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BARseq 2.5

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We use this protocol and it's working

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Abstract

BARseq is a high throughput in situ transcriptomics method utilizing designed barcodes incorporated in the amplification process for multiplexed gene detection. Readout methods include: 1) In situ sequencing detecting endogenous gene transcripts for identifying cell types at subclass level; 2) in situ sequencing of exogenous barcoded gene, such as transcripts derived from Sindbis virus; 3) In situ probe hybridization directly labeling 1-4 significant marker genes of interest.

BARseq2.5 Protocol is an updated version of BARseq2.0 with an overall higher detection sensitivity and quality. At the Allen Institute For Neural Dynamics, this protocol is implemented for the purpose of profiling enhancer AAVs designed to target specific cell types of interest. Although not specifically included in this protocol, BARseq2.5 has also been used for other purposes such as axonal projection mapping.

Guidelines

- Unsealed, sectioned samples kept more than 18 months in freezer is not recommended for BARseq as endogenous gene readout yields poor signals and lower spot counts.

- This protocol does not include the sequencing of axonal projection barcodes. However there is no conflict to incorporate that part of the protocol (as described in BARseq2.0 protocol) into BARseq2.5 and will work as usual.

- Volume of various reaction mixtures depends on the numbers of slides running the experiment, this protocol uses 4 slides (8 chambers) as an example.

- For the rest of this protocol, a single wash in and out of chambers is indicated in syntax as such: *0.5% PBST Wash ; 5 sec ; 25 °C.* For this example it means to pipette 0.5% PBST into chamber and immediately suction out of the chamber after 5 seconds sitting on an aluminum block at room temperature. Pipetting and suctioning should be as gentle as possible to prevent sample being left dried.

Materials

Note

Any materials used in this protocol not listed specifically implies using any generic brand/type of the item likely doesn't have significant effect on reproducibility.

Reagents

- 20% PFA - 20% Paraformaldehyde (formaldehyde) aqueous solution, 10×10 mL **Electron Microscopy Sciences Catalog #**15713
- Nuclease-Free Water (not DEPC-Treated) **Thermo Fisher Scientific Catalog #**AM9932
- Nuclease-Free Water (not DEPC-Treated) 100 mL **Thermo Fisher Scientific Catalog #**AM9938
- KCl (2 M), RNase-free **Thermo Fisher Scientific Catalog #**AM9640G
- PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free **Thermo Fisher Scientific Catalog #**AM9625
- SSC (20X), RNase-free **Thermo Fisher Scientific Catalog #**AM9763
- TWEEN® 20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P1379
- Tris-HCl (1 M), pH 8.0, RNase-free **Thermo Fisher Scientific Catalog #**AM9856
- Dimethyl sulfoxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**276855
- Invitrogen™ Formamide (Deionized) **Thermo Fisher Scientific Catalog #**AM9342 (AM9344)
- RevertAid H Minus Reverse Transcriptase (200 U/µL) **Thermo Fisher Scientific Catalog #**EP0452

Note

Including:
- RT (Reverse Transcriptase)
- RT Buffer (Reverse Transcriptase Buffer)

- RiboLock RNase Inhibitor (40 U/µL) **Thermo Fisher Scientific Catalog #**EO0381
- Recombinant Albumin, Molecular Biology Grade **New England Biolabs Catalog #**B9200S
- RNase H (5000 U) **Qiagen Catalog #**Y9220L
- Ampligase Thermostable DNA Ligase **LGC biosearch Catalog #**A0102K
- Ampligase 10X Reaction buffer **LGC biosearch Catalog #**A1905B
- Aminoallyl-dUTP Solution (50 mM) **Thermo Fisher Scientific Catalog #**R1101

phi29-XT RCA Kit **New England Biolabs** **Catalog #**E1603L

Note

Including:
- Phi29XT polymerase
- Phi29XT polymerase buffer
- dNTP

phi29 DNA Polymerase (10 U/µL) **Thermo Fisher Scientific** **Catalog #**EP0094

Note

Including:
- Phi29 polymerase
- Phi29 polymerase buffer

Iodoacetamide, No-Weigh™ Format **Thermo Fisher Scientific** **Catalog #**A39271

MiSeq Reagent Nano Kit v2 (300-cycles) **Illumina, Inc.** **Catalog #**MS-103-1001

Note

Including:
- USM (SRE) - Imaging/scan Mix
- IRM (IMS) - Incorporation Mix
- CRM (CMS) - Cleavage Mix
- Incorporation buffer

DAPI (4,6-diamidino-2-phenylindole, dihydrochloride) **Thermo Fisher Scientific** **Catalog #**D1306

BS(PEG)9 **Thermo Fisher Scientific** **Catalog #**21582

Glycerol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G6279

Ethyl Alcohol **Thermo Fisher Scientific** **Catalog #**UN1170

dNTP Mix (10 mM each) (deoxynucleotide triphosphate mixtures containing dATP, dCTP, dGTP and dTTP) **Thermo Fisher Scientific** **Catalog #**R0194

Other Consumables

HybriWell™ Sealing System - Hybridization Cover - HBW2222FL **Grace Bio-Labs Catalog #**611204

Fisherbrand™ Tissue Path Superfrost™ Plus Gold Slides 144/PK **Thermo Fisher Scientific Catalog #**15-188-48

Thermo Scientific™ RNase AWAY™ Surface Decontaminant **Thermo Fisher Scientific Catalog #**21-402-178

Falcon® 50 mL High Clarity PP Centrifuge Tube Conical Bottom Sterile 25/Rack 500/Case **Corning Catalog #**352098, 430776, 431123

Kimberly-Clark Professional™ Kimtech Science™ Kimwipes™ Delicate Task Wipers, 1-Ply **Fisher Scientific Catalog #**06-666A

Start Kit PL-LTS 2,20,200,1000µL 30386597 - Pipette (Mettler-Toledo Rainin, LLC) **Rainin Catalog #**30386597

Parafilm® M Sealing Film **VWR International (Avantor) Catalog #**52858-000

Equipment

Vactrap™ 2 Vacuum Trap System 2 1 L HDPE Bottle Assembly with Tray, 0.2 µm Vent Filter, Not Autoclav **VWR International (Avantor) Catalog #**76207-602

RapidFISH Slide Hybridizer 115V **Boekel Scientific Catalog #**240200 (40 °C) (45 °C)

SureTemp ™ Dual-Convection Incubators, 40L **Benchmark Scientific Catalog #**H2505-40 (37 °C)

UVP HB-1000 Hybridization Incubator **Thermo Fisher Scientific Catalog #**UVP95003001 (60 °C)

StainTray™ - Slide Staining System - 10 Slide Staining Tray With Black Lid **Electron Microscopy Sciences Catalog #**71396-B

VWR® Modular Heating Blocks, Solid - Double 15.1 × 9.5 × 5.7 cm **VWR International (Avantor) Catalog #**13259-297

VWR® Standard 1000 Standard Orbital Shaker **VWR International (Avantor) Catalog #**89032-088

PPE

- Gloves
- Mask
- Lab Coat

Microscope

Nikon inverted research microscope ECLIPSE Ti2-E/Ti2-E/B

Channel	Laser	Filter Block	Emission Filter	Excitation Filter
A	637 nm	Peak: 405/514/635 ; Range: 10 nm	Peak: 676 ; Range: 29 nm	None
T	545 nm	Peak: 421/491/567/659/776 ; Range: 10 nm	410-445/492-526/590-662	None
C	637 nm	Peak: 405/514/635 ; Range: 10 nm	Peak: 775 ; Range: 140 nm	None
G	518 nm	Peak: 405/514/635 ; Range: 10 nm	Peak: 565 ; Range: 24 nm	None

Table 1. Laser and filter settings used for imaging the endogenous sequencing cycles

Channel	Laser	Filter Block	Emission Filter	Excitation Filter
DAPI	405 nm	Peak: 421/491/567/659/776 ; Range: 10 nm	410-445/492-526/590-662	None
Alexa 488	477 nm	Peak: 421/491/572 ; Range: 10 nm	410-445/492-526/590-662	None
Alexa 532	518 nm	Peak: 405/514/635 ; Range: 10 nm	Peak: 565 ; Range: 24 nm	None
Atto 565	545 nm	Peak: 421/491/572 ; Range: 10 nm	410-445/492-526/590-662	None
Alexa 647	637 nm	Peak: 405/514/635 ; Range: 10 nm	Peak: 676 ; Range: 29 nm	None
Alexa 750	748 nm	Peak: 421/491/567/659/776 ; Range: 10 nm	Peak: 441/511/593/684/817 ; Range: 10 nm	None

Table 2. Laser and filter settings used for imaging the hybridization cycle

Safety warnings

General PPE required (Gloves, Lab coat). Extra Caution When Handling The Following Liquids:

Item	GHS
Iodoacetamide	
Paraformaldehyde (PFA)	
Incorporation Buffer	
Formamide	

Before start

Start Day 1 from carrying initial processing of sample slices already mounted onto slides stored in a -80°C freezer.

Library Preparation Day 1

1 **Initial Preparation**
Working environment must be RNase free until Day 2 where mRNA has been reverse transcribed into more stable cDNA.

1.1 Wearing a mask throughout Day 1 is highly recommended to minimize mRNA degradation of samples

1.2 Clean lab bench/workstation area with RNase Away spray

1.3 Regularly replace pipette tips attached to vacuum system

2 **Fixation**

2.1 Make 4% Paraformaldehyde (PFA) in 1X Phosphate Buffered Saline (PBS) in a 50mL sterile, conical tube (Need 1 tube for every 2 slides)

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
10X-PBS	Invitrogen	AM9625	10	X	5	mL/Tube	1	X
20%-PFA	EMS	15710	20	%	10	mL/Tube	4	%
Nuclease Free Water	Invitrogen	AM9932	---	---	35	mL/Tube	---	---

2.2 Make 1X PBS in a 50mL sterile, conical tube (Need 1 tube for every 2 slides)

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
10X-PBS	Invitrogen	AM9625	10	X	5	mL/Tube	1	X
Nuclease Free Water	Invitrogen	AM9932	---	---	45	mL/Tube	---	---

2.3 Fetch slides from -80 °C freezer

2.4 Immerse slides immediately in 4% PFA (2 slides positioned back to back in each tube)

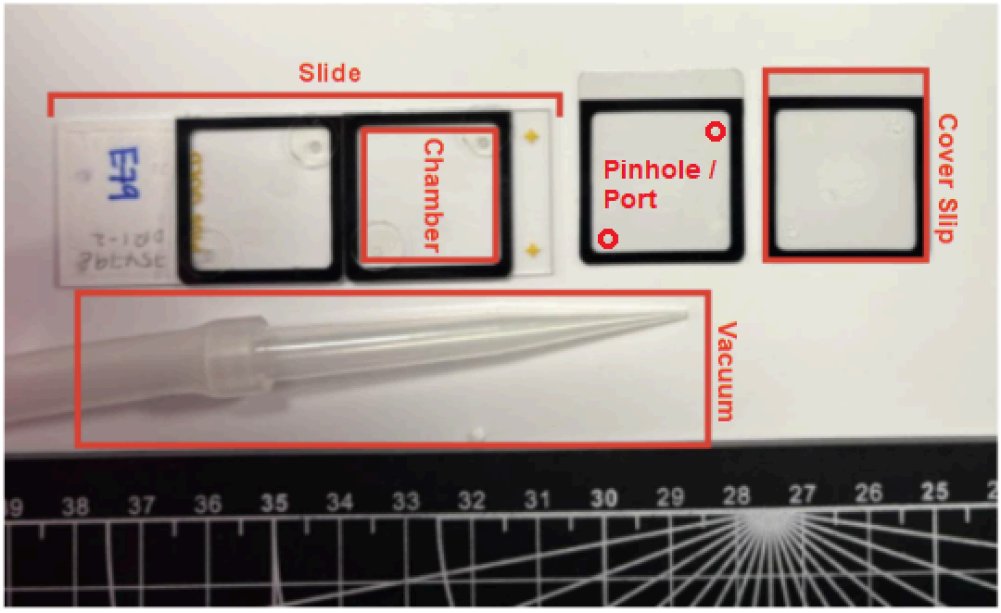
2.5 Place tube with slides on a shaker at low speed (~80 rpm), make sure the slides inside are sitting horizontally to avoid bubbles

2.6 Incubate for 1 hour at room temperature (20-25 °C)

2.7 Use forceps to transfer slides from 4% PFA into 1X PBS (full immersion the same way to stop reaction)

2.8 Dispose the 4% PFA into a dedicated liquid waste

3 **Chamber Installation**
The goal of this step is to place 2 HybriWell covers onto each slide to create chambers for further chemistry to take place.



Vacuum setup: insert a pipette tip (no filter) into a vacuum line

3.1 Make 0.5% Phosphate Buffered Saline with Tween (PBST) in a 50mL sterile, conical tube (Used throughout library preparation)

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
10X-PBS	Invitrogen	AM9625	10	X	5	mL/Tube	1	X
Tween-20	Sigma-Aldrich	P1379	---	---	250	uL/Tube	0.5	%
Nuclease Free Water	Invitrogen	AM9932	---	---	~45	mL/Tube	---	---

3.2 Use forceps to take out the slides from the PBS tube

3.3 Dry the back of the slide by placing it on a Kimwipe

3.4 Dry the edges/perimeter around the sections of the front of the slide with another folded Kimwipe

3.5 Take out 2 Grace bio-lab hybriwell-FL (hybriwell) covers per slide

3.6 For each slide, pair up every 2 hybriwells, touching side by side on the table

3.7 Peel off the sticker (thin film) of the hybriwell

3.8 Hold up the slide horizontally with the label part of the slide on your left hand

3.9 Have the slide facing down towards the table, and directly stick onto the hybriwells. Press around the edges from the slide side to make sure good contact.

3.10 Pipette/inject 0.5% PBST via the lower right pin hole of the hybriwells into the chamber until it is full (~150 uL)

4 Dehydration

4.1 Make 70% Ethanol in a 50mL sterile, conical tube

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
Ethanol	Thermo Fisher	UN1170	100	%	35	mL/Tube	70	%
Nuclease free water	Invitrogen	AM9932	---	---	15	mL/Tube	---	---

4.2 Make 85% Ethanol in a 50mL sterile, conical tube

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
Ethanol	Thermo Fisher	UN1170	100	%	42.5	mL/Tube	85	%
Nuclease free water	Invitrogen	AM9932	---	---	7.5	mL/Tube	---	---

* Optional to make 1mL which is sufficient for 4 slides

4.3 Pick up the slide with one hand, press finger on the transparent tap, vacuum out the 0.5% PBST

4.4 Pipette 0.5% PBST again for a second wash, set it aside and move onto the next step

4.5 Fetch a slide holder box, clean the holder box using RNase away spray bottle, set it aside

4.6 Vacuum out the 0.5% PBST

4.7 70% Ethanol Wash ; 5 min ; 25 °C

4.8 85% Ethanol Wash ; 5 min ; 25 °C

4.9 100% Ethanol Wash ; 5 min ; 25 °C

4.10 Inject 100% ethanol into the chamber

4.11 Add a drop or two of the 100% ethanol onto the surface of the hybriwell (chamber)

4.12 Cut out 1 rectangular piece of parafilm for each slide

4.13 Gently place parafilm on top of the chambers

4.14 Place the slides into the slide holder box

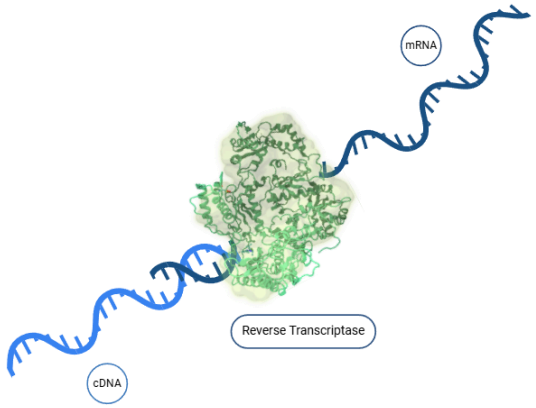
Note

It's important to keep holder box dry. Optionally, seal the slide holder box in a plastic bag or place Kimwipes sprayed with 100% Ethanol inside the box. Do not use any water or other liquid to wet Kimwipes that will add moisture.

4.15 Place the holder box in the 4C fridge and let it incubate for at minimum of 1.5 hours (up to 3 hrs max)

5 Reverse Transcription

Reverse transcribe mRNA to generate more stable cDNA (Template broken down with RNase H on Day 2 after crosslinking)



Schematics of reverse transcription

5.1 Thaw the following: Primers (ordered custom), RT Buffer, dNTP

5.2 Take out the slide box from the 4 °C fridge

5.3 Vacuum out the 100% ethanol

5.4 Pipette 0.5% PBST into the chamber, let it sit for 5 minutes

5.5 Vacuum out half of the 0.5% PBST, and then refill, let it sit for 5 minutes

5.6 Vacuum out the 0.5% PBST

5.7 Repeat 0.5% PBST Wash ; 5 sec ; 25 °C for 3~5 times until liquid can enter/exist chamber smoothly

5.8 Pipette 0.5% PBST, set it aside and move onto the next step

5.9 Prepare RT Mix in a RNAse-free microcentrifuge tube in the following concentration and order:

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
N20 Primer (20 random bases)	IDT	---	1000	uM	65	uL	50	uM
Additional RT Primer	IDT	---	100	uM	13	uL	1	uM
Additional RT Primer	IDT	---	100	uM	13	uL	1	uM
Additional RT Primer	IDT	---	100	uM	13	uL	1	uM
Additional RT Primer	IDT	---	100	uM	13	uL	1	uM
Nuclease Free Water	Invitrogen	AM9938	100	%	683	uL	52.5	%
RT buffer	Thermo Fisher	EP0451	5	X	260	uL	1	X
dNTPs	Thermo Fisher	R0192	10	mM	65	uL	0.5	mM
BSA (Recombinant Albumin)	NEB	B9200S	20	mg/mL	13	uL	0.2	mg/mL
Ribolock NRAse Inhibitor	Thermo Fisher	EO0381	40	U/uL	32.5	uL	1	U/uL
RevertAid H Minus M-MulV RT	Thermo Fisher	EP0452	200	U/uL	130	uL	20	U/uL

The following reagents should be kept in a benchtop cooler or in dry ice when taken out of the freezer: BSA, Ribolock RNase Inhibitor, RevertAid H Minus Reverse Transcriptase. Flick and spin down the solution. Avoid vortexing the enzyme mix.

5.10 Vacuum out the 0.5% PBST

5.11 Carefully Pipette the RT mix into the chambers

5.12 Place the slides back into the holder box

5.13 Fold four Kimwipes and place in the holder box

5.14 Add ~7.5 mL of water into the slide holder box to fully wet the Kimwipes for humidification

5.15 Place the entire holder box into a 37 °C oven, incubate for +12 hours overnight

Library Preparation Day 3

5h 45m

6 **Fixate cDNA**

Be careful not to over suction until section dried out and appears white. To avoid drying out the section, vacuum liquid from chambers slowly by pressing one finger on the transparent tab of the Hybriwell to control flow rate.

6.1 Thaw BS(PEG)9 Crosslinker

> Note
>
> *Please note that BS(PEG) can occasionally differ from batch to batch, in which could affect experimental outcome (i.e. blurry signal)

6.2 Add 460 uL of anhydrous DMSO (Dimethyl Sulfoxide) into 100 mg of BS(PEG)9 vial

> Note
>
> Any excess dissolved BS(PEG)9 can be stored in -20 °C freezer. Seal the vial with parafilm, and place in a desiccator. Opened BS(PEG)9 should not be used if stored more than a week.

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
BS(PEG)9	Thermo Fisher	21582	100	mg	---	---	---	---
DMSO	Sigma-Aldrich	276855	---	---	460	uL	---	---

6.3 Take out slides from 37 °C oven, vacuum RT mixture and replace with 0.5% PBST

6.4 Prepare the following reaction mixture:

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
BS(PEG)9 (DMSO Reconstituted)	Thermo Fisher	21582	0.22	mg/uL	200	uL	0.04	mg/uL
0.5% PBST	---	---	---	---	800	uL	---	---

6.5 Carefully vacuum 0.5% PBST from chamber

6.6 Pipette the crosslinker mixture in, place slides back into the holder box with the lid on

6.7 Incubate at room temperature for 01:00:00 1h

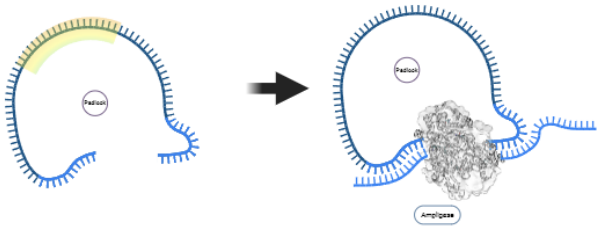
6.8 Vacuum cross linker mix, inject 1M Tris-HCl (pH 8.0) into the chambers to stop reaction

6.9 Place slide back into the holder box

6.10 Incubate for at least 00:30:00 at room temperature (can incubate up to 2 hours maximum)

7 **Padlock Annealing**

Anneal designed padlocks (carrying barcode sequence) targeting genes of interest. Ampligase is used for ligating the padlock to form a complete loop as template for the rolling circle amplification (RCA) (Step 8)



Schematics of Padlock hybridization and DNA ligation

7.1 Thaw the following: all padlocks (ordered custom), ampligase buffer, formamide

7.2 Prepare the following padlock mix for example:

Number of Chambers							8	Chamber
Total Volume							1000	uL
Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
Padlock 1	IDT	---	4.35	uM	4.60	uL	0.02	uM
Padlock 2	IDT	---	4.17	uM	4.80	uL	0.02	uM
Padlock 3	IDT	---	4.17	uM	4.80	uL	0.02	uM
Nuclease Free Water	Invitrogen	AM9932	100	%	550.8	uL	55.08	%
Ampligase buffer	LGC	A1905B	10	X	100	uL	1	X
KCl stock solution	Invitrogen	AM9640G	2	M	25	uL	0.05	M
Formamide	Ambion	AM9342	---	---	200	uL	---	---
Ampligase	LGC	A0102K	100	U/uL	5	uL	0.5	U/uL
RiboLock Rnase Inhibitor	Thermo Fisher	EO0381	40	U/uL	25	uL	1	U/uL
Rnase H	Enzymatics	Y9220L	5000	U/mL	80	uL	400	U/mL

The following reagents should be kept in a benchtop cooler or in dry ice when taken out of the freezer: Ampligase, Ribolock RNase Inhibitor, RNase H.

7.3 Set the mixture aside, vacuum Tris-HCl from the chambers, and replace with 0.5% PBST

7.4 Repeat with another 0.5% PBST Wash ; 5 sec ; 25 °C

7.5 Remove 0.5% PBST, inject chambers with the mixture

7.6 Place slide back into the holder box

7.7 Transfer the entire holder box inside a 37 °C oven

7.8 Incubate at 37 °C for exactly 00:30:00 30m

7.9 Immediately transfer the slides into a 45 °C hyb oven

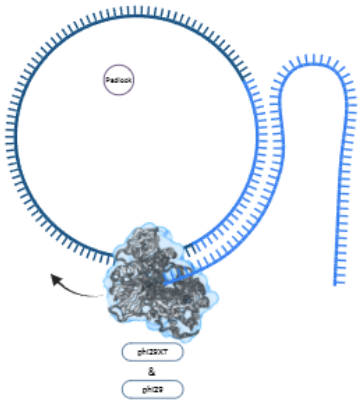
7.10 Continue the incubation at 45 °C for 00:45:00 (can last up to 1 hour) 45m

Note

Start preparing the RCA mix (next step) 15 minutes before the 45 °C incubation is about to end. Prolonged time interval between step 7 and step 8 could lead to poor signal quality.

8 **Rolling Circle Amplification**

Amplify copies of the padlock into rolonies for signal detection purposes. The RCA is a two step incubation using polymerase phi29XT followed by phi29.



Schematics of rolling circle amplification

8.1 Thaw the following: dNTP (phi29XT Kit), phi29XT polymerase buffer

8.2 Prepare the phi29XT polymerase reaction mix as follows:

Number of Chambers					8	Chamber
Total Volume					1000	uL

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
Nuclease Free Water	Invitrogen	AM9938	100	%	600	uL	60	%
Phi29XT polymerase 5x buffer	NEB	B0572S	5	X	200	uL	1	X
dNTPs (XT Kit)	NEB	N0447X	10	mM	100	uL	1	mM
Phi29XT polymersae	NEB	M0572L	10	X	100	uL	1	X

The following reagents should be kept in a benchtop cooler or in dry ice when taken out of the freezer: Phi29XT polymerase

8.3 Take slides out of the oven, remove the padlock mix

8.4 0.5% PBST Wash ; 5 sec ; 25 °C (Repeat 3 times)

8.5 Pipette the phi29XT reaction mix into the chamber

8.6 Transfer slides into 40 °C oven, let it incubate for 02:00:00 2h

8.7 Thaw the following: dNTP, aadUTP, phi29 polymerase, phi29 polymerase buffer

8.8 Prepare phi29 reaction mix as follows:

Number of Chambers					8	Chamber
Total Volume					1000	uL

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
Nuclease Free Water	Invitrogen	AM9938	100	%	662.5	uL	66.25	%
50% glycerol	Sigma-Aldrich	G6279	100	%	100	uL	10	%
phi29 polymerase 10x buffer	Thermo Fisher	EP0094	10	X	100	uL	1	X
aadUTP	Thermo Fisher	R1101	50	mM	2.5	uL	0.125	mM
dNTPs	Thermo Fisher	R0192	10	mM	25	uL	0.25	mM
BSA (Recombinant Albumin)	NEB	B9200S	20	mg/mL	10	uL	0.2	mg/mL
phi29 polymersae	Thermo Fisher	EP0094	10	U/uL	100	uL	1	U/uL

The following reagents should be kept in a benchtop cooler or in dry ice when taken out of the freezer: BSA, Phi29 polymerase

8.9 Vacuum out the previous phi29XT mix, and inject the phi29 mix

8.10 Place slide back into the holder box

8.11 Incubate at room temperature overnight (>12 hours)

Library Preparation Day 3

5h 45m

9 **Fixation**
Final fixation for securing the RCA products in place using BS(PEG)9

9.1 Thaw BS(PEG)9 Crosslinker

9.2 If BS(PEG)9 stock has not been prepared, perform the following step:
Add 460 uL of DMSO into 100 mg of BS(PEG)9 vial

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
BS(PEG)9	Thermo Fisher	21582	100	mg	---	---	---	---
DMSO	Sigma-Aldrich	276855	---	---	460	uL	---	---

9.3 Prepare the following reaction mixture:

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
BS(PEG)9 (DMSO Reconstituted)	Thermo Fisher	21582	0.22	mg/uL	200	uL	0.04	mg/uL
0.5% PBST	---	---	---	---	800	uL	---	---

9.4 Vacuum out the phi29 mix from chamber and replace with 0.5% PBST

9.5 Vacuum out the 0.5% PBST, inject the reaction mix into chambers

9.6 Place slides back into the holder box

9.7 Incubate at room temperature for 01:00:00

1h

9.8 Stop reaction by vacuuming out the reaction mix and inject 1M Tris-HCl (pH 8.0) into chambers

9.9 Incubate at room temperature for 00:30:00 (can incubate up to 2 hours maximum) 30m

10 **Storage**

10.1 Vacuum out Tris-HCl from chambers

10.2 Inject 0.5% PBST into the chambers

10.3 Store slides in 4°C fridge before use (Ideally < 1 week before moving onto the next step for imaging) Overnight

Hybridization Cycle [1 Cycle] 15m

11 **Reagents Preparation**

11.1 Thaw the following: USM, DAPI stock solution, all Hyb labeling probes

11.2 Make FISH Wash (10% Formamide) in a sterile, conical 50mL tube (opaque or covered with aluminum foil)

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
Formamide	Ambion	AM9342	>99.5	%	5	mL	10	%
20X-SSC	Invitrogen	AM9763	20	X	5	mL	2	X
Nuclease Free water	Invitrogen	AM9932	---	---	40	mL	---	---

Note: FISH (fluorescent in-situ hybridization) Wash is

11.3 Make 1:500 DAPI diluted working solution in a 1.5 mL microcentrifuge tube

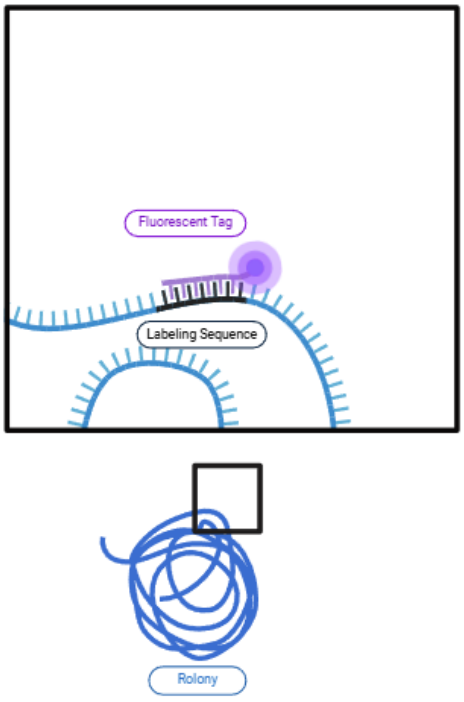
Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
DAPI Stock	---	---	1	mg/mL	1	uL	0.002	mg/mL
2% PBST	---	---	---	---	499	uL	---	---

12 **Labeling**

This step directly labels the presence of enhancer AAV transcripts and 1-4 optional marker genes of interest.

Note

- For the rest of this protocol, a single wash in and out of chambers is indicated in syntax as such: *0.5% PBST Wash ; 5 sec ; 25 °C.* For this example it means to pipette 0.5% PBST into chamber and immediately suction out of the chamber after 5 seconds sitting on a aluminum block at room temperature. Pipetting and suctioning should be as gentle as possible to prevent sample being left dried.
- During gene sequencing, slides must be placed immediately onto an aluminum block for quick cooling down back to room temperature after any heating incubation, unless specified otherwise. In situ sequencing uses reagents from illumina MiSeq kit containing: IRM (IMS), CRM (CMS), USM (SRE), & incorporation buffer.
- The following example uses 4 hybridization oligonucleotides, but this should be adjusted to each user's needs.



Schematics of fluorophore conjugated probe hybridization

12.1 Vacuum out 0.5% PBST and pipette FISH wash into the chambers

12.2 Set slides aside, move onto preparing the labeling mix:

Number of Chambers							8	Chamber
Total Volume							1000	uL

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
Atto[565] (All Gene)	IDT	---	100	uM	2.5	uL	0.25	uM
Alexa[488] (Slc17a7)	IDT	---	100	uM	2.5	uL	0.25	uM
Alexa[532] (Gad1)	IDT	---	100	uM	2.5	uL	0.25	uM
Alexa[647] (sYFP)	IDT	---	100	uM	2.5	uL	0.25	uM
FISH Wash	---	---	100	uM	990	mL	---	---

12.3 Vacuum out FISH wash and inject the labeling mixture in

12.4 Incubate slides in 60°C UVA Hyb oven for 00:02:00 on heat block 2m

12.5 Place slides inside a slide box with chamber. Ensure the slides do not touch anything to allow for air-cooling

12.6 With the slide box lid on, let slides incubate at room temperature for 00:10:00 in the dark 10m

12.7 FISH Wash ; 5 sec ; 25 °C

12.8 Pipette diluted 1:500 DAPI working solution into chambers

12.9 Let slide incubate in the slide box in dark at room temperature for 00:03:00 3m

12.10 Vacuum out DAPI and wipe both sides of the slides using Kimwipe with a bit of ethanol

12.11 Pipette USM into the chambers

12.12 Image slides

Strip [Between Hybridization & Sequencing Cycles] 30m

13 **Reagents Preparation**

13.1 Make Strip Buffer in a 50mL sterile conical tube (opaque or covered with aluminum foil)

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
Formamide	Ambion	AM9342	>99.5	---	30	mL	60	%
20X-SSC	Invitrogen	AM9763	20	X	5	mL	2	X
Tween-20	Sigma-Aldrich	P1379	---	---	500	uL	1	%
Nuclease Free water	Invitrogen	AM9932	---	---	14.5	mL	---	---

14 **Stripping** 15m

14.1 Vacuum USM from chambers

14.2 Strip Buffer ; 5 min ; 60 °C (UVA Hyb Oven) 00:05:00 (Repeat 3 times) 15m

14.3 Vacuum out strip buffer and add PBST 0.5%

14.4 Place the slides under the microscope to check if previous signals are gone, if not repeat 1x more wash

Gene Sequencing Cycle [7 Cycles] 12m

15 **Reagents Preparation**

15.1 Make 0.5% PBST in a 50mL sterile conical tube

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
10X-PBS	Invitrogen	AM9625	10	X	5	mL	1	X
Tween-20	Sigma-Aldrich	P1379	---	---	250	uL	0.5	%
Nuclease Free Water	Invitrogen	AM9932	---	---	~45	mL	---	---

15.2 Make 2% PBST in a 50mL sterile conical tube

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
10X-PBS	Invitrogen	AM9625	10	X	5	mL	1	X
Tween-20	Sigma-Aldrich	P1379	---	---	1	mL	2	%
Nuclease Free Water	Invitrogen	AM9932	---	---	44	mL	---	---

15.3 Make FISH Wash in a 50 mL sterile conical tube (opaque or covered with aluminum foil)

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
Formamide	Ambion	AM9342	>99.5	%	5	mL	10	%
20X-SSC	Invitrogen	AM9763	20	X	5	mL	2	X
Nuclease Free water	Invitrogen	AM9932	---	---	40	mL	---	---

15.4 Add 3 mL of 2%PBST to reconstitute Iodoacetamide in a 5 mL microcentrifuge tube

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
Iodoacetamide	Thermo Fisher	A39271	9.3	mg	---	---	3.1	mg/mL
2% PBST	---	---	---	---	3	mL	---	---

Store solution away from light. Make 3 mL of fresh Iodoacetamide dissolved in 2% PBST for every 12 hours. Don't use any dissolved Iodoacetamide stored overnight, Iodoacetamide is reactive and unstable when dissolved.

15.5 Thaw the following: USM, IRM, CRM, sequencing primer (YS220. Custom, ordered form IDT)

16 Primer Annealing

16.1 Vacuum out previous 0.5% PBST from the chambers

16.2 0.5% PBST ; 5 sec ; 25 °C

16.3 FISH Wash ; 5 sec ; 25 °C

16.4 Set slides aside, move onto preparing the sequencing primer working solution as follows:

Number of Chambers	8	Chamber
Total Volume	1000	uL

Reagent	Vendor	Catolog #	[Stock]		Applied Volume		[Working]	
FISH Wash	---	---	---	---	975	uL	---	---
YS220	IDT	---	100	uM	25	uL	2.5	uM

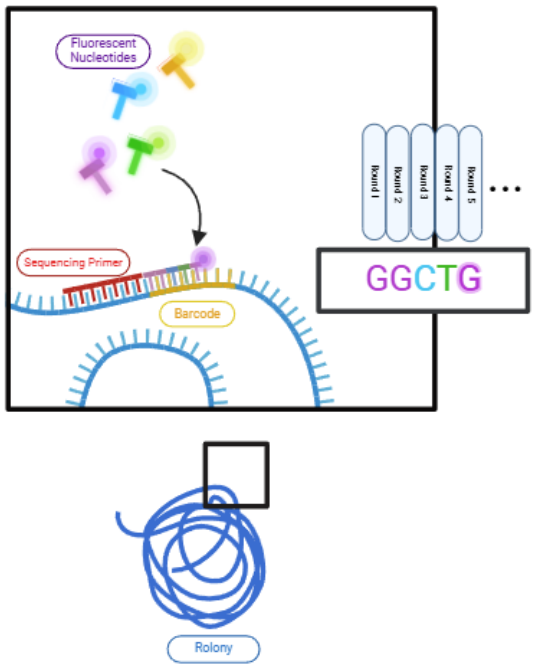
16.5 Place slides inside the 60 °C UVA Hyb Oven and incubate for 00:02:00 on heat block 2m

16.6 Place slides inside a slide box with chamber. Ensure the slides do not touch anything to allow for air-cooling

16.7 With the slide box lid on, let slides incubate at room temperature for 00:10:00 in the dark 10m

17 **Initial Cycle Sequencing (cycle 1)**

In situ sequencing - This section of steps is to simultaneously detects multiple endogenous gene transcripts via 7 rounds of sequencing and imaging the barcodes, utilizing the MiSeq Reagent Nano Kit v2 (300-cycles) from Illumina.



Schematics of in situ sequencing

17.1 Incorporation Buffer ; 3 min ; 60 °C

17.2 2% PBST ; 5 sec ; 25 °C

17.3 Iodoacetamide ; 3 min ; 60 °C

17.4 2% PBST ; 5 sec ; 25 °C

17.5 Incorporation Buffer ; 5 sec ; 25 °C (Repeat 2 times)

17.6 IRM ; 3 min ; 60 °C (Repeat 2 times)

17.7 2% PBST ; 5 sec ; 25 °C

17.8 2% PBST ; 3 min ; 60 °C (Repeat 4 times)

17.9 Wipe both sides of the slides using Kimwipe with a bit of ethanol

17.10 Pipette USM into the chambers

17.11 Image slides

18 **Subsequent Cycles Sequencing (Repeat this step for cycle 2,3,4,5,6,7)**
Barcode is 7 nucleic bases long. The 8th base is "C" for all barcode, thus it can be sequenced up to 8th cycle in which should give signal only in the "C" channel when imaging as a measure to confirm that each sequencing cycle was performed at high efficiency.

18.1 Incorporation Buffer ; 5 sec ; 25 °C (Repeat 2 times)

18.2 CRM ; 3 min ; 60 °C (Repeat 2 times)

18.3 Incorporation Buffer ; 5 sec ; 25 °C

18.4 2% PBST ; 5 sec ; 25 °C

18.5 Iodoacetamide ; 3 min ; 60 °C

18.6 2% PBST ; 5 sec ; 25 °C

18.7 Incorporation Buffer ; 5 sec ; 25 °C (Repeat 2 times)

18.8 IRM ; 3 min ; 60 °C (Repeat 2 times)

18.9 2% PBST ; 5 sec ; 60 °C (Repeat 4 times)

18.10 Wipe both sides of the slides using Kimwipe with a bit of ethanol

18.11 Pipette USM into the chambers

18.12 Image slides

Protocol references

Version:
BARseq Version 2.0 (Xiaoyin Chen, Anthony M. Zador, Mara Rue)

Citation:
Xiaoyin Chen, Anthony M. Zador, Mararue 2023. BARseq - high-throughput cell typing with in situ sequencing. **protocols.io**https://dx.doi.org/10.17504/protocols.io.81wgbp4j3vpk/v2Version created by **Xiaoyin Chen**

Figures Created in BioRender. W, J. (2025) https://BioRender.com/63mitxi

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