Dec 29, 2023 Version 1

DoTA-seq V3.1 V.1

Forked from a private protocol

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol describes the process of DoTA-seq generating a single cell sequencing library from a cell suspension. This workflow can be performed in two days, with the PCR step happening overnight. Before beginning this workflow make sure to have:

1. The necessary microfluidics devices prepared and ready to go
2. The multiplex DoTA-seq target primers validated to work together without generating large molecular weight primer dimers.

Please read the publication for further details.

Guidelines

Strongly recommend all pre-PCR steps (setting up reagents, washing gels) to be done in a PCR Clean hood. This has two purposes:

1. Reduce PCR contamination of templates which can strongly effect single-cell PCR reactions.
2. Reduce dust contamination of reagents which can clog devices and cause failures.

Materials

- ddPCR Supermix for probes (no dUTP) **Bio-Rad Laboratories** **Catalog #**1863024
- MetaPolyzyme **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**MAC4L-5MG
- Lysozyme from chicken egg white **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**L6876
- HFE 7500 Perfluorinated Oil
- Perfluorooctanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**370533
- TCEP-HCl **Gold Biotechnology** **Catalog #**TCEP
- NN′-Bis(acryloyl)cystamine **Santa Cruz Biotechnology** **Catalog #**sc-215506
- Ammonium persulfate **Catalog #**A3678
- Acrylamide **P212121**
- TEMED (Tetramethyl-ethulenediamine) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T9281
- Biorad Evagreen Droplet Oil **Bio-Rad Laboratories** **Catalog ##**1864005
- DNA Clean & Concentrator™-5 **Zymo Research** **Catalog #**D4003
- DNA Clean & Concentrator™-5 **Zymo Research** **Catalog #**D4003
- Axygen® 0.2 mL Maxymum Recovery® Thin Wall PCR Tubes **Corning** **Catalog #**PCR-02-L-C
- NEBNext Library Quant Kit for Illumina - 100 rxns **New England Biolabs** **Catalog #**E7630S
- SYBR Green **Thermo Fisher Scientific**
- Proteinase K solution, 20 mg ml – 1 **Ambion** **Catalog #**AM2546
- Lysozyme from chicken egg white **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**L6876
- Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1%

Safety information

Unpolymerized Acrylamide is toxic, handle with care and dispose according to regulations

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- DNA Clean & Concentrator™-5 **Zymo Research** **Catalog #**D4003
- HFE 7500 Perfluorinated Oil
- PBS 0.1% Tween20
- PBS 0.1% Tween 20
- SYBR Green **Thermo Fisher Scientific**
- Cellbrite Fix 555 **Biotium** **Catalog ##**30088
- Acrylamide **P212121**
- NN′-Bis(acryloyl)cystamine **Santa Cruz Biotechnology** **Catalog #**sc-215506
- Ammonium persulfate **Catalog #**A3678
- Biorad Evagreen Droplet Oil **Bio-Rad Laboratories** **Catalog ##**1864005
- TEMED (Tetramethyl-ethulenediamine) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T9281
- Biorad Evagreen Droplet Oil **Bio-Rad Laboratories** **Catalog ##**1864005
- Perfluorooctanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**370533

Acetone
Isopropanol
PBS EDTA 1mM Tween20 2% w/v
1X PBS (Phosphate-buffered saline)
Lysozyme from chicken egg white **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L6876
MetaPolyzyme **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**MAC4L-5MG
1X PBS (Phosphate-buffered saline)
PBS 0.1% Tween20
Proteinase K solution, 20 mg ml – 1 **Ambion Catalog #**AM2546
10% SDS **Bio-Rad Laboratories Catalog #**161-0146
TE Buffer (Tris 10mM EDTA 1mM pH 8)
TE 2% Tween-20
TE 2% Tween-20
Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1%
Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1%
ddPCR Supermix for probes (no dUTP) **Bio-Rad Laboratories Catalog #**1863024
DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D0632
Biorad Evagreen Droplet Oil **Bio-Rad Laboratories Catalog ##**1864005
Axygen® 0.2 mL Maxymum Recovery® Thin Wall PCR Tubes **Corning Catalog #**PCR-02-L-C
EDTA (0.5 M), pH 8.0 **Life Technologies Catalog #**AM9260G
HFE 7500 Perfluorinated Oil
Perfluorooctanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**370533
TCEP-HCl **Gold Biotechnology Catalog #**TCEP
DNA Clean & Concentrator™-5 **Zymo Research Catalog #**D4003
SPRIselect reagent kit **Beckman Coulter Catalog #**B23317
High Sensitivity D1000 ScreenTape **Agilent Technologies Catalog #**5067-5584
NEBNext Library Quant Kit for Illumina - 100 rxns **New England Biolabs Catalog #**E7630S
HFE 7500 Perfluorinated Oil

Troubleshooting

Preparing Cells

10m

1 Prepare a cell suspension by washing twice in 1mL of PBS 0.1% Tween20 by spinning down at 5000 x g, 00:01:00

2 Resuspend cells in 100 µL PBS 0.1% Tween 20

3 Add 1 µL SYBR Green **Thermo Fisher Scientific** 10,000X dye to the cells to stain them

4 Count cells using a hemacytometer using the SYBR signal, calculate concentration of the cell suspension.

5 (Optional) Stain with Cellbrite Fix 555 **Biotium Catalog** ##30088 to get a cell membrane/wall stain

Preparing Gel

30m

6 Make 200 µL Hydrogel Precursor Solution - Mix together in a tube:
100 µL Acrylamide **P212121** monomer in water [M] 25 Mass / % volume
15 µL
NN'-Bis(acryloyl)cystamine **Santa Cruz Biotechnology Catalog** #sc-215506 in Methanol [M] 5 Mass / % volume
10 µL Ammonium persulfate **Catalog** #A3678 [M] 10 Mass / % volume
75 µL Cell suspension diluted in PBS (**a total of 7e6 cells** to achieve a final concentration of **3.5e7 cells/mL** in the total solution)

Vortex Vigorously to Mix

Generate Gel Droplets

10m

7 Prepare and Load the Syringes with the gel sample and 600 µL
Biorad Evagreen Droplet Oil **Bio-Rad Laboratories Catalog** ##1864005 and connect to the microfluidic devices by following this protocol

Protocol

NAME

Loading Syringes to Inject into Microfluidics Device

CREATED BY

freeman.lan

Preview

8 Connect the syringes to the inlets of the **DoTA-seq Step 1** Microfluidics Device and Run the syringe pumps at **500uL/hr** for the gel syringe, and **900uL/hr** for the oil syringe.

9 Collect gel droplets for 00:20:00 in a 1mL tube. 20m

Note

Sometimes the initial droplet formation produces polydisperse droplets. In this case, wait 2 min for the bad emulsion to leave the outlet tubing into a waste tube, then begin collecting in the collection tube.

10 Make 200 µL Gel Polymerization Oil - mix together in a tube:

195 µL

Biorad Evagreen Droplet Oil **Bio-Rad Laboratories Catalog** ##1864005

5 µL

TEMED (Tetramethyl-ethulenediamine) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T9281

11 Add the Gel polymerization oil to the collected droplets, invert slowly 3 times to mix, and Incubate the tube containing droplets at 37 °C for 00:10:00 to complete polymerization of the gel matrix. 10m

Note

You can now look at the emulsion under the microscope using Countess slides **Thermo Fisher Scientific Catalog #**C10228 to determine the encapsulation ratio of your cells. SYBRGreen and CF555 signal should be concordant and correspond to cells.

Breaking out gels from emulsion

12 Pulse spin the emulsion in a centrifuge to close pack the emulsion and drain the oil to the bottom of the tube.

13 Use a pipette to remove the oil from the bottom of the tube, leaving just the emulsion

14 Add 200 µL Perfluorooctanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**370533 to break the emulsion 1m
Vortex, then Wait 00:01:00 for the emulsion to break.

15 Pulse spin again and remove the oil in the bottom of the tube with a pipette.

16 Add 1000 µL of HFE 7500 Perfluorinated Oil to the tube. 10s
Mix by inverting 5 times, Wait 00:00:10 then remove with a pipette.

17 Add 1000 µL of Acetone to the tube. 10s
Mix by inverting 5 times, Wait 00:00:10 then remove with a pipette.
The gels should begin to flocculate and dehydrate.

18 Add 1000 µL of Isopropanol 10s
Mix by inverting 5 times, Wait 00:00:10 then remove with a pipette.
The gels should dehydrate and become hard.

Note: Do not wait too long as it could cause the gels to irreversibly aggregate into clumps.

19 Resuspend in 1000 µL PBS EDTA 1mM Tween20 2% w/v

The gels can be stored at 4 °C for several days without changing DoTA-seq results.

Note

You can now look at the gels under the microscope using Countess slides **Thermo Fisher Scientific Catalog #**C10228 to determine the encapsulation ratio of your cells.

For **SYBR Staining,** you should first wash a small aliquot in PBS 2% Tween to remove background SYBR+ oil droplets before visualization on the microscope.

You should see some loss in CF555 signal as the acetone and alcohol wash removes some bacterial membranes.

Lysing Bacteria

20 Wash gels 3 times in 1000 µL 1X PBS (Phosphate-buffered saline) **(No Tween)** by centrifugation at 500 x g, 00:00:30 each time 30s

21 Make a Enzymatic Lysis Solution by adding:

20 mg

Lysozyme from chicken egg white **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L6876

100 µL [M] 1 mg/mL

MetaPolyzyme **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**MAC4L-5MG

900 µL 1X PBS (Phosphate-buffered saline) **(No Tween)**

22 Resuspend the gels in this lysis solution. Incubate at 37 °C for 02:00:00 2h

23 Wash the gels 3 times in 1000 µL PBS 0.1% Tween20

24 Make a SDS Lysis solution by adding:

20 µL [M] 20 mg/mL

Proteinase K solution, 20 mg ml – 1 **Ambion Catalog #**AM2546

100 µL | 10% SDS **Bio-Rad Laboratories Catalog #**161-0146
880 µL | TE Buffer (Tris 10mM EDTA 1mM pH 8)

25 Resuspend the gels in this SDS Lysis solution, incubate at 55 °C for 01:00:00 1h

26 Wash the gels three times in 1000 µL | TE 2% Tween-20
Note: Use **2% Tween 20,** not 0.1% Tween
These gels can be stored at 4 °C in TE 2% Tween-20 for several days without impacting DoTA-seq results.

Note

You can now look at the gels under the microscope using Countess slides **Thermo Fisher Scientific Catalog #**C10228 to determine the encapsulation ratio and lysis efficiency of your cells.

It is advised to restain with SYBR and CF555 to get best signal. Lysed cells should exhibit SYBR signal but no CF555 Signal.

Barcoding the Cells 7m

27 Wash the gels three times in 1000 µL
Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1%

28 Resuspend gels in 100 µL
Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1%

29 Load the gels into a syringe following the protocol described in this excellent visual protocol.

Citation

Demaree B, Weisgerber D, Lan F, Abate AR (2018)
. An Ultrahigh-throughput Microfluidic Platform for Single-cell Genome Sequencing..
Journal of visualized experiments : JoVE.

https://doi.org/10.3791/57598 LINK

Alternatively, for a simpler version, you can also use a P200 pipette to directly pipette the gel into a syringe backfilled with HFE7500.

30 Generate a PCR Master Mix (This mix gives about ~10,000 cells per library - Scale up as required) 20m

25 µL

ddPCR Supermix for probes (no dUTP) **Bio-Rad Laboratories Catalog #**1863024

0.4 µL [M] 50 micromolar (µM) P7 Primer mixed with Barrev primer at [M] 5 micromolar (µM)

0.4 µL [M] 50 micromolar (µM) P5 Primer with appropriate I5 index

0.2 µL Variable [M] 10 micromolar (µM) DoTA-seq multiplex primer mix (**10-20nM final concentration per prime**r)

0.2 µL Variable [M] 10 micromolar (µM) 16S DoTA-seq primers (**10-20nM final concentration**)

0.5 µL Variable [M] 1 picomolar (pM) Freshly diluted from 500pM stock Barcode Oligo

0.25 µL [M] 500 millimolar (mM) Single use aliquots

DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D0632

Note

The ratio of 16S to DoTA-seq target primers mix can be varied depending on the relative amplification efficiencies. The best way to determine is to start from equal concentrations, then adjust based on the sequencing results (do most cells contain more 16S reads than target reads?)

Note

IMPORTANT - BARCODE CONCENTRATIONS MAY NEED TO BE MEASURED
Typically, 0.5uL of 1pM barcode will give approximately 1 barcode for every 10 droplets. However, it is best to measure the barcode encapsulation rate by making PCR droplets containing the barcodes at the expected dilution and P7 and Barrev primers targeting the barcode for amplification. Visualize the resulting PCR emulsion using SYBRgreen staining under the microscope to obtain the real encapsulation ratio. Typically, the real barcode concentration can be ~5 fold off from the expected concentration based on manufacturer's labelling.

Note

Barcode oligos should always be freshly diluted from 500pM to 1pM before use, as we have found gradual loss of barcodes over time in a 1pM solution.

31 Load the PCR mastermix into the syringe following this protocol

Load 500 µL of Biorad Evagreen Droplet Oil **Bio-Rad Laboratories Catalog** ##1864005 into a syringe following this protocol

Protocol

NAME
Loading Syringes to Inject into Microfluidics Device
CREATED BY
freeman.lan
Preview

32 Connect the syringes to the **DoTA-seq Step 2** microfluidics device. 7m

Run the syringe pumps at **200uL/hr** for the gel and PCR mastermix, and **800uL/hr** for the oil syringe.

Collect droplets in an
Axygen® 0.2 mL Maxymum Recovery® Thin Wall PCR Tubes **Corning** **Catalog** #PCR-02-L-C
for 00:07:00 for every 25 µL of PCR mastermix or until the PCR mastermix runs out.

33 Use a pipette to remove the oil in the PCR tube, leaving just the emulsion layer (it's okay to have a little bit of oil remaining).

34 Thermocycle the PCR emulsion as follows: 4h

95 °C 5 min

40 cycles of:
95 °C 30s
72 °C 10s
60 °C 5 min
72 °C 30s

Final incubation of:
72 °C 10min
12 °C Hold
All ramp times are at 1 °C per second

PCR Cleanup 1m

35 Keep the emulsion on ice to prevent polymerase activity

Add 25 µL EDTA (0.5 M), pH 8.0 **Life Technologies** **Catalog** #AM9260G to the emulsion
Vortex the emulsion to mix

36 Add 25 µL HFE 7500 Perfluorinated Oil to the emulsion

Add 25 µL
Perfluorooctanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**370533 to the emulsion
Vortex the emulsion to mix

37 Wait 00:01:00 , then pulse centrifuge to separate the PCR mix from the oil. 1m

Note: If you do not see a clear separation of two clear phases and no emulsion remaining, repeat step 35.

Transfer the top aqueous phase to a new 1mL tube.

38 Add 20 µL [M] 1 Molarity (M)
TCEP-HCl **Gold Biotechnology Catalog #**TCEP to the tube and vortex to completely decrosslink any remaining gels.

> Note
>
> You should be unable to obtain any "jellyish" substance by centrifugation! If there is any jellyish substance left it is not fully de-crosslinked. **ADD MORE TCEP.**

39 Clean up the PCR reaction using the 10m
DNA Clean & Concentrator™-5 **Zymo Research Catalog #**D4003 kit.
Elute in 50 µL Elution Buffer.

40 Remove primer dimers and free barcodes using the 10m
SPRIselect reagent kit **Beckman Coulter Catalog #**B23317 with 0.7X volume of beads.

41 Check the resulting library for primer dimers using 10m

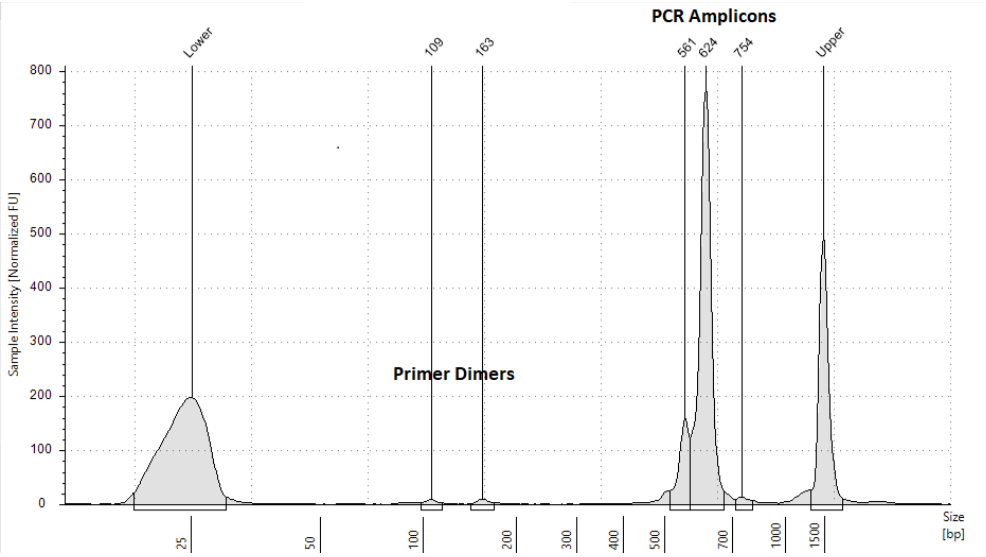
Equipment	
TapeStation	NAME
Agilent	BRAND
G2991AA	SKU
https://www.agilent.com/en/product/tapestation-automated-electrophoresis/tapestation-instruments/4200-tapestation-system-228263	LINK

with a

High Sensitivity D1000 ScreenTape **Agilent Technologies Catalog #**5067-5584

Other high sensitivity capillary electrophoresis methods will also work.

There should be minimal primer dimers on the trace. Below is an example of an acceptable trace.



Example of an acceptable Tapestation trace.

42 Quantify the library using a qPCR library quantification kit such as 1h

NEBNext Library Quant Kit for Illumina - 100 rxns **New England Biolabs Catalog #**E7630S

Note

Note that you must use a PCR based library quantification kit as not all amplicons contain all the adaptors for sequencing and therefore will throw off sequence non-specific forms of quantification!

43 Sequence the library on an Illumina sequencer using Custom Sequencing Primers listed here. DoTA-seq-Oligo-Sequences.xlsx

Citations

Step 29

Demaree B, Weisgerber D, Lan F, Abate AR. An Ultrahigh-throughput Microfluidic Platform for Single-cell Genome Sequencing.
https://doi.org/10.3791/57598