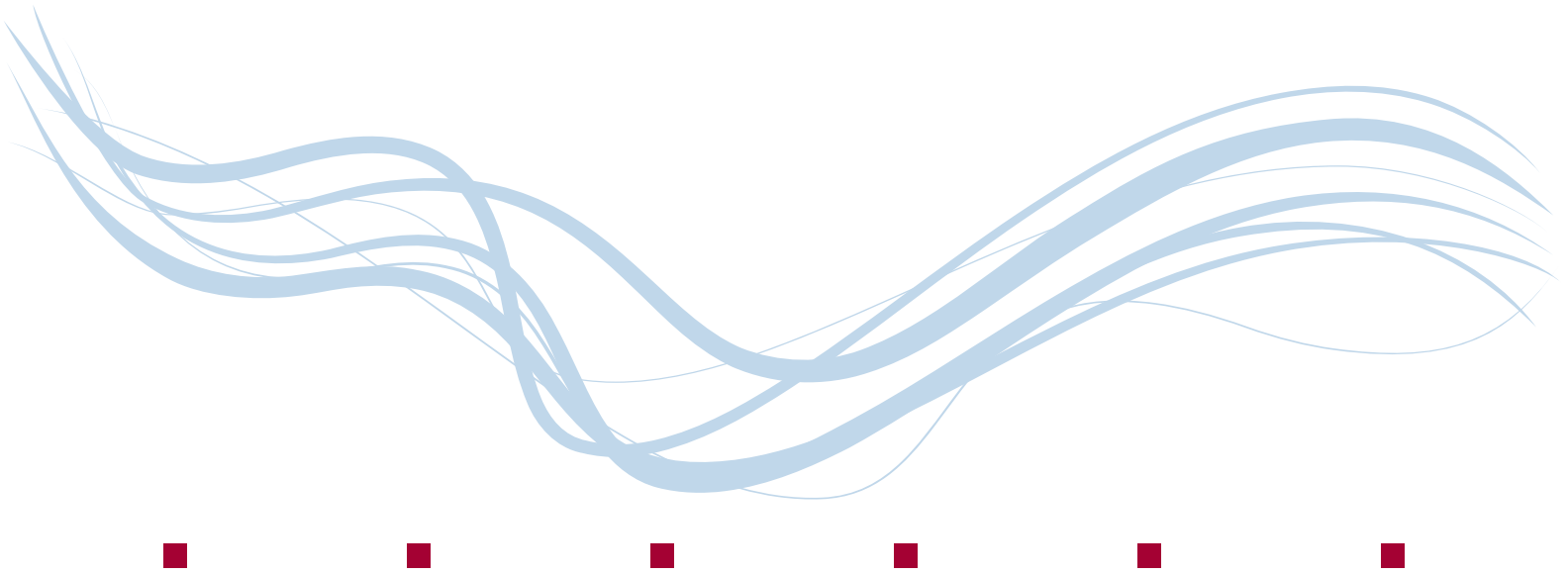
integenX

Apollo 324™ System

PrepX SPIA® RNA-Seq Library

Protocol



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WARRANTY

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IntegenX Inc.
5720 Stoneridge Drive, Suite 300
Pleasanton, CA 94588

925.701.3400

Email: support@integenX.com

www.IntegenX.com

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Chapter 1

Overview

In this chapter:

This *PrepX SPIA RNA-Seq Library Protocol* provides the basic information necessary to use your Apollo 324™ System to prepare amplified cDNA from Total RNA samples for RNA-Seq applications. The protocol uses NuGEN® SPIA® reagents for generating the amplified cDNA from Total RNA.

This document assumes that you know how to use the Apollo 324™ System and the touchscreen interface. For details on using the system, refer to the *Apollo 324 User Guide*.

IMPORTANT: IntegenX recommends that first-time users take advantage of user training offered with the installation of the system.

About the Protocol

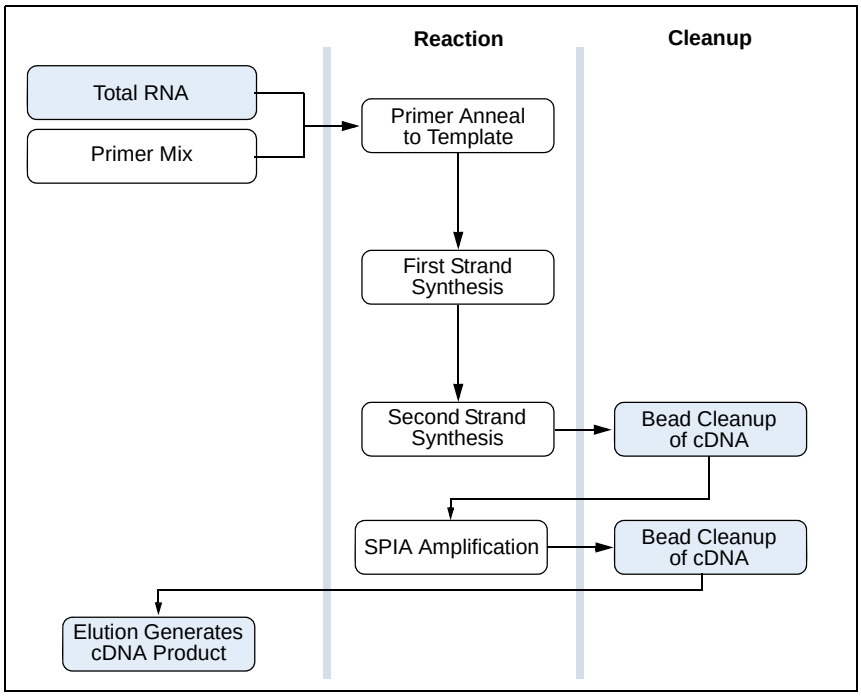
The PrepX RNA Library protocol reagent kit provides researchers the flexibility to optimize the performance of the Apollo 324 System to prepare cDNA in one run. The entire process to prepare the cDNA is completed in approximately 5.5 hours, with the products ready for generating up to eight DNA libraries in another run using the standard PrepX reagent kit.

The kit includes enzymes, primers, buffers, and 1X beads, plus mineral oil and molecular biology grade water, to process 24 samples.

Workflow

1. Press the PrepX SPIA RNA-Seq Library button on the touchscreen to activate the Peltier heating/cooling units.
2. Place consumables, reagents, magnetic beads and Total RNA samples in the racks on the work surface.
3. Start the protocol run, using the touchscreen interface.
4. The eight samples undergo processing:
   a. Anneal primer to the Total RNA sample
   b. First strand synthesis
   c. Second strand synthesis
   d. Bead-based purification (cleanup) of the double-stranded cDNA
   e. SPIA® amplification
5. Final bead-based purification of amplified double-stranded cDNA.
6. cDNA samples are now ready for use to prepare DNA libraries using one of the PrepX DNA protocols.

Processing Schematic



Chapter 2

Preparing Samples and Reagents

In this chapter:

Overview

This chapter describes how to prepare samples and reagents for the PrepX SPIA RNA-Seq Kit protocol.

IMPORTANT: We only guarantee the Apollo 324 System to perform using the recommended supplies and materials listed in this document.

Materials for Operation

Reagent Kit

The following reagents are provided in the PrepX SPIA RNA-Seq Library Kit. The kit provides sufficient reagents for three 8-sample runs (24 samples).

- Buffers and first strand cDNA synthesis primers
  - 24 Buffer and primer strips (four tubes in each strip, magenta labeled seal)
- Enzymes and NuGEN SPIA primers
  - 24 Enzyme and primer strips (four tubes in each strip, black labeled seal)
- Paramagnetic beads and other reagents
  - RNAClean® XP beads (four tubes)
  - Mineral oil (25 mL) (five 5 mL bottles)
  - Molecular biology grade water (50 mL) (one bottle)

Instruments

The following items are required.

Item	Part Number	Supplier
Centrifuge for 0.2 mL 8-tube strips	various	various
Vortex mixer for preparing reagents and samples	various	various

Customer-Supplied Reagents and Consumables

The following reagents and consumables are recommended to run the Apollo 324 System.

Customer-Supplied Consumables	Part Number	Supplier
1.1 mL 12-tube strips (Axygen®)	89005-580	VWR®
0.2 mL 8-tube strips (Axygen)	10011-764	VWR
0.2 mL 8-cap strips (Axygen)	10011-786	VWR
Eppendorf 96-well skirted microtiter plate, conical, 150 µL	47744-122	VWR
50 mL Falcon test tube	21008-940	VWR
15 mL Falcon test tube	21008-929	VWR
2 mL screw cap test tube	16466-042	VWR
Aluminum heat seal	AB1720	Thermo Scientific®
Piercing tips	300028	IntegenX
Dispensing filter tips	300027	IntegenX
Reservoirs	300031	IntegenX
Micropipette tips (20 µL, 200 µL and 1000 µL)	various	various
Micropipettes (20 µL, 200 µL and 1000 µL)	various	various
Customer-Supplied Reagents	**Part Number**	**Supplier**
100%, 200 proof EtOH	E7023	Sigma-Aldrich®
RNaseZap®	AM9780, AM9782	Ambion®

Decontaminating the Lab Space and Pipettes

1. Wipe the lab space work surfaces with a 10% bleach solution.
2. Wipe the work surfaces with RNaseZap decontamination solution.
3. Wipe the work surfaces with 70% EtOH.

IMPORTANT: We recommend using a set of pipettes dedicated for working with RNA.

Decontaminating the Instrument Work Surface

1. Wipe the instrument work surface with RNaseZap decontamination solution sprayed onto a laboratory wipe.
2. Wipe the instrument work surface with 70% EtOH.

Preparing the SPIA RNA-Seq Reagent Kit Reagents

1. Retrieve Box 1 (buffers and primers) and Box 2 (enzymes and primers) from the freezer.
2. Retrieve the bottles of RNAClean XP beads from the refrigerator to bring them to room temperature.
3. Thaw the frozen reagents on ice. Thaw only the reagents that you will be using for the run. Keep all reagents on ice.
4. Once thawed, spin down reagents briefly prior to use.

   We recommend using a centrifuge with a swinging bucket for the reagent tubes.
5. Visually inspect to be sure that the entire volume is at the bottom of each vial with no air pockets.

Preparing 70% v/v EtOH

Combine 28 mL of 100% EtOH and 12 mL of molecular biology grade water in a sterile 50 mL Falcon tube and mix well. You will require 15 mL for each run.

Preparing the Mineral Oil Aliquot

Pipette 8 mL of mineral oil into a sterile 15 mL Falcon tube.

Preparing the Molecular Biology Grade Water Aliquot

Pipette 15 mL of molecular biology grade water into a sterile 50 mL Falcon tube.

Preparing the Total RNA Samples

IMPORTANT: The lowest recommended input concentration for the NuGEN reagents used in this protocol is 400 pg/µL, providing 2 ng of Total RNA per 5 µL of the Total RNA sample for the run. The current working range is 2 ng to 10 ng of Total RNA.

The cDNA product of the protocol run must have a concentration of at least 7ng/µL in order to be used to prepare libraries using the PrepX DNA Library protocol. For details on processing the cDNA, see “Processing the cDNA for Library Preparation” in Chapter 3.

NOTE: We recommend using color-coded Axygen® strip tubes to ensure correct setup.

1. Dilute your Total RNA sample to the current working concentration of 2 ng/µL.
2. Pipette 5 µL of Total RNA sample into each tube of an Axygen 8-tube strip.
3. Spin down the thawed SPIA RNA-Seq reagents briefly prior to use. Visually inspect to be sure that the entire volume is at the bottom of each vial with no air pockets.

   We recommend using a centrifuge with a swinging bucket for the reagent tubes.

4. At the lab bench and on ice, use a piercing tip to manually pierce only the A1 primer tubes (first strand cDNA synthesis primer) in the magenta sealed strip. This is the position closest to the arrow.

Strip 1

Material	(µL)
A3 (RT-Enzyme)	1.5
B2 (Enzyme)	1.5
C3 (Enzyme)	10.0
C1 (Primer)	10.0

Strip 2

Material	(µL)
C2 (Buffer)	20
B1 (Buffer)	9.7
A2 (Buffer)	2.5
A1 (Primer)	2.0

5. Very carefully, pipette 5 µL of the diluted Total RNA sample into the 2 µL of primer in the A1 tube.

Be very careful when pipetting. Do not allow any air pockets to form under the sample. If there are air pockets, the robot arm will pick up air instead of fluid.

6. Mix well with a pipette tip.
7. Place the samples on ice until ready to load onto the Apollo 324 work surface.

IMPORTANT: The instrument is calibrated for Axygen tubes only. Using other types might result in run failure.

Loading the Beads in the Microtiter Plate

1. At the lab bench, at room temperature, vortex mix the RNAClean XP beads for 10 to 15 seconds to ensure that they are uniformly mixed and in suspension.
2. Centrifuge the beads briefly.
3. Pipette 130 µL of the beads into each well in row 1 of a clean microtiter plate. Be certain that no air is trapped underneath the bead volume.

NOTE: Just before starting the protocol, you will aliquot the beads into the microtiter plate. This is done later in order to avoid bead settling.

Chapter 3

Setting Up and Running the Protocol

In this chapter:

Overview

This chapter describes how to set up and run the protocol, providing guidance for placing the reagents, samples and consumables on the work surface, and then running the protocol. The work surface setup window in the touchscreen interface is mapped to the work surface blocks on the instrument. The window provides guidance for placing reagents, samples and consumables, and then running the protocol.

The default setup for 8 samples fills the available blocks on the work surface.

For details on preparing the samples and reagents before loading them on the instrument, see Chapter 2, “Preparing Samples and Reagents.”

For details on placing:

- Consumables, see “Placing Consumables on the Work Surface.”
- Samples and reagents, see “Loading Samples and Reagents.”

An accumulation of discarded pipettes and tips in the waste tip box can cause a run to fail. Be sure to open the waste tip box access door and check that the box has been emptied.

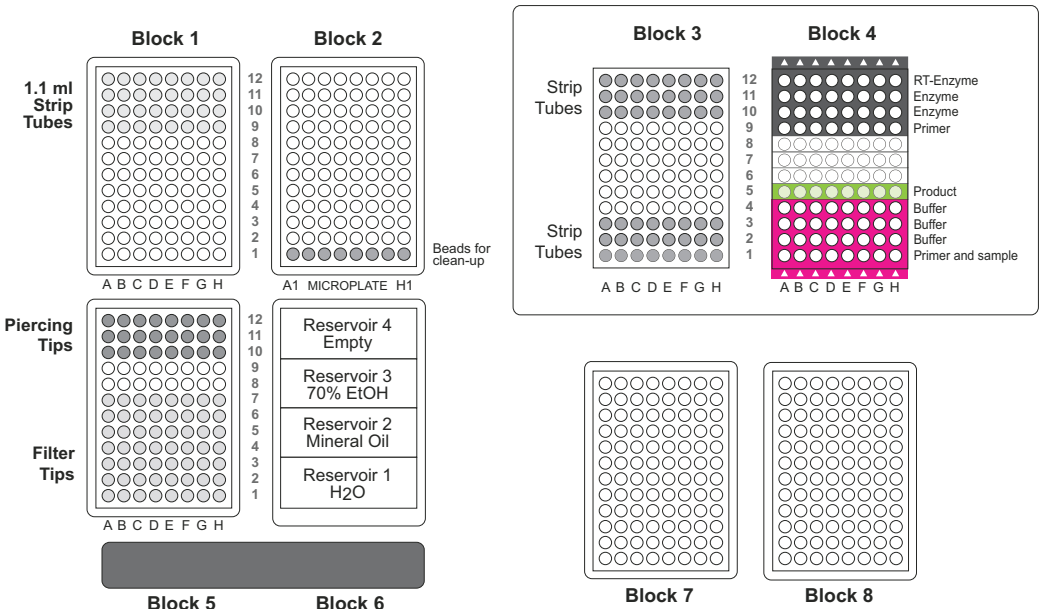
The cDNA that is prepared by this protocol can then be used to construct libraries, using the PrepX-DNA Library protocol.

Setting Up a Run

This section describes how to load items on the instrument, using the setup window to verify placement.

Work Surface Layout

The following illustration shows the layout of the work surface and placement of the consumables, reagents and samples for the protocol run.



Setup Window

The setup window in the touchscreen interface provides guidance for setting up runs. In the setup window, the NEXT and BACK buttons enable you to navigate so you can easily view the setup for any block.

- **NEXT** magnifies the next sequential block.
- **BACK** returns to the last magnified block or the Start window.

For details on using the touchscreen interface, refer to the *Apollo 324 User Guide*.

Launching the Software

1. Power on the instrument.

   The software start-up window is displayed for a few seconds, and then the IntegenX splash screen appears. When you power on the instrument, the temperature in the heating/cooling units (Blocks 3 and 4) adjusts to 18 °C.

   After the splash screen disappears, the initial Start window is displayed, showing the available protocols.

   After the software is launched, the pipette head of the Apollo 324 System will initialize.

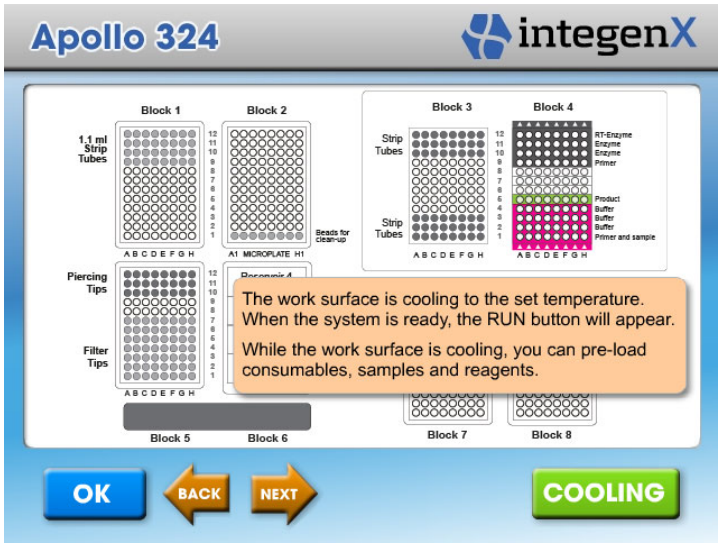
IMPORTANT: Pressing in the center of the window or pressing and holding any of the buttons for several seconds will initiate calibration of the touchscreen. For details, see the *Apollo 324 System User Guide*.

2. To select the protocol, in the Start window, press **PrepX SPIA RNA-Seq Library**.

   The work surface setup window is displayed.

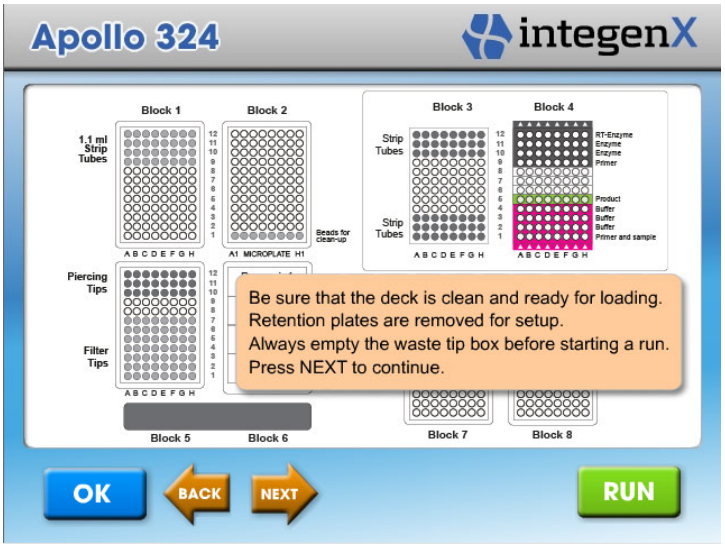
   On the instrument work surface, the left-hand heating/cooling unit (Block 3) remains at 18 °C, while the right-hand unit (Block 4) cools to 4 °C.

   While Block 4 is cooling, the **COOLING** button is displayed.



During cooling of Block 4

When Block 4 reaches 4 °C, the **RUN** button appears.



When Block 4 reaches 4 °C

3. While the unit is cooling, pre-load consumables, samples and reagents on the work surface.

   NOTE: You can place the consumables (tubes, plates, reservoirs and tips) on the work surface before starting the software and loading the samples and reagents. For details, see "Placing Consumables on the Work Surface."

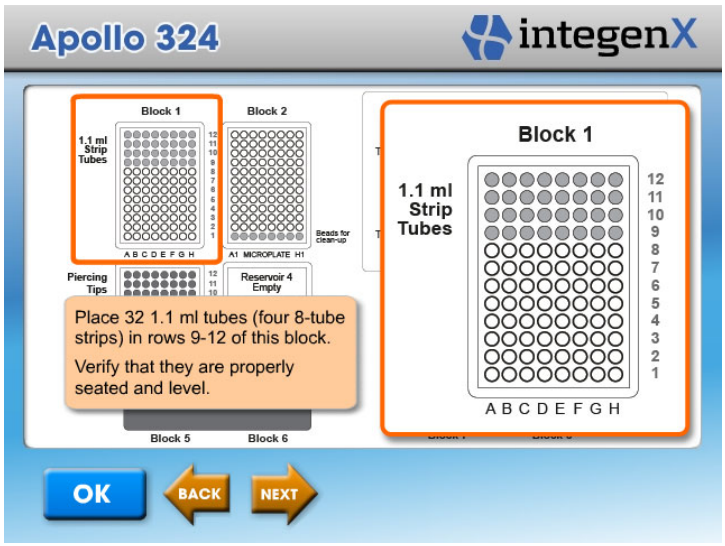
   For instructions on loading reagents and samples, see "Loading Samples and Reagents."

Placing Consumables on the Work Surface

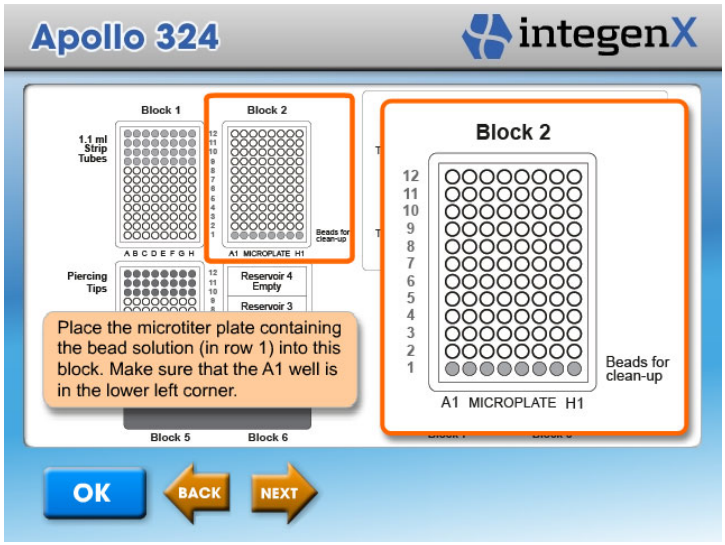
Placing the consumables on the work surface can be done in advance.

1. Place 32 1.1 mL tubes (four strips of 8-tube strips) in rows 9–12 of Block 1.

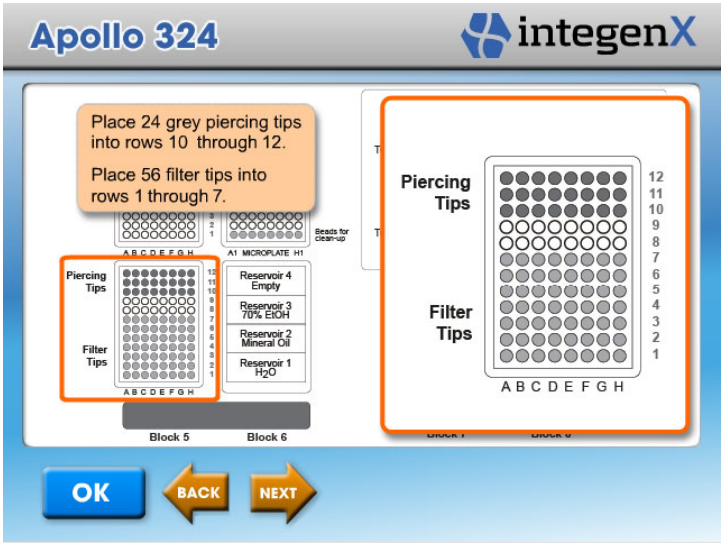
   Verify that they are properly seated and level. These tubes will be filled during the run.



2. In Block 2, place the 96-well microtiter plate containing 130 µL of the NuGEN bead solution (in row 1). Make sure that the A1 well is in the lower left corner.



3. Place the piercing tips and dispensing filter tips into Block 5 as follows:
   a. Place 24 grey piercing tips into rows 10–12 of Block 5.
   b. Place 56 dispensing filter tips into rows 1–7 of Block 5.



NOTE: Do not use the plastic carrier tray in Block 5, as it might cause tips to stick during automatic operation.

4. Place four empty reservoirs in Block 6. You will fill these later.

   Make sure that the reservoirs are seated properly.

5. Blocks 7 and 8 are not used and remain empty.

6. Verify that the waste tip box is empty.

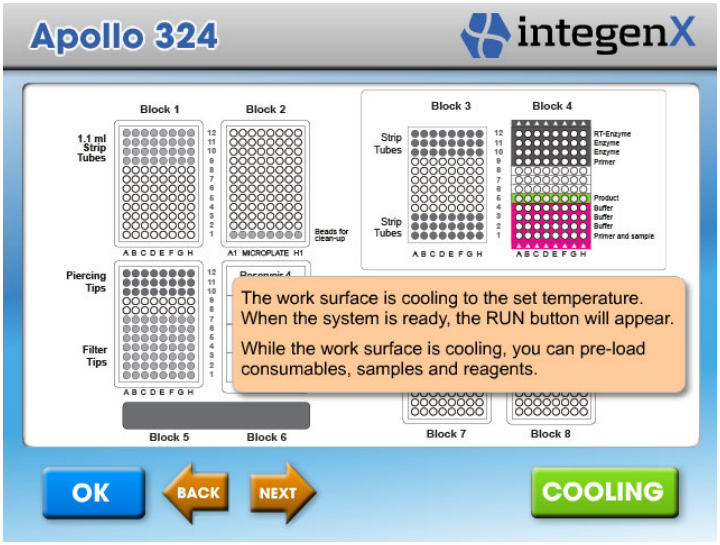
Loading Samples and Reagents

1. If you have not already done so, press the **PrepX SPIA RNA-Seq Library** button in the Start window to initiate the protocol.

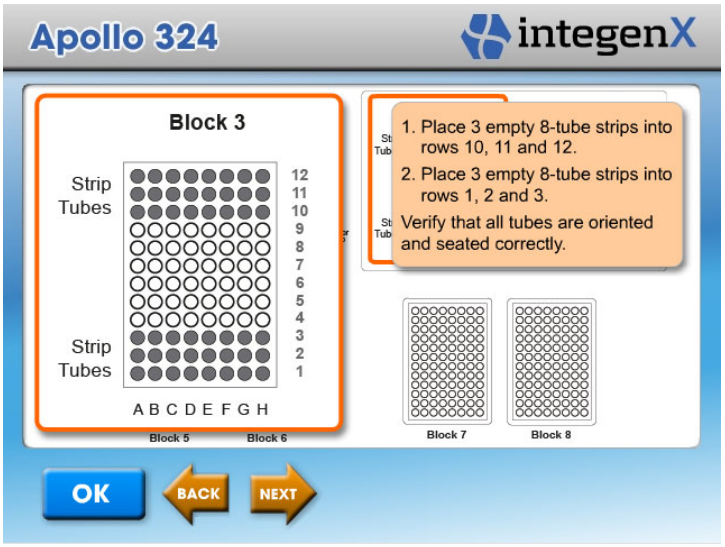
   The work surface setup window is displayed.

   On the instrument work surface, the left-hand heating/cooling unit (Block 3) remains at 18 °C, while the right-hand unit (Block 4) cools to 4 °C. While it is cooling, the **COOLING** button is displayed; when it reaches 4 °C, the **RUN** button appears.

   While the unit is cooling, you can place samples and reagents on the work surface.

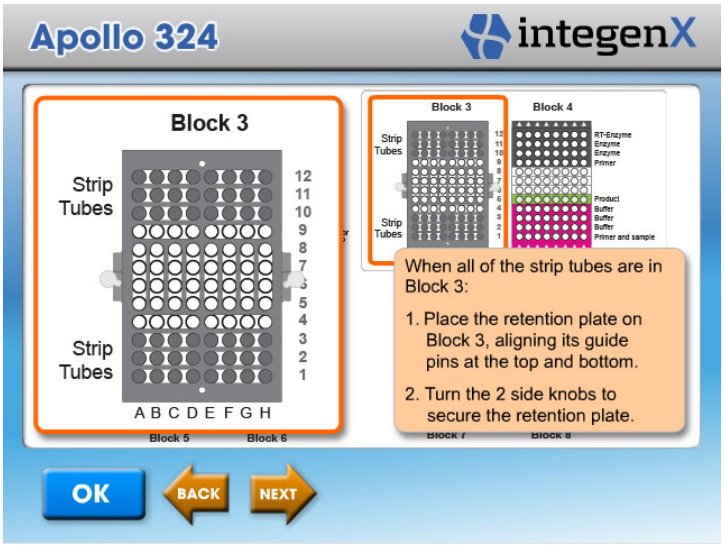


2. Load empty tubes into Block 3 as follows:
   a. Place three empty 8-tube strips into rows 10, 11 and 12.
   b. Place three empty 8-tube strips into rows 1, 2 and 3.

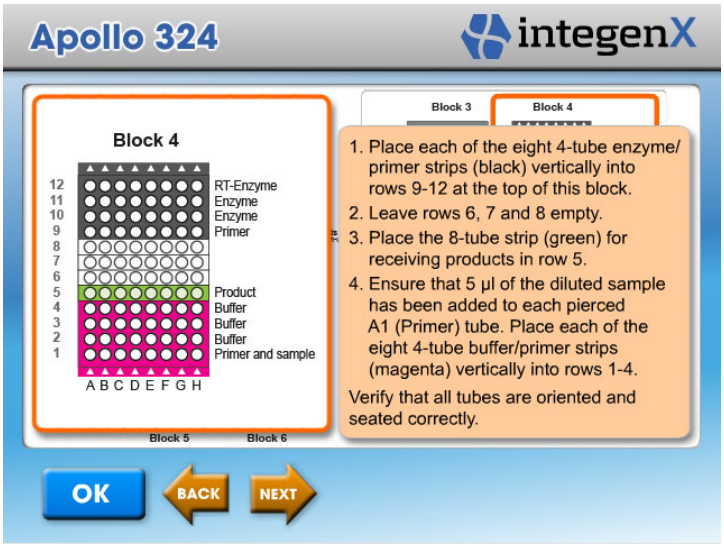


NOTE: Verify that all tubes are oriented and seated correctly.

3. Install the metal retention plate over Block 3 to secure the tubes and keep them stable, aligning the guide pins at the top and bottom.
   Rotate the side knobs to lock the retention plate in place.



4. Load the prepared sample and reagents into Block 4 as follows:
   a. Place each of the eight 4-tube enzyme/primer strips (black) vertically into rows 9–12 at the top of the block so that the arrows point up toward the rear of the work surface.
   b. Leave rows 6, 7 and 8 empty.
   c. Place an empty 8-tube strip for receiving products in row 5.
      NOTE: Label the tubes with details about the run and sample.
   d. Place each of the eight 4-tube buffer/primer strips (magenta) vertically into rows 1–4 at the bottom of the block so that the arrows point up toward the rear of the work surface.



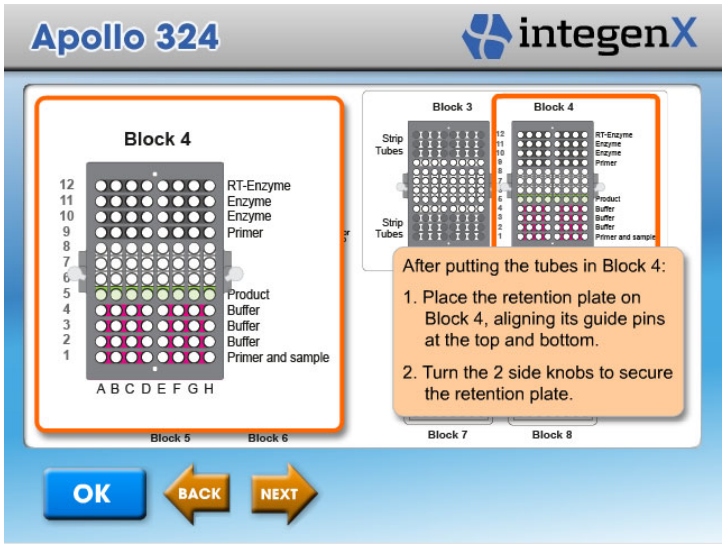
NOTE: Verify that all tubes are oriented and seated correctly.

IMPORTANT: Ensure that 5 µL of the diluted Total RNA sample has been added to the A1 (Primer) tubes.

CAUTION Visually inspect the tubes as you place them in the block to ensure that the entire volume is in the bottom of the tubes, without droplets on the side walls, bubbles or void volume. If necessary, centrifuge briefly before placing the tubes.

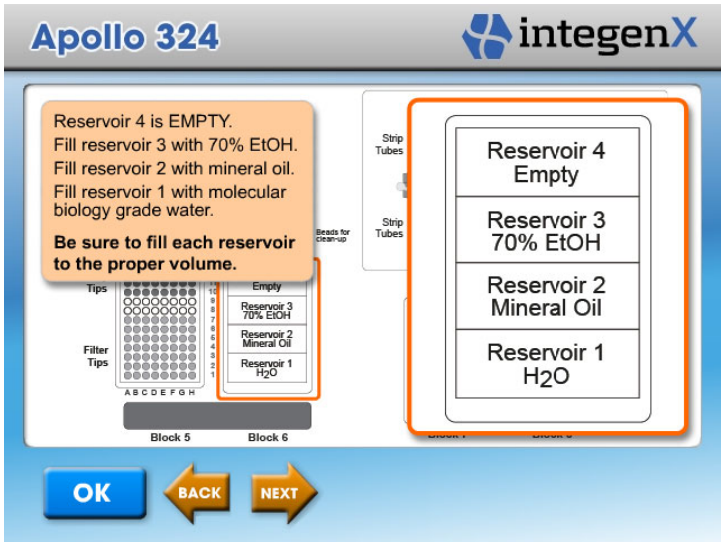
5. Install the metal retention plate over Block 4 to secure the tubes and keep them stable, aligning the guide pins at the top and bottom.

   Rotate the side knobs to lock the retention plate in place.

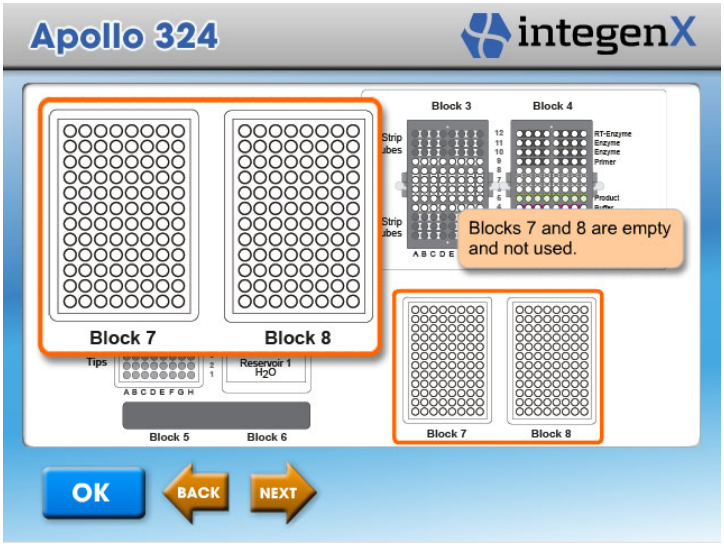


6. Fill the reagent reservoirs in Block 6 as follows:
   a. Dispense 15 mL of molecular biology grade water into Reservoir 1.
   b. Dispense 8 mL of mineral oil into Reservoir 2.
   c. Dispense 15 mL of freshly prepared 70% v/v EtOH into Reservoir 3.

   NOTE: Reservoir 4 remains empty for this configuration.



7. Blocks 7 and 8 are not used and remain empty.



8. Verify placement of all reagents and consumables and check that all tubes, plates and reservoirs are seated properly.
9. Verify that all components are installed according to the setup window.

   You are now ready to start the run. For instructions, see “Starting a Run.”

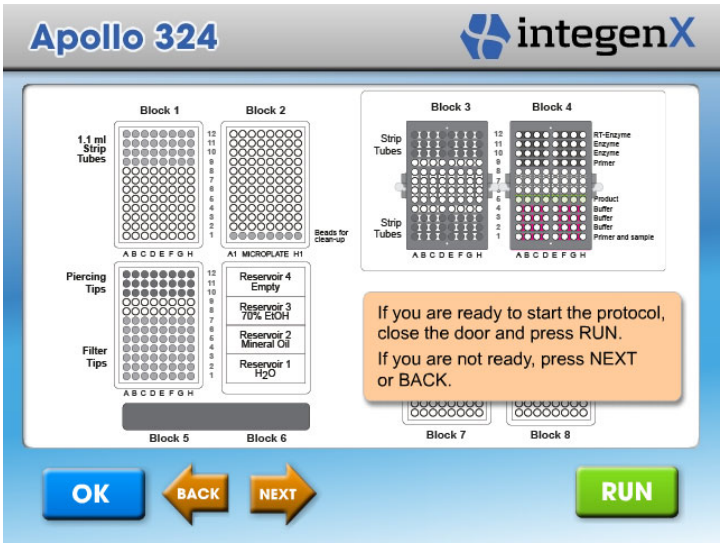
Running the Protocol

Starting a Run

When all of the items are on the work surface, close the instrument door.

IMPORTANT: You must close the door in order to start the run.

If you are ready to start the protocol, press **RUN**. If you are not ready, press **NEXT**, **BACK** or any block to review the setup and make changes to items on the work surface.

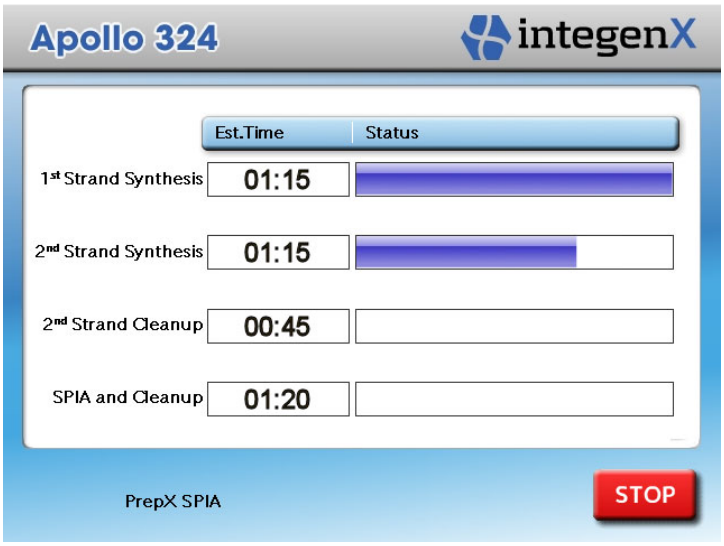


After you press **RUN**, the front door of the instrument locks and the run starts. The status of each step of the run is displayed in a progress bar.

Monitoring a Run

After a run begins, the progress window shows the status of each step. A countdown timer shows how much time (minutes and seconds) is left for each step.

From this window, you can stop a run completely. The **STOP** button appears only after a run has started.



Finishing a Protocol

After the products have been eluted off the beads, the protocol is finished. A message informs you when the protocol is complete.

Press **OK** to display the Start window. At this point, the door unlocks.

DANGER

Do NOT attempt to open the instrument door until the “Protocol Finished” message is displayed. Do NOT assume that a protocol is complete until this message is displayed.

Retrieving and Handling the Processed cDNA

1. Open the door and remove the 8-tube product strip (in row 5, Block 4).
2. Cap the processed samples immediately and store them on ice or at -20 °C.
3. Remove and discard used reagents and consumables.

The cDNA samples are now ready for further processing.

Processing the cDNA for Library Preparation

The cDNA prepared by this protocol can be used to construct DNA libraries by running the PrepX-DNA Library protocol on the Apollo 324 System. The cDNA sample **MUST** be sheared before it can be used to construct libraries.

IMPORTANT: A cDNA concentration of at least 20ng/µL is recommended for optimal results before proceeding to prepare libraries using the PrepX DNA Library protocol. If the cDNA concentration is lower than 20ng/µL, we recommend using a magnetic bead based DNA capture procedure to concentrate the sheared cDNA prior to running the PrepX DNA Library protocol.

After shearing and concentrating, the cDNA samples will be ready for PrepX DNA Library processing on the Apollo 324 System. For instructions on running the PrepX DNA Library protocol and recommendations for PCR and post-PCR conditions, see the *PrepX DNA Library Protocol*.

Shearing the cDNA

We recommend using Covaris® systems to shear the cDNA produced from the NuGEN protocol. For instructions on shearing the cDNA, refer to the Covaris documentation. We recommend the following settings on the Covaris S2:

Setting	Value
Mode	Frequency Sweeping
Number of Cycles	5
Bath Temperature Limit	10.0
Total Process Time	5:00
Treatment 1	Duty Cycle: 10% Intensity: 5 Cycles/Burst: 100 Time: 60 seconds
Treatment 2	Duty Cycle: 0.5% Intensity: 0.1 Cycles/Burst: 50 Time: 0 seconds
Treatment 3	Duty Cycle: 0.5% Intensity: 0.1 Cycles/Burst: 50 Time: 0 seconds
Treatment 4	Duty Cycle: 0.5% Intensity: 0.1 Cycles/Burst: 50 Time: 0 seconds

Adjusting the cDNA Concentration

Be sure that the concentration of the processed cDNA sample matches the input requirements for high-sensitivity chip analysis (such as on the Agilent® Bioanalyzer). It might be necessary to perform a dilution of the sheared sample to avoid overloading the high-sensitivity chips.

Determining the Proper Concentration

1. Use a UV-Vis spectrophotometer to obtain a quick reading of each sheared cDNA sample. Perform two readings of each sample and record the concentration (ng/µL).

   Always blank the spectrophotometer with molecular biology grade water prior to taking the reads.

2. Based on the spectrophotometer readings, determine the proper dilution, if required, to prepare an aliquot of the sheared cDNA at approximately 1-10ng/µL in order to properly run on high-sensitivity chips.

   If required, prepare a diluted aliquot of the sheared cDNA with molecular biology grade water and record the dilution factor and concentration (pg/µL).

3. Run a high sensitivity chip analysis of the sheared cDNA (diluted if required) by following the guidelines of the protocol supplied with the high sensitivity kit. The analysis will indicate the proper concentration for your samples.

   If the cDNA requires magnetic bead based capture for concentrating the samples, proceed to the next section.

Concentrating the cDNA Samples

Perform magnetic bead based capture to concentrate low yielding samples.

1. In a 1.5 mL Eppendorf tube, add 1.8 µL of AMPure® XP beads per 1.0 µL of sheared cDNA sample. Mix thoroughly with a pipette, ensuring that the beads and sample are mixed uniformly.
2. Incubate for 10 minutes to allow complete capture.
3. Pellet the beads for about two minutes, using a magnet to capture the beads to one side of the tube. Carefully remove and discard the supernatant.
4. Wash the bead pellet as follows:
   a. Resuspend the pellet with 250 µL freshly prepared 70% EtOH, carefully bouncing it from side to side using a magnet until it is uniformly dispersed. **DO NOT VORTEX**.
   b. Re-pellet the beads for about two minutes, using a magnet to capture the beads to one side of the tube.
   c. Carefully remove and discard the 70% EtOH.
5. Repeat the wash step (step 4) once, for a total of two washes.
6. After the second wash, re-pellet the beads for about two minutes, using a magnet and a 10 µL pipette to remove as much of the remaining EtOH as possible from the bottom of the tube.
7. Allow the bead pellet to air dry for approximately five minutes (and no longer than 10 minutes).

   The beads should lighten in color and form cracks as they dry.
8. Resuspend the cDNA sample as follows:
   a. Dispense an appropriate volume of molecular biology grade water into the sample tube to achieve the desired concentration.
   b. Mix with a pipette, vortex and spin to resuspend the bead pellet. This might require scraping beads from the tube side walls and several aspirations in order to mix well. If the beads are not well-suspended or if they are in clumps, the DNA will not elute properly. Vortex and spin if desired.
9. Pellet the beads, using a magnet, for one to two minutes, until the liquid is clear. Visually confirm that all beads are pelleted.
10. Transfer the concentrated sample to clean tubes and discard the bead pellet.
11. Use a UV spectrophotometer to ensure that the concentration is now above 20ng/µL.

The cDNA samples are now ready for core PrepX DNA library processing. For instructions on running the PrepX DNA Library protocol, refer to the *Apollo 324 System User Guide*.

Recommendations for PCR and Post-PCR Conditions

IMPORTANT: After PrepX DNA Library processing, the DNA prepared from cDNA might not amplify as well as a standard DNA library. To enrich the DNA library, we recommend using 18 cycles of PCR with 1 ng of sample DNA. For recommendations and instructions on PCR and post-PCR conditions, refer to the *Apollo 324 System User Guide.*

Reagent and Reaction Locations

Block 1
Axygen 1.1 mL Tubes

Row	Description
12	70% EtOH (700 µL) 2nd synthesis bead wash
11	70% EtOH (700 µL) Final bead wash
10	Dirty water (700 µL) Clean EtOH
9	Elution water (700 µL) Elute final product
8	
7	
6	
5	
4	
3	
2	
1	

Block 2
Microtiter Plate

Row	Description
12	Bead wash 1, 2 - Synth wash
11	Bead wash 3, 4 - Synth wash
10	Bead wash 1, 2 - Final wash
9	Bead wash 3, 4 - Final wash
8	Waste water - Final wash water
7	
6	
5	
4	
3	
2	
1	5X beads (130 µL) for synth cleanup

Block 3
0.2 mL PCR Tubes (PCR 0)

Row	Material	Description
12	Empty strip tube	Mineral oil (60 µL)
11	Empty strip tube	Mineral oil (170 µL)
10	Empty strip tube	Mineral oil (30 µL)
9		
8		
7		
6		
5		
4		
3	Empty strip tube	SPIA amplification
2	Empty strip tube	Cycling for 2nd synthesis
1	Empty strip tube	Cycling for 1st synthesis

Block 4
0.2 mL PCR Tubes (PCR 1)

Row	Description
12	A3 (RT-enzyme) (1.5 µL) 1st synthesis
11	B2 (Enzyme) (1.5 µL) 2nd synthesis
10	C3 (Enzyme) (10 µL) SPIA amplification
9	C1 (Primer) (10 µL) SPIA amplification
8	
7	
6	
5	Product (50 µL) Final product
4	C2 (Buffer) (20 µL) SPIA amplification
3	B1 (Buffer) (9.7 µL) 2nd synthesis
2	A2 (Buffer) (2.5 µL) 1st synthesis
1	A1 (Primer+sample) user sample input

Block 5
Tip Rack

Row	Description
12	Piercing tip - 1st synthesis
11	Piercing tip - 2nd synthesis
10	Piercing tip - SPIA
9	
8	
7	Filter tip - Mineral oil fill
6	Filter tip - 70% EtOH fill
5	Filter tip - Final cleanup
4	Filter tip - SPIA amplification
3	Filter tip - Synthesis cleanup
2	Filter tip - 2nd synthesis
1	Filter tip - 1st synthesis

Block 6
Reservoirs

Material
Empty
70% EtOH (15 mL) for wash
Mineral Oil (8 mL) for sample overlay
H_2O (15 mL) for elution/wash

Block 7
Empty, Not Used

Block 8
Empty, Not Used

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IntegenX Inc.
5720 Stoneridge Drive, Suite 300
Pleasanton, CA 94588

925.701.3400

support@integenX.com

www.IntegenX.com

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