Generation of A549 PPM1H BromoTag CRISPR/CAS9 knock-in cell line

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We use this protocol and it's working

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ABSTRACT

This protocol details the generation of A549 PPM1H BromoTag CRISPR/CAS9 knock-in cell line.

ATTACHMENTS

845-2182.docx

MATERIALS

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Materials

Consumables

- Nunc™ Cell-Culture Treated 6-well plates (ThermoFisher Scientific #140675)
- Nunc™ Cell Culture/Petri Dishes (ThermoFisher Scientific #150318)
- Autoclaved 1.5 ml Eppendorf tubes (Eppendorf #0030120086)
- Spin-X Centrifuge tube filters, 0.22µm cellulose acetate membrane (Costar #8161)
- 1.5 ml Eppendorf tubes rack
- Marker pen
- Pipette set (1 ml, 200 µl, 20 µl, 10 µl)
- 15 ml falcon tubes

Reagents

- **Lysis buffer**:

	A	B
	Tris-HCl pH 7.5	50 mM
	Triton X-100	1% (v/v)
	Glycerol	10%
	EDTA pH 8.0	5 mM
	NaCl	150 mM
	sodium orthovanadate	1 mM
	sodium glycerophosphate	10 mM
	sodium pyrophosphate	10 mM
	mycrocystin-LR	1 µg/ml
	complete EDTA-free protease inhibitor cocktail	

- Anti-PPM1H antibody (sheep polyclonal antibody, MRC PPU Reagents and Services, DA018)
- Anti-BromoTag (sheep polyclonal antibody, MRC PPU Reagents and Services, SA599)
- IRDye® 800CW Donkey anti-Goat IgG Secondary Antibody (Licor #926-32214)
- DMEM, high glucose (ThermoFisher Scientific #11965092)
- Trypsin-EDTA (0.05%), phenol red (ThermoFisher Scientific # 25300054)
- Lipofectamine™ LTX Reagent with PLUS™ Reagent (ThermoFisher Scientific #A12621)

- Opti-MEM™ I Reduced Serum Medium (ThermoFisher Scientific #31985062)
- KOD Hot Start DNA Polymerase (Sigma #71086)
- StrataClone Blunt PCR Cloning Kit (Agilent #240218)
- FastDigest EcoRI (ThermoFisher Scientific #FD0274)
- DNeasy® Blood & Tissue Kit (Qiagen #69504)
- QIAprep Spin Miniprep Kit (Qiagen #27106)
- BromoTag® AGB1 compound (Tocris #7686)
- BromoTag® cis-AGB1 compound (Tocris #7687)

Equipment

- Eppendorf Thermomixer
- PCR machine
- Incubator 37°C, supplemented with 5% CO_2
- Block heater

Nunc™ Cell-Culture Treated Multidishes, 6 well **Thermo Fisher Catalog #140675**

Nunc™ Cell Culture/Petri Dishes, 8.8cm2, Nunclon Delta treated, lid **Thermo Fisher Catalog #150318**

1.5ml Safe-lock tubes **Eppendorf Catalog #0030120086**

IRDye® 800CW Donkey anti-Goat IgG Secondary Antibody **LI-COR Catalog #926-32214**

DMEM, high glucose **Thermo Fisher Scientific Catalog #11965092**

0.05% Trypsin-EDTA, phenol red **Invitrogen - Thermo Fisher Catalog #25300054**

Lipofectamine™ LTX Reagent with PLUS™ Reagent **Thermo Fisher Catalog #A12621**

Opti-MEM (Reduced Serum Medium) **Thermo Fisher Scientific Catalog #31985062**

KOD Hot Start DNA Polymerase **Merck MilliporeSigma (Sigma-Aldrich) Catalog #71086-3**

StrataClone Ultra Blunt PCR Cloning Kit. **Agilent Technologies Catalog #240218**

FastDigest EcoRI **Thermo Fisher Catalog #FD0274**

QIAgen DNeasy Blood and Tissue Kit, 50 rxn **Qiagen Catalog #69504**

QIAprep Spin Miniprep Kit Qiagen Catalog #27106

BromoTag® cis-AGB1 Tocris Catalog #7687

BromoTag® AGB1 Tocris Catalog #7686

Transfection for PPM1H BromoTag

5m

1

Note

Note: Complementary oligos for the optimal guide pair A (sense guide 5'-GAAATGGCCCAGGGGATTGGG and anti-sense guide 5'- GAGCTTGTTTCCATGTATTAA) were designed to target the C-terminus of PPM1H locus (ENSG00000111110) (Fig1B). The sense guide is cloned into the pBabeD P U6 plasmid, and the anti-sense guide is cloned into the pX335 plasmid. The donor DNA containing IRES2 GFP for cell sorting purpose is cloned into the pMK-RQ plasmid (Fig1A-B).

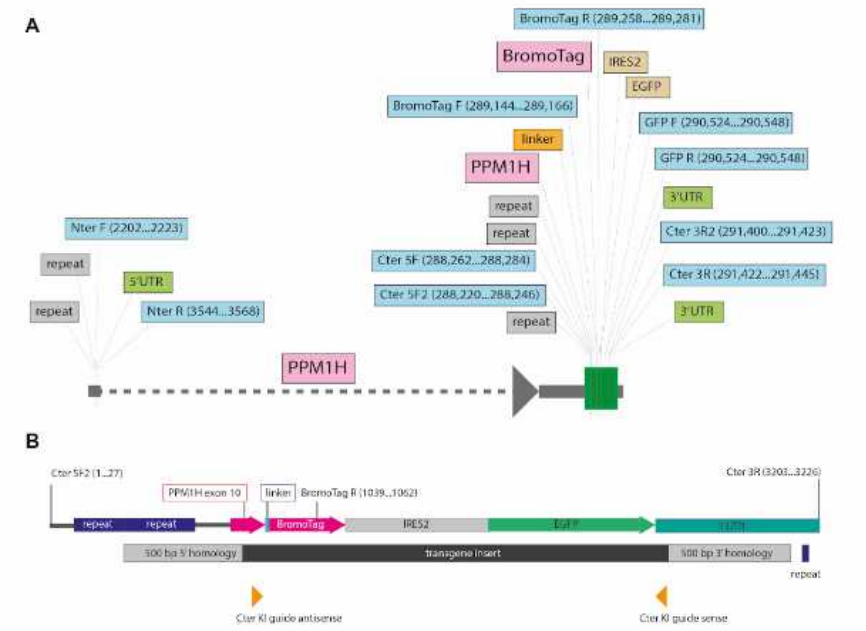

Figure 1. Homozygous selection for A549 PPM1H BromoTag clones. (A) The map of PPM1H locus containing C-terminal BromoTag and IRES2 GFP. (B) The map shows the homology arms and positions of the genotyping primers for PPM1H BromoTag clones.

Seed wild type A549 cells approximately 60 – 70% confluency in 3 mL media/well (DMEM + 10% FBS (Foetal Bovine Serum) + 1% penicillin/streptomycin + 1% L-glutamine) in 6-well plates. Preferably plate 2 wells per guide + donor DNA (one well for cell growing and one for immunoblotting).

Note

Note: prepare 2 extra wells as a control with no DNA transfection.

2 After 16 – 20 hours of cell seeding, perform transfection using LipofectamineLTX.

Note

Note: 1 µg DNA used in total at a 1:1:2 ratio (sense guide 0.25 µg : anti-sense guide 0.25 µg : donor DNA 0.5 µg).

2.1 If use only one guide, then use at a ratio 1:1 (guide 0.5 µg : donor DNA 0.5 µg).

- DNA : LipofectamineLTX ratio = 1 µg : 3.5 µL
- DNA : PLUS Reagent ratio = 1 µg : 1 µL
- Transfection (per well):

Mix A:

	A	B
	Opti-MEM	150 µl
	LipofectamineLTX	3.5 µl

Mix B:

	A	B
	DNA in total	1 µg
	PLUS Reagent	1 µl

2.2 Add mix A to mix B, incubate at Room temperature for 00:05:00, add gently 300 µL mix dropwise to cells. 5m

3 After 24 hours of transfection, aspirate old media and add 3 mL fresh media/well containing puromycin at 3 µg /ml final concentration. Treat cells for 2 days before adding fresh media.

Note

Note: Un-transfected cells (control) should die, and transfected cells should have some cells remaining due to puromycin resistance gene.

4 Allow cells to grow until confluent. Spit cells from 6-well plates into 10 cm petri dishes, then let cells grow until confluent. Cells from 1 dish are stored in freeze media (10% DMSO + 90% FBS) at -80 °C for single cell sorting later, cells from the other dish are used for immunoprecipitation to confirm the presence of PPM1H BromoTag.

Immunoprecipitation of PPM1H from the pools

2h 25m

5 Aspirate media from 10cm dish, wash cells twice with PBS. Add 300 µL lysis buffer into the dish and scrape it using a suitable scrapper. Transfer the lysate into a 1.5ml Eppendorf tube.

6 Centrifuge the lysates at 17000 x g, 4°C, 00:10:00. Transfer supernatant into a new 1.5ml Eppendorf tube. 10m

7 Quantify protein concentration using Bradford assay.

8 Immuno-precipitate PPM1H using anti-PPM1H coupled beads. 500 µg of lysates is incubated with 10 µL of beads for 02:00:00 in the cold room. 2h

Note

Note:

- Anti-PPM1H antibody is covalently coupled with A/G agarose beads at a ratio 1:1 (1 µg antibody : 1 µL resin).
- Lyse cells and immunoprecipitate PPM1H from wild type A549 cells as a control.

9 Wash the beads 3 times with PBS, then add 20 µL of 2X SDS-PAGE loading buffer, incubate for 00:10:00 at 70 °C. 10m

10 Collect the supernatant by centrifuging through a 0.22 µm Spin-X Centrifuge tube filters.

11 Add beta-mercaptoethanol to the eluate to a final concentration 1%.

12 Boil the eluate at 95 °C for 00:05:00, then subjected to immunoblot analysis. 5m

Note

Note: prepare 2 gels for immunoblotting, 1 gel will be used for anti-PPM1H antibody, the other gel will be used for anti-BromoTag antibody. The secondary antibody used is IRDye® 800CW donkey anti-goat. In the membrane blotted with anti-PPM1H antibody, the wild type PPM1H is detected at a size of approximately 56 kDa, and a band of PPM1H BromoTag is detected around 71 kDa. In the membrane blotted with anti-BromoTag antibody, there is no band detected in control samples (wild type A549 cells), while a band of approximately 71 kDa is detected in transfected cells.

Single cell sorting 4m

13 After confirming the presence of PPM1H BromoTag in transfected cells, re-grow cells stored at -80 °C from the section Transfection for PPM1H BromoTag in 10 cm plate in 10 mL complete media.

Complete media

	A
	DMEM
	10%FBS
	1% penicillin/streptomycin
	1% L-glutamine

14 One day before cell sorting: add 200 µL media (DMEM + 20%FBS + 1% penicillin/streptomycin + 1% L-glutamine) into a 96-well plate, keep in 37 °C incubator supplemented with 5% CO_2.

15 On the day of cell sorting:

15.1 Trypsinise cells (3 mL /dish), stop trypsinisation with 10 mL media (DMEM + 10%FBS + 1% penicillin/streptomycin + 1% L-glutamine), transfer to a 15ml falcon tube.

15.2 Spin down at 250 x g, 00:03:00 . 3m

15.3 Remove media, resuspend cells in 2 mL of media (DMEM + 1%FBS + 1% penicillin/streptomycin + 1% L-glutamine).

15.4 Sort single cells with positive GFP signal into a 96-well plate prepared one day before.

Note

Note: wild type A549 cells used as a negative control (without GFP).

15.5 After finishing cell sorting, spin down the 96-well plate at 250 x g, 00:01:00 to ensure cells are at the bottom of the plate. 1m

15.6 Leave cells to grow for approximately 2 weeks in 37 °C incubator supplemented with 5% CO_2.

Growing clones

16 Check every clone under microscopy. After approximately 2 weeks, a single cell will grow into a colony.

17 Split growing cells from single clones into 6-well plates (2 mL media/well, DMEM + 10%FBS + 1% penicillin/streptomycin + 1% L-glutamine). Let them grow until confluent.

18 Split cells from single clones into 2 wells of 6-well plates, wait until they are confluent.

19 Lyse cells from one well for screening (section Screening positive clones), and store cells from another well in freeze media (10% DMSO + 90% FBS) at -80 °C for short term storage.

Note

Note: cell lysates and cells stored at -80 °C from the same single clone should be labelled with the same name (ideally labelled as numbers, eg: 1,2,3, etc...).

Screening positive clones

4h 1m

20 Lyse cells from individual clones recovered from cell sorting with 150 µL of lysis buffer.

21 Analyse the lysates by immunoblotting with anti-PPM1H or anti-BromoTag antibodies (Fig1C). Wild type cells used as a control for wild type PPM1H with molecular weight approximately 56 kDa. PPM1H BromoTag protein runs at a size around 71 kDa.

22 Select clones not showing a band of wild type PPM1H for further analysis. To confirm the presence of PPM1H BromoTag in these clones.

22.1 Seed A549 wild type and knock-in cells at ~ 70% confluency in 6-well plates Overnight. 1m

22.2 Treat cells with [M] 300 nanomolar (nM) AGB1 or [M] 300 nanomolar (nM) cis-AGB1 (epimer inactive degron) compounds for 04:00:00. 4h

22.3 Lyse cells, then analyse by immunoblotting with anti-PPM1H antibody.

Note

Note: under treatment of 300 nM AGB1 compound, PPM1H BromoTag expression is reduced dramatically ~98% after 4 hours, while no reduction of expression is observed with the inactive degron compound. No reduction of PPM1H expression is seen as well in wild type cells with both active and inactive compounds.

DNA sequence to characterise homozygous A549 knock-in PPM.

23 Extract genomic DNA from several positive clones using Qiangen kit according to the manufacturer's instructions.

24 Perform PCR to confirm the presence of PPM1H BromoTag in potential homozygous clones (Fig1D).

- A pair of primers (Cter 5F2: 5’- CTTGCTGAACTTACATTGGTCAAGAGG and BromoTag R: 5’-ACTTGATTGTGCTCATGTCCATGG) are used to amplify an amplicon with expected size at 1062 bp (Fig1A-B).
- Water and wild-type (WT) A549 should be included as negative controls.
- KOD Hot Start Polymerase kit is used as described below.

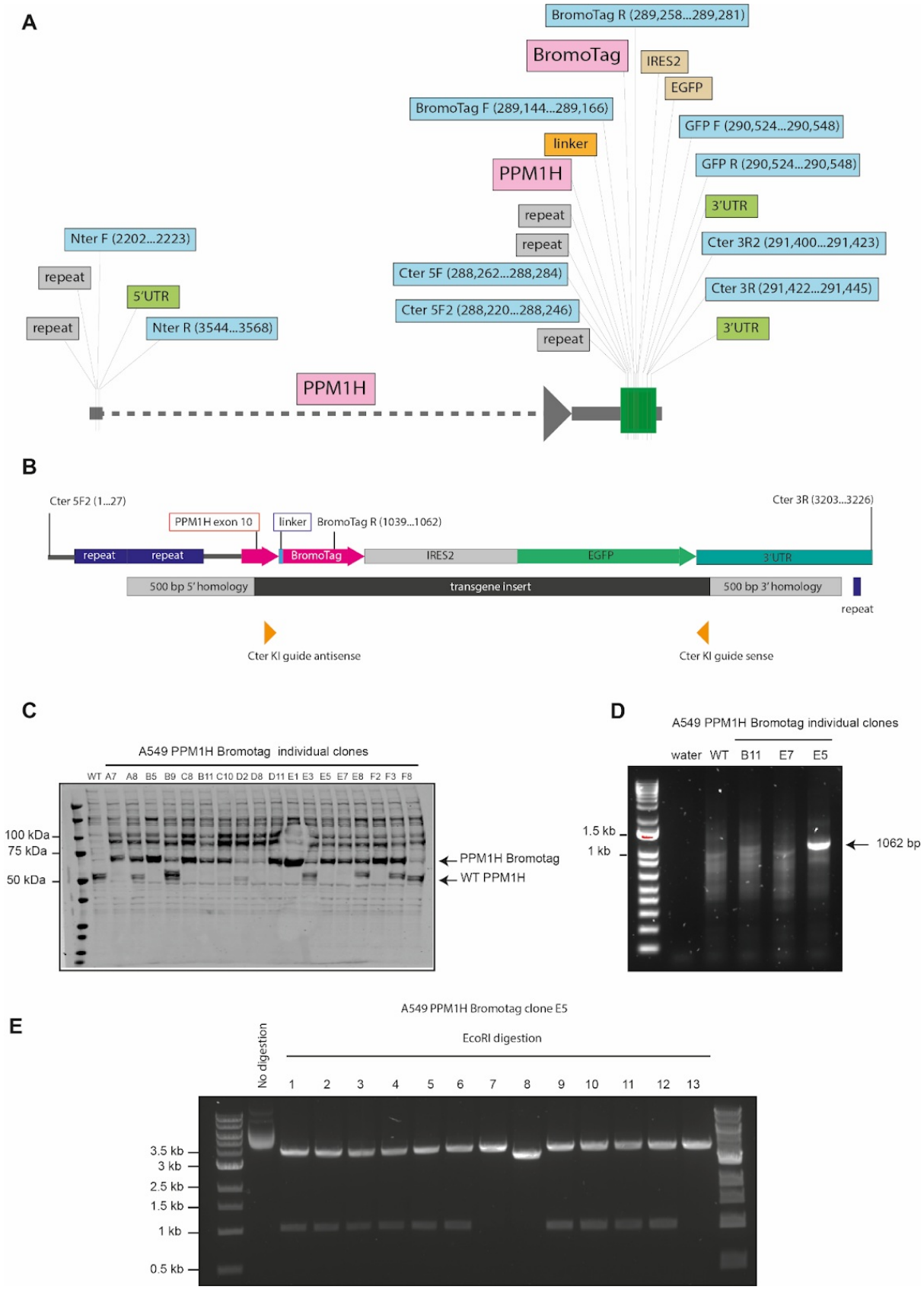

Figure 1. Homozygous selection for A549 PPM1H BromoTag clones. (A) The map of PPM1H locus containing C-terminal BromoTag and IRES2 GFP. (B) The map shows the homology arms and positions of the genotyping primers for PPM1H BromoTag clones (C) Immunoblotting was performed to screen individual clones of PPM1H BromoTag using anti-

PPM1H antibody at concentration 1 µg/ml (sheep polyclonal antibody, MRC PPU Reagents and Services, DA018). Wild-type (WT) A549 cells were included as a control. (D) PCR was performed to confirm the presence of PPM1H BromoTag in potential homozygous clones observed from immunoblotting. A pair of primers (Cter 5F2 and BromoTag R) were used to amplify an amplicon with expected size at 1062 bp. Water and wild-type (WT) A549 were included as negative controls. (E) The clone E5 was selected to clone into the StrataClone PCR cloning vector pSC-B-amp/kan according to the manufacturer's instructions (Stratagene). Thirteen random positive colonies were digested with EcoRI restriction enzyme. All thirteen plasmids were sent for sequencing with M13 forward and M13 reverse primers. The plasmids 1, 2, 3, 4, 5, 6, 9, 10, 11, 12 showed sequences matching with the template of PPM1H BromoTag. In contrast, the plasmids 7, 8, 13 did not show any match with the template, indicating that the fragments cloned into these plasmids are just non-specific PCR products. These results confirm that PPM1H BromoTag clone E5 is a homozygous clone.

PCR reaction setup

25

	A	B
	Reagents	**Volume (µl)**
	10X buffer	2
	dNTP (2mM each)	2
	25mM MgSO4	1.2
	DMSO	1.2
	10 µM forward primer	0.6
	10 µM reverse primer	0.6
	Genomic DNA (75ng)	1
	KOD polymerase	0.4
	Nuclease-free water	11
	Total volume	20

Cycling conditions

4h

26

	A	B	C
	Step	**Time**	
	98°C	2 min	
	98°C	10 s	35 cycles
	60°C	20 s	
	70°C	20 s/kb	
	70°C	5 min	
	4°C	hold	

27 Select clones showing a band at expected size (1062bp) for further analysis (Fig1D). Clone PCR products from the positive clones into the StrataClone PCR cloning vector pSC-B-amp/kan according to the manufacturer's instructions (Stratagene).

28 After overnight incubation at 37 °C, pick randomly 12-20 bacterial white colonies for plasmid DNA analysis and grow in 3 mL LB amp media Overnight. 4h

Note

Note: do not pick blue colonies.

29 Prepare miniprep DNA plasmids from the selected colonies according to the manufacturer's instructions (Qiagen).

30 Identify plasmids containing the PCR product insert (1062bp) by restriction analysis with EcoRI digestion according to the manufacturer's instructions (ThermoFisher Scientific) (Fig1E).

31 Send all the miniprep plasmids for DNA sequence with M13 forward primer (5'-GTAAAACGACGGCCAGTG) and M13 reverse primer (5'- GGAAACAGCTATGACCATG) to identify homozygous knock-in clones.

32 Store homozygous A549 PPM1H BromoTag clones in liquid nitrogen for long-term storage.