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Generation of stable ATG3-mCherry wild-type or mutans HeLa cells with HaloTag-LC3B using lentivirus

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We use this protocol and it's working

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Abstract

This protocol details the generation of stable ATG3-mCherry wild-type or mutant HeLa cells with HaloTag-LC3B using lentivirus.

Attachments

PDF

737-1853.pdf

117KB

Materials

Buffers and reagents:

- XL10-Gold competent E. coli (home made)
- Stbl3 competent E. coli (MacroLab, UC Berkeley)
- Growth broth: LB broth
- DMEM medium with GlutaMAX containing 10% FBS and 10% Pen-Strep.

pVSV-G **addgene Catalog #**#138479

pCMVR8.74 **addgene Catalog #**22036

pCDH1-CMV-HT-LC3-SV40-Hygro **addgene Catalog #**182045

Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #**M0491L

NheI-HF - 5,000 units **New England Biolabs Catalog #**R3131L

BamHI-HF - 50,000 units **New England Biolabs Catalog #**R3136L

EZ-10 Spin Column DNA Gel Extraction Kit **Bio Basic Inc. Catalog #**BS654.SIZE.250preps

EZ-10 Spin Column Plasmid DNA Min-preps Kit **Bio Basic Inc. Catalog #**BS614.SIZE.250preps

Qiagen Hi-Speed MidiPrep kit **Qiagen Catalog #**12643

DMEM, high glucose, GlutaMAX™ Supplement **Thermo Fisher Catalog #**10566016

Gibco™ Fetal Bovine Serum value heat inactivated (formerly USDA-approved in North America or quali **Fisher Scientific Catalog #**A5256801

Penicillin-Streptomycin (10,000 U/mL) **Thermo Fisher Scientific Catalog #**15140122

Opti-MEM™ I Reduced Serum Medium **Thermo Fisher Catalog #**31985070

TransIT®-LT1 Transfection Reagent **Mirus Bio Catalog #**MIR 2300

Lenti-X™ Concentrator **Takara Bio Inc.** **Catalog #**631231

15-Dimethyl-15-diazaundecamethylene polymethobromide Polybrene **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**H9268

Hygromycin B (50 mg/mL) **Thermo Fisher** **Catalog #**10687010

Troubleshooting

Cloning pCDH1-CMV-ATG3-mCherry-Hygro lentiviral vector (ATG3 wild-type or mutants)

1 Amplify the coding sequence for human ATG3 (1-125), mCherry or ATG3 (126-314) fragment using Q5 High Fidelity DNA Polymerase and purify PCR products using Gel extraction kit (Bio Basic).

2 PCR products in step 1 are used as templates to run the second step PCR to amplify ATG3 (1- 125)-mCherry-ATG3(126-314) and gel extracted. Primers include a NheI restriction site at the 5′ end and a BamHI site at the 3′ end.

3 Digest the ATG3-mCherry PCR product and pCDH1-CMV-HT-LC3-SV40-Hygro plasmid using NheI and BamHI restriction enzymes (New England BioLabs).

4 Purify the digested DNAs using Gel extraction kit (Bio Basic).

5 Ligate the digested ATG3-mCherry and linearized pCDH1-CMV-hygro backbone using T4 DNA ligase (New England BioLabs) according to manufacturer instructions.

6 Transform ligation product into XL10-Gold competent *E. coli* and plate on Ampicillin resistant plates. Incubate the plate at 37 °C for 16:00:00 . 16h

7 Inoculate one single colony in 5 mL LB medium with ampicillin at 37 °C Overnight . 16h

8 Miniprep plasmids (Bio Basic) and send for sequencing.

HEK293T lentiviral transfection

9 Each plasmid for lentiviral transfection is transformed into Stbl3 competent cells for the propagation. Next day, pick up one colony to inoculate overnight culture in LB medium with ampicillin.

10 Midi prep the cultures to purify plasmids (Qiagen).

11 Plate 5 × 10^6 HEK 293T cells on a 10 cm plate in DMEM medium.

12 HEK293T transfection:

12.1 Add retroviral packaging plasmids (pVSV-G, pCMV R8.74) and pCDH1-CMV-ATG3-mCherryHygro (ATG3 wild-type or mutants), 5 µg each in 1.5 mL warm Opti-MEM medium.

12.2 Add 45 µL of TransIT-LT1 transfection reagent (Mirus) and swirl.

12.3 Incubate at Room temperature for 00:15:00 . 15m

12.4 Add 1.5 mL dropwise into 10 cm HEK293T plate.

12.5 At 72 hours post-transfection, collect retroviral supernatant into a falcon tube.

13 Concentrate retroviral supernatant to 1 mL using Lenti-X concentrator (Takara) with the manufacturer instruction.

Transduction of HeLa cells with pCDH-CMV-ATG3-mCherry lentivirus

14 Plate 1 × 10^5 ATG3 KO/HaloTag-LC3B HeLa cells into 12-well plate one day before.

15 Next day, titrate 100 µL , 200 µL , 400 µL of concentrated retroviral solution with 8 µg/mL Polybrene (Sigma) into target HeLa cells.

16 At 24 hours post-transduction, remove retroviral supernatant and replace with fresh DMEM complete medium with 0.5 mg/mL hygromycin B (Gibco).

17 After one week of hygromycin B selection, sort HeLa cells by FACS to enrich for mCherry positive cells using Wolf G2 cell sorter (Nanocellect).