

Nov 08, 2018

High-throughput miniaturized 16S rRNA amplicon library preparation

 [mSystems](#)

DOI

dx.doi.org/10.17504/protocols.io.u2ieyce

Jeremiah Minich¹, Greg Humphrey², Rodolfo A Salido Benitez², Jon Sanders², Austin Swafford³, Eric Allen¹, Rob Knight²

¹Marine Biology Research Division. UC San Diego; ²Department of Pediatrics. UC San Diego;

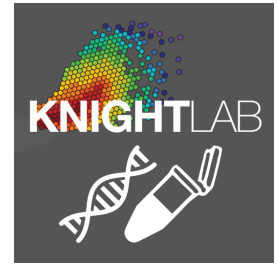
³Center for Microbiome Innovation. UC San Diego

Earth Microbiome Project



Rodolfo A Salido Benitez

Knight Lab @ UC San Diego Health



OPEN  ACCESS



DOI: dx.doi.org/10.17504/protocols.io.u2ieyce

External link: <https://msystems.asm.org/content/3/6/e00166-18>

Protocol Citation: Jeremiah Minich, Greg Humphrey, Rodolfo A Salido Benitez, Jon Sanders, Austin Swafford, Eric Allen, Rob Knight 2018. High-throughput miniaturized 16S rRNA amplicon library preparation. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.u2ieyce>

Manuscript citation:

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

License: This is an open access protocol distributed under the terms of the **[Creative Commons Attribution License](#)**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: October 26, 2018

Last Modified: November 08, 2018

Protocol Integer ID: 17194

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 μ L), 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 μ L reactions to triplicate 5 μ L reactions) are large enough to cover a MiSeq sequencing run for 768 samples, while preserving accurate microbiome measurements.

Materials

MATERIALS

✕ Eppendorf twin.tec® PCR 96-well plate, skirted **Eppendorf Catalog #951020401**

✕ Platinum Hot Start PCR Master Mix (2x) **Thermo Fisher Scientific Catalog #13000014**

✕ ep T.I.P.S. Motion Racks 20 - 300 μ L w/ filter **Eppendorf Catalog #0030014456**

✕ 384-Well Low Dead Volume (LDV) Microplate **Catalog #LP-0200**

✕ 384-Well Polypropylene Microplate **Catalog #P-05525**

✕ KingFisher Microplate **Thermo Fisher Scientific Catalog #97002540**

✕ ep T.I.P.S 0.2 - 10 μ L w/filter **Eppendorf Catalog #0030015193**

✕ twin.tec PCR Plate 384 **Eppendorf Catalog #951020729**

✕ epMotion Reservoir 10 mL **Eppendorf Catalog #0030126521**

✕ ep T.I.P.S 1 - 50 μ L w/filter **Eppendorf Catalog #0030015215**

✕ IDT Lyophilized Primer Plate **Integrated DNA Technologies, Inc. (IDT)**

✕ epMotion Reservoir 30 mL **Eppendorf Catalog #960051009**

✕ IDT Lyophilized Primer in vial



Before start

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



NAME

16S Illumina Primer Plate Resuspension (100 μ M)

CREATED BY

Rodolfo A Salido Benitez

PREVIEW

- 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

NAME

Liquid Handling

TYPE

Eppendorf

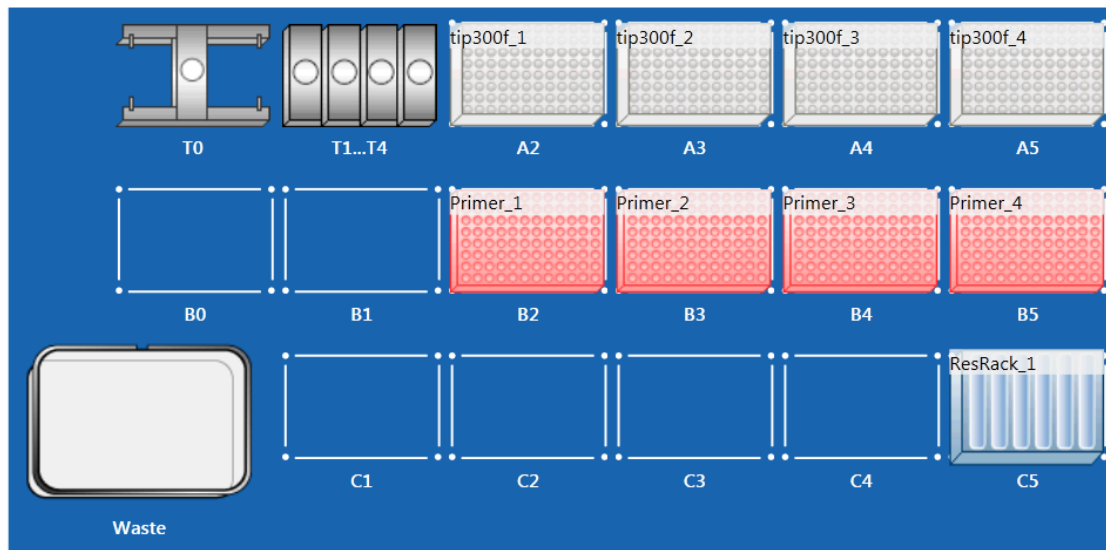
BRAND

5075000962

SKU

Follow the diagram below while setting up the epMotion worktable.

Worktable



Place (4) boxes of ep.T.I.P.S. Motion Racks 20 - 300 μ L 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 Reservoir 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_Prime...

The automated protocol transfers 30 μ L 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 μ M)

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 (5 μ M each) in 96-Well plates using the following protocol



Protocol



NAME

16S Illumina Primer Plate Aliquoting (working plates 5µM Forw & Rev)

CREATED BY

Rodolfo A Salido Benitez

PREVIEW

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

NAME

Liquid Handling

TYPE

Eppendorf

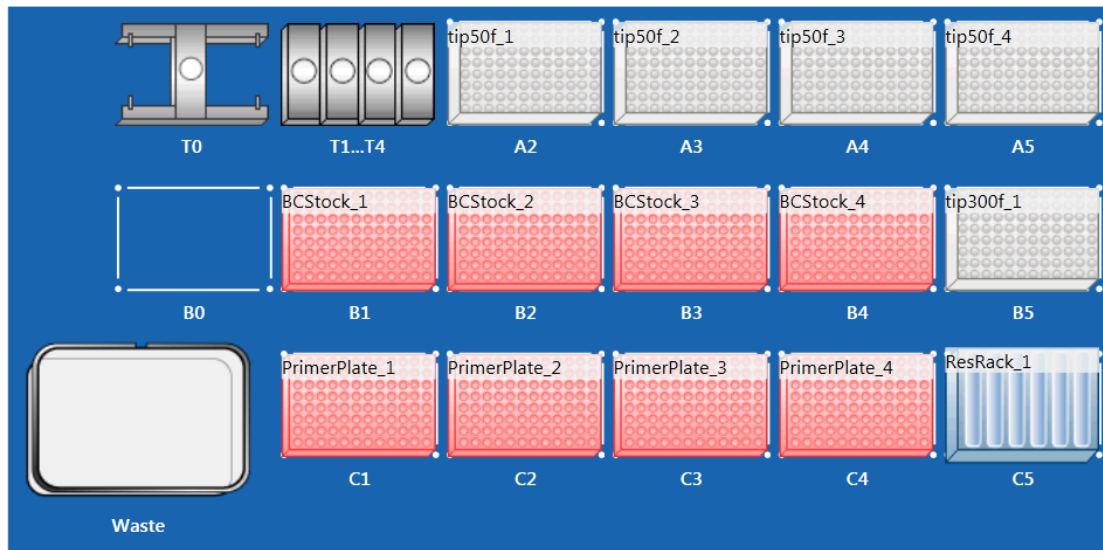
BRAND

5075000962

SKU

Follow the diagram bellow while setting up the epMotion worktable

Worktable



Place (4) boxes of ep.T.I.P.S. Motion Racks 1 - 50 μ L w/ filter in slots A2-A5
 Place (1) box of ep. T.I.P.S. Motion Racks 20 - 300 μ L w/ filter in slot B5
 Place (4) 96-Well plates of resuspended primers (100 μ M) 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 μ M) in slot 1 of the Reservoir 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_181...

The automated protocol transfers 38 μ L of reverse primer (5.26 μ M) into the (4) different 96-Well Primer Plates using the multidispense feature of the epBlue software.
 Then it transfer 2 μ L of barcoded forward primers (100 μ M) 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 interweaved layout using the following protocol

Fully interweaved layout is described in abstract of protocol

Protocol



NAME

16S Illumina Primer Plate Compression

CREATED BY

Rodolfo A Salido Benitez

PREVIEW

3.1 Thaw and centrifuge primer plates.

3.2 Appropriately label destination plate.

3.3

Equipment

epMotion 5075

NAME

Liquid Handling

TYPE

Eppendorf

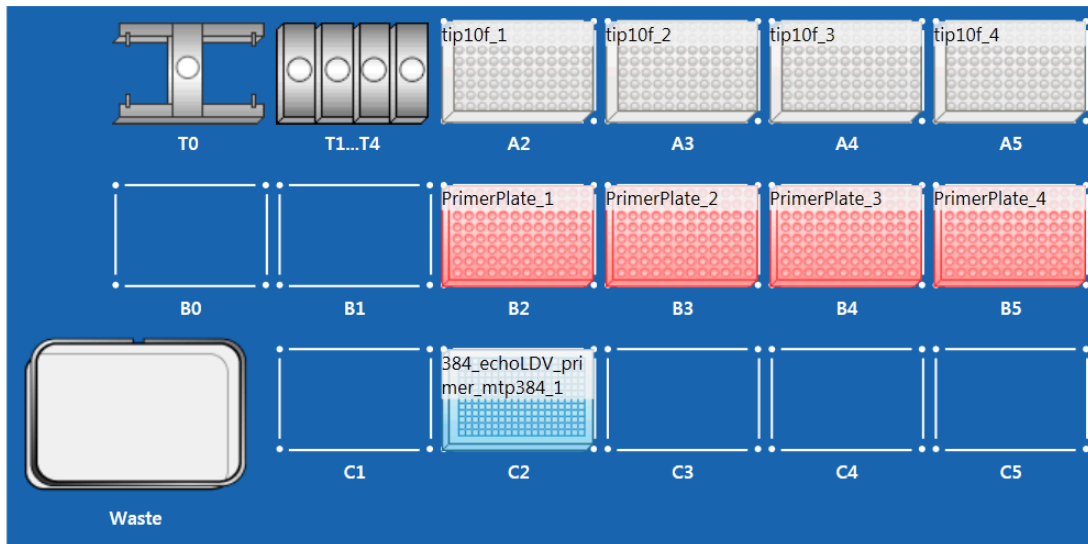
BRAND

5075000962

SKU

Follow the diagram below while setting up the epMotion worktable.

Worktable



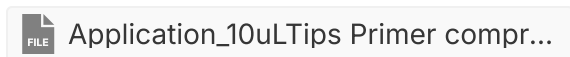
Place (4) boxes of 0.2 - 10µL epT.I.P.S. w/filters on deck slots A2-A5

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*)



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 interweaved layout using the following protocol.

Fully interweaved layout is described in abstract of protocol

Protocol



NAME

Extracted gDNA Plate Compression

CREATED BY

Rodolfo A Salido Benitez

PREVIEW

4.1 Thaw and centrifuge gDNA plates.

4.2 Appropriately label destination plate.

4.3

Equipment

epMotion 5075

NAME

Liquid Handling

TYPE

Eppendorf

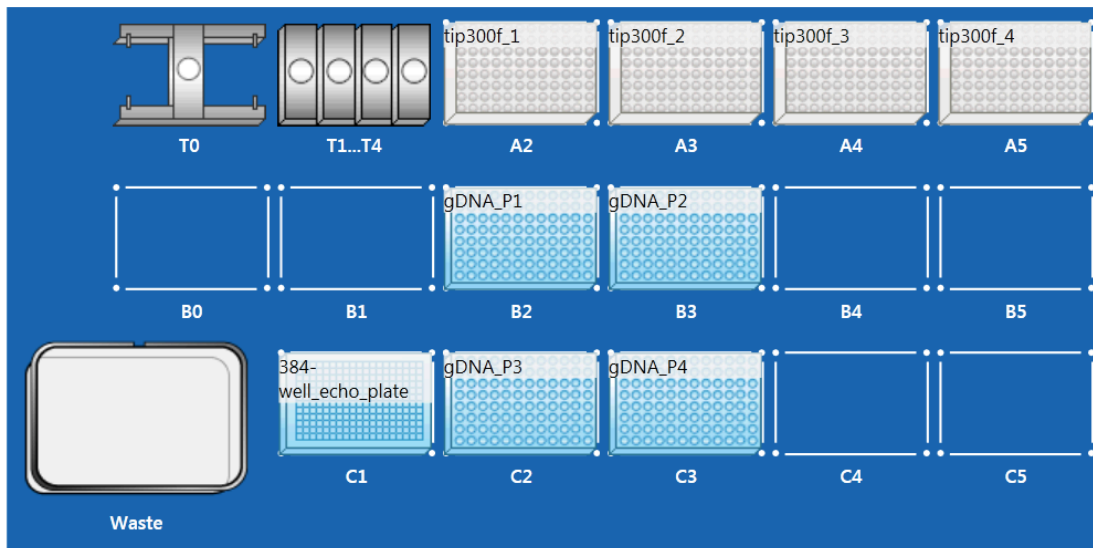
BRAND

5075000962

SKU

Follow the diagram below while setting up the epMotion worktable.

Worktable



Place (4) boxes of 20 - 300 μ L 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 compressi...

The automated protocol transfers 60 μ L 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



NAME

PCR Master Mix Aliquoting

CREATED BY

Rodolfo A Salido Benitez

PREVIEW

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

 2534 μ L 2X PCR Master Mix 3295 μ L PCR Clean Water

Mix pipette.

- 5.2

Equipment

epMotion 5075

NAME

Liquid Handling

TYPE

Eppendorf

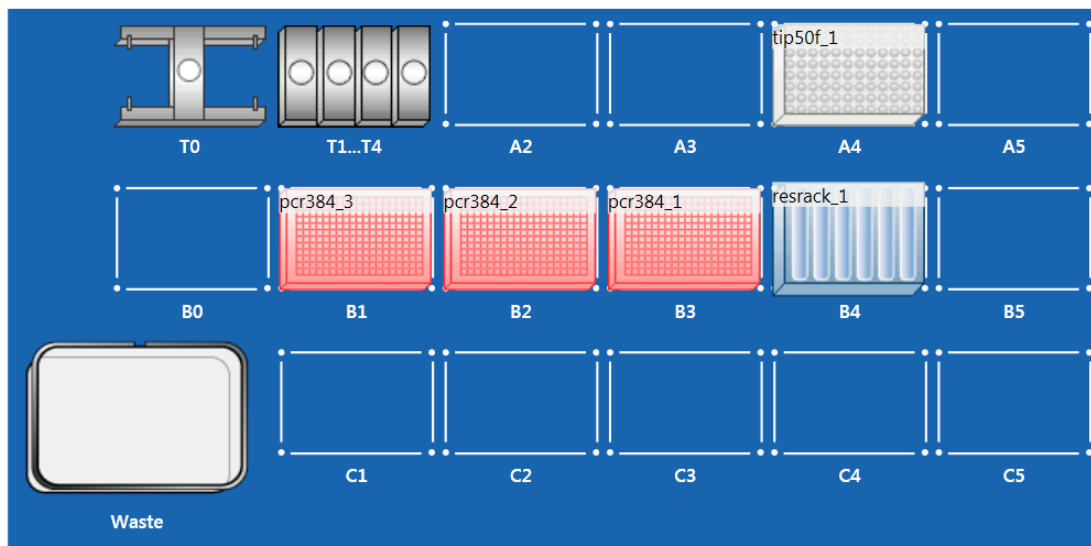
BRAND

5075000962

SKU

Follow the diagram below while setting up the epMotion worktable.

Worktable



Place (1) box of 1-50 μ L epT.I.P.S. w/filters on deck slot A4

Place empty 384 PCR Primer Plates 1-3 on deck slots 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 aliq...

The automated protocol transfers 4.6 μ L 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



NAME

Sub-microliter Primer and gDNA Dispense

CREATED BY

Rodolfo A Salido Benitez

PREVIEW

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**

NAME

Liquid Handling

TYPE

Labcyte

BRAND

GEN-27

SKU

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 transfer 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



NAME

3X PCR Replicate Pooling

CREATED BY

Rodolfo A Salido Benitez

PREVIEW

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

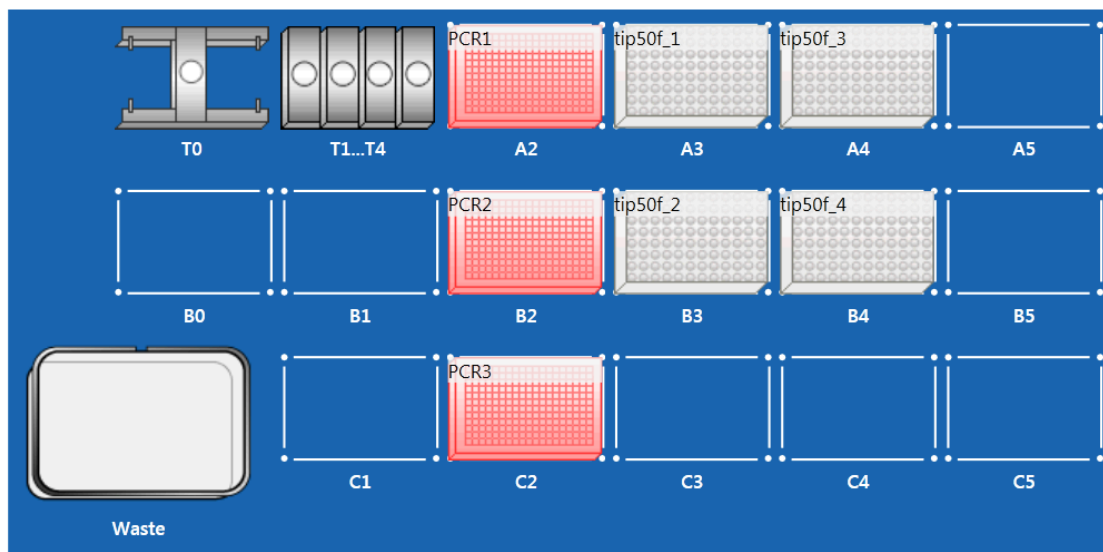
8.2

Equipment

| | |
|-----------------|-------|
| epMotion 5075 | NAME |
| Liquid Handling | TYPE |
| Eppendorf | BRAND |
| 5075000962 | SKU |

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-...

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.