scATAC Pre-Indexing Kit

(24-Plex)

Laboratory Protocol

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Introduction

The ScaleBio scATAC Pre-Indexing Kit provides a complementary system to increase sample and cell throughput for on-market single cell analysis systems. When used in combination with the Chromium Next GEM Single Cell ATAC Reagent Kit (1000175/1000176) the scATAC Pre-Indexing Kit can enable users to pool up to 24 samples and load up to 100,000 nuclei per channel of 10X Chromium while maintaining a low effective doublet rate. This is accomplished by indexing samples upstream of the 10X workflow using indexed tagmentation, followed by super loading of the pooled and indexed nuclei onto the 10X Genomics Chromium platform, then purifying and sequencing libraries in accordance with the Chromium Next GEM Single Cell ATAC manual (Figure 2). Importantly, in addition to sample identification the Scale indexed tagmentation allows users to distinguish molecules from two or more indexed nuclei within the same droplet, allowing users to super load each 10X Chromium channel and producing a final output of up to 300,000 nuclei from one Scale Pre-Indexing Kit.

Figure 1. Overview of the split-pool scheme for scATAC-seq using ScaleBio

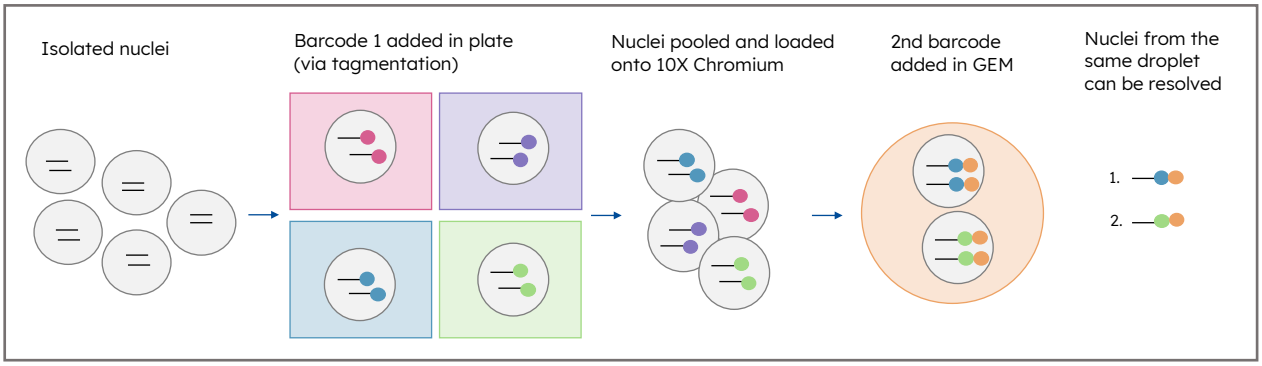
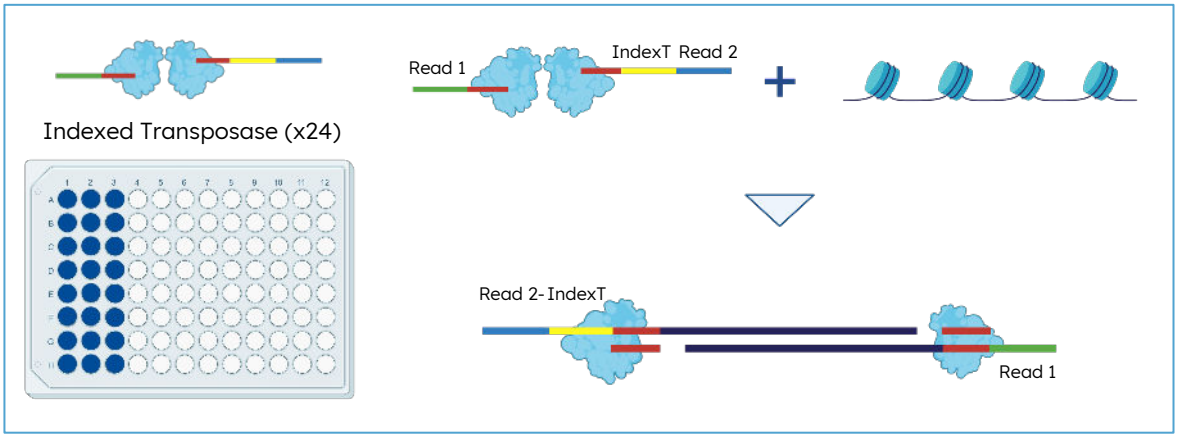
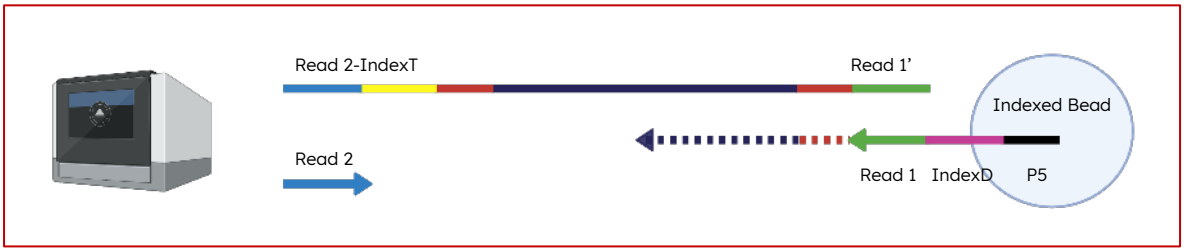


Figure 2. Schematic of the scATAC Pre-Indexing kit with the 10X Chromium Next GEM Single-cell ATAC Reagent Kit and the Chromium™ system.

Indexed Tagmentation



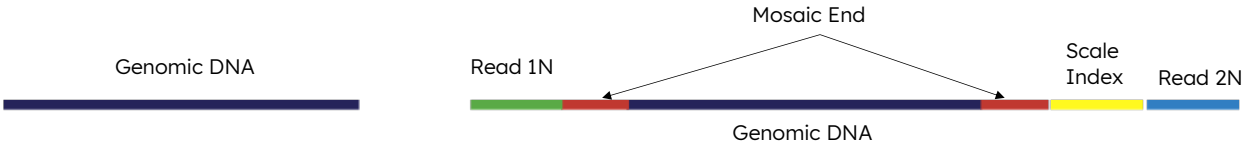
Indexing on 10X Chromium



Sequencing library PCR amplification

Background

Following nuclei extraction, tagmentation is performed with a set of indexed transposomes (TSMs) containing a DNA transposon adaptor. ScaleBio plates contain 24 uniquely indexed wells; users can load up to 50,000 nuclei in each well.



Following tagmentation nuclei are pooled and up to 100,000 nuclei are loaded into each channel of the Chromium Next GEM Chip. Once encapsulated within GEMs a complementa11ry sequence on the TSM allows for capture and addition of a 10X Barcode specific to the GEM.

GEMs from each channel are then processed in accordance with the Chromium Next GEM Single Cell ATAC protocol. Finally, libraries are PCR amplified with the indexed primers supplied with the ScaleBio scATAC Pre-Indexing kit, allowing for pooling of channels during sequencing.

After sequencing of the libraries, the ScaleBio bioinformatics pipeline can be used to process sequencing data, assigning reads to a sample and an individual nucleus, and providing basic ATAC-seq QC metrics. The combination of the Scale Index and 10X Barcode allow multiple nuclei captured within the same GEM to be distinguished the majority of the time. Output files from the pipeline can then be used for further downstream analysis.

scATAC Pre-Indexing Kit Contents

Item	Number/Volume	Part Number	Storage Temp
Indexed Tagmentation Plate (ITP)	1 plate/5 µl per well	110011	-20°C
3x Tagmentation Buffer (ETB3)	1/600 µl	110021	-20°C
Loading Buffer (LB)	1/300 µl	110041	-20°C

Additional items – ScaleBio

Item	Number/Volume	Part Number	Storage Temp
Set of is700 primers	8/25 µl per tube	110101	-20°C

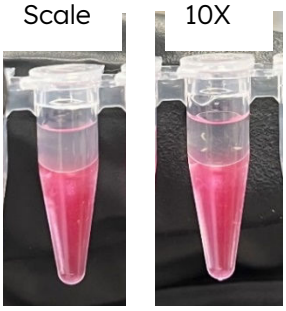
Additional Requirements

The following materials are required for tagmentation and Chromium indexing. Note that this list does not include items required for nuclei extraction. Similar items from other suppliers may also be appropriate, however optimization may be required.

Consumables		
Item	**Supplier**	**Part Number**
Chromium Next GEM Single Cell ATAC Library & Gel Bead Kit v1.1 or v2	10X Genomics	16 rxns, 1000175 4 rxns, 1000176
Chromium Next GEM Chip H Single Cell Kit	10X Genomics	48 rxns, 1000161 16 rxns, 1000162
Nuclease free water	Ambion	AM9932
Countess cell counting chamber slides	ThermoFisher	C10228
High Sensitivity DNA kit	Agilent	5067-4626
8-well strip tubes	USA Scientific	1402-4700
DNA LoBind 1.5 mL tubes	Eppendorf	022431021
DNA LoBind 2.0 mL tubes	Eppendorf	022431048
15 mL conical tubes	VWR	10025-686
Flowmi 40 µm cell strainers for 1000 µl pipette tips	Bel-Art	H13680-0040
Equipment		
Item	**Supplier**	**Part Number**
Countess II FL Automated Cell Counter	ThermoFisher	AMQAF1000
Centrifuge for plates with temperature control	Eppendorf Sorvall	022627040 75004240
Benchtop centrifuge with temperature control	Fisher Scientific	13-100-676
Bioanalyzer	Agilent	G2939BA
Disposable reagent reservoir	VWR	89094-658
Magnetic stand for bead collection	10X Genomics	120250
Thermal cycler for 96-well plates	Bio-Rad	1861096
Microseal B plate sealer	Bio-Rad	MSB1001
Plate shaker	IKA	0003319000
Vortex Genie 2	Scientific Industries	SI-0236
Metal or plastic 96-well PCR rack	Various	Various

Notes/Before you begin

1. Transfer of ITP contents to alternative plates/tubes prior to tagmentation is not recommended as this can decrease assay sensitivity. Additionally, storage of contents from unused wells is not recommended as additional freeze-thaw cycles may decrease tagmentation efficiency.
2. 10X Genomics 20X Nuclei Buffer (2000207) is recommended but nuclei may be maintained on ice in a variety of buffers depending on cell type and chosen extraction method. If using an alternative buffer, please contact ScaleBio to confirm downstream compatibility.
3. If extensive clumping is observed while quantifying nuclei with 0.04% Trypan blue, attempt quantification without the use of Trypan blue as this may reduce clumping and improve accuracy.
4. Filtering of nuclei prior to tagmentation (if not performed during nuclei extraction) and prior to loading of nuclei into 10X Chromium is optional if significant clumping is observed during quantification. Pass nuclei through Flowmi 40 µm filter and quantify again. Note that this will result in some nuclei loss.
5. For ease of workflow, if using all 24 wells for a single sample do not add 5 µl ETB3 to each well. Instead combine 150 µl of nuclei at the appropriate dilution with 150 µl ETB3, then distribute 10 µl of this combination to each well.
6. To ensure even distribution of nuclei, gently flick the tube containing nuclei prior to pipetting and pipette from the center of the volume.
7. Different cell types may require different centrifugation conditions due to size or other variables. It is recommended to optimize centrifugation speed and time for your cell type prior to starting the protocol. Keep in mind that tagmentation also affects nuclei sedimentation properties and we recommend centrifugation at 300 x g for 7 minutes as a safe starting point. Use of a fixed angle centrifuge following tagmentation is recommended to increase visibility of pellet.
8. Be careful to not disturb pellet when aspirating supernatant. Always aspirate with a pipette, not vacuum aspirators. First aspirate majority of supernatant leaving behind a small quantity, then switch to a lower volume pipette to aspirate the remaining volume. If pellet is aspirated, dispense supernatant back into original tube and repeat centrifugation, or cell recovery will be decreased significantly.
9. Addition of Recovery Agent (Post Gem Incubation Cleanup Step 3.1a) may cause the aqueous phase to appear cloudy after mixing, this is normal when using the scATAC Pre-Indexing Kit.



Nuclei Extraction

Nuclei extraction procedures will differ based upon cell source and type. Please refer to the following materials for best practices:

Isolation of nuclei from frozen tissue for ATAC-seq and other epigenomic assays (protocols.io)

Single Cell ATAC - 10x Genomics

CG000212_SingleCellATAC_Nuclei_Isolation_MouseBrain_DemonstratedProtocol_RevB.pdf (ctfassets.net)

CG000169_DemonstratedProtocol_NucleiIsolation_ATAC_Sequencing_RevD.pdf (ctfassets.net)

CG000053_CellPrepGuide_RevC.pdf (ctfassets.net)

CG000209_Chromium_NextGEM_SingleCell_ATAC_ReagentKits_v1.1_UserGuide_RevD.pdf (ctfassets.net)

Tagmentation and Indexing

A. Tagmentation

Item	Part Number	Preparation	Storage Temp
Indexed Tagmentation Plate (ITP)	110011	• Thaw on ice • Vortex 15 sec • Spin 2000 x g, 5 sec • Keep on ice	-20°C
3x Tagmentation Buffer (ETB3)	110021	• Thaw on ice • Vortex 15 sec • Spin 2000 x g, 5 sec • Keep on ice	-20°C
Nuclei	N/A	Keep on ice	N/A

1. Prepare aliquot for counting. Dilute nuclei 10-fold by adding 2 µl of nuclei to 18 µl of 1X Diluted Nuclei Buffer. (If using an alternative nuclei buffer, see Note 2 on page 6). Mix with 20 µl 0.4% trypan blue solution prior to quantifying nuclei using cell counter or hemocytometer.

   ! **Important:** Perform 2 counts to achieve an accurate estimate. See Notes 3 and 4 on page 6.

2. Dilute nuclei to desired concentration in 1X Diluted Nuclei Buffer (see Nuclei concentration table, page X).
3. Remove the ITP plate from ice and place it into a 96-well PCR rack for improved grip during seal removal.
4. Carefully remove the aluminum seal, holding the edge of the ITP plate firmly while pulling back diagonally. Place plate back on ice.
5. Add 5 µl ETB3 to each well followed by 5 µl nuclei dilution (See Notes 5 and 6). Mix by gentle pipetting up and down, 3 times.

   ! **Caution:** Change pipette tips between wells to prevent cross-contamination of tagmentation indices.

6. Seal the ITP plate using a Microseal B. **A poor seal can result in evaporation and loss of sample.**
7. Incubate at 37°C for 60 minutes in thermocycler block with a heated lid (47°C).
8. While incubating the plate, thaw the Loading Buffer (LB) on ice.

B. Stop Tagmentation

Item	Part Number	Preparation	Storage Temp
Loading Buffer (LB)	110041	• Thaw on ice • Vortex 15 sec • Spin 2000 x g, 5 sec • Keep on ice	-20°C

1. Remove the ITP plate from the thermocycler.
2. Incubate the plate for 5 minutes before removing the seal from the plate.
3. Pipette up and down 3 times to resuspend nuclei prior to removing and pooling contents of each well. Transfer all the volume to a fresh 1.7 mL microcentrifuge tube.

There is no need to change tips during pooling.

4. Centrifuge nuclei at 300 x g for 5 minutes at 4°C. (See Note 7)
5. Completely remove supernatant **without disturbing the pellet**. (See Note 8).
6. Resuspend nuclei pellet in LB by inverting and gently flicking. Use 4 µl LB per 100,000 nuclei originally loaded into the ITP plate. If the pellet is not fully resuspended, perform gently pipetting up and down 5-10 times.
7. Prepare aliquot for counting. Dilute nuclei 10-fold by adding 2 µl of nuclei to 18 µl of LB Buffer. Mix with 20 µl 0.4% trypan blue solution prior to quantifying nuclei using cell counter or hemocytometer.

Important: Perform 2 counts to achieve an accurate estimate. See Notes 3 and 4 on page 6.

8. Prepare 100,000 nuclei for loading in 15 µl of LB buffer by preparing a nuclei dilution of 7,142 nuclei/µl. This 15 µl will be combined with 60 µl of 10X Master Mix prior to loading on the 10X Chromium.

If not loading 100,000 per channel, divide desired loading by 14 to calculate the appropriate nuclei concentration per µl.

C. 10X Chromium Indexing

Proceed from Step 2, page 24 of Chromium Next GEM Single Cell ATAC Reagent Kits v1.1/v2 protocol with the following modifications:

1. Step 2.5a: Limit the number of cycles to **4**.
2. Step 3.1p: **Do Not Pipette Mix**, briefly vortex to mix.
3. Step 3.2a/k: **Do Not Pipette Mix**, briefly vortex to mix.
4. Step 3.2l: Incubate for **5 minutes** at room temperature.
5. Step 4.1c: Replace 10X single Index N Set A reagent with 2.5 µl of is700P primer. Use one ScaleBio is700P primer per 10X Chromium channel. This will allow for samples from different channels to be pooled and sequenced in the same sequencing run.
6. Step 4.1d: Limit the number of PCR cycles to **8**.
7. Step 4.2a/e/n: **Do Not Pipette Mix**, briefly vortex to mix.
8. Step 4.2o: Incubate for **5 minutes** at room temperature.

Appendix

Nuclei Concentration Table

Number of nuclei per well	Required nuclei concentration (nuclei/ µl)	Approximate final output of well (desired output)
20,000	4,000	5,000
25,000	5,000	6,250
30,000	6,000	7,500
35,000	7,000	8,750
40,000	8,000	10,000
45,000	9,000	11,250
50,000	10,000	12,500

Comparative results are obtained with 20,000 – 50,000 nuclei per ITP well, exceeding these limits is not recommended.

iTSM Index Layout

	1	2	3
A	GAACCGCG	AGGTTATA	TCATCCTT
B	TGGCCGGT	CAATTAAC	ATAATGTG
C	TCTGTTGG	CTCACCAA	TATTAGCT
D	ATGTAAGT	GCACGGAC	GGTACCTT
E	ATCCACTG	GCTTGTCA	CAAGCTAG
F	TAAGTGGT	CGGACAAC	ATATGGAT
G	GCTCATTG	ATCTGCCA	CTTGGTAT
H	GATCTATC	AGCTCGCT	CGGAACTG

ScaleBio Indexing Amplification Primers

Each S70XP primer contains an equal mix of 4 indexed primers to ensure equal base distribution and optimize sequencing quality.

Primer	Sequence 1	Sequence 2	Sequence 3	Sequence 4	Part Number
S701P	GGTCACCT	TAGACATC	CTCTGTGA	ACAGTGAG	110111
S702P	CAGAGAAT	AGCGCTTG	GTTCACGA	TCATTGCC	110121
S703P	AGGCCGAA	CCATAAGC	TACAGCTT	GTTGTTCG	110131
S704P	TATACTGA	CCGTGGAT	GGACACTG	ATCGTACC	110141
S705P	GGTCCAGA	CAAGGTCT	ACCTTGTG	TTGAACAC	110151
S706P	GGAACTAG	CTTCGAGC	TCCTAGCT	AAGGTCTA	110161
S707P	TCGGTACA	GGAACGGT	CATTGCTG	ATCCATAC	110171
S708P	GGTTCATG	CTAAGGAT	TACCATCA	ACGGTCGC	110181

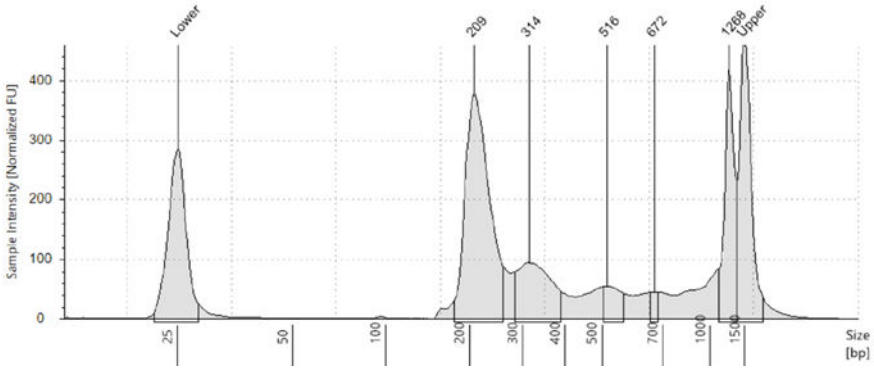
Post-Library QC

Representative Scale ATAC library size distribution on TapeStation High Sensitivity D1000 ScreenTape

Dilute samples based upon manufacturer's recommendations. Addition of an undiluted sample can better ensure visibility of mono- and di-nucleosome peaks.

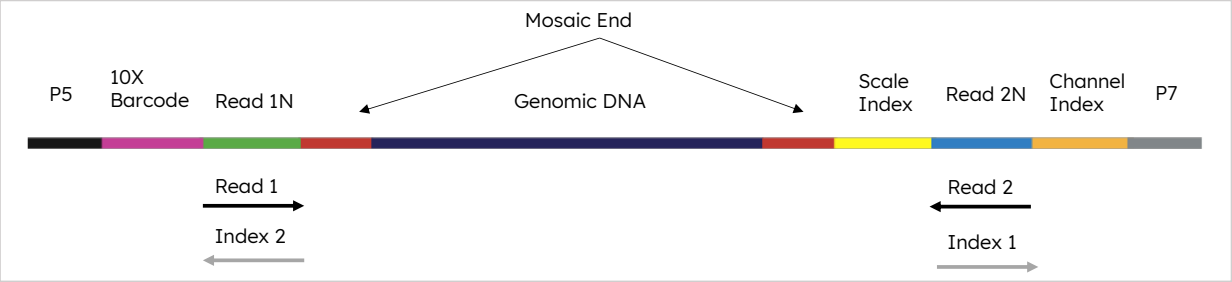
Approximate library concentrations may range between 2-10 ng/µl, but this will vary by nuclei loading and sample type.

Example TapeStation trace



Sequencing Diagram and Recommendations

Standard sequencing recipe with standard sequencing primers, no PhiX spike in.



Read	Read 1	Index 1 (i7)	Index 2 (i5)	Read 2	Total
Purpose	Genomic DNA	Channel Index	10X Barcode	Scale Index, ME, Genomic DNA	
Length (bp)	36	8	16	(8+19+36) = 63	123

Analysis Considerations

- Each single cell corresponds to a unique combination of *Scale Index* and *10X Barcode*
    - A single partition can contain multiple unique cells with different *Scale Index* sequences
    - If multiple *Channel Index* sequences are used for one library, these do not define single cells. The reads should be pooled (as in the 10X Chromium default workflow).
- All barcode sequences should be error-corrected allowing for at least 1 mismatch (*Hamming distance*) between read and barcode sequence
- The fixed 19 bp *ME* ` sequence should be trimmed from read 2 before alignment
- Unique fragments are based on the cell barcode (*Scale index* + *10X Barcode* sequence) and mapping position of both reads.
- The *10X Barcode* is read in the opposite direction from the 10X Chromium default; hence the barcode sequence list should be reverse complemented if using your own pipeline.