

May 31, 2024

Generation of Flp-In™ T-REx™ 293 cells stably expressing VPS13C^mClover under a tetracycline inducible promoter

DOI

dx.doi.org/10.17504/protocols.io.36wgqnnnxgk5/v1

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DOI: dx.doi.org/10.17504/protocols.io.36wgqnnnxgk5/v1

Protocol Citation: Xinbo Wang, Shujun Cai, Will Hancock-Cerutti, Pietro De Camilli 2024. Generation of Flp-In™ T-REx™ 293 cells stably expressing VPS13C^mClover under a tetracycline inducible promoter. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.36wgqnnnxgk5/v1>

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Protocol status: Working

We use this protocol and it's working

Created: May 22, 2024

Last Modified: May 31, 2024

Protocol Integer ID: 100990

Keywords: ASAPCRN

Abstract

This method describes the generation of Flp-In™ T-REx™ 293 cells stably expressing internally mClover tagged VPS13C under a tetracycline inducible promoter.

Materials

Specialized Reagents:

✕ Flp-In™ T-REx™ 293 Cell Line **Thermo Fisher Scientific Catalog #R78007**

✕ VPS13C^mClover3 **addgene Catalog #118760**

✕ pcDNA™5/FRT/TO Vector Kit **Thermo Fisher Catalog #V652020**

✕ pOG44 Flp-Recombinase Expression Vector **Thermo Fisher Catalog #V600520**

✕ Blasticidin S HCl (10 mg/mL) **Thermo Fisher Catalog #A1113903**

✕ Zeocin™ Selection Reagent **Thermo Fisher Catalog #R25001**

Hygromycin B (ThermoFisher Catalog # J67371.8EQ)

Tetracycline (ThermoFisher Catalog # A39246)

Reagents to prepare

Culture media:

A	B
Dulbecco's modified Eagle's medium (DMEM)	
fetal bovine serum (FBS)	10%
zeocin	200 µg/ml
blasticidin S.	15 µg/ml

Post transfection media:

A	B
Dulbecco's modified Eagle's medium (DMEM)	
fetal bovine serum (FBS)	10%
blasticidin S.	15 µg/ml

Selection media:

A	B
Dulbecco's modified Eagle's medium (DMEM)	
fetal bovine serum (FBS)	10%
blasticidin S	15 µg/ml
hygromycin B	200 µg/ml

**Expression media:**

A	B
Dulbecco's modified Eagle's medium (DMEM)	
fetal bovine serum (FBS)	10%
blasticidin S	15 µg/ml
hygromycin B	200 µg/ml
tetracycline	0.1 µg/ml

Cloning:

- Takara Catalog # 638947
- Gibco manual (Catalog # R780-07)

Safety warnings

 All appropriate biosafety precautions should be observed when handling Cell culture and recombinant DNA.



Cloning










- 1 Clone gene of interest into pcDNA5/FRT/TO. For VPS13C^mClover the In-Fusion system (Takara Catalog # 638947) was used.
- 2 Sequence plasmid.

Cell culture

- 3 Thaw and culture Flp-In™ T-REx™ 293 cells according to Gibco manual (Catalog # R780-07) in a 10cm dish. Once cells are 90% Confluent, split.
- 4 Freeze some cells according to Gibco manual protocol for future use.

Transfection and selection of stably integrated cells

15m



- 5 Plate cells in 6 well format to ~30% confluence in media containing no antibiotics.
- 6 The next day, transfect cells using Fugene HD and a ratio of 9:1 pOG44: pcDNA5/FRT/TO-VPS13C^mClover.
- 7 Mix  2 µg total DNA ( 0.2 µg pcDNA5/FRT/TO-VPS13C^mClover,  1.8 µg pOG44) in  100 µL opti-MEM. 
- 8 Add  8 µL Fugene HD to DNA mixture. Let sit  00:15:00 . 15m
- 9 Add Fugene/DNA mixture to cells in a dropwise fashion.
- 10 At 24h post transfection, wash cells and add fresh post-transfection media containing Blasticidin (no Zeocin or Hygromycin). 
- 11 At 48h post-transfection, split cells and plate at <25% confluence in selection medium containing Blasticidin and  200 µg/ml Hygromycin B (no zeocin).



12 Feed the cells with Hygromycin B selection medium every 3-4 days.

13 When foci become apparent, pool Foci and allow cells to expand.

Note



- In our experience, at  200 µg/ml Hygromycin B, it may appear that no colonies remain viable, however after 1-2 weeks a small amount of colonies were observed in which the gene of interest was stably integrated.
- If all cells are nonviable under these conditions, it may be necessary to reduce the concentration of Hygromycin B in the selection media to  100 µg/ml and/or optimize transfection conditions.

Per manufacturers protocol, these surviving foci should be isogenic for the inserted gene.

14 Pooled cells can then be split, frozen, and/or used for experiments.

15 Freeze cells according to Gibco manual.

Expression of VPS13C

16 Plate cells at desired confluence in format appropriate for experiment in expression media containing  0.1 µg/ml -  1 µg/ml tetracycline.

Note

In our hands, we observed no difference in expression levels of VPS13C^{mClover} protein between 0.1 µg/ml and 1 µg/ml and thus we used the lower concentration, however the Gibco manual suggests using 1 µg/ml.

17 After 24 hours, cells may be lysed for western blot or used for microscopy.