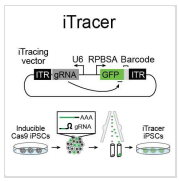

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Amplify iTracer Barcode and Scars from 10x cDNA

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protocol .

QuadBio


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Induced pluripotent stem cell (iPSC)-derived organoids provide models to study human organ development. Single-cell transcriptomics enable highly resolved descriptions of cell states within these systems; however, approaches are needed to directly measure lineage relationships. Here we establish iTracer, a lineage recorder that combines reporter barcodes with inducible CRISPR–Cas9 scarring and is compatible with single-cell and spatial transcriptomics. We apply iTracer to explore clonality and lineage dynamics during cerebral organoid development and identify a time window of fate restriction as well as variation in neurogenic dynamics between progenitor neuron families. We incorporate gene perturbation (iTracer-perturb) and assess the effect of mosaic *TSC2* mutations on cerebral organoid development. Our data shed light on how lineages and fates are established during cerebral organoid formation. More broadly, our techniques can be adapted in any iPSC-derived culture system to dissect lineage alterations during normal or perturbed development.

This protocol describes barcode and scar detection from single-cell cDNA:

Barcode and scar regions were amplified from 60-70ng of cDNA remaining from the single-cell RNAseq preparation with three separate PCR reactions. First cDNA was amplified via PCR broadly targeting a region containing both the scar and barcode. Subsequently, the reaction was split equally and we performed a nested PCR separately targeting the barcode and scar regions. Lastly, we added Illumina sequencing adapters (10x Genomics). Following every PCR reaction the samples were cleaned-up using magnetic beads (Beckman Coulter). Libraries are then ready to be sequenced on Illumina sequencer.

Ashley Maynard, Sophie Jansen, Giovanna Brancati 2022. Amplify iTracer Barcode and Scars from 10x cDNA. **protocols.io**
https://protocols.io/view/amplify-itracer-barcode-and-scars-from-10x-cdna-b63grgjw

protocol

He, Z., Maynard, A., Jain, A. et al. Lineage recording in human cerebral organoids. Nat Methods 19, 90–99 (2022). https://doi.org/10.1038/s41592-021-01344-8

lineage tracing, genomic lineage tracer, iTracer, barcodes

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Olgios needed:

Primer Name	Assay	Sequence
10x_Root_RFP_F	scRNAseq amplified libraries	cggcacgctgatctacaagg
10x_Nest_RFP_F	scRNAseq amplified libraries	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTgagttcaagaccatctacatggcc
10x_Root_GFP_F	scRNAseq amplified libraries	gacgacggcaactacaagacc
10x_Nest_GFP_F	scRNAseq amplified libraries	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaggtgaacttcaagatccgcc
10x_Universal_R	scRNAseq amplified libraries	CTACACGACGCTCTTCCGATCT
10x_Nest_Barcode_GFP_F	scRNAseq amplified libraries	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTggatcactctcggcatgga
10x_Nest_Barcode_RFP_F	scRNAseq amplified libraries	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtggaacagtacgagcgctc

**if using the GFP iTracer please use oligos with _G or _GFP
** if using the RFP iTracer, please use the oligos with _R or _RFP

Set up Reaction 1 (Root PCR)

1 Make reaction master mix:

A	B
Component	**1x rxn**
100% DMSO	1.5uL
10uM 10x Root PCR GFP F Primer	2.5uL
10uM 10x Universal R Primer	2.5uL
2x Phusion Ready Mix	25uL
EvaGreen	0.75uL
cDNA product (50ng)	17.75uL total
H2O	

2 Add **30.75 µL** per well of reaction master mix to qPCR plates, then add a total of **17.75 µL** of sample.

3 Run PCR according to the following program:

A	B	C	D
Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98 ºC	30 sec	1
Denaturation	98 ºC	15 sec	45 cycles & stop when saturated
Annealing	66 ºC	15 sec	
Extension	72 ºC	20 sec	
Final extension	72 ºC	2 min	1

Volume **50 µL** + **105 °C** lid temperature.

4 Stop reaction in the exponential phase before the curve levels off (see example)

Example of when to stop PCR reactions.

5 Clean up reaction with 1:1 SPRI beads (**50 µL** beads added). Elute in **30 µL** EB buffer

6 Check concentrations of reactions on Nanodrop.

Set up Reaction 2a (Nested PCR for Barcodes)

7 Make reaction master mix:

A	B
Component	**1x rxn**
2x Phusion Ready Mix	25uL
10uM Nest Barcode GFP Primer	2.5uL
10uM 10x Universal R Primer	2.5uL
EvaGreen	0.75uL
H20	14.25uL
Root PCR Product	5uL

8 Add **45 µL** per well of reaction master mix to qPCR plates

9 Run PCR according to the following program:

A	B	C	D
Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98 ºC	30 sec	1
Denaturation	98 ºC	15 sec	30 cycles & stop when saturated
Annealing	65 ºC	15 sec	
Extension	72 ºC	20 sec	
Final extension	72 ºC	60 sec	1

Volume **50 µL** + **105 °C** lid temperature

10 Stop reaction in the exponential phase before the curve levels off.

11 Clean up reaction with 1:1 SPRI beads:

11.1 Vortex to resuspend the SPRIselect reagent. Add **50 µL** SPRIselect Reagent (1X) to each sample. Pipette mix 15x (pipette set to **150 µL**). Incubate **00:05:00** at **Room temperature** .

11.2 Place the magnet on High until the solution clears. Remove **165 µL** supernatant. **DO NOT discard any beads.**

11.3 With the tube still in the magnet, add **200 µL** 80% ethanol to the pellet. Wait **00:00:30** . Remove the ethanol. Repeat steps i and j for a total of 2 washes.

11.4 Centrifuge briefly. Place on the magnet on Low. Remove remaining ethanol.

11.5 Remove from the magnet. Add **30 µL** Buffer EB. Pipette mix 15x. Incubate 2 min at **Room temperature** .

11.6 Place on the magnet on Low until the solution clears. Transfer **30 µL** to a new tube strip.

12 Check concentrations of reactions on Nanodrop.

Set up Reaction 2b (Nested PCR for Scars)

13 Make reaction master mix:

A	B
Component	**1x rxn**
2x Phusion Ready Mix	25uL
10uM Nest GFP Primer	2.5uL
10uM 10x Universal R Primer	2.5uL
EvaGreen	0.75uL
H20	14.25uL
Root PCR Product	5uL

14 Add **45 µL** per well of reaction master mix to qPCR plates.

15 Run PCR according to the following program:

A	B	C	D
Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98 ºC	30 sec	1
Denaturation	98 ºC	15 sec	30 cycles & stop when saturated
Annealing	66 ºC	15 sec	
Extension	72 ºC	20 sec	
Final extension	72 ºC	60 sec	1

Volume **50 µL** + **105 °C** lid temperature

16 Stop reaction in the exponential phase before the curve levels off.

17 Clean up reaction with 1:1 SPRI beads:

17.1 Vortex to resuspend the SPRIselect reagent. Add **50 µL** SPRIselect Reagent (1X) to each sample. Pipette mix 15x (pipette set to **150 µL**). Incubate **00:05:00** at **Room temperature** .

17.2 Place the magnet on High until the solution clears. Remove **165 µL** supernatant. **DO NOT discard any beads.**

17.3 With the tube still in the magnet, add **200 µL** 80% ethanol to the pellet. Wait **00:00:30** . Remove the ethanol. Repeat steps i and j for a total of 2 washes.

17.4 Centrifuge briefly. Place on the magnet on Low. Remove remaining ethanol.

17.5 Remove from the magnet. Add **30 µL** Buffer EB. Pipette mix 15x. Incubate 2 min at **Room temperature** .

17.6 Place on the magnet on Low until the solution clears. Transfer **30 µL** to a new tube strip.

18 Check concentrations of reactions on Nanodrop.

Reaction 3 (Indexing PCR with 10x indexes) 3d 0h 10m 30s

19 Prepare reaction mix:

A	B
Component	**1x rxn**
2x Phusion Ready Mix	25ul
10x SI Primer	1ul

20 Prepare the DNA+H20, normalize PCR products to **60 ng** , and fill to **19 µL** with water

21 Add **26 µL** of reaction mix to each sample.

22 Add 10x Index primers **5 µL** .

23 Run PCR according to the following program:

A	B	C	D
Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98 ºC	30 sec	1
Denaturation	98 ºC	15 sec	30 cycles & stop when saturated
Annealing	55 ºC	15 sec	
Extension	72 ºC	20 sec	
Final extension	72 ºC	2 min	1

Volume **50 µL** + **105 °C** lid temperature

24 SPRI Select bead clean-up (double sided clean-up as performed at 10x final clean up, v3.1 revD step 3.6)

24.1 Add **50 µL** of EB to the PCR reactions to have **100 µL** total volume.

24.2 Vortex to resuspend the SPRIselect reagent. Add **60 µL** SPRIselect Reagent (0.6X) to each sample. Pipette mix 15x (pipette set to **150 µL**). Incubate **00:05:00** at **Room temperature** . 5m

24.3 ⚠

Place the magnet on High until the solution clears. **DO NOT discard supernatant.** Transfer **150 µL** supernatant to a new tube strip.

24.4 Vortex to resuspend the SPRIselect reagent. Add **20 µL** SPRIselect Reagent (0.8X) to each sample. Pipette mix 15x (pipette set to **150 µL**). Incubate **00:05:00** at **Room temperature** . 5m

24.5 ⚠

Place the magnet on High until the solution clears. Remove **165 µL** supernatant. **DO NOT discard any beads.**

24.6 With the tube still in the magnet, add **200 µL** 80% ethanol to the pellet. Wait **00:00:30** . Remove the ethanol. Repeat steps i and j for a total of 2 washes. 30s

24.7 Centrifuge briefly. Place on the magnet on Low. Remove remaining ethanol.

24.8 Remove from the magnet. Add **35.5 µL** Buffer EB. Pipette mix 15x. Incubate 2 min at **Room temperature** .

24.9 Place on the magnet on Low until the solution clears. Transfer **35 µL** to a new tube strip.

24.10 Run Bioanalyzer (1:10 diluted).

24.11 Store at **4 °C** for up to **72:00:00** or at **-20 °C** for long-term storage. **Libraries are ready for sequencing!** 3d