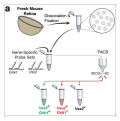

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Probe-Seq V.3

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1 Works for me dx.doi.org/10.17504/protocols.io.baeiibce

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ABSTRACT

Recent transcriptional profiling technologies are uncovering previously-undefined cell populations and molecular markers at an unprecedented pace. While single cell RNA (scRNA) sequencing is an attractive approach for unbiased transcriptional profiling of all cell types, a complementary method to isolate and sequence specific cell populations from heterogeneous tissue remains challenging. Here, we developed Probe-Seq, which allows deep transcriptional profiling of specific cell types isolated using RNA as the defining feature. Dissociated cells are labelled using fluorescent *in situ* hybridization (FISH) for RNA, and then isolated by fluorescent activated cell sorting (FACS). We used Probe-Seq to purify and profile specific cell types from mouse, human, and chick retinas, as well as the *Drosophila* midgut. Probe-Seq is compatible with frozen nuclei, making cell types within archival tissue immediately accessible. As it can be multiplexed, combinations of markers can be used to create specificity. Multiplexing also allows for the isolation of multiple cell types from one cell preparation. Probe-Seq should enable RNA profiling of specific cell types from any organism.

EXTERNAL LINK

https://elifesciences.org/articles/51452

Probe-Seq v1.3.pdf

MATERIALS TEXT

HBSS/FBS:
HBSS 900 µL (Thermo Fisher Scientific, cat. #14025092)
FBS 100 µL (Thermo Fisher Scientific, cat. #10437028)
Total Volume 1000 µL

DMEM/BSA:
DMEM 3000 µL (Thermo Fisher Scientific, cat. #11995065)
BSA 12 mg (MilliporeSigma cat. #A9418)
Total Volume 3000 µL

Papain Mix:
HBSS 315 µL
1M HEPES pH 7 35 µL (Thermo Fisher Scientific, cat. #15630080)
50mM Cysteine 20 µL (MilliporeSigma, cat. # 168149)
0.5M EDTA pH 8 0.4 µL (Thermo Fisher Scientific, cat. #AM9260G)
Nuclease Free Water 19.6 µL
Papain 10 µL (Worthington, cat. #LS0003126)
Total Volume 400 µL

4% PFA

*4% PFA can be stored at 4^{o}C for one month

Permeabilization Buffer:
PBS 989 µL
Hoechst 33342 1 µL (Thermo Fisher Scientific, cat. #H3570)
10% Triton X-100 10 µL
Total Volume 1000 µL

* Make the above solutions fresh (except 4% PFA) before the experiment.

* Keep HBSS/FBS and DMEM/BSA at room temp. Put 4% PFA and Permeabilization Buffer on ice.

* The papain mix should be incubated at 37^{O}C for 10 minutes before use.

* Add 1 µL of RNasin Plus (Promega, cat. #N2615) for every 1 mL of every solution used. Incubate for at least 10 minutes with the RNasin before use.

FISH Reagents

The details of the SABER reagents can be found in Kishi, Lapan, Beliveau, West et al., (2019) *Nature Methods* paper. For detailed protocol for gene-specific probe set design, see the saber.fish website. Here are the reagents that you will need for Probe-Seq:

40% wHyb:
20x SSC 5 mL (Thermo Fisher Scientific, cat. #15557044)
UltraPure Water 25 mL
Deionized formamide 20 mL (MilliporeSigma, cat. #S4117) *Aliquot and store at -20^{O}C
Total Volume 50 mL

Store at -20.
Pre-warm to 43^{O}C before use.

Hyb1:
20x SSC 1 mL
UltraPure Water 1 mL
Deionized formamide 4 mL
50% Dextran sulfate 2 mL (MilliporeSigma cat. #D8906) *Dissolve powdered dextran sulfate in water overnight to make 50%. Stored at -20^{O}C
Total Volume 8 mL

Store aliquots of 120 µL at -20^{O}C.

Probe Mix:
Hyb1 96 µL
Probes 1 µg
UltraPure Water up to 120 µL

Pre-warm to 43^{O}C before use.

Fluorescent Oligo Mix:
PBS 100 µL
10 µM Fluorescent oligo 2 µL (each)

Pre-warm to 37^{O}C before use.

* Add 1 µL of RNasin Plus (Promega, cat. #N2615) for every 1 mL of every solution used. Incubate for at least 10 minutes with the RNasin before use.

1 For a more detailed step-by-step guide for Probe-Seq, please download the PDF version of the protocol. It can be found under the Abstract tab.

2 Prepare all solutions.

3 Dissect retinas in HBSS at RT.

4 Place retina in microcentrifuge tube and remove most of the HBSS.

5 Add 400 µL of the pre-warmed Papain Mix.

6 Incubate for 7 minutes at 37^{O}C with no agitation.

7 Spin 600xg for 2.5 minutes at RT.

8 Remove supernatant and add 1 mL of HBSS/FBS without agitation.

9 Spin 600xg for 2.5 minutes at RT.

10 Remove supernatant and add 600 µL of DMEM/BSA.

11 Dissociate by triturating the tissue with a P1000 pipette. ~15 times in/out pipetting should be sufficient to dissociate into single cells.

12 Transfer homogenate to a 5 mL polypropylene tube (Thermo Fisher Scientific cat. #14-959-11A). Make sure to seal the cap by pushing in.

13 Spin 600xg for 5 minutes at 4^{O}C.

14 Remove supernatant and add 1 mL of 4% PFA. Resuspend. Incubate for 15 minutes at 4^{O}C with rocking.

15 Spin 2000xg for 5 minutes at 4^{O}C.

16 Remove supernatant and add 1 mL of Permeabilization Buffer. Resuspend. Incubate for 10 minutes at 4^{O}C with rocking.

17 Spin 2000xg for 5 minutes at 4^{O}C.

18 Remove supernatant and add 500 µL of 40% wHyb. Resuspend. Incubate for at least 30 minutes at 43^{O}C in the oven.

19 Spin 2000xg for 5 minutes at RT.

20 Remove supernatant and add 100 µL of pre-warmed (43^{O}C) Probe Mix. Resuspend. Incubate for at least 16 hours at 43^{O}C in the oven.

21 Add 500 µL of pre-warmed (43^{O}C) 40% wHyb, resuspend, and spin 2000xg for 5 minutes at RT.

22 Remove supernatant and add 500 µL of pre-warmed (43^{O}C) 40% wHyb. Resuspend. Incubate for 15 minutes at 43^{O}C.

23 Spin 2000xg for 5 minutes at RT.

24 Remove supernatant and add 1 mL of pre-warmed (43^{O}C) 2x SSC. Resuspend. Incubate for 5 minutes at 43^{O}C.

25 Spin 2000xg for 5 minutes at RT.

26 Remove supernatant, Resuspend, and add 500 µL of pre-warmed (37^{O}C) PBS.

27 Spin 2000xg for 5 minutes at RT.

28 Remove supernatant and add 100 µL of pre-warmed (37^{O}C) Fluorescent Oligo Mix. Resuspend. Incubate for 10 minutes at 37^{O}C.

29 Add 500 µL of pre-warmed (37^{O}C) PBS, resuspend, and spin 2000xg for 5 minutes at RT.

30 Remove supernatant and add 500 µL of pre-warmed (37^{O}C) PBS. Resuspend. Incubate for 5 minutes at 37^{O}C.

31 Spin 2000xg for 5 minutes at RT.

32 Resuspend in 500 – 1000 µL of PBS. Keep cells on ice.

33 Filter and proceed to FACS.

34 Gate based on Hoechst histogram, as shown below. This step will ensure that you get 2N nuclei. Make sure not to include 4N nuclei. You can also gate based on FSC/SSC for cells vs. debris, but this step is likely unnecessary.

35 Gate using a 2D plot with appropriate wavelengths. The negative population will likely run diagonal. The positive population will be left- or right-shifted compared to the diagonal events (i.e. the $Vsx2^{+}$ cells below). Often, the separation will not be as obvious as GFP or well characterized cell surface markers. Thus, it's a good idea to run a negative control (fluorescent oligo only control).

36 FACS isolated cells are sorted into 1% BSA/PBS and kept at 4^{O}C.

37 Spin at 3000xg for 7 minutes at 4^{O}C.

38 Remove as much supernatant as possible. Usually, there is ~40-50 µL left.

39 From the Recoverall RNA/DNA Isolation Kit (Thermo Fisher Scientific AM1975), mix 100 µL of Digestion Buffer and 4 µL of protease for each sample.

40 Incubate at 50^{O}C for 3 hours (wrap the lid with parafilm). Note that this step differs from the manufacturer's protocol.

41 The samples can be stored at -80^{O}C indefinitely after incubation or proceed to next steps according to the kit protocol.

42 Prep Isolation Additive/Ethanol mix (for 100 µL digest, 120 µL isolation + 275 µL 100% Ethanol. Usually, there is ~140 µL so adjust accordingly).

43 Add Isolation/Ethanol Mix and Pipet to mix.

44 Add onto filter and spin 10000xg for 30 seconds at RT.

45 Discard flow-through. Wash with 700 µL of Wash 1. Spin 10000xg for 30 seconds at RT.

46 Discard flow-through. Wash with 500 µL of Wash 2. Spin 10000xg for 30 seconds at RT.

47 Discard flow-through. Spin 10000xg for 30 seconds at RT.

48 Prep DNase Mix (6 µL 10x DNase Buffer, 4 µL DNase, 50 µL UltraPure Water).

49 Add 60 µL of DNase Mix to center of cartridge. Close cap and incubate for 30 minutes at RT.

50 Add 700 µL of Wash 1, incubate 1 minute at RT. Spin 10000xg for 30 seconds at RT.

51 Discard flow-through. Wash twice with 500 µL of Wash 2.

52 Spin 10000xg for 1 minute to remove residual fluid.

53 Elute in ~17 µL of UltraPure water (Use more water if lower concentration desired. Eluting with 17 µL will leave you with ~12 µL of RNA, which is enough for SMART-Seq v.4, Qubit, and/or BioAnalyzer).

54 Store RNA at -80^{O}C. ** Qubit or BioAnalyzer can be used to estimate RNA concentration. If less than 10,000 cells, concentration may not be available by these methods.

55 Proceed to the SMART-Seq v.4 protocol for cDNA synthesis and amplification. Depending on the number of cells, you will use 9.5 µL of RNA for cDNA synthesis. Use 150 pg of cDNA for Nextera XT library kit.