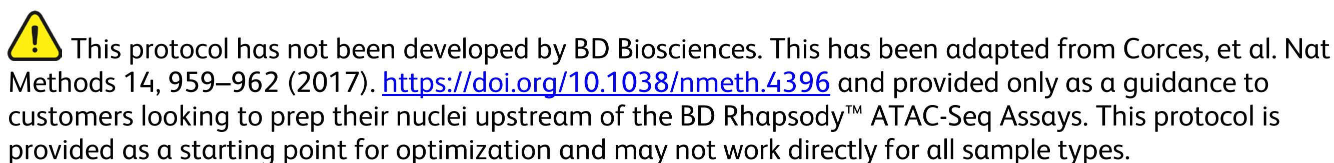
Protocol for isolation of nuclei from frozen tissues for ATAC-Seq


This protocol has not been developed by BD Biosciences. This has been adapted from Corces, et al. Nat Methods 14, 959–962 (2017). https://doi.org/10.1038/nmeth.4396 and provided only as a guidance to customers looking to prep their nuclei upstream of the BD Rhapsody™ ATAC-Seq Assays. This protocol is provided as a starting point for optimization and may not work directly for all sample types.

Protocol Notes

1. All steps should be performed on ice or at 4C. Pre-chill a centrifuge to 4C.
2. Make sure to add the correct amount of protease inhibitor tablets to the 6x HB unstable solution
3. See the buffer composition and order list at the end of the protocol for suppliers and catalog numbers
4. RNAse inhibitor(with 1mM DTT) is critical for ATAC+RNA assays, do not reduce concentration below 1U/ul in any buffer

Protocol

1. Place frozen tissue into a pre-chilled 1 ml Dounce with 1 ml cold 1x Homogenization Buffer (HB)
2. Allow frozen tissue to thaw for 5 minutes. Often the tissue chunk will sink to the bottom of the Dounce once thawed.
3. Dounce with A pestle until resistance goes away (~ 10 strokes). If large chunks of connective tissue are still present, you may need to pre-clear by filtration via a 100 um nylon mesh filter.
4. Dounce with B pestle for 20 strokes, wash Dounce with 1mL, obtain 2mL of suspension.
5. Pre-clear larger chunks by pelleting at 100 g for 1 min in a pre-chilled centrifuge.
6. Avoiding pelleted chunks of connective tissue, transfer 400 ul to a round bottom 2 ml Lo- Bind Eppendorf tube
7. Add 1 volume (400 ul) of 50% Iodixanol solution to give a final concentration of 25% Iodixanol and mix by pipetting.
8. Layer 600 ul of 29% Iodixanol solution under the 25% mixture. Avoid mixing of layers.
9. Layer 600 ul of 35% Iodixanol solution under the 29% mixture. Avoid mixing of layers. This step requires gradual removal of the pipette tip during pipetting to avoid excessive volume displacement.
10. In a swinging bucket centrifuge, spin for 20 min at 3,000 RCF with the brake off (5 acceleration, 0 deceleration). Total time: ~35mins.
11. Aspirate the top layers down to within 300 ul of the nuclei band
12. Setting the pipette to 200 ul volume, collect the nuclei band and transfer it to a fresh tube (collect the interphase between 29% and 35% Iodixanol)
13. Resuspend nuclei band in 2 ml of nuclei wash buffer. Spin at 500g for 5mins.
14. Discard supernatant without disturbing the pellet. Resuspend the nuclei pellet in 25uL *Modified Nuclei Buffer.*
15. Pipet 95 µL of *cold Sample Buffer* into a new 2 mL LoBind tube.
16. Ensure the nuclei are well suspended by gently pipet-mixing with a wide-bore pipette tip and pipet 5 µL of nuclei suspension (from step 14) and into the tube with 95 µL *cold Sample Buffer*.
17. Keep the remaining nuclei on ice.
18. Pipet 0.5 µL of 5 mM DyeCycle Green and transfer to the tube.
19. Gently pipet-mix with a wide bore tip 10 times and incubate on ice for 5 minutes to stain the nuclei, protected from light.
20. Count the stained nuclei immediately using the BD Rhapsody™ Scanner. Refer to the BD Rhapsody™ HT Single-Cell Capture and Analysis System Single-Cell Capture and cDNA Synthesis Protocol (Doc ID: 23-24252). The steps are described below briefly:
    - Pipet 10 µL of well-mixed nuclei suspension into the INCYTO disposable hemocytometer and count with the scanner.

- Multiply the reading by 20 to calculate the concentration of unstained nuclei.
- If the unstained nuclei concentration is >10,000 nuclei/µL, dilute the nuclei to 10,000 nuclei/µL with *Modified Nuclei Buffer a*nd keep on ice.
- If the unstained nuclei concentration is <10,000 nuclei/µL, keep on ice. Adjust the nuclei volume and nuclease-free water in the tagmentation reaction.

21. Proceed immediately to the tagmentation step by following protocols Single-Cell ATAC-Seq and mRNA Whole Transcriptome Analysis Library Preparation Protocol Doc ID: 23-24474 (for WTA+ATAC) or Single-Cell ATAC-Seq Library Preparation Protocol Doc ID: 23-24473(for ATAC only)

Stock Buffer Preparations

Prepare these two buffers in advance. These buffers are stable at room temperature. Sterile filtration is recommended, especially for sucrose.

6x Homogenization Buffer Stable Master Mix (1200 ul total volume)

Reagent	Final Conc.	Volume
1M CaCl2	30 mM	36uL
1M Mg (Ac)2	18 mM	21.6uL
1M Tris pH 7.8	60 mM	72uL
H2O		1070.4uL

1M Sucrose

Add 34.23 g sucrose to 78.5 ml water.

Same Day Buffer Preparations

Prepare these buffers on the day of processing. Maintain on ice. Volumes listed are for one sample and with 20% excess, unless noted otherwise.

6x Homogenization Buffer Unstable Solution (650 ul per sample)

Reagent	Final Conc.	Volume (in ul)
6x Homogenization Buffer Stable	6x	778.61
100 mM PMSF	0.1 mM	1.30
14.3 M β-mercaptoethanol	1 mM	0.10

1x Homogenization Buffer Unstable Solution (2 ml per sample)

Reagent	Final Conc.	Volume (in ul)
6x Homogenization Buffer Unstable	1x	400
1M Sucrose	320 mM	768
500 mM EDTA	0.1 mM	0.48
10% IGEPAL	0.1%	24
40 U/ul RNase inhibitor *(needs to be purchased from 3rd party vendor)*	*1U/uL*	60
1M DTT *(needs to be purchased from 3rd party vendor)*	*1mM*	2.4
H2O		1145

50% Iodixanol Solution (400 ul per sample)

Reagent	Final Conc.	Volume in ul
6x Homogenization Buffer Unstable	1x	80
60% Iodixanol Solution	50%	400

29% Iodixanol Solution (600 ul per sample)

Reagent	Final Conc.	Volume in ul
6x Homogenization Buffer Unstable	1x	120
1M Sucrose	160 mM	115.2
60% Iodixanol Solution	29%	348
40 U/ul RNase inhibitor *(needs to be purchased from 3rd party vendor)*	1U/uL	18
1M DTT *(needs to be purchased from 3rd party vendor)*	*1mM*	0.72
H2O		118.08

35% Iodixanol Solution (600 ul per sample)

Reagent	Final Conc.	Volume in ul
6x Homogenization Buffer Unstable	1x	120
1M Sucrose	160 mM	115.2
60% Iodixanol Solution	35%	420
40U/ul RNase inhibitor *(needs to be purchased from 3rd party vendor)*	1U/uL	18
1M DTT *(needs to be purchased from 3rd party vendor)*	*1mM*	0.72
H2O		46.08

Nuclei Wash Buffer: (2 ml per sample)

Reagent	Final Conc.	Volume in ul
1 M Tris-HCl, pH 7.4	10 mM	24
5M NaCl	10 mM	4.8
1M MgCl2	3mM	7.2
10% Tween-20	0.1%	24
40 U/ul RNase inhibitor *(needs to be purchased from 3rd party vendor)*	1U/uL	60
1M DTT *(needs to be purchased from 3rd party vendor)*	*1mM*	2.4
H2O		2277.6

Modified Nuclei Buffer (makes 200 ul total volume, use as needed in the protocol):

Reagent	BD Product Number	Volume
Nuclei Buffer (provided by BD)	51-9023091	193 µL
RNase inhibitor (provided by BD)	51-9024039	5 µL
0.1M DTT (provided by BD)	51-9022688	2 µL

To save costs, RNase inhibitor and DTT can be left out of all buffers above if you are interested in standalone ATAC only and not interested in the RNA(WTA) readout. Do not forget to replace the volume of RNase inhibitor and DTT with equal volume of nuclease-free water.

If you are interested in standalone ATAC only and not interested in the RNA(WTA) readout, *modified nuclei buffer* can be replaced by just the nuclei buffer (BD Product Number 51-**9023091) at the same volume.**

Order List

Item	Supplier	Cat Number
Eppendorf 2 ml Lo-Bind tubes	Sigma	Z666556-250EA
Iodixanol (aka Optiprep)	Sigma	D1556-250ML
Sucrose	Sigma	S7903-250G
IGEPAL CA-630	Sigma	i8896
EDTA	Ambion (Thermo)	AM9261
β-mercaptoethanol	Sigma	M6250-100ML
PMSF	Sigma	P7626-1G
$CaCl_2$	Sigma	21115-100ML
$Mg(Ac)_2$	Sigma	63052-100ML
Tris pH 7.8	Sigma	T2569
Tris pH 7.4	Sigma	T2194
$MgCl_2$	Ambion (Thermo)	AM9530G
Nuclease free water	Invitrogen or any major supplier	10977-015
2 ml Dounce Tissue Grinder Set	Sigma	D8938-1SET
150 ml, 0.2 um PES filters	Thermo	124-0045
Tween-20, 10%	Sigma/Roche	11332465001
Sodium Chloride Solution, 5 M	Sigma	59222C
RNase Inhibitor (40 U/µL) *	NEB	M0314L
	Watchmaker Genomics	*7K0088-500UL*
1 M DTT*	Sigma	646563
Disposable hemocytometer	INCYTO	DHC-N01-5
5 mM DyeCycle™Green	ThermoFisher Scientific	V35004

<u>Gather these reagents provided by BD Biosciences</u>

- Sample Buffer (650000062) from the BD Rhapsody™ Enhanced Cartridge Reagent Kit V3(667052)
- Nuclei Buffer(51-9023091) from the BD Rhapsody™ ATAC-Seq Tagmentation and Supplemental Reagents Kit(571201)
- 0.1M DTT(51-9022688)* and RNase Inhibitor(51-9024039)* from the BD Rhapsody™ Multiomic ATAC-Seq Amplification Kit(571361)

** Not needed for standalone ATAC protocol (no WTA/RNA readout)*

Protocol for Isolation of nuclei from whole cells (PBMCs and Cell Lines) for ATAC-Seq

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Protocol Notes

1. All steps should be performed on ice or at 4C. Pre-chill a centrifuge to 4C.
2. See the buffer composition and order list at the end of the protocol for suppliers and catalog numbers
3. Make sure your cells are viable! We recommend viability above 90% and preferably around 95%.
    - For samples with viability between 80-90%, treat cells in culture medium with DNase (Worthington cat# LS002007) at a final concentration of 200 U/ml. Resuspend DNase in Hanks Balanced Salt Solution. DNase needs divalent cations so treat cells in culture media that lacks EDTA. Treat for 30 minutes at 37°C. Wash thoroughly with 1x PBS(No Ca/Mg) to remove DNase prior to proceeding to ATAC-seq transposition reaction.
    - If viability is still a problem, either sort for live cells or use a magnetic bead depletion based on Annexin V (Miltenyi cat# 130-090-201). Be sure to verify sample viability after sorting or bead based to make sure the desired viability has been achieved.
4. RNAse inhibitor (with 1mM DTT) is critical for ATAC+RNA assays, do not reduce concentration below 1U/ul.

Protocol

1. Transfer cell suspension containing 500K cells (> 90% viable) into a 2-mL Lo-Bind tube.
2. Centrifuge the cells at 300 x g for 5 minutes at 4 °C.
3. Carefully remove the tube from the centrifuge, remove and discard the supernatant without disturbing the cell pellet.
4. Resuspend the cell pellet with 1 mL of 1X PBS without Ca/Mg.
5. Centrifuge the cells at 300 x g for 5 minutes at 4 °C.
6. Carefully remove the tube from the centrifuge, completely remove the supernatant without disturbing the cell pellet.
7. On ice, add 200 µL of freshly prepared Nuclei Lysis Buffer into the tube. Make sure the cell pellet is completely submerged in Lysis Buffer.
8. Resuspend the cells by gentle pipetting up and down for 5 times, and then incubate on ice for 5 minutes (for cell lines) and 3 min (for PBMCs).

Different cell lines may require different lysis times. The lysis times listed here should be used a starting point and should be optimized for your sample type

9. At the end of lysis time, immediately add 2 mL of freshly prepared Nuclei Wash Buffer into the tube to stop the lysis reaction
10. Mix by inverting the tube 5 times.
11. Centrifuge the tube at 500 x g for 5 minutes at 4 °C.
12. Carefully remove the tube from the centrifuge and discard supernatant without disturbing the nuclei pellet.

Some residual buffer should be left behind to avoid accidentally aspirating removing the nuclei pellet.

13. Add 25 µL of the Modified Nuclei Buffer.
14. Resuspend the nuclei pellet by gently pipetting up and down 10 times with a wide-bore tip. Keep it on ice constantly.
15. Pipet 95 µL of cold sample buffer into a new 2-mL LoBind tube.
16. Ensure the nuclei are well suspended by gently pipet-mixing with a wide-bore pipette tip and pipet 5 µL of nuclei suspension (from step 14) and transfer into the tube with 95 µL cold sample buffer
17. Keep the remaining nuclei on ice.
18. Pipet 0.5 µL of 5 mM DyeCycle Green and transfer to the tube.
19. Gently pipet-mix with a wide bore tip 10 times and incubate on ice for 5 minutes to stain the nuclei, protected from light.
20. Count the stained nuclei immediately using the BD Rhapsody™ Scanner. Refer to the BD Rhapsody™ HT Single-Cell Capture and Analysis System Single-Cell Capture and cDNA Synthesis Protocol (Doc ID: 23-24252). The steps are described below briefly:
21. Pipet 10 µL of well-mixed nuclei suspension into the INCYTO disposable hemocytometer and count with the scanner.
22. Multiply the reading by 20 to calculate the concentration of unstained nuclei.
23. If the unstained nuclei concentration is >10,000 nuclei/µL, dilute the nuclei to 10,000 nuclei/µL with Modified Nuclei Buffer and keep on ice.
24. If the unstained nuclei concentration is <10,000 nuclei/µL, keep on ice. Adjust the nuclei volume and nuclease-free water in the Tagmentation reaction.
25. Proceed immediately to the tagementation step by following protocols Single-Cell ATAC-Seq and mRNA Whole Transcriptome Analysis Library Preparation Protocol Doc ID: 23-24474 (for WTA+ATAC) or Single-Cell ATAC-Seq Library Preparation Protocol Doc ID: 23-24473(for ATAC only)

Buffers and Reagents:

Prepare these buffers on the day of processing. Maintain on ice. Volumes are for one sample and with 20% excess, unless noted otherwise.

Nuclei Lysis Buffer (200 ul per sample)

Reagent	**Final Conc.**	**Volume (uL)**
1 M Tris-HCl, pH 7.4	10 mM	2.4
5M NaCl	10 mM	0.48
1M $MgCl_2$	3mM	0.72
10% IGEPAL	0.1%	2.4
10% Tween20	0.1%	2.4
2% Digitonin	0.01%	1.2
40 U/ul RNase inhibitor *(needs to be purchased from 3rd party vendor)*	1U/uL	6
1M DTT *(needs to be purchased from 3rd party vendor)*	*1mM*	0.24
H2O		224.16

Nuclei Wash Buffer: (2 ml per sample)

Reagent	**Final Conc.**	**Volume in ul**
1 M Tris-HCl, pH 7.4	10 mM	24
5M NaCl	10 mM	4.8
1M $MgCl_2$	3mM	7.2
10% Tween-20	0.1%	24
40 U/ul RNase inhibitor *(needs to be purchased from 3rd party vendor)*	1U/uL	60
1M DTT *(needs to be purchased from 3rd party vendor)*	*1mM*	2.4
H2O		2277.6

Modified Nuclei Buffer (makes 200 ul total volume, use as needed in the protocol):

Reagent	**BD Product Number**	**Volume**
Nuclei Buffer (provided by BD)	51-9023091	193 µL
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If you are interested in standalone ATAC only and not interested in the RNA(WTA) readout, *modified nuclei buffer* can be replaced by just the *nuclei buffer* (BD Product Number 51-9023091) at the same volume.

Order List

Item	Supplier	Cat Number
Eppendorf 2 ml Lo-Bind tubes	Sigma	Z666556-250EA
$MgCl_2$	Ambion (Thermo)	AM9530G
Nuclease free water	Invitrogen or any major supplier	10977-015
Tween-20, 10%	Sigma/Roche	11332465001
Sodium Chloride Solution, 5 M	Sigma	59222C
RNase Inhibitor (40 U/µL) *	NEB	M0314L
	Watchmaker Genomics	*7K0088-500UL*
1 M DTT*	Sigma	646563
Disposable hemocytometer	INCYTO	DHC-N01-5
Tris pH 7.4	Sigma	T2194
IGEPAL CA-630	Sigma	i8896
2% Digitonin	Promega	G9441
5 mM DyeCycle™Green	ThermoFisher Scientific	V35004
DPBS, no calcium, no magnesium	ThermoFisher Scientific	14190144

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- Nuclei Buffer (51-9023091) from the BD Rhapsody™ ATAC-Seq Tagmentation and Supplemental Reagents Kit(571201)
- 0.1M DTT (51-9022688)* and RNase Inhibitor(51-9024039)* from the BD Rhapsody™ Multiomic ATAC-Seq Amplification Kit(571361)

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