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We use this protocol and it's working

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DeRisi Lab RNA Library Prep 96-well Protocol on Echo 550

Forked from CZ Biohub RNA Library Prep Protocol on Echo 550

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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.

This protocol has been optimized for bronchoalveolar lavage fluid (BALF), nasopharyngeal swabs, RNA PAXgene blood, and plasma samples (see protocol variations for sample types needing RNA depletion).

Keywords: automated, miniaturized, protocol, NGS, nMGS, library prep, library preparation, Labcyte Echo, sequencing, Illumina, ligation

GUIDELINES

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.
- Take Ampure beads out of 4C and room temp 30 minutes before beads cleans.

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 96-well PCR plate with 5uL water and spin at 37ºC-38ºC 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 PP Plus Echo Qualified source well working volume: 20 - 65uL (dead volume of ~20uL)

MATERIALS

REAGENTS

Agencourt AMPure XP SPRI beads **Beckman Coulter Catalog #A63881**

ERCC RNA Spike-In Mix **Invitrogen - Thermo Fisher Catalog #4456740**

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

NEBNext Adaptor for Illumina **New England Biolabs Catalog #E7337AA**

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

PLATES

Equipment	
384-well PP 2.0 Plus Microplate Echo Qualified	NAME
Plate	TYPE
Labcyte	BRAND
PPL-0200	SKU
https://www.beckman.com/supplies/echo-plates/001-14622	LINK
Sterile	SPECIFICATIONS

Equipment	
Echo Qualified resevoir 2x3 Well Polypropylene Microplate	NAME
Plate	TYPE
Labcyte	BRAND
ER-0050	SKU
https://www.beckman.com/supplies/echo-plates/001-11101	LINK
RNase/DNase free	SPECIFICATIONS

BEFORE START INSTRUCTIONS

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 or obtain a 96-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 96-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.

GeneVac

1 Warming up GeneVac: Turn to "aqueous" setting and set temperature to ~40-42ºC and let run for ~30min before use.

2 Spin plate of sample RNA in vacuum evaporator at the appropriate temperature and time settings to dry completely (approximately 37-38ºC for 1 hour, depending on number of samples and machine used).

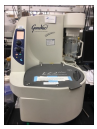
Equipment

new equipment NAME

Genevac EZ-2 BRAND

EZ-2 SKU

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



PREPARE MASTER MIX REAGENTS (DAY 1)

3 Prepare enough fragmentation, first strand, and second strand master mixes for each sample. For whole blood, plasma or other samples requiring rRNA/globin RNA depletion, see fragmentation modifications below.

Note

Most clinical respiratory samples do not require FastSelect depletion, but user should verify with desired sample type.

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.

Equipment

384-well PP 2.0 Plus Microplate Echo Qualified	NAME
Plate	TYPE
Labcyte	BRAND
PPL-0200	SKU
https://www.beckman.com/supplies/echo-plates/001-14622	LINK
Sterile	SPECIFICATIONS

	A	B
	***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

	A	B
	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 **New England Biolabs Catalog #E7770S/L**

Step 3 includes a Step case.

FastSelect

step case

FastSelect

	A	B
	RNA FRAGMENTATION (BP)	
		1 rxn
	Reagent	vol (uL)
	RNA (USE ERCC @ 50pg/uL)	0.5
	(pink) First SS Reaction Buffer 5x	0.4
	(pink) Random Primers	0.1
	rRNA + Globin FastSelect (1:10)	0.1
	Total volume	1.1
		uL/rxn

4 Load reagents into a 384PP Plus Echo source plate by pipetting reagents into each required well and seal with a foil seal. Pipet carefully to avoid formation of bubbles. Ensure that wells with master mix are noted to make Echo CSV with transfer specifications for each well.

5 Spin the Echo source plate in the centrifuge for 5 minutes at 2000 rpm to rid source wells of any bubbles produced in manual pipette transfer of master mixes. Reagents should be at room temp before Echo transfer.

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 (1100nL for FastSelect)*** into each sample well.

Note

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

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

FRAGMENTATION INCUBATION (for FastSelect only)

9 This step is ONLY for samples with FastSelect depletion. **For all other sample types, proceed to Step 10.**

	A
	FRAGMENTATION INCUBATION (heated lid set to 105ºC)
	1 minutes at 94°
	2 minutes at 75°C
	2 minutes at 70C
	2 minutes at 65°C
	2 minutes at 60°C

A
2 minutes at 55°C
5 minutes at 37°C
5 minutes at 25°C

DISPENSING FIRST STRAND SYNTHESIS REAGENTS

10 Remove foil seal, and load into Echo. Using the GP 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 INCUBATION

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 GP 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: HAND BEAD CLEAN

16 Bead clean 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 45m

A
Prepare 80% ethanol (fresh, 50 mL reservoir) and beads (50 mL reservoir, left out at RT for 30 min prior to use)
• Add 12 uL nuclease-free water to sample wells to bring up to 20 uL. Adding water to increase overall volume helps when eluting beads.
• Add 28 uL beads to sample plate, mix, and incubate 5 minutes (visually confirm sufficient mixing)
• Move sample plate to magnet and incubate 5 minutes
• Remove 48 uL supernatant and transfer to waste
• Wash with 150uL 80% ethanol, pause 30 seconds, remove and move to waste
• Repeat ethanol wash step. Ensure wells are free of ethanol using a p20—this is crucial
• Air dry beads for 10 minutes *do not overdry and visually ensure no ethanol is present*
• Move sample plate off magnet
• Resuspend beads in 6uL eluent (water), mix, and incubate 5 minutes (visually confirm sufficient mixing)
• Move sample plate to magnet and incubate 3-5 minutes

	A
	• Aspirate 5uL of supernatant (containing cDNA) and transfer into a new clean PCR plate
	• Seal final plate and place in -20 until day 2.

PREPARE MASTER MIX REAGENTS (DAY 2)

17 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. USER/PCR mix is very viscous and easily bubbles, do not go to second stop when dispensing into 6-Res Echo source plate.

Equipment

384-well PP 2.0 Plus Microplate Echo Qualified	NAME
Plate	TYPE
Labcyte	BRAND
PPL-0200	SKU
https://www.beckman.com/supplies/echo-plates/001-14622	LINK
Sterile	SPECIFICATIONS

Equipment

Echo Qualified resevoir 2x3 Well Polypropylene Microplate	NAME
Plate	TYPE
Labcyte	BRAND
ER-0050	SKU
https://www.beckman.com/supplies/echo-plates/001-11101	LINK
RNase/DNase free	SPECIFICATIONS

	A	B
	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 (1:100)	0.25

	A	B
	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 **New England Biolabs Catalog #E7337AA**

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

18 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.

Note

End Prep and Adaptor master mixes should be loaded into 384 PP Plus plate. **USER/PCR** master mix should be loaded into a 6-Res plate.

19 Spin the Echo source plate in the centrifuge for 5 minutes at 2000 rpm to rid source wells of any bubbles produced in pipette transfer of master mixes.

DISPENSING END PREP REAGENTS

20 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.

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

Note

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

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

END PREP INCUBATION

23

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

DISPENSING ADAPTOR LIGATION REAGENTS

24 Remove sample plate from thermocycler, remove foil seal, and load into Echo. Using the GP setting, proceed with **2 transfers.** First, transfer **3100nL of adaptor ligation master mix.** Then, transfer **250nL of appropriately diluted adaptor** to each sample well. Make sure to add these reagents separately; this will prevent excessive adaptor dimer.

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

ADAPTOR LIGATION INCUBATION

26

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

USER/PCR SETUP - REAGENT PLATE

27 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.

Note

MUST be done before starting the bead clean-up.

28 Load a **new, sterile**, empty PCR plate into the Echo destination port. Using the GP setting, dispense **10,900nL of USER/PCR master mix** from a 6-Res plate into each sample well.

NUCLEIC ACID PURIFICATION: HAND BEAD CLEAN

29 Take beads out of 4C 30 min before bead cleaning to bring to room temp. Bead clean the end-prepped, adaptor-ligated cDNA using a sample to Ampure bead solution ratio of **0.8x**.

A

	A
	Prepare 80% ethanol (fresh, 50 mL reservoir) and beads (50 mL reservoir)
	• Add 25.65 uL nuclease-free water to sample wells to bring up to 35 uL. Adding water to the sample to increase volume helps when eluting beads.
	• Add 28 uL beads to sample plate, mix, and incubate for 5 minutes (visually confirm sufficient mixing)
	• Move sample plate to magnet and incubate 5 minutes
	• Remove 64 uL supernatant and transfer to waste
	• Wash with 150uL 80% ethanol, pause 30 seconds, remove and move to waste
	• Repeat ethanol wash step. Remove remaining ethanol with a p20—this is crucial for proper drying.
	• Air dry beads for 10 minutes *do not overdry but ensure wells are free of ethanol*
	• Move sample plate off magnet
	• Resuspend beads in 6uL eluent (barcodes), mix, and incubate 5 minutes (visually confirm sufficient mixing)
	• Move sample plate to magnet and incubate 5 minutes
	• Aspirate 5uL of supernatant (containing adaptor-ligated cDNA and barcodes) and transfer into the final plate prepared with USER/PCR master mix
	• Gently vortex and quick-spin plate to mix

Note

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

USER/PCR INCUBATION

30

	A	B	C
	USER/PCR INCUBATION (heated lid set to 105C)		
		cycle #	
	37ºC for 15 mins	1	
	98ºC for 30s	1	

	A	B	C
	98ºC for 10s	12-18*	*NOTE: number of PCR cycles should be chosen based on approximate RNA sample input. PAXgene and NP: 14; BAL: 16-18; Plasma: 16
	65ºC for 75s		
	65ºC for 5mins	1	
	Hold at 4ºC	∞	

NUCLEIC ACID PURIFICATION: HAND BEAD CLEAN

31 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.

	A
	*The following bead clean should be done on the bench or anywhere BUT the pre-PCR hood
	Prepare 80% ethanol (fresh, 50 mL reservoir) and beads (50 mL reservoir)
	• Add 12.72 uL beads to sample plate, mix, and incubate 5 minutes (visually confirm sufficient mixing)
	• Move sample plate to magnet and incubate 5 minutes
	• Remove 27.5 uL supernatant and transfer to waste
	• Wash with 150uL 80% ethanol, pause 30 seconds, remove and move to waste
	• Repeat ethanol wash step
	• Air dry beads for 10 minutes *do not overdry*
	• Move sample plate off magnet

	A
	• Resuspend beads in 33uL eluent (water), mix, and incubate 5 minutes (visually confirm sufficient mixing)
	• Move sample plate to magnet and incubate 5 minutes
	• Aspirate 31uL of supernatant (containing cDNA) and transfer into a new clean Echo source plate
	• Seal final plate and freeze for downstream use - using an Echo plate for the final plate means pooling can be done with the Echo
	• use a regular 384PP source plate rather than an LDV plate - LDV plates seem to bind DNA and cause loss of library
	• Remaining 2uL in the bead plate can be recovered and used for QuBit and Tapestation (to test several samples for successful library prep)

Note

Elutions volume may vary per application, but if continuing to automated pooling step elute in 30uL and recover 28uL to account for Labcyte Echo dead volume.

FINISHED LIBRARIES

32 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.

Protocol

NAME

High-Throughput Library Pooling for NGS

CREATED BY

Madeline Y Mayday

PREVIEW

32.1 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.

Note

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

32.2 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).

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

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

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

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

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).

Note

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.

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

32.7 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.

Note

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

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

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