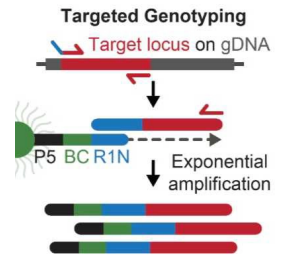


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GoT-ChA: Genotyping of Targeted loci with single-cell Chromatin Accessibility

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External link: <https://github.com/landau-lab/Gotcha>

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Protocol status: Working

We use this protocol and it's working

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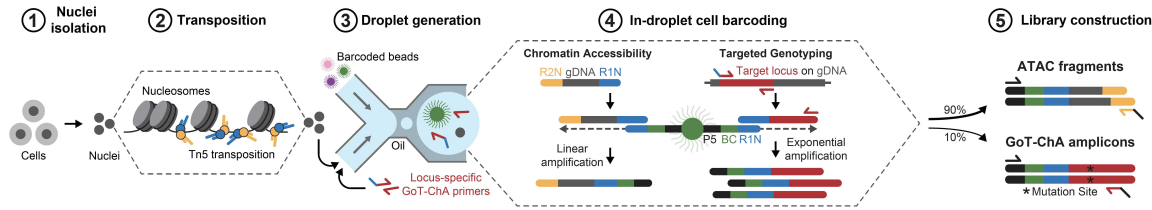
Abstract

Somatic mutations are crucial for cancer initiation and evolution, and have been identified across a number of healthy tissues in the human body. These mutations can disrupt normal cellular functions, leading to aberrant clonal expansions via acquired fitness advantages or skewed differentiation topologies. GoT-ChA and similar methods (e.g. GoT, TARGET-seq) aim to pair targeted genotyping with single-cell sequencing approaches in order to understand the impact of somatic mutations directly in human patient samples, in both malignant and non-malignant contexts.

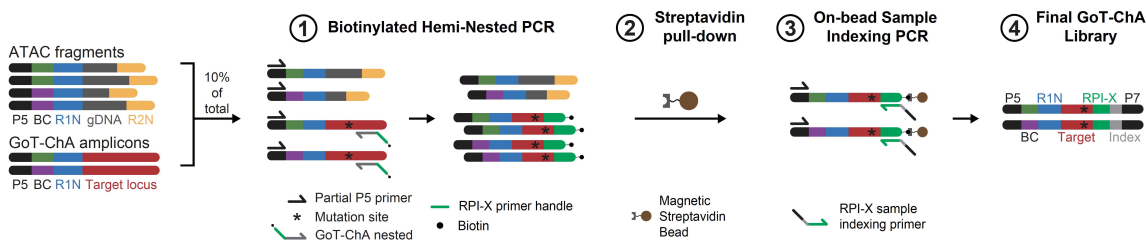
GoT-ChA is unique in that it is a high-throughput single-cell method that pairs targeted genotyping with chromatin accessibility measurements based on the broadly utilized scATAC-seq platform from 10x Genomics. Previous single-cell genotyping approaches were largely based on scRNA-seq protocols, utilizing expressed and captured mRNA transcripts as sources for genotyping information. This results in a limiting dependence on gene expression and mutation location (due to 3' end bias of most scRNA-seq methods), precluding the usage of such technologies on lowly expressed or inconveniently located loci of interest. GoT-ChA surmounts these limitations via direct utilization of genomic DNA for genotyping, whilst simultaneously assaying chromatin accessibility.

Guidelines

How does GoT-ChA work?



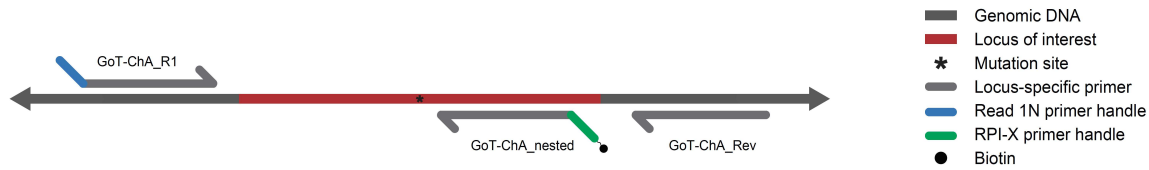
In order to capture genotypes within droplet-based scATAC-seq, two GoT-ChA primers are added into the cell barcoding PCR reaction that are designed to flank the locus of interest. One primer contains the partial Nextera Read 1N sequence in its handle, which allows for GoT-ChA amplicons to interact with the 10x Genomics gel bead oligonucleotide and obtain a unique cell barcode, just as tagmented chromatin fragments do. Further, the second GoT-ChA primer allows for exponential amplification of GoT-ChA amplicons while tagmented chromatin fragments are only linearly amplified.



After the single-cell emulsion is broken, a small portion of the sample is taken for GoT-ChA library construction, comprised of a hemi-nested PCR, biotin-streptavidin pull-down, and an on-bead sample indexing PCR. The final GoT-ChA library can be pooled with scATAC-seq libraries and sequenced together using standard ATAC sequencing parameters.

Before start

Designing a GoT-ChA experiment.



To utilize GoT-ChA, three primers need to be designed that flank the genomic region of interest. GoT-ChA_R1 (containing the partial Nextera Read1 sequence in its handle) and GoT-ChA_Rev flank the loci of interest, ideally forming an amplicon between 200-500 bps in size. GoT-ChA_nested is utilized in the hemi-nested PCR during GoT-ChA library construction, and crucially needs to bind within 50bp (inclusive of binding site) of the mutation to be covered with standard scATAC-seq sequencing parameters.



Buffer Preparations

- 1 Pre-chill swing-bucket centrifuge to 4°C
- 2 Pre-heat block to 65°C for later use dissolving digitonin

for GoT-ChA on Isolated Nuclei

3 Lysis Buffer

A	B	C	D	E
reagent	stock	final	μL for 1 mL (whole cell)	μL for 1 mL (Nuclei)
Tris-HCl (pH 7.4)	1 M	10 mM	10	10
NaCl	5 M	10 mM	2	2
MgCl ₂	1 M	3 mM	3	3
NP-40	10%	0.1%	10	10
BSA	10%	1%	100	100
Tween20	10%	0.1%	-	10ul
Digitonin	5%	0.01%	-	2ul
Nucelase-free H ₂ O			875	863

prepare fresh, keep at 4°C

4 Wash buffer

A	B	C	D	E
reagent	stock	final	μL for 1 mL	μL for 10 mL
Tris-HCl (pH 7.4)	1 M	10 mM	10	100
NaCl	5 M	10 mM	2	20
MgCl ₂	1 M	3 mM	3	30
BSA	10%	1%	100	1000
Tween 20 (for Nuclei prep only)	10%	0.1%	10	100



A	B	C	D	E
Nuclease-free H ₂ O			875/865	8750/8650

prepare fresh, keep at 4°C

5 **PBS + 0.04% BSA**

A	B	C	D
reagent	stock	final	µL for 1 mL
BSA	10%	0.04%	4
PBS			996

GoT-ChA on Permeabilized Whole Cells

- 6 Obtain all single cell suspensions (filter if needed) and measure viability and density (if viability is <90% proceed with live cell enrichment and/or use best judgement depending on sample source / importance / rarity).
- 7 Resuspend cells in 450 µl PBS.

if low cell number, scale down accordingly.
- 8 Add 30 µl 16% formaldehyde (1% f.c) and incubate 10min at room temperature, swirl, invert occasionally
- 9 Quench by adding glycine to 0.125M f.c.
- 10 Wash with 1x ice-cold PBS by filling up the tube, invert 5 times
- 11 Spin 5 minutes 400g at 4 °C.
- 12 Discard supernatant and repeat wash with 1ml 1x ice-cold PBS
- 13 Spin 5 minutes 400g at 4 °C, discard supernatant.





- 14 Resuspended cell pellet in 100 μ L chilled lysis buffer, mix by pipetting.
- 15 Incubate on ice for 3min (primary cells), 5min (cell lines)
- 16 Add 1 ml chilled wash buffer to the lysed cells, mix by pipetting
- 17 Spin 5 minutes 500g at 4 °C.
- 18 Remove supernatant, resuspend in 150 μ L 1x nuclei buffer (10x Genomics)
- 19 Filter through 40 μ m strainers
- 20 Use 2 μ L nuclei suspension mixed with 6 μ L Diluted Nuclei Buffer and 2 μ L trypan blue to determine nuclei concentration
- 21 Calculate amount of μ L for target number of recovered cells
(max volume for loading is 5 μ L for 10x transposition reaction)
- 22 Carry with Transposition for sc-experiment using 10x kit and follow the protocol. **(remember to spike in 1 μ L of combined Forward and Reverse (each at 22.5 μ M conc) to the emulsion Master Mix)**
corresponding to step 2.1 from 10X genomics ATAC user guide

GoT-ChA on Isolated Nuclei

- 23 Obtain all single cell suspensions (filter if needed) and measure viability and density (if viability is <90% proceed with live cell enrichment and/or use best judgement depending on sample source / importance / rarity).
- 24 Spin down at 300 x g for 5 minutes at 4 °C in PCR tubes if the number of cells are very low.
- 25 Remove supernatant and resuspend in 100 μ L room temperature PBS + BSA



if low number of cells, scale done volume

- 26 Spin down at 300 x g for 5 minutes at 4 °C
- 27 Resuspend cell pellet in 100 µL chilled lysis buffer and mix via pipetting
- 28 Incubate on ice for 3 minutes (primary cells) or 5 minutes (cell lines)
- 29 Add 1 ml chilled wash buffer to the lysed cells and mix via pipetting
- 30 Spin 5 minutes at 700 x g at 4 °C
- 31 Remove supernatant in two steps to not touch pellet
- 32 Resuspend the pellet in X µL of chilled Diluted Nuclei Buffer for a final volume close to 7-8 µL
- 33 Use 2 µL nuclei suspension mixed with 6 µL Diluted Nuclei Buffer and 2 µL trypan blue to determine nuclei concentration
- 34 Calculate amount of µL for target number of recovered cells
(max volume for loading is 5 µL for 10x transposition reaction)
- 35 Carry with Transposition for sc-experiment using 10x kit and follow the protocol. **(remember to spike in 1 µL of combined Forward and Reverse (each at 22.5µM conc) to the emulsion Master Mix)**
corresponding to step 2.1 from 10X genomics ATAC user guide

Emulsion generation

- 36 As per 10x protocol. **(remember to spike in Gotcha primers (22.5uM - 1ul each) per reaction during emulsion generation step)**



10X ATAC-seq		
	Temp	Duration
	72	5 min
	98	30s
12x	98	10s
	59	30s
	72	1 min
	15	Hold

GoT-ChA in Nuclei		
	Temp	Duration
	72	5 min
	98	30s
6x	98	10s
	59	2 mins 30s
	72	1 min
	15	Hold

GoT-ChA in Whole cells		
	Temp	Duration
	72	5 min
	98	40s
6x	59	2 mins 30s
	72	1 min
6x	98	10s
	59	30s
	72	1 min
	15	Hold

Step 2.5 from the 10X Genomics ATAC User guide

SPRI Clean up

- 37 Continue with SPRI clean up and elute DNA with 44.5ul of EB buffer. (40ul for ATAC and 4ul for GOTCHA)

Step 3.2 from the 10X Genomics ATAC User guide

- 38 Proceed to ATAC-library process as per 10x protocol with 40ul of eluted DNA.

Nested PCR

- 39 Perform 10-20 cycles of PCR with the following conditions **(with nested primer)**

40 GoTChA Nested PCR

A	B
KAPA reagents	1X (in µL)
DNA input	20 (4 DNA+16 Water)
p5 generic (10 µM)	2.5
GoTChA nested primer (10 µM)	2.5



A	B
M) Biotylated	
KAPA (2X)	25
<i>total volume:</i>	50

41 **GoTChA Nested PCR thermocycler program**

A	B	C
1 cycle	3 min	95°C
10-20 cycles	20 sec	95°C
	30 sec	65°C
	1 min	72°C
1 cycle	2 min	72°C
	hold	4°C

42 SPRI clean up with 1.2X. Elute in 40 µL.

Biotin- Pull Down

43 Allocate 15 µL of streptavidin M-280 beads per sample (+10%)

44 Wash beads three times with 100ul of 1x SSPE buffer

45 Resuspend in 5X SSPE buffer for 10 µL per sample (+10%)

46 Add 10 µL of beads to 40 µL of SPRI'd sample

47 Incubate at room temperature for 15 minutes

48 Wash two times with 100ul of 1x SSPE buffer

49 Wash one time with 100ul of 10 mM Tris HCl (pH 8.0)



50 Resuspend beads in 20 μ L

On-bead Sample Indexing PCR

51 Perform 6-10 cycles of PCR with the following conditions:

52 GoTChA Sample Index PCR

A	B
KAPA reagents	1X (in μ L)
DNA input	20
p5 generic (10 μ M)	2.5
RPI-X (10 μ M)	2.5
KAPA (2X)	25
amt to add:	30
total volume:	50

53 GoTChA Sample Index thermocycler program

A	B	C
1 cycle	3 min	95°C
10-20 cycles	20 sec	95°C
	30 sec	65°C
	1 min	72°C
1 cycle	2 min	72°C
	hold	4°C

54 Run an e-gel to assess correct product formation. Use 5 μ L (10%) of total sample

55 Purify libraries using AMPureXP beads at a 1.2X ratio

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1000000

14 JULY 2004



14 JULY 2005

1000000

1000000



Protocol references

Izzo F, Myers RM, Ganesan S, Mekerishvili L, Kottapalli S, Prieto T, Eton EO, Botella T, Dunbar AJ, Bowman RL, Sotelo J, Potenski C, Mimitou EP, Stahl M, El Ghaity-Beckley S, Arandela J, Raviram R, Choi DC, Hoffman R, Chaligné R, Abdel-Wahab O, Smibert P, Ghobrial IM, Scandura JM, Marcellino B, Levine RL, Landau DA. Mapping genotypes to chromatin accessibility profiles in single cells. *Nature*. 2024 May;629(8014):1149-1157. doi: 10.1038/s41586-024-07388-y. Epub 2024 May 8. PMID: 38720070; PMCID: PMC11139586.