DEC 29, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.81wgbxx3ylpk/v1

Protocol Citation: freeman.lan 2023. DoTA-seq V3.1. **protocols.io** https://dx.doi.org/10.17504/protocols.io.81wgbxx3ylpk/v1

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

Created: Aug 24, 2023

Last Modified: Dec 29, 2023

DoTA-seq V3.1

Forked from DoTA-seq V3.1

freeman.lan[1]

[1]UW Madison

DoTA-seq

freeman.lan

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) **BioRad Sciences Catalog #1863024**

MetaPolyzyme **Sigma Aldrich Catalog #MAC4L-5MG**

Lysozyme from chicken egg white **Sigma Aldrich Catalog #L6876**

PROTOCOL integer ID:
86909

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**

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

Biorad Evagreen Droplet Oil **BioRad Sciences 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 **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

DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D0632** Step 30

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

Materials, Step 10

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

Materials, Step 24

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

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

HFE 7500 Perfluorinated Oil Materials, Step 16

Acetone Step 17

TE Buffer (Tris 10mM EDTA 1mM pH 8) Step 24

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

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

Materials, Step 21

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

Step 41

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

In Materials, Materials, Step 39

PBS EDTA 1mM Tween20 2% w/v Step 19

TE 2% Tween-20 In 2 steps

Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1% Step 28

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

Materials

PBS 0.1% Tween 20 Step 2

Acrylamide **P212121** Materials, Step 6

PBS 0.1% Tween20 Step 1

Biorad Evagreen Droplet Oil **Bio-Rad Laboratories Catalog ##1864005** Step 10

PBS 0.1% Tween20 Step 23

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

In Materials and 2 steps

10% SDS **Bio-Rad Laboratories Catalog #161-0146** Step 24

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

Step 32

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

Materials

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

Materials, Step 42

Biorad Evagreen Droplet Oil **Bio-Rad Laboratories Catalog ##1864005** Step 7

Biorad Evagreen Droplet Oil **Bio-Rad Laboratories Catalog ##1864005** Step 31

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

Materials, Step 30

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

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

Materials

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

In Materials, Materials, Step 21

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

NN'-Bis(acryloyl)cystamine **Santa Cruz Biotechnology Catalog #sc-215506**
Step 6

Isopropanol Step 18

Pre-injection buffer 10mM HEPES pH 7.5 Pluronic 0.1% Step 27

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

HFE 7500 Perfluorinated Oil Step 36

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

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** 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 **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 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 **Sigma Aldrich Catalog ##1864005**

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

11 Add the Gel polymerization oil to the collected droplets, invert slowly 3 times to mix, and Incubate the 10m tube containing droplets at 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 from 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.

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

15

16 10s

Add 1000 µL of HFE 7500 Perfluorinated Oil **Contributed by users** to the tube.

Mix by inverting 5 times, Wait 00:00:10 then remove with a pipette.

17 10s

Add 1000 µL of Acetone **Sigma Aldrich** to the tube.

Mix by inverting 5 times, Wait 00:00:10 then remove with a pipette.

The gels should begin to flocculate and dehydrate.

18 10s

Add 1000 µL of Isopropanol **Sigma Aldrich**

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 **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.

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) **Sigma Aldrich** **(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 **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)**

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 **Sigma Aldrich**

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 **Contributed by users** 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% **Sigma Aldrich**

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

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

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 primer**)

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 **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 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 **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 (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 **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

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 **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.

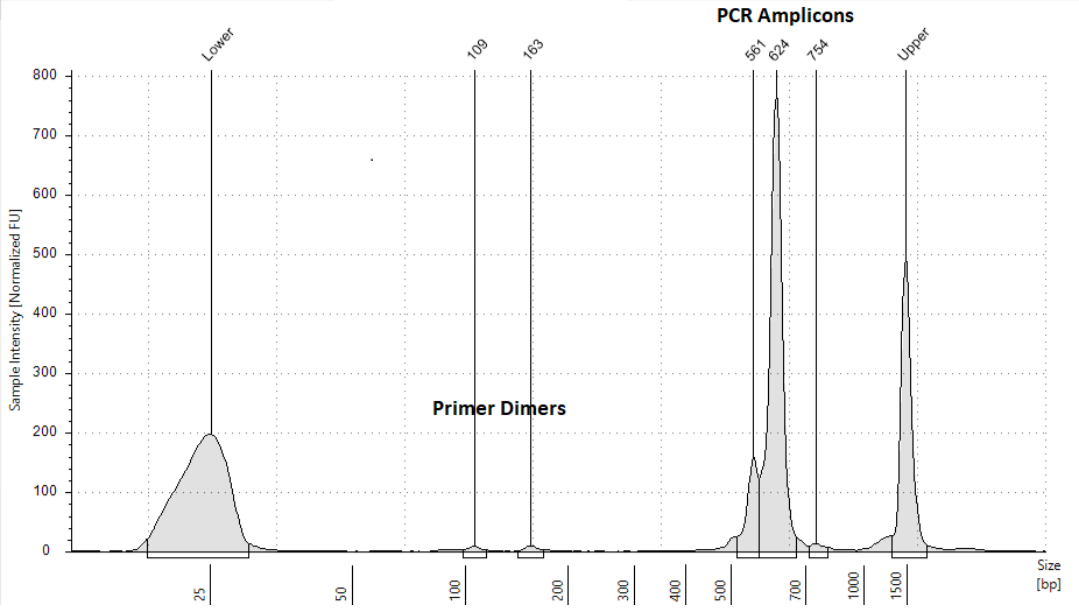
40 Remove primer dimers and free barcodes using the SPRIselect reagent kit **Sigma Aldrich Catalog #B23317** with 0.7X volume of beads. 10m

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 **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