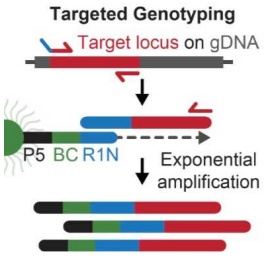
Oct 05, 2024

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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We use this protocol and it's working

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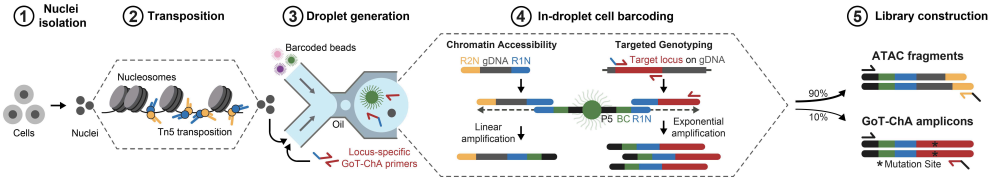
Abstract

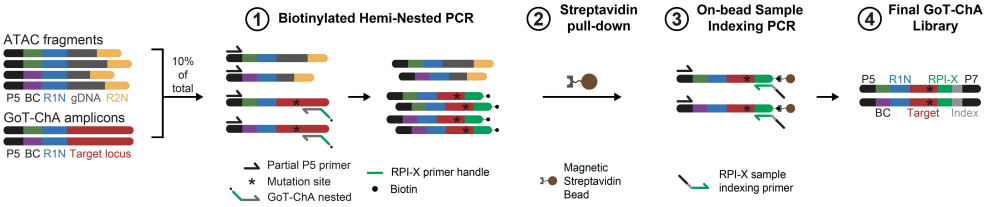
Somatic mutations are crucial for cancer initiation and evolution, and have been identified across a number of healthy tissues in the human body. These mutations can disrupt normal cellular functions, leading to aberrant clonal expansions via acquired fitness advantages or skewed differentiation topologies. 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.

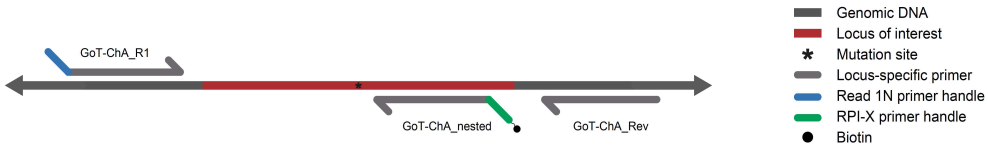


After the single-cell emulsion is broken, a small portion of the sample is taken for GoT-ChA library construction, comprised of a hemi-nested PCR, biotin-streptavidin pull-down, and an on-bead sample indexing PCR. The final GoT-ChA library can be pooled with scATAC-seq libraries and sequenced together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

Troubleshooting

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
	MgCl2	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 H2O			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

	A	B	C	D	E
	MgCl2	1 M	3 mM	3	30
	BSA	10%	1%	100	1000
	Tween 20 (for Nuclei prep only)	10%	0.1%	10	100
	Nuclease-free H20			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℃.

12 Discard supernatant and repeat wash with 1ml 1x ice-cold PBS

13 Spin 5 minutes 400g at 4℃, discard supernatant.

14 Resuspended cell pellet in 100 µl 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℃.

18 Remove supernatant, resuspend in 150 µl 1x nuclei buffer (10x Genomics)

19 Filter through 40 µm strainers

20 Use 2 µL nuclei suspension mixed with 6 µL Diluted Nuclei Buffer and 2 µL trypan blue to determine nuclei concentration

21 Calculate amount of µL for target number of recovered cells

(max volume for loading is 5 µL for 10x transposition reaction)

22 Carry with Transposition for sc-experiment using 10x kit and follow the protocol. **(remember to spike in 1µL 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

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 µL 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 1µL 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
6x	98	10s
	59	30s
	72	1 min
	15	Hold

GoT-ChA in Whole cells		
	Temp	Duration
	72	5 min
6x	98	40s
	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) Biotiylated	2.5
	KAPA (2X)	25
	total volume:	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 µL

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 µL)
	DNA input	20
	p5 generic (10 µM)	2.5

	A	B
	RPI-X (10 µM)	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 µL (10%) of total sample

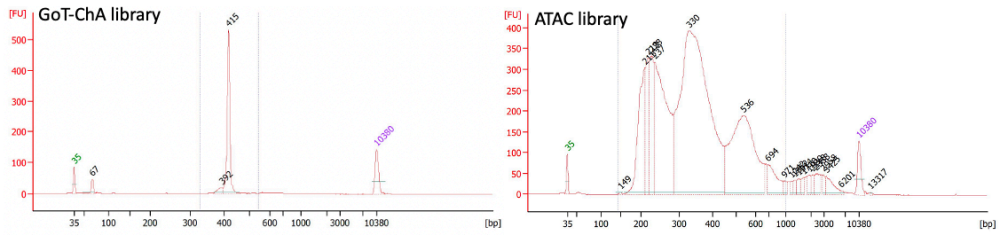
55 Purify libraries using AMPureXP beads at a 1.2X ratio

56 Elute in 20 µL of EB or Water

Assessing library quality

57 Run a BioA

Expected results:



Representative bioanalyzer traces of GoT-ChA genotyping (left) and GoT-ChA scATAC (rigth) libraries. FU, fluorescent units.

58 Measure samples via Qubit

59 Dilute samples for sequencing. GoT-ChA libraries can be sequenced together with ATAC libraries assuming i7 indexes do not clash. Sequencing parameters are as follows Read 1N - 50 cycles, i7 index -8 cycles, i5 Index - 16 cycles. Read 2N - 50 cycles.

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.