Encore® Complete RNA-Seq Library SystemsRNA

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USER GUIDE

Encore® Complete RNA-Seq Library Systems

PART NOS. 0311, 0312, 0313, 0333 AND 0334

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I. Introduction

A. Background

The Encore® Complete RNA-Seq Library Systems provide an end-to-end solution for strand-specific RNA-Seq library construction using as little as 100 ng of total RNA, or poly(A)+ RNA that has been isolated from this amount of total RNA. The core technology used in this product enriches for non-rRNA in NGS libraries during cDNA synthesis and can be applied to transcriptomes extracted from a broad range of higher eukaryotes. The cDNA synthesis is carried out using proprietary primers to create double-stranded cDNA which retains RNA strand 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 in the same kit. The Encore Complete RNA-Seq IL Multiplex Systems 1–8 and 9–16 and DR Multiplex Systems 1–8 and 9–16 provide optional barcoding to further optimize efficiencies and cost savings in transcriptome sequencing.

The Encore 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 also 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 (see Figure 1).

NuGEN offers three configurations of the Encore Complete RNA-Seq Library Systems:

- **Encore Complete RNA-Seq Library System (Part No. 0311)** contains reagents for production of non-barcoded libraries.
- **Encore Complete RNA-Seq IL Multiplex Systems 1–8 (Part No. 0312) and 9–16 (Part No. 0313)** each provide eight inline barcode adaptors. These two kits should be used together to prepare libraries for 16-plex sequencing.
- **Encore Complete RNA-Seq DR Multiplex Systems 1–8 (Part No. 0333) and 9–16 (Part No. 0334)** each provide 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.

The Encore Complete RNA-Seq Library System (Part No. 0311) contains reagents for production of non-barcoded libraries starting with total RNA. The Encore Complete RNA-Seq IL Multiplex Systems 1–8 (Part No. 0312) and 9–16 (Part No. 0313) each provide eight unique barcoded adaptors to be used in combination to prepare libraries for 16-plex sequencing. The Encore Complete RNA-Seq DR Multiplex Systems 1–8 (Part No. 0333) and 9–16 (Part No. 0334) each provide eight unique dedicated read adaptors to prepare libraries for multiplex sequencing using a dedicated read design strategy with a second sequencing primer. In combination each of these multiplex kit pairs enables up to 16-plex sequencing using either inline or dedicated read barcode designs.

NuGEN offers a growing number of products for key applications on leading NGS platforms, including the Ovation® RNA-Seq System V2 (Part No. 7102), Ovation RNA-Seq FFPE System (Part No. 7150), Ovation 3′-DGE System (Part No. 7200), Ovation WGA

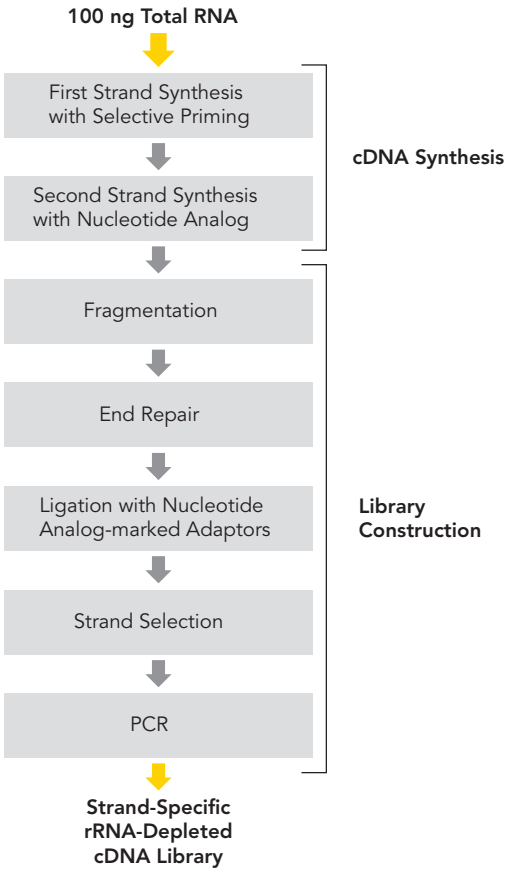
I. Introduction

FFPE System (Part No. 6200), Encore ds-DNA Module (Part No. 2500), Encore Library Spike-in Controls (Part No. 0310), Encore Rapid Library Systems (Part Nos. 0316, 0317, 0318, 0319, 0320, 0328), and Ovation Ultralow Library Systems (Part Nos. 0303, 0304, 0305, 0329, 0330, 0331) and Encore Target Capture Module (Part No. 0332).

B. Workflow

As shown in Figure 1, the streamlined workflow consists of double-stranded cDNA generation using selective priming, fragmentation of double-stranded cDNA, end repair to generate blunt ends, adaptor ligation, strand selection via nucleotide analog-targeted degradation and PCR amplification to produce the final library. The entire workflow, including fragmentation, can be completed in as little as seven hours, and yields DNA libraries ready for cluster formation for either single read or paired-end sequencing. As an alternative to direct input of total RNA, researchers who are only interested in mature coding transcripts can first isolate poly(A)+ RNA from 100 to 500 ng total RNA for input to the Encore Complete RNA-Seq Library Systems.

Figure 1. Encore Complete RNA-Seq Library Systems workflow.



I. Introduction

C. Performance Specifications

The Encore Complete RNA-Seq Library Systems are designed to generate DNA libraries suitable for either single read or paired-end sequencing on Illumina Genome Analyzer IIx/IIe (GAII.), MiSeq, HiScan SQ or HiSeq 2000/2500 NGS platforms. They are fast, simple and robust systems capable of starting with 100 ng of total RNA to generate libraries suitable for use on the Illumina cBot Cluster Generation System in about seven hours.

D. Quality Control

Every lot of the Encore Complete RNA-Seq Library Systems undergoes functional testing to meet specifications for library generation performance.

E. Storage and Stability

The Encore Complete RNA-Seq Library Systems are shipped on dry ice and should be unpacked immediately upon receipt.

Note: This product contains components with multiple storage temperature requirements. Vials labeled Agencourt® RNAClean® XP Beads (clear cap) should be removed from the top of the shipping carton upon delivery and stored at 4°C. All other components should be stored at –20°C on internal shelves of a freezer without a defrost cycle.

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

F. Material Safety Data Sheet (MSDS)

An MSDS for this product is available from NuGEN Technical Service by calling 888-654-6544, by sending an email to techserv@nugeninc.com or by download at www.nugeninc.com/nugen/index.cfm/resources/support/user-guides/.

II. Components

A. Reagents Provided

Table 1. Encore Complete RNA-Seq Library System Reagents (Part No. 0311-08)

0311-08 PART NUMBER	0311-08 DESCRIPTION	0311-08 VIAL NUMBER
S01504	First Strand Primer Mix	A1 VER 9
S01505	First Strand Buffer Mix	A2 VER 8
S01506	First Strand Enzyme Mix	A3 VER 4
S01507	Second Strand Buffer Mix	B1 VER 6
S01508	Second Strand Enzyme Mix	B2 VER 4
S01553	Second Strand Stop Buffer	B3 VER 2
S01509	End Repair Buffer Mix	ER1 VER 4
S01510	End Repair Enzyme Mix	ER2 VER 4
S01466	Ligation Buffer Mix	L1 VER 4
S01517	Ligation Adaptor Mix	L2 VER 6
S01467	Ligation Enzyme Mix	L3 VER 4
S01511	Strand Selection Buffer Mix I	SS1
S01512	Strand Selection Enzyme Mix I	SS2
S01513	Strand Selection Buffer Mix II	SS3
S01514	Strand Selection Enzyme Mix II	SS4
S01515	Amplification Buffer Mix	P1 VER 3
S01543	Amplification Primer Mix	P2 VER 3
S01316	Amplification Enzyme Mix	P3
S01317	DMSO	P4
S01001	Nuclease-free Water	D1
S01307	Agencourt RNAClean XP Beads	—

II. Components

Table 2. Encore Complete RNA-Seq IL Multiplex System 1–8 Reagents (Part No. 0312-32)

0312-32 PART NUMBER	0312-32 DESCRIPTION	0312-32 VIAL NUMBER
S01527	First Strand Primer Mix	A1 VER 9
S01528	First Strand Buffer Mix	A2 VER 8
S01529	First Strand Enzyme Mix	A3 VER 4
S01530	Second Strand Buffer Mix	B1 VER 6
S01531	Second Strand Enzyme Mix	B2 VER 4
S01554	Second Strand Stop Buffer	B3 VER 2
S01532	End Repair Buffer Mix	ER1 VER 4
S01533	End Repair Enzyme Mix	ER2 VER 4
S01534	Ligation Buffer Mix	L1 VER 4
S01518	Ligation Adaptor Mix	L2V6-BC1
S01519	Ligation Adaptor Mix	L2V6-BC2
S01520	Ligation Adaptor Mix	L2V6-BC3
S01521	Ligation Adaptor Mix	L2V6-BC4
S01522	Ligation Adaptor Mix	L2V6-BC5
S01523	Ligation Adaptor Mix	L2V6-BC6
S01524	Ligation Adaptor Mix	L2V6-BC7
S01525	Ligation Adaptor Mix	L2V6-BC8
S01535	Ligation Enzyme Mix	L3 VER 4
S01536	Strand Selection Buffer Mix I	SS1
S01537	Strand Selection Enzyme Mix I	SS2
S01538	Strand Selection Buffer Mix II	SS3
S01539	Strand Selection Enzyme Mix II	SS4
S01540	Amplification Buffer Mix	P1 VER 3

Encore Complete RNA-Seq IL Multiplex System 1–8 Reagents (Part No. 0312-32), *continued*		
0312-32 PART NUMBER	**0312-32 DESCRIPTION**	**0312-32 VIAL NUMBER**
S01544	Amplification Primer Mix	P2 VER 3
S01541	Amplification Enzyme Mix	P3
S01542	DMSO	P4
S01001	Nuclease-free Water	D1
S01502	Agencourt RNAClean XP Beads	—

Table 3. Encore Complete RNA-Seq IL Multiplex System 9–16 Reagents (Part No. 0313-32)

0313-32 PART NUMBER	0313-32 DESCRIPTION	0313-32 VIAL NUMBER
S01527	First Strand Primer Mix	A1 VER 9
S01528	First Strand Buffer Mix	A2 VER 8
S01529	First Strand Enzyme Mix	A3 VER 4
S01530	Second Strand Buffer Mix	B1 VER 6
S01531	Second Strand Enzyme Mix	B2 VER 4
S01554	Second Strand Stop Buffer	B3 VER 2
S01532	End Repair Buffer Mix	ER1 VER 4
S01533	End Repair Enzyme Mix	ER2 VER 4
S01534	Ligation Buffer Mix	L1 VER 4
S01545	Ligation Adaptor Mix	L2V6-BC9
S01546	Ligation Adaptor Mix	L2V6-BC10
S01547	Ligation Adaptor Mix	L2V6-BC11
S01548	Ligation Adaptor Mix	L2V6-BC12
S01549	Ligation Adaptor Mix	L2V6-BC13

Encore Complete RNA-Seq IL Multiplex System 9–16 Reagents (Part No. 0313-32), *continued*		
0313-32 PART NUMBER	**0313-32 DESCRIPTION**	**0313-32 VIAL NUMBER**
S01550	Ligation Adaptor Mix	L2V6-BC14
S01551	Ligation Adaptor Mix	L2V6-BC15
S01552	Ligation Adaptor Mix	L2V6-BC16
S01535	Ligation Enzyme Mix	L3 VER 4
S01536	Strand Selection Buffer Mix I	SS1
S01537	Strand Selection Enzyme Mix I	SS2
S01538	Strand Selection Buffer Mix II	SS3
S01539	Strand Selection Enzyme Mix II	SS4
S01540	Amplification Buffer Mix	P1 VER 3
S01544	Amplification Primer Mix	P2 VER 3
S01541	Amplification Enzyme Mix	P3
S01542	DMSO	P4
S01001	Nuclease-free Water	D1
S01502	Agencourt RNAClean XP Beads	—

Table 4. Encore Complete RNA-Seq DR Multiplex System 1–8 Reagents (Part No. 0333-32)

0333-32 PART NUMBER	0333-32 DESCRIPTION	0333-32 VIAL NUMBER
S01527	First Strand Primer Mix	A1 VER 9
S01528	First Strand Buffer Mix	A2 VER 8
S01529	First Strand Enzyme Mix	A3 VER 4
S01530	Second Strand Buffer Mix	B1 VER 6
S01531	Second Strand Enzyme Mix	B2 VER 4

II. Components

Encore Complete RNA-Seq DR Multiplex System 1–8 Reagents (Part No. 0333-32), *continued*		
0333-32 PART NUMBER	**0333-32 DESCRIPTION**	**0333-32 VIAL NUMBER**
S01554	Second Strand Stop Buffer	B3 VER 2
S01532	End Repair Buffer Mix	ER1 VER 4
S01533	End Repair Enzyme Mix	ER2 VER 4
S01534	Ligation Buffer Mix	L1 VER 4
S01609	Ligation Adaptor Mix	L2V6DR-BC1
S01610	Ligation Adaptor Mix	L2V6DR-BC2
S01611	Ligation Adaptor Mix	L2V6DR-BC3
S01612	Ligation Adaptor Mix	L2V6DR-BC4
S01613	Ligation Adaptor Mix	L2V6DR-BC5
S01614	Ligation Adaptor Mix	L2V6DR-BC6
S01615	Ligation Adaptor Mix	L2V6DR-BC7
S01616	Ligation Adaptor Mix	L2V6DR-BC8
S01535	Ligation Enzyme Mix	L3 VER 4
S01536	Strand Selection Buffer Mix I	SS1
S01537	Strand Selection Enzyme Mix I	SS2
S01538	Strand Selection Buffer Mix II	SS3
S01539	Strand Selection Enzyme Mix II	SS4
S01540	Amplification Buffer Mix	P1 VER 3
S01608	Amplification Primer Mix	P2 VER 6
S01541	Amplification Enzyme Mix	P3
S01542	DMSO	P4
S01001	Nuclease-free Water	D1
S01502	Agencourt RNAClean XP Beads	—

II. Components

Table 5. Encore Complete RNA-Seq DR Multiplex System 9–16 Reagents (Part No. 0334-32)

0334-32 PART NUMBER	0334-32 DESCRIPTION	0334-32 VIAL NUMBER
S01527	First Strand Primer Mix	A1 VER 9
S01528	First Strand Buffer Mix	A2 VER 8
S01529	First Strand Enzyme Mix	A3 VER 4
S01530	Second Strand Buffer Mix	B1 VER 6
S01531	Second Strand Enzyme Mix	B2 VER 4
S01554	Second Strand Stop Buffer	B3 VER 2
S01532	End Repair Buffer Mix	ER1 VER 4
S01533	End Repair Enzyme Mix	ER2 VER 4
S01534	Ligation Buffer Mix	L1 VER 4
S01617	Ligation Adaptor Mix	L2V6DR-BC9
S01618	Ligation Adaptor Mix	L2V6DR-BC10
S01619	Ligation Adaptor Mix	L2V6DR-BC11
S01620	Ligation Adaptor Mix	L2V6DR-BC12
S01621	Ligation Adaptor Mix	L2V6DR-BC13
S01622	Ligation Adaptor Mix	L2V6DR-BC14
S01623	Ligation Adaptor Mix	L2V6DR-BC15
S01624	Ligation Adaptor Mix	L2V6DR-BC16
S01535	Ligation Enzyme Mix	L3 VER 4
S01536	Strand Selection Buffer Mix I	SS1
S01537	Strand Selection Enzyme Mix I	SS2
S01538	Strand Selection Buffer Mix II	SS3
S01539	Strand Selection Enzyme Mix II	SS4
S01540	Amplification Buffer Mix	P1 VER 3

II. Components

Encore Complete RNA-Seq DR Multiplex System 9–16 Reagents (Part No. 0334-32), *continued*		
0334-32 PART NUMBER	**0334-32 DESCRIPTION**	**0334-32 VIAL NUMBER**
S01608	Amplification Primer Mix	P2 VER 6
S01541	Amplification Enzyme Mix	P3
S01542	DMSO	P4
S01001	Nuclease-free Water	D1
S01502	Agencourt RNAClean XP Beads	—

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - Covaris S-series Sonication System
  - Agilent 2100 Bioanalyzer or materials and equipment for electrophoretic analysis of nucleic acids
  - 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 (Sigma-Aldrich, Cat. #E7023), for purification steps
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - 0.2 mL individual thin-wall PCR tubes or 8 X 0.2 mL strip PCR tubes or 0.2 mL thin-wall PCR plates
  - Magnetic separation device options:
    - Agencourt SPRIPlate Ring Super Magnet Plate (Beckman Coulter Genomics, Cat. #A32782)
    - Agencourt SPRIStand (Beckman Coulter Genomics, Cat. #A29182)
    - MagnaBot® II Magnetic Separation Device (Promega, Cat. #V8351)
    - DynaMag™-96 Bottom, Side, or Side Skirted (Invitrogen, Cat. #123-32D, 123-31D, 120-27)
  - Disposable gloves

II. Components

- Kimwipes
- Ice bucket
- Cleaning solutions such as DNA-OFF™ (MP Biomedicals, Cat. #QD0500)
- OPTIONAL: MinElute Reaction Cleanup Kit (QIAGEN, Cat. #28204)
- OPTIONAL: PhiX Control (Illumina, Cat. #FC-110-3001)

To Order

- Affymetrix USB Products, www.affymetrix.com
- Beckman Coulter Genomics, www.beckmangenomics.com
- Covaris, www.covarisinc.com
- Illumina, www.illumina.com
- Invitrogen, www.invitrogen.com
- MP Biomedicals, www.mpbio.com
- Promega, www.promega.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com

III. Planning the Experiment

A. Input RNA Requirements

1. RNA Quantity

Total RNA input must be between 100 ng and 150 ng. If poly(A)+ RNA is used, this material should be isolated from 100 to 500 ng of total RNA and the entire poly(A)+ fraction input to the workflow. Inputs outside of this range may affect reaction stoichiometry, resulting in sub-optimal libraries. Lower input amounts will potentially result in insufficient yields depending on the requirements of the analytical platform. We strongly recommend quantification 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. The instrument provides a sensitive and rapid way 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.

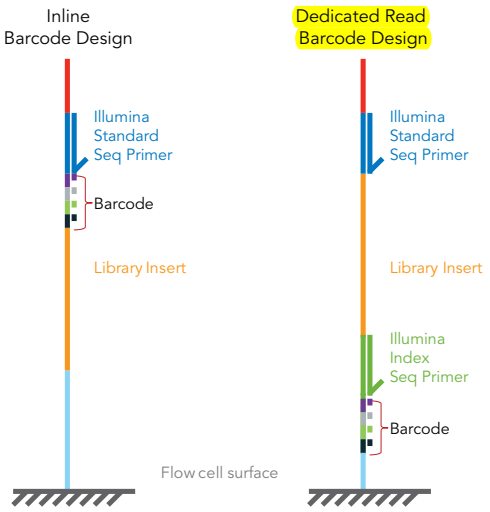
4. DNase Treatment

It is highly recommended to use DNase I-treated RNA with this system. The presence of genomic DNA in the RNA sample may potentially have adverse effects on downstream analytical platforms. Genomic DNA may be processed along with the RNA. Also, 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.

B. Using Encore Complete RNA-Seq Libraries on Illumina NGS Platforms

The Encore Complete RNA-Seq Library Systems use two different approaches for multiplex sequencing, providing users the flexibility to choose between an inline barcode strategy or a dedicated read (second sequencing primer) strategy. Figure 2 depicts the two different multiplex barcode strategies.

Figure 2. Multiplexing strategy used by the Encore Complete RNA-Seq Library Systems.



Depending on the multiplex kits being used and the number of libraries run in the same sequencing lane, there are important considerations for setting up the experiments, as outlined below.

1. Encore Complete RNA-Seq IL Multiplex Systems

The Encore Complete RNA-Seq IL Multiplex Systems 1–8 and 9–16 employ a novel approach to multiplexing that differs from the standard Illumina method in that the barcode sequence is read from the initial cycles of the first read (Figure 2), as opposed to a second independently primed read, as in the Illumina multiplexing strategy. We recommend combining these two kits and performing no less than a 16-plex experiment. When using the Encore Complete RNA-Seq IL Multiplex Systems 1–8 and 9–16, reads are parsed offline after the sequencing run is completed. It is recommended to use barcodes 1–16 to ensure sufficient diversity in the first few bases to enable efficient base calling.

We recommend sequencing libraries produced with the Encore Complete RNA-Seq IL Multiplex Systems 1–8 and 9–16 using the Illumina standard recipe (the workflow for

! *We recommend combining these two kits and performing no less than a 16-plex experiment.*

non-multiplexed sequencing), rather than the multiplex recipe, when sequencing on Illumina NGS platforms. This applies to both single read and paired-end runs.

If it is necessary to sequence an Encore Complete RNA-Seq IL Multiplex Systems library using the Illumina multiplex recipe, the barcodes will be preserved in read 1. However, the index read—a separate read carried out after read 1—will not generate meaningful barcode data in Encore Complete RNA-Seq IL Multiplex System libraries.

It may be necessary to run Encore Complete RNA-Seq IL Multiplex System libraries on the same flow cell with Illumina multiplexed libraries from time to time. If this is the case, use the multiplex recipe then parse the multiplexed reads offline after the run. In some cases the reads generated by multiplexed libraries may be sorted into an "Undetermined indices" folder by the Illumina system because the Encore Complete RNA-Seq IL Multiplex System libraries do not employ the Illumina multiplexing strategy.

2. Encore Complete RNA-Seq DR Multiplex Systems

The Encore Complete RNA-Seq DR Multiplex Systems 1–8 and 9–16 use the same approach to multiplexing found in the standard Illumina method and have no minimum multiplexing requirement. 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

Amplified libraries may be stored at –20°C.

D. Data Analysis and Parsing Multiplex Libraries

Data analysis for Next Generation Sequencing is an evolving field. The number of analysis strategies and software tools is growing rapidly. The specific analysis workflow for a given experiment will depend on many variables, including the type of experiment (DNA-Seq, Exome-Seq, RNA-Seq, etc.) and the goals of the particular study.

The first step in the post-sequencing data analysis for Encore Complete RNA-Seq IL Multiplex Systems 1–8 and 9–16 reads is to parse the reads by sample, based on the barcode sequence. For the inline barcode system, this must be done offline after the sequencing run. Select an analysis software package that includes a barcode splitter function capable of parsing the barcodes used in the Encore Complete RNA-Seq IL

Multiplex Systems 1–8 and 9–16. Refer to the FAQ section in Appendix E of this user guide for specific recommendations on inline barcode splitter software.

For the Encore Complete RNA-Seq DR Multiplex Systems 1–8 and 9–16, follow the recommendations in the Illumina technical support documentation on parsing barcodes. The sequence of the Encore Complete RNA-Seq DR Multiplex Systems 1–8 and 9–16 barcodes will need to be entered prior to parsing. These sequences are found in Appendix E of this user guide.

Once the data have been parsed according to sample, additional sample specific data analysis may be employed according to the requirements of the experiment.

IV. Protocol

A. Overview

The library preparation process used in the Encore Complete RNA-Seq Library Systems is performed in the following stages:

1. cDNA generation	1.5 hours
2. Fragmentation and purification	1.25 hour
3. End repair	0.75 hours
4. Adaptor ligation	0.5 hours
5. Strand selection and purification	1.5 hours
6. Library amplification and purification	1.5 hours
Total time to prepare amplified library	**~7 hours**

Components in the Encore Complete RNA-Seq Library Systems are color-coded, with each color linked to a specific stage of the process. Each stage requires making a master mix then adding it to the reaction, followed by incubation. Master mixes are prepared by mixing components provided for that stage.

B. Protocol Notes

- We recommend the routine use of a positive control RNA. Especially the first time you set up a reaction, the use of a positive control RNA will allow the establishment of a baseline of performance and provide the opportunity to become familiar with the bead purification steps. 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 no-template control (NTC) is recommended to monitor the work environment for potential carryover of previous libraries.
- Set up no fewer than 4 reactions at a time. This ensures sufficient reagent recoveries for the full nominal number of amplifications from the kit. Making master mixes for fewer than 4 samples at a time may affect reagent recovery volumes.
- Thaw components used in each step and immediately place them on ice. It is best not to thaw all reagents at once.
- 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.
- 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, 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.
- When preparing master mixes, use the minimal amount of extra material to ensure 8 reactions in the kit.
- Components and reagents from other NuGEN products should not be used with this product.
- Use only fresh ethanol stocks to make ethanol for washes in the cDNA purification protocols. Make the ethanol mixes fresh as well. Lower concentrations of ethanol in wash solutions will result in loss of yield, as the higher aqueous content will dissolve the cDNA and wash it off the beads or column.

C. Agencourt® RNAClean® XP Purification Beads

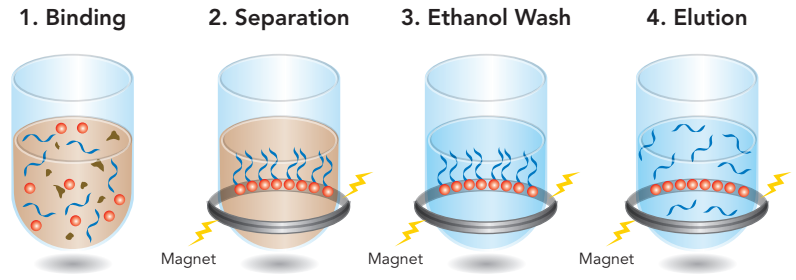
Tips and Notes

There are significant modifications to the Agencourt RNAClean XP beads standard procedure; therefore, you must follow the protocols outlined in this user guide for the use of these beads. However, you may review the Beckman Coulter Genomics user guide to become familiar with the manufacturer's recommendations.

The bead purification processes used in this kit consist of the following steps:

1. Binding of DNA to Agencourt RNAClean XP beads
2. Magnetic separation of beads from supernatant
3. Ethanol wash of bound beads to remove contaminants
4. Elution of bound DNA from beads

Figure 3. Agencourt® RNAClean® XP Bead purification process overview.



Reproduced from original picture from Agencourt/Beckman Coulter Genomics

Additional Tips and Notes

- Remove beads from 4°C and leave at room temperature for at least 15 minutes before use, ensure that they have completely reached room temperature. Cold beads reduce recovery.
- Fully resuspend beads by inverting and tapping before adding to sample.
- Note that ratio of RNAClean XP bead volume to sample volume varies at each step of the protocol. The bead:sample ratios used differ from the standard Agencourt protocol.
- It is critical to let the beads separate on the magnet for a full five minutes. Removing binding buffer before the beads have completely separated will impact DNA yields.
- After completing the binding step, it is important to minimize bead loss when removing the binding buffer. With the samples placed on the magnet, remove the specified quantity of binding buffer from each sample. Some liquid will remain at the bottom of the tube, but this will minimize bead loss.
- Any significant loss of beads during the ethanol washes will impact DNA yields, so make certain to minimize bead loss throughout the procedure.
- Ensure that the ethanol wash is freshly prepared from fresh ethanol stocks at the indicated concentration. Lower percent ethanol mixes will reduce recovery.
- During the ethanol washes, keep the samples on the magnet. The beads should not be allowed to disperse; the magnet will keep the beads on the walls of sample wells or tubes in a small ring. It is critical that all residual ethanol be removed prior to continuing with the next step. Therefore, when removing the final ethanol wash, first remove most of the ethanol, then allow the excess to collect at the bottom of the tube before removing the remaining ethanol. This reduces the required bead air drying time.
- After drying the beads for the time specified in the protocol, inspect each tube carefully and make certain that all the ethanol has evaporated before proceeding.
- It is strongly recommended that strip tubes or partial plates are firmly placed when used with the magnetic plate. We don't advise the use of individual tubes as they are difficult to position stably on the magnetic plates.

D. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes, equipped with a heated lid, and with a capacity of 100 µL reaction volume. Prepare the programs shown in Table 6, following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature to 100°C only when sample temperature reaches above 30°C. For thermal cyclers with a fixed temperature heated lid (e.g., ABI GeneAmp® PCR 9600 and 9700 models), use the default settings (typically 100 to 105°C).

Table 6. Thermal Cycler Programming

PRIMER ANNEALING	
Program 1 Primer Annealing	65°C – 5 min
FIRST STRAND	
Program 2 First Strand	40°C – 30 min, hold at 4°C
SECOND STRAND	
Program 3 Second Strand	16°C – 60 min, hold at 4°C
END REPAIR	
Program 4 End Repair	25°C – 30 min, 70°C – 10 min, hold at 4°C
LIGATION	
Program 5 Ligation	25°C – 30 min, hold at 4°C
Strand Selection I	
Program 6 Strand Selection I	72°C – 10 min, hold at 4°C
Strand Selection II	
Program 7 Strand Selection II	37°C – 30 min; 95°C – 30 sec, hold at 4°C
AMPLIFICATION	
Program 8 Library Amplification	5 cycles (94°C – 30 sec, 55°C – 30 sec, 72°C – 1 min); 15 cycles (94°C – 30 sec, 63°C – 30 sec, 72°C – 1 min); 72°C – 5 min, hold at 4°C

V. Protocol for the Encore Complete RNA-Seq Library Systems

A. First Strand cDNA Synthesis

1. Remove the First Strand Primer Mix (blue: A1), First Strand Buffer Mix (blue: A2), First Strand Enzyme Mix (blue: A3) and the Nuclease-free Water (green: D1) from the –20°C storage.
2. Spin down the contents of A3 and place on ice.
3. Thaw the other reagents at room temperature, mix by vortexing, spin and place on ice. Leave the Nuclease-free Water at room temperature.
4. Add 2 µL of A1 to a 0.2 mL PCR tube.
5. Add 5 µL of total RNA sample (100 ng to 150 ng) to the primer.
6. Mix by pipetting 5 times, spin and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 6):

   65°C – 5 min
8. Immediately remove the tubes from the thermal cycler and snap chill on ice.
9. Once Primer Annealing (Step 7) is complete, prepare a master mix by combining A2 and A3 in a 0.5 mL capped tube, according to the volumes shown in Table 7.

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

FIRST STRAND BUFFER MIX (BLUE: A2 VER 8)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 4)
2.5 µL	0.5 µL

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

10. Add 3 µL of the First Strand Master Mix to each tube.
11. Mix by pipetting, spin and place on ice.
12. Place the tubes in a warmed thermal cycler programmed to run Program 2 (First Strand cDNA Synthesis; see Table 6):

    40°C – 30 min, hold at 4°C
13. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
14. Continue immediately with the Second Strand cDNA Synthesis protocol.

B. Second Strand cDNA Synthesis

1. Remove the Second Strand Buffer Mix (yellow: B1), Second Strand Enzyme Mix (yellow: B2), and Second Strand Stop Mix (yellow: B3) from –20°C storage.
2. Spin down the contents of B2 and place on ice.
3. Thaw reagents B1 and B3 at room temperature, mix by vortexing, spin and place on ice.
4. Make a master mix by combining B1 and B2 in a 0.5 mL capped tube, according to the volumes shown in Table 8.

Table 8. Second Strand Master Mix (volumes listed are for a single reaction)

SECOND STRAND BUFFER MIX (YELLOW: B1 VER 6)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER 4)
63 µL	2 µL

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

5. Add 65 µL of the Second Strand Master Mix to each First Strand reaction tube.
6. Mix by pipetting, spin and place on ice.
7. Place the tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand cDNA Synthesis; see Table 6):

   16°C – 60 min, hold at 4°C
8. Remove the tubes from the thermal cycler and spin to collect condensation.
9. Add 45 µL of the Second Strand Stop Mix B3. Mix by pipetting and spin.
10. Continue immediately with cDNA Fragmentation or store samples at –20°C.

C. cDNA Fragmentation

1. Treat all the DNA samples with the Covaris S-Series System according to the manufacturer's recommendations using the settings shown in Table 9 or other user-defined settings that produce fragmented DNA with a median size of 200 bp.

Table 9. Covaris S-Series System Settings

PARAMETER	VALUE
Duty Cycle	10%
Intensity	5
Cycles/Burst	200
Time(s)	180
Temperature (Water Bath)	6–8°C
Power Mode Frequency	Sweeping
Degassing Mode	Continuous
Sample Volume	120 µL
Water (FILL/RUN)	S2 – level 12 E210 – level 6
AFA Intensifier	Yes

2. Continue with cDNA Purification or store samples at –20°C.

D. cDNA Purification

1. Ensure the Agencourt RNAClean XP beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. Resuspend the beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. Prepare a 70% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content which may reduce yield. (Sufficient wash solution should be prepared for all bead purification steps.)
4. At room temperature, add 180 µL (1.8 volumes) of the bead suspension to 100 µL of fragmented cDNA. Mix thoroughly by pipetting up and down.
5. Split each sample into two 140 µL aliquots.
6. Incubate at room temperature for 10 minutes.

7. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
8. Carefully remove 125 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of DNA carried into end repair, so ensure beads are not removed with the binding buffer or the wash.
9. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
10. Remove the 70% ethanol wash using a pipette.
11. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two 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.
12. Air dry the beads on the magnet for 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
13. Remove the tubes from the magnet.
14. Add 12 µL room temperature Nuclease-free Water (green: D1) to the first aliquot of dried beads. Mix thoroughly to ensure all the beads are resuspended.
15. Add the first aliquot of resuspended beads to the second aliquot of dried beads for each sample. Mix thoroughly to ensure all the beads are resuspended and let stand on the bench top for 3 minutes.
16. Transfer the tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.
17. Carefully remove 10 µL of the eluate, ensuring as few beads as possible are carried over, and transfer to a fresh set of PCR tubes and place on ice.
18. Continue immediately to the End Repair protocol.

E. End Repair

1. Remove End Repair Buffer Mix (blue: ER1), End Repair Enzyme Mix (blue: ER2) and Nuclease-free Water (green: D1) from the components stored at –20°C.
2. Thaw ER1 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down contents of ER2, place on ice.
4. Leave water, D1, to thaw at room temperature.

5. Prepare a master mix by combining ER1 and ER2 in a 0.5 mL capped tube, according to the volumes shown in Table 10.

Table 10. End Repair Master Mix (volumes listed are for a single reaction)

END REPAIR BUFFER MIX (BLUE: ER1 VER 4)	END REPAIR ENZYME MIX (BLUE: ER2 VER 4)
2.5 µL	0.5 µL

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

6. Add 3 µL of the End Repair Master Mix to 10 µL of each sample.
7. Mix by pipetting, cap and spin the tubes and place on ice.
8. Place the tubes in a pre-warmed thermal cycler programmed to run Program 4 (End Repair; see Table 6):

   25°C – 30 min, 70°C – 10 min, hold at 4°C
9. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
10. Continue immediately to the Ligation protocol.

F. Ligation

1. Remove Ligation Buffer Mix (yellow: L1), Ligation Adaptor Mix (yellow: L2), and Ligation Enzyme Mix (yellow: L3) from –20°C storage.
2. Thaw L1 and L2 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down L3 Ligation Enzyme and place on ice.
4. Add 3 µL of the appropriate Ligation Adaptor Mix (L2) to each sample. Mix by pipetting thoroughly. If multiplexing, make sure a unique barcode is used for each sample.

   Note: For more information about running a multiplex experiment, see Appendix B of this user guide.
5. Make a master mix by combining water D1, L1, and L3 in a 0.5 mL capped tube, according to the volumes shown in Table 11. Mix by pipetting slowly, without introducing bubbles, spin and place on ice.

   Note: The L1 Ligation Buffer Mix is very viscous. Please be sure to pipet this reagent slowly.

Table 11.Ligation Master Mix (volumes listed are for a single reaction)

WATER (GREEN: D1)	LIGATION BUFFER MIX (YELLOW: L1 VER 4)	LIGATION ENZYME MIX (YELLOW: L3 VER 4)
6.5 µL	6.0 µL	1.5 µL

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

6. Add 14 µL Ligation Master Mix to each reaction tube. Mix thoroughly by pipetting slowly and gently, spin and place on ice. Proceed immediately with the incubation.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 5 (Ligation; see Table 6):

   25°C – 30 min, hold at 4°C
8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Continue immediately to the Strand Selection I protocol.

G. Strand Selection I

1. Remove Strand Selection Buffer Mix I (purple: SS1), Strand Selection Enzyme Mix I (purple: SS2), Strand Selection Buffer Mix II (purple: SS3) and Strand Selection Enzyme Mix II (purple: SS4) from –20°C storage.
2. Thaw SS1 and SS3 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down SS2 and SS4 and place on ice.
4. Make a master mix by combining SS1 and SS2 in a 0.5 mL capped tube, according to the volumes shown in Table 12.

Table 12.Strand Selection I Master Mix (volumes listed are for a single reaction)

STRAND SELECTION I BUFFER MIX (PURPLE: SS1)	STRAND SELECTION I ENZYME MIX (PURPLE: SS2)
69 µL	1 µL

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

5. Add 70 µL of the Strand Selection I Master Mix to 30 µL of each sample.
6. Mix by pipetting, cap and spin tubes and place on ice.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 6 (Strand Selection I; see Table 6):

   72°C – 10 min, hold at 4°C
8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Continue immediately to the Strand Selection I Purification protocol.

H. Strand Selection I Purification

1. Ensure the Agencourt RNAClean XP beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. Resuspend beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. At room temperature, add 180 µL (1.8 volumes) of the bead suspension to the Strand Selection I reaction product. Mix thoroughly by pipetting up and down.
4. Split each sample into two 140 µL aliquots.
5. Incubate at room temperature for 10 minutes.
6. Transfer tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
7. Carefully remove 125 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of DNA carried into Strand Selection II, so ensure beads are not removed with the binding buffer or the wash.
8. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
9. Remove the 70% ethanol wash using a pipette.
10. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two 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 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
12. Remove tubes from magnet.
13. Add 25 µL room temperature Nuclease-free Water (D1) to the first aliquot of dried beads. Mix thoroughly to ensure all the beads are resuspended.
14. Add the first aliquot of resuspended beads to the second aliquot of dried beads for each sample. Mix thoroughly to ensure all the beads are resuspended and let stand on the bench top for 3 minutes.
15. Transfer tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.

16. Carefully remove 21.5 µL of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh set of PCR tubes and place on ice.
17. Continue immediately to the Strand Selection II protocol.

I. Strand Selection II

1. Make a master mix by combining SS3 and SS4 in a 0.5 mL capped tube, according to the volumes shown in Table 13.

Table 13.Strand Selection II Master Mix (volumes listed are for a single reaction)

STRAND SELECTION II BUFFER MIX (PURPLE: SS3)	STRAND SELECTION II ENZYME MIX (PURPLE: SS4)
2.5 µL	1 µL

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

2. Add 3.5 µL of the Strand Selection II Master Mix to 21.5 µL of each sample.
3. Mix by pipetting, cap and spin tubes and place on ice.
4. Place tubes in a pre-warmed thermal cycler programmed to run Program 7 (Strand Selection II; see Table 6):

   37°C – 30 min; 95°C – 30 sec, hold at 4°C
5. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
6. Continue immediately to the Library Amplification protocol.

J. Library Amplification

1. Remove Amplification Buffer Mix (red: P1), Amplification Primer Mix (red: P2 VER 3 or P2 VER 6), Amplification Enzyme Mix (red: P3) and DMSO (red: P4) from –20°C storage.
2. Thaw P1 and P2 at room temperature. Mix each by vortexing, spin and place on ice.
3. Thaw P4 at room temperature. Keep at room temperature until use.
4. Spin down P3 Amplification Enzyme Mix and place on ice.
5. Make a master mix by sequentially combining P1, P2 and P4 in an appropriately sized capped tube according to the volumes shown in Table 6. Add P3 Enzyme Mix at the last moment and mix well by pipetting taking care to avoid bubbles. Spin the tubes and place on ice.

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

Table 14. Amplification Master Mix (volumes listed are for a single reaction)

AMP BUFFER MIX (RED: P1 VER 3)	AMP PRIMER MIX (RED: P2 VER 3 or P2 VER 6)	DMSO (RED: P4)	AMP ENZYME MIX (RED: P3)
42 µL	8 µL	4 µL	1 µL

6. On ice, add 55 µL of the Amplification Master Mix to 25 µL of each sample.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 8 (Library Amplification; see Table 6):

   5 cycles (94°C – 30 sec, 55°C – 30 sec, 72°C – 1 min); 15 cycles (94°C – 30 sec, 63°C – 30 sec, 72°C – 1 min); 72°C – 5 min, hold at 4°C
8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Proceed to Bead Purification of the Amplified Material or store at –20°C.

K. Bead Purification of the Amplified Material

1. Ensure the RNAClean XP beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. Resuspend beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. At room temperature, add 96 µL (1.2 volumes) of the bead suspension to each reaction.
4. Mix thoroughly by pipetting up and down. It may be helpful to use a multichannel pipettor to ensure the incubation times are uniform.
5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to the magnet plate and let stand 5 minutes to completely clear the solution of beads.
7. Carefully remove only 160 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

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

10. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two 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 a minimum of 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.

12. Remove the tubes from the magnet.

13. Add 30 µL room temperature Nuclease-free Water (D1) to the dried beads. Mix thoroughly to ensure all the beads are resuspended.

14. Incubate at room temperature for 5 minutes.

15. Transfer the tubes to the magnet and let stand for 2 minutes.

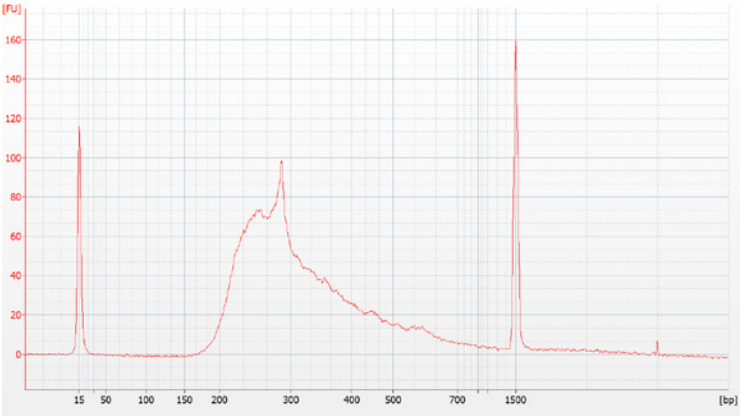
16. Carefully remove 25 µL of the eluate, ensuring as few beads as possible are carried over and transfer to a fresh set of tubes. When pipetting any portion of this eluted library downstream, be sure to use a magnet stand to minimize bead carryover into any ensuing reactions.

17. Proceed to Quantitative and Qualitative Assessment of the Library.

L. Quantitative and Qualitative Assessment of the Library

1. Run the samples on the Bioanalyzer DNA Chip 1000. Fragment distribution should be as shown in Figure 4.

Figure 4. Fragment distribution on Bioanalyzer DNA Chip 1000 from 100 ng human brain total RNA input.



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. Protocol for the Encore Complete IL Multiplex Systems 1–8 and 9–16 (Part Nos. 0312 and 0313)

The following protocol steps are intended for use with the Encore Complete IL Multiplex Systems 1–8 and 9–16 (Part Nos. 0312-32, 0313-32). Each kit contains eight different barcoded adaptors to be used in combination for 16-plex experiments.

Setting up an Encore Complete IL Multiplex Experiment

It is important when performing multiplex sequencing to produce each library independently, and not to mix adaptors during the actual library construction protocol. Multiplexing is achieved by mixing the amplified libraries prior to cluster generation.

We recommend using the Encore Complete RNA-Seq IL Multiplex Systems 1-8 and 9-16 in concert for experiments using no less than 16-plex sequencing. If multiplexing below 16-plex with these kits, you must include Illumina's PhiX Control (Cat. #FC-110-3001) per the manufacturer's instructions and reduce library concentration to obtain optimal cluster density. For more information, consult the Illumina Technical Note, "Using a PhiX Control for HiSeq Sequencing Runs" (Pub.No. 770-2011-041).

A. First Strand cDNA Synthesis

1. Remove the First Strand Primer Mix (blue: A1), First Strand Buffer Mix (blue: A2), First Strand Enzyme Mix (blue: A3) and the Nuclease-free Water (green: D1) from the –20°C storage.
2. Spin down the contents of A3 and place on ice.
3. Thaw the other reagents at room temperature, mix by vortexing, spin and place on ice. Leave the Nuclease-free Water at room temperature.
4. Add 2 µL of A1 to a 0.2 mL PCR tube.
5. Add 5 µL of total RNA sample (100 ng to 150 ng) to the primer.
6. Mix by pipetting 5 times, spin and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 6):

   65°C – 5 min
8. Immediately remove the tubes from the thermal cycler and snap chill on ice.
9. Once Primer Annealing (Step 7) is complete, prepare a master mix by combining A2 and A3 in a 0.5 mL capped tube, according to the volumes shown in Table 15.

VI. Protocol for the Encore Complete IL Multiplex Systems 1–8 and 9–16 (Part Nos. 0312 and 0313)

Table 15.First Strand Master Mix (volumes listed are for a single reaction)

FIRST STRAND BUFFER MIX (BLUE: A2 VER 8)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 4)
2.5 µL	0.5 µL

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

10. Add 3 µL of the First Strand Master Mix to each tube.
11. Mix by pipetting, spin and place on ice.
12. Place the tubes in a warmed thermal cycler programmed to run Program 2 (First Strand cDNA Synthesis; see Table 6):

    40°C – 30 min, hold at 4°C
13. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
14. Continue immediately with the Second Strand cDNA Synthesis protocol.

B. Second Strand cDNA Synthesis

1. Remove the Second Strand Buffer Mix (yellow: B1), Second Strand Enzyme Mix (yellow: B2), and Second Strand Stop Mix (yellow: B3) from –20°C storage.
2. Spin down the contents of B2 and place on ice.
3. Thaw reagents B1 and B3 at room temperature, mix by vortexing, spin and place on ice.
4. Make a master mix by combining B1 and B2 in a 0.5 mL capped tube, according to the volumes shown in Table 16.

Table 16.Second Strand Master Mix (volumes listed are for a single reaction)

SECOND STRAND BUFFER MIX (YELLOW: B1 VER 6)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER 4)
63 µL	2 µL

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

5. Add 65 µL of the Second Strand Master Mix to each First Strand reaction tube.
6. Mix by pipetting, spin and place on ice.
7. Place the tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand cDNA Synthesis; see Table 6):

    16°C – 60 min, hold at 4°C

8. Remove the tubes from the thermal cycler and spin to collect condensation.
9. Add 45 µL of the Second Strand Stop Mix B3. Mix by pipetting and spin.
10. Continue immediately with cDNA Fragmentation or store samples at –20°C.

C. cDNA Fragmentation

1. Treat all the DNA samples with the Covaris S-Series System according to the manufacturer's recommendations using the settings shown in Table 17 or other user-defined settings that produce fragmented DNA with a median size of 150 bp.

Table 17.Covaris S-Series System Settings

PARAMETER	VALUE
Duty Cycle	10%
Intensity	5
Cycles/Burst	200
Time(s)	180
Temperature (Water Bath)	6–8°C
Power Mode Frequency	Sweeping
Degassing Mode	Continuous
Sample Volume	120 µL
Water (FILL/RUN)	S2 – level 12 E210 – level 6
AFA Intensifier	Yes

2. Continue with cDNA Purification or store samples at –20°C.

D. cDNA Purification

1. Ensure the Agencourt RNAClean XP beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. Resuspend the beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads.

(An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)

3. Prepare a 70% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content which may reduce yield. (Sufficient wash solution should be prepared for all bead purification steps.)
4. At room temperature, add 180 µL (1.8 volumes) of the bead suspension to 100 µL of fragmented cDNA. Mix thoroughly by pipetting up and down.
5. Split each sample into two 140 µL aliquots.
6. Incubate at room temperature for 10 minutes.
7. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
8. Carefully remove 125 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of DNA carried into end repair, so ensure beads are not removed with the binding buffer or the wash.
9. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
10. Remove the 70% ethanol wash using a pipette.
11. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two 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.
12. Air dry the beads on the magnet for 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
13. Remove the tubes from the magnet.
14. Add 12 µL room temperature Nuclease-free Water (green: D1) to the first aliquot of dried beads. Mix thoroughly to ensure all the beads are resuspended.
15. Add the first aliquot of resuspended beads to the second aliquot of dried beads for each sample. Mix thoroughly to ensure all the beads are resuspended and let stand on the bench top for 3 minutes.
16. Transfer the tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.

VI. Protocol for the Encore Complete IL Multiplex Systems 1–8 and 9–16 (Part Nos. 0312 and 0313)

17. Carefully remove 10 µL of the eluate, ensuring as few beads as possible are carried over, and transfer to a fresh set of PCR tubes and place on ice.
18. Continue immediately to the End Repair protocol.

E. End Repair

1. Remove End Repair Buffer Mix (blue: ER1), End Repair Enzyme Mix (blue: ER2) and Nuclease-free Water (green: D1) from the components stored at –20°C.
2. Thaw ER1 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down contents of ER2, place on ice.
4. Leave water, D1, to thaw at room temperature.
5. Prepare a master mix by combining ER1 and ER2 in a 0.5 mL capped tube, according to the volumes shown in Table 18.

Table 18. End Repair Master Mix (volumes listed are for a single reaction)

END REPAIR BUFFER MIX (BLUE: ER1 VER 4)	END REPAIR ENZYME MIX (BLUE: ER2 VER 4)
2.5 µL	0.5 µL

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

6. Add 3 µL of the End Repair Master Mix to 10 µL of each sample.
7. Mix by pipetting, cap and spin tubes and place on ice.
8. Place tubes in a pre-warmed thermal cycler programmed to run Program 4 (End Repair; see Table 6):

   25°C – 30 min, 70°C – 10 min, hold at 4°C
9. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
10. Continue immediately to the Ligation protocol.

F. Ligation

1. Remove Ligation Buffer Mix (yellow: L1), Ligation Adaptor Mix (yellow: L2V6-BC1 through L2V6-BC16), and Ligation Enzyme Mix (yellow: L3) from –20°C storage.
2. Thaw L1 and L2 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down L3 Ligation Enzyme and place on ice.

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4. Add 3 µL of the appropriate Ligation Adaptor Mix (L2) to each sample. Mix by pipetting thoroughly. Make sure a unique barcode is used for each sample to be used on a single flowcell lane.
5. Make a master mix by combining water D1, L1, and L3 in a 0.5 mL capped tube, according to the volumes shown in Table 19. Mix by pipetting slowly, without introducing bubbles, spin and place on ice.

   Note: The L1 Ligation Buffer Mix is very viscous. Please be sure to pipet this reagent slowly.

Table 19.Ligation Master Mix (volumes listed are for a single reaction)

WATER (GREEN: D1)	LIGATION BUFFER MIX (YELLOW: L1 VER 4)	LIGATION ENZYME MIX (YELLOW: L3 VER 4)
6.5 µL	6.0 µL	1.5 µL

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

6. Add 14 µL Ligation Master Mix to each reaction tube. Mix thoroughly by pipetting slowly and gently, spin and place on ice. Proceed immediately with the incubation.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 5 (Ligation; see Table 6):

   25°C – 30 min, hold at 4°C
8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Continue immediately to the Strand Selection I protocol.

G. Strand Selection I

1. Remove Strand Selection Buffer Mix I (purple: SS1), Strand Selection Enzyme Mix I (purple: SS2), Strand Selection Buffer Mix II (purple: SS3) and Strand Selection Enzyme Mix II (purple: SS4) from –20°C storage.
2. Thaw SS1 and SS3 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down SS2 and SS4 and place on ice.
4. Make a master mix by combining SS1 and SS2 in a 0.5 mL capped tube, according to the volumes shown in Table 20.

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

Table 20.Strand Selection I Master Mix (volumes listed are for a single reaction)

STRAND SELECTION I BUFFER MIX (PURPLE: SS1)	STRAND SELECTION I ENZYME MIX (PURPLE: SS2)
69 µL	1 µL

5. Add 70 µL of the Strand Selection I Master Mix to 30 µL of each sample.
6. Mix by pipetting, cap and spin tubes and place on ice.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 6 (Strand Selection I; see Table 6):

   72°C – 10 min, hold at 4°C
8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Continue immediately to the Strand Selection I Purification protocol.

H. Strand Selection I Purification

1. Ensure the Agencourt RNAClean XP beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. Resuspend the beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. At room temperature, add 180 µL (1.8 volumes) of the bead suspension to the Strand Selection I reaction product. Mix thoroughly by pipetting up and down.
4. Split each sample into two 140 µL aliquots.
5. Incubate at room temperature for 10 minutes.
6. Transfer tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
7. Carefully remove 125 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of DNA carried into Strand Selection II, so ensure beads are not removed with the binding buffer or the wash.
8. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
9. Remove the 70% ethanol wash using a pipette.

10. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two 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 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
12. Remove the tubes from the magnet.
13. Add 25 µL room temperature Nuclease-free Water (D1) to the first aliquot of dried beads. Mix thoroughly to ensure all the beads are resuspended.
14. Add the first aliquot of resuspended beads to the second aliquot of dried beads for each sample. Mix thoroughly to ensure all the beads are resuspended and let stand on the bench top for 3 minutes.
15. Transfer the tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.
16. Carefully remove 21.5 µL of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh set of PCR tubes and place on ice.
17. Continue immediately to the Strand Selection II protocol.

I. Strand Selection II

1. Make a master mix by combining SS3 and SS4 in a 0.5 mL capped tube, according to the volumes shown in Table 21.

Table 21.Strand Selection II Master Mix (volumes listed are for a single reaction)

STRAND SELECTION II BUFFER MIX (PURPLE: SS3)	STRAND SELECTION II ENZYME MIX (PURPLE: SS4)
2.5 µL	1 µL

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

2. Add 3.5 µL of the Strand Selection II Master Mix to 21.5 µL of each sample.
3. Mix by pipetting, cap and spin tubes and place on ice.
4. Place tubes in a pre-warmed thermal cycler programmed to run Program 7 (Strand Selection II; see Table 6):

   37°C – 30 min; 95°C – 30 sec, hold at 4°C

5. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
6. Continue immediately to the Library Amplification protocol.

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J. Library Amplification

1. Remove Amplification Buffer Mix (red: P1), Amplification Primer Mix (red: P2 VER 3), Amplification Enzyme Mix (red: P3) and DMSO (red: P4) from –20°C storage.
2. Thaw P1 and P2 at room temperature. Mix each by vortexing, spin and place on ice.
3. Thaw P4 at room temperature. Keep at room temperature until use.
4. Spin down P3 Amplification Enzyme Mix and place on ice.
5. Make a master mix by sequentially combining P1, P2 and P4 in an appropriately sized capped tube according to the volumes shown in Table 22. Add P3 Enzyme Mix at the last moment and mix well by pipetting taking care to avoid bubbles. Spin the tubes and place on ice.

Table 22.Amplification Master Mix (volumes listed are for a single reaction)

AMP BUFFER MIX (RED: P1 VER 3)	AMP PRIMER MIX (RED: P2 VER 3)	DMSO (RED: P4)	AMP ENZYME MIX (RED: P3)
42 µL	8 µL	4 µL	1 µL

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

6. On ice, add 55 µL of the Amplification Master Mix to 25 µL of each sample.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 8 (Library Amplification; see Table 6):

   5 cycles (94°C – 30 sec, 55°C – 30 sec, 72°C – 1 min); 15 cycles (94°C – 30 sec, 63°C – 30 sec, 72°C – 1 min); 72°C – 5 min, hold at 4°C
8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Proceed to Bead Purification of the Amplified Material or store at –20°C.

K. Bead Purification of the Amplified Material

1. Ensure the RNAClean XP beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. Resuspend the beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. At room temperature, add 96 µL (1.2 volumes) of the bead suspension to each reaction.
4. Mix thoroughly by pipetting up and down. It may be helpful to use a multichannel pipettor to ensure the incubation times are uniform.

5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to the magnet plate and let stand 5 minutes to completely clear the solution of beads.
7. Carefully remove only 160 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

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

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two 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 a minimum of 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
12. Remove the tubes from the magnet.
13. Add 30 µL room temperature Nuclease-free Water (D1) to the dried beads. Mix thoroughly to ensure all the beads are resuspended.
14. Incubate at room temperature for 5 minutes.
15. Transfer tubes to magnet and let stand for 2 minutes.
16. Carefully remove 25 µL of the eluate, ensuring as few beads as possible are carried over and transfer to a fresh set of tubes. When pipetting any portion of this eluted library downstream, be sure to use a magnet stand to minimize bead carryover into any ensuing reactions.
17. Proceed to Quantitative and Qualitative Assessment of the Library.

L. Quantitative and Qualitative Assessment of the Library

1. Run the samples on the Bioanalyzer DNA Chip 1000. Fragment distribution should be as shown in Figure 5.

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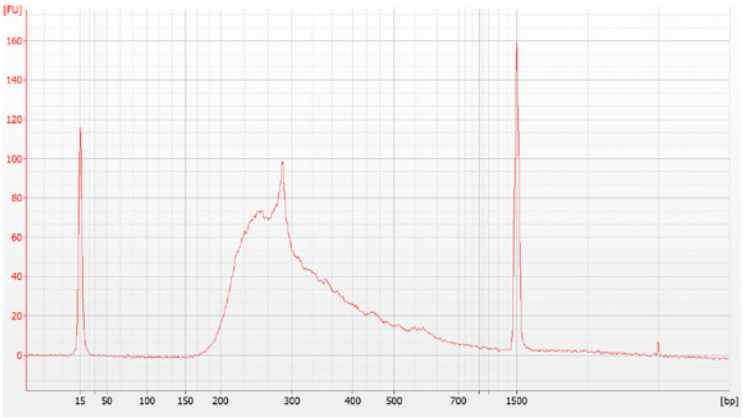
Figure 5. Fragment distribution on Bioanalyzer DNA Chip 1000 from 100 ng human brain total RNA input.



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).

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The following protocol steps are intended for use with the Encore Complete DR Multiplex Systems 1–8 and 9–16 (Part Nos. 0333-32, 0334-32). Each kit contains eight different barcoded adaptors for multiplex sequencing that can be used to interrogate up to 16 independently generated libraries on a single lane of the Illumina NGS platforms.

A. Setting up an Ovation Ultralow DR Multiplex Experiment

It is important when performing multiplex sequencing to produce each library independently, and not to mix adaptors during the actual library construction protocol. Multiplexing is achieved by mixing the amplified libraries prior to adding to the cBot instrument.

B. First Strand cDNA Synthesis

1. Remove the First Strand Primer Mix (blue: A1), First Strand Buffer Mix (blue: A2), First Strand Enzyme Mix (blue: A3) and the Nuclease-free Water (green: D1) from the –20°C storage.
2. Spin down the contents of A3 and place on ice.
3. Thaw the other reagents at room temperature, mix by vortexing, spin and place on ice. Leave the Nuclease-free Water at room temperature.
4. Add 2 µL of A1 to a 0.2 mL PCR tube.
5. Add 5 µL of total RNA sample (100 ng to 150 ng) to the primer.
6. Mix by pipetting 5 times, spin and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 6):

   65°C – 5 min
8. Immediately remove the tubes from the thermal cycler and snap chill on ice.
9. Once Primer Annealing (Step 7) is complete, prepare a master mix by combining A2 and A3 in a 0.5 mL capped tube, according to the volumes shown in Table 23.

Table 23. First Strand Master Mix (volumes listed are for a single reaction)

FIRST STRAND BUFFER MIX (BLUE: A2 VER 8)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 4)
2.5 µL	0.5 µL

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

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

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11. Mix by pipetting, spin and place on ice.
12. Place the tubes in a warmed thermal cycler programmed to run Program 2 (First Strand cDNA Synthesis; see Table 6):

    40°C – 30 min, hold at 4°C
13. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
14. Continue immediately with the Second Strand cDNA Synthesis protocol.

C. Second Strand cDNA Synthesis

1. Remove the Second Strand Buffer Mix (yellow: B1), Second Strand Enzyme Mix (yellow: B2), and Second Strand Stop Mix (yellow: B3) from –20°C storage.
2. Spin down the contents of B2 and place on ice.
3. Thaw reagents B1 and B3 at room temperature, mix by vortexing, spin and place on ice.
4. Make a master mix by combining B1 and B2 in a 0.5 mL capped tube, according to the volumes shown in Table 24.

Table 24.Second Strand Master Mix (volumes listed are for a single reaction)

SECOND STRAND BUFFER MIX (YELLOW: B1 VER 6)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER 4)
63 µL	2 µL

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

5. Add 65 µL of the Second Strand Master Mix to each First Strand reaction tube.
6. Mix by pipetting, spin and place on ice.
7. Place the tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand cDNA Synthesis; see Table 6):

    16°C – 60 min, hold at 4°C
8. Remove the tubes from the thermal cycler and spin to collect condensation.
9. Add 45 µL of the Second Strand Stop Mix B3. Mix by pipetting and spin.
10. Continue immediately with cDNA Fragmentation or store samples at –20°C.

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D. cDNA Fragmentation

1. Treat all the DNA samples with the Covaris S-Series System according to the manufacturer's recommendations using the settings shown in Table 25 or other user-defined settings that produce fragmented DNA with a median size of 150 bp.

Table 25.Covaris S-Series System Settings

PARAMETER	VALUE
Duty Cycle	10%
Intensity	5
Cycles/Burst	200
Time(s)	180
Temperature (Water Bath)	6–8°C
Power Mode Frequency	Sweeping
Degassing Mode	Continuous
Sample Volume	120 µL
Water (FILL/RUN)	S2 – level 12 E210 – level 6
AFA Intensifier	Yes

2. Continue with cDNA Purification or store samples at –20°C.

E. cDNA Purification

1. Ensure the Agencourt RNAClean XP beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. Resuspend the beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. Prepare a 70% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content which may

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reduce yield. (Sufficient wash solution should be prepared for all bead purification steps.)

4. At room temperature, add 180 µL (1.8 volumes) of the bead suspension to 100 µL of fragmented cDNA. Mix thoroughly by pipetting up and down.
5. Split each sample into two 140 µL aliquots.
6. Incubate at room temperature for 10 minutes.
7. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
8. Carefully remove 125 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of DNA carried into end repair, so ensure beads are not removed with the binding buffer or the wash.
9. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
10. Remove the 70% ethanol wash using a pipette.
11. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two 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.
12. Air dry the beads on the magnet for 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
13. Remove the tubes from the magnet.
14. Add 12 µL room temperature Nuclease-free Water (green: D1) to the first aliquot of dried beads. Mix thoroughly to ensure all the beads are resuspended.
15. Add the first aliquot of resuspended beads to the second aliquot of dried beads for each sample. Mix thoroughly to ensure all the beads are resuspended and let stand on the bench top for 3 minutes.
16. Transfer the tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.
17. Carefully remove 10 µL of the eluate, ensuring as few beads as possible are carried over, and transfer to a fresh set of PCR tubes and place on ice.
18. Continue immediately to the End Repair protocol.

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F. End Repair

1. Remove End Repair Buffer Mix (blue: ER1), End Repair Enzyme Mix (blue: ER2) and Nuclease-free Water (green: D1) from the components stored at –20°C.
2. Thaw ER1 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down contents of ER2, place on ice.
4. Leave water, D1, to thaw at room temperature.
5. Prepare a master mix by combining ER1 and ER2 in a 0.5 mL capped tube, according to the volumes shown in Table 26.

Table 26. End Repair Master Mix (volumes listed are for a single reaction)

END REPAIR BUFFER MIX (BLUE: ER1 VER 4)	END REPAIR ENZYME MIX (BLUE: ER2 VER 4)
2.5 µL	0.5 µL

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

6. Add 3 µL of the End Repair Master Mix to 10 µL of each sample.
7. Mix by pipetting, cap and spin tubes and place on ice.
8. Place tubes in a pre-warmed thermal cycler programmed to run Program 4 (End Repair; see Table 6):

   25°C – 30 min, 70°C – 10 min, hold at 4°C
9. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
10. Continue immediately to the Ligation protocol.

G. Ligation

1. Remove Ligation Buffer Mix (yellow: L1), Ligation Adaptor Mix (yellow: L2DR-BC1 through L2DR-BC16), and Ligation Enzyme Mix (yellow: L3) from –20°C storage.
2. Thaw L1 and L2 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down L3 Ligation Enzyme and place on ice.
4. Add 3 µL of the appropriate Ligation Adaptor Mix (L2) to each sample. Mix by pipetting thoroughly. Make sure a unique barcode is used for each sample to be used on a single flowcell lane.
5. Make a master mix by combining water D1, L1, and L3 in a 0.5 mL capped tube, according to the volumes shown in Table 27. Mix by pipetting slowly, without introducing bubbles, spin and place on ice.

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Note: The L1 Ligation Buffer Mix is very viscous. Please be sure to pipet this reagent slowly.

Table 27. Ligation Master Mix (volumes listed are for a single reaction)

WATER (GREEN: D1)	LIGATION BUFFER MIX (YELLOW: L1 VER 4)	LIGATION ENZYME MIX (YELLOW: L3 VER 4)
6.5 µL	6.0 µL	1.5 µL

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

6. Add 14 µL Ligation Master Mix to each reaction tube. Mix thoroughly by pipetting slowly and gently, spin and place on ice. Proceed immediately with the incubation.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 5 (Ligation; see Table 6):

   25°C – 30 min, hold at 4°C
8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Continue immediately to the Strand Selection I protocol.

H. Strand Selection I

1. Remove Strand Selection Buffer Mix I (purple: SS1), Strand Selection Enzyme Mix I (purple: SS2), Strand Selection Buffer Mix II (purple: SS3) and Strand Selection Enzyme Mix II (purple: SS4) from –20°C storage.
2. Thaw SS1 and SS3 at room temperature. Mix by vortexing, spin and place on ice.
3. Spin down SS2 and SS4 and place on ice.
4. Make a master mix by combining SS1 and SS2 in a 0.5 mL capped tube, according to the volumes shown in Table 28.

Table 28. Strand Selection I Master Mix (volumes listed are for a single reaction)

STRAND SELECTION I BUFFER MIX (PURPLE: SS1)	STRAND SELECTION I ENZYME MIX (PURPLE: SS2)
69 µL	1 µL

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

5. Add 70 µL of the Strand Selection I Master Mix to 30 µL of each sample.
6. Mix by pipetting, cap and spin tubes and place on ice.

7. Place tubes in a pre-warmed thermal cycler programmed to run Program 6 (Strand Selection I; see Table 6):

   72°C – 10 min, hold at 4°C

8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Continue immediately to the Strand Selection I Purification protocol.

I. Strand Selection I Purification

1. Ensure the Agencourt RNAClean XP beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. Resuspend the beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. At room temperature, add 180 µL (1.8 volumes) of the bead suspension to the Strand Selection I reaction product. Mix thoroughly by pipetting up and down.
4. Split each sample into two 140 µL aliquots.
5. Incubate at room temperature for 10 minutes.
6. Transfer tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
7. Carefully remove 125 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of DNA carried into Strand Selection II, so ensure beads are not removed with the binding buffer or the wash.

8. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
9. Remove the 70% ethanol wash using a pipette.
10. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two 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 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
12. Remove the tubes from the magnet.

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13. Add 25 µL room temperature Nuclease-free Water (D1) to the first aliquot of dried beads. Mix thoroughly to ensure all the beads are resuspended.
14. Add the first aliquot of resuspended beads to the second aliquot of dried beads for each sample. Mix thoroughly to ensure all the beads are resuspended and let stand on the bench top for 3 minutes.
15. Transfer the tubes to the magnet and let stand for 3 minutes for the beads to clear the solution.
16. Carefully remove 21.5 µL of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh set of PCR tubes and place on ice.
17. Continue immediately to the Strand Selection II protocol.

J. Strand Selection II

1. Make a master mix by combining SS3 and SS4 in a 0.5 mL capped tube, according to the volumes shown in Table 29.

Table 29.Strand Selection II Master Mix (volumes listed are for a single reaction)

STRAND SELECTION II BUFFER MIX (PURPLE: SS3)	STRAND SELECTION II ENZYME MIX (PURPLE: SS4)
2.5 µL	1 µL

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

2. Add 3.5 µL of the Strand Selection II Master Mix to 21.5 µL of each sample.
3. Mix by pipetting, cap and spin tubes and place on ice.
4. Place tubes in a pre-warmed thermal cycler programmed to run Program 7 (Strand Selection II; see Table 6):

   37°C – 30 min; 95°C – 30 sec, hold at 4°C
5. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
6. Continue immediately to the Library Amplification protocol.

K. Library Amplification

1. Remove Amplification Buffer Mix (red: P1), Amplification Primer Mix (red: P2 VER 6), Amplification Enzyme Mix (red: P3) and DMSO (red: P4) from –20°C storage.
2. Thaw P1 and P2 at room temperature. Mix each by vortexing, spin and place on ice.
3. Thaw P4 at room temperature. Keep at room temperature until use.

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4. Spin down P3 Amplification Enzyme Mix and place on ice.
5. Make a master mix by sequentially combining P1, P2 and P4 in an appropriately sized capped tube according to the volumes shown in Table 30. Add P3 Enzyme Mix at the last moment and mix well by pipetting taking care to avoid bubbles. Spin the tubes and place on ice.

Table 30. Amplification Master Mix (volumes listed are for a single reaction)

AMP BUFFER MIX (RED: P1 VER 3)	AMP PRIMER MIX (RED: P2 VER 6)	DMSO (RED: P4)	AMP ENZYME MIX (RED: P3)
42 µL	8 µL	4 µL	1 µL

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

6. On ice, add 55 µL of the Amplification Master Mix to 25 µL of each sample.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 8 (Library Amplification; see Table 6):

   5 cycles (94°C – 30 sec, 55°C – 30 sec, 72°C – 1 min); 15 cycles (94°C – 30 sec, 63°C – 30 sec, 72°C – 1 min); 72°C – 5 min, hold at 4°C
8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Proceed to Bead Purification of the Amplified Material or store at –20°C.

L. Bead Purification of the Amplified Material

1. Ensure the RNAClean XP beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. Resuspend the beads by inverting and tapping the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)
3. At room temperature, add 96 µL (1.2 volumes) of the bead suspension to each reaction.
4. Mix thoroughly by pipetting up and down. It may be helpful to use a multichannel pipettor to ensure the incubation times are uniform.
5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to the magnet plate and let stand 5 minutes to completely clear the solution of beads.
7. Carefully remove only 160 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

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

8. With the plate still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
9. Remove the 70% ethanol wash using a pipette.
10. Repeat the 70% ethanol wash one more time, for a total of two washes.

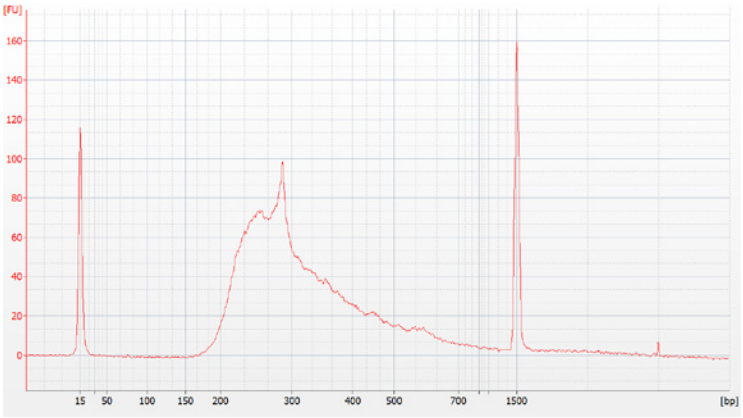
    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.
11. Air dry the beads on the magnet for a minimum of 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
12. Remove the tubes from the magnet.
13. Add 30 µL room temperature Nuclease-free Water (D1) to the dried beads. Mix thoroughly to ensure all the beads are resuspended.
14. Incubate at room temperature for 5 minutes.
15. Transfer tubes to magnet and let stand for 2 minutes.
16. Carefully remove 25 µL of the eluate, ensuring as few beads as possible are carried over and transfer to a fresh set of tubes. When pipetting any portion of this eluted library downstream, be sure to use a magnet stand to minimize bead carryover into any ensuing reactions.
17. Proceed to Quantitative and Qualitative Assessment of the Library.

M. Quantitative and Qualitative Assessment of the Library

1. Run the samples on the Bioanalyzer DNA Chip 1000. Fragment distribution should be as shown in Figure 6.

VII. Protocol for the Encore Complete DR Multiplex Systems 1–8 and 9–16 (Part Nos. 0333 and 0334)

Figure 6. Fragment distribution on Bioanalyzer DNA Chip 1000 from 100 ng human brain total RNA input.



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).

VIII. Technical Support

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

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

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

IX. Appendix

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 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 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
8. Add 10 µL DNase I to 70 µL Buffer RDD. Gently invert the tube to mix.

   Note: Other DNase I enzymes we recommend for use in this step are the Shrimp DNase (recombinant) from USB Corp. (use 10 µL) or the DNase I (RNase- free) from New England BioLabs (use 10 µL).
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 minutes.
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 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 RNase-free water (D1, green cap) to bring the final volume to 25 µL.
3. Incubate at 25°C for 15 minutes followed by 37°C for 15 minutes and return to ice.
4. After the DNase treatment, the sample must be purified. We recommend either of the two purification procedures below:

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

   - 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 ≥8000 X g (≥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 ≥8000 X g (≥10,000 rpm). Discard the flow-through.
   - Add 200 µL fresh 80% ethanol, close cap and spin for 30 seconds at ≥8000 X g (≥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: Do not use cold water!
   - 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)

- Add 80 µL ice-cold Nuclease-free Water (D1, green cap) to the sample on ice.
- Add 350 µL Buffer RLT and mix by pipetting.
- Add 250 µL 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 µL sample to the column.
- After closing the column, spin for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
- 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.
- 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.
- Spin for 2 minutes at ≥8000 X g (≥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 ≥8000 X g (≥10,000 rpm) and discard the flow-through.
- Place the RNeasy MinElute Spin Column in a fresh 1.5 mL collection tube.
- Add 14 µL Nuclease-free Water (D1, green cap) directly to the center of the filter in the tube and close the cap. Do not use cold water!
- Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

B. PCR Enrichment Artifacts

In some instances, PCR enrichment may create artifacts in the downstream library size analysis which appear as high molecular weight species during Bioanalyzer or gel analysis (Figure 7). This phenomenon is due to the amplification of diverse library molecules that have the same adaptor sequences at their termini. As the concentration of library molecules increases during PCR, the adaptor ends begin to compete with the PCR primers for hybridization, resulting in partially hybridized species. Although this may impact PCR efficiency, it does not impact library quality for subsequent sequencing, nor does it affect quantitation by qPCR.

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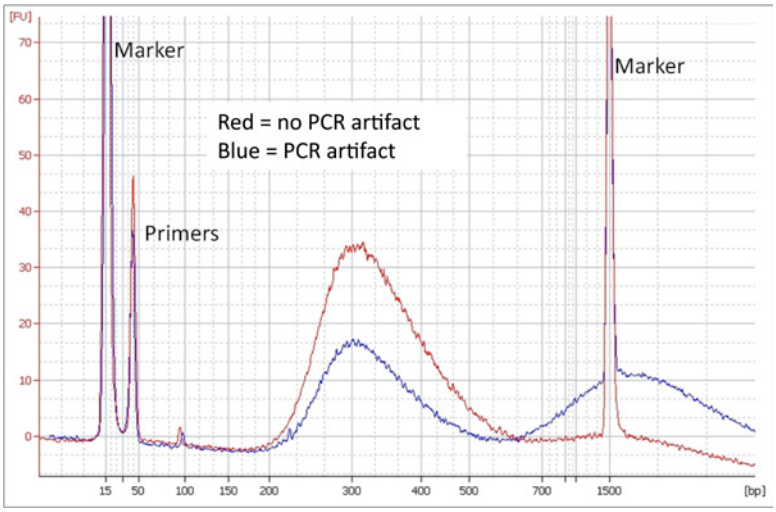
Figure 7. Fragment distribution on Bioanalyzer Chip 1000 when PCR overamplification is present.



If desired, performing a single round of PCR in the presence of excess primer will resolve the material to a single peak of the correct library size.

When quantifying libraries that may have been subject to PCR artifacts, use the lower molecular weight peak to estimate library size and qPCR to determine concentration.

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C. Sequences of the Barcodes in the Multiplexed Reactions

Barcode sequences for adaptors used in Encore Complete RNA-Seq IL Multiplex Systems 1–8 and 9–16 are shown in Table 31. Barcode sequences for adaptors used in Encore Complete RNA-Seq DR Multiplex Systems 1–8 and 9–16 can be found in Table 32.

Table 31. Barcode sequences for inline (IL) adaptors.

LIGATION ADAPTOR MIX	BARCODE SEQUENCE
L2V6-BC1	ACCC
L2V6-BC2	CGTA
L2V6-BC3	GAGT
L2V6-BC4	TTAG
L2V6-BC5	AGGG
L2V6-BC6	CCAT
L2V6-BC7	GTCA
L2V6-BC8	TATC
L2V6-BC9	AAAA
L2V6-BC10	CTGC
L2V6-BC11	GCTG
L2V6-BC12	TGCT
L2V6-BC13	ATTT
L2V6-BC14	CACG
L2V6-BC15	GGAC
L2V6-BC16	TCGA

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Table 32. Barcode sequences for dedicated read (DR) adaptors.

LIGATION ADAPTOR MIX	BARCODE SEQUENCE
L2V6DR-BC1	AAGGGA
L2V6DR-BC2	CCTTCA
L2V6DR-BC3	GGACCC
L2V6DR-BC4	TTCAGC
L2V6DR-BC5	AAGACG
L2V6DR-BC6	CCTCGG
L2V6DR-BC7	GGATGT
L2V6DR-BC8	TTCGCT
L2V6DR-BC9	ACACGA
L2V6DR-BC10	CACACA
L2V6DR-BC11	GTGTTA
L2V6DR-BC12	TGTGAA
L2V6DR-BC13	ACAAAC
L2V6DR-BC14	CACCTC
L2V6DR-BC15	GTGGCC
L2V6DR-BC16	TGTTGC

D. Capture of Smaller RNA Species

The standard protocol for the Encore Complete RNA-Seq Library Systems found in Section V of this user guide will effectively capture and create libraries from intact mRNA species. In order to capture smaller species that are approximately 75 nt or larger, it is recommended to change the procedure for the cDNA Purification protocol (section V.D).

Instead of using the Agencourt RNA Clean XP Beads for purification, we recommend using the MinElute Reaction Cleanup Kit (QIAGEN, Cat. #28204), according to the manufacturer's protocol. Elute the cDNA product using 10 µL room temperature Nuclease-free Water (D1). The materials for this purification are not included with the system, so it is necessary to purchase the purification kit separately. Performance of other purification systems has not been evaluated.

IX. Appendix

E. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the Encore Complete RNA-Seq Library Systems?
The Encore Complete RNA-Seq Library Systems provide all necessary buffers, primers, enzymes and purification beads. The kit also provides nuclease-free water for purification elution steps.

Q2. Does this system contain a SPIA®-based amplification?
No. The cDNA is generated with selective primers, but no SPIA-based amplification is used.

Q3. What equipment is required or will be useful?
A comprehensive list of required and recommended equipment can be found in Section II.B of this user guide.

Q4. Can I use the Encore Complete RNA-Seq 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 other organisms may vary.

Q5. Do I need to use high-quality total RNA?
The Encore Complete RNA-Seq 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.

Q6. Do I need to perform an rRNA depletion or Poly(A) enrichment step before processing with the Encore Complete RNA-Seq Library Systems?
The system is designed to use total RNA as input. rRNA depletion or Poly(A) enrichment is not necessary.

Q7. Can I use poly(A)+ RNA as an alternative to total RNA?
Yes, if you are primarily interested in sequencing reads from mature coding transcripts then poly(A)+ RNA may be substituted for total RNA. NuGEN has demonstrated that poly(A)+ RNA can be isolated from 100 to 500 ng of total RNA using MPG mRNA Purification Kit (PureBiotech Cat. #MRRK 1010) and the poly(A)+ fraction input to the Encore Complete RNA-Seq Library Systems protocol with good results.

Q8. How much total RNA do I need for amplification?
The selective priming process is designed to deplete rRNA from 100–150 ng total RNA input.

Q9. How does your protocol improve the efficiency of ligation and avoid adaptor dimer formation?
The Encore Complete RNA-Seq Library Systems utilize 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 Complete RNA-Seq Library Systems utilize 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. When using the poly(A)+ fraction as input to the workflow the percentage of total rRNA reads is <10%. *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 Complete RNA-Seq Library Systems were designed to create libraries of intact mRNA species. The protocol can be modified to capture smaller RNA species, such as snoRNAs, with a minimum length of approximately 75 bases. This modification involves use of an alternate purification method using the QIAGEN MinElute Reaction Cleanup kit to purify the fragmented cDNA. The details of this protocol enhancement can be found in Appendix D of this user guide. The performance of other purification systems have not been evaluated.

Q13. Can contaminating genomic DNA interfere with the Encore Complete RNA-Seq Library Systems performance?
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 DNase treatment of RNA samples, refer to Appendix D for guidelines.

Q14. Is this system compatible with target enrichment strategies?
Yes, the Encore Complete RNA-Seq 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. Can this system be used with other library preparation workflows?
The Encore Complete RNA-Seq Library Systems are an end-to-end solution designed to generate Illumina libraries starting from total RNA and has not been tested with alternative library preparation systems.

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Q16. How do I measure my amplified cDNA product yield? Can I use an Agilent Bioanalyzer to evaluate the product?
Yes. Refer to section V.L of the user guide for guidelines on Quantitative and Qualitative Assessment.

Q17. Where can I safely stop in the protocol?
Samples can be placed in short-term storage at –20°C after B. Second Strand Synthesis, after C. cDNA Fragmentation, or after any of the bead purification steps.

Q18. Does NuGEN provide reagents for performing the fragmentation step of the protocol?
We recommend using the Covaris instrument for cDNA fragmentation, as suggested in the "materials" section of this user guide. NuGEN does not provide the reagents used in the fragmentation steps, but the user guide does specify suggested settings for the Covaris instrument.

Q19. I don't have access to a Covaris instrument, can I use alternative fragmentation methods?
We have evaluated only Covaris fragmented DNA during the development of these systems. Other mechanical means of fragmentation, such as sonication, may be suitable as long as the method generates a tight size distribution of DNA fragments with a median size of 200 bp.

Q20. Can I use alternative magnetic separation devices?
Due to the large number of commercially available magnets, we do not have a comprehensive list of compatible products. However, if many magnets are compatible, and as long as the magnet is strong enough to clear the solution of magnetic beads, it can be applied to the system. We have the following guidelines for selecting a magnetic separation device:

1. Use of a magnet designed for 0.2 mL tubes (PCR tubes) can help improve performance. Compared to magnets that are designed for 1.5 mL tubes, these minimize loss that can occur when samples are transferred from one tube to another.
2. Prior to purchasing, check the manufacturer's specifications for minimum and maximum volumes that can be effectively treated. For the Encore Complete RNA-Seq Library System, the minimum is 13 µL and the maximum is 145 µL.
3. Test the magnet with a mock purification to ensure the magnet will effectively clear the solution under the conditions in the NuGEN workflow. This is also helpful to gain familiarity with the purification workflow.

Q21. 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.

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Q22. Do the Encore Complete RNA-Seq Library Systems work with the Illumina Cluster Station (predecessor of the cBot instrument)?
Yes, the Systems are also compatible with the Illumina Cluster Station.

Q23. Can I use the Encore Complete RNA-Seq IL Multiplex Systems 1–8 and 9–16 for experiments using less than 16-plex sequencing?
Yes, but if you choose to use these kits for anything lower than 16-plex experiments, you must use Illumina's PhiX Control (Cat. #FC-110-3001), per the manufacturer's recommendations and reduce library concentration to obtain optimal cluster density. For more information, consult the Illumina Technical Note, "Using a PhiX Control for HiSeq Sequencing Runs" (Pub.No. 770-2011-041).

Q24. What kind of error correction is used to minimize the impact of sequencing errors in the barcodes?
Our balanced 4-base barcodes differ by more than a single nucleotide, so no single base error will result in an incorrect barcode assignment. Again, we recommend that only perfect match barcode reads be used for binning.

For experiments using the Encore Complete RNA-Seq DR Multiplex Systems 1–8 and 9–16 with dedicated read barcodes, please follow the Illumina recommendations on parsing barcodes. The NuGEN dedicated read barcodes are six-base unique barcode tags. The sequences of these NuGEN barcodes must be input prior to parsing.

Q25. Can I combine the barcoded libraries prior to amplification?
The stoichiometry of barcoded libraries may be adversely affected by this modification to the Encore Complete RNA-Seq Library Systems workflow. We suggest that the libraries be amplified and quantitated independently before being balanced and pooled for use on the cBot or Cluster Station.

Q26. What kind of sequencing primers can I use with your library?
The Encore Complete RNA-Seq Library Systems are designed for use with the standard Illumina sequencing primers for both single end and paired-end sequencing applications.

Q27. Can the Encore Complete RNA-Seq 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 for fragments with an average size of 150 bases. That corresponds to the expected distance between the 5′-most and 3′-most coordinates of paired-end reads.

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Q28. Do I need to perform a separate sequencing read for the multiplex adaptors?
The Encore Complete RNA-Seq DR Multiplex Systems barcoding strategy requires a separate, "dedicated" sequencing read to identify the barcode. In contrast, the Encore Complete RNA-Seq IL Library Systems barcoding strategy utilizes an inline barcode that is sequenced as part of the same read as the library insert (see Figure 2, page 13). As a result, the Encore Complete RNA-Seq IL Multiplex System libraries can be treated as a non-barcoded library for the sequencing run (a standard recipe). The barcodes are parsed out (binned) bioinformatically after the sequences are obtained.

Q29. How should I analyze the data from a multiplex experiment using your barcodes? Can you recommend software for segregating sequence reads based on your barcodes?
We suggest that only perfect matches of all barcoded bases be considered for read binning. We recommend that specialized software be utilized for splitting barcoded reads into the appropriate groups. We have successfully used the following software tools for multiplex experiments:

Single End Parsing:
1. Fastx Barcode splitter http://hannonlab.cshl.edu/fastx_toolkit/
2. BARTAB http://www.biomedcentral.com/1471-2105/10/362
3. Fastx-multx utility http://code.google.com/p/ea-utils/

Paired-End Parsing:
1. fastq-multx utility from ea-utils package
   http://code.google.com/p/ea-utils/

Q30. How many bases do the Encore Complete RNA-Seq Library System adaptors add to the library?
The adaptors add 119 bp to the library in Encore Complete RNA-Seq Library System, 123 bp in the Encore Complete RNA-Seq IL Multiplex Systems 1–8 and 9–16, and 122 bp to the Encore Complete RNA-Seq DR Multiplex Systems 1–8 and 9–16.

Q31. What is the expected yield of the amplified DNA library using the Encore Complete RNA-Seq Library Systems?
The expected yield is 0.5–2 µg, 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.

Q32. Are the Encore Library Spike-In Controls included with the Encore Complete RNA-Seq Library Systems?
No. The Encore Library Spike-In Controls (Part No. 0310) are sold separately. Please visit the product webpage for more information: http://www.nugeninc.com/ngsspikes.

Q33. 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. Greater than 95% of reads from Encore Complete RNA-Seq libraries will align in the sense strand orientation relative to the RNA template.

F. Update History

This document, the Encore Complete RNA-Seq Library Systems User Guide (M01244 v4) is an update to address the following topics:

Description (Version)	Section	Page(s)
Added recommendation to perform no less than 16-plex multiplexing with Encore Complete RNA-Seq IL Multiplex Systems 1–8 and 9–16.	I.A., III.B.1., VII.B.,E.	1, 13, 35,40
Addition of description and instructions for the Encore Complete RNA-Seq DR Multiplex Systems 1–8 and 9–16	I.A., II.A. Tables 4 and 5, III.B.C., V.F., VII.B.C.E.	1, 7–9, 13, 14, 24, 33–41
Added recommendation to use Illumina PhiX Control if performing less than 16-plex multiplexing with Encore Complete RNA-Seq IL Multiplex Systems 1–8 and 9–16.	VII.B.,E.	35, 40
Removed content regarding balancing of barcodes.	III.C., VII.B., VII.E.	14, 33–34, 40
Changed median DNA fragment size from 150 bp to 200 bp.	V.C., VII.E.	21, 39
Added section regarding PCR overamplification.	V.L.	30
Added bulleted list of Encore Complete RNA-Seq Library Systems products for clarity (v4)	I.A.	1
Added text to indicate that Encore Complete RNA-Seq DR Multiplex Systems do not require 16-plex multiplexing (v4)	III.B.	14
Added new sections specific to IL and DR barcodes (v4)	VI, VII	31–52
Replaced content in Appendix B to reflect new recommendations for setting up multiplex experiments (v4)	IX.B.	56–57

IX. Appendix

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