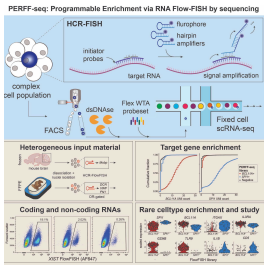
Mar 25, 2024

2. PERFF-seq: HCR Flow-FISH and Polymer Disassembly Protocol



DOI
dx.doi.org/10.17504/protocols.io.8epv5x8r4g1b/v1

Tsion Abay[1,2], Robert Stickels[1,2], Meril Takizawa[3], Ronan Chaligne[3], Caleb Lareau[4]

[1]Gladstone-UCSF Institute of Genomic Immunology, San Francisco, CA, USA;
[2]Department of Pathology, Stanford University, Stanford CA, USA;
[3]Single-cell Analytics Innovation Lab, Memorial Sloan Kettering Cancer Center, New York, NY, USA;
[4]Computational and Systems Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA

T Tsion Abay
Gladstone Institute

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.8epv5x8r4g1b/v1

Protocol Citation: Tsion Abay, Robert Stickels, Meril Takizawa, Ronan Chaligne, Caleb Lareau 2024. 2. PERFF-seq: HCR Flow-FISH and Polymer Disassembly Protocol . **protocols.io https://dx.doi.org/10.17504/protocols.io.8epv5x8r4g1b/v1**

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

Created: February 03, 2024

Last Modified: March 25, 2024

Protocol Integer ID: 94653

Keywords: PERFF-seq, enrichment, HCR Flow-FISH

Abstract

This protocol can be used for performing HCR Flow-FISH followed by cell/nuclei enrichment via FACS and polymer disassembly on fixed and permeabilized cells and tissue derived nuclei. This protocol should be performed after finalizing "PERFF-seq: Cell and Nuclei Preparation" for the desired sample type.

Guidelines

- Maintain RNAse free environment when preparing buffers and throughout the protocol by spraying bench with RNase*Zap* and using molecular grade reagents when possible.
- To increase cell recovery, opt for a swinging bucket rotor when pelleting and leave a few uL of liquid behind when aspirating buffers.
- When possible, use low-binding plasticware.

Materials

1. Hybridization Buffer (Molecular Instruments)
2. Wash Buffer (Molecular Instruments)
3. Amplification Buffer (Molecular Instruments)
4. Probes and Complementary Hairpins (Molecular Instruments)
5. SSC-T: 5X SSC, 0.1% Tween-20
6. Sorting & Collection Buffer: 1x PBS + 5% BSA(Gibco Catalog No: 15260037) + 0.133 U/uL RNase Inhibitor (Sigma Aldrich Catalog No: 15260037)
7. dsDNAse Thermofisher Lot No: EN0771
8. Storage Buffer: Water + 1x Quenching Buffer + 0.1 Enhancer (warmed for 10 minutes at 65C)
9. Long Term Storage Buffer: Water + 1x Quenching Buffer + 10% Glycerol (Millipore Sigma Catalog No: G5516-100ML) + 0.1 Enhancer (warmed for 10 minutes at 65C)
10. Post Storage Processing buffer: 0.5x PBS + 0.02% BSA and 0.2U/uL RNase Inhibitor
11. Thermomixer with Heated Lid or a Rotating Hybridization Oven
12. Swinging Bucket Rotor

Safety warnings

Hybridization and Wash buffers contain formamide which is a hazardous material.

Before start

Calculate the starting cell count and volume of probes and hairpins needed for your experiment by putting these factors into account:

- Proportion of the specific population of interest within the total cell/nuclei population.
- Final cell count should be >50,000
  i.e specific population of interest to sort
- Approximately 50% of starting cells/nuclei are lost during the wash steps of this protocol.
- Unstained controls and single color controls (if multiplexing)

$$Starting\ Cell\ Count = \frac{Final\ Cell\ Count}{Percentage\ of\ subpopulation\ of\ interest} * 200$$

Detection Stage

1 After performing cell/nuclei preparation, pre-warm hybridization buffer and thermomixer with a heated lid to 37 °C .

2 Add 400uL of pre-warmed hybridization buffer per 1 million (1M) cells/nuclei.
For cells, incubate in a thermomixer at 37 °C for 30 minutes (300rpm shaking) or a rotating hybridization oven.
For nuclei, incubate samples at 37 °C **with no shaking.**

3 In the meantime, prepare probe solution for 1M cells/nuclei by adding 8uL of each probe set (8uL from the 1uM probe stock) and top off pre-warmed hybridization buffer to a final volume of 100uL. Increase per cell count and per probe set used.

- *Example: For 5 million cells/nuclei and 2 probe sets:*

*Probe solution = 80uL probes (2 probe sets * 8uL * 5 million cells) + 420uL of hybridization buffer*

4 After incubating, add 100uL of the prepared probe mix into each sample for a final probe concentration of 32nM.

5 Incubate for 16-24 hours.
For cells, incubate in a thermomixer at 37 °C (300rpm).
For nuclei, incubate samples at 37 °C **with no shaking**.

6 After overnight incubation, add 500uL of SSC-T into samples and centrifuge at 850xg for 10 minutes.

7 Gently remove supernatant (Leaving few uLs behind to make sure not to perturb fragile pellet).

8 Add 500uL of wash buffer per 1M cells/nuclei and incubate at 37 °C for 10 minutes.

9 Centrifuge at 850xg for 5 minutes and remove supernatant.

10 Repeat steps 8 and 9, 3 more times for a total of 4 washes.

11 Add 500uL of SSC-T per 1M cells/nuclei and incubate for 5 minutes at room temperature to remove formamide from sample.

12 Centrifuge at 850xg for 5 minutes and remove supernatant.

13 **Stopping point:**
Samples can be resuspended in long term storage buffer (1mL per 5 million, see Materials section or 10XG protocol for more details).
Cells or nuclei can be stored for up to 3 weeks at 4 °C .

Amplification Stage

14 Add 150uL of amplification buffer per 1M cells/nuclei and incubate for 30 minutes at room temperature.

15 In the meantime, transfer 5uL of H1 and H2 hairpins per 1M cells/nuclei for each probe set (i.e. 5uL from the 3uM hairpin stock).

- *Example: for 5M cells/nuclei and 2 probe sets:*

*Transfer 25uL(5M * 5uL) of h1 and h2 hairpin for each probe set (total of 4 hairpin stocks)*

- *H1 and H2 hairpins are kept in separate tubes at this step.*

16 Heat shock at 95 °C for 90 seconds using a thermocycler.

17 Remove strip-tubes from thermocycler and incubate at room temperature in the dark for 30 minutes.

18 After incubation, prepare hairpin solution by combining h1 and h2 and adding SSC-T for final hairpin solution of 100uL per 1M cells/nuclei.

- *Example: For 5M cells/nuclei and 2 probe sets:*

*Hairpin solution = 100uL of hairpins (25uL*4 hairpin stocks) + 400uL of amplification buffer*

19 Add 100uL of hairpin solution to each sample for a final hairpin concentration of 60nM and incubate at least 4 hours or overnight at 25 °C or Room temperature .

For cells, 300rpm shaking
For nuclei, **no shaking**

20 To wash unbound hairpins, add 500uL of SSC-T and centrifuge at 850xg for 5 minutes.

21 Repeat step 20, 4 more times for a total of 5 washes.

Sample Enrichment Using FACS

22 If fluorophore multiplexing is performed, compensation should be done using single color control samples and unstained cells/nuclei that went through the PERFF-seq protocol.

23 Re-suspend cells/nuclei in collection buffer.
- Ideally, cells/nuclei should be filtered through a 35um filter before sorting.

24 **Stopping point:**
Alternatively, samples can be resuspended in storage buffer (1mL per 5 million, see Materials section or 10XG protocol for more details).
Cells or nuclei can be stored for up to 1 week at 4 °C .

HCR Polymer Disassembly

25 After sorting, centrifuge cells/nuclei at 850xg for 5 minutes and gently remove supernatant. Resuspend in 275uL of 1x DNase I buffer at Room temperature for 15 minutes.

- Record the amount of cells/nuclei recovered from sorting.
- 30uL 10x DNase I buffer + 270uL of RNase free water --> Add 275uL to sample

26 Add 25uL of DNase I enzyme (0.5U/ul enzyme in 1x buffer) to solution and incubate at 37 °C for 2 hours.

27 Inactivate DNase I by incubating the sample at 55 °C for 5 minutes in the presence of 10 mM DTT (final).

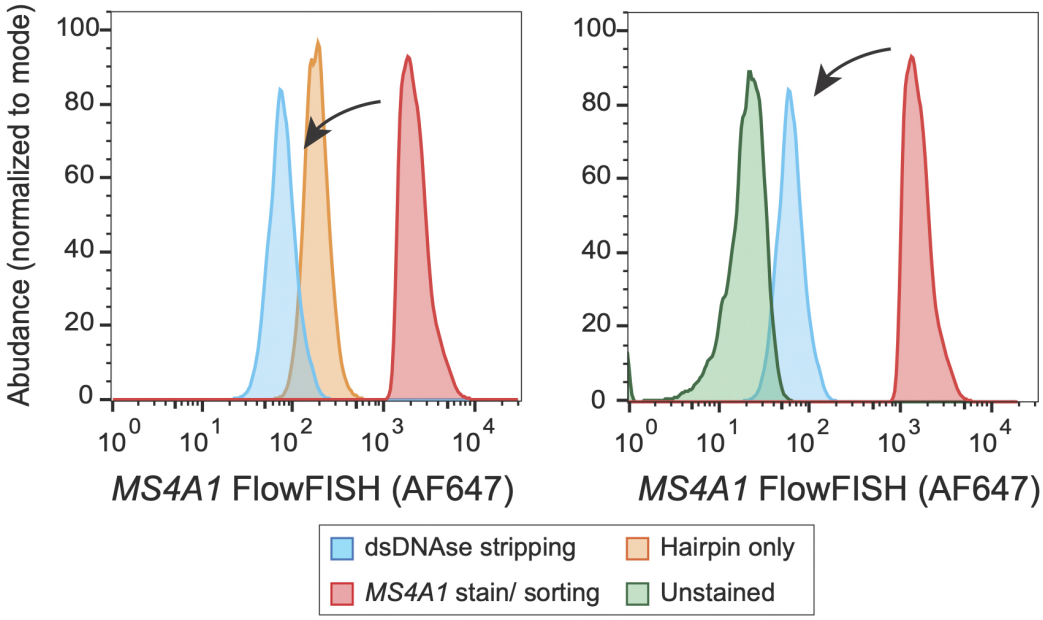
28 Centrifuge at 850xg for 5 minutes to pellet cells/nuclei.

29 Re-suspend in 500uL wash buffer and incubate at 37 °C for 10 minutes.

30 Repeat steps 28 and 29 for a total of 2 washes.

31 Resuspend in 500uL PBS-T and incubate for 5 minutes at Room temperature .
Optional: Resuspend in 1x PBS buffer and analyze on flow cytometer to ensure stripping.

Expected result



Expected Result from dsDNase Stripping

32 For 10XG Library Prep:
Wash 2 times with post-storage processing buffer and proceed immediately with probe hybridization step of the 10x genomics Chromium fixed RNA profiling protocol.

33 **Stopping point:**
Alternatively, samples can be resuspended in storage buffer (1mL per 5 million, see Materials section or 10XG protocol for more details).
Cells or nuclei can be stored for up to 1 week at 4 °C .

Protocol references

1. Reilly, S. K. et al. Direct characterization of cis-regulatory elements and functional dissection of complex genetic associations using HCR-FlowFISH. Nat. Genet. 53, 1166–1176 (2021).
2. HCR™ RNA flow cytometry protocol for mammalian cells in suspension, Molecular Instruments.
3. Chromium Fixed RNA Profiling Protocol, 10x Genomics
4. PERFF-seq: Cell and Nuclei Preparation