# Laboratory Operating Procedure

# *AGNOSTIC ION TORRENT SEQUENCING*

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## Introduction

The procedure described here is used for the preparation of libraries to be sequenced on the Ion Torrent S5XL platform. Random priming allows detection of viral sequences present in the sample without prior knowledge and may be useful in virus discovery in samples where the composition is unknown, e.g., sewage samples.

## Sample Material

This procedure has been successfully used for the preparation of sequencing libraries from 3 sample types (sewage filtrate, cell culture supernatant and stool). Other sample types may require modification of the procedure.

## Equipment and Reagents

## Equipment

- Laminar flow safety hood
- UV safety cabinet for preparation of mastermix and addition of templates (Grant Instruments™ Stainless Steel PCR UV Cabinet, Product Code 11441048)
- Heating block with two heating units
- Table top centrifuge (Thermo Fisher Micro CL17 Microcentrifuge, 120V, with 24x 1.5/2 ml rotor, Catalog No. 75002451)
- Thermocycler with heated lid
- Vortex
- Magnetic Particle Concentrator (DynaMag™-2 Magnet, Catalog No. 12321D)
- Pipettes: P2, P10, P20, P200, P1000 (Rainin Pipet –Lite XLS)
- Real Time PCR System (Applied Biosystems 7500)

## Reagents and consumables

- Nuclease free water (Zymo Research, Catalog No. W1001-6)
- Disposable gloves
- 0.2ml sterile, nuclease free PCR tubes
- 1.5ml microcentrifuge tubes (Safe-Lock, Eppendorf®**,** Catalog No. 0030120086)
- Sterile, nuclease free pipette tips with aerosol barriers (to prevent cross contamination)
- Turbo DNase (Invitrogen, Catalog No. AM2238)
- Roche High Pure RNA Isolation Kit (Roche, Catalog No. 11828665001)
- Random hexamers
- dNTPs (Roche, Catalog No. 11969064001)
- Superscript II Reverse Transcriptase (Invitrogen, Catalog No. 18064014)
- Klenow polymerase 3’-5’exonuclease (NEB, Catalog No. M0212S)
- RNase H (NEB, Catalog No. M0297S)
- Ampure Beads (Beckman Coulter, Catalog No. A63881)
- Absolute Ethanol (Merck Product No. 1.02371.2500)
- Ion Xpress™ Plus Fragment Library Kit (Thermo Fisher Scientific, Catalog No. 4471269)
- Ion Xpress™ Barcode Adapters 1-32 (Thermo Fisher Scientific, Catalog Nos. 4471250 and 4474009)
- Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific, Catalog No. 4468802)

## General remarks

- Ensure two separate work stations (UV safety cabinets) to prepare master mix (designated as ‘clean’ hood) and addition of template (designated as ‘template’ hood)
- Change gloves and lab coats between the two stations to avoid contamination of reagents
- Store small aliquots of dNTPs to avoid multiple freeze-thaw cycles
- Have designated work areas for pre and post-amplification procedures
- Plan experiment to have a unidirectional work flow to avoid contamination between post and pre-amplification work areas
- Use sample specific barcodes
- Record barcodes and corresponding sample ID
- Avoid opening multiple barcode tubes at the same time
- Do not use the same barcodes in consecutive library preparations

## Reagent Details

- TURBO™ DNase Product Information Sheet (Invitrogen, Catalog No. AM2238) https://assets.thermofisher.com/TFS-Assets/LSG/manuals/4393900B.pdf
- Roche High Pure RNA Isolation Kit protocol (Roche, Catalog No. 11828665001) https://lifescience.roche.com/documents/High-Pure-RNA-Isolation-Kit.pdf
- Ion Library TaqMan® Quantitation Kit user guide (Thermo Fisher Scientific, Catalog No. 4468802) https://tools.thermofisher.com/content/sfs/manuals/MAN0015802_IonLibrary_Taqman_Quantitation_Kit_UG.pdf
- Ion Xpress™ Plus Fragment Library Kit user bulletin (Thermo Fisher Scientific, Catalog No. 4471269)
- https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0007044_PrepAmpliconLibrFragment_using_IonXpressPlusFragKit_UB.pdf
- Endoh D, Mizutani T, Kirisawa R, et al. Species-independent detection of RNA virus by representational difference analysis using non-ribosomal hexanucleotides for reverse transcription. *Nucleic Acids Research*. 2005;33(6):e65. doi:10.1093/nar/gni064.

## Before getting started

- Thaw reagents completely before making mastermix
- Ensure temperature profiles are set and saved on the thermocycler
- Aliquot Ampure XP Beads in convenient volumes (~2ml) to avoid multiple cycles of warming and cooling of the original bottle
- Ampure XP Beads must be brought to ambient temperature for at least 30 minutes before purification
- Mix Ampure XP: carefully mix an aliquot of Ampure XP beads by hand at room temperature to obtain a homogenous mixture

## Procedure

The workflow procedure is as follows and is described in Sections 1-5 of this document.

1. Turbo DNase Treatment (Section 1)
2. Nucleic acid (NA) Isolation (Section 2)
3. Reverse Transcription (Section 3)
4. Second strand synthesis (Section 4)
5. Library preparation with the Ion Xpress™ Plus Fragment Library Kit (Section 5)
    - 5.1 Fragmentation
    - 5.2 Adaptor Ligation
    - 5.3 Amplification
    - 5.4 Library Quantitation

All steps until Section 5.3 should be performed in the pre-PCR work area and subsequently in the post-amplification work area.

## SECTION 1 – DNase TREATMENT USING TURBO DNase

| DNase TREATMENT | STOCK CONCENTRATION | VOLUME 1 rxn (µl) |
|---|---|---|
| Turbo DNase | 2U/µl | 20 |
| DNase Buffer | 10X | 24 |
| | **TOTAL VOLUME** | 44 |
| | Sample volume | 200 |

- DNase Buffer is from the Turbo DNase kit (Invitrogen, Catalog No. AM2238)
- Add DNase mixture (44 µl) to sample (200 µl) under the safety flow hood
- Pipet 5 times to mix thoroughly
- Incubate at 37°C for 30 minutes in a heat block
- Spin down after incubation to collect condensation on lid
- Proceed to nucleic acid isolation (Section 2)

## SECTION 2 – NUCLEIC ACID (NA) ISOLATION

- Use the Roche High Pure RNA Isolation Kit (as described below) to extract NA
    - Add 400 µl of Lysis buffer and vortex for 15 seconds
    - Transfer the entire sample to a High Pure Filter Tube (Filter tube inserted into a collection tube)
    - Centrifuge filter tube assembly in a tabletop microcentrifuge (e.g Thermo Fisher Micro CL17 Microcentrifuge) for 15 secs at 8000x g
    - Discard flow-through and combine filter tube with collection tube
    - Add 500 µl of Wash Buffer I to the upper reservoir of the filter tube assembly and centrifuge at 8,000x g for 15 secs
    - Discard flow-through and combine filter tube with collection tube
    - Add 500 µl of Wash Buffer II to the upper reservoir of the filter tube assembly and centrifuge at 8,000x g for 15 secs. Discard flow-through and combine filter tube with collection tube
    - Add 200 µl of Wash Buffer II to the upper reservoir of the filter tube assembly and centrifuge at maximum speed, approximately 13,000x g for 2 min. This removes any residual wash buffer.
    - Discard collection tube and insert filter tube into a 1.5 ml microcentrifuge tube
    - Add **50 µl** of nuclease free water at 37°C to the filter tube
    - Incubate at room temperature for 2 minutes
    - Centrifuge filter tube – 1.5 ml microcentrifuge tube assembly in a tabletop microcentrifuge at 8,000x g for 1 minute to collect the eluate.
    - Discard filter column. The eluate contains the extracted nucleic acid.
- Proceed with the next steps or store the eluted nucleic acid extract at -80°C until required

**PRECAUTIONS**

- Perform all steps up to and including the addition of lysis buffer in the safety flow hood
- Do not perform the DNase step as indicated in the Roche High Pure RNA Isolation Kit protocol. This is because a DNase step has been performed with Turbo DNase prior to extraction.
- Add the 50 µl nuclease free water to the center of the filter column membrane to ensure optimal recovery

## SECTION 3 – REVERSE TRANSCRIPTION

| **RT Mix -I** | **STOCK CONCENTRATION** | **VOLUME 1 rxn (µl)** |
|---|---|---|
| Random Hexamers | 100µM | 4.6 |
| E. coli ligase buffer | 10X | 3 |
| $MgCl_2$ | 100mM | 2.4 |
| | **TOTAL VOLUME** | 10 |

| **RT Mix -II** | **STOCK CONCENTRATION** | **VOLUME 1 rxn (µl)** |
|---|---|---|
| Nuclease free $H_2O$ | | 15.2 |
| E. coli ligase buffer | 10X | 2 |
| dNTPs | 25mM each | 0.8 |
| DTT | 0,1 M | 1 |
| Superscript II | 200 U/ µl | 1 |
| | **TOTAL VOLUME** | 20 |

- The random hexamers used are described in Endoh et al., Nucleic Acids Research. 2005;33(6):e65. doi:10.1093/nar/gni064
- E. coli ligase buffer is from the New England Biolabs https://international.neb.com/products/b0202-t4-dna-ligase-reaction-buffer#Product%20Information
- Add 20 µl of NA extract (from Section 2) to RT mix- I (10 µl). Final reaction volume 30µl
- Incubate 2 mins at room temperature
- After incubation, add 20 µl RT mix - II to the mixture
- Incubate 90 mins at 37°C followed by 20 mins at 70°C in a heat block

- Proceed immediately to Second strand synthesis (Section 4)

**PRECAUTIONS**

- Pulse spin the tubes between incubations to collect condensation on lid

## SECTION 4 – SECOND STRAND SYNTHESIS

| SECOND STRAND SYNTHESIS | STOCK CONCENTRATION | VOLUME 1 rxn (µl) |
|---|---|---|
| Nuclease free $H_2O$ | | 86.5 |
| NEBuffer 2[1] | 10X | 10 |
| Klenow polymerase 3' - 5' exo | 5U/µl | 1 |
| RNaseH | 5U/µl | 1.5 |
| dNTPs | 25mM each of dATP, dCTP, dGTP and dTTP | 1 |
| | **TOTAL VOLUME** | 100 |
| | RT - PCR Product | 50 |
| | **FINAL VOLUME** | 150 |

[1]NEBuffer 2 is from New England Biolabs and is supplied with the Klenow enzyme

- Mix 100 µl of second strand synthesis mixture with 50 µl of RT PCR product
- Incubate at 37°C for 90 minutes in a heat block
- Proceed immediately to Section 4.1 or store at -80°C

## SECTION 4.1 – AMPURE BEADS PURIFICATION

- To each reaction, add 150 µl of Ampure Beads
- Pipet to mix, pulse spin and incubate at RT for 5 min
- Place tubes in magnetic rack
- Wait 3 minutes for solution to turn clear and beads to bind
- Discard supernatant careful not to disturb beads
- Add 500 µl 70% Ethanol. Incubate 30 sec, rotate tube at least twice in rack with 30 seconds between rotation. After last 30 second interval, carefully remove 70% Ethanolsupernatant
- Repeat wash step
- Pulse spin the tube, place in magnetic rack and remove all liquid using a P20 pipette

- Air dry the beads at RT for 3-5 minutes. Dry the beads until the reflective shine of the ethanol disappears but not the point of the beads cracking.
- Remove the tubes from the magnetic rack and add **15 µl** of low TE
- Pipet to mix and vortex for 10 sec to completely re-suspend the beads
- Pulse spin and place on magnetic rack till solution is completely clear
- Transfer supernatant to a new 1.5 ml tube
- Proceed directly to Library preparation using Ion Xpress Plus Fragment Library Kit

**PRECAUTIONS**

- Equilibrate the Ampure Beads to room temperature for at least 30 minutes prior to purification
- Prepare fresh 70% Ethanol
- Allow the beads to bind completely to the magnet before discarding supernatant
- Do not over dry the beads. Do not let the beads crack

## SECTION 5 – LIBRARY PREPARATION USING ION XPRESS PLUS FRAGMENT LIBRARY KIT

### SECTION 5.1 – FRAGMENTATION

| FRAGMENTATION MIX | STOCK LOT NO. | VOLUME 1 rxn (µl) |
|---|---|---|
| Ion Shear 10x Reaction buffer[1] | | 5 |
| Nuclease free water | | 20 |
| | **TOTAL VOLUME** | 25 |
| | Input DNA | 15 |
| | **REACTION VOLUME** | 40 |

[1]Reagents used in Section 5.1 – 5.3 are components of the Ion Xpress™ Plus Fragment Library Kit (Thermo Fisher Scientific, Catalog No. 4471269). Reagents in Section 5.4 are components of the Ion Library TaqMan® Quantitation Kit (Thermo Fisher Scientific, Catalog No. 4468802)

- Label 3 tubes per sample with the sample ID and incubation time (1,3 and 5 minutes)
- Add 1.66 µl of Stop Buffer (LOT NO. _____) in each tube
- Add 25 µl of Fragmentation mix into the tube with the input DNA
- Add 10 µl of Ion Shear Plus Enzyme Mix II (LOT NO. _____) to the tube with DNA and Fragmentation Mix
- Rapidly pipet to mix and incubate at 37°C in a heat block
- After 1 minute of incubation, remove 16.66 µl of the mixture into the tube labelled with SAMPLE ID+1 minute. Vortex for 5 secs and place on ice
- After 3 minutes of incubation, remove 16.66 µl of the mixture into the tube labelled with SAMPLE ID+3 minute. Vortex for 5 secs and place on ice

- After 5 minutes of incubation, remove entire remaining volume of mixture into the tube SAMPLE ID+5 minutes. Vortex for 5 secs and place on ice
- Pool the tubes of different incubation times into one tube

**PRECAUTIONS**

- Record the lot number of reagents in the kit to help troubleshoot
- Prepare an ice box and a timer in advance
- Work in pairs to help co-ordinate the incubation times

## SECTION 5.1.1 – AMPURE BEADS PURIFICATION

- Add 45µl of water to the sample
- Add 85µl of Ampure beads and mix by pipetting
- Perform wash steps with 70% Ethanol as described in Section 4.1
- Elute in 25µl low TE. This is the Fragmented DNA to which the adaptors are ligated.
- Proceed to Adaptor Ligation (Section 5.2)

## SECTION 5.2 – ADAPTOR LIGATION

| ADAPTOR LIGATION | STOCK LOT NO. | VOLUME 1 rxn (µl) |
|---|---|---|
| Nuclease free water | | 51 |
| 10x Ligase buffer[1] | | 10 |
| dNTP mix | | 2 |
| DNA Ligase | | 2 |
| Nick repair polymerase | | 8 |
| Adapter P1 | | 1 |
| | **TOTAL VOLUME** | 74 |
| **SAMPLE SPECIFIC! ADD INDIVIDUALLY** | Barcode adapter | 1 |
| | **FRAGMENTED DNA** | 25 |
| | **REACTION VOLUME** | 100 |

[1]The 10X Ligase buffer, dNTP mix, DNA Ligase, Nick repair polymerase and Adapter P1 are from the Ion Xpress Plus Fragment Library kit.
Barcode adapters are from Thermo Fisher

- Make a mastermix of all the reagents except the sample specific barcode adapter
- Add the sample specific barcode adapter and record corresponding sample ID
- Incubate in a thermocycler set to the following program
    - 25°C for 15 min

- 72°C for 5 minutes
- 4°C up to 1h

- Transfer to a fresh 1.5 ml tube
- Proceed immediately with Ampure Purification (Section 5.2.1)

**PRECAUTIONS**

- Use sample-specific barcode
- Record barcode used and corresponding sample ID
- Open only one barcode tube at a time to avoid cross contamination

### SECTION 5.2.1 – AMPURE BEADS PURIFICATION

- Add 85 µl of Ampure beads and mix by pipetting
- Perform wash steps with 70% Ethanol as described in Section 4.1
- Elute in **100 µl low TE**
- Proceed to Section 5.2.2

### SECTION 5.2.2 – AMPURE BEADS PURIFICATION

- Add 85 µl of Ampure beads and mix by pipetting
- Perform wash steps with 70% Ethanol as described in Section 4.1
- Elute in **30 µl low TE**

### SECTION 5.3 – LIBRARY AMPLIFICATION

| ION TORRENT PCR AMPLIFICATION | STOCK LOT NO. | VOLUME 1 rxn (µl) |
|---|---|---|
| Platinum PCR supermix high fidelity[1] | | 100 |
| Library amplification primer mix | | 5 |
| | **TOTAL VOLUME** | 105 |
| | Barcoded DNA | 25 |

[1]Platinum PCR supermix high fidelity and Library amplification primer mix are component of the Ion Xpress Plus fragment library kit.

- Incubate in a thermocycler set to the following program
  - 95°C for 5 minutes
    95°C for 15 sec
    58°C for 15 sec } 15 cycles
    70°C for 1 min
    4°C up to 1 hour
- Proceed to Ampure purification (Section 5.3.1)

## SECTION 5.3.1 – AMPURE BEADS PURIFICATION

- Add 111 µl of Ampure beads and mix by pipetting
- Perform wash steps with 70% Ethanol as described in Section 4.1
- Elute in **30 µl low TE**
- Proceed to quantify the amplified libraries using the Ion Library TaqMan Quantitation Kit (Section 5.4) or store at -20°C until required

## SECTION 5.4 – LIBRARY QUANTITATION

| ION TORRENT LIBRARY QUANTITATION | STOCK LOT NO. | VOLUME 1 rxn (µl) |
|---|---|---|
| qPCR Mix 2x | | 5 |
| Quantitation Assay 20x | | 0.5 |
| | **TOTAL VOLUME** | 5.5 |

- Make serial dilutions (1:100, 1:1000, 1:10000) of each library
    - 1:100 - 1µl undiluted library in 99µl water
    - 1:1000 – 5µl 1:100 library in 45µl water
    - 1:10000 - 5µ 1:1000 library in 45µl water
- Make serial dilutions of E.coli DH10B Control Library
    - C1: 5 µl stock E.coli + 45 µl water (Final conc: 6.8 pM)
    - C2: 5 µl C1 +45 µl water (Final conc: 0.68 pM)
    - C3: 5 µl C2 +45 µl water (Final conc: 0.068 pM)
    - C4: 5 µl C3 +45 µl water (Final conc: 0.0068 pM)
    - C5: 5 µl C4 +45 µl water (Final conc: 0.00068 pM)
- Load each sample and standard in duplicate
- Use 4.5 µl of each library dilution and standard. Final reaction volume of 10 µl
- Use the following temperature profile for the reaction
    - 50°C for 2 minutes
    - 95°C for 1 minute
    - 95°C for 20sec
    - 60°C for 30sec

    } 40 cycles
- Analyse the standard curves and $C_t$ readings of the samples to measure the quantitation of the prepared libraries
- Normalize libraries to 40 pM before pooling for sequencing
- If libraries have a concentration of <40 pM, perform another round of amplification as described in Section 5.3 until a concentration > 40 pM is obtained.