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HyPR Protocol

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1 *Works for me* This document is published without a DOI.

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ABSTRACT

Single-cell quantification of RNAs is important for understanding cellular heterogeneity and gene regulation, yet current approaches suffer from low sensitivity for individual transcripts, limiting their utility for many applications. Here we present Hybridization of Probes to RNA for sequencing (HyPR-seq), a method to sensitively quantify the expression of up to 100 chosen genes in single cells. HyPR-seq involves hybridizing DNA probes to RNA, distributing cells into nanoliter droplets, amplifying the probes with PCR, and sequencing the amplicons to quantify the expression of chosen genes. HyPR-seq achieves high sensitivity for individual transcripts, detects nonpolyadenylated and low-abundance transcripts, and can profile more than 100,000 single cells. We demonstrate how HyPR-seq can profile the effects of CRISPR perturbations in pooled screens, detect time-resolved changes in gene expression via measurements of gene introns, and detect rare transcripts and quantify cell type frequencies in tissue using low-abundance marker genes. By directing sequencing power to genes of interest and sensitively quantifying individual transcripts, HyPR-seq reduces costs by up to 100-fold compared to whole-transcriptome scRNA-seq, making HyPR-seq a powerful method for targeted RNA profiling in single cells.

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ABSTRACT

Single-cell quantification of RNAs is important for understanding cellular heterogeneity and gene regulation, yet current approaches suffer from low sensitivity for individual transcripts, limiting their utility for many applications. Here we present Hybridization of Probes to RNA for sequencing (HyPR-seq), a method to sensitively quantify the expression of up to 100 chosen genes in single cells. HyPR-seq involves hybridizing DNA probes to RNA, distributing cells into nanoliter droplets, amplifying the probes with PCR, and sequencing the amplicons to quantify the expression of chosen genes. HyPR-seq achieves high sensitivity for individual transcripts, detects nonpolyadenylated and low-abundance transcripts, and can profile more than 100,000 single cells. We demonstrate how HyPR-seq can profile the effects of CRISPR perturbations in pooled screens, detect time-resolved changes in gene expression via measurements of gene introns, and detect rare transcripts and quantify cell type frequencies in tissue using low-abundance marker genes. By directing sequencing power to genes of interest and sensitively quantifying individual transcripts, HyPR-seq reduces costs by up to 100-fold compared to whole-transcriptome scRNA-seq, making HyPR-seq a powerful method for targeted RNA profiling in single cells.

Preparation

-Chill the centrifuge
-Make sure 70% EtOH is in the -20
-Centrifuge at 850g unless otherwise specified

Protocol

1.Transfer cells (3-5 million per condition) to a 15mL conical tube and centrifuge for 5 min at 350g at 4 degrees
2.Aspirate supernatant, wash cell pellet in cold 3-5ml PBS, spin 5 min at 350g at 4 degrees
3.Aspirate supernatant and resuspend cells in 5ml 4% formaldehyde in PBST (3-5 million cells)
4.Fix cells for at least 1 hr at room temperature while rotating
5.Centrifuge for 5min at 850g and aspirate supernatant
6.Wash cells with 3-5ml PBST
7. Wash cells with 3-5ml PBST
8.Centrifuge for 5min at 850g and aspirate supernatant
9.Resuspend cells in 3-5ml cold 70% ethanol
10.Store at 4C for 10min
11.Centrifuge for 5min at 850g and aspirate supernatant.
12.Wash cells 2x with 3-5ml of PBST. Centrifuge for 5min to remove supernatant.
13.Transfer cells to 2ml wide bottom tubes and centrifuge for 5min at 850g and aspirate supernatant.
15.Re-suspend the pellet with 500µL of 30% probe hybridization buffer and pre-hybridize for 5 min at 37 °C.

CAUTION: probe hybridization buffer contains formamide, a hazardous material.

16.In the meantime, prepare probe solution by adding final 200nM total concentration probe mix (20nM per probe) to 500µL of 30%probe hybridization buffer.
17.Centrifuge for 5min to remove supernatant and add probe solution.
18.Incubate the sample overnight at 37 °C.
19.Centrifuge for 5min to remove probe solution.
20.Re-suspend the cell pellet with 500µL of 30% probe wash buffer.

CAUTION: probe wash buffer contains formamide, a hazardous material.

21.Incubate for 10min at 37 °C and remove the wash solution by centrifugation for 5 min.
22.Repeat steps 20 and 21 for three additional times.
23.Centrifuge for 5min to aspirate supernatant.
24.Re-suspend the cell pellet with 500µL of 5X SSCT.
25.Incubate for 5min at room temperature.
26.Centrifuge for 5min to pellet the cells.
27.Prepare 15 pmol of each hairpin by snap cooling 5µL of 3µM stock in hairpin storage buffer (heat at 95 °C for 90 seconds and

cool to room temperature in a dark drawer for 30 min).
28. Prepare 1st hairpin mixture by adding 5µL of H1 (from step 27) to 200µL of amplification buffer at room temperature.
29. Centrifuge for 5 min to pellet the cells, aspirate supernatant and add the hairpin mixture directly to the sample.
30. Incubate the sample for 1 hour at RT.
31. Centrifuge for 5min and remove the hairpin solution.
32. Wash pellet with 500µL of 5XSSCT.
33. Centrifuge for 5min and remove supernatant.
34. Wash pellet with 500µL of 5XSSCT.
35. Prepare 2nd hairpin mixture by adding 5µL of 0B H2 to 200µL of amplification buffer at room temperature.
36. Centrifuge for 5 min to pellet the cells, aspirate supernatant and add the hairpin mixture directly to the sample.
37. Incubate the sample for 1 hour at RT.
38. Centrifuge for 5 min to pellet the cells, aspirate supernatant and resuspend the cell pellet with 500µL of 5XSSCT.
39. Without incubation, remove the wash solution by centrifugation for 5 min.
40. Wash 3x in 500µL of 5XSSCT
41. Wash in 200µL 1xT4 Buffer.
42. Centrifuge for 5 min to pellet the cells, aspirate supernatant and incubate the cells in 200µL T4 ligation mixture for 1 hours at RT.
    T4 Ligation Mixture
    10x T4 Buffer
    1:50 T4 DNA Ligase
    Rest of volume in water
43. Wash cells 2x in 400µL 0.2% PBST
44. Resuspend cells in 400µL 0.2% PBST, filter cells (20um pluriStrainer cat. 43-0020-01)
45. Count cells and proceed to Bulk or EM PCRs

PCR

Emulsions:

50ul 2x Evagreen (Biorad cat 186-4033)
12.5ul beads at 8000 beads/ul (Chemgenes; 5'-Bead-linker-PC-linker
CAAGCAGAAGACGGCATACGAGATJJJJJJJJJJJJJGTTGGCACC
AGGCTTACGGATGTTGCACCAGC- 3')
62.5ul MM/tube

5ul ad1 primer (ad1.3-1.10)
10,000 cells (max vol 16.25)
32.5- vol of cells Water

Bulk:

10ul 2x Evagreen (Biorad cat 186-4033)
3ul Water
13ul master mix/tube

1ul ad1 primer
1ul ad2 primer
5ul 100 cells (8ul max)

Cycling Conditions:

All steps at a 50% ramp rate

94°C 30sec

Followed by 30 cycles of:

94°C 5sec

64°C 30sec

73°C 30sec

Followed by

75°C 5min

Steps:

1. Put Bulk in PCR machine.
2. For EM, proceed to loading Droplets into DG8 cartridge (Biorad cat 186-4008; with gasket cat 186-3009)
   70ul Oil (bottom, med sized) (Biorad cat 186-4006)

20ul PCR (middle, small sized)-> do replicates for each condition

3. For EM, after droplets are formed, put under UV light for 2min (4 wells at a time)
4. For EM, seal plate (Biorad cat 12001925) and load in the PCR machine
5. Do 1.8x SPRI according to manufacturer instructions to prepare DNA for sequencing

Illumina Read Structure
Read1 42
Read2 0
Index1 14
Index2 8

Bulks 1-2M reads per sample
EM 60M reads per sample

Buffers:

4% formaldehyde in PBST (50ml)
4% PFA (stored in 4 degrees)
50ul Tween-20

0.2% PBST (50ml)
100ul Tween-20
50ml PBS

Probe Hyb/Wash Buffer (50ml)
12.5ml 20x SSC (5x)
15ml formamide (30%, stored at 4 degrees)
50ul Tween-20 (0.1%)
22.5ml Water

5xSSCT (50ml)
12.5ml 20x SSC (5x)
50ul Tween-20 (0.1%)
37.5ml Water