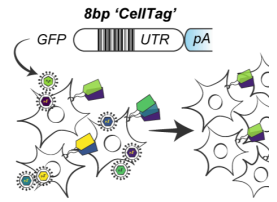


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# 🌐 Single-cell mapping of lineage and identity via CellTagging V.4

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**Protocol status:** Working

**We use this protocol and it's working**

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**Keywords:** cell barcoding, lineage tracing, single-cell analysis, direct lineage reprogramming

## 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.

## Materials

### STEP MATERIALS

-  Pooled CellTag Library V1 **addgene Catalog #115643**
-  Pooled CellTag Library V2 **addgene Catalog #115644**
-  Pooled CellTag Library V3 **addgene Catalog #115645**
-  QIAGEN Plasmid Plus Mega Kit **Qiagen Catalog #12981**
-  Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #FC-131-1096**
-  Agencourt Ampure XP **Beckman Coulter Catalog #A63880**
-  High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592**
-  High Sensitivity D5000 Reagents **Agilent Technologies Catalog #5067-5593**
-  293T cell line **ATCC Catalog #CRL-3216**
-  X-tremeGENE™ 9 DNA Transfection Reagent **Merck MilliporeSigma (Sigma-Aldrich) Catalog #6365779001**
-  pCMV-VSV-G **addgene Catalog #8454**
-  pCMV-dR8.2 dvpr **addgene Catalog #8455**
-  Protamine Sulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P3369-10G**
-  TrypLE™ Express Enzyme **Thermo Fisher Scientific Catalog #12604013**
-  Stellar Competent Cells **Takara Bio Inc. Catalog #636763**

## Protocol materials

-  Pooled CellTag Library V2 **addgene Catalog #115644**
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## 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 CellTags by liquid culture to maintain library complexity. CellTag libraries are available from Addgene: <https://www.addgene.org/pooled-library/morris-lab-celltag/>. For analysis of clonal dynamics with a single round of CellTagging, we recommend the V1 pooled library. For multiple rounds of CellTagging to support lineage reconstruction, pooled libraries V2 and V3 should also be obtained.


 Pooled CellTag Library V1 **addgene Catalog #115643**

 Pooled CellTag Library V2 **addgene Catalog #115644**


 Pooled CellTag Library V3 **addgene Catalog #115645**


2 Thaw Stellar Competent Cells in an ice bath just before use.


 Stellar Competent Cells **Takara Bio Inc. Catalog #636763**

3 After thawing, mix  100  $\mu\text{L}$  of cells with 10-50ng of pooled CellTag DNA in a 1.5-mL microcentrifuge tube.







4 Place transformation mixture on ice for  00:30:00

5 Heat shock the cells for 60 seconds at  42  $^{\circ}\text{C}$

6 Place tube on ice for  00:01:00

7 Add SOC medium to bring the final volume to  1000  $\mu\text{L}$



- 8 Incubate by shaking (~250 rpm) for  01:00:00 at  37 °C
- 9 Take  5 µL 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.

#### Note


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 **Qiagen Catalog #12981**

## Assessment of CellTag library complexity via sequencing

- 13

### 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




 Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #FC-131-1096**

(Adapted from dropseq.org):


Preheat a thermocycler to  55 °C

14 For each sample, combine 600pg of CellTag plasmid prep with H<sub>2</sub>O in a total volume of

 5 µL

15 To each tube, add  10 µL of Nextera TD buffer and  5 µL of Amplicon Tagment enzyme (the total volume of the reaction is now  20 µL ). Mix by pipetting ~5 times. Spin down.


16 Incubate at  55 °C for  00:05:00

17 Add  5 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:

 15 µL of Nextera PCR mix

 8 µL H<sub>2</sub>O

 1 µL of 10 µM CellTag-PCR hybrid oligo

 1 µL of 10 µM Nextera N701 oligo

**Note**

CellTag-PCR hybrid oligo sequence:

CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCT  
TCCGATCTGGCATGGACGAGCTGTACAAGTAA

Nextera N701 oligo sequence:

CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG

- 20 Run this PCR program:

 95 °C  00:00:30

12 cycles of:

 95 °C  00:00:10

 55 °C  00:00:30

 72 °C  00:00:30

Then:



 72 °C  00:05:00


 4 °C forever

- 21 Purification of the tagmented library and analysis on the Tapestation:

Vortex the bottle of AMPure beads to mix.

 Agencourt Ampure XP **Beckman Coulter Catalog #A63880**

- 22 Add  30 µL of room temperature AMPure XP beads to each PCR tube of sample. This is a .6x beads to sample ratio. Purify according to manufacturer's instructions and Elute in  10 µL H<sub>2</sub>O.

- 23 Run a Tapestation High Sensitivity d5000 tape according to the manufacturer's instructions. Use  1 µL of the purified cDNA sample as input.

 High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592**

 High Sensitivity D5000 Reagents **Agilent Technologies Catalog #5067-5593**

- 24 Sequence on Illumina MiSeq, according to the manufacturer's instructions.





- 25 Sequence analysis to generate CellTag Whitelist, see <https://github.com/morris-lab> for code and tutorials.

## Production of CellTag lentivirus

26

### Safety information

Follow BSL2 safety precautions.

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



 293T cell line **ATCC Catalog #CRL-3216**


### Note


293T cell culture medium:  
DMEM  
10% FBS

- 28 Day 1: change media ~ two hours prior to transfection.

- 29 Transfection: prepare two 1.5ml Eppendorf tubes with the following:

(1)  200  $\mu$ L DMEM  $\leftarrow$  add  15  $\mu$ L X-tremeGENE9 directly to the media


(2)  200  $\mu$ L DMEM + CellTag plasmid ( 2ug ) + pCMV-VSV-G (200 ng) + pCMV-dR8.2 dvpr (2 ug)

 X-tremeGENE™ 9 DNA Transfection Reagent **Merck MilliporeSigma (Sigma-Aldrich) Catalog #6365779001**



 pCMV-VSV-G **addgene Catalog #8454**



 pCMV-dR8.2 dvpr addgene Catalog #8455

- 30 Transfer DMEM+DNA to DMEM+X-tremeGENE9 and mix by pipetting, incubate at room temperature for  00:15:00
- 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

## Production of CellTag lentivirus

- 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.

## Production of CellTag lentivirus

- 35 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 0.1% 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 CellTagged 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 **Merck MilliporeSigma (Sigma-Aldrich)** Catalog #P3369-10G

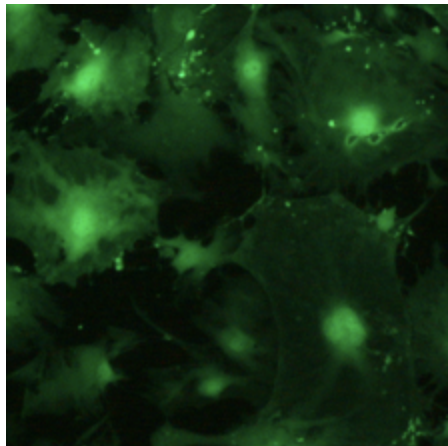
### Note

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 ~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 CellTag 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

40

**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.

- 41 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.

 TrypLE™ Express Enzyme **Thermo Fisher Scientific Catalog #12604013**

- 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>

**Note**

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 ~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.

- 43 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.

- 44 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-rna-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:



## Dataset

Biddy et al., Single-cell mapping of lineage and identity in dir<sup>NAME</sup>

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE99915>

LINK