

USER GUIDE

Encore® SP+ Complete Library Systems

PART NOS. 8151-32 AND 8152-32

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I. Introduction

A. Background

The Encore® SP+ Complete Library Systems are complete reagent, cartridge and protocol kits for the simple automation of RNA-Seq library preparation protocols using the Mondrian™ SP+ Workstation. These systems enable RNA-Seq library construction using as little as 100 ng of total RNA. The core technology used in this product enriches for coding and regulatory transcripts in NGS libraries during cDNA synthesis and can be applied to transcriptomes from a broad range of higher eukaryotes. The cDNA synthesis is carried out using proprietary primers to create double stranded cDNA that retains strand-specific expression information. The resulting sequencing reads can be aligned to the strand from which the RNA originated, enabling detection of both sense and antisense expression. No dedicated steps are required to reduce rRNA levels in the final NGS library. The resulting cDNA is converted to NGS libraries using reagents and adaptors provided as part of the Encore SP+ Complete Library Systems kit.

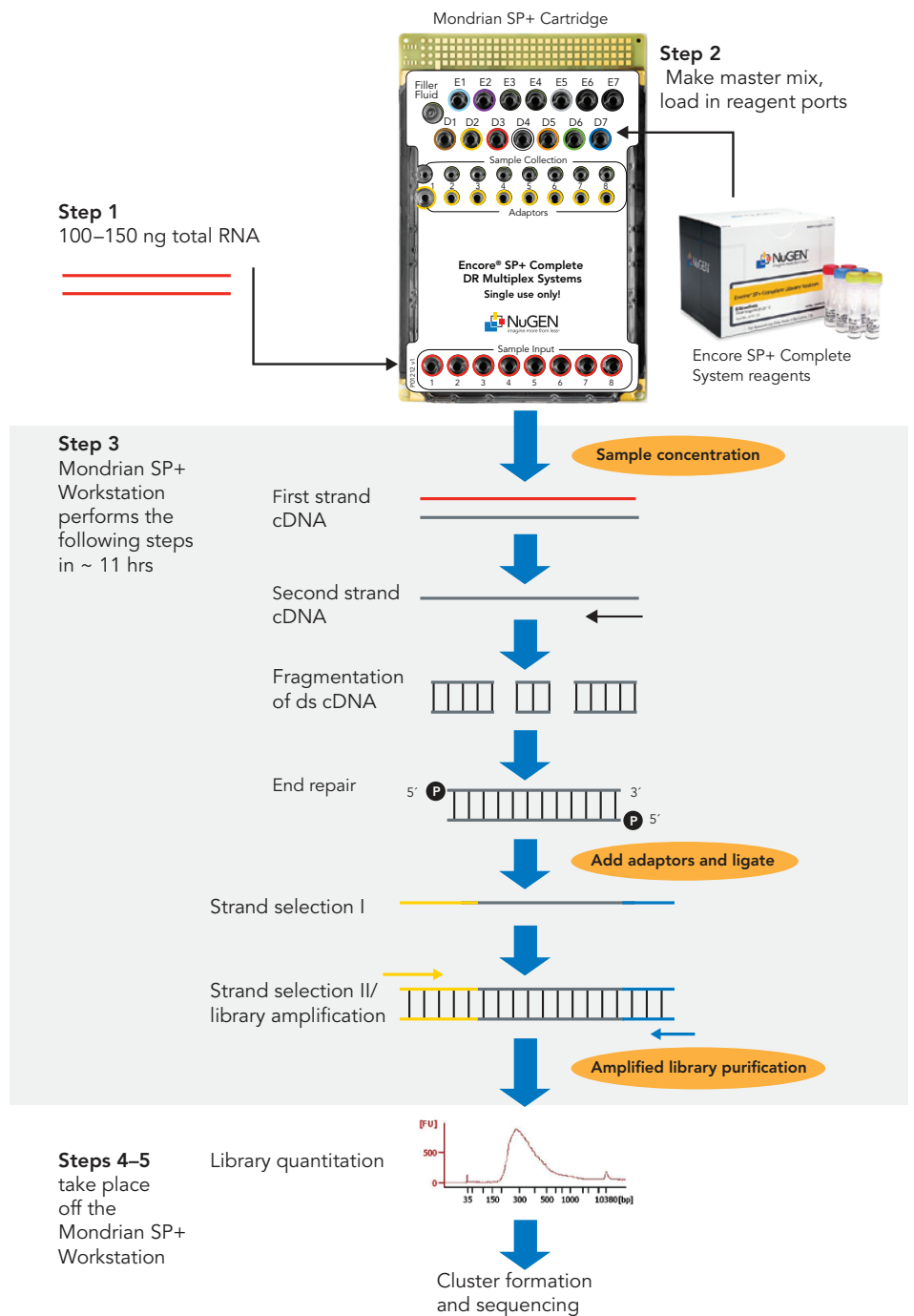
The Encore SP+ Complete RNA-Seq Library Systems have been designed for strand-specific expression analysis by incorporation of a nucleotide analog during the second strand cDNA synthesis and subsequent ligation to a pair of double-stranded adaptors containing the same analog in one strand. After ligation, the cDNA strand and adaptor containing the analog are selectively removed (Strand Selection), leaving only one cDNA strand with both adaptor sequences attached. This product is then converted into a sequence-ready library by PCR amplification.

The workflow consists of three steps:

1. Hands-free automation of the following assay steps on the Mondrian SP+ Workstation (see Figure 1):
 - RNA sample concentration
 - cDNA generation
 - cDNA fragmentation
 - Library construction
 - End repair
 - Adaptor ligation
 - Strand selection
 - Library amplification
 - Sample purification
2. Sample collection from the Mondrian SP+ Cartridge
3. Quantitation, cluster formation and sequencing

I. Introduction

Figure 1. The three steps of the Encore SP+ Complete Library Systems workflow.



I. Introduction

Starting with 100–150 ng of total RNA, the Encore SP+ Complete protocol can be completed in approximately 11 hours and yields libraries ready for quantitation, cluster formation and either single read or paired-end sequencing.

NuGEN offers a two configurations of the Encore SP+ Complete Library Systems: Encore SP+ Complete DR Multiplex System 1–8 (Part No. 8151) and Encore SP+ Complete DR Multiplex System 9–16 (Part No. 8152). Each kit provides eight unique dedicated read adaptors to prepare libraries for multiplex sequencing using a dedicated read design strategy with a second sequencing primer. Either kit may be used for up to 8-plex sequencing or the two kits may be used together to multiplex up to 16 samples.

Important: Please familiarize yourself with the instructions in the Mondrian SP+ Cartridge User Guide (M01344) and the Mondrian SP+ Workstation User Manual (M01322) prior to beginning any work with the Encore SP+ Complete Library Systems.

B. Performance Specifications

The Encore SP+ Complete Library Systems are designed to generate DNA libraries suitable for either single read or paired-end sequencing on Illumina Genome Analyzer IIx/IIe (GAIIx), MiSeq, HiScan SQ or HiSeq 2000/2500 NGS platforms. This simple, hands-free and robust system is capable of starting with 100 ng of total RNA to generate libraries ready for quantitation and cluster formation in about 11 hours.

C. Quality Control

Every lot of the Encore SP+ Complete Library Systems undergoes functional testing to confirm that the products meet the specifications for library generation performance.

We recommend the use of control samples when beginning experiments and/or using a new source of samples. For RNA-based experiments, such as RNA-Seq, we recommend using the Microarray Quality Control (MAQC) reference samples A and B.

D. Storage and Stability

The Encore SP+ Complete Library Systems reagents are shipped in two boxes. Box 1 is shipped on dry ice and, upon receipt, should be stored at -20°C on an internal shelf of a freezer without a defrost cycle. Box 2 is shipped at room temperature, but contains components with multiple storage temperature requirements and should be unpacked immediately upon receipt.



This product contains components with multiple storage temperatures.

I. Introduction

- Vials labeled Agencourt® RNAClean® XP Beads (clear cap) and Bead Additive (S01825) should be removed from the top of the Box 2 shipping carton upon delivery and stored at 4°C.
- All other Box 2 components should be stored at room temperature.

The kit has been tested to perform to specifications after as many as four freeze/thaw cycles. Kits handled and stored according to the above guidelines will perform to specifications for at least six months.

E. Material Safety Data Sheet (MSDS)

An MSDS for this product is available on the NuGEN website at www.nugen.com/nugen/index.cfm/support/user-guides/

II. Components

A. Reagents Provided

Table 1. Encore SP+ Complete DR Multiplex System 1–8 (Part No. 8151-32)

8151 PART NUMBER	8151 DESCRIPTION	8151 BOX	8151 VIAL CAP	8151 VIAL NUMBER
S01711	First Strand Primer Mix	1 of 2	Blue	A1 VER 11
S01712	First Strand Buffer Mix	1 of 2	Blue	A2 VER 9
S01713	First Strand Enzyme Mix	1 of 2	Blue	A3 VER 5
S01714	Second Strand Buffer Mix	1 of 2	Yellow	B1 VER 7
S01715	Second Strand Enzyme Mix	1 of 2	Yellow	B2 VER 4
S01717	Fragmentation Enzyme Mix	1 of 2	Orange	F2 VER 3
S01716	Fragmentation Buffer Mix	1 of 2	Orange	F1 VER 3
S01627	End Repair Buffer Mix	1 of 2	Blue	ER1 VER 5
S01510	End Repair Enzyme Mix	1 of 2	Blue	ER2 VER 4
S01662	Ligation Buffer Mix	1 of 2	Yellow	L1 VER 5
S01722 S01723 S01724 S01725 S01726 S01727 S01728 S01729	DR Multiplex Ligation Adaptor Mixes 1–8	1 of 2	Yellow	L2V10DR-BC1 L2V10DR-BC2 L2V10DR-BC3 L2V10DR-BC4 L2V10DR-BC5 L2V10DR-BC6 L2V10DR-BC7 L2V10DR-BC8
S01467	Ligation Enzyme Mix	1 of 2	Yellow	L3 VER 4
S01718	Strand Selection Buffer Mix I	1 of 2	Purple	SS1 VER 2
S01512	Strand Selection Enzyme Mix I	1 of 2	Purple	SS2
S01738	Strand Selection Enzyme Mix II	1 of 2	Purple	SS4
S01807	Strand Selection II/ Amplification Buffer Mix	1 of 2	Red	SS3/P1
S01765	Amplification Primer Mix	1 of 2	Red	P2 VER 9

II. Components

Encore SP+ Complete DR Multiplex System 1–8 Components and Reagents (Part No. 8151) <i>continued</i>				
8151 PART NUMBER	8151 DESCRIPTION	8151 BOX	8151 VIAL CAP	8151 VIAL NUMBER
S01764	Amplification Enzyme Mix	1 of 2	Red	P3 VER 2
S01001	Nuclease-Free Water	1 of 2	Green	D1
P01208	Mondrian SP+ Cartridges (x4)	2 of 2	N/A	N/A
P01212	Encore SP+ Complete Library Systems Cartridge Loading Guide (x4)	2 of 2	N/A	N/A
S01719	Mondrian SP+ Filler Fluid (x4)	2 of 2	N/A	N/A
S01587	Sample Concentration Solution	2 of 2	Clear	N/A
S01588	Bead Binding Solution	2 of 2	Clear	N/A
S01589	Bead Wash Solution	2 of 2	Clear	N/A
S01590	Elution Buffer	2 of 2	Clear	N/A
S01825	Bead Additive	Shipped separately	Clear	N/A
S01698	Agencourt RNAClean XP Beads	Shipped separately	Clear	N/A

Table 2. Encore SP+ Complete DR Multiplex System 9–16 Components and Reagents (Part No. 8152)

8152 PART NUMBER	8152 DESCRIPTION	8152 BOX	8152 VIAL CAP	8152 VIAL NUMBER
S01711	First Strand Primer Mix	1 of 2	Blue	A1 VER 11
S01712	First Strand Buffer Mix	1 of 2	Blue	A2 VER 9
S01713	First Strand Enzyme Mix	1 of 2	Blue	A3 VER 5
S01714	Second Strand Buffer Mix	1 of 2	Yellow	B1 VER 7

II. Components

Encore SP+ Complete DR Multiplex System 9–16 Components and Reagents (Part No. 8152) <i>continued</i>				
8152 PART NUMBER	8152 DESCRIPTION	8152 BOX	8152 VIAL CAP	8152 VIAL NUMBER
S01715	Second Strand Enzyme Mix	1 of 2	Yellow	B2 VER 4
S01717	Fragmentation Enzyme Mix	1 of 2	Orange	F2 VER 3
S01716	Fragmentation Buffer Mix	1 of 2	Orange	F1 VER 3
S01627	End Repair Buffer Mix	1 of 2	Blue	ER1 VER 5
S01510	End Repair Enzyme Mix	1 of 2	Blue	ER2 VER 4
S01662	Ligation Buffer Mix	1 of 2	Yellow	L1 VER 5
S01730 S01731 S01732 S01733 S01734 S01735 S01736 S01737	DR Multiplex Ligation Adaptor Mixes 9–16	1 of 2	Yellow	L2V10DR-BC9 L2V10DR-BC10 L2V10DR-BC11 L2V10DR-BC12 L2V10DR-BC13 L2V10DR-BC14 L2V10DR-BC15 L2V10DR-BC16
S01467	Ligation Enzyme Mix	1 of 2	Yellow	L3 VER 4
S01718	Strand Selection Buffer Mix I	1 of 2	Purple	SS1 VER 2
S01512	Strand Selection Enzyme Mix I	1 of 2	Purple	SS2
S01738	Strand Selection Enzyme Mix II	1 of 2	Purple	SS4
S01807	Strand Selection II/ Amplification Buffer Mix	1 of 2	Red	SS3/P1
S01765	Amplification Primer Mix	1 of 2	Red	P2 VER 9
S01764	Amplification Enzyme Mix	1 of 2	Red	P3 VER 2
S01001	Nuclease-Free Water	1 of 2	Green	D1
P01208	Mondrian SP+ Cartridges (x4)	2 of 2	N/A	N/A
P01212	Encore SP+ Complete Library Systems Cartridge Loading Guide (x4)	2 of 2	N/A	N/A

II. Components

Encore SP+ Complete DR Multiplex System 9-16 Components and Reagents (Part No. 8152) <i>continued</i>				
8152 PART NUMBER	8152 DESCRIPTION	8152 BOX	8152 VIAL CAP	8152 VIAL NUMBER
S01719	Mondrian SP+ Filler Fluid (x4)	2 of 2	N/A	N/A
S01587	Sample Concentration Solution	2 of 2	Clear	N/A
S01588	Bead Binding Solution	2 of 2	Clear	N/A
S01589	Bead Wash Solution	2 of 2	Clear	N/A
S01590	Elution Buffer	2 of 2	Clear	N/A
S01825	Bead Additive	Shipped separately	Clear	N/A
S01698	Agencourt RNAClean XP Beads	Shipped separately	Clear	N/A

II. Components

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**

- Mondrian SP+ Workstation (Part No. 8100)
- Agilent 2100 Bioanalyzer or materials and equipment for electrophoretic analysis of nucleic acids
- Microcentrifuge for individual 0.5 mL and 0.2 mL tubes
- 0.5–10 μ L pipette, 2–20 μ L pipette, 20–200 μ L pipette, 200–1000 μ L pipette
- Vortexer

- **Reagents**

- Isopropyl alcohol
- Low EDTA TE buffer, pH 8.0 (Affymetrix, Cat. #75793)

- **Supplies and Labware**

- Nuclease-free pipette tips
- 0.5 mL and 0.2 mL RNase-free microcentrifuge tubes
- Disposable gloves
- Lint-free wipes such as: Kimwipes™ or Berkshire Super PolX® 1200 Wipers (VWR, Cat. #21914)
- Canned air
- Ice bucket
- Cleaning solutions such as RNaseZAP® (Life Technologies, Cat. #AM9780) and DNA-OFF™ (MP Biomedicals, Cat. #QD0500)
- OPTIONAL: Products for DNase treatment and RNA template clean-up
 - QIAGEN MinElute Reaction Cleanup Kit (QIAGEN, Cat. #28204)
 - QIAGEN RNase-Free DNase Set (QIAGEN Cat. # 79254)
 - QIAGEN RNeasy Mini RNA Purification Kit (QIAGEN Cat. # 74104)
 - RNA Clean & Concentrator-5 (Zymo Research, Cat. #R1015)
 - QIAGEN RNeasy MinElute Cleanup Columns (QIAGEN, Cat. #74204)

To Order:

- Affymetrix, www.affymetrix.com
- Agilent, www.agilent.com
- Life Technologies, www.lifetechnologies.com
- MP Biomedicals, www.mpbio.com
- QIAGEN, www.qiagen.com
- VWR, www.vwr.com
- Zymo Research, www.zymoresearch.com

III. Planning the Experiment

A. Input RNA Requirements

1. RNA Quantity

Total RNA input must be between 100 ng and 150 ng. Inputs outside of this range may affect reaction stoichiometry, resulting in sub-optimal libraries. Low input amounts may result in insufficient yields depending on the requirements of your analytical platform. We strongly recommend quantitation of total RNA to ensure the minimum input requirement is met.

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. When preparing small amounts of RNA, we recommend using a commercially available system that does not require organic solvents. If using a method such as Trizol, we recommend column purification after isolation. One measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The A260:A280 ratio for RNA samples should be in excess of 1.8.

3. RNA Integrity

RNA samples of high molecular weight with little or no evidence of degradation will perform very well with this product. In many samples, RNA integrity can be determined using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip® or RNA 6000 Pico LabChip. These instruments provide sensitive and rapid ways of confirming RNA integrity prior to processing. While it is impossible to guarantee satisfactory results with all degraded samples, this system can work with many samples that are moderately degraded.

4. DNase Treatment

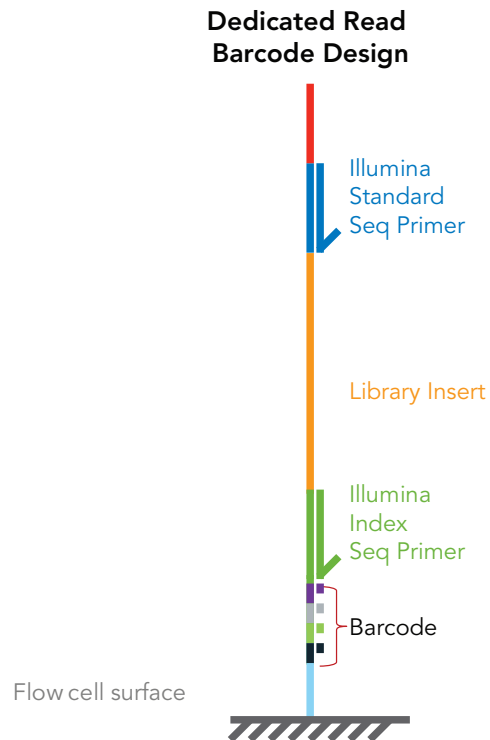
We highly recommend using DNase I-treated RNA with this system. The presence of genomic DNA in the RNA sample may have adverse effects on downstream analytical platforms. If the total RNA sample contains a significant amount of genomic DNA, it may be difficult to accurately quantify the true RNA concentration. The RNA input quantity may, therefore, be overestimated based on an absorbance measurement. Since it is important that RNA input be between 100 ng and 150 ng, we recommend using a DNase treatment that will remove genomic DNA during RNA purification. See Appendix A for recommendations and protocols.

B. Using the Encore SP+ Complete Library Systems on Illumina NGS Platforms

The Encore SP+ Complete Library Systems use a 'Dedicated Read' (DR) design with a second sequencing primer for multiplex sequencing. Figure 2 illustrates the DR multiplex barcode strategy.

III. Planning the Experiment

Figure 2. Dedicated read multiplexing strategy used by the Encore SP+ Complete Library Systems.



The Encore SP+ Complete Library Systems use the same approach to multiplexing found in the standard Illumina method. These libraries should be sequenced using the Illumina protocol for multiplex sequencing. The DR Barcode sequences are found in Appendix C of this user guide and must be entered into the Illumina software prior to the analysis.

C. Amplified Library Storage

Purified and amplified libraries may be stored at -20°C prior to proceeding with quantitation, cluster generation and sequencing.

IV. Protocol

A. Overview

The Encore SP+ Complete Library Systems use a fully automated process on the Mondrian SP+ Workstation. The total time to prepare a purified and enriched library, ready for quantitation and sequencing, is about 11 hours.

The Encore SP+ Complete Library Systems are sold only with DR barcodes. However, these barcodes may also be used for non-multiplex sequencing.

B. Protocol Notes

- The Encore SP+ Complete Library Systems are designed and intended for processing eight samples at a time. Do not attempt to prepare smaller volume master mixes or process fewer than eight samples using the Encore SP+ Complete Library Systems.
- We recommend the routine use of a positive control RNA. Especially the first time you set up a reaction, using a positive control will allow you to establish a performance baseline.
- Use the Nuclease-free Water provided with the kit (D1, green) or an alternate source of nuclease-free water. We do not recommend the use of DEPC-treated water with this protocol.
- Thaw the components used in each step and immediately place them on ice. Always keep thawed reagents and reaction tubes on ice unless otherwise instructed.
- After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve the precipitate 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.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer.
- When instructed to pipet mix a solution or master mix, gently aspirate and dispense a volume that is at least half the total volume of the solution/master mix.
- Components and reagents from other NuGEN kits and systems should not be used with the Encore SP+ Complete Library Systems.

C. Cartridge Quality Control Check

The Mondrian SP+ Cartridge QC protocol confirms the basic functionality of the Mondrian SP+ Cartridge prior to use. We recommend running this protocol with each cartridge prior to preparing or adding samples and reagents.

1. We recommend cleaning the contact pins on the deck of the workstation prior to running the Mondrian SP+ Cartridge QC Protocol. The cleaning procedure is detailed in Appendix C.
2. On the bench top, fill the cartridge with Filler Fluid via the Filler Fluid port, according to the instructions in Appendix B.

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3. Carefully transport the cartridge to the workstation and insert it into the deck.
4. Pull the cartridge lever of the Mondrian SP+ Workstation forward to the locked position.
5. Place the Cartridge Loading Guide on the cartridge or use the guide as a reference to identify the correct port for loading Elution Buffer.
6. Pipette 50 μ L of Elution Buffer into port E5 of the Mondrian SP+ Cartridge. Insert the pipette tip into the port all the way to the bottom of the cartridge. When the tip contacts the bottom, withdraw the pipette tip slightly to allow space for dispensing. Slowly depress the plunger to dispense the reagent, but do not depress the plunger completely (blow out) as this could introduce bubbles into the cartridge. Slowly withdraw the pipette tip from the port, performing a final blowout while the tip is within the upper cylinder of the port.

Note: Do NOT add any samples or other reagents to the cartridge at this time. Ensure that only Elution Buffer has been loaded.

7. Close the lid.
8. Select 'Run' on the touch screen menu, choose the Mondrian SP+ Cartridge QC protocol from the list of protocols and then select 'Next' to proceed to the Protocol Information screen.
9. Select 'Next' to proceed to the Run Information screen.
10. **Optional:** Enter run details on the Run Information screen. We recommend recording the serial number of the cartridge on this screen. The cartridge serial number can be found on the front of each cartridge for easy reference.
11. Select 'Next' and then select 'Start Run'.

The Mondrian SP+ Cartridge QC protocol will take about 15 minutes to complete. During this test, Elution Buffer droplets will be dispensed from the E5 port and transported around the cartridge prior to being returned to port E5. The purpose of this test is to confirm the basic performance of the cartridge.

IV. Protocol

At the end of the protocol, the instrument will display the Run Complete screen and one of the following messages:

MESSAGE	MEANING	NEXT STEP
Mondrian™ SP+ Cartridge passed. Continue to intended protocol.	No errors were detected. Droplet transport was normal. Cartridge is ready to run a protocol	Press "OK" on the Run Complete screen to return to the main menu. Proceed to the intended protocol.
Mondrian™ SP+ Cartridge failed. Remove cartridge from instrument deck and set aside prior to contacting NuGEN Technical Support.	A problem was detected with droplet transport within the cartridge.	Press 'OK' on the Run Complete screen to return to the main menu. Carefully remove the cartridge from the deck of the workstation, setting aside the cartridge for possible return to NuGEN. Begin with a new cartridge and contact NuGEN Technical Support to request a replacement for the failed cartridge.

D. Protocol for Encore SP+ Complete Library Systems on the Mondrian SP+ Cartridge

1. Sample Solution Mix Preparation

Prepare the Sample Solution Mix for loading onto the cartridge. This is done on a per sample basis and not as a master mix. You must prepare and process no fewer than eight samples on each cartridge.

Table 3. Sample Solution Mix (volumes given are for one sample)

COMPONENT	VOLUME
100 to 150 ng of total RNA	23.5 µL
Agencourt RNAClean XP beads	4.0 µL
Sample Concentration Solution	27.5 µL
Total volume	55 µL

Ensure the Agencourt RNAClean XP beads are at room temperature and completely resuspended prior to use. Keep the Agencourt RNAClean XP beads at room temp after

IV. Protocol

using them as they will be used again in the Agencourt RNAClean XP Bead Master Mix. Each Sample Solution Mix should be mixed well and incubated at room temperature (approximately 23°C) for 10 minutes. The above recipe is meant for a single sample. Prepare one Sample Solution Mix for each sample to be loaded into the cartridge.

2. Encore SP+ Complete Reagent Master Mix Preparation

Prepare Bead Binding Solution, Bead Wash Solution and Elution Buffer

1. Remove the Bead Binding Solution, Bead Wash Solution and Elution Buffer from room temperature reagent box.
2. Vortex to mix and spin down briefly. Leave tubes at room temperature while preparing the additional master mixes below.

Prepare DR Multiplex Barcode Adaptors

1. Thaw DR Multiplex Ligation Adaptor Mixes (L2V10DR-BC1–8 or L2V10DR-BC9–16) at room temperature.
2. Vortex well to mix and spin down briefly. Leave tubes at room temperature while preparing the additional master mixes below.

Prepare First Strand Primer Mix and First Strand cDNA Master Mix

1. Remove the First Strand Primer Mix (blue: A1 VER 11) from –20°C storage, spin down briefly and place on ice to thaw. Leave the First Strand Primer Mix on ice until ready to load into the Mondrian SP+ Cartridge. (See Step 2 of the Mondrian SP+ Cartridge Loading Instructions, below).
2. Thaw the First Strand Buffer Mix (blue: A2 VER 9) at room temperature and vortex to mix well. Briefly spin down the First Strand Enzyme Mix (A3 VER 5) and place on ice.
3. Prepare the First Strand cDNA Master Mix in a 0.5 mL microcentrifuge tube or 0.2 mL PCR tube according to the volumes shown in Table 4. Label the tube D2.

Table 4. First Strand cDNA Master Mix

COMPONENT	VIAL CAP	VOLUME
First Strand Buffer Mix (A2 VER 9)	Blue	7.7 µL
First Strand Enzyme Mix (A3 VER 5)	Blue	2.3 µL
Total volume		10.0 µL



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

IV. Protocol



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

Prepare Second Strand cDNA Master Mix

1. Remove the Second Strand Enzyme Mix (yellow: B2 VER 4) from -20°C storage, spin down briefly and place on ice to thaw.
2. Thaw the Second Strand Buffer Mix (yellow: B1 VER 7) at room temperature, vortex to mix well and spin down briefly. Place on ice.
3. Prepare the Second Strand cDNA Master Mix in a 0.5 mL microcentrifuge tube or 0.2 mL PCR tube according to the volumes shown in Table 5. Label the tube D3.

Table 5. Second Strand cDNA Master Mix

COMPONENT	VIAL CAP	VOLUME
Second Strand Buffer Mix (B1 VER 7)	Yellow	6.0 μL
Second Strand Enzyme Mix (B2 VER 4)	Yellow	4.0 μL
Total volume		10.0 μL

Prepare Strand Selection I Master Mix

1. Thaw the Strand Selection Buffer Mix I (purple: SS1 VER 2) at room temp and vortex to mix well. Keep Strand Selection Enzyme Mix I (purple: SS2) on ice.
2. Prepare the Strand Selection I Master Mix in a 0.5 mL microcentrifuge tube or a 0.2 mL PCR tube according to the volumes shown in Table 6. Label the tube D4.

Table 6. Strand Selection I Master Mix

COMPONENT	VIAL CAP	VOLUME
Strand Selection Buffer Mix I (SS1 VER 2)	Purple	8.5 μL
Strand Selection Enzyme Mix I (SS2)	Purple	1.5 μL
Total volume		10.0 μL



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

Prepare Ligation Master Mix

1. Thaw Ligation Buffer Mix (yellow: L1 VER 5) at room temp and vortex to mix well. Spin and place the Ligation Enzyme Mix (yellow: L3 VER 4) on ice.
2. Prepare the Ligation Master Mix in a 0.5 mL microcentrifuge tube or a 0.2 mL PCR tube according to the volumes shown in Table 7. Label the tube D5.

IV. Protocol



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

Table 7. Ligation Master Mix

COMPONENT	VIAL CAP	VOLUME
Ligation Buffer Mix (L1 VER 5)	Yellow	7.0 μ L
Ligation Enzyme Mix (L3 VER 4)	Yellow	3.0 μ L
Total volume		10.0 μL

Prepare End Repair Master Mix

1. Thaw the End Repair Buffer Mix (blue: ER1 VER 5) at room temp and vortex to mix well. Spin and place the End Repair Enzyme Mix (blue: ER2 VER 4) on ice.
2. Prepare the End Repair Master Mix in a 0.5 mL microcentrifuge tube or a 0.2 mL PCR tube according to the volumes shown in Table 8. Label the tube D6.

Table 8. End Repair Master Mix

COMPONENT	VIAL CAP	VOLUME
End Repair Buffer Mix (ER1 VER 5)	Blue	9.0 μ L
End Repair Enzyme Mix (ER2 VER 4)	Blue	1.0 μ L
Total volume		10.0 μL

Prepare Fragmentation Master Mix

1. Thaw the Fragmentation Buffer Mix (orange: F1 VER 3) at room temp and vortex to mix well. Spin and place the Fragmentation Enzyme Mix (orange: F2 VER 3) on ice.
2. Prepare the Fragmentation Master Mix in a 0.5 mL microcentrifuge tube or a 0.2 mL PCR tube according to the volumes shown in Table 9. Label the tube D7.

Table 9. Fragmentation Master Mix

COMPONENT	VIAL CAP	VOLUME
Fragmentation Buffer Mix (F1 VER 3)	Orange	8.7 μ L
Fragmentation Enzyme Mix (F2 VER 3)	Orange	1.3 μ L
Total volume		10.0 μL



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



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

IV. Protocol



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

Prepare Strand Selection II/Library Amplification Master Mix

1. Thaw the Strand Selection II/Amplification Buffer Mix (red: SS3/P1) and Amplification Primer Mix (red: P2 VER 9) at room temp and vortex to mix well. Spin and place the Strand Selection Enzyme Mix II (purple: SS4) and the Amplification Enzyme Mix (red: P3 VER 2) on ice.
2. Prepare the Strand Selection II/Library Amplification Master Mix in a 0.5 mL microcentrifuge tube or a 0.2 mL PCR tube according to the volumes shown in Table 10. Label the tube E1.

Table 10. Strand Selection II/Library Amplification Master Mix

COMPONENT	VIAL CAP	VOLUME
Strand Selection II/Amplification Buffer Mix (SS3/P1)	Red	17.5 μ L
Strand Selection Enzyme II Mix (SS4)	Purple	2.5 μ L
Amplification Primer Mix (P2 VER 9)	Red	2.0 μ L
Amplification Enzyme Mix (P3 VER 2)	Red	6.0 μ L
Total volume		28.0 μL

Prepare Bead Master Mix

1. Vortex the Agencourt RNAClean XP Beads, which have been resting at room temperature, and vortex to mix well. Keep at room temperature.
2. Remove the Bead Additive from the room temperature box and vortex to mix well. Keep at room temperature.
3. Prepare the Bead Master Mix in a 0.5 mL microcentrifuge tube or a 0.2 mL PCR tube according to the volumes shown in Table 11. Mix well by pipetting. Label the tube E2.

IV. Protocol

Important: Use caution to ensure no additional bead solution is transferred on the outside of the pipette tip.

! Mix well by pipetting.

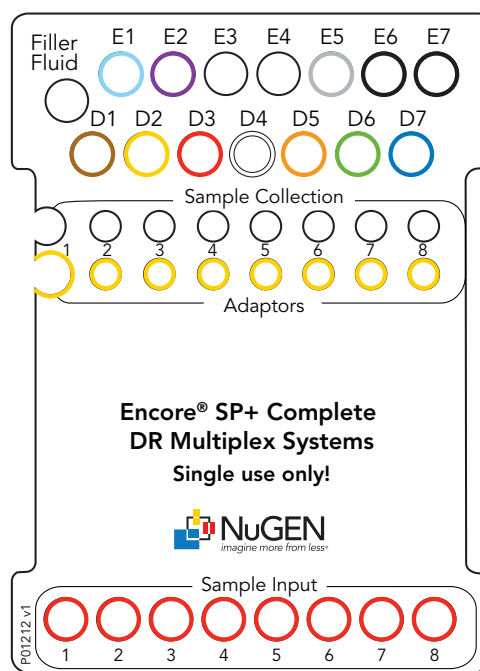
Table 11. Bead Master Mix

COMPONENT	VIAL CAP	VOLUME
Agencourt RNAClean XP Beads	N/A	35.0 μ L
Bead Additive	N/A	1.0 μ L
Total volume		36.0 μL

3. Mondrian SP+ Cartridge Loading Instructions

For detailed instructions on how to prepare the Mondrian SP+ Cartridge for reagent and sample loading, refer to Mondrian SP+ Cartridge User Guide (M01344).

Figure 3. Mondrian SP+ Cartridge loading guide for the Encore SP+ Complete Library System protocol.



IV. Protocol

Loading Reagents and Samples in the Mondrian SP+ Cartridge

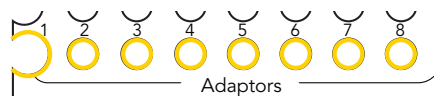
Using Figure 3 as a guide, follow the instructions below to load reagents into their appropriate cartridge ports.

- Use a 10 μL pipette to add DR Multiplex Ligation Adaptor Mixes, First and Second Strand Master Mixes, Ligation Master Mix, End Repair Master Mix, and Fragmentation Master Mix.

Important: It is critical that a 10 or 20 μL pipette be used for adding any volumes of $\leq 10 \mu\text{L}$. Do not use 2 μL pipettes to add reagents to the cartridge as they will not generate sufficient force to expel the reagents from the pipette tip into the Filler Fluid-containing cartridge.

- Use a 100 or 200 μL pipette for adding the Sample Solution Mixes, Strand Selection II/Library Amplification Master Mix, Bead Binding Solution, Elution Buffer, Bead Master Mix, and Bead Wash Solution.
- Load the ports in a steady manner to avoid overflow of the Filler Fluid.
- When adding samples and reagents, insert the pipette tip into the port until it contacts the bottom of the cartridge, then withdraw the pipette tip slightly to allow space for dispensing. Slowly depress the plunger to dispense the reagent, but do not depress the plunger completely (blow out) as this could introduce bubbles into the cartridge.

1. Load 1.5 μL of each DR Multiplex Ligation Adaptor Mix (L2V10DR-BC1–8 or L2V10DR-BC9–16) into the appropriate port (A1 through A8) matching the sample to be barcoded. Ensure that the reagent is carefully dispensed at the very bottom of the port. Adaptor ports (A1 through A8) are highlighted with yellow rims).












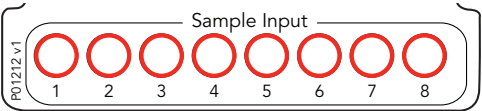
2. Load 25 μL Strand Selection II/Library Amplification Master Mix (tube E1) into E1. Port E1 is highlighted with a light blue rim.



3. Vortex the Agencourt RNAClean XP Bead Master Mix (tube E2) to ensure the beads are resuspended, and load 30 μL Bead Master Mix into E2. Port E2 is highlighted with a purple rim.



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4. Load 50 μ L Bead Binding Solution into E3 and E4. Ports E3 and E4 are not highlighted in any way.	E3 E4 
5. If Cartridge QC has been completed, skip this step and proceed to step 6. If Cartridge QC has not been completed, load 50 μ L Elution Buffer into E5. Port E5 is highlighted with a grey rim.	E5 
6. Load 50 μ L Bead Wash Solution into E6 and E7. Ports E6 and E7 are highlighted with a black rim.	E6 E7 
7. Load 8 μ L First Strand Primer Mix (blue: A1 VER 11) into D1. Port D1 is highlighted with a brown rim.	D1 
8. Load 8 μ L First Strand cDNA Master Mix (tube D2) into D2. Port D2 is highlighted with a yellow rim.	D2 
9. Load 8 μ L Second Strand cDNA Master Mix (tube D3) into D3. Port D3 is highlighted with a red rim.	D3 
10. Load 8 μ L Strand Selection I Master Mix (tube D4) into D4. Port D4 is highlighted with a white rim, outlined in black.	D4 
11. Load 8 μ L Ligation Master Mix (tube D5) into D5. Port D5 is highlighted with an orange rim.	D5 
12. Load 8 μ L End Repair Master Mix (tube D6) into D6. Port D6 is highlighted with a green rim.	D6 
13. Load 8 μ L Fragmentation Master Mix (tube D7) into D7. Port D7 is highlighted with a dark blue rim.	D7 
<p>14. Load 50 μL of sample mix (from Step 1, above) into S1–S8. Ensure that the 10-minute incubation step described above is performed prior to loading onto the cartridge. Sample input ports are highlighted in red.</p> 	

IV. Protocol

15. If the cartridge is not already inserted into the Mondrian SP+ Workstation deck, insert the cartridge into the deck and pull the locking lever forward to engage the cartridge with the control electronics and close the lid.

Mondrian SP+ Workstation Initialization Instructions

1. If the workstation is not already ON, locate the workstation ON/OFF switch at the back of the workstation and turn switch to the ON position.
2. Press the 'On' button on the front of the workstation.
3. Select 'Run' from the Main Menu touch screen, select the Encore SP+ Complete protocol from the Protocol Selection menu and press 'Next'. Follow the instructions on the screen to begin the run.

Library Collection from the Mondrian SP+ Cartridge

Note: A short instructional video on 'Mondrian Sample Collection' is available on the Mondrian Support page at www.nugen.com/mondriansupport.

1. Place eight 0.2 mL or 0.5 mL microcentrifuge tubes in a rack. Add 15 μ L of Nuclease-free Water (green: D1) to each tube.
2. Use a 100 or 200 μ L pipette set to 20 μ L. Depress the plunger on the pipette and insert the tip all the way to the bottom of the sample collection port, perpendicular to the cartridge so as to make a seal between the cartridge and the pipette tip.
3. Maintaining the seal formed between the pipette tip and the bottom of the cartridge, release the plunger. Immediately lift the pipette slightly off the bottom of the cartridge to release the seal, rapidly drawing Filler Fluid and the sample droplet into the pipette tip.
4. Examine the pipette tip to ensure that the appropriately sized droplet is suspended in the Filler Fluid in the pipette tip.
5. Repeat steps 2–4 above if the prepared library droplet was not captured the first time. If no sample droplet is observed, dispense the contents of the pipette tip into the sample collection vessel and repeat the sample collection procedure for the port. Note that the droplet occasionally breaks into pieces and becomes more difficult to observe. The excess Filler Fluid collected during the procedure will not interfere with downstream molecular processes.
6. Continue to the next sample collection port and repeat this process until all eight libraries have been collected and placed in separate tubes.
7. Remove the cartridge from the workstation and dispose of as appropriate in laboratory waste.

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8. Cap the library droplet-containing PCR tubes and vortex briefly to mix. Spin down briefly to bring the aqueous phase to the bottom of the tube. The Filler Fluid oil should remain as a separate layer above the aqueous phase.
9. Set a 20 μL pipette to 18 μL , and remove and discard the Filler Fluid oil layer. A very thin layer of Filler Fluid oil may remain in the tube on top of the aqueous phase. If the tube contains more than 20 μL of oil, repeat this oil removal step until little or no oil remains visible in the tube.
10. The liquid remaining in the tubes contains the purified and amplified RNA-Seq library. Store the library at -20°C or proceed immediately to Section VI. Quantitative and Qualitative Assessment of the Purified Amplified Libraries.

V. Quantitative and Qualitative Assessment of the Purified Amplified Libraries

A. Overview

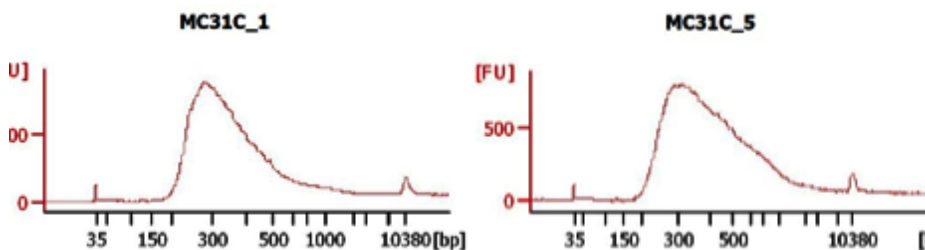
The Quantitative and Qualitative Assessment is used to confirm the average size of the library inserts and calculate library concentration. This information is required prior to loading the cluster-generation workstation.

B. Recommendations for the Quantitative Assessment of the Purified, Amplified RNA-Seq Libraries

1. Run a 1 μ L aliquot of the purified amplified libraries on the Bioanalyzer HS DNA Chip 1000. The typical distribution of 200 bp inserts is shown in Figure 4. Note that the actual size of the libraries appears closer to 300 bp due to the additional length conferred by the adaptors and primers ligated onto the initial \sim 200 bp fragment.

The distribution pattern of the amplified library inserts depends upon the initial fragment size used to construct the library. Following the Encore SP+ Complete protocol effectively eliminates adaptor dimer formation. Any deviation from the protocol may result in dimer formation, which will appear as low molecular weight spikes of approximately 120 bp on the Bioanalyzer trace.

Figure 4. Library Insert Distribution. Distribution of amplified, purified Encore SP+ Complete Library System library inserts on a Bioanalyzer HS DNA Chip 1000. Library MC31C_1 was made from 100 ng human brain total RNA and library MC31C_5 was made from 100 ng Universal Human Reference total RNA.



2. Validate the library as described in Illumina user guides for DNA library construction, e.g., Genomic DNA Sample Prep Manual (Cat. #FC-102-1001).

VI. Technical Support

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

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

In all other locations, contact your NuGEN distributor for technical support.

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A. DNase Treatment of RNA

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

1. Homogenize sample in RLT buffer including β -mercaptoethanol according to the type of sample as described in the RNeasy Mini Kit protocol.
2. Add 1X volume of 70% ethanol to the homogenized lysate, pipet up and down to mix sample well. Do not centrifuge.
3. Place an RNeasy mini column in a 2 mL collection tube.
4. Apply the sample (up to 700 μ L), including any precipitate that may have formed, to the column.
5. Close the tube gently and centrifuge for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
6. For volumes greater than 700 μ L, load aliquots onto the RNeasy column successively and centrifuge as before.
7. Add 350 μ L Buffer RW1 into the RNeasy mini column to wash, and centrifuge for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
8. Add 10 μ L DNase I to 70 μ L Buffer RDD. Gently invert the tube to mix.
Note: Other DNase I enzymes we recommend for use in this step are the Shrimp DNase (recombinant) from USB Corp. (use 10 μ L) or the DNase I (RNase- free) from New England BioLabs (use 10 μ L).
9. Pipet the DNase I incubation mix (80 μ L) directly onto the membrane inside the RNeasy mini column. Incubate at the bench top ($\sim 25^{\circ}\text{C}$) for 15 minutes.
10. Add 350 μ L Buffer RW1 into the RNeasy mini column and centrifuge for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash. Discard the flow-through.
11. Transfer the RNeasy column to a fresh 2 mL collection tube. Add 500 μ L Buffer RPE (with the added ethanol) to the RNeasy column.
12. Close the tube gently, and centrifuge for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
13. Add another 500 μ L Buffer RPE to the RNeasy column.
14. Close the tube gently, and centrifuge for 2 minutes at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
15. Transfer the RNeasy column to a new 1.5 mL collection tube.
16. Pipet 30–50 μ L Nuclease-free Water (D1, green cap) directly onto the RNeasy membrane.
17. Close the tube gently and centrifuge for 1 minute at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute.

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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 or USB Cat. #78316) with 1 µL rDNase (10 Units Roche Cat. #04716728001 or 2 Units USB Cat. #78311).
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.
4. After the DNase treatment, the sample must be purified. We recommend either of the two purification procedures below:

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

- Add 4 volumes (100 µL) of RNA binding buffer to the sample.
- Obtain one RNA Clean & Concentrator-5 Kit column and apply the sample to the column.
- Spin column for 30 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
- Add 200 µL wash buffer (with ethanol added as per vendor's specifications).
- After closing the column, spin for 30 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
- Add 200 µL fresh 80% ethanol, close cap and spin for 30 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
- Place the RNA Clean & Concentrator-5 Kit column in a fresh 1.5 mL collection tube.
- Add 10 µL Nuclease-free Water (green: D1) directly to the center of the filter in the tube and close the cap.

Important: Allow the nuclease-free water to come to room temperature prior to use!

- Spin for 1 minute at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to collect the purified RNA.

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

- Add 80 μ L ice-cold Nuclease-free Water (D1, green cap) to the sample on ice.
- Add 350 μ L Buffer RLT and mix by pipetting.
- Add 250 μ L 96 to 100% ethanol and mix thoroughly by pipetting.
- Place an RNeasy MinElute Spin Column into a 2 mL collection tube (one column per sample) and apply the 700 μ L sample to the column.
- After closing the column, spin for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
- Place the RNeasy MinElute Spin Column into a fresh 2 mL collection tube. Add 500 μ L Buffer RPE to the column and close the tube. Spin for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through, keeping the same collection tube.
- Add 500 μ L 80% ethanol to the RNeasy MinElute Spin Column and close the tube.
Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
- Spin for 2 minutes at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.
- 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 $\geq 8000 \times g$ ($\geq 10,000$ rpm) and discard the flow-through.
- Place the RNeasy MinElute Spin Column in a fresh 1.5 mL collection tube.
- Add 14 μ L Nuclease-free Water (D1, green cap) directly to the center of the filter in the tube and close the cap. Do not use cold water.
- Spin for 1 minute at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to collect the purified RNA.

B. Mondrian SP+ Cartridge Handling

Background

The Mondrian™ SP+ Cartridge is used with the Mondrian SP+ System and offers a convenient 8-sample batch size, simple reagent loading and easy sample recovery. Reagents are contained in discrete droplets encased within Filler Fluid, isolating the reactions from the lab environment and carryover contamination.

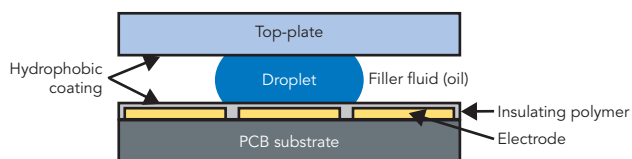
Construction

When prepared for use, cartridges consist of an oil layer (Filler Fluid) sandwiched between a PCB substrate and a clear top plate. The PCB substrate is patterned with insulated electrodes. By changing the relative voltages of the patterned electrodes,

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aqueous droplets are manipulated to perform complex assays. Droplets are dispensed from loading ports, transported to various locations on the cartridge, mixed, incubated and collected using only software control. Figure 5 shows a cutaway side view schematic of a cartridge.

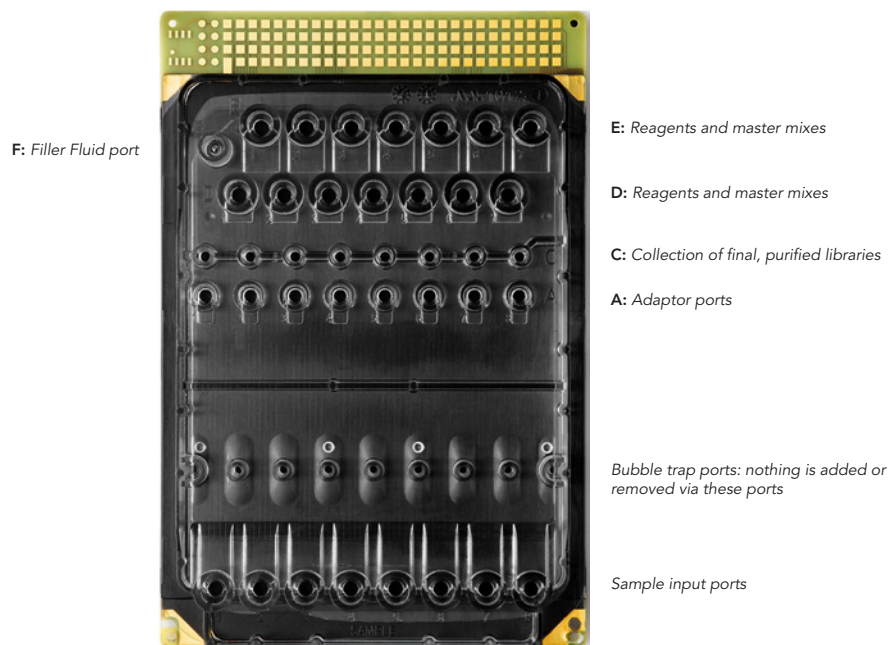
Figure 5. Cartridge cutaway side view.



Cartridges

Cartridges are one-time-use only. Cartridge re-use is not supported, even if a run is canceled prior to completion.

Figure 6. Top view of a Mondrian SP+ Cartridge. Sample ports along the bottom edge are labeled S1–8. Other reagent port rows are labeled A–E. Note that there is no row B. There are eight ports in rows A and C, and seven in rows D and E. The port labeled “F” is sized for a standard Luer-lock connector, through which the Filler Fluid is added to the cartridge. The cartridge electrodes are visible as rows of small gold squares at the top of the cartridge.



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Important: Take care when removing the cartridge from the packaging. Handle the cartridge by the sides and the non-electrode end. Do not handle the cartridge by the electrodes.

Filling the Cartridge

SP+ Cartridges must be filled with the provided Filler Fluid prior to loading with reagents. This process should be carried out on the bench top.

1. Use scissors to cut open the Mondrian SP+ Cartridge pouch. Remove the Mondrian SP+ Cartridge.

Note: Do **not** attempt to tear open the pouch as this may damage the cartridge.

2. Lay the cartridge on a level bench top and place the appropriate SP+ Library Systems Cartridge Loading Guide on the cartridge.
3. Remove the empty Filler Fluid tube and the SP+ Filler Fluid bottle from the NuGEN SP+ Library System kit.
4. Carefully grasp the tab on the red aluminium seal of the SP+ Filler Fluid bottle and pull up and back to remove the seal.
5. Remove the top and bottom caps from the empty Filler Fluid tube.
6. Attach the empty Filler Fluid tube to the SP+ Cartridge by inserting the tip of the Filler Fluid tube into the port marked "Filler Fluid" on the cartridge while gently twisting the tube in a clockwise direction to secure the Filler Fluid tube on the cartridge.
7. Remove the rubber stopper from the bottle of SP+ Filler Fluid.
8. Slowly pour the SP+ Filler Fluid into the Filler Fluid tube. Be careful not to pour too quickly as this may introduce bubbles into the fluid. Air bubbles entering the cartridge may interfere with droplet movement. During the cartridge filling process, you will begin to see the Filler Fluid rising in the sample and reagent ports. You should see Filler Fluid in all sample and reagent ports by the end of the filling process.
9. Once the entire volume of Filler Fluid has been delivered, carefully remove the Filler Fluid tube from the cartridge.



The SP+ Filler Fluid has been degassed and packaged in a sealed, single-use vial. Do not open the vial until ready for use.

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Cartridge Insertion

1. Raise the lid of the Mondrian SP+ Workstation to reveal the cartridge deck.



2. With the cartridge lever in the up position, as shown above, hold the cartridge level by the sides with the ports facing up and the gold colored electrodes pointing towards the workstation. Carefully insert the cartridge into the cartridge deck flush with the surface of the deck, allowing the side rails to guide it into place.



3. If you encounter any resistance to the cartridge moving into the deck, inspect for foreign objects that may be impeding the cartridge and remove them. If the cartridge appears to be catching on the edge of the heater bars or Peltier cooling plate, you may gently press down on them to allow the cartridge to move forward. The heater bars and cooling unit are designed to be slightly flexible in order to engage the cartridge. However, do not press too hard on the heater bars or Peltier unit as they may become misaligned.

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4. Confirm that the cartridge is fully inserted into the deck. When the cartridge is properly inserted, the electrodes are no longer visible and the three guide arrows etched into the front of the deck should be flush with the edge of the cartridge.



5. Pull the locking lever forward as illustrated above to engage the control electronics and lock the cartridge into place.

Note: Once a run has started, do not move the locking lever as this will result in an unrecoverable interruption of the running protocol. The Mondrian SP+ Workstation will not run with the lever in the up position.

C. Cleaning the Workstation Contact Pins

The interface pins on the workstation/cartridge interface may become dirty and cause performance issues. If this happens, you will need to clean the pins.

Materials

- Lint-free wipes such as Kimwipes or Super PolX 1200 Wipers. Do not use cotton or any material that may leave particles behind
- Isopropyl alcohol
- Canned air

Procedure

1. Turn the workstation off and unplug from the power source.
2. Soak the wipe in isopropyl alcohol.
3. Firmly rub all pins with the wipe.
4. Wait 2 minutes.
5. Blow the area dry with canned air.

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6. Plug the workstation back in and turn it on.

Note: We recommend cleaning the pins once a week, after any prolonged storage of the workstation, after a Filler Fluid spill, or if the Mondrian SP+ Cartridge QC protocol returns a 'Mondrian SP+ Cartridge Failed' or a 'Mondrian SP+ Cartridge Status Undetermined' message.

D. Sequences of the DR Barcodes in the Multiplexed Reactions

Barcode sequences for adaptors used in the Encore SP+ Complete Library Systems can be found in Tables 12 and 13. These six-nucleotide barcode adaptor sequences must be entered into the Illumina Sequencing System prior to parsing of the data. You may combine between 2 and 8 barcoded adaptors to allow for a range of multiplex sequencing.

Table 12. Barcode sequences for dedicated read (DR) adaptors used in Encore SP+ Complete Multiplex System 1–8 (Part No. 8151)

Ligation Adaptor Mix	6 nt Barcode Sequence as Read by the Sequencer	Barcode Adaptor Pairing for 2-plex Multiplex Sequencing	Barcode Adaptor Pairing for 3-plex Through 8-plex Multiplex Sequencing
L2V10DR-BC1	AACCAG	Duplex Set 1	One of the duplex sets from the column to the left must be used in combination with any of the other remaining six individual barcodes.
L2V10DR-BC2	TGGTGA		
L2V10DR-BC3	AGTGAG	Duplex Set 2	
L2V10DR-BC4	GCACTA		
L2V10DR-BC5	ACCTCA	Duplex Set 3	
L2V10DR-BC6	GTGCTT		
L2V10DR-BC7	AAGCCT	Duplex Set 4	
L2V10DR-BC8	GTCGTA		

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Table 13. Barcode sequences for dedicated read (DR) adaptors used in Encore SP+ Complete Multiplex System 9–16 (Part No. 8152)

Ligation Adaptor Mix	6 nt Barcode Sequence as Read by the Sequencer	Barcode Adaptor Pairing for 2-plex Multiplex Sequencing	Barcode Adaptor Pairing for 3-plex Through 8-plex Multiplex Sequencing
L2V10DR-BC9	AAGAGG	Duplex Set 1	One of the duplex sets from the column to the left must be used in combination with any of the other remaining six individual barcodes.
L2V10DR-BC10	GGAGAA		
L2V10DR-BC11	AGCATG	Duplex Set 2	
L2V10DR-BC12	GAGTCA		
L2V10DR-BC13	CGTAGA	Duplex Set 3	
L2V10DR-BC14	TCAGAG		
L2V10DR-BC15	CACAGT	Duplex Set 4	
L2V10DR-BC16	TTGGCA		

For additional information on the design strategy and sequence of the barcode adaptors please refer to the FAQs in Appendix D.

E. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the Encore SP+ Complete Library Systems?

The Encore SP+ Complete Library Systems kits provide all necessary buffers, primers, enzymes and purification beads.

Q2. Does this system contain a SPIA®-based amplification?

No. The cDNA is generated with selective primers, but without SPIA-based amplification.

Q3. I don't want to do multiplex sequencing but the Encore SP+ Complete Library Systems are available only with multiplex barcodes. How can I perform non-multiplexed sequencing?

The Encore SP+ Complete Library Systems kit can be used for non-multiplexed sequencing. If you do not wish to perform multiplex sequencing, follow the protocol as outlined in Section IV.D. but do not combine samples prior to sequencing.

Q4. What equipment is required or will be useful when using the Encore SP+ Complete Library Systems?

A comprehensive list of required and recommended equipment can be found in Section III.B. of this user guide.

Q5. Can I use the Encore SP+ Complete Library Systems with RNA from any organism?

This system has been designed specifically for higher vertebrates, such as human, mouse, rat, frog, zebrafish and chicken. Performance with RNA from other organisms may vary and should be empirically tested prior to beginning a project.

Q6. Do I need to use high-quality total RNA?

The Encore SP+ Complete Library Systems are 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 yields and may encounter affected sequencing metrics. Degraded samples must be empirically tested to determine their performance with the Encore SP+ Complete Library Systems.

Q7. Do I need to perform an rRNA depletion or Poly(A) enrichment step before processing RNA samples with the Encore SP+ Complete Library Systems?

No. The system is designed to use total RNA as input. rRNA depletion or Poly(A) enrichment is not necessary.

Q8. How much total RNA do I need for Encore SP+ Complete?

100–150 ng total RNA input.

Q9. How does your protocol improve the efficiency of ligation and avoid adaptor dimer formation?

The Encore SP+ Complete Library Systems use optimized chemistries to increase the efficiency of blunt-end adaptor ligation and minimize the amount of adaptor dimer in the library.

Q10. How does your protocol enable strand retention?

The Encore SP+ Complete Library Systems use targeted degradation of an incorporated modified nucleotide to ensure library inserts all carry the same directionality.

Q11. What percentage of rRNA reads can I expect in my data?

Use of high quality higher vertebrate total RNA, including human, rat and mouse, has routinely achieved <25% of total reads aligning to rRNA and mitochondrial sequences. *In silico* analysis suggests similar percentages can be achieved with chicken and zebrafish total RNA. Use with lower eukaryotes, such as *D. melanogaster* or *C. elegans* may result in higher rRNA read percentages.

Q12. Will this system capture small RNA species?

The Encore SP+ Complete Library Systems were designed to create libraries of intact mRNA species. We have not tested any modifications to Encore SP+ Complete Library Systems protocol that enable the capture of smaller RNA species.

Q13. Can contaminating genomic DNA interfere with the Encore SP+ Complete Library Systems performance?

Yes. When using purified total RNA samples, contaminating genomic DNA may be incorporated into libraries. For this reason we recommend DNase treatment during RNA purification. For an explanation of DNase requirements see Section III.A.4. For guidelines on DNase treatment of RNA samples, refer to Appendix A.

Q14. Is this system compatible with target enrichment strategies?

Yes. The Encore SP+ Complete Library Systems are compatible with downstream target enrichment. Specific blocking sequences may be necessary, depending upon the selection technology. Please contact NuGEN Technical Support for additional information.

Q15. How should I measure my amplified cDNA product yield? Can I use an Agilent Bioanalyzer to evaluate the product?

Yes. Refer to Section VI of the user guide for guidelines on Quantitative and Qualitative Assessment.

Q16. Where can I safely stop in the protocol?

Purified and amplified libraries collected from the Mondrian SP+ Workstation can be placed in short-term storage at -20°C prior to proceeding with quantitation, cluster generation and sequencing.

Q17. Does NuGEN provide reagents for performing the fragmentation step of the protocol?

Yes, the Encore SP+ Complete Library Systems provide the cDNA fragmentation reagents as part of the kit.

Q18. Can I use alternative fragmentation methods, such as Covaris shearing, instead of using the fragmentation reagents provided in the kit?

NuGEN does not support or recommend the use of alternative fragmentation methods, such as Covaris shearing of RNA, in lieu of the fragmentation reagents provided in the Encore SP+ Complete Library Systems. Using alternative fragmentation methods will impact the performance of the system and invalidate the warranty on the product.

Q19. How much material should I load into the cBot?

Please follow manufacturer's recommendations for library QC, quantitation, balancing and loading of the amplified library on the cBot.

Q20. Do the Encore SP+ Complete Library Systems work with the Illumina Cluster Station, predecessor to the cBot instrument?

Yes. The systems are also compatible with the Illumina Cluster Station.

Q21. What kind of error correction is used to minimize the impact of sequencing errors in the barcodes?

Each of the DR Barcode sequences shown in Tables 12 and 13 have an edit distance of three, meaning that three 'events', such as nucleotide substitution, insertion, or mutation, must occur before any of the barcodes is converted into another barcode sequence. For example, three different events/ edits would have to occur in the sequence of barcode L2V10DR-BC4 before its sequence would be the same as any other barcode included in the Encore SP+ Complete Library Systems. Users must decide whether or not to configure their barcode parsing software to accept barcode sequences with mismatches between the reference sequence of the barcode from Tables 12 and 13 and the actual sequence results. For a detailed explanation of the error correction approach used for the DR barcodes, please refer to: Faircloth BC, Glenn TC (2012) Not All Sequence Tags Are Created Equal: Designing and Validating Sequence Identification Tags Robust to Indels. *PLoS ONE* 7(8): e42543. doi:10.1371/journal.pone.0042543.

Q22. What kind of sequencing primers can I use with libraries generated using the Encore SP+ Complete Library Systems?

The Encore SP+ Complete Library Systems are designed for use with the standard Illumina sequencing primers for both single end and paired-end sequencing applications.

Q23. Can the Encore SP+ Complete Library Systems be used with paired-end sequencing?

Yes. They can be used for both single end and paired-end sequencing. Special consideration should be given to the expected insert size in the paired-end assay. The workflow generates libraries with an average insert size of 200 bases. This length corresponds to the expected distance between the 5'-most and 3'-most coordinates of paired-end reads.

Q24. Do I need to perform a separate sequencing read for the multiplex adaptors?

Yes. A separate sequencing read is required to sequence the multiplex adaptors. The Encore SP+ Complete Library Systems barcoding strategy requires a separate, "dedicated" sequencing read to identify the barcode.

VII. Appendix

Q25. How many bases do the Encore SP+ Complete Library Systems multiplex adaptors add to the library?

The adaptors add 122 bp to the library inserts generated from the Encore SP+ Complete Library Systems.

Q26. What is the expected yield of the enriched DNA library using the Encore SP+ Complete Library Systems?

The expected yield is between 100–300 ng, depending on the quality and quantity of the input RNA. This amount is a large excess over the amount of DNA required for use on the cBot or Cluster Station.

Q27. Can I use standard alignment algorithms to analyze strand-specific sequencing data?

Yes. Strand-specific reads can be processed and mapped to reference sequences using the same methods used for other RNA-Seq libraries. Of the reads from Encore SP+ Complete Library Systems libraries, >90% will align in the sense strand orientation relative to the RNA template.

F. Update History

This document, the Encore SP+ Complete Library System User Guide (M01331 v4) is an update to address the following topics:

Description	Section	Page(s)
Updated volumes in Table 11	IV.D.	17
Updated mixing directions for Bead Master Mix	IV.D.	19



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