Sperm-seq wet lab protocol: sperm preparation and droplet-based sequencing library generation

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Method Article

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Abstract

Sperm-seq is a high-throughput, droplet-based single-sperm sequencing technology capable of generating thousands of cell-barcoded single-sperm sequencing libraries at one time. This protocol describes Sperm-seq library generation, featuring a full protocol for sperm preparation and suggestions for droplet-based sequencing methods from 10X Genomics to employ. Sperm preparation can be completed in half a day and the full protocol can be completed in 2-3 days, with several wait times and break points.

Introduction

Reagents

Buffer SMT *(freeze-thaw buffer adapted from Montag et al. 1992; Samocha-Bone et al. 1998):*

250 mM sucrose (from 1M sucrose, Sigma S7903)

5 mM $MgCl_2$ (from 1M $MgCl_2$, Sigma M8266)

10 mM Tris pH 7.5 (from 1 M Tris ph 7.5, ThermoFisher Scientific 15567027)

Example recipe using stocks above (10 mL): 2500 uL sucrose, 50 uL $MgCl_2$, 100 uL Tris pH 7.5, 7350 uL ultrapure water

Buffer DB *(salt decondensation buffer adapted from Montag et al. 1992; Samocha-Bone et al. 1998):*

113 mM KCl (potassium chloride) (from 1 M KCl Sigma P5405)

12.5 mM KH_2PO_4 (monobasic potassium phosphate) (from 1 M KH_2PO_4, Sigma P9791)

2.5 mM Na_2HPO_4 (disodium phosphate) (from 1 M Na_2HPO_4, Sigma S3264)

2.5 mM $MgCl_2$ (magnesium chloride) (from 25 uL 1 M $MgCl_2$, Sigma M8266)

20 mM Tris pH 7.5 (from 1 M Tris ph 7.5, ThermoFisher Scientific 15567027)

Example recipe using stocks above (will make 10 mL total when heparin and beta-mercaptoethanol that are added during experiments are taken into account): 1130 uL KCl, 125 uL KH_2PO_4, 25 uL Na_2HPO_4, 25 uL $MgCl_2$, 200 mM Tris pH 7.5, 8145 uL ultrapure water

10X Genomics reagents compatible with single-cell DNA sequencing:

Option 1 (early version of reagents): 10X version 1 GemCode reagents, as described in 10X GemCode User Guide Rev C “Required Materials and Equipment” section: GemCode Gel-Bead Kit; GemCode Chip Kit;

GemCode Library Kit

Option 2 (currently sold reagents): 10X Chromium Single Cell DNA Reagent kits, as described in 10X Chromium Single Cell DNA User Guide Rev B “Introduction” section: Chromium Single Cell DNA Library Kit, 16 rxns PN-1000025; Chromium Single Cell DNA Gel Bead Kit, 16 rxns PN-1000026; DynabeadsTM MyOneTM SILANE, 16 rxns PN-2000048; Chromium Single Cell DNA Cell Bead Reagent Kit, 16 rxns PN-1000023; Chromium Single Cell DNA Cell Bead Polymer Kit, 16 rxns PN-1000031; Chromium i7 Multiplex Kit, 96 rxns PN-120262; Chromium Chip C Single Cell DNA Kit, 48 rxns PN-1000022; Chromium Chip D Single Cell DNA Kit, 48 rxns PN-1000042

Other required reagents:

1X PBS pH 7.4 (Life Technologies 10010023)

10 mM (180 mg/mL) heparin, from Heparin Sodium Salt from Porcine (Sigma H3393), diluted in ultrapure water

100 mM beta-mercaptoethanol (BME) diluted from 14.3 M BME (Sigma M6250) (**use in fume hood**)

0.1 units/uL Heparinase 1 (Sigma H2519) diluted in ultrapure water (following Taylor 1997; store at -20 degrees Celcius)

10X SYBR Green I (diluted from 10,000X, ThermoFisher Scientific S7563)

Equipment

Nonstick 1.5 mL microfuge tubes (ThermoFisher Scientific AM12450)

Fuchs-Rosenthal C-chip hemocytometer (VitaScientific NANC07005) (or other hemocytometer)

10X Genomics equipment compatible with single-cell DNA sequencing:

Option 1 (early version of reagents): 10X GemCode controller and other equipment described in 10X GemCode User Guide Rev C “Required Materials and Equipment” section and other equipment described therein

Option 2 (currently sold reagents): 10X Chromium controller; FlowmiTM Filters, 50 rxns, PN-1000055; and other Chromium accessories and equipment described in 10X Chromium Single Cell DNA User Guide Rev B “Introduction” section: as described therein

Procedure

1. Thaw sperm aliquot(s) containing at least 250,000 sperm cells (typically 10 uL) at room temperature.

2. Quick spin sperm aliquot and transfer to nonstick 1.5 mL tube.

3. Spin for 10 minutes at 400g at 4 degrees Celsius. Remove supernatant.

4. Resuspend sperm pellet in 10 uL PBS.

5. Repeat spin from (3). Resuspend sperm pellet in 2.5 uL Buffer SMT.

6. Submerge tube for 5-10 seconds in liquid nitrogen. Uncap tube to de-pressurize. Hold tube in fist until thawed.

7. Repeat (6) for a total of 3 freeze-thaw cycles.

8. Make 100 uL Buffer DB with freshly added heparin and BME (enough for 4 samples): combine 1.5 uL 10 mM heparin, 2 uL 100 mM BME, and 96.5 uL Buffer DB.

9. Add 22.5 uL of Buffer DB with heparin and BME from (8) to each sperm sample.

10. Incubate at 37 degrees Celsius for 45 minutes. Thaw heparinase on ice during the latter half of this incubation.

11. Remove sample(s) to room temperature. Add 5 uL freshly-thawed heparinase and pipette gently to mix.

12. Incubate sample with added heparinase at room temperature for 2 hours before preceding immediately to next steps.

13. Generate a DNA-stained 1:100 dilution of sperm (for counting under fluorescence microscope) by combining 1 uL sperm mixture, 10 uL 10X SYBR I, and 89 uL PBS. Allow to incubate in the dark at room temperature, with or without gentle rotation, for 5-10 minutes.

14. Add 20 uL diluted, stained sperm to Fuchs-Rosenthal C-chip hemocytometer and count the number of sperm present in 5 big squares under green fluorescent light. (Sperm appear as bright fluorescent dots; un-decondensed sperm are compact dots while sperm at this state, after decondensation, are bigger circles.) Sperm concentration per microliter is the number of sperm in 5 big squares multiplied by 100. (*Or, use fluorescence to count sperm via a known and trusted method.)*

15. **If using 10X Genomics GemCode (old) reagents *(for other reagents continue to step 16)*:**

a. Dilute sperm to a final number of 10,833 in 5 uL ultrapure water.

b. Make master mix: per sample, combine 32.5 uL GemCode reagent mix, 1.5 uL primer release agent, 9.2 uL GemCode polymerase, and 16.8 uL ultrapure water.

c. Add 60 uL of master mix to each sperm sample, pipetting gently with wide bore pipettes to mix.

d. Vortex GemCode beads at full spead for 25 seconds.

e. Dilute vortexed GemCode beads 1:11 ultrapure water (to a total volume of at least 90 uL per sample).

f. Prepare chip for droplet generation (per 10X Genomics' protocol): add 60 uL of sample-master mix mixture in the sample wells, then 85 uL of freshly-pipette-mixed 1:11 diluted-bead mixture to the bead wells, then 150 uL droplet generation oil to the oil wells of the chip.

g. Generate and process droplets following 10X GemCode User Guide Rev C Steps 5.36-5.5.2.

h. Complete library generation following 10X GemCode User Guide Rev C Steps 5.6-6.2.6.

16. **If using 10X Genomics Chromium Single-cell DNA (new) reagents *(if using previous reagents follow step 15 instead)*:** Follow 10X Chromium Single Cell DNA User Guide Rev B "Chromium Single Cell DNA Reagents kits" protocol for generating single-cell sequencing libraries (Step 1- "Cell Bead Generation," Step 2 "Cell Bead Processing," Step 3 "GEM Generation & Barcoding," Step 4 "Post GEM Incubation Cleanup & QC", and Step 5 "Library Construction"), using the prepared sperm as input. Target 2000 cells for recovery. This process can be paused at any point recommended by 10X.

17. Sequence according to 10X's and Illumina sequencer instruction. (Generally, sequence yield is improved by increasing the input library concentration slightly from that recommended by Illumina.)

18. Process sequence data as described in Bell *et al* "Insights about variation in meiosis from 31,228 human sperm genomes" (*Nature* 2020).

Troubleshooting

If sequence data is not of sufficient quality (uneven coverage across the genome; unclear boundary between beads associated with whole sperm cells and beads associated with ambient DNA), the following modifications may be helpful to troubleshoot sperm decondensation:

Make fresh buffers (prior to step 1)

Vary BME amount – try 1mM in final sperm treatment buffer rather than 2mM (step 8)

Decrease the number of freeze-thaw cycles (steps 6-7)

Pipette gently using wide-bore pipette tips (all steps where sperm are mixed)

Time Taken

Sperm preparation: ~3.5 hours

Sperm quantification: ~15 minutes (more for more samples)

Library preparation: 1-3 days, depending on kit/approach used and when breaks are taken

Anticipated Results

References

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