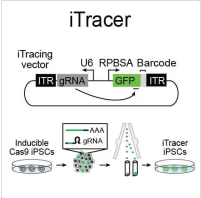

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iTracer Plasmid Prep

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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 Plasmid Prep. **protocols.io** https://protocols.io/view/itracer-plasmid-prep-b63ergje

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-G
pSBbi-iTracer-R

Order barcodes

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

barcode template:
gacgagctgtacaagtgatccgWNNNNNNNNNWcacccagctttcttgtacaaagtggctgtg
barcode forward primer: gacgagctgtacaagtgatc
barcode reverse primer: cacagccactttgtacaaga

Amplify barcodes

3 Resuspend all oligos 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)**

4 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

5 Run PCR program 7m 50s

Step 5.1: **98 °C** for **00:02:00**

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**

Step 5.5: Repeat steps #5.2-#5.4 x24 times (25 cycles total)

Step 5.6: **72 °C** for **00:05:00**

Step 5.7: **4 °C** until ready to proceed.

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

7 Clean up barcodes with column clean up (Macherey-Nagel #REF740609.50) and nanodrop.

Amplify plasmids 1h 1m 45s

8 Transform component cells with: 1h 1m 45s

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

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

10 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.

EcoRI Digest of iTracer plasmid

11 To cut the plasmid for barcode insertion first digest the plasmid with EcoRI-HF (NEB #R3101S) by combining in a PCR tube:

5 µg iTracer plasmid (from step #9)

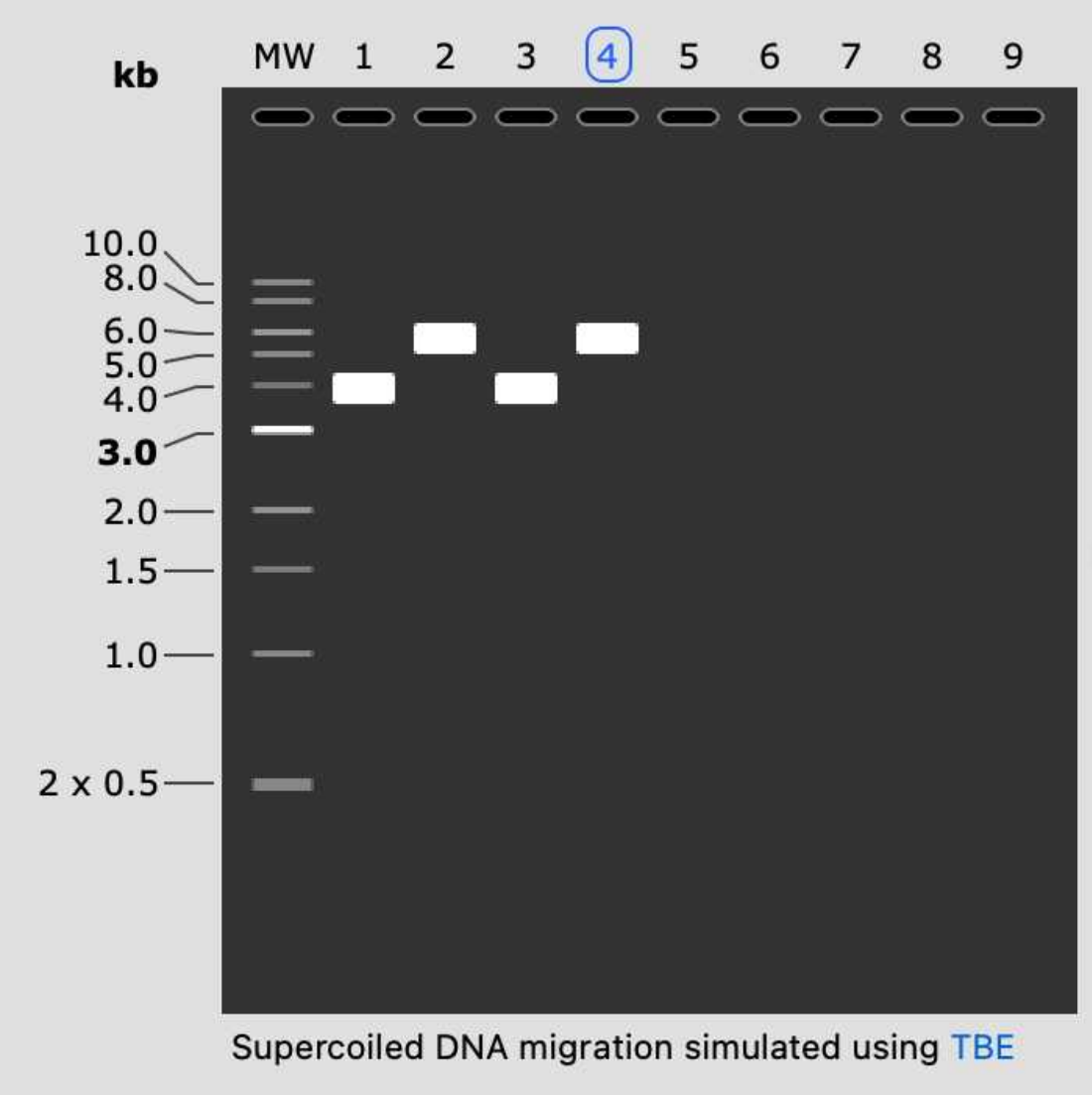
5 µL Cutsmart buffer (NEB #B7204)

3 µL EcoRI-HF (NEB #R3101S)

fill to **50 µL** with water

12 Incubate **37 °C** **Overnight**

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



Simulated Gel from SnapGene. Supercoiled (uncut) plasmid will run faster than EcoRI digested plasmid. MW: 1 kb ladder, lane 1: pSBbi-iTracer-G (supercoiled), lane 2: pSBbi-iTracer-G EcoRI digested, lane 3: pSBbi-iTracer-R (supercoiled), lane 4: pSBbi-iTracer-R EcoRI digested.

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

Gibson Assembly of Barcodes into iTracer plasmid 15m

15 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

16 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

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

Prepare transformation reactions 1h 1m 45s

1h 1m 45s

18 For both the control and plasmid + barcode reactions (preform x2 reactions for plasmid + barcode and x1 reaction for control):

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 for **00:45:00** at **37 °C**

19 For the plasmid + barcode reaction: combine the reaction and equally plate the total reaction volume (~273uL each) on Ampicillin plates (x3 15cm square plates). Add extra SOC buffer to help evenly spread the volume on the plate. 16h

For the control plasmid only reaction: plate ~273ul of total reaction volume, add extra SOC buffer to help evenly spread the volume on the plate.

Incubate plates **Overnight** (~16hrat **37 °C**

Collect final plasmids

20 Inspect control plate vs plasmid+barcode plates. There should be a notable difference, where the control plate has few colonies compared to the plasmid+barcode reaction plates. If this is observed continue to step #19.

If this is not the case you may have an error and will need to troubleshoot (control plates with many colonies may have plasmid carryover from from the backbone fragment clean up Step #14, do these steps and repeat).

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

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

23 Perform x2 Qiagen Midiprep (Qiagen #12143) reactions, elute in **200 µL**

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