USER GUIDE

Encore® NGS Library Automation Solutions

PART NOS. 0300-A01 AND 0315A-0315E

NuGEN
imagine more from less®

Patents, Licensing and Trademarks

The purchase of this product conveys to the buyer the limited, non-exclusive, non-transferable right (without the right to modify, reverse engineer, resell, repackage or further sublicense) under these patent applications and any patents issuing from these patent applications to use this product and methods, accompanying this package insert, for research and development purposes solely in accordance with the intended use described and the written instructions provided in this user guide. No license to make or sell products by use of this product is granted to the buyer whether expressly, by implication, by estoppels or otherwise. In particular, the purchase of this product does not include or carry any right or license to use, develop or otherwise exploit this product commercially and no rights are conveyed to the buyer to use the product or components of the product for purposes including commercial services or clinical diagnostics.

For information on purchasing a license to the NuGEN patents for uses other than in conjunction with this product or to use this product for purposes other than research, please contact NuGEN Technologies, Inc., 201 Industrial Road, Suite 310, San Carlos, CA 94070. Phone 888-654-6544 or 650-590-3600; FAX 888-296-6544 or 650-590-3630.

Warranty

NuGEN warrants that this product meets the performance standards described in the Company's product and technical literature for a period of six months from the date of purchase, provided that the product is handled and stored according to published instructions, and that the product is not altered or misused. If the product fails to meet these performance standards, NuGEN will replace the product free of charge or issue a credit for the purchase price.

NuGEN's liability under this warranty shall not exceed the purchase price of the product. NuGEN shall assume no liability for direct, indirect, consequential or incidental damages arising from the use, results of use, or inability to use its products. NuGEN reserves the right to change, alter, or modify any product to enhance its performance and design.

NuGEN's products are developed, designed and sold FOR RESEARCH USE ONLY. This product is not to be used for diagnostic or therapeutic purposes, nor is it to be administered to humans or animals.

Except as expressly set forth herein, no right to modify, reverse engineer, distribute, offer to sell or sell NuGEN's product is conveyed or implied by buyer's purchase of this NuGEN product. The buyer agrees to use NuGEN products accompanying the product insert in accordance with the intended use and the written instructions provided.

Table of Contents

Contents

I. Introduction

A. Background

The Encore® NGS Library Automation Solution products (Part Nos. 0300-A01 and 0315A–0315E) provide streamlined workflows for NGS library construction from 200 ng of fragmented double-stranded DNA (dsDNA) optimized for use on laboratory automation platforms. Sequencing libraries can be constructed for up to 384-plex multiplexing experiments. These products are suitable for use on the Illumina Genome Analyzer IIx/IIe, HiScan™ SQ, MiSeq™ and HiSeq™ Systems.

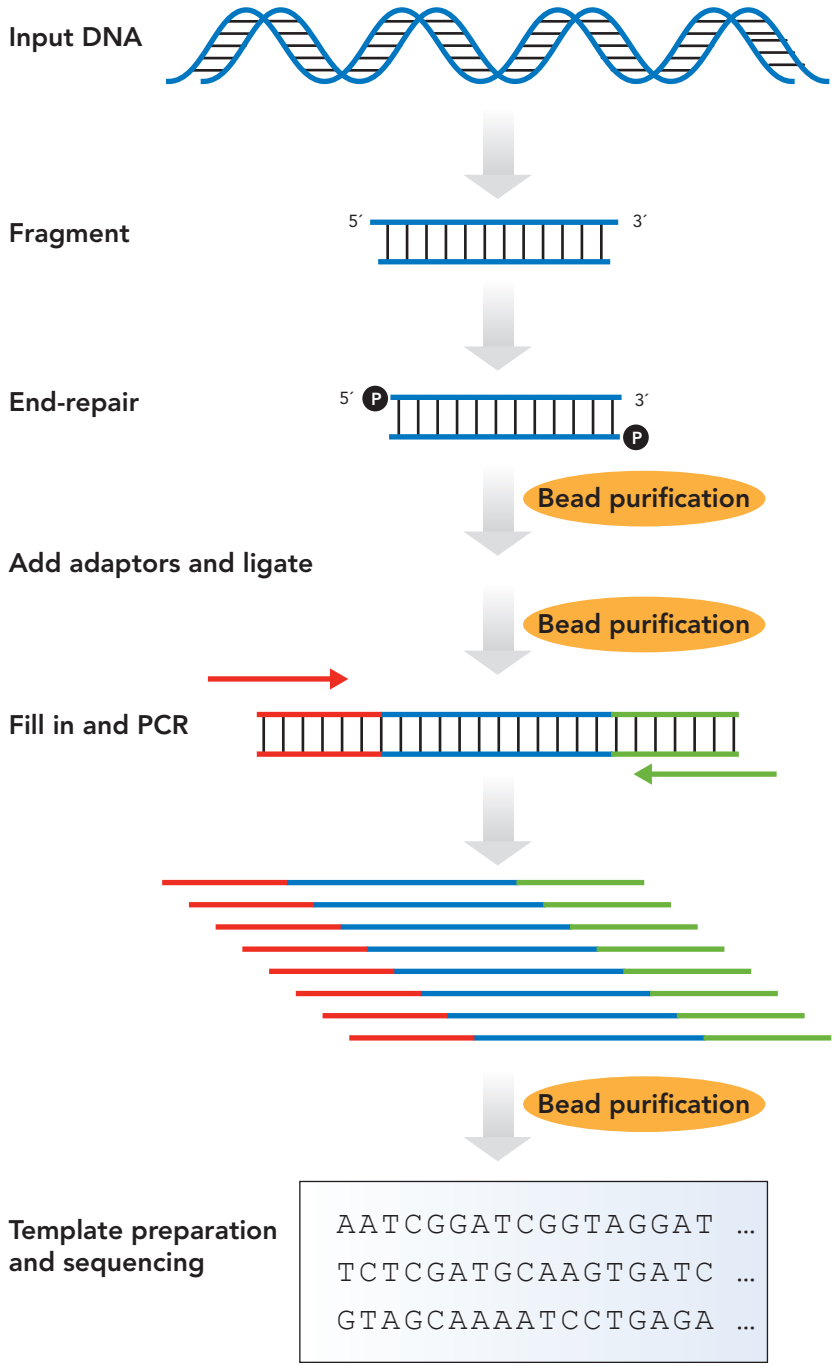
NuGEN offers a complete portfolio of sample preparation solutions for leading NGS platforms, including the Encore NGS Library Automation Solutions as well as the Ovation® RNA-Seq System V2 (Part No. 7102), Ovation RNA-Seq FFPE System (Part No. 7150), Ovation 3'-DGE System (Part No. 7200), Ovation WGA FFPE System (Part No. 6200) and Encore ds-DNA Module (Part No. 2500).

B. Library Construction Process

The workflow (Figure 1) consists of four main steps: fragmentation of double-stranded DNA, end repair to generate blunt ends, ligation of adaptor molecules (with optional inline barcodes provided with some Encore NGS Library Automation Solution products) to the 5' and 3' ends of each fragment, and amplification of fragments with ligated adaptor sequences to produce the final library.

I. Introduction

Figure 1. The Encore NGS Library Automation Solutions library construction process.



I. Introduction

C. Performance Specifications

The Encore NGS Library Automation Solutions comprise fast, simple and robust systems designed to produce DNA libraries suitable for sequencing on Illumina NGS platforms. Starting from 200 ng of fragmented DNA, the system yields libraries ready for cluster formation and either single read or paired-end sequencing in about 5 hours.

D. Quality Control

Each lot of the Encore NGS Library Automation Solutions is tested to meet specifications for library generation performance.

E. Storage and Stability

Encore NGS Library Automation Solutions products are shipped on dry ice and should be unpacked immediately upon receipt.

All components should be stored at –20°C on the internal shelves of a freezer without a defrost cycle.

These products have 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. NuGEN has not yet established long-term storage conditions for Encore NGS Library Automation Solutions.

F. Material Safety Data Sheet (MSDS)

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

II. Kit Components

A. Reagents Provided

Table 1. Encore NGS Library System I Reagents (Part No. 0300-A01)

COMPONENT	0300-A01 PART NO.	VIAL CAP	VIAL NUMBER	MINIMUM RECOVERABLE VOLUME
End Repair Buffer Mix	S01437	Blue	ER1	1553 µL
End Repair Enzyme Mix	S01433	Blue	ER2	311 µL
Ligation Buffer Mix	S01438	Yellow	L1 VER 3	1911 µL
Adaptor Mix	S01492	Yellow	L2 VER 5	2624 µL
Ligation Enzyme Mix	S01434	Yellow	L3	337 µL
Amplification Buffer Mix	S01439	Red	P1	8200 µL
Amplification Primer Mix	S01440	Red	P2	385 µL
Amplification Enzyme Mix	S01435	Red	P3	128 µL
DMSO	S01436	Red	P4	585 µL

! *The L2 VER 5 Adaptor Mix supplied with the Encore NGS Library System I is intended for use only in non-multiplexed libraries as it does not include a barcode. The multiplex Adaptor Mixes supplied with the Encore 384 Multiplex System are to be substituted for L2 VER 5 when running multiplex experiments.*

II. Kit Components

Table 2. Encore 384 Multiplex System Reagents (Part Nos. 0315A, 0315B, 0315C, 0315D and 0315E)

The Encore NGS Library System I (Part No. 0300-A01) serves as a core reagent set and is included with the Encore 384 Multiplex System. In addition to the core reagent set, the Encore 384 Multiplex System includes barcoded adaptors in a 96-well plate.

Note: Discard the L2 VER 5 Ligation Adaptor Mix contained in the Encore NGS Library System I kit. L2 VER 5 is for use only in non-multiplex experiments.

The barcode sequences are available for download from www.nugeninc.com. This file provides the following information:

- Plate (A through D)
- Position (row and column defining a single well on the plate)
- Adaptor ID (a unique name for each Ligation Adaptor Mix)
- Barcode (actual 8-base barcode sequence).

Part Nos. 0315A–0315D each include a single Encore 384 Multiplex Plate (correspondingly Plates A through D) and sufficient reagents for 96 library preps.

Part No. 0315E includes Encore 384 Multiplex Plates A through D (the complete 384 adaptor set) and sufficient reagents for 384 library preps.

COMPONENT	0315A-0315E PART NO.	MINIMUM RECOVERABLE VOLUME
Encore NGS Library System I	0300-A01	-
Encore 384 Multiplex Plate A	S01460	5 µL per well
Encore 384 Multiplex Plate B	S01461	5 µL per well
Encore 384 Multiplex Plate C	S01462	5 µL per well
Encore 384 Multiplex Plate D	S01463	5 µL per well

II. Kit Components

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
  - Multichannel pipettes
  - 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
  - Agencourt® RNAClean® XP Kit (Beckman Coulter Genomics, Cat. #A63987)
  - 1X TE buffer, pH=8.0
  - 1X TE buffer (low EDTA), pH = 8.0 (Affymetrix, Cat. #75793)
  - GlycoBlue (Ambion, Cat. # AM9515)
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - 0.2 mL thin-wall PCR plates or 8 X 0.2 mL thin-wall PCR-strip tubes
  - Agencourt SPRIPlate Ring Super Magnet Plate (Beckman Coulter Genomics, Cat. #A32782)
  - Disposable gloves
  - Kimwipes
  - Ice bucket
  - DNA-OFF™ (MP Biomedicals, Cat. #QD0500)

To Order:

- Agilent, www.agilent.com
- Affymetrix, www.affymetrix.com
- Ambion, www.ambion.com
- Beckman Coulter Genomics, www.beckmangenomics.com
- Covaris, www.covarisinc.com
- MP Biomedicals, www.mpbio.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com

III. Planning the Experiment

A. Input DNA Requirements

The Encore NGS Library Automation Solutions are designed to work with 200 ng of high quality fragmented genomic dsDNA or ds-cDNA with a median size of 150–200 bp (refer to Appendix B for a description of the method recommended for performing DNA Fragmentation). DNA samples must be free of contaminating proteins, RNA, organic solvents (including phenol and ethanol) and salts. Use of a commercially available system for DNA/cDNA isolation is recommended. The A260:A280 ratio for DNA samples should be in excess of 1.8. Use of DNA samples with lower ratios may result in poor performance.

B. Barcode Sequences

The Encore NGS Library Automation Solutions utilize either a 4-base or 8-base barcode sequence for multiplexing. The barcode sequences are available for download in a tab delimited text format from www.nugeninc.com. In addition, the 4-base barcodes are shown in Appendix A. Due to the high level of complexity, the 8-base barcode sequences are not reproduced here.

C. Working with Encore 384 Multiplex Plates

Each Encore 384 Multiplex Plate contains 96 Ligation Adaptor Mixes, each with a unique 8-base barcode, for use in multiplex sequencing experiments. There are four plates (designated A, B, C and D) for a total of 384 unique adaptor mixes designed to enable up to 384-plex multiplex sequencing. Due to the Illumina cluster recognition algorithm, NuGEN does not advise preparing multiplexed library pools at a level of less than 16-plex. Each well of the 96-well plate contains sufficient recoverable volume for a single library prep.

The Encore 384 Multiplex Plates are sealed with a pierceable foil seal. This seal is designed to provide for airtight storage, but is easily pierceable with a pipette tip in either automation settings or when used in a manual workflow. Each well is designed for a single use. Once pierced, the wells are not intended to be resealed.

Prior to use, thaw the Encore 384 Multiplex Plate on ice or at room temperature, mix briefly by vortexing, then spin in a centrifuge with an appropriate rotor for microwell plates.

We recommend using the Illumina standard recipe, and not the multiplex recipe, when sequencing Encore multiplexed libraries on Illumina NGS systems.

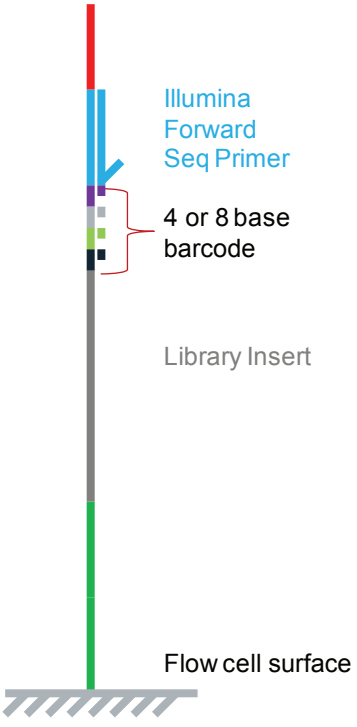
D. Using the Encore Multiplex Libraries on Illumina NGS Systems

We recommend sequencing Encore NGS Library Automation Solutions libraries using the Illumina standard recipe (the workflow for non-multiplexed sequencing) rather than the multiplex recipe, when sequencing on Illumina NGS systems. This applies to both single read and paired end runs. The Encore NGS Library Automation Solutions 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. When multiplexing using the Encore NGS Library Automation Solutions, reads are parsed offline after the sequencing run is completed.

If it is necessary to sequence an Encore NGS Library Automation Solutions library using the Illumina multiplex recipe, the barcodes will still be preserved in read 1; however, the index read (which is a separate read carried out after read 1) will not generate meaningful barcode data in Encore multiplexed libraries. It may be necessary to run Encore multiplexed 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 Encore multiplexed reads offline after the run.

In some cases the reads generated by Encore multiplexed libraries may be sorted into an "Undetermined indices" folder by the Illumina system. This is due to the fact that the Encore libraries do not employ the Illumina multiplexing strategy.

Figure 2. Encore NGS Automation Solutions Multiplex Barcoding Strategy.



E. Selecting Balanced Adaptor Sets for Multiplexing

The Illumina NGS platforms require that the initial base reads show balanced representation of the four bases as a QC measure. The multiplexing strategy used by the Encore NGS Library Automation Solutions places the barcode at the begining of the read (Figure 2), so it is important to use a balanced barcode set when setting up multiplex experiments. Refer to Appendix A for more details on selecting balanced barcode sets for multiplexing.

F. Amplified Library Storage

Amplified libraries may be stored at –20°C.

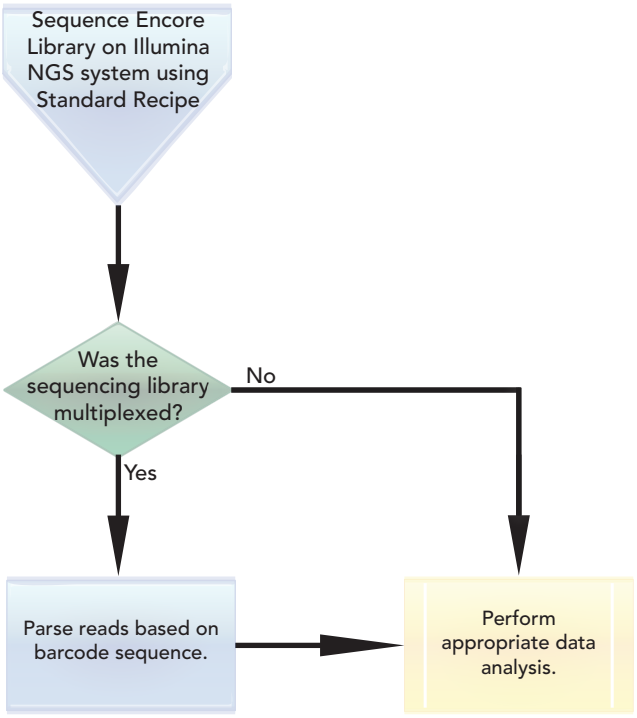
G. Data Analysis Guidelines

Data analysis in the Next Generation Sequencing space 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 workflow will be to parse the reads by sample, based on the barcode sequence (Figure 3). Select an analysis software package that includes a barcode splitter function capable of parsing the 4- and 8-base barcodes used in the Encore NGS Library Automation Solutions products. Reference files containing the barcode sequences for the 4-base or 8-base barcodes used by the Encore NGS Library Automation Solutions products are available for download from www.nugeninc.com to facilitate this step.

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

Figure 3. Recommended Data Analysis Workflow.
Use the standard recipe (non-multiplexed) when using Encore NGS Library Automation Solutions libraries on the Illumina NGS system whether the experiment is multiplex or not. If it is necessary to use the Illumina multiplex recipe due to the presence of Illumina multiplexed libraries on the same flow cell with the Encore multiplex libraries, no Illumina barcode will be detected with the Encore libraries. Encore library reads will be parsed offline after the sequencing run has been completed based on the barcode contained in read 1.



IV. Automation Guidelines

A. Overview

The library preparation process used in an Encore NGS Library Automation Solution is performed in three stages:

1. DNA end repair and purification	1.5 hours
2. Adaptor ligation and purification	1.5 hours
3. Amplification and purification	2.0 hours
Total time to prepare amplified library	**~5 hours**

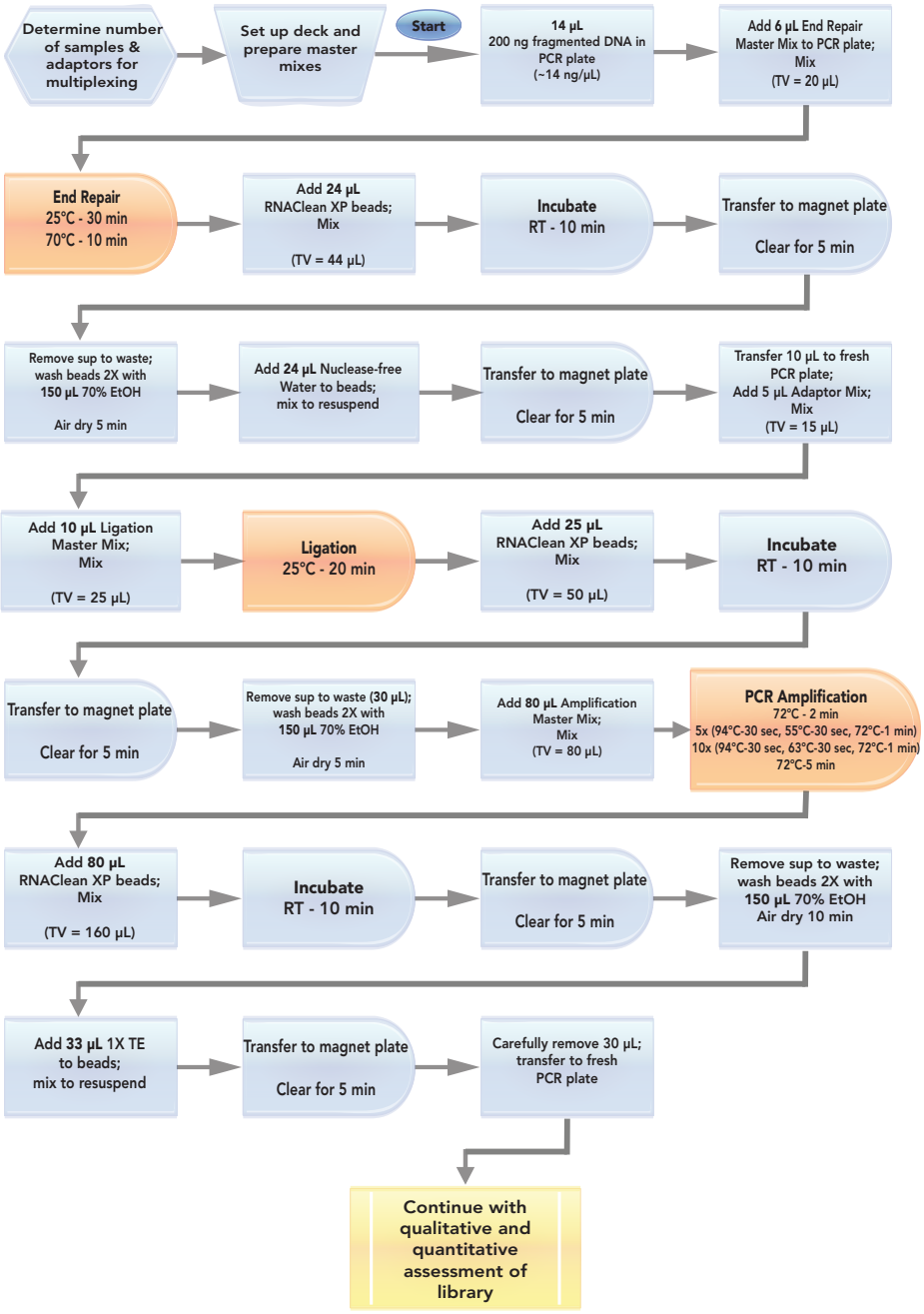
Components in the Encore NGS Library Automation Solutions are color coded, with each color linked to a specific stage of the process. Performing each stage requires making a master mix then adding it to the reaction, followed by incubation. Master mixes are prepared by mixing components provided for that stage.

B. Constructing the Automation Script

The Encore NGS Library Automation Solutions products have been designed to enable the construction of sequencing libraries in 96 sample batches. Reagent volumes have been scaled accordingly, taking into account the overages required for automation protocols. To assist in constructing automation scripts we have provided a Minimum Recoverable Volume for each component (refer to Table 2). This volume can be used as a guideline to determine the feasibility of your particular automation schema. Take care in developing automation scripts that minimize reagent consumption above the nominal volume requirements (dead volumes) in order to ensure 96 full reactions are obtained.

IV. Automation Guidelines

Figure 4. The Encore NGS Library Automation Solutions workflow.



Abbreviations used: TV = Total Volume, RT = Room Temperature.

C. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for PCR plate or 0.2 mL PCR tubes, equipped with a heated lid, and with a capacity of 100 µL reaction volume. Prepare the programs shown in Table 3, following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature to 100°C. Disable the heated lid when incubation temperature is below 30°C (i.e., Program 2 below) . 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). Do not use the heated lid during the Ligation protocol (Program 2).

Table 3. Thermal Cycler Programming

END REPAIR	
Program 1 End Repair	25°C – 30 min, 70°C – 10 min, hold at 4°C
ADAPTOR LIGATION	
Program 2 Adaptor Ligation	25°C – 20 min, hold at 4°C **Note:** Do not use heated lid for this step!
LIBRARY AMPLIFICATION	
Program 3 Library Amplification	72°C – 2 min, 5 cycles of (94°C – 30 sec, 55°C – 30 sec, 72°C – 1 min), 10 cycles of (94°C – 30 sec, 63°C – 30 sec, 72°C – 1 min), 72°C – 5 min, hold at 10°C

D. Master Mix Composition

The composition of the required master mixes on a per reaction basis is given in the tables below.

Table 4. Master Mix Composition (volumes listed are for a single reaction)

END REPAIR MASTER MIX	
END REPAIR BUFFER MIX (BLUE: ER1)	END REPAIR ENZYME MIX (BLUE: ER2)
5 µL	1 µL

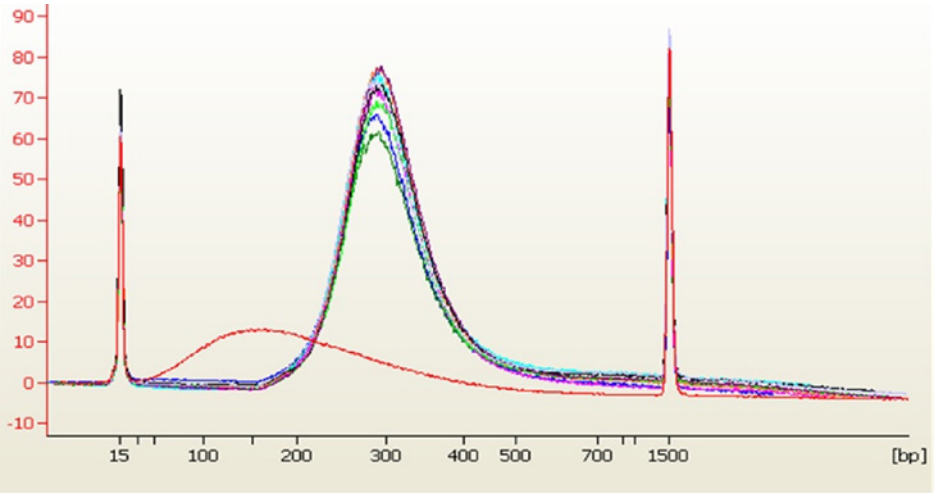
LIGATION MASTER MIX	
LIGATION BUFFER MIX (YELLOW: L1 VER 3)	LIGATION ENZYME MIX (YELLOW: L3)
8.5 µL	1.5 µL

PCR AMPLIFICATION MASTER MIX				
AMPLIFICATION BUFFER MIX (RED: P1)	AMPLIFICATION PRIMER MIX (RED: P2)	DMSO (RED: P4)	NUCLEASE-FREE WATER (GREEN: D1)	AMPLIFICATION ENZYME MIX (RED: P3)
64 µL	3 µL	4 µL	8 µL	1 µL

E. Quantitative and Qualitative Assessment

Run the samples on the Agilent Bioanalyzer using the DNA 1000 LabChip according to the manufacturer's recommendations. A representative fragment distribution pattern is shown in Figure 5.

Figure 5. Fragment distribution for sequencing library preps run on the Agilent Bioanalyzer using the DNA 1000 LabChip.

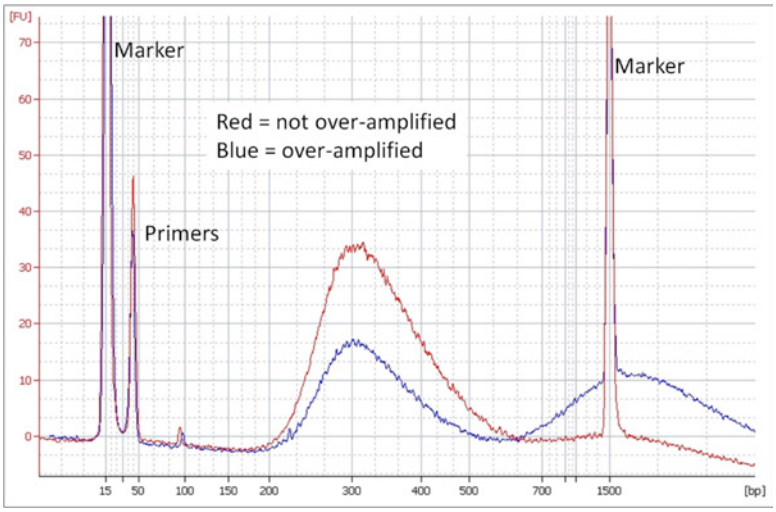


Fragmented template DNA is shown in red. Other traces represent typical sequencing library preps.

F. Potential for Bioanalyzer Artifacts

In some instances, PCR amplification may create artifacts in the downstream library size analysis that appear as high molecular weight species during Bioanalyzer or gel analysis (Figure 6. 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.

Figure 6. Apparent fragment distribution on Bioanalyzer Chip 1000 of a library exhibiting the PCR amplification artifact.



IIf desired, performing a single round of PCR in the presence of excess primer will resolve the material to a single peak.

When quantifying libraries that display the described high molecular weight PCR artifact, use the lower molecular weight peak to estimate library size and qPCR to determine concentration.

V. 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 europe@nugeninc.com.

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

VI. Appendix

A. Considerations for Setting up Multiplex Sequencing Experiments

The NuGEN NGS Library Automation Solutions utilize either a 4-base or 8-base barcode sequence. The barcode sequences are available for download from www.nugeninc.com.

We recommend using the Illumina standard recipe, and not the multiplex recipe, when sequencing Encore multiplexed libraries on Illumina NGS systems.

We recommend sequencing Encore NGS Library Automation Solutions libraries using the Illumina standard recipe (the workflow for non-multiplexed sequencing) rather than the multiplex recipe, when sequencing on Illumina NGS systems. This applies to both single read and paired end runs. The Encore NGS Library Automation Solutions 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, which is the case for the Illumina multiplexing strategy. When multiplexing using the Encore NGS Library Automation Solutions, reads are parsed offline after the sequencing run is completed.

If it is necessary to sequence an Encore NGS Library Automation Solutions library using the Illumina multiplex recipe, the Encore barcodes will still be preserved in read 1; however, the index read (which is a separate read carried out after read 1) will not generate meaningful barcode data in Encore multiplexed libraries. It may be necessary to run Encore multiplexed 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 Encore multiplexed reads offline after the run.

In some cases the reads generated by Encore multiplexed libraries may be sorted into an "Undetermined indices" folder by the Illumina system. This is due to the fact that the Encore libraries do not employ the Illumina multiplexing strategy.

Selecting Balanced Adaptor Sets With the Encore 384 Multiplex System (Part Nos. 0315A-0315E)

The Encore 384 Multiplex System includes sequencing adaptor mixes (Ligation Adaptor Mixes) containing 384 unique 8-base barcodes for use in multiplex sequencing experiments, provided as a set of four NGS 384 Multiplex Plates. Each plate is available independently or they can be ordered as a complete set. As many as 384 different samples can be run in a single flow cell lane using the complete adaptor set. We recommend running no less than 16-plex sequencing experiments using this kit. 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).

Since the barcode sequences comprise the first 8 bases read, it is important that a base-balanced set of adaptor mixes be used in each flow cell lane. Each NGS 384 Multiplex Plate has been designed to contain a balanced set of adaptor mixes. When performing experiments using less than a full plate of 96 adaptor mixes, it is important to select base-balanced adaptor mix subsets to avoid the potential for introducing base bias in the initial reads, which may interfere with the cluster identification algorithm used by the Illumina Systems. Each NGS 384 Multiplex Plate has been config-

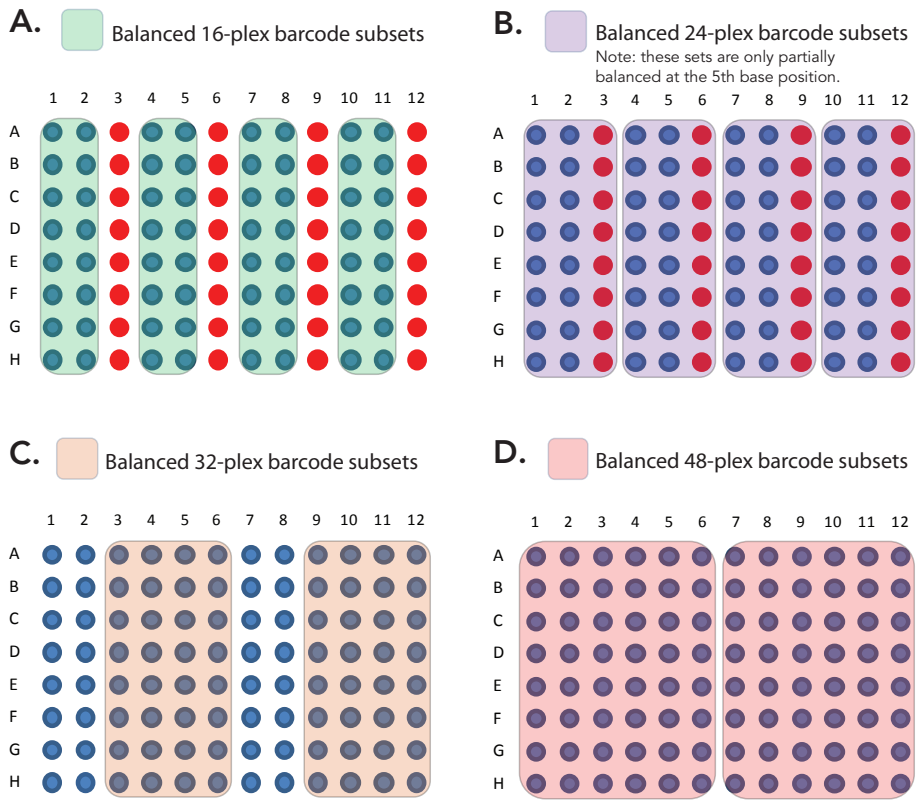
VI. Appendix

ured to facilitate easy identification of balanced adaptor subsets for various levels of multiplexing.

Figure 7 illustrates how each NGS 384 Multiplex Plate is laid out for various levels of multiplexing. For example, Panel A shows the options for 16-plex multiplexing adaptor mix sets. In order to select a base-balanced subset from a given plate, simply choose one of the sets highlighted in yellow. Using these adaptors as a set in a given flow cell lane will ensure that each of the first 5 base positions will have a balanced representation of the four bases. Other levels of multiplexing are also shown in Figure 7.

Note that perfect balance of adaptors becomes progressively less important as the degree of multiplexing increases. Thus in panel D, where the 24-plex sets contain a slight imbalance at the 5th base position, this slight imbalance should not interfere with sequencing performance.

Figure 7. Balanced barcode subsets for various levels of multiplexing with the Encore 384 Multiplex System (Part Nos. 0315A–0315E).



B. DNA Fragmentation

Input DNA must be fragmented prior to use with Encore NGS Library Automation Solutions products. We recommend using the Covaris Adaptive Focused Acoustics method for DNA fragmentation prior to initiation of the library protocol. The protocol below includes an example of settings for producing fragments of approximately 150–200 bp. Consult the Covaris System documentation for guidance on producing larger DNA fragments.

1. Dilute 1–5 µg of each DNA sample in 120 µL 1X TE (low EDTA).
2. Treat all the DNA samples with the Covaris S-Series System according to the manufacturer's recommendations using the settings shown in Table 5 or other user-defined settings that produce fragmented DNA with a median size of 150–200 bp:

! *The Covaris parameters shown here are designed to produce a median fragment size of 150-200 bp.*

Table 5. Covaris S-Series System Settings

PARAMETER	VALUE
Duty Cycle	10%
Intensity	5
Cycles/Burst	100
Time	1 minute
Sample Volume	120 µL

3. If the DNA concentration is less than ~14 ng/µL, it will be necessary to concentrate the fragmented DNA sample before proceeding to the End Repair protocol. The sample can be concentrated with the QIAquick PCR Purification Kit (Cat. # 28104) or the Agencourt RNAClean XP beads. Ethanol precipitation may also be done using GlycoBlue according to the manufacturer's protocol.

   Notes: If the QIAquick concentration method is chosen, follow the manufacturer's protocol and elute in 30 µL of 1X TE (low EDTA).

   If the RNAClean XP concentration method is chosen, follow the protocol given in the End Repair Purification protocol on page 22 but in step 4 use a ratio of 1 volume of sample to 1.2 volumes of beads. For example, if your sample volume is 120 µL, you will add 144 µL of beads. Elute in 30 µL of 1X TE (low EDTA). Beads are not provided for this step.

4. Quantify the fragmented DNA sample by UV/Vis spectrophotometry and evaluate the size distribution on an Agilent Bioanalyzer using the HS DNA method (or agarose gel) to verify the median fragment size.

5. Continue immediately with the Encore NGS Library Automation Solutions protocol or store the fragmented DNA at –20°C.

C. Manual Protocol

While the Encore NGS Library Automation Solutions products are designed for use on laboratory automation platforms, it is also possible to carry out manual preparation of sequencing libraries. For manual processing, please follow the protocol given below. This protocol should be used as a guideline for constructing automation scripts.

End Repair

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

Do not vortex any enzyme mixes.

1. Remove the RNAClean XP purification beads from 4°C storage and place on the bench top for use in the next step.
2. Obtain the End Repair Buffer Mix (blue: ER1), End Repair Enzyme Mix (blue: ER2) and the Nuclease-free Water (green: D1) from –20°C storage:
3. Spin down the contents of ER2, place on ice.
4. Thaw ER1 at room temperature, mix by vortexing, spin and place on ice. Leave the Nuclease-free Water at room temperature.
5. In a 0.2 mL PCR tube, dilute 200 ng of the fragmented DNA to a volume of 14 µL (~14 ng/µL) in the supplied Nuclease-free Water (green: D1).
6. Prepare a master mix by combining ER1 and ER2 in a 0.5 mL capped tube, according to the volumes shown in Table 6.

Table 6. End Repair Master Mix (volumes listed are for a single reaction)

END REPAIR BUFFER MIX (BLUE: ER1)	END REPAIR ENZYME MIX (BLUE: ER2)
5 µL	1 µL

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

7. Add 6 µL of the End Repair Master Mix to each tube.
8. Mix by pipetting 5 times, spin and place on ice.
9. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (End Repair; see Table 3):

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

VI. Appendix

End Repair Purification

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

1. Ensure the Agencourt RNAClean XP beads have completely reached room temperature before proceeding.
2. Ensure that the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.

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

3. Prepare at least 1 mL of 70% ethanol wash solution for each sample. This wash will be used again in a later step and preparing at least 1 mL per sample will ensure sufficient wash is available for both purification steps.

   Note: 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.

4. At room temperature, add 24 µL (1.2 volumes) of the bead suspension to each reaction.
5. Mix thoroughly by pipetting 10 times.

   Note: It may be helpful to use a multi-channel pipettor for this and subsequent steps both for ease of use and to ensure the incubation times are uniform.

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.

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

8. Carefully remove only 30 µL of the supernatant 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 ligation, so ensure beads are not removed with the binding buffer or the wash.

9. With the tubes still on the magnet, add 150 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.

    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 5 minutes.

Ensure that all residual ethanol is removed prior to continuing.

13. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.

14. Remove the tubes from the magnet.
15. Add 24 µL Nuclease-free Water (green: D1) to the dried beads. Mix thoroughly by pipetting to ensure all the beads are resuspended.
16. Transfer the tubes to the magnet and let stand for 5 minutes.
17. Carefully remove 10 µL of the eluate ensuring beads are not carried over, transfer to a fresh set of 0.2 mL PCR tubes and place on ice.
18. Set aside the Agencourt RNAClean XP beads and the remaining 70% ethanol at room temperature for use in a later step.

    Note: Store the ethanol in a tightly sealed container.
19. Continue immediately with the Adaptor Ligation protocol.

Important Note: Prepare the Library Amplification Master Mix during the Adaptor Ligation incubation step (below). Reserve the addition of the P3 Enzyme Mix until the ligation reactions have been completed and you are ready to add the Library Amplification Master Mix to the reactions. It is critical that the ligation product does not remain on ice for more than 10 minutes before the amplification reaction begins.

Adaptor Ligation

! *If multiplexing, plan your Ligation Adaptor Mix selection carefully. Make sure you have understood the material in Appendix A regarding selection of base balanced barcode sets prior to setting up the ligations.*

1. Obtain the Ligation Buffer Mix (yellow: L1), Ligation Adaptor Mixes (yellow: L2 VER 5 for non-multiplexed libraries; L2V2 or NGS 384 Multiplex Plate(s) for multiplexing), Ligation Enzyme Mix (yellow: L3), Amplification Buffer Mix (red: P1), Amplification Primer Mix (red: P2) and DMSO (red: P4) from –20°C storage.
2. Spin down L3 and place on ice.
3. Thaw the remaining reagents at room temperature, mix by vortexing, spin and place on ice. Set aside P1, P2 and P4 for use in the Library Amplification protocol immediately after the completion of Adaptor Ligation.

   Note: Place P4 at room temperature, as the DMSO freezes if kept on ice.

! *Make sure you are using the appropriate Ligation Adaptor Mixes for your experiment.*

4. Add 5 µL of the appropriate Ligation Adaptor Mix to each sample. Mix by pipetting thoroughly with a pipette set to 10 µL.

   Note: The L2 VER 5 supplied with the Encore NGS Library System I is intended for use only in non-multiplexed libraries as it does not include a barcode sequence.
5. Make a master mix by combining L1 and L3 in a 0.5 mL capped tube, according to the volumes shown in Table 7. Mix by pipetting slowly, without introducing bubbles, spin and place on ice. Use immediately.

Mix by pipetting slowly, without introducing bubbles, spin for 2 seconds and place on ice. Use mix immediately.

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

LIGATION BUFFER MIX (YELLOW: L1)	LIGATION ENZYME MIX (YELLOW: L3
8.5 µL	1.5 µL

6. Add 10 µL of the Ligation Master Mix to each reaction tube.
7. Mix by pipetting 5 times at a 20 µL setting, spin and place on ice.
8. Place the tubes in a pre-warmed thermal cycler programmed to run Program 2 (Adaptor Ligation; see Table 3):

   25°C – 20 min, hold at 4°C

   Note: Do not use the heated lid for this step. Leave the thermal cycler lid open.
9. During the Adaptor Ligation incubation, make a master mix by combining P1, P2, P4 and D1 in an appropriately sized, capped tube according to the volumes shown in Table 8 (page 26). Do not add the P3 enzyme mix at this point.
10. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
11. Continue immediately with the Ligation Purification protocol.

Ligation Purification

Note: Ensure the Agencourt RNAClean XP beads are still at room temperature before proceeding.

1. Ensure that the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.

   Note: 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.
2. At room temperature, add 25 µL (1.0 volumes) of the bead suspension to each reaction.
3. Mix thoroughly by pipetting 10 times.

   Note: It may be helpful to use a multi-channel pipettor for this and subsequent steps both for ease of use and to ensure the incubation times are uniform.
4. Incubate at room temperature for 10 minutes.
5. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.

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

! *Minimize bead loss by leaving a residual volume of supernatant after completion of the binding step.*

6. Carefully remove only 35 µL of the supernatant 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 ligation, so ensure beads are not removed with the binding buffer or the wash.

7. With the tubes still on the magnet, add 150 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
8. Remove the 70% ethanol wash using a pipette.
9. Repeat the 70% ethanol wash.

   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.

10. Air-dry the beads on the magnet for 5 minutes.

! *Ensure that all residual ethanol is removed prior to continuing.*

11. 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. Set aside the Agencourt RNAClean XP beads and the remaining 70% ethanol at room temperature for use in a later step.

    Note: Store the ethanol in a tightly sealed container.

14. Continue immediately with the Library Amplification protocol with the ligation product still bound to the beads.

Library Amplification

1. Obtain the Amplification Enzyme Mix (red: P3) from –20°C storage.
2. Spin down P3 and place on ice.
3. Add P3 to the Amplification Master Mix (prepared previously) according to the volume shown in Table 1. Mix well by pipetting taking care to avoid bubbles, spin and place on ice.

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

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

AMPLIFICATION BUFFER MIX (RED: P1)	AMPLIFICATION PRIMER MIX (RED: P2)	DMSO (RED: P4)	NUCLEASE-FREE WATER (GREEN: D1)	AMPLIFICATION ENZYME MIX (RED: P3)
64 µL	3 µL	4 µL	8 µL	1 µL

4. On ice, add 80 µL of the Amplification Master Mix to each tube containing the dried beads. Mix thoroughly by pipetting at least 8–10 times. Attempt to get the majority of the beads in suspension and remove most of the beads from the tube walls.

   Note: The beads may not form a perfectly uniform suspension, but this will not affect the reaction. The addition of Amplification Master Mix will elute the cDNA from the beads.

5. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (Library Amplification; see Table 3):

   72°C – 2 min,
   5 cycles of (94°C – 30 sec, 55°C – 30 sec, 72°C – 1 min),
   10 cycles of (94°C – 30 sec, 63°C – 30 sec, 72°C – 1 min),
   72°C – 5 min, hold at 10°C

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

7. Continue immediately with the Purification of the Amplified Library protocol.

Purification of the Amplified Library

1. Obtain the RNAClean XP beads and 70% ethanol set aside earlier. Ensure both are at room temperature before proceeding.

2. Ensure that the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.

3. At room temperature, add 80 µL (1 volume) of the bead suspension to each reaction.

4. Mix thoroughly by pipetting 10 times.

   Note: It may be helpful to use a multi-channel pipettor for this and subsequent steps both for ease of use and to ensure the incubation times are uniform.

5. Incubate at room temperature for 10 minutes.

6. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.

! *Minimize bead loss by leaving a residual volume of supernatant after completion of the binding step.*

7. Carefully remove only 140 µL of the supernatant 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 ligation, so ensure beads are not removed with the binding buffer or the wash.

8. With the plate still on the magnet, add 150 µL of the 70% ethanol and allow to stand for 30 seconds.
9. Remove the 70% ethanol wash using a pipette.
10. Repeat the 70% ethanol wash.

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

11. Air dry the beads on the magnet for a minimum of 10 minutes.

! *Ensure that all residual ethanol is removed prior to continuing.*

12. 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 33 µL of 1X TE to the dried beads. Mix thoroughly to ensure all the beads are resuspended.
15. Transfer the tubes to magnet and let stand for 2 minutes.
16. Carefully remove 30 µL of the eluate ensuring as few beads as possible are carried over and transfer to a fresh set of tubes.

    Note: When using the library in subsequent methods, replace the tubes on the magnet for a few minutes prior to sampling to minimize bead carryover.

17. Continue immediately with the Quantitative and Qualitative Assessment protocol or store the purified sequencing libraries at –20°C.

D. Frequently Asked Questions (FAQs)

Q1. What kind of sequencing primers can I use with your library?
The Encore NGS Library Automation Solutions are designed for use with the standard Illumina sequencing primers.

Q2. Do the Encore NGS Library Automation Solutions work with the Illumina Cluster Station (predecessor of the cBot instrument)?
Yes, the Systems are also compatible with the Illumina Cluster Station.

VI. Appendix

Q3. Why did I receive an Encore NGS Library System I (Part No. 0300-A01) when I ordered a different kit?
The Encore NGS Library System I kit provides the reagents necessary for library construction. Specific Ligation Adaptor Mixes are provided when ordering an Encore NGS Library Automation Solutions multiplex product, which will be substituted for the L2 VER 5 Ligation Adaptor Mixes supplied with the Encore NGS Library System I kit.

Q4. Are the Agencourt RNAClean XP purification beads provided with Encore NGS Library Automation Solutions products?
No. Due to the high variability in bead volume requirements for various automation platforms, RNAClean XP purification beads are not provided and should be purchased separately.

Q5. I don't have access to a Covaris instrument, can I use alternative fragmentation methods?
We have only evaluated Covaris fragmented DNA during the development of the Encore NGS Library Automation Solutions. 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 fragment that is suitable from your application.

Q6. How does your protocol improve the efficiency of ligation and avoid adaptor dimer formation?
The Encore NGS Library Automation Solutions utilize optimized chemistries to increase the efficiency of blunt-end adaptor ligation and minimize the amount of adaptor dimer in the library.

Q7. Does NuGEN provide reagents for performing the fragmentation step of the protocol?
We suggest that the end users utilize the Covaris instrument and consult the Covaris System documentation for guidance on producing DNA fragments of desired size range. NuGEN does not provide the reagents used in the fragmentation steps.

Q8. What is the expected yield of the amplified DNA library using the Encore NGS Library Automation Solutions?
The expected yield is 0.2–2 µg, depending on the quantity and quality of the cDNA or genomic DNA.

Q9. Can I use the Encore 384 Multiplex System 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 (Catalog # 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).

VI. Appendix

E. Update History

This document, the Encore NGS Library Automation Solutions User Guide (M01229 v3) is an update to address the following topics.

Description	Section(s)	Page(s)
Added recommendation to perform no less than 16-plex multiplexing Encore NGS Multiplex System I. (v2)	II.A., VI.A.	5, 19
Added recommendation to perform no less than 16-plex multiplexing Encore 384 Multiplex System. (v2)	III.C., VI.A.	8, 21
Removed figures illustrating balancing of barcodes for less than 16-plex multiplexing. (v2)	VI.A.Fig.6	22
Added recommendation to use Illumina PhiX Control when performing less than 16-plex multiplexing with Encore NGS Multiplex System I or Encore 384 Multiplex System. (v2)	VI.A., VI.D.Q10.	19, 31
Removed content regarding balancing of barcodes for Encore NGS Library System I. (v2)	VI. A.	20
Added sections regarding PCR overamplification. (v2)	IV.E.	16-17
Remove recommendations for Encore NGS Multiplex System I, Part No. 0314-A01. (v3)	Throughout	Throughout
Remove recommendations for Encore Spike-in Controls. (v3)	Throughout	Throughout

NuGEN Technologies, Inc.

Headquarters USA

201 Industrial Road, Suite 310
San Carlos, CA 94070 USA
Toll Free Tel: 888.654.6544
Toll Free Fax: 888.296.6544
custserv@nugeninc.com
techserv@nugeninc.com

Europe

P.O. Box 109
9350 AC Leek
The Netherlands
Tel: +31-13-5780215
Fax: +31-13-5780216
europe@nugeninc.com

For our international distributors contact information, visit our website

www.nugeninc.com