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DoTA-seq V3 V.1

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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.

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

Protocol materials

- HFE 7500 Perfluorinated Oil
- NN′-Bis(acryloyl)cystamine **Santa Cruz Biotechnology** **Catalog #**sc-215506
- Ammonium persulfate **Catalog #**A3678
- ddPCR Supermix for probes (no dUTP) **Bio-Rad Laboratories** **Catalog #**1863024
- SYBR Green **Thermo Fisher Scientific**
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- DNA Clean & Concentrator™-5 **Zymo Research** **Catalog #**D4003
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- Proteinase K solution, 20 mg ml – 1 **Ambion** **Catalog #**AM2546
- Perfluorooctanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**370533
- DNA Clean & Concentrator™-5 **Zymo Research** **Catalog #**D4003
- Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1%
- PBS 0.1% Tween20
- SYBR Green **Thermo Fisher Scientific**
- PBS 0.1% Tween 20
- Cellbrite Fix 555 **Biotium** **Catalog ##**30088
- Ammonium persulfate **Catalog #**A3678
- Acrylamide **P212121**
- NN′-Bis(acryloyl)cystamine **Santa Cruz Biotechnology** **Catalog #**sc-215506
- Biorad Evagreen Droplet Oil **Bio-Rad Laboratories** **Catalog ##**1864005
- Perfluorooctanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**370533
- Acetone
- Isopropanol

PBS EDTA 1mM 0.1% w/v Tween 20

1X PBS (Phosphate-buffered saline)

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

1X PBS (Phosphate-buffered saline)

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

PBS 0.1% Tween20

10% SDS **Bio-Rad Laboratories** **Catalog #**161-0146

1X PBS (Phosphate-buffered saline)

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

PBS 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%

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

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

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

Perfluorooctanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**370533

HFE 7500 Perfluorinated Oil

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

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

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

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**)

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

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Preview

8 Run the syringe pumps at **600uL/hr** for the gel, and **1000uL/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 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 at 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 Acetone to the tube. 5s
Wait 00:00:05 then remove with a pipette.
The gels should begin to flocculate and dehydrate.

17 Add 1000 µL of Isopropanol 5s
Wait 00:00:05 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.

18 Resuspend in 1000 µL PBS EDTA 1mM 0.1% w/v Tween 20

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.

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

Lysing Bacteria

19 Wash gels 3 times in 1000 µL 1X PBS (Phosphate-buffered saline) **(No Tween)** by centrifugation at 500 x g, 00:00:15 each time 15s

20 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)**

21 Resuspend the gels in this lysis solution. Incubate at 37 °C for 01:00:00 1h

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

23 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 1X PBS (Phosphate-buffered saline)

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

25 Wash the gels three times in 1000 µL PBS 2% Tween 20

Note: Use PBS **2% Tween 20,** not 0.1% Tween

These gels can be stored at 4 °C 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

26 Wash the gels three times in 1000 µL

Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1%

27 Resuspend gels in 100 µL

Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1%

28 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

29 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

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 (10uM concentration per primer)

0.2 µL Variable [M] 10 micromolar (µM) 16S DoTA-seq primers

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

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 an estimated 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.

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.

30 Load the PCR mastermix into the syringe following this protocol

Protocol

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Preview

31 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

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Preview

32 Run the syringe pumps at 200uL/hr for the gel and PCR mastermix, and 900uL/hr for the oil syringe. 7m

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

34 Thermocycle the PCR emulsion as follows: 4h

95 °C 5 min

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

20 cycles of:
95 °C 30s
72 °C 10s
60 °C 90s
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 Transfer the top aqueous phase to a new 1mL tube. 1m

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 DNA Clean & Concentrator™-5 **Zymo Research Catalog #**D4003 kit. 10m
Elute in 50 µL Elution Buffer.

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

41 Check the resulting library for primer dimers using 10m

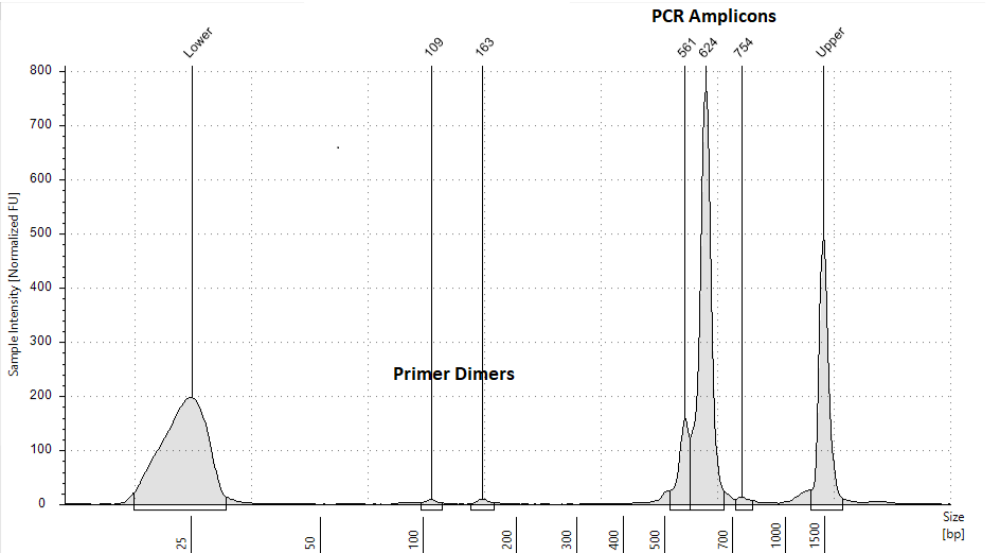
Equipment

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

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

1h

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 28

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