DoTA-seq V3 V.1

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DoTA-seq | Droplet Microfluidics

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VERSION 1

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OPEN ACCESS

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

Created: Aug 22, 2022

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) **BioRad Sciences Catalog #1863024**
- MetaPolyzyme **Sigma Aldrich Catalog #MAC4L-5MG**
- Lysozyme from chicken egg white **Sigma Aldrich Catalog #L6876**
- HFE 7500 Perfluorinated Oil
- Perfluorooctanol **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**

Last Modified: Dec 29, 2023

PROTOCOL integer ID: 69011

TEMED (Tetramethyl-ethulenediamine) **Sigma-aldrich Catalog #T9281**

Biorad Evagreen Droplet Oil **BioRad Sciences Catalog ##1864005**

DNA Clean & Concentrator™-5 **Zymo Research Catalog #D4003**

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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 **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

Axygen® 0.2 mL Maxymum Recovery® Thin Wall PCR Tubes **Corning Catalog #PCR-02-L-C**

Materials, Step 32

EDTA (0.5 M), pH 8.0 **Life Technologies Catalog #AM9260G** Step 35

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

Step 41

PBS 2% Tween 20 Step 25

DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D0632** Step 29

SYBR Green **Thermo Fisher Scientific** Materials, Step 3

TCEP-HCl **Gold Biotechnology Catalog #TCEP** Materials, Step 38

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

In Materials and 3 steps

PBS EDTA 1mM 0.1% w/v Tween 20 Step 18

SPRIselect reagent kit **Beckman Coulter Catalog #B23317** Step 40

Ammonium persulfate **Catalog #A3678** Materials, Step 6

Acrylamide **P212121** Materials, Step 6

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

Materials, Step 10

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

Materials, Step 20

PBS 0.1% Tween20 In 2 steps

PBS 0.1% Tween 20 Step 2

Cellbrite Fix 555 **Biotium Catalog ##30088** Step 5

Acetone Step 16

10% SDS **Bio-Rad Laboratories Catalog #161-0146** Step 23

DNA Clean & Concentrator™-5 **Zymo Research Catalog #D4003**

In Materials, Materials, Step 39

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

Materials, Step 23

HFE 7500 Perfluorinated Oil Materials, Step 36

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

Materials, Step 42

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

In Materials and 2 steps

Isopropanol Step 17

NN'-Bis(acryloyl)cystamine **Santa Cruz Biotechnology Catalog #sc-215506**

Materials, Step 6

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

Materials, Step 29

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

In Materials, Materials, Step 20

Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1% In Materials and 2 steps

Preparing Cells

10m

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

2 Resuspend cells in 100 µL PBS 0.1% Tween 20 **Sigma Aldrich**

3 Add 1 µL SYBR Green **Sigma Aldrich** 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 **Sigma Aldrich 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 **Sigma Aldrich** monomer in water [M] 25 Mass / % volume

15 µL NN'-Bis(acryloyl)cystamine **Sigma Aldrich Catalog #sc-215506** in Methanol

[M] 5 Mass / % volume

10 µL Ammonium persulfate **Sigma Aldrich 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 **Sigma Aldrich 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 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 **Sigma Aldrich Catalog ##1864005**

5 µL TEMED (Tetramethyl-ethulenediamine) **Sigma Aldrich Catalog #T9281**

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

> **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 **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 **Sigma Aldrich** 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 **Sigma Aldrich** 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 **Sigma Aldrich**

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) **Sigma Aldrich** **(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 **Sigma Aldrich Catalog #L6876**

100 µL [M] 1 mg/mL MetaPolyzyme **Sigma Aldrich Catalog #MAC4L-5MG**

900 µL 1X PBS (Phosphate-buffered saline) **Sigma Aldrich** **(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 **Sigma Aldrich**

23 Make a SDS Lysis solution by adding:

20 µL [M] 20 mg/mL Proteinase K solution, 20 mg ml − 1 **Sigma Aldrich Catalog #AM2546**

100 µL 10% SDS **Sigma Aldrich Catalog #161-0146**

880 µL 1X PBS (Phosphate-buffered saline) **Sigma Aldrich**

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 **Sigma Aldrich**

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% **Sigma Aldrich**

27 Resuspend gels in 100 µL Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1% **Sigma Aldrich**

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

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) **Sigma Aldrich 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 **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

NAME

Loading Syringes to Inject into Microfluidics Device

CREATED BY

freeman.lan

PREVIEW

31 Load 500 µL of Biorad Evagreen Droplet Oil **Sigma Aldrich Catalog ##1864005** into a syringe following this protocol

Protocol

NAME

Loading Syringes to Inject into Microfluidics Device

CREATED BY

freeman.lan

PREVIEW

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

Axygen® 0.2 mL Maxymum Recovery® Thin Wall PCR Tubes **Sigma Aldrich 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 **Sigma Aldrich Catalog #AM9260G** to the emulsion
Vortex the emulsion to mix

36 Add 25 µL HFE 7500 Perfluorinated Oil **Sigma Aldrich** to the emulsion
Add 25 µL Perfluorooctanol **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
Transfer the top aqueous phase to a new 1mL tube.

38 Add 20 µL [M] 1 Molarity (M) TCEP-HCl **Sigma Aldrich 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 **Sigma Aldrich Catalog #D4003** 10m

kit.
Elute in 50 µL Elution Buffer.

40 Remove primer dimers and free barcodes using the 10m

SPRIselect reagent kit **Sigma Aldrich Catalog #B23317** with 0.7X volume of beads.

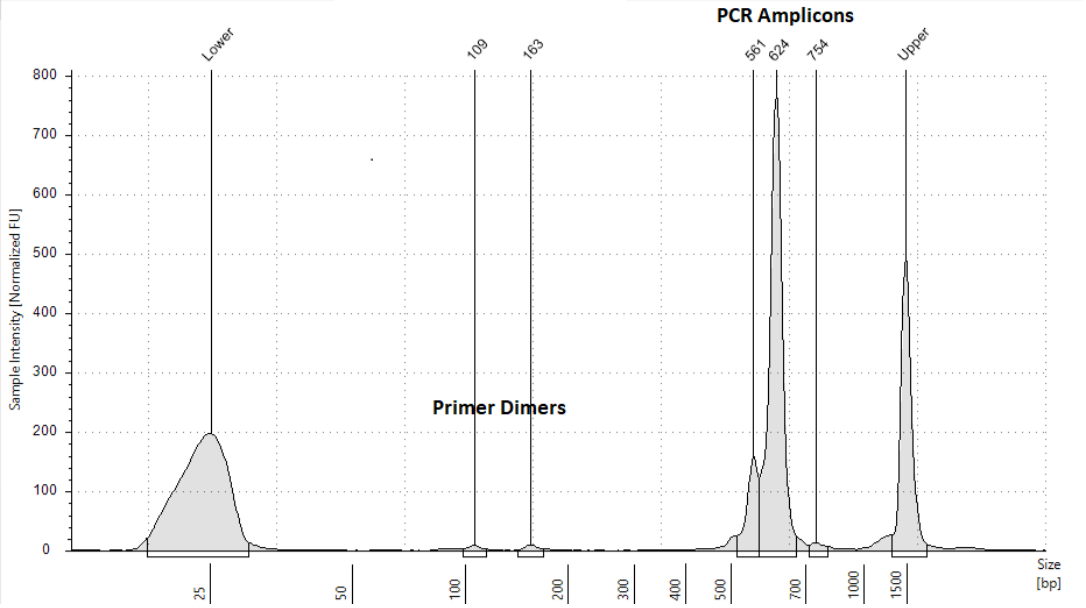
41 Check the resulting library for primer dimers using 10m

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 **Sigma Aldrich 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 **Sigma Aldrich 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