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

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We use this protocol and it's working

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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)	

	A	B
	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)

Troubleshooting

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.