre- and post-amplification areas (tips, columns, wash solutions from beads and columns, tubes, everything) in sealable plastic bags. Seal and dispose of them promptly after each experiment to avoid waste spillage.

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

3. Avoid running negative controls (i.e., no RNA input reactions). Instead use low-template controls (inputs of 50 pg to 100 pg) in order to detect and monitor any non-specific amplification issues. The clearest indication that non-specific amplification is taking place is the appearance of higher than expected yields or irregular bioanalyzer traces in a low template control (LTC) reaction.

   a. Typical amplification performance:

      i. LTC yields for Crescendo cDNA Synthesis amplifications should be significantly lower than yields for RNA inputs within the recommended input range of 500 pg to 50 ng.

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

   b. Atypical amplification performance:

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

      ii. The Bioanalyzer traces of amplification products may look significantly different than the typical Crescendo cDNA Synthesis reaction traces. The LTC reaction is designed to be an especially sensitive indicator of atypical amplification performance.

      iii. Contact Tecan NGS Technical Support when atypical performance is suspected.

F. Frequently Asked Questions (FAQs)

Getting Started

Q1. What materials are provided with the Crescendo cDNA Synthesis kit?
The Crescendo cDNA Synthesis kit provides all necessary buffers, primers and enzymes for first strand synthesis, second strand synthesis and amplification. Beckman Coulter Agencourt magnetic beads for cDNA purification and SPIA cDNA purification steps are not provided.

Q2. What equipment is required or will be useful?
Required equipment includes a microcentrifuge, pipettes, vortexer, a thermal cycler, a spectrophotometer and magnetic plate. An Agilent Bioanalyzer may also be useful for optional analytical tests.

Q3. What additional consumables are needed?
For the purification steps, we recommend using Agencourt beads. Agencourt beads are not provided with the kit and should be purchased separately.

Input Recommendations

Q4. How much total RNA do I need for amplification?
Crescendo cDNA Synthesis can be used with purified total RNA in the range of 500 pg to 50 ng. Input amounts outside this range may produce unsatisfactory and variable results.

Q5. Do I need to use high-quality total RNA?
The Crescendo cDNA Synthesis kit is designed to work with purified total RNA. When using purified total RNA, samples should be of high molecular weight with little or no evidence of degradation. While it is impossible to guarantee the highest levels of performance when using RNA of lower quality, this system should allow the successful analysis of somewhat degraded samples. With such samples, users may experience lower amplification yields and should plan to use relatively higher RNA inputs.

Q6. Can DNA be used as input for the Crescendo cDNA Synthesis kit?
No. Crescendo cDNA Synthesis is designed to convert and amplify RNA, not DNA.

Q7. Can contaminating genomic DNA interfere with the Crescendo cDNA Synthesis kit performance?
When using purified total RNA samples, large amounts of contaminating genomic DNA may amplify during the process. For this reason we recommend DNase treatment during RNA purification.

Q8. Do you recommend DNase treatment of purified total RNA samples?
Yes. For an explanation of DNase requirements see page 6. For DNase treatment of RNA samples, refer to **Appendix C** for guidelines.

Q9. Can I use Crescendo cDNA Synthesis on bacterial RNA samples?
The SPIA amplification process theoretically will work with many bacterial RNAs; however, the kit has not been optimized for this purpose.

Q10. Are there any tissues that will not work with the Crescendo cDNA Synthesis?
We have not encountered any specific RNA sources that will not work with this kit.

General Workflow

Q11. Has Tecan performed reproducibility studies on the Crescendo cDNA Synthesis kit?
Yes. Sample-to-sample, lot-to-lot, and operator-to-operator reproducibility studies are routinely conducted.

Q12. Can I perform fewer than 8 reactions at a time?
We recommend a minimum batch size of 8 reactions. Smaller batch sizes may result in difficulty pipetting small volumes and lead to poor performance.

Q13. How much overage is recommended when making reagent master mixes for each step?
A minimum amount of overage should be used in master mixes to ensure the full nominal number of reactions in the kit. The amount of overage needed depends on sample batch size, pipetting accuracy, and viscosity of reagents. We have found that 10% extra volume in the master mixes in this kit is sufficient for most experiments.

Q14. Does Crescendo cDNA Synthesis generate product in the absence of RNA input?
In the complete absence of input RNA, approximately 0.3 µg or less of non-specific product is generated. However, in the presence of even a very small amount of RNA the amplified cDNA has been demonstrated to be specific. It is not recommended to run reactions without input RNA (i.e. no-template controls).

Q15. How many rounds of amplification are performed with the Crescendo cDNA Synthesis kit?
This system performs a single round of amplification and is not designed to support multiple rounds of amplification.

Q16. Do I need to order specific primers for the amplification?
No. The DNA/RNA primers provided in the Crescendo cDNA Synthesis kit are universal. No gene-specific primers are required.

Q17. Do I have to use the supplied DNA/RNA primers?
Yes. The Crescendo cDNA Synthesis kit will not work properly with other primers.

Q18. Where can I safely stop in the protocol?
It is safe to stop after the SPIA Amplification protocol prior to final cleanup at the point specifically noted in the protocol. Store reaction products at –20 °C.

Q19. What purification methods do you recommend?
We recommend using Agencourt magnetic beads for the double-stranded cDNA purification and final SPIA cDNA purification steps.

Quantification and Qualification

Q20. What size cDNA is generated by the Crescendo cDNA Synthesis kit?
Figure 2 and Figure 3 (page 19 and 20) shows a representative fragment distribution pattern obtained with 2 ng of high-quality Universal Human Reference and human brain total RNA samples.

Q21. How much SPIA cDNA can I expect from a single reaction?
You should expect micrograms of SPIA cDNA from a starting input of 500 pg of good quality total RNA.

Q22. How do I measure my SPIA cDNA yield?
Refer to SPIA cDNA Yield and Purity protocol on page 23 in this User Guide for guidance.

Q23. Is the cDNA yield dependent upon the quantity of total RNA input?
Yes, higher inputs of RNA will lead to somewhat higher amplification yields.

Q24. Can I use an Agilent Bioanalyzer to evaluate the amplification products?
Yes. Refer to **Appendix B** of this User Guide for guidelines.

Downstream Applications

Q25. Can I use the final purified SPIA cDNA for qPCR analysis?
Yes. qPCR can be performed on the final SPIA cDNA before or after purification. Guidelines for qPCR analysis of SPIA cDNA can be found in **Appendix C**.

For Research Use Only. Not for use in diagnostic procedures.

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