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CZ Biohub RNA Library Prep Protocol on Echo 550

Forked from a private protocol

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ABSTRACT

Preparation of high-quality sequencing libraries is a costly and time-consuming component of metagenomic next generation sequencing (mNGS). While the overall cost of sequencing has dropped significantly over recent years, the reagents needed to prepare sequencing samples are likely to become the dominant expense in the process. Furthermore, libraries prepared by hand are subject to human variability and needless waste due to limitations of manual pipetting volumes. Reduction of reaction volumes, combined with sub-microliter automated dispensing of reagents without consumable pipette tips, has the potential to provide significant advantages.

Here, we describe the integration of several instruments, including the Labcyte Echo 550 acoustic liquid handler and the iSeq and NovaSeq Illumina sequencing platforms, to miniaturize and automate mNGS library preparation, significantly reducing the cost and the time required to prepare samples. Through the use of External RNA Controls Consortium (ERCC) spike-in RNAs, we demonstrated the fidelity of the miniaturized preparation to be equivalent to full volume reactions. Furthermore, detection of viral and microbial species from cell culture and patient samples was also maintained in the miniaturized libraries. For 384-well mNGS library preparations, we achieved a savings of over 80% in materials and reagents alone, and reduced preparation time by 90% compared to manual approaches, without compromising quality or representation within the library.

GUIDELINES

4042 Pre-PCR BSL-1 Guidelines

- Absolutely no amplicons allowed in this room, thermocyclers should **NOT** be used for PCR
- Although a lab coat is not necessary for all BSL-1 work, **always** wear a lab coat and gloves when doing library prep or working with patient samples and any isolated nucleic acid prior to the final amplification stage
- Wipe down workspace with 70% ethanol and RNAse-ZAP before commensing work
- Clean up lab space after work is complete

Protocol Specific Guidelines

- Prepare master mix reagents in a hood the day of use to preserve efficiency of all enzymes.
- Only use consumables and pipettes that have are designated for hood use

Equipment Guidelines

Vacuum Evaporators

- Different model vacuum evaporators vary in sample dehydration times. To confirm the minimal drying time for each instrument, fill each well of a 384-well PCR plate with 5uL water and spin at 40ºC-45ºC until completely dry.
- Variations in the manufacturing of different brands of PCR plates may also inhibit efficient sample drying. To prevent excessive drying times and potential compromise of RNA, test the desired brand of PCR plate by filling each well with 5uL water and ensuring it dries within 20-30 minutes. Over-drying and over-heating may cause degradation of the RNA.

Labcyte Echo 525

- When calculating master mix volumes, be sure to make a sufficient amount of each reagent to account for the minimum and maximum working volume of source plates.
- 384 Echo Qualified source well working volume: 20 - 65uL (dead volume of ~20uL)

Equipment Calendars

Reserve the following equipment you will need ahead of time via google calendar. If you do not have access, speak to Saharai

(saharai.caldera@czbiohub.org).
Pre-PCR Hood: https://calendar.google.com/calendar?cid=Y3piaW9odWIub3JnXzNxaXQ5dmtkcWtpbTllaTg3dDAxYXY5dGkwQGdyb3VwLmNhbGVuZGFyLmdvb2dsZS5jb20

Echo 550 calendar: https://calendar.google.com/calendar?cid=Y3piaW9odWIub3JnXzNvM2MyYXIwbGZhY3FwNzJqMzAzYXJzNWRnQGdyb3VwLmNhbGVuZGFyLmdvb2dsZS5jb20

Batman Bravo calendar: https://calendar.google.com/calendar?cid=Y3piaW9odWIub3JnXzU3a2MwczFxMnR0dnRpZjVxMXA4dTZucTIwQGdyb3VwLmNhbGVuZGFyLmdvb2dsZS5jb20

Woody Bravo calendar: https://calendar.google.com/calendar?cid=Y3piaW9odWIub3JnX2huM2YzNG04MTR2Z2ZnazlhdGtpaDBtMDlrQGdyb3VwLmNhbGVuZGFyLmdvb2dsZS5jb20

Genenvac calendar: https://calendar.google.com/calendar?cid=Y3piaW9odWIub3JnXzBrNmhzZjBzc2VlNTh1Zm10NnU4MnF2aGdrQGdyb3VwLmNhbGVuZGFyLmdvb2dsZS5jb20

MATERIALS

NAME	CATALOG #	VENDOR
Agencourt AMPure XP SPRI beads	A63881	Beckman Coulter
ERCC RNA Spike-In Mix	4456740	Invitrogen - Thermo Fisher

STEPS MATERIALS

NAME	CATALOG #	VENDOR
NEBNext Ultra II RNA Library Prep Kit for Illumina	E7770S/L	New England Biolabs
NEBNext Adaptor for Illumina	E7337AA	New England Biolabs
NEB USER® Enzyme	M5505S/L	New England Biolabs

BEFORE STARTING

Prep once:

A. Calibrating the vacuum concentrator.

- Test and calibrate all new plate brands or lot numbers by drying 5uL of water in each well to confirm plates dry appropriately (within 20-30 minutes).
- Temperature settings should be between 40-45 °C (could vary slightly depending on model).
- Test for minimum drying time.

B. Calibrating all plates for liquid handlers.

- It is vital to input and test labware definitions of all plates and confirm all liquid handlers have appropriate liquid transfer settings.

C. Prep ERCCs.

- External RNA Control Consortium (ERCC) RNA Spike-In Mix (Cat No. 4456740) was used in this study as a control for quality of sequencing libraries. Stock concentrations were quantified using Qubit RNA HS assay and diluted in nuclease free water to a target stock concentration of 50pg/uL. This stock was divided into small aliquots and stored at -80ºC for one time use.

Prep every time:

A. Prep master mix calculations and sample sheets.

- In order to program the Echo dispense protocols to reflect the right transfer volumes and destination wells, it is helpful to prepare a sample sheet listing the well location of each sample.

Prep before PCR amplification step:

A. Barcode plate.

- Prepare a 384-well PCR plate with at least 10uL of 5uM unique dual indexing primers in each necessary well. This plate must be prepared before the post-ligation bead clean as it is used for the elution step.

B. USER/PCR plate.

- Using the Echo, prepare a 384-well PCR plate with 10.9uL of USER/PCR master mix in each necessary well. This plate must be prepared before the post-ligation bead clean as it is used as the final plate.

PREPARE INITIAL RNA SAMPLE PLATE

1 Load 5uL of sample RNA into a 384-well PCR plate. Repeat with all desired samples. Record well location of each sample.

It is convenient to organize the 384-well plate into 4 quadrants of 96 samples. This will allow for multichannel dispensing of samples using a pipette, providing an easy transition between manual and automated steps.

2 Spin plate of sample RNA in vacuum evaporator at the appropriate temperature and time settings to dry completely (approximately 40ºC for 25-30 minutes, depending on number of samples and machine used).

Genevac EZ-2 EZ-2
https://www.spscientific.com/Products/Centrifugal_Evaporators___Sample_Concentrators/Genevac/EZ-2_Series/EZ-2_Series/

PREPARE MASTER MIX REAGENTS

3 Prepare enough fragmentation, first strand, and second strand master mixes for each sample.

Be sure to take into account the dead volume and the maximum working volume of the source plate being used. For example, in a 384-well Echo-qualified source plate, the volume of reagent in each well must be between 20uL and 65uL to ensure accurate dispensing.

Fragmentation Master Mix (1x)	
Reagent (lilac tubes)	**Volume (uL per sample)**
ERCC (stock concentration 50pg/uL)	0.5
First Strand Synthesis Reaction Buffer	0.4
Random Primers	0.1
Total Reaction Volume	**1.0**
First Strand Synthesis Master Mix (1x)	
Reagent (lilac tubes)	**Volume (uL per sample)**
Fragmentation Reaction Volume	1.0
NEBNext First Strand Synthesis Enzyme Mix	0.2
Nuclease Free Water	0.8
Total Reaction Volume	**2.0**
Second Strand Synthesis Master Mix (1x)	
Reagent (orange tubes)	**Volume (uL per sample)**
First Strand Reaction Volume	2.0
Second Strand Synthesis Reaction Buffer	0.8
Second Strand Synthesis Enzyme Mix	0.4
Nuclease Free Water	4.8
Total Reaction Volume	**8.0**

NEBNext Ultra II RNA Library Prep Kit for Illumina
by New England Biolabs
Catalog #: E7770S/L

4 Load reagents into Echo source plates by pipetting reagents into each required well and seal with a foil seal. Pipet carefully to avoid formation of bubbles.

5 Spin the Echo source plate in the centrifuge for 5 minutes at 1500 rcf to rid source wells of any bubbles produced in manual pipette transfer of master mixes.

Time and rpm was per recommendation by Howard Lee at Labcyte.

Reagents must come to room temperature before dispensing on the Echo.

DISPENSING FRAGMENTATION REAGENTS

6 For 384-well source plates, use the Echo's “Survey” setting to determine volume in each source well to ensure volume is 20<x<65uL to account for the minimum and maximum working volume.

7 Using the BP setting, dispense ***1000nL of fragmentation master mix*** into each sample well.

Ensure there are no bubbles present in the source plates after spinning.

8 Remove sample plate and seal with a foil seal. Gently vortex plate to mix, followed by quick spin.

FRAGMENTATION INCUBATION

9

Fragmentation Incubation	
Heated Lid: 105 °C	
Temperature (°C)	Time (mins)
94	12
4	Hold

DISPENSING FIRST STRAND SYNTHESIS REAGENTS

10 Remove sample plate from thermocycler, remove foil seal, and load into Echo. Using the BP setting, dispense ***1000nL of first strand synthesis master mix*** into each sample well needed for reaction.

11 Remove sample plate and seal with a foil seal. Gently vortex plate to mix, followed by quick spin.

FIRST STRAND SYNTHESIS INCUB

12

First Strand Synthesis Incubation	
Heated Lid: 105 °C	
Temperature (°C)	**Time (mins)**
25	10
42	15
70	15
4	Hold

DISPENSING SECOND STRAND SYNTHESIS REAGENTS

13 Remove sample plate from thermocycler, remove foil seal, and load into Echo. Using the BP2 setting, dispense ***6000nL second strand synthesis master mix*** into each sample well.

14 Remove sample plate and seal with a foil seal. Gently vortex plate to mix, followed by quick spin.

SECOND STRAND SYNTHESIS INCUBATION

15

Second Strand Synthesis Incubation	
Heated Lid: Off	
Temperature (°C)	**Time (mins)**
16	60
10	Hold

NUCLEIC ACID PURIFICATION: BEAD CLEAN

45m

16 Bead clean on Biomek using a **1.4x** Ampure bead-to-sample ratio. If you make your own SPRI beads, you will need to adjust the ratio to match the 1.4x Ampure bead

Use **nuclease free water** to elute sample from beads.

Post Second Strand Bead Clean (1.4x)	
Bead Clean Steps	**Volumes per sample(uL)**
Initial Sample Volume	8.0
Ampure Bead Volume	11.2
Elution Volume	6.0
Recover Volume	5.0

17

PREPARE MASTER MIX REAGENTS

18 Prepare enough end prep, adaptor ligation, USER/PCR master mixes, and adaptor dilutions for each sample.

Be sure to take into account the dead volume and the maximum working volume of the source plate. For example, in a 384-well Echo source plate, the volume of reagent in each well must be between 20uL and 65uL to ensure accurate dispensing.

End Prep Master Mix (1x)	
Reagent(green tubes)	**Volume per sample(uL)**
Post-Second Strand Synthsis Bead Clean Volume	*5.0*
Ultra II End Prep Reaction Buffer	0.7
Ultra II End Prep Enzyme Mix	0.3
Total Volume	6.0
Adaptor Ligation Master Mix (1x)	
Reagent(red tubes)	**Volume per sample(uL)**
End Prep Reaciton Volume	*6.0*
NEBNext Ultra II Ligation Master Mix	3.0
NEBNext Ligation Enhancer	0.1
Total Volume	9.1
Adaptor Master Mix (1x)	
Reagent(red tube)	**Volume per sample(uL)**
Diluted Adaptor	0.25
Note: Adaptor should be diluted based on approximate sample input and should not be added to adaptor ligation master mix to avoid adaptor-dimers.	
USER/PCR Master Mix (1x)	
Reagent(USER- white tube; Q5- blue tube)	**Volume per sample(uL)**
Adaptor Ligation Reaction Volume	*5.0*
Nuclease Free Water	2.5
NEB USER Enzyme	0.9
NEBNext Ultra II Q5 Master Mix	7.5
Total Volume	15.9

NEBNext Adaptor for Illumina
by New England Biolabs
Catalog #: E7337AA

NEB USER® Enzyme
by New England Biolabs
Catalog #: M5505S/L

19 Load reagents into Echo source plates by pipetting reagents into each required well and seal with a foil seal. Pipet carefully to avoid formation of bubbles.

Echo Qualified 384-Well Polypropylene PP-0200
https://www.labcyte.com/products/consumables/echo-qualified-source-plates

Labcyte Echo Qualified Reservoir ER-0050
https://www.labcyte.com/products/consumables/echo-qualified-reservoir

For reagent master mixes that require more than 12 source wells of a 384-well Echo source plate (PP-200), it may be prudent to use a 6-well reservoir source plate (ER-0050) to minimize dead volume. For example, in this prep, samples require a large volume of second strand synthesis master mix. Using a 6-well Echo reservoir plate for this step reduces dead volume and therefore requires less volume of reagents.

20 Spin the Echo source plate in the centrifuge for 5 minutes at 400g to rid source wells of any bubbles produced in pipette transfer of master mixes.

Time and rpm was per recommendation by Labcyte.

Reagents must come to room temperature before dispensing on the Echo.

DISPENSING END PREP REAGENTS

21 For 384-well source plates, use the Echo's “Survey” setting to determine volume in each source well to ensure volume is 20<x<65uL to account for the minimum and maximum working volume.

22 Using the BP setting, dispense **1000nL of end prep master mix** into each sample well.

Ensure there are no bubbles present in the source plates after spinning.

Labcyte Echo 525 LIQUID HANDLER
https://www.labcyte.com/products/liquid-handling/echo-525-liquid-handler

23 Remove sample plate and seal with a foil seal. Gently vortex plate to mix, followed by quick spin.

END PREP INCUBATION

24

End Prep Incubation	
Heated Lid: >75 °C	
Temperature (°C)	**Time (mins)**
20	30
65	30
10	Hold

DISPENSING ADAPTOR LIGATION REAGENTS

25 Remove sample plate from thermocycler, remove foil seal, and load into Echo. Using the GP setting, transfer **3100nL of adaptor ligation master mix** and **250nL of appropriately diluted adaptor** to each sample well. Make sure to add these reagent separately; this will prevent excessive adaptor dimer.

Labcyte Echo 525 LIQUID HANDLER
https://www.labcyte.com/products/liquid-handling/echo-525-liquid-handler

26 Remove sample plate and seal with a foil seal. Gently vortex plate to mix, followed by quick spin.

ADAPTOR LIGATION INCUBATION

27

Adaptor Ligation	
Heated Lid: Off	
Temperature (°C)	**Time (mins)**
20	15
10	Hold

USER/PCR SETUP - REAGENT PLATE

28 Before bead cleaning the sample post-adaptor ligation, prepare the USER/PCR master mix plate. It will be used as the final destination of cDNA during post-adaptor ligation bead clean.

MUST be done before starting the bead clean-up.

Beckman Coulter Biomek NXp

29 Load a **new, sterile**, empty PCR plate into the Echo destination port. Using the GP setting, dispense **10,900nL of USER/PCR master mix** into each sample well.

Labcyte Echo 525 LIQUID HANDLER
https://www.labcyte.com/products/liquid-handling/echo-525-liquid-handler

NUCLEIC ACID PURIFICATION: BEAD CLEAN

30 Bead clean the end-prepped, adaptor-ligated cDNA on the Biomek using a sample to Ampure bead solution ratio of **0.8x**.

Use **5uM TRUESEQ BARCODES** to elute sample from beads. At least 10uL of barcode should be in each well of a 384-well PCR plate in order to elute correctly.

Use **USER/PCR master mix plate** as final destination plate of bead clean-up.

Post Adaptor Ligation Bead Clean (0.8x)	
Bead Clean Steps	**Volumes per sample(uL)**
Initial Sample Volume	9.35
Ampure Bead Volume	7.28
Elution Volume	6.0
Recover Volume	5.0

USER/PCR master mix plate **MUST** be prepared before bead cleaning the sample. This plate is used to collect the final elution.

USER/PCR PLATE PREPARATION

31 **Ensure FINAL PLATE of the bead clean plate contains:**

sample cDNA
barcodes
USER/PCR master mix

Remove from Bravo, and seal with a foil seal. Gently vortex plate to mix, followed by quick spin.

USER/PCR INCUBATION

32

USER/PCR Incubation		
Heated Lid: 105 °C		
Cycles	**Temperature (°C)**	**Time**
1	37	15 m
1	98	30 s
19	98	10 s
	65	75 s
1	65	5 m
1	10	Hold

PCR cycle number is dependent on RNA input per sample in the first step of this protocol. For samples with undetectable RNA, we recommend a maximum of 19 cycles.

NUCLEIC ACID PURIFICATION: BEAD CLEAN

33 Bead clean the post USER/PCR cDNA on the Biomek using a sample to Ampure bead solution ratio of **0.8x**.

Use **Nuclease-free water** to elute sample from beads.

Post PCR Bead Clean (0.8x)	
Bead Clean Steps	**Volumes per sample(uL)**
Initial Sample Volume	15.9
Ampure Bead Volume	12.72
Elution Volume	11.0 - 26.0
Recover Volume	10.0 - 25.0

Elutions volume may vary per application, but if contiuing to automated pooling step elute in 25uL and recover 24uL to account for Labcyte Echo dead volume.

OPTIONAL NUCLEIC ACID PURIFICATION: BEAD CLEAN

34 To remove additional primer- and adaptor-dimers, it may be necesssary to perform a second bead clean to remove the smaller fragments at **0.8x** of Ampure beads to sample volume. This should be done after a QC to determine if there is significant dimers in libraries.

Use **Nuclease-free water** to elute sample from beads.

2x Post PCR Bead Clean (0.8x)	
Bead Clean Steps	**Volumes per sample(uL)**
Initial Sample Volume	10
Ampure Bead Volume	8
Elution Volume	11.0 - 26.0
Recover Volume	10.0 - 25.0

Beckman Coulter Biomek NXP
https://www.beckman.com/liquid-handlers/biomek-nxp

Elution volume may vary depending on downstream applications, but if continuing to automated pooling step, elute in 25uL and recover 24uL to account for the Echo's dead volume.

FINISHED LIBRARIES

35 Finished libraries are now ready to be quality checked, and/or undergo equal volume pooling to determine representation of each library in the pool. This can be used to determine the volume of each sample needed for equal pooling.

High-Throughput Library Pooling for NGS PREVIEW RUN
by Madeline Mayday,
University of California, San Francisco

35.1 Transfer the libraries into a new 384 well Echo source plate. Skip if samples already complete. Make sure to dilute samples if needed to contain enough dead volume. Samples should be between 22-25uL to be able to dispense accurately using the Echo.

Wells of the source plate containing samples should have a dead volume of 20uL for the Echo to be able to pool accurately.

35.2 Using the Labcyte Echo, pool 0.5uL of each library into a 384-well destination plate.

Use a new 384 PCR plate, or a 96 well PCR plate, as the ***destination plate*** to dispense the appropriate number of samples into the necessary number of wells. Be sure to take into account of the maximum working volume per destination plate. For example, for a 384 well plate, do not dispense more than 12uL into each well (i.e. 500nL of 22 samples per well).

35.3 Manually combine the multiple wells of the pool into one single DNA lo-bind tube. Mix.

35.4 Sequence pool on an Illumina iSeq, or a low-throughput sequencer.

35.5 Using the output from equal volume pooling, determine the approximate representation of each library in the total pool.

$$T = N \sum (\frac{1}{x})$$

x = percent of reads per sample
T = final volume of total pool
N = normalization factor

The sum of the inverse of the percent fraction of each sample from the iSeq run should give a normalization factor. This normalization factor will be used to determine the volume of each sample to be pooled.

Needed information:

1. ratio of reads of each sample to total number of reads (percent of reads) = x
2. desired total volume of pool necessary for sequencing submission = T (uL)

Use this information to solve for N (or the normalization factor).

The normalization factor is an estimation that can be used to pool evenly. It can be adjusted for various reasons to fit the criteria per batch.

35.6 Use the normalization factor to determine the volume to pool for each sample.

35.7 Use the Echo to dispense the original sequencing libraries to the final pool using the calculated volumes.

Use a new 384 PCR plate, or a 96 well PCR plate, as the destination plate to dispense the appropriate number of samples into the necessary number of wells. Take in account the maximum working volumes.

Reminder: For 384, do not dispense more than 11uL into each well total.

35.8 Manually combine the multiple wells of the pool into one single DNA lo-bind tube. Mix.

35.9 Equimolar pool of libraries are ready to be sequenced on a high throughput sequencer like the Illumina HiSeq or NovaSeq.