Feb 20, 2022

Intranuclear CITE-seq (inCITE-seq): joint single-cell measurements of multiplexed nuclear proteins and RNA

Hattie Chung[1], Emma Magee[1]

[1]Broad Institute

1

protocol .

Hattie Chung

This protocol allows for intranuclear antibody staining of fixed nuclei in suspension. Nuclei suspensions are suitable for CITE-seq and 10X Genomic applications.

Hattie Chung, Emma Magee 2022. Intranuclear CITE-seq (inCITE-seq): joint single-cell measurements of multiplexed nuclear proteins and RNA . **protocols.io** https://protocols.io/view/intranuclear-cite-seq-incite-seq-joint-single-cell-bt7mnrk6

protocol ,

Apr 13, 2021

Feb 20, 2022

49101

STOCK BUFFERS

- *All buffers should be fresh each time, except 0.2% PBST which can be made in advance, until ready for use.*
- *Prepare all buffers prior to starting the protocol .*
- *All buffer volumes below are for calculated for one sample + dead volume, except PBST which can be made in stock*
- *Pre-chill and store all buffers* **On ice** .

0.2% PBST

A	B	C	D
Stock Conc.	Name	Final Conc.	Volume (mL)
10%	Tween-20	0.2%	10
-	PBS	-	500
		Total Volume (mL)	510

EZ Lysis Buffer

A	B	C	D
Stock Conc.	Name	Final Conc.	Volume (mL)
5,000U	Recombinant RNase Inhibitor (RRI)	~1 U/µL	0.011
	EZ Lysis Buffer	-	11
		Total Volume (mL)	11.011

Resuspension Buffer (RSB)

A	B	C	D
Stock Conc.	Name	Final Conc.	Volume (µL)
1M	MgCl2	3mM	3.3
5,000U	Recombinant RNase Inhibitor (RRI)	~1 U/µL	1.1
-	PBS	-	1,095.6
		Total Volume (µL)	1,100.00

FA-NT Buffer

A	B	C	D
Stock Conc.	Name	Final Conc.	Volume (µL)
16%	Formaldehyde	1.33%	257.69
10%	Tween-20	0.1%	31
10%	NP-40	0.2%	62
1M	MgCl2	3mM	9.3
-	PBS	-	2,740.01

Blocking Buffer

A	B	C	D
Stock Conc.	Name	Final Conc.	Volume (µL)
1,000U	TruStain FcX™ PLUS Blocking Reagent	1:100	10
5%	UltraPure BSA	1%	200
10%	Dextran Sulfate (10% stock solution)	0.05%	5
1M	MgCl2	3mM	3
5,000U	Recombinant RNase Inhibitor (RRI)	~1 U/µL	1
-	PBST	-	781
		Total Volume (µL)	1,000.00

Reagents used in this protocol:

Dounce homogenizers

- **Sigma Catalog #D8938-1SET**

Falcon™ 15mL Conical Centrifuge Tubes **Fisher**

- **Scientific Catalog #14-959-53A**
-

☒ Falcon® 5 mL Round Bottom Polystyrene Test Tube with Cell Strainer Snap Cap **Falcon Catalog # 352235**

☒ Eppendorf tubes 1.5 mL uncolored **Eppendorf**

- **Centrifuge Catalog #022363204**

  ☒ pluriStrainer® 20

- µm **pluriSelect Catalog #43-50020-03**

  ☒ C-Chip disposable

- hemacytometer **INCYTO Catalog #DHC-N01**

  ☒ RNase Zap **Sigma**

- **Aldrich Catalog #R2020-250ML**

  ☒ Nuclei EZ Lysis

- Buffer **Sigma Catalog #N-3408**

  ☒ Recombinant RNAse

- Inhibitor **Takarabio Catalog #2313A**

- ☒ 1X PBS, cell culture grade **Thermo Fisher Scientific**

  ☒ 1 M Magnesium Chloride (MgCl2) **Sigma**

- **Aldrich Catalog #M8266**

  ☒ 10% Tween-20

- Solution **Teknova Catalog #T0710**

  ☒ NP-40 Surfact-Amps™ Detergent Solution **Thermo**

- **Fisher Catalog #28324**

  ☒ Pierce™ 16% Formaldehyde (w/v) Methanol-free **Thermo Fisher**

- **Scientific Catalog #28906**

  ☒ TruStain FcX™ PLUS (anti-mouse CD16/32)

- Antibody **BioLegend Catalog #156603**

  ☒ UltraPure™ BSA (50 mg/mL) **Thermo**

- **Fisher Catalog #AM2618**

Formaldehyde and glacial acetic acid should be used in the chemical fume hood

Before you start the protocol 20m

1 All steps should be performed **On ice** or at **4 °C** . Cool a swinging bucket centrifuge to **4 °C** . Prepare all reagents and chill **On ice** .

2 Clean one set of douncers (mortar, pestle A and pestle B) for every sample as follows. Make sure to thoroughly wash inside the mortar and at the end of each pestle where the sample will come in contact: 2m

- Rinse with distilled water.
- Spray with 70% ethanol. Let sit for ~ **00:01:00**
- Rinse with distilled water
- Spray with RNase Zap. Let sit for **00:01:00**
- Rinse with distilled water
- Rinse with DNase and RNase free double distilled water
- Let air dry on kimewipe

Once dry, pre-chill on **On ice** .

3 Pre-chill all tubes **On ice** . For each sample, you will need:

- 3 x 15mL Falcon Tubes
- 1 x 1.5mL Eppendorf tube
- 2 x 35µm filter-cap FACS tubes
- 2 x 20µm filters

Nuclei Extraction 20m

4 Remove frozen tissue sample from **-80 °C** storage and place on dry ice until ready.

5 Place sample into a clean, pre-chilled mortar filled with **2 mL** of EZ Lysis Buffer.

6 Dounce with pestle "A" until resistance subsides (~40 strokes) **On ice** .

7 Place pestle "A" in 50mL Falcon tube to hold until ready for cleaning.

8 Douce with pestle "B" until resistance subsides (~40 strokes) **On ice** .

9 Place pestle "B" in 50mL Falcon tube to hold until ready for cleaning.

10 Transfer the **2 mL** homogenate to a pre-chilled 15mL Falcon Tube.

11 Add **3 mL** of EZ Lysis Buffer to raise the sample volume to **5 mL** total -- volumes can be added to wash out the mortar before being added, to maximize nuclei transfer.

12 Incubate for **00:05:00** **On ice** . 5m

12.1 During this incubation, spray mortars and pestles with 10% bleach, let sit, and rinse with distilled water in order to clean off remaining tissue. After rinsing, soak mortars and pestles in 10% bleach and store until next use.

13 Spin down nuclei at **500 x g, 4°C, 00:05:00** in pre-cooled swinging bucket centrifuge. 5m

14 Carefully remove and discard supernatent.

15 Resuspend pellet in **1 mL** of EZ Lysis Buffer using a P1000 pipette. Mix carefully and thoroughly. Add another
4 mL of EZ Lysis Buffer. Mix carefully and thoroughly. The total volume should be **5 mL** .

16 Incubate for **00:05:00** **On ice** . 5m

17 Spin down nuclei at **500 x g, 4°C, 00:05:00** in pre-cooled swinging bucket centrifuge. 5m

18 Carefully remove and discard supernatent.

19 Resuspend pellet in **1 mL** of RSB Buffer.

20 Filter **1 mL** of nuclei suspension through a pre-chilled **35 µm** filter cap FACS tubes.

Fixation and Permeabilization

21 Transfer **1 mL** of nuclei suspension from the filter tube into a pre-chilled 15mL Falcon tube.

22 Add **1 mL** of FA-NT solution using a P1000 pipette, mixing the first **1 mL** carefully and thoroughly with the nuclei suspension. Add **2 mL** more of FA-NT solution. The total volume in the tube should be **4 mL** .

23 Immediately spike **3 µL** of glacial acetic acid to nuclei suspension.

24 Incubate for **00:10:00** at **4 °C** while rocking. 10m

25 Immediately after, quench the fixation reaction by adding **3 µL** of **1 M** Glycine. Use P1000 pipette to mix sample thoroughly to ensure equal distribution of glycine throughout solution.

26 Filter through a **20 µm** filter into new, pre-chilled 15mL Falcon Tube.

27 Spin down nuclei at **850 x g, 4°C, 00:05:00** . 5m

28 Remove and discard supernatent.

Primary Antibody Stain

29 Resuspend pellet in **500 µL** of Blocking Buffer. Pipette up and down multiple times using a P200 pipette to ensure the sample is mixed thoroughly.

30 Incubate for **00:15:00** at **4 °C** while rocking. 15m

31 Spin down nuclei at **850 x g, 4°C, 00:05:00** . 5m

Remove and discard supernatent.

32

33 Resuspend pellet in **200 µL** of primary antibody diluted to the appropriate concentration in Blocking Buffer.

34 Incubate for **01:00:00** at **4 °C** while rocking. 1h

35 Spin down nuclei at **850 x g, 4°C, 00:05:00** . 5m

36 Carefully remove and discard supernatent.

37 Resuspend pellet in **500 µL** of PBST.

38 Incubate **00:05:00** **On ice** . 5m

39 Repeat wash steps 34-37 to to ensure removal of any excess antibodies. 10m

40 Spin down nuclei at **850 x g, 4°C, 00:05:00** . 5m

41 Remove and discard supernatent. If preforming a secondary antibody stain, proceed to step 42. If loading onto 10x, proceed to step 50.

[OPTIONAL] Secondary Antibody Staining

42 Resuspend pellet in **200 µL** of secondary antibody diluted 1:1000 in Blocking Buffer.

43 Incubate for **00:20:00** at **4 °C** while rocking. 20m

44 After incubation, spike in **2 µL** of 100x DAPI into each sample. Mix carefully and thoroughly using a P200 pipette.

45 Repeat wash steps 34-37 two times to ensure removal of any excess antibodies in solution. 10m

46 Spin down nuclei at **850 x g, 4°C, 00:05:00** . 5m

47 Resuspend pellet in **500 µL** of PBST.

48 filter through a 20 **20 µm** filter into a pre-chilled FACS tube.

49 Keep samples in dark **On ice** until processing via flow cytometry.

preparation for 10x loading

50 Resuspend pellet in **100 µL** of pre-chilled RSB Buffer (ensure that there is no Tween-20 in this solution!) using a P200 pipette. Mix carefully and thoroughly. Add **200 µL** more of RSB using a P1000 pipette. Mix carefully and thoroughly. Total volume should be **300 µL** .

51 Filter through a **20 µm** filter into a pre-chilled FACS tube.

52 Count nuclei using hemocytometer chamber. Keep nuclei **On ice** until ready for 10X loading.