BARseq - high-throughput cell typing with in situ sequencing V.2

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ABSTRACT

This protocol describes the application of BARseq as a standalone in situ sequencing method to achieve multiplexed interrogation of endogenous genes. In this variation, BARseq is similar to in situ sequencing (ISS), but uses Illuimna SBS for sequencing readout.

GUIDELINES

Standard precautions with RNA samples should be taken to reduce RNA degradation during tissue processing and library preparation. Pipetting and suctioning should be gentle throughout the whole procedure, and sample should not be left dried.

MATERIALS

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DNA oligos:

	A	B	C
	XC2757	/5AmMC12/NNNNNNNNNNNNNNNNNNN	N20 for random priming
	YS220	GATCGTCGGACTGTAGAACTCTGAACCTGTCG	sequencing primer
	YS221	/5Alex594N/GATCGTCGGACTGTAGAACTCTGAACCTGTCG	Hybridization probe, for RPI gene detection
	XC2758	/5Alex488N/AGTCAGCGTCGAGCACGCGGCACTTATTGCA	Hybridization probe, Slc17a7
	XC2759	/5Alex532N/TGAGTAGAGTTGACTAAGAGCCGTTAGATGCC	Hybridization probe, Gad1
	XC2760	/5Alex647N/TCGCTGTACTAATAGTTGTCGACAGATCGTCA	Hybridization probe, Slc30

Reagents:

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

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Keywords: BARseq, in situ sequencing, spatial transcriptomics

- Phusion high-fidelity PCR kit **Thermo Scientific Catalog #F553S**
- Tween-20 **Sigma-aldrich Catalog #P-7949**
- BS(PEG)9, 100 mg (Note: BS(PEG)9 loses its effectiveness 1 month after reconstitutic in DMSO. Prepare a fresh batch every month, especially if it has been frozen and thawed repeatedly. **Thermo Scientific Catalog #21582**
- Formamide **Thermo Fisher Scientific Catalog #AM9342**
- 10x PBS **Thermo Fisher Scientific Catalog #AM9624**
- RNase-free water **Contributed by users**
- dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10mM) **Thermo Fisher Scientific Catalog #R0192**
- phi29 DNA Polymerase (10 U/µL) **Thermo Scientific Catalog #EP0091**
- RNAse H **Enzymatics Catalog #Y9220L**
- Glycerol **Sigma Aldrich Catalog #G5516**
- Ethanol **Merck Millipore Catalog #100983**
- Pierce™ MMTS (methyl methanethiosulfonate) **Thermo Fisher Catalog #23011**
- SSC (20X), RNase-free **Thermo Fisher Catalog #AM9770**
- RiboLock RNase Inhibitor (40 U/µL) **Thermo Fisher Catalog #EO0381**
- RevertAid H Minus Reverse Transcriptase (200 U/µL) **Thermo Fisher Catalog #EP0452**
- Paraformaldehyde 20% **Electron Microscopy Sciences Catalog #15713**
- BSA Molecular grade **New England Biolabs Catalog #B9000S**
- Ampligase DNA Ligase Kit **Lucigen Catalog #A8101**
- KCl (2 M) RNase-free **Thermo Fisher Scientific Catalog #AM9640G**
- Aminoallyl-dUTP Solution (50 mM) **Thermo Fisher Scientific Catalog #R1101**

Tris (1 M) pH 8.0 RNase-free **Thermo Fisher Scientific Catalog #AM9855G**

HiSeq SBS Kit v4 **illumina Catalog #FC-401-4003**

Grace Bio-Labs HybriWell-FL™ sealing system Fluor-friendly adhesive chamber **Sigma Aldrich Catalog #GBL612204**

Other equipment required include incubators set at 37 "C, 45 "C, and 60 "C. All tubes should be RNase-free. RNase-free filter tips should be used. A Crest Xlight v3 spinning disk confocal on an Nikon Ti2E with Photometrics Kinetix, and Lumencor Celesta was used for imaging the sequencing steps. The filters and lasers used are indicated in Table 1.

	A	B	C	D
	Channels	Laser	Dichroic	Emission filter
	G/YFP	514	Zt405/514/635rpc	FF01-565/24
	T/RFP	561	FF421/491/567/659/776-Di01	FF01-441/511/593/684/817
	A	640	Zt405/514/635rpc	FF01-676/29
	C	640	Zt405/514/635rpc	FF01-775/140
	GFP	488	FF421/491/572-Di01	69401m
	DAPI	405	FF421/491/572-Di01	69401m
	TexasRed	561	FF421/491/572-Di01	69401m
	Cy5	640	Zt405/514/635rpc	ZET532/640m

Table 1. Laser and filter settings for sequencing imaging.

SAFETY WARNINGS

Use caution when handling liquids containing formaldehyde and formamide.

Library preparation

1 Tissues with barcoded neurons should be cryo-sectioned to 20 µm and mounted on slides.

Slides can be stored at -80 °C for up to a month.

2 **DAY 1**

Take slide(s) out of -80 °C and immerse immediately in 4% paraformaldehyde in 1x PBS (2 slides per 50mL falcon tube, back-to-back)

3 Incubate for 1 hour at room temperature on slow shaker

4 Wash the slides by immersing in 1x PBS (2 slides per 50ml falcon tube, back to back)

5 Wipe excess PBS off the surface of the chamber, then stick on the Hybriwell-FL chambers. Note that the ports on the chamber should be placed as far away from the tissue slices as possible.

6 Wash twice in PBST (1x PBS + 0.5% Tween-20)

7 Wash in 70% Ethanol for 5 mins

8 Wash in 85% Ethanol for 5 mins

9 Wash in 100% Ethanol for 5 mins

10 Replace with new 100% Ethanol, drop extra 100% Ethanol on top of slides and cover with ParaFilm to avoid evaporation. Incubate for at least 1.5 hrs at 4 °C (up to 3 hours)

11 Wash in PBST for 4-6 times, until all bubbles are cleared in the chamber and PBST flows into and out of the chamber smoothly.

12 Make reverse transription mix: 50 µM N20 primer (XC2757), 20 U/µL RevertAid H Minus M-MuLV reverse transcriptase, 500 µM dNTP, 0.2 µg/µL BSA, 1 U/µL RiboLock RNase Inhibitor, 1x RevertAid RT buffer.

13 Incubate in reverse transcription mix overnight at 37 °C. Create a humidity chamber to avoid the slides drying out using kim-wipes and DI water.

14 **DAY 2:**

Wash with PBST once

15 Incubate in a mixture of 1µL BS(PEG)9 per 4 µL PBST (e.g. 200ul BS(PEG)9 and 800ul PBST) for one hour at room temperature

16 Wash with 1M Tris pH 8.0, then incubate in new 1M Tris pH 8.0 for 30 mins

17 Wash twice in PBST

18 Make ligation mix: 1x Ampligase buffer, 100 nM padlock probe each, 0.5 U/µL Ampligase, 0.4 U/µL RNase H, 1 U/µL RiboLock RNase Inhibitor, 50 mM KCl (extra of those already provided by the ampligase buffer), 20% formamide.

19 Incubate in ligation mix for at least 30 mins at 37 °C (can go longer but not shorter), then at least 45 mins at 45 °C (can go longer but not shorter).

20 Wash twice in PBST

21 Make RCA mix: 1 U/µL phi29 DNA polymerase, 1x phi29 polymerase buffer, 0.25 mM dNTP, 0.2 µg/µL BSA, 5% glycerol (extra of those from the enzymes), 125 µM aminoallyl dUTP

22 Incubate in RCA mix overnight at room temperature

23 **DAY 3:**

Wash with PBST once

24 Incubate in a mixture of 1µL BS(PEG)9 per 4 µL PBST (e.g. 200ul BS(PEG)9 and 800ul PBST) for one hour at room temperature

25 Wash with 1M Tris pH 8.0, then incubate in new 1M Tris pH 8.0 for 30 mins

26 Wash twice in PBST

Sequencing

27 **Hybridization of Gene sequencing primer:**

Wash with FISH wash (2x SSC with 10% formamide)

28 Hybridize sequencing primer (YS220) with a primer concentration of 1 µM in FISH wash for 10 mins at room temperature

29 Wash with FISH wash three times, 2 mins each

30 Wash with PBST twice

31 **Sequence first cycle:**
Do the following incubations. Unless noted with incubation temperature, each step is performed at room temperature. For steps without incubation time, treat these as quick washes. large flat metal blocks can be used to place sample slides to quickly cycle through high and low temperatures. This version uses MiSeq Nano v2 kit:
https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/miseq-reagent-kit-v2.html

31.1 Incorporation Buffer 60 °C 3 mins x1

31.2 2% PBST x1

31.3 Idoacetamide blocker: For 9.3mg vial dilute pellet in between 2.5-3.5mL 2% PBST. Make fresh tube daily and store out of light.

Idoacetamide blocker 60 °C 3 mins x1

31.4 2% PBST x1

31.5 Incorporation Buffer x2

31.6 IRM 60 °C 3 mins x2

31.7 2% PBST x1

31.8 2% PBST 60 °C 3 mins x4

31.9 Replace 2% PBST with USM and Image **if slides are dirty, clean with 70% Ethanol before adding USM**

32 **Sequence subsequent cycles:**Do the following incubations. Unless noted with incubation temperature, each step is performed at room temperature. For steps without incubation time, treat these as quick washes. large flat metal blocks can be used to place sample slides to quickly cycle through high and low temperatures.

32.1 Incorporation buffer x2

32.2 CRM 60 °C 3 mins x2

32.3 Incorporation buffer x1 - Wipe ports after adding the incorporation buffer, to ensure that no CRM is left on the slide's surface

32.4 2% PBST x1

32.5 Idoacetamide blocker: For 9.3mg vial dilute pellet in between 2.5-3.5mL 2% PBST. Make fresh tube daily and store out of light.

Idoacetamide blocker 60 °C 3 mins x1

32.6 2% PBST x1

32.7 Incorporation buffer x2

32.8 IRM 60 °C 3 mins x2

32.9 2% PBST x1

32.10 2% PBST 60 °C 3 mins x4

32.11 Replace 2% PBST with USM and image **if slides are dirty, clean with 70% Ethanol before adding USM**

33 Hybridization cycle

33.1 **Hybridize probes:**
Make strip buffer: 60% formamide 2xSSC 0.01% Tween20

Strip buffer 60 ˚C 5 mins x3
Cool down quickly on metal plates between washes, place on metal plates in 60 ˚C oven to heat up quickly.

33.2 FISH wash (2x SSC with 10% formamide) 1x

33.3 Hybridize probes (YS221, XC2758, XC2759, XC2760) with a primer concentration of 1 µM in FISH wash at 60 ˚C for 2 minutes, then for 10 mins at room temperature. Rotate plates in holder to ensure they cool down slowly.

33.4 FISH wash x1

33.5 0.002 mg/ML DAPI in 2% PBST, room temperature for 5 mins

33.6 Replace PBST with USM and image **if slides are dirty, clean with 70% Ethanol before adding USM**

33.7

33.8