USER GUIDE

Encore® NGS Library Systems *for* Ion Torrent™

PART NOS. 0306, 0307 AND 0308

NuGEN
imagine more from less®

Patents, Licensing and Trademarks

 The Ovation® and Applause® families of products and methods are covered by U.S. Patent Nos. 6,692,918, 6,251,639, 6,946,251, 7,354,717, 7,771,946, and 8,071,311 and other issued and pending patents in the U.S. and other countries. NuGEN, Ovation, SPIA, Ribo-SPIA, Applause, Encore, Prelude, Mondrian and Imagine More From Less are trademarks or registered trademarks of NuGEN Technologies, Inc. Other marks appearing in these materials are marks of their respective owners.

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 1
- A. Background 1
- B. Library Construction Process 1
- C. Performance Specifications 3
- D. Quality Control 3
- E. Storage and Stability 3
- F. Material Safety Data Sheet (MSDS) 3

II. Kit Components 4
- A. Reagents Provided 4
- B. Additional Equipment, Reagents and Labware 7

III. Planning the Experiment 9
- A. Input DNA Requirements 9
- B. Amplified Library Storage 9

IV. Protocol 10
- A. Overview 10
- B. Protocol Notes 10
- C. Agencourt® RNAClean® XP Purification Beads 11
- D. Programming the Thermal Cycler 13
- E. DNA Fragmentation 13
- F. Size Selection 15
- G. End Repair 16
- H. Adaptor Ligation 17
- I. Ligation Product Purification 18
- J. Library Amplification 19
- K. Purification of the Amplified Library 20
- L. Quantitative and Qualitative Assessment 21

V. Technical Support 23

VI. Appendix 24
- A. Considerations for Multiplex Experiments 24
- B. Frequently Asked Questions (FAQs) 26
- C. Update History 27

I. Introduction

A. Background

The Encore® NGS Library Systems *for* Ion Torrent™ (Part Nos. 0306, 0307 and 0308) provide a streamlined workflow for NGS library construction from a minimum of 100 ng of fragmented double-stranded DNA (dsDNA) that can be completed in as little as four hours, with only two purification steps. Kit configurations supporting both multiplexed and non-multiplexed sequencing applications are available. These libraries are suitable for sequencing on the Ion Personal Genome Machine™ (PGM™) from Life Technologies.

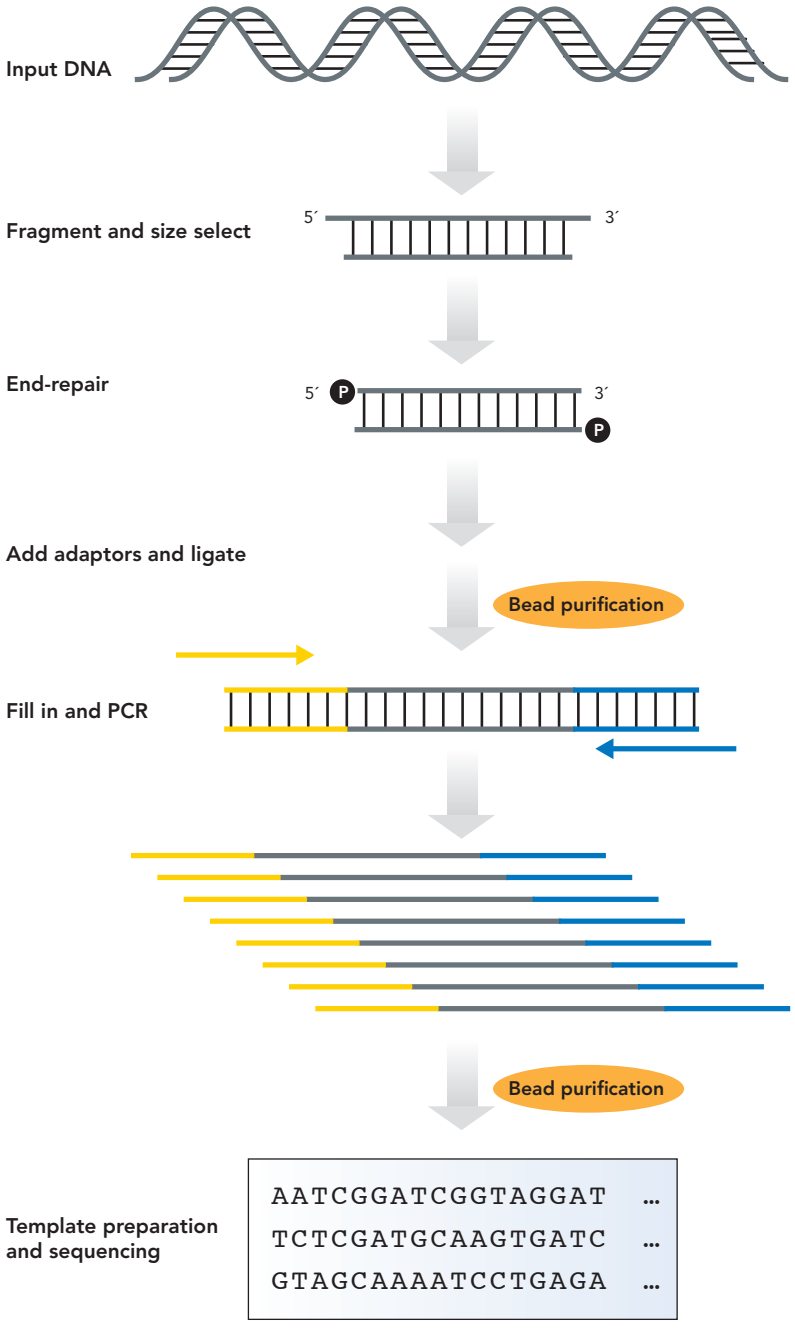
This product, in combination with the Ovation® RNA-Seq System V2 (Part No. 7102), Ovation RNA-Seq FFPE System (Part No. 7150), Ovation 3'-DGE System (Part No. 7200), Ovation WGA FFPE System (Part No. 6200), Encore NGS Library Systems (Automation, Part No. 0314), Ovation Ultralow Library Systems (Part Nos. 0303, 0304, 0305), Encore Complete RNA-Seq Systems (Part Nos. 0311, 0312, 0313), and Encore 384 Library System (Part No. 0315), is a component in an expanding portfolio of NGS sample preparation solutions from NuGEN enabling a wide range of key applications on leading NGS platforms.

B. Library Construction Process

The workflow (Figure 1) consists of four main steps: (1) Fragmented double-stranded DNA is size selected using an innovative, automation friendly magnetic bead based procedure to isolate fragments with a median size of 150 base pairs (bp), (2) The ends of these fragments are repaired to generate blunt ends, (3) Adaptor molecules are ligated, placing specific adaptors on the 5′ and 3′ end of each fragment, and (4) Fragments with ligated adaptor sequences are generated by a fill-in reaction and simultaneous PCR amplification to produce the final library and allow for the optional incorporation of barcodes for multiplexing sequencing.

I. Introduction

Figure 1. The Encore NGS Library Systems *for* Ion Torrent process.



I. Introduction

C. Performance Specifications

The Encore NGS Library Systems *for* Ion Torrent are fast, simple and robust, designed to produce DNA libraries suitable for sequencing on the Ion PGM System. Starting with 100 ng to 1 µg of fragmented DNA, the systems generate libraries suitable for template preparation in about four hours.

D. Quality Control

Each Encore NGS Library System *for* Ion Torrent lot is tested to meet specifications for library generation performance.

E. Storage and Stability

This product contains components with multiple storage temperatures.

The Encore NGS Library Systems *for* Ion Torrent are shipped on dry ice and should be unpacked immediately upon receipt.

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

This product has been tested to perform to specifications after as many as six freeze/thaw cycles. Kits handled and stored according to the above guidelines will perform to specifications for at least six months. NuGEN has not yet established long-term storage conditions for the Encore NGS Library Systems *for* Ion Torrent.

F. Material Safety Data Sheet (MSDS)

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

II. Kit Components

A. Reagents Provided

Table 1. Encore NGS Library System *for* Ion Torrent Reagents (Part No. 0306)

COMPONENT	0306-08 PART NUMBER	VIAL CAP	VIAL NUMBER
End Repair Buffer Mix	S01464	Blue	ER1 VER 3
End Repair Enzyme Mix	S01510	Blue	ER2 VER 4
End Repair Enhancer	S01563	Blue	ER3
Ligation Buffer Mix	S01311	Yellow	L1
Ligation Adaptor Mix	S01441	Yellow	L2 VER 3
Ligation Enzyme Mix	S01313	Yellow	L3
Amplification Buffer Mix	S01314	Red	P1
Amplification Primer Mix	S01442	Red	P2 VER 2
Amplification Enzyme Mix	S01316	Red	P3
DMSO	S01317	Red	P4
Nuclease-free Water	S01001	Green	D1
Agencourt RNAClean XP Beads	S01307	Clear	-

II. Kit Components

Table 2. Encore NGS Multiplex Library System I *for* Ion Torrent Reagents (Part No. 0307)

COMPONENT	0307-32 PART NUMBER	VIAL CAP	VIAL NUMBER
End Repair Buffer Mix	S01464	Blue	ER1 VER 3
End Repair Enzyme Mix	S01510	Blue	ER2 VER 4
End Repair Enhancer	S01563	Blue	ER3
Ligation Buffer Mix	S01311	Yellow	L1
L2V3-BC1 Ligation Adaptor Mix	S01443	Yellow	L2V3–BC1
L2V3-BC2 Ligation Adaptor Mix	S01444	Yellow	L2V3–BC2
L2V3-BC3 Ligation Adaptor Mix	S01445	Yellow	L2V3–BC3
L2V3-BC4 Ligation Adaptor Mix	S01446	Yellow	L2V3–BC4
L2V3-BC5 Ligation Adaptor Mix	S01447	Yellow	L2V3–BC5
L2V3-BC6 Ligation Adaptor Mix	S01448	Yellow	L2V3–BC6
L2V3-BC7 Ligation Adaptor Mix	S01449	Yellow	L2V3–BC7
L2V3-BC8 Ligation Adaptor Mix	S01450	Yellow	L2V3–BC8
Ligation Enzyme Mix	S01313	Yellow	L3
Amplification Buffer Mix	S01314	Red	P1
Amplification Primer Mix	S01459	Red	P2 VER 2
Amplification Enzyme Mix	S01316	Red	P3
DMSO	S01317	Red	P4
Nuclease-free Water	S01001	Green	D1
Agencourt RNAClean XP Beads	S01307	Clear	-

II. Kit Components

Table 3. Encore NGS Multiplex Library System IB *for* Ion Torrent Reagents (Part No. 0308)

COMPONENT	0308-32 PART NUMBER	VIAL CAP	VIAL NUMBER
End Repair Buffer Mix	S01464	Blue	ER1 VER 3
End Repair Enzyme Mix	S01510	Blue	ER2 VER 4
End Repair Enhancer	S01563	Blue	ER3
Ligation Buffer Mix	S01311	Yellow	L1
L2V3-BC9 Ligation Adaptor Mix	S01451	Yellow	L2V3–BC9
L2V3-BC10 Ligation Adaptor Mix	S01452	Yellow	L2V3–BC10
L2V3-BC11 Ligation Adaptor Mix	S01453	Yellow	L2V3–BC11
L2V3-BC12 Ligation Adaptor Mix	S01454	Yellow	L2V3–BC12
L2V3-BC13 Ligation Adaptor Mix	S01455	Yellow	L2V3–BC13
L2V3-BC14 Ligation Adaptor Mix	S01456	Yellow	L2V3–BC14
L2V3-BC15 Ligation Adaptor Mix	S01457	Yellow	L2V3–BC15
L2V3-BC16 Ligation Adaptor Mix	S01458	Yellow	L2V3–BC16
Ligation Enzyme Mix	S01313	Yellow	L3
Amplification Buffer Mix	S01314	Red	P1
Amplification Primer Mix	S01459	Red	P2 VER 2
Amplification Enzyme Mix	S01316	Red	P3
DMSO	S01317	Red	P4
Nuclease-free Water	S01001	Green	D1
Agencourt RNAClean XP Beads	S01307	Clear	-

II. Kit Components

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - 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
  - (optional) Diagenode® Bioruptor® NGS or Covaris® S-series Sonication System
  - (optional) Spin-Vac Concentrator
- **Reagents**
  - Ethanol (Sigma-Aldrich, Cat. # E7023), for purification steps
  - 1X TE buffer, pH=8.0
  - 1X TE buffer (low EDTA), pH = 8.0 (Affymetrix USB Products, Part No. 75793)
- **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 for the Size Selection protocol and the End Repair Product Bead Purification protocol (select one option):
    - MagnaBot® II Magnetic Separation Device (Promega, Cat. # V8351)
    - Agencourt SPRIStand (Beckman Coulter Genomics, Cat. # A29182)
  - Magnetic separation device for the Purification of the Amplified Library protocol (select one option):
    - Agencourt SPRIPlate® 96R Ring Magnet Plate (Beckman Coulter Genomics, Cat. # A29164)
    - Agencourt SPRIPlate Ring Super Magnet Plate (Beckman Coulter Genomics, Cat. # A32782)
    - Agencourt SPRIStand (Beckman Coulter Genomics, Cat. # A29182).
  - (optional) Microcon YM-30 Microconcentrator Column (Millipore, Cat. #42422)
  - Disposable gloves
  - Kimwipes
  - Ice bucket
  - Cleaning solutions such as DNA-OFF™ (MP Biomedicals, Cat. # QD0500)

II. Kit Components

To Order:

- Affymetrix USB Products, www.affymetrix.com
- Beckman Coulter Genomics, www.beckmangenomics.com
- Covaris, www.covarisinc.com
- Diagenode, www.diagenode.com
- Millipore Corporation, www.millipore.com
- MP Biomedicals, www.mpbio.com
- Promega, www.promega.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com

III. Planning the Experiment

A. Input DNA Requirements

The Encore NGS Library Systems *for* Ion Torrent are designed to work with 100 ng to 1 µg of high quality genomic DNA or ds-cDNA. 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. Amplified Library Storage

Amplified libraries may be stored at –20°C.

IV. Protocol

A. Overview

The library preparation process used in the Encore NGS Library Systems *for* Ion Torrent is performed in four stages:

1. DNA size selection	1 hour
2. DNA end repair	0.5 hours
3. Adaptor ligation and purification	1 hour
4. Amplification and purification	1.5 hours
Total time to prepare amplified library	**~4 hours**

Components in the Encore NGS Library Systems *for* Ion Torrent 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. Protocol Notes

- We recommend the routine use of a positive control DNA. Especially the first time you set up a reaction, the use of a positive control DNA will allow the establishment of a baseline of performance and provide the opportunity to become familiar with the bead purification steps.
- Use the Nuclease-free 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.
- When preparing libraries using amplified cDNA from the Ovation RNA-Seq, Ovation RNA-Seq V2, Ovation RNA-Seq FFPE or Ovation 3'-DGE Systems it is important to use Master Mix B as indicated in Table 6B. Do not include component ER3 (End Repair Enhancer) in the master mix. Instead, substitute 0.6 uL of water (D1) in the place of ER3 as indicated.
- Setting up a minimum of four reactions at a time ensures that you are not pipetting very small volumes.
- Thaw components used in each step as described in the protocol and immediately place them on ice. Do not thaw all reagents at once.
- 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 it completely prior to use. You may gently warm the buffer mix for two 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 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 recovery of the maximum number of reactions.
- Components and reagents from other NuGEN kits should not be used with the Encore NGS Library Systems *for* Ion Torrent.
- Use only fresh ethanol stocks to make 70% ethanol used in the purification protocols. Make the ethanol mixes fresh as well, carefully measuring both the ethanol and water with pipettes. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the beads.

C. Agencourt® RNAClean® XP Purification Beads

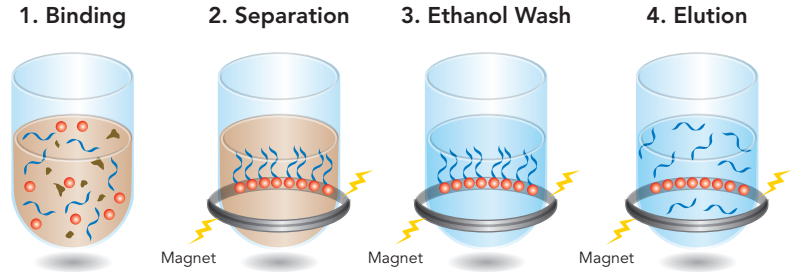
Tips and Notes

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 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 2. Agencourt® RNAClean® XP Beads process overview.



Reproduced from original picture from Agencourt/Beckman Coulter Genomics

IV. Protocol

Additional Tips and Notes

- Remove the 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 the beads by inverting and tapping before adding to sample.
- Note that ratio of RNAClean XP bead volume to sample volume varies depending on the specific protocol. The bead to sample ratios described are critical to the requirements of the specific protocol and deviating from the stated recommendations may result in poor performance.
- It is important to let the beads separate on the magnet for the full time interval defined in the protocol. Continuing with the next step before the beads have completely separated will impact DNA yields.
- After completing the binding step, it is critical to minimize bead loss when removing the binding buffer. With the samples placed on the magnet, remove only the indicated volume of supernatant from each sample. In some cases, liquid will remain at the bottom of the tube. This is by design and is intended to 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 do not advise the use of individual tubes as they are difficult to position stably on the magnet.

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 4, 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). Do not use the heated lid during the Ligation protocol (Program 2).

Table 4. 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 – 10 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

E. DNA Fragmentation

DNA samples must be fragmented to a median fragment length of 150 bp prior to sequencing library construction. There is a variety of physical and enzymatic methods that can theoretically be used for fragmentation. NuGEN has not carried out any exhaustive review of the suitability of these various methods for use with this kit; however, in practice many may prove to be compatible.

Below we describe two methods which have been used successfully: the Bioruptor® NGS Fragmentation Method and the Covaris Fragmentation Method.

The Bioruptor NGS Fragmentation Method

The Diagenode Bioruptor NGS sonication device has been successfully used in conjunction with the Encore NGS Library Systems *for* Ion Torrent. Follow the manufacturer's recommendations to produce fragmented DNA with a median size of 150 bp.

IV. Protocol

The Covaris Fragmentation Method

1. Dilute each DNA sample (100 ng to 1 µg) to 130 µL in 1X TE Buffer (low EDTA).

   Note: If using less than 433 ng of DNA input, it will be necessary to concentrate the fragmented DNA prior to size selection.

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 bp.

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

Table 5. Covaris S-Series System Settings

PARAMETER	VALUE
Duty Cycle	10%
Intensity	5
Cycles/burst	100
Time	430 seconds
Temperature (water bath)	6-8°C
Power Mode	Frequency Sweeping
Degassing Mode	Continuous
Sample volume	130 µL
Buffer	1X TE Buffer (low EDTA)
DNA Mass	<5 µg
Starting Material	ds-cDNA or gDNA
Water (FILL/RUN)	S2 – level 12 E210 – level 6
AFA Intensifier	Yes

3. If less than 433 ng of DNA is used, concentrate the fragmented DNA using a Spin-Vac Concentrator (without heating) or Microcon YM-30 column to a volume of 30 µL or less.

4. Bring concentrated DNA samples up to 30 µL volume by adding 1X TE Buffer (low EDTA) if necessary.
5. Continue immediately with the Size Selection protocol or store fragmented DNA at –20°C.

F. Size Selection

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.

Note: For this step we strongly recommend using either the Promega MagnaBot II Magnetic Separation Device or the Agencourt SPRIStand due to the relatively small volume.

1. Ensure the Agencourt RNAClean XP beads have completely reached room temperature before proceeding.
2. Prepare at least 2 mL of 70% ethanol wash solution for each sample. This wash will be used again in later steps and preparing at least 2 mL per sample will ensure sufficient wash is available for all steps.

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

   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.
3. Resuspend the beads by inverting and tapping the tube. Ensure that the beads are fully resuspended before adding to sample. After resuspending, do not spin the beads.
4. Add 30 µL of each DNA sample (100 ng to 1 µg) to a 0.2 mL PCR tube.
5. At room temperature, add 42 µL (1.4 volumes) of the bead suspension to 30 µL fragmented genomic DNA. Mix by pipetting 10 times.

   Use of the described sample to bead volume ratios is critical to proper performance of the Size Selection protocol.
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. Keeping the tubes on the magnet, carefully remove exactly 60 µL of the supernatant and transfer to a fresh set of tubes.
9. At room temperature, add 60 µL (1.0 volumes) of the bead suspension to the supernatant. Mix by pipetting 10 times. The total volume will be 120 µL.
10. Incubate at room temperature for 10 minutes.
11. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
12. Keeping the tubes on the magnet, carefully remove only 100 µL of the supernatant and discard it. Leaving some of the volume behind minimizes bead loss at this step.

13. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.

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

14. Remove the 70% ethanol wash using a pipette.
15. Repeat the wash, 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 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.

16. Air dry the beads on the magnet for a minimum of 5 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.
17. Remove the tubes from the magnet.
18. Add 10 µL Nuclease-free Water (green: D1) to the dried beads. Mix thoroughly by pipetting to ensure all the beads are resuspended.
19. Transfer the tubes to the magnet and let stand for 2 minutes to completely clear the solution of beads.
20. Carefully transfer 7 µL of the size selected DNA to a fresh 0.2 mL PCR tube and place on ice. Set aside the remainder of the DNA/bead mixture on ice.
21. 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.

22. Continue immediately with the End Repair protocol or store the purified DNA at –20°C.

G. End Repair

1. Obtain the End Repair Buffer Mix (blue: ER1), End Repair Enzyme Mix (blue: ER2), End Repair Enhancer (blue: ER3) and the Nuclease-free Water (green: D1) from –20°C storage.

! *Do not vortex any enzyme mixes.*

2. Spin down the contents of ER2 and ER3, place on ice.
3. Thaw ER1 at room temperature, mix by vortexing, spin and place on ice. Leave the Nuclease-free Water at room temperature.

4. Prepare a master mix by combining ER1, ER2 and ER3 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 MASTER MIX FOR gDNA SAMPLES		
END REPAIR BUFFER MIX (BLUE: ER1 VER 3)	**END REPAIR ENZYME MIX (BLUE: ER2 VER 4)**	**END REPAIR ENHANCER (BLUE: ER3)**
2.5 µL	0.4 µL	0.6 µL

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

5. Add 3.5 µL of the End Repair Master Mix to each size selected DNA sample.
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 (End Repair; see Table 4):

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

H. Adaptor Ligation

1. Obtain the Ligation Buffer Mix (yellow: L1), appropriate Ligation Adaptor Mix(es) (yellow: L2), 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.

! *It is critical to proceed to the Library Amplification protocol immediately after completion of Adaptor Ligation.*

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.
4. Add 1 µL of the appropriate Ligation Adaptor Mix (L2) to each sample. Mix by pipetting thoroughly with a pipette set to 5 µL. If multiplxing, make sure a unique barcode is used for each sample.
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.

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

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

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

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

6. Add 14 µL of the Ligation Master Mix to each reaction tube.
7. Mix by pipetting slowly and gently 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 4):

   25°C – 10 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 and P4 in an appropriately sized capped tube according to the volumes shown in Table 8 (below). 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 Product Purification protocol.

I. Ligation Product 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.

Note: For this step we strongly recommend using either the Promega MagnaBot II Magnetic Separation Device or the Agencourt SPRIStand due to the relatively small volume.

1. Obtain the RNAClean XP beads and 70% ethanol set aside earlier. Ensure both are at room temperature before proceeding.
2. Resuspend the beads by inverting and tapping the tube. Ensure that the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.
3. At room temperature, add 60 µL of Nuclease-free Water to each reaction.
4. Mix by pipeting 5 times then spin.
5. Add 68 µL (0.8 volumes) of the bead suspension to each reaction.
6. 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.

7. Incubate at room temperature for 10 minutes.
8. 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.*

9. 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 will impact the amount of DNA carried into the next stage, so ensure beads are not removed with the binding buffer or the wash.

10. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
11. Remove the 70% ethanol wash using a pipette.
12. 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.

13. Air dry the beads on the magnet for 5 minutes.

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

14. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
15. Remove the tubes from the magnet.
16. Add 10 µL of Nuclease-free Water to the dried beads. Mix thoroughly to ensure all the beads are resuspended.
17. Incubate at room temperature for 2 minutes.
18. Transfer the tubes to the magnet and let stand for 2 minutes.
19. Carefully remove 8 µL of the eluate ensuring that none of the beads are carried over and transfer to a fresh set of 0.2 mL PCR tubes. Place on ice.
20. 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.

21. Continue immediately with the Library Amplification protocol.

J. 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 8. Mix well by pipetting taking care to avoid bubbles, spin and place on ice.

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

AMP BUFFER MIX (RED: P1)	AMP PRIMER MIX (RED: P2 VER 2)	DMSO (RED: P4)	AMP ENZYME MIX (RED: P3)
64 µL	3 µL	4 µL	1 µL

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

4. On ice, add 72 µL of the Amplification Master Mix to a 0.2 mL PCR tube for each sample.
5. Add 8 µL of the Adaptor Ligation product to each tube, being sure to wash the tip out into the master mix several times to completely dispense. Mix thoroughly by pipetting at the 72 µL setting, spin and place on ice. Continue immediately with Step 6 below.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (Library Amplification; see Table 4):

   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
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
8. Continue immediately with the Purification of the Amplified Library protocol.

K. Purification of the Amplified Library

Note: For this step we strongly recommend using either an Agencourt SPRIPlate Ring Magnet or the Agencourt SPRIStand.

1. Obtain the RNAClean XP beads and 70% ethanol set aside earlier. Ensure both are at room temperature before proceeding.
2. Resuspend the beads by inverting and tapping the tube. Ensure that the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.
3. At room temperature, add 70 µL 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 yield.

8. With the plate still on the magnet, add 200 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 2 more times, for a total of 3 washes.

    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 Buffer 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.

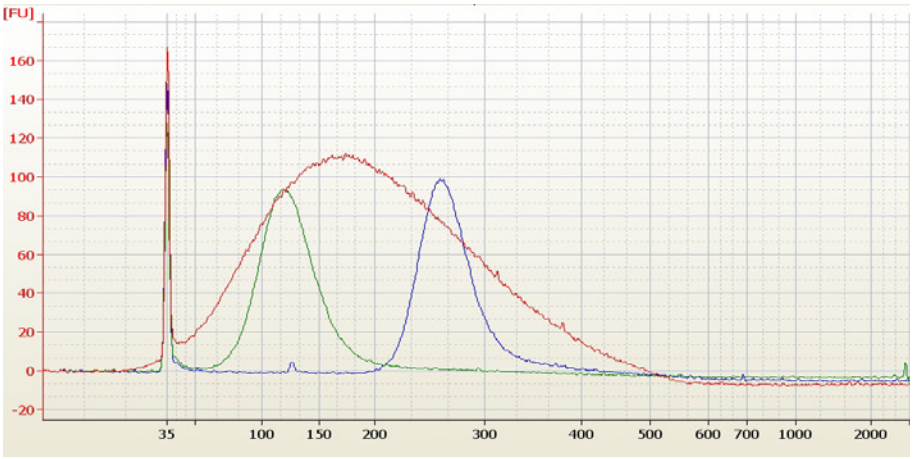
17. Continue immediately with the Quantitative and Qualitative Assessment protocol or store the purified sequencing libraries at –20°C.

L. Quantitative and Qualitative Assessment

Run the samples on the Agilent Bioanalyzer using the High Sensitivity DNA Kit (Agilent Cat. #5067-4627) following the manufacturer's recommendations. The fragment distribution may vary depending upon the input DNA sample.

Figure 3 shows a representative fragment distribution for the Encore NGS Library Systems *for* Ion Torrent product (blue trace). Note this distribution has a median fragment size of approximately 230 bp, appropriate for use on the Ion PGM System. A significantly broader size distribution may result in sub-optimal performance on the Ion PGM System.

Figure 3. Fragment distribution of sequencing libraries and intermediaries prepared with the Encore NGS Library Systems *for* Ion Torrent.



Fragmented DNA Size Selected DNA
Encore NGS Library Systems *for* Ion Torrent Sequencing Library

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 Multiplex Experiments

The Encore NGS Multiplex Library Systems *for* Ion Torrent (Part Nos. 0307 and 0308) include sequencing adaptor mixes (Ligation Adaptor Mixes) containing 16 unique 8-base barcodes for use in multiplex sequencing experiments (Table 9). Anywhere from 2-plex to as many as 16-plex multiplex sequencing runs can be carried out per Ion chip using any combination of the available Ligation Adaptor Mixes. Our multiplexing strategy has been designed to enable use of automated parsing and alignment workflows provided by the Torrent Server software. Because the barcode sequences are unique to this system, please consult the Ion Sequencing Kit user guide for information on adding a custom barcode set to the Torrent Server. The custom barcode set will allow you to define the barcode sequences used by the Encore NGS Library Systems *for* Ion Torrent, and will be called up in order to enable the use of the automated workflows on the Torrent Server.

Alternatively, the reads can be parsed using third party applications after sequencing. Barcode sequences will be found in the 5' most positions of the reads and should be used to separate the reads into the appropriate pools (parsing). We recommend that only perfect matches be considered for read parsing. After parsing, the barcode sequences should be trimmed from the reads prior to alignment.

VI. Appendix

Table 9. Adaptor Barcodes for multiplexing in the Encore NGS Multiplex Library Systems *for* Ion Torrent (Part Nos. 0307 and 0308).

LIGATION ADAPTOR MIX	BARCODE SEQUENCE
L2-BC1	TCTGTCTG
L2-BC2	TCCTCGAG
L2-BC3	TCTATGTG
L2-BC4	TGTCGATG
L2-BC5	TCGATACG
L2-BC6	TTGCAGAG
L2-BC7	TCGCTCAG
L2-BC8	TAGCATAG
L2-BC9	TAGCGATG
L2-BC10	TCAGCGCG
L2-BC11	TAGTCGCG
L2-BC12	TCACACTG
L2-BC13	TGCCGTCG
L2-BC14	TACTCGTG
L2-BC15	TATCATCG
L2-BC16	TGAGACAG

VI. Appendix

B. Frequently Asked Questions (FAQs)

Q1. What kind of sequencing primers can I use with your library?
The Encore NGS Library Systems *for* Ion Torrent are designed for use with the standard Ion Torrent sequencing primers.

Q2. What Ion PGM System chip types are compatible with the sequencing libraries produced using the Encore NGS Library Systems *for* Ion Torrent?
The Encore NGS Library Systems *for* Ion Torrent are compatible with the Ion 314 Chip and the Ion 316 Chips.

Q3. Do I need to perform any additional size selection prior to using the library on the Ion PGM System?
The Encore NGS Library Systems *for* Ion Torrent protocol includes optimized size-selection steps designed to produce an appropriate fragment size distribution for use on the Ion PGM System. It is not necessary to perform any additional size selection.

Q4. How many bases do the Encore NGS Library Systems *for* Ion Torrent adaptors add to the library?
The adaptors add 118 bp to the library in Encore NGS Library System *for* Ion Torrent and 126 bp in the Encore NGS Multiplex Library Systems *for* Ion Torrent. Figure 3 shows a typical sequencing library size distribution.

Q5. Can I use alternative fragmentation methods?
We have evaluated two fragmentation methods, the Bioruptor NGS and the-Covaris method, during the development of the Encore NGS Library Systems *for* Ion Torrent. These methods are suitable as long as the protocol generates a tight size distribution of DNA fragments with a 150 bp median fragment.

Q6. How does your protocol improve the efficiency of ligation and avoid adaptor dimer formation?
The Encore NGS Library Systems *for* Ion Torrent utilize proprietary 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 Bioruptor NGS or the Covaris instrument, 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.

Q8. What is the expected yield of the amplified DNA library using the Encore NGS Library Systems *for* Ion Torrent?
The expected yield is 0.2–2 µg, depending on the quantity and quality of the cDNA or genomic DNA used.

VI. Appendix

C. Update History

This document, the Encore NGS Library Systems *for* Ion Torrent user guide (M01216 v3), is an update to address the following topics.

Description	Section	Page(s)
Add support for the Ovation Ultralow Library Systems. (v2)	I.A.	1
Add support for Encore Complete RNA-Seq Systems. (v2)	I.A.	1
Revise Components lists. (v2)	II.A. Tables 1, 2 and 3	4 - 6
Revise End Repair protocol to provide guidance for processing gDNA samples vs. RNA-Seq samples. (v2)	IV.G.	16 - 17
Correct typos. (v2)	Throughout	Throughout
Update trademark references. (v2)	Throughout	Throughout
Remove reference to discontinued product Encore NGS Library Systems (0300, 0301 and 0302). (v3)	I.A.	1
Remove End Repair Master Mix for RNA-Seq Library Prep instructions. (v3)	IV.G. Table 6B	17

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