High-throughput miniaturized 16S rRNA amplicon library preparation

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Earth Microbiome Project

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ABSTRACT

Next Generation Sequencing technologies have enabled many advances across biology with microbial ecology benefiting primarily through expanded sample sizes. Although the cost of running sequencing instruments has decreased substantially over time, the price of library preparation methods has largely remained unchanged. We developed a low cost, miniaturized (5 µL), high-throughput (384-sample), amplicon library preparation method with the acoustic liquid handler, Echo 550. Our method reduces costs of library preparation to $1.42 USD per sample, a 58% reduction compared to existing automated methods and a 21-fold reduction from commercial kits, without compromising sequencing success or distorting the microbial community composition analysis. The cost savings of implementing the miniaturized library preparation (going from triplicate 25 µL reactions to triplicate 5 µL reactions) are large enough to cover a MiSeq sequencing run for 768 samples, while preserving accurate microbiome measurements.

EXTERNAL LINK

https://msystems.asm.org/content/3/6/e00166-18

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

High-Throughput Miniaturized 16S rRNA Amplicon Library Preparation Reduces Costs while Preserving Microbiome Integrity Jeremiah J. Minich, Greg Humphrey, Rodolfo A. S. Benitez, Jon Sanders, Austin Swafford, Eric E. Allen, Rob Knight mSystems Nov 2018, 3 (6) e00166-18; DOI: 10.1128/mSystems.00166-18

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

MATERIALS

NAME	CATALOG #	VENDOR
Eppendorf twin.tec® PCR 96-well plate, skirted	951020401	Eppendorf
Platinum Hot Start PCR Master Mix (2x)	13000014	Thermo Fisher Scientific
ep T.I.P.S. Motion Racks 20 - 300 µL w/ filter	0030014456	Eppendorf
384-Well Low Dead Volume (LDV) Microplate	LP-0200	
384-Well Polypropylene Microplate	P-05525	
KingFisher Microplate	97002540	Thermo Fisher Scientific
ep T.I.P.S 0.2 - 10 µL w/filter	0030015193	Eppendorf
twin.tec PCR Plate 384	951020729	Eppendorf
epMotion Reservoir 10 mL	0030126521	Eppendorf
ep T.I.P.S 1 - 50 µL w/filter	0030015215	Eppendorf

NAME	CATALOG #	VENDOR
IDT Lyophilized Primer Plate	View	Integrated DNA Technologies
epMotion Reservoir 30 mL	960051009	Eppendorf
IDT Lyophilized Primer in vial	View	

BEFORE STARTING

Please wear at least the minimum required personal protective equipment.

Ensure that all necessary kit components are available as well as user-supplied consumables.

Remove nuclease and nucleotide contamination from work surfaces and instruments prior to starting using an appropriate solution, such as RNase AWAY™ (Thermo Scientific™ catalogue: 700511), followed by wiping with 70% to 100% molecular biology grade ethanol to remove additional contaminants.

Make primer working plates.

1 Resuspend lyophilized primers from 96-Well plates shipped from Integrated DNA Technologies (IDT) at 3nmol per well using the following protocol

PROTOCOL

16S Illumina Primer Plate Resuspension (100 µM)
by Rodolfo Salido Benitez,
Knight Lab @ UC San Diego Health

PREVIEW START EXPERIMENT

1.1 In a sterile 30mL reservoir, add 11.725 ml (required minimum volume) of PCR Clean Water to resuspend (4) 96-Well plates.

11.725 ml PCR Clean Water

1.2 Centrifuge all 96-Well primer plates to ensure lyophilized sample is at the bottom of the well. (3nmol primer per well)

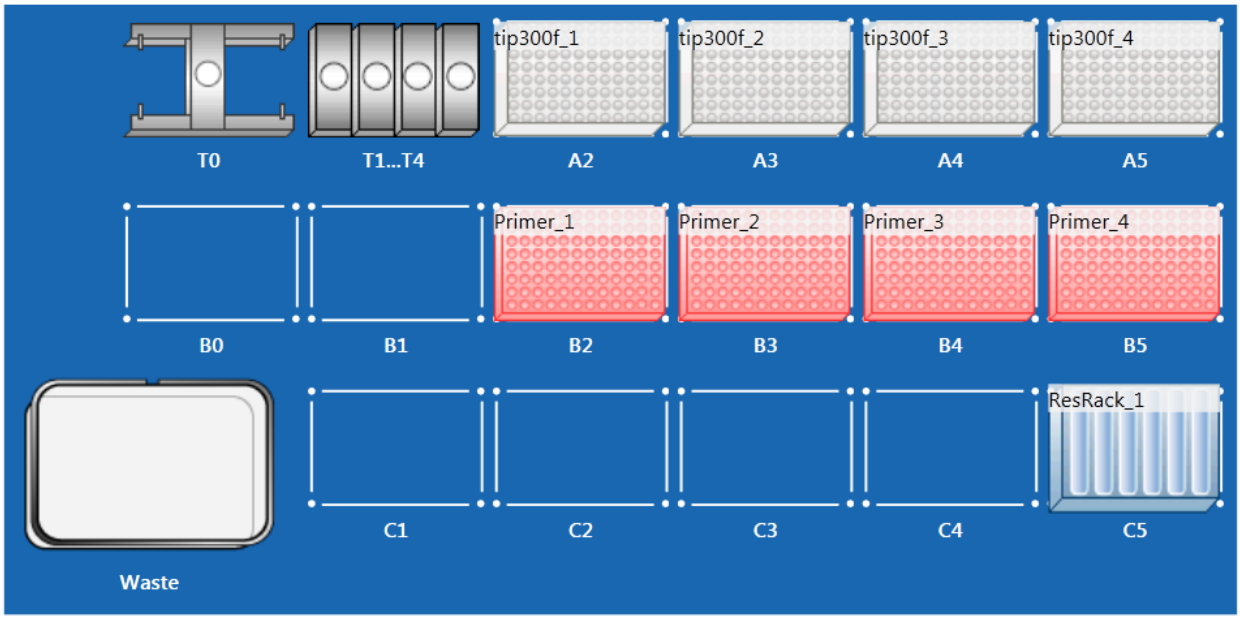
1.3

EQUIPMENT

epMotion 5075
Liquid Handling
Eppendorf 5075000962

Follow the diagram below while setting up the epMotion worktable.

Worktable



Place (4) boxes of ep.T.I.P.S. Motion Racks 20 - 300 µL w/ filter in slots A2-A5
Place (4) 96-Well plates of lyophilized primers from IDT in slots B2-B5
Place 30 mL reservoir with PCR Clean Water in slot 1 of the Resevoir Rack and place Rack in slot C5

1.4 Remove box lids and plate foils and execute protocol.

(Protocol must be imported to epBlue software prior to attempting to execute it. *epBlue 40.6 or later*)

Application_4_Resus_100uM_Primers_181003_105824.export6

The automated protocol transfers 30µL of PCR Clean Water into the (4) different 96-Well Primer Plates and mix pipettes for 5 cycles to resuspend lyophilized primers to a 100 µM)

1.5 Remove plates from worktable and seal with storage aluminum foils.

2 Aliquot and mix forward (barcoded) and reverse primer pairs to working concentrations (5µM each) in 96-Well plates using the following protocol

PROTOCOL

16S Illumina Primer Plate Aliquoting (working plates 5µM Forw & Rev)
by Rodolfo Salido Benitez,
Knight Lab @ UC San Diego Health

PREVIEW START EXPERIMENT

2.1 Resuspend Reverse Primer from lyophilized sample vial to a final concentration of 100 µM

2.2 In a sterile 30mL reservoir, add 15,840 µL of PCR Clean Water and 880 µL of Reverse Primer (100 µM) to make (4) 96-Well primer working plates.

15840 µl PCR Clean Water **880 µl Reverse Primer (100µM)**

2.3 Thaw and centrifuge 96-Well resuspended primer plates.

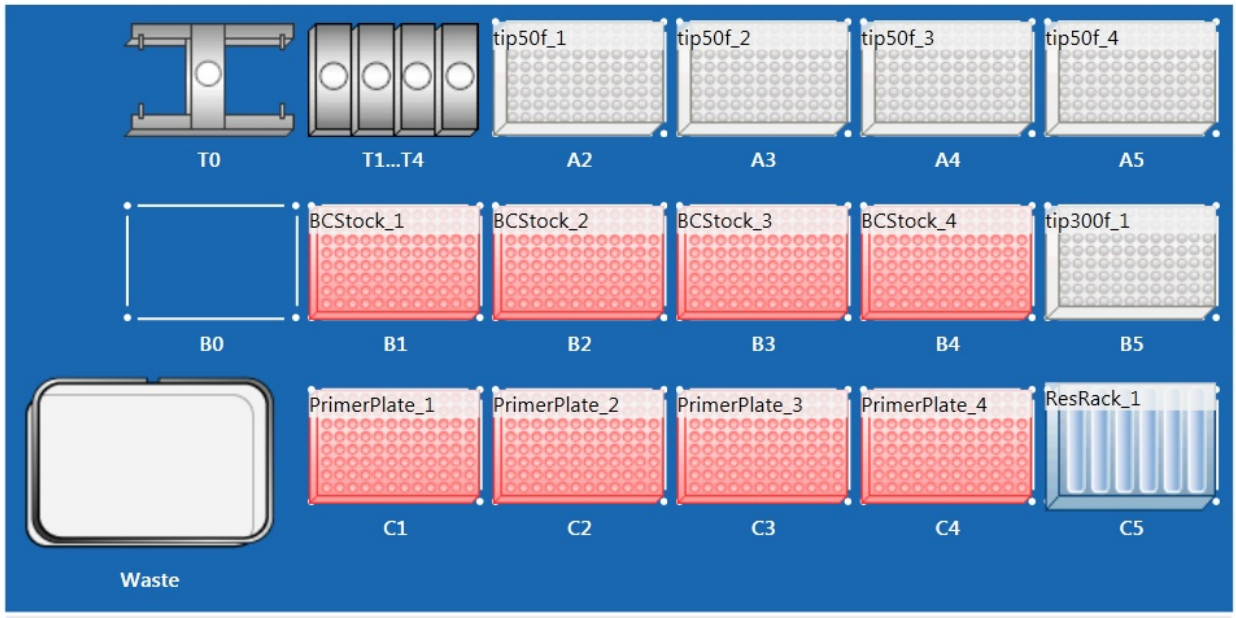
2.4 Appropriately label destination plates.

2.5

EQUIPMENT
epMotion 5075
Liquid Handling
Eppendorf 5075000962

Follow the diagram bellow while setting up the epMotion worktable

Worktable



Place (4) boxes of ep.T.I.P.S. Motion Racks 1 - 50 µL w/ filter in slots A2-A5
Place (1) box of ep. T.I.P.S. Motion Racks 20 - 300 µL w/ filter in slot B5
Place (4) 96-Well plates of resuspended primers (100µM) from IDT in slots B1-B4
Place (4) 96-Well twin.tec plates in slots C1-C4.
Place 30 mL reservoir with reverse primer (5.26 µM) in slot 1 of the Resevoir Rack and place Rack in slot C5

2.6 Remove box lids and plate foils and execute protocol.

(Protocol must be imported to epBlue software prior to attempting to execute it. *epBlue 40.6 or later*)

Application_4_5uM_Primer_40ul_181003_105836.export6

The automated protocol transfers 38µL of reverse primer (5.26µM) into the (4) different 96-Well Primer Plates using the multidispense feature of the epBlue software.
Then it transfer 2µL of barcoded forward primers (100µM) from IDT stock plates into the (4) different 96-Well Primer Plates (working plates)

2.7 Remove plates from worktable and seal with storage aluminum foils.

Make acoustic droplet ejection compatible plates

3 Compress (4) 96-Well working primer plates into (1) 384-Well echo compatible primer plate in a fully interweived layout using the following protocol

Fully interweived layout is described in abstract of protocol

PROTOCOL

16S Illumina Primer Plate Compression
by Rodolfo Salido Benitez,
Knight Lab @ UC San Diego Health

PREVIEW START EXPERIMENT

3.1 Thaw and centrifuge primer plates.

3.2 Appropriately label destination plate.

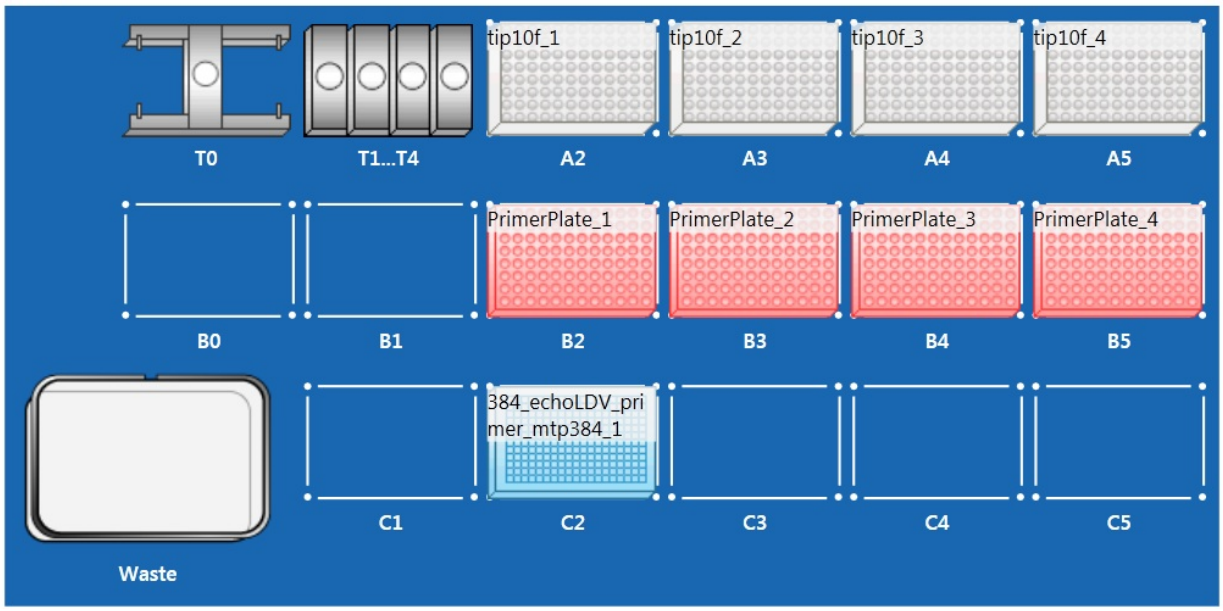
3.3

EQUIPMENT

epMotion 5075
Liquid Handling
Eppendorf 5075000962

Follow the diagram below while setting up the epMotion worktable.

Worktable



Place (4) boxes of 0.2 - 10µL epT.I.P.S. w/filters on deck slots A2-A5
Place Primer Plates 1-4 on deck slots B2-B5
Place LDV Destination Plate on slot C2

3.4 Remove box lids and plate foils and execute protocol.

(Protocol must be imported to epBlue software prior to attempting to execute it. *epBlue 40.6 or later*)

Application_10uLTips Primer compression 4-96 to 1-384 LDV_181003_115538.export6

The automated protocol transfers 9µL of each primer into the destination plate following the plate layout outlined in the description of this protocol.io

4 Compress (4) 96-Well gDNA plates into (1) 384-Well echo compatible gDNA plate in a fully interweived layout using the follwing protocol.

Fully interweived layout is described in abstract of protocol

PROTOCOL

Extracted gDNA Plate Compression
by Rodolfo Salido Benitez,
Knight Lab @ UC San Diego Health

PREVIEW START EXPERIMENT

4.1 Thaw and centrifuge gDNA plates.

4.2 Appropriately label destination plate.

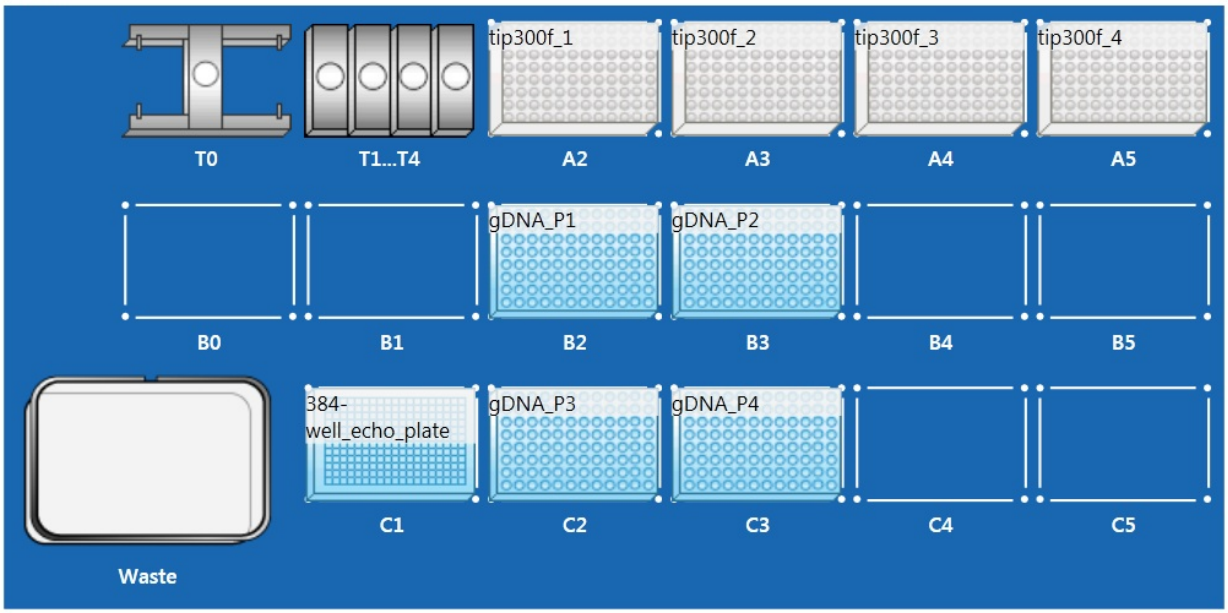
4.3

EQUIPMENT

epMotion 5075
Liquid Handling
Eppendorf 5075000962

Follow the diagram below while setting up the epMotion worktable.

Worktable



Place (4) boxes of 20 - 300 µL epT.I.P.S. w/filters on deck slots A2-A5.
Place 96-well gDNA Plates 1-4 on deck slots B2-B3 and C2-C3 respectively.
Place 384-well PP or LDV Destination Plate on slot C1

4.4 Remove box lids and plate foils and execute protocol.

(Protocol must be imported to epBlue software prior to attempting to execute it. *epBlue 40.6 or later*)

Application_gDNA_plate compression_4-96 to 1-384 pp_181003_105135.export6

The automated protocol transfers 60µL of extracted gDNA from each source well into the destination plate following the plate layout outlined in the description of this protocol.io

Prepare PCR Plates

5 Dispense PCR master mix into triplicate 384-Well plates using the following protocol.

PROTOCOL

PCR Master Mix Aliquoting
by Rodolfo Salido Benitez, Knight Lab @ UC San Diego Health

PREVIEW START EXPERIMENT

5.1 In a sterile 10mL reservoir, add 2,534 µl of Platinum Hot Start PCR 2X Master Mix and 3,295 µL of PCR Clean Water per set of 384 samples

2534 µl 2X PCR Master Mix **3295 µl PCR Clean Water**

Mix pipette.

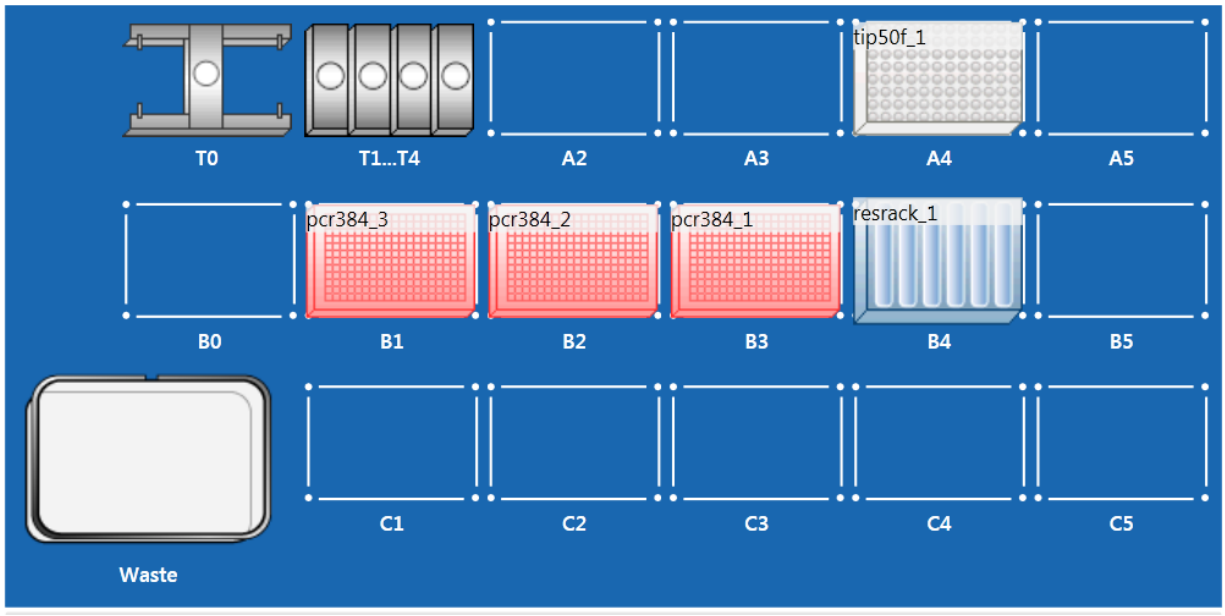
5.2

EQUIPMENT

epMotion 5075
Liquid Handling
Eppendorf 5075000962

Follow the diagram below while setting up the epMotion worktable.

Worktable



Place (1) box of 1-50µL epT.I.P.S. w/filters on deck slot A4
Place empty 384 PCR Primer Plates 1-3 on deck stols B1-B3
Place 10mL reservoir with PCR Master Mix in the first slot of the reservoir rack, then place reservoir rack on deck slot B4.

5.3 Remove box lids and execute protocol.

(Protocol must be imported to epBlue software prior to attempting to execute it. *epBlue 40.6 or later*)

Application_3XPCR_mastermix aliquot 4ul_384eppendorf_181003_105306.export6

The automated protocol transfers 4.6 µL of PCR Master Mix into (3) 384-well PCR plates using the multidispense feature of the epBlue software.

6 Execute sub-microliter dispenses using Echo 555 into triplicate 384-Well plates using the following protocol.

PROTOCOL

Sub-microliter Primer and gDNA Dispense
by Rodolfo Salido Benitez,
Knight Lab @ UC San Diego Health

PREVIEW START EXPERIMENT

6.1 Thaw and centrifuge primer, gDNA, and PCR plates.

SAFETY INFORMATION

Centrifugation speed for echo qualified Low Dead Volume (LDV) plates **must not** exceed 1500rpm.

6.2

NOTE

The following steps are optional. The Knight Lab performs them to ensure enough source material is present in source plates before executing the protocol.

Survey source plates to ensure the Primer and gDNA source plates have enough material to execute the protocol to completion.

Open Echo 550 Liquid Handler software. Go to Diagnostics tab. Click Source Plate Out. Place plate to be surveyed in source plate tray

ensuring that the instrument has the appropriate plate insert, then click Source Plate In. Select appropriate plate profile when prompted. Under Miscellaneous, click the dropdown menu and select Survey, then click Launch. A window will pop up. Click Go in the new window to start the plate survey.

NOTE

384-Well Polypropylene (PP) plate expects the 2.10 mm insert
The Knight Lab uses the following plate profile for the PP plate: 384PP_AQ_BP2_HT
Working range of volumes for the PP plate is 15 - 65 µL.

384-Well Low Dead Volume (LDV) plate expects the 4.50 mm insert
The Knight Lab uses the following plate profile for the LDV plate: 384LDV_AQ_B2_HT
Working range of volumes for the LDV plate is 3-12 µL

6.3

EQUIPMENT

Echo 550
Liquid Handling
Labcyte GEN-27

Execute the following Echo 550 protocol to transfer 16S primers. You'll need the Echo Plate Reformat software.

Mini-PCR_primer_dispense.epr

The protocol will transfer 200 nL of primers from each source well of the LDV source plate to the corresponding destination well for a total of 3 copies of the twin tec PCR destination plates.

6.4 Execute the following Echo 550 protocol to trasnfer gDNA.

Mini-PCR_gDNA_dispense.epr

The protocol will transfer 200 nL of gDNA from each source well of the PP source plate to the corresponding destination well for a total of 3 copies of the same twin tec PCR destination plates used in last step.

6.5 Seal all source plates with storage aluminum foils.

Seal all destination plates with thermo-cycler compatible aluminum foils and centrifuge them.

Amplify PCR Plates

7

Thermocycler conditions

- Primers: 16S V4 515F–806R
- Amplicon size: ~390 bp
- Cycle times are longer for 384-well thermocyclers.

Temperature	Time, 384 Well	Repeat
94 °C	3 min	
94 °C	60 s	x35
50 °C	60 s	x35
72 °C	105 s	x35
72 °C	10 min	

4 °C	hold	

Thermocycler conditions

Pool and store PCR products

8 Pool PCR products from triplicate 384-Well plates using the following protocol

PROTOCOL

3X PCR Replicate Pooling
by Rodolfo Salido Benitez,
Knight Lab @ UC San Diego Health

PREVIEW START EXPERIMENT

8.1 If needed, thaw PCR plates, then centrifuge them.

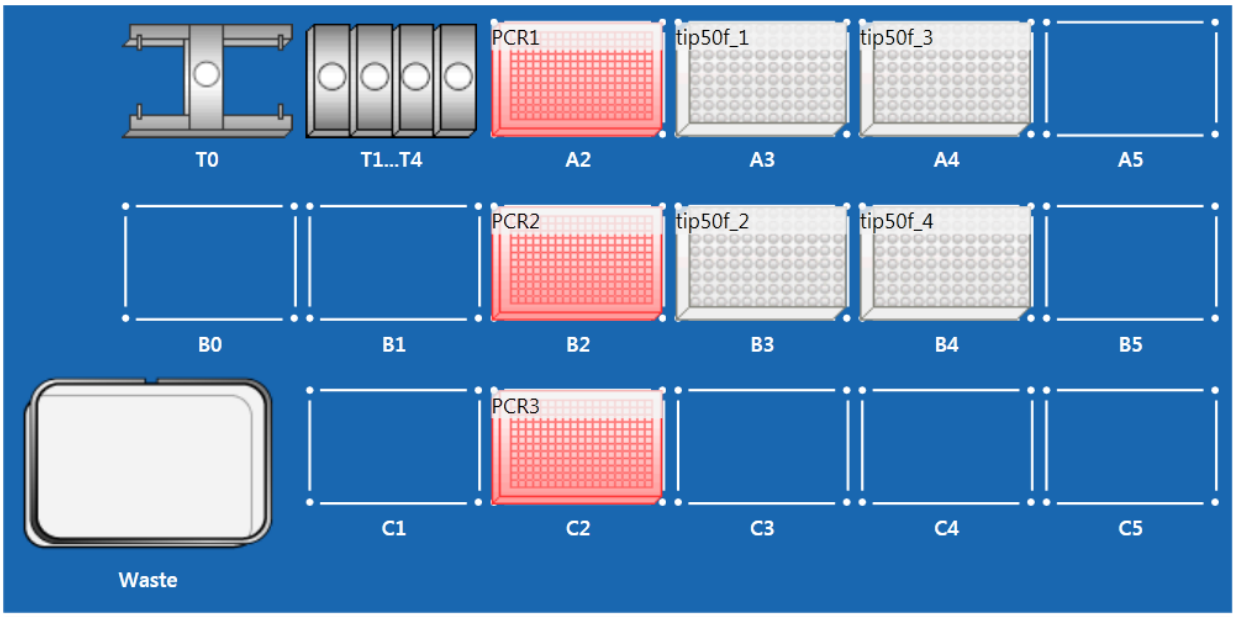
8.2

EQUIPMENT

epMotion 5075
Liquid Handling
Eppendorf 5075000962

Follow the diagram while setting up the epMotion worktable.

Worktable



Place (4) boxes of 1-50µL ep T.I.P.S. w/filter on slots A3-A4 and B3-B4 respectively.
Place PCR plates on slots A2, B2, and C2.

8.3 Remove box lids and plate foils and execute protocol.

(Protocol must be imported to epBlue software prior to attempting to execute it. *epBlue 40.6 or later*)

Application_3X_pooling_3-384 to 1-384_181003_105035.export6

The automated protocol uses the multiaspirate feature of the epBlue software to pool PCR products from plates PCR1 and PCR2 and dispense them into plate PCR3. The command aspirates 7µL from each plate which will result in some air being aspirated.

9 Seal plate with storage aluminum foil and store in freezer.