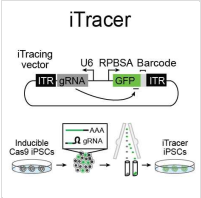

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iTracer-perturb Plasmid Prep

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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 the creation of the iTracer vectors.

pSBbi-iTracer-G.dna pSBbi-iTracer-R.dna

Ashley Maynard, Hsiu-Chuan Lin 2022. iTracer-perturb Plasmid Prep. **protocols.io** https://protocols.io/view/itracer-perturb-plasmid-prep-b66drha6

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, barcodes, genomic lineage tracer, iTracer

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Order iTracer plasmids

1 Please order iTracer from the European Plasmid Repository (https://www.plasmids.eu/).

pSBbi-iTracer-perturb-R2AG-gG
pSBbi-iTracer-perturb-B2AG-gG

Order and amplify the guide donor plasmid

2 Order plasmid: pMJ114 from addgene (https://www.addgene.org/85995/?gclid=CjwKCAjw0a-SBhBkEiwApljU0o4BqNfCIJ0XWMbzs8XS691A3nujA1zPVsyWJjiVOAHEKqRWnAoSQxoCzXoQAvD_BwE)

3 Amplify pMJ114 by taking a non-filtered p10 tip and dipping into glycerol stock of pMJ114. Add this tip to **3 mL** of 2yt buffer + Ampicillin

4 Incubate **Overnight** (~16hr) at **37 °C** .

5 Harvest the cells from the liquid culture and use a Qiagen Miniprep (Qiagen #27106X4) column to purify the plasmid and elute in **35 µL** . Nanodrop the purified plasmid to obtain the concentration.

Digest the pMJ114 plasmid

6 BstXI followed by BlpI digestion (removes the "filler" guide")

7 Run a 1% agarose gel and preform a gel extraction clean up to obtain a clean plasmid fragment.

Order custom guide

8 To add your custom guide first order your guide, replacing **[YOURgRNA]** sections with the sequences you wish to target.

forward: atgg **[YOURgRNA]** gtttaagagc
reverse: ttagctcttaaac **[YOURgRNA(complement and reverse)]** ccataaac

Ligate your custom gRNA into pMJ114 plasmid

9 Phosphorylate and anneal the oligo pairs from step 8:

1 µL 10uM forward primer

1 µL 10uM reverse primer

1 µL of 10x T4-Ligase Buffer

6.5 µL water

0.5 µL T4 PNK (https://international.neb.com/products/m0201-t4-polynucleotide-kinase#Product%20Information)

10 Ligate (T4 DNA ligase, NEB #M0202S)

100 ng of digested plasmid from step 7

1 µL of phosphorlated and annealed oligo pairs from step 9

3 µL of 10x ligase buffer

1 µL of T4 DNA ligase

add water to **30 µL**

Incubate at **Room temperature** for at least 1hr

11 Transform component cells with:

50 µL cells + **10 µL** reaction mix

On ice **00:15:00**

Heat shock the cells for **00:00:45** at **42 °C**

Place tube **On ice** for **00:01:00**

Add **350 µL** SOC

Incubate by shaking (~ **250 rpm**) for **00:45:00** at **37 °C**

Plate cells on Amp plates and incubate overnight at **37 °C**

12 Select a subset of colonies for sequencing for quality control and for mini prep. Here you will split the colony between your choice of sequencing platform to check for incorporation of your guide and amplifying your colony by taking a non-filtered p10 tip and dipping into colony and adding this tip to **3 mL** of 2yt buffer + Ampicillin.

13 Mini prep (Qiagen #27106X4) the clone(s) that sequencing confirms has the incorporated guide.

14 PCR amplify the guide area using:

forward primer: ggggtcggcaattgaaaccggtcgtgaccgagcttgtctgccc
reverse prmer: gggaaataggccctcgcacatcgtgatagtcagactggaaaaaaagcacc

Amplify barcodes by combing the following in a PCR tube:

1.25 µL 10uM forward primer
1.25 µL 10uM reverse primer
1.25 µL guide template
12.5 µL of Phusion Master Mix
9.5 µL water

15 Run PCR program 7m 50s

98 °C for **00:02:00**

(25 cycles total)
Step 5.2: **98 °C** for **00:00:10**
Step 5.3: **58 °C** for **00:00:20**
Step 5.4: **72 °C** for **00:00:20**

72 °C for **00:05:00**
4 °C until ready to proceed.

16 Run a 1% agarose gel and preform a gel extraction clean up to obtain a clean PCR product (guide product).

Amplify iTracer-perturb plasmid 1h 1m 45s

1h 1m 45s

17 Transform component cells with:

50 µL cells + **2 µL** plasmid

On ice **00:15:00**

Heat shock the cells for **00:00:45** at **42 °C**

Place tube **On ice** for **00:01:00**

Add **350 µL** SOC

Incubate by shaking (~ **250 rpm**) for **00:45:00** at **37 °C**

Add reaction mix to **3 mL** of 2yt buffer + Ampicillin

18 Incubate **Overnight** (~16hr) at **37 °C** .

19 Harvest the cells from the liquid culture and use a Qiagen Miniprep (Qiagen #27106X4) column to purify the plasmid and elute in **35 µL** . Nanodrop the purified plasmid to obtain the concentration.

AgeI digest of iTracer-perturb plasmid

20 To cut the plasmid for guide-product insertion first digest the plasmid with AgeI by combining in a PCR tube:

5 µg iTracer-perturb plasmid

5 µL Cutsmart buffer (NEB #B7204)

3 µL AgeI

fill to **50 µL** with water

21 Incubate **37 °C** **Overnight**

22 Run 1% agarose gel with digested and undigested plasmid (control) to check for complete cutting.

23 Perform a column clean up (Macherey-Nagel #REF740609.50) of AgeI fragmented plasmid

and nanodrop.

Gibson assembly of guide product and iTracer-perturb

24 Calculate the pmol of 1ul of the guide-product and the pmol of 1ul of the iTracer-perturb plasmid fragment (this calculator can help https://ch.promega.com/resources/tools/biomath/)

Example:

guide amplicon = 500bp with 0.18ug/ul = 0.55pmol DNA (in 1ul)
plasmid fragment = 5613bp with 0.2044ug/ul = 0.055pmol (in 1ul)

guide product should be 10 fold the molar excess of an insert, therefore:
10 x 0.055 pmol (the plasmid fragment) = 0.55 pmol (the molarity we need of our barcodes)

In this example we will use 1ul of guide-product for 1ul of vector in the gibson assembly

25 Combine in a PCR tube:

calculated amount of guide-product (shown above)
1uL iTracer-perturb (AgeI fragmented plasmid)
10 µL Gibson Assembly Mix (NEB #E2611S)
fill to **20 µL** with water

** control run same reaction without guide product.

15m

26 Incubate **00:15:00** at **50 °C** . Then place **On ice** .

27 **50 µL** cells + **10 µL** reaction mix
On ice **00:15:00**
Heat shock the cells for **00:00:45** at **42 °C**
Place tube **On ice** for **00:01:00**
Add **350 µL** SOC

Incubate by shaking (~ **250 rpm**) for **00:45:00** at **37 °C**

28 Plate the reaction volume on Ampicillin plates and incubate **Overnight** at **37 °C**
Plate the control reaction on Ampicillin plates and incubate **Overnight** at **37 °C**

29 Select a subset of colonies for sequencing for quality control and for mini prep. Here you will split the colony between your choice of sequencing platform to check for incorporation of your guide and amplifying your colony by taking a non-filtered p10 tip and dipping into colony and adding this tip to **3 mL** of 2yt buffer + Ampicillin.

30 Mini prep (Qiagen #27106X4) the clone(s) that sequencing confirms has the incorporated guide. (iTracer-perturb+guide plasmid)

Order barcodes

31 To ensure the greatest barcode diversity please order the barcode components below:

barcode template:
GGACGAGCTGTACAAGTAAGCTGATCCWNNNNNNNNNWCCAAGCgcccttgagcatctgacttc
barcode forward primer: GGACGAGCTGTACAAGTAAGCTG
barcode reverse primer: gaagtcagatgctcaagggcGCTTG

Amplify barcodes

32 Resuspend all primers to concentration of **100 micromolar (µM)** , you can store these stocks at **-20 °C**

Dilute stock primers to **10 micromolar (µM)**
Dilute barcode template **50 micromolar (µM)**

33 Amplify barcodes by combing the following in a PCR tube:

1.25 µL diluted barcode forward primer (from step #3)
1.25 µL diluted barcode reverse primer (from step #3)
0.5 µL diluted barcode template (from step #3)
9.5 µL water

34 Run PCR program 7m 50s

98 °C for **00:02:00**

(25 cycles total)
Step 5.2: **98 °C** for **00:00:10**
Step 5.3: **58 °C** for **00:00:20**
Step 5.4: **72 °C** for **00:00:20**

72 °C for **00:05:00**

4 °C until ready to proceed.

35 Run 1% agarose gel with **1 µL** of amplified barcode product to check for successful amplification.

NotI Digest of iTracer-perturb+guide plasmid

36 To cut the plasmid for barcode insertion first digest the plasmid with NotI-HF by combining in a PCR tube:

5 µg iTracer-perturb+guide plasmid
5 µL Cutsmart buffer (NEB #B7204)
3 µL NotI-HF
fill to **50 µL** with water

37 Incubate **37 °C** **Overnight**

38 Run 0.5% agarose gel with digested and undigested plasmid (control) to check for complete cutting.

39 Perform a get extraction clean up (Macherey-Nagel #REF740609.50) of EcoRI fragmented plasmid and nanodrop.

Gibson Assembly of Barcodes into iTracer plasmid 15m

40 Calculate the pmol of 1ul of the barcodes (step #7) and the pmol of 1ul of the plasmid fragment (step #13) (this calculator can help https://ch.promega.com/resources/tools/biomath/)

Example:

barcodes = 63bp with 0.0238ug/ul = 0.57pmol DNA (in 1ul)
plasmid fragment (iTracer-R) = 5613bp with 0.2044ug/ul = 0.055pmol (in 1ul)

barcodes should be 10 fold the molar excess of an insert, therefore:
10 x 0.055 pmol (the plasmid fragment) = 0.55 pmol (the molarity we need of our barcodes)

In this example we will use 1ul of barcodes for 1ul of vector in the gibson assembly

41 Combine in a PCR tube:

1ul of the calculated volume of EcoRI fragmented plasmid
1ul of the calculated volume of barcodes from step #15
10 µL NEBuilder®HiFi DNA Assembly Master Mix (NEB #E2621S)
fill to **20 µL** with water

** Make sure to set-up a control following the same reaction conditions above but without barcodes, instead add water. We will use this reaction to estimate self ligation of the plasmid.

15m

42 Incubate **00:15:00** at **50 °C** . Then place **On ice** .

1h 1m 45s

43 **50 µL** cells + **10 µL** reaction mix
On ice **00:15:00**
Heat shock the cells for **00:00:45** at **42 °C**
Place tube **On ice** for **00:01:00**
Add **350 µL** SOC

Incubate by shaking (~ **250 rpm**) for **00:45:00** at **37 °C**

44 Plate the total reaction volume on Ampicillin plates (x3 15cm square plates) and incubate **Overnight** at **37 °C**
Plate 30% of total volume of the control reaction (to be comparable to experimental above) and incubate **Overnight** at **37 °C**

Collect final plasmids

45 To collect Gibson assembled plasmids use **10 mL** of 2yt buffer and a scraper to collect all the colonies.

46 Wash the plate with **5-7 mL** 2yt buffer and collect in the same tube as the collection in step #18.

47 Perform x3 Qiagen Midiprep (Qiagen #12143) reactions, elute in **200 µL**

48 Pool final midiprep reactions (final volume ~ **600 µL**) and nanodrop for final concentration.
You now have **barcoded iTracer-perturb** plasmids ready for electroporation into your cells!