



Single-cell mapping of lineage and identity via CellTagging

Version 3

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dx.doi.org/10.17504/protocols.io.vawe2fe

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ABSTRACT

Single-cell technologies are offering unprecedented insight into complex biology, revealing the behavior of rare cell populations that are typically masked in bulk population analyses. One current limitation of single-cell approaches is that lineage relationships are lost as a result of cell processing, restricting interpretations of the data collected. Elegant computational approaches have been developed in an effort to infer these missing observations, but it remains a challenge to reconstruct true reprogramming trajectories using these tools. Although sophisticated lineage tracing solutions to connect cell history with fate are emerging, these protocols are either not compatible with high-throughput scRNA-seq, or require genome editing strategies that are not readily deployed in some systems. Here, our protocol describes a single-cell resolution clonal tracking approach, 'CellTagging', based on combinatorial cell indexing, permitting the parallel capture of lineage information and cell identity. CellTagging integrates with high-throughput single-cell RNA-sequencing, where iterative rounds of cell labeling enable the construction of multi-level lineage trees. This straightforward lentiviral-labeling approach can be applied to an array of cell biological applications to simultaneously profile lineage and identity, at single-cell resolution.

TAGS

single-cell rna-seq

clonal analysis

Show tags

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

https://www.biorxiv.org/content/early/2017/04/28/127860

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

STEPS MATERIALS

NAME ~	CATALOG # ~	VENDOR V
Pooled CellTag Library V1	115643	€ addgene
Pooled CellTag Library V2	115644	
Pooled CellTag Library V3	115645	€ addgene
Stbl3 competent cells	C7373-03	Thermo Fisher
		Scientific
QIAGEN Plasmid Plus Mega Kit	12981	Qiagen
Nextera XT DNA Library Preparation Kit	FC-131-1096	illumina
Agencourt Ampure XP	A63880	Beckman Coulter

NAME V	CATALOG #	VENDOR V
High Sensitivity D5000 ScreenTape	5067-5592	Agilent Technologies
High Sensitivity D5000 Reagents	5067-5593	Agilent Technologies
293T cell line	CRL-3216	ATCC
X-tremeGENE™ 9 DNA Transfection Reagent	6365779001	Sigma Aldrich
pCMV-VSV-G	8454	€ addgene
pCMV-dR8.2 dvpr	8455	€ addgene
Protamine Sulfate	P3369-10G	Sigma Aldrich
TrypLE™ Express Enzyme	12604013	Thermo Fisher Scientific

SAFETY WARNINGS

For generation of lentivirus, follow BSL2 safety precautions.

Amplification of pooled CellTag libraries

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NOTE

In this first part of the protocol, we describe the amplification of pooled CellT ags by liquid culture to maintain library complexity. CellT ag libraries are available from Addgene: https://www.addgene.org/pooled-library/morris-lab-celltag/. For analysis of clonal dynamics with a single round of CellT agging, we recommend the V1 pooled library. For multiple rounds of CellT agging to support lineage reconstruction, pooled libraries V2 and V3 should also be obtained.







7 Thaw Stbl3 Competent Cells in an ice bath just before use.



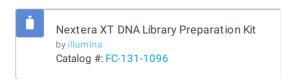
3	After thawing, mix
4	Place transformation mixture on ice for © 00:30:00
5	Heat shock the cells for 60 seconds at 8 42 °C
6	Place tube on ice for © 00:01:00
7	Add SOC medium to bring the final volume to $\ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ $
8	Incubate by shaking (~250 rpm) for © 01:00:00 at 8 37 °C
9	Take of the recovery. Prepare serial dilutions from 1:10 to 1:1000 and plate onto LB + Ampicillin plates. Spread the sample evenly over the plate so that the bacterial colonies are easy to count. Grow overnight at 37 °C
10	Add the rest of the recovery to 500 ml of LB + Ampicillin. Grow overnight while shaking (~250 rpm) at 37 °C
11	Following overnight incubation, count the number of colonies on the plates to calculate the number of colony forming units (CFUs). To maintain CellTag library complexity, aim for 100-200 CFUs per unique CellTag in the library.
	Number of unique CellTags contained in each pooled library from Addgene: CellTag-V1: 19,973 CellTags CellTag-V2: 4,934 CellTags CellTag-V3: 5,737 CellTags
12	Harvest the cells from the liquid culture and use Qiagen Megaprep columns (Or multiple Maxiprep columns) to purify the library.
	QIAGEN Plasmid Plus Mega Kit by Qiagen Catalog #: 12981

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NOTE

In this next phase of the protocol, the above CellTag library is prepared and sequenced to assess complexity. This step is recommended in order to create a 'whitelist' of CellTags existing in the library, facilitating downstream analysis to enhance sensitivity and specificity of clone calling and lineage reconstruction.

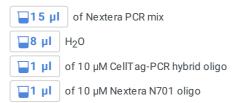
Tagmentation of cDNA with



(Adapted from dropseq.org):

Preheat a thermocycler to 8 55 °C

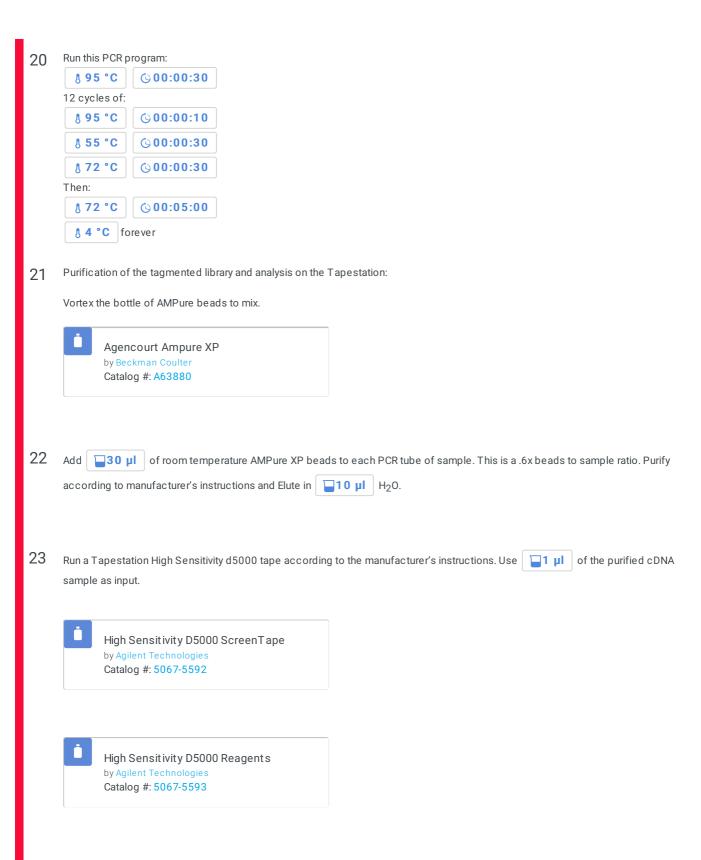
- To each tube, add $\boxed{}$ 10 μ l of Nextera TD buffer and $\boxed{}$ 5 μ l of Amplicon Tagment enzyme (the total volume of the reaction is now $\boxed{}$ 20 μ l). Mix by pipetting ~5 times. Spin down.
- 16 Incubate at **§ 55 °C** for **© 00:05:00**
- 17 Add J5 µl of Neutralization Buffer. Mix by pipetting ~5 times. Spin down. Bubbles are normal.
- 18 Incubate at room temperature for © 00:05:00
- 19 Add to each PCR tube in the following order:



NOTE

CellTag-PCR hybrid oligo sequence: CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCATGGACGAGCTGTACAAGTAA

Nextera N701 oligo sequence: CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG



- $24 \qquad \text{Sequence on Illumina MiSeq, according to the manufacturer's instructions}.$
- $25 \quad \text{Sequence analysis to generate CellTag Whitelist, see} \ \ \underline{\text{https://github.com/morris-lab}} \ \text{for code and tutorials}.$

Production of CellTag lentivirus

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Follow BSL2 safety precautions.

 $27\,$ Day 0: Plate 293T cells at 50-60% confluency, on a 10cm plate.



NOTE

293T cell culture medium:

DMEM

10% FBS

- 28 Day 1: change media ~ two hours prior to transfection.
- $29 \quad \text{Transfection: prepare two 1.5ml Eppendorf tubes with the following:} \\$
 - (1) 200 µl DMEM <- add 15 µl X-tremeGENE9 directly to the media
 - (2) DMEM + CellTag plasmid (2ug) + pCMV-VSV-G (200 ng) + pCMV-dR8.2 dvpr (2 ug)
 - X-tremeGENE™ 9 DNA Transfection
 Reagent
 by Sigma Aldrich
 Catalog #: 6365779001
 - pCMV-VSV-G by addgene Catalog #: 8454
 - pCMV-dR8.2 dvpr by addgene Catalog #: 8455

Transfer DMEM+DNA to DMEM+X-treme GENE9 and mix by pipetting, incubate at room temperature for 600:15:00

- 31 Add DMEM+DNA+X-tremeGENE9 mix dropwise to the cell culture plate. Gently push plate back-and-forth, side-to-side to evenly distribute the transfection reagents.
- 32 Day 2: Change media
- 33 Day 3: First virus harvest

Collect cell supernatant and filter through a low-protein-binding 0.45uM syringe filter to remove cell debris.

Add fresh media to cells

34 Day 4: Second virus harvest

Collect cell supernatant and filter through a low-protein-binding 0.45uM syringe filter to remove cell debris.

Discard cells

Virus is ideally used as fresh as possible. It can be stored at | § 4 °C | for a few days or at | § -80 °C | for longer-term storage.

Titre virus according to https://www.addgene.org/protocols/fluorescence-titering-assay/. Flow cytometry can be used to more accurately assess virus titre. Alternatively, our imaging-based titration software can be used: see https://github.com/morris-lab for code and tutorials.

Transduction of cells with CellTag lentivirus

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NOTE

In this phase of the protocol, we outline the transduction of mouse embryonic fibroblasts (MEFs) with the CellTag virus libraries, generated in the prior steps. These following steps are highly-dependent on the properties of the cells to be CellTagged. We will note major considerations to make in terms of experimental design. So far, we have successfully CellTagged and traced MEFs, mouse endoderm progenitors, human embryonic kidney, human endothelial, and mouse pre-B cells.

37 Day 1

Plate MEFs at a density of 50,000 cells in a well of a 6-well plate, on 1% gelatin.

■NOTE

The starting number of cells is an extremely important consideration: to maximize the number and size of clones that can be detected and traced, we recommend keeping the starting cell population to be CellT agged relatively small. The downstream choice of single-cell capture platform is also important to consider here. For example, platforms with a higher cell capture efficiency require fewer cells to be loaded, therefore supporting the plating and culture of smaller numbers of cells in these early stages.

38 Day 2

Transduce cells with the CellTag viral library overnight. We transduce cells with fresh viral supernatant, with the addition of protamine sulfate. Polybrene can also be used to enhance transduction efficiency.



Protamine Sulfate

by Sigma Aldrich

Catalog #: P3369-10G

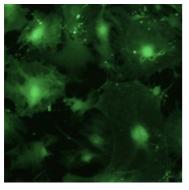
NOTE

We transduce MEFs at a multiplicity of infection (MOI) of around 3-4. This results in each cell expressing a unique combination of CellTags, increasing the confidence of downstream clone calling. We do not track cells expressing fewer than 2 CellTags. With an MOI of \sim 3, we find that around 70% of MEFs express 2 or more CellTags.

We use fresh viral supernatant for this step. For some cell types, the media is not compatible, or the cells are sensitive to supernatant. In these cases, we recommend concentration of CellT ag viral particles via ultracentrifugation, followed by resuspension in fresh media. In our experience, MEFs, 293Ts, and B-cells respond well to viral transduction with supernatant. Difficult to transduce cells may also benefit from viral concentration and spinfection.

39 Day 3

Change media. At this stage, GFP expression should start to be visible. Culture cells for a further 48 hours at which point almost all cells should be GFP positive.



CellTagged fibroblasts expressing GFP, 48 hours post-transduction

NOTE

In initial experiments, we recommend a 'trial run' to assess cell response to CellTagging and any potential viral silencing. Culturing MEFs over a 10 week period, we observe that CellTag expression becomes weaker but is not completely silenced. This can be assessed visually, or via flow cytometry, or sequencing as outlined below.

Cell harvest and replating for clonal tracking

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NOTE

In this phase of the protocol, cells are cultured and portions periodically harvested for single-cell RNA-sequencing. The remaining cells should be replated to support clonal expansion. Here, the frequency of sampling and detection of clones is highly dependent on the growth properties of the cells being studied. For example, many clones will be detected from early stages in fast-growing cells. For slow-growing cells, or protocols involving transition of cells to post-mitotic cells, fewer and smaller clones will be detected.

Harvest cells for single-cell RNA-sequencing. For MEFs, we wash cells in calcium- and magnesium-free PBS, followed by gentle dissociation in TryplE Express, followed by washing in DMEM+10% FBS.



42 Following cell counting, methanol fix minimum of 10,000 cells. Replate the remaining cells for continued clonal expansion.

Methanol fixation protocol:

Alles, J. et al. Cell fixation and preservation for droplet-based single-cell transcriptomics. BMC Biol. 15, 44 (2017) (https://bmcbiol.biomedcentral.com/articles/10.1186/s12915-017-0383-5)

See also: https://support.10xgenomics.com/single-cell-gene-expression/sample-prep/doc/demonstrated-protocol-methanol-fixation-of-cells-for-single-cell-rna-sequencing

ENOTE

For timecourse analyses, we recommend fixation of cells followed by single-cell processing and library preparation within the same batch.

For single-cell processing via 10x Genomics, we methanol fix a minimum of 10,000 cells per sample. Ideally, 25,000 cells are fixed to yield \sim 10,000 single-cell transcriptomes per sample. For Drop-seq, we fix a minimum of 100,000 per sample. We have found that methanol fixation works well for MEFs, B-cells, and 293Ts but performance can vary depending on cell type. We recommend that this is assessed for each cell type.

To support lineage reconstruction, replated cells can be further CellTagged with V2 and V3 libraries. For MEFs, we CellTagged cells with the V2 library 5 days following V1 CellTagging. We followed this with the V3 library 15 days after V1 CellTagging. Again, this is highly cell-type and protocol dependent.

NOTE

Analysis of clonal expansion can be achieved with one round of tagging with the CellTag V1 pooled library. For more complex lineage reconstruction to support the detection of lineage bifurcations, we recommend subsequent CellTagging with V2 and V3 pooled libraries. For lineage reconstruction, it is critical to use these different libraries which contain unique motifs to support CellTag demultiplexing and reconstruction in downstream analyses.

Following harvest of all samples, cells should be processed for single-cell capture, library preparation, and sequencing using 10x Genomics or Drop-seq platforms, according to the standard protocols. CellTag transcripts are effectively captured via these standard workflows and no additional steps are required.

For fixed cell rehydration, please refer to: https://support.10xgenomics.com/single-cell-gene-expression/sample-prep/doc/demonstrated-protocol-methanol-fixation-of-cells-for-single-cell-ma-sequencing

NOTE

On both the 10x Genomics and Drop-seq platforms, we aim to sequence cells to a depth of at least 30,000 reads per cell. Overall, keeping the cell population size relatively small and the proportion of these cells sequenced high, this will increase the number and size of clones detected.

Single-cell analysis, clone-calling, and lineage reconstruction

45 See https://github.com/morris-lab for code and tutorials.

Visit http://celltag.org/ to explore our dataset on the dynamics of reprogramming, reconstructed via CellTagging.

Our raw data is available here:

III DAT ASET

Biddy et al., Single-cell mapping of lineage and identity in dir [©]

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