Generation of stable amber suppression cell lines

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Abstract

Unnatural amino acid mutagenesis via amber suppression provides the means to tailor proteins inside living cells. A wide range of unnatural amino acids has been incorporated using the *Methanococcus* pyrrolysyl-tRNA synthetase/tRNACUA (PylRS/PylT) in mammalian cell systems in proof of principle experiments, for (i) minimal genetically encoded fluorescence or affinity tags, (ii) photo-control of enzymes, (iii) genetically encoded post-translational protein modifications. We have developed a general and efficient method to genomically integrate the PylRS/PylT amber suppression machinery using PiggyBac-mediated transposition. Using a versatile plasmid system, we are able to achieve homogenous and highly efficient amber suppression in a wide range of cell lines. A general protocol for the generation of stable amber suppression cell lines is described here.

Key words

Genetic code expansion, Mammalian cells, Unnatural amio acid, tRNA, Aminoacyl-tRNA-Synthetase, PiggyBac transposase, Site-specific protein labeling,

1. Introduction

A successful method for stable amber suppression in mammalian cells entails high expression of PylT and PylRS genes from one or more integrated copies in the genome. To accomplish this, we have established a versatile vector system for PiggyBac-mediated transgenesis in mammalian cells [1]. PiggyBac is a short inverted terminal repeats (ITR) mobile element isolated from the moth *Trichoplusia ni* [2]. It integrates and excises with high precision at genomic TTAA nucleotide sequences. The insert size flanked by ITRs is flexible and a single protein, termed PiggyBac Transposase (PBase), mediates transposition, making the system easily adaptable for genetic engineering. PiggyBac transposase is highly active in mammalian cells, achieving high (1-30%) integration efficiency of a single and multiple targeting vectors in mouse embryonic stem cells (mESC) [3,4].

To allow flexible combination of various PylRS variants and protein of interest (POI), we have designed targeting cassettes that encode for PylRS and POI and complementary antibiotic selection markers on two independent vectors (Figure 1). Each vector contains a Pol II transcript driven by an EF1-α promoter and a tandem repeat of 4 PylT genes driven by U6 Pol III promoters, arranged head-to-head (Figure 1). A Puromycin or Neomycin resistance marker is expressed from an IRES in the Pol II transcript. The expression cassette is flanked by universal chicken β-globin insulators and PiggyBac ITRs. PiggyBac transposase is supplied from a third non-integrating plasmid (named pPBase here). The integration process entails introducing all three plasmids into the target cells using a suitable transfection or electroporation method. PiggyBac transposase, transiently expressed from pPBase, mediates reversible integration of both targeting cassettes at genomic TTAA nucleotide sequences. Although a high copy number of donor plasmid in the transfected cell skews the reversible process towards integration, the genomic copies remain unstable as long as PiggyBac transposase remains active. Thus, care has to be taken to apply a sufficiently long drug selection period until the transposition activity has

ceased. Each targeting cassette may be integrated in one or several copies at distinct location, a process that is tunable by varying plasmid ratios in the transfection and stringency of selection conditions. Of note, the repeated process of integration and excision of individual copies does not introduce mutations in the genomic TTAA targeting sites because of the high precision of cutting and pasting of the transposase enzyme. Once integrated, we have observed long-term stable expression and amber suppression efficiency, facilitated both by the silencing-resistant EF1-α promoter and flanking insulator sequence.

Here, the process of cloning the gene for the POI into a PiggyBac targeting vector, transfecting and selecting cells is described, and an optimized protocol for mESC is provided.

2. Materials

1. Chemically competent *E.coli* Stbl3 or NEB® Stable cells
2. Standard cloning enzymes; e.g. *Nhe*I, *Not*I, DNA polymerase, T4 DNA Ligase
3. Standard media and plates for *E. coli* transformation
4. Agarose gel electrophoresis equipment
5. Mini and endotoxin-free Maxi plasmid preparation kits
6. EF1_up primer: AACTGGGAAAGTGATGTCG
7. IRES_down primer: AGACCCCTAGGAATGCTCGT
8. Puromycin (10mg/mL stock solution, store at -20˚C)
9. G418 Sulfate (50mg/mL stock solution, store at +4˚C)
10. 1mg/mL Polyethyleneimine (PEI) transfection solution (see **Note 1**)
11. Primary cell transfection reagent TransIT-X2® (Mirus)
12. Cell culture medium (depends on requirements of cell line)
13. ES cell culture medium; 500mL KO DMEM, 6ml of Ala-Gln (100x), 6ml of 100µM 2-mercaptoethanol stock solution (35µl of 2-mercaptoethanol in 50mL PBS, sterile

filtered, stored at 4˚C), 6mls of non-essential amino acids (100x), 60uL LIF (10 Mio U/mL Leukaemia inhibitory factor), 95mL ES grade FBS

14. OPTI-MEM I Reduced Serum Medium
15. Super PiggyBac Transposase Expression Vector pPBase (PB200PA, System Bioscience Inc)
16. PiggyBac targeting vector pPB 4xPylT/PylRS with Puromycin resistance marker (see **Note 2**)
17. PiggyBac targeting vector for amber suppression reporter or gene of interest with Neomycin resistance marker, and positive control e.g. pPB 4xPylT/mCherry-TAG-EGFP

3. Methods

3.1. Cloning the gene of interest into PiggyBac Targeting Vector

Two vectors are needed to accomplish stable integration of both the Pyrrolysyl-tRNA-Synthetase and the gene of interest containing an amber codon in the desired position. The overall design of the two vectors is identical, but two complementary antibiotic resistance markers are used to select for integration (Figure 1). Importantly, the use of two independent vectors allows step-wise generation of cell lines and adjustment of expression levels of the PylRS and gene of interest by varying antibiotic concentrations. New PylRS variants and genes of interest can be easily cloned into the targeting vectors by restriction cloning, replacing the existing inserts.

1. Design external primers to amplify your gene of interest (GOI) with overhangs matching the restriction site in the PiggyBac targeting vector (e.g. *Nhe*I and *Not*I). Include a Kozak sequence and the Met start codon (e.g. CCACCATG). To simplify detection of the amber suppressed protein, include a N- or C-terminal peptide tag (e.g. HA) (see **Note 3**).
2. Design internal primers for an overlap extension PCR reaction to replace a sense codon with an amber codon at the desired position of the unnatural amino acid (see **Note 4**).

3. Perform two PCR reactions using primer pairs to amplify the two fragments of the GOI 3' and 5' of the introduced TAG codon. As a control, amplify full-length GOI without amber codon using external primers only.
4. Purify amplified products using a PCR purification column.
5. To produce full-length GOI insert with amber codon, perform a second PCR reaction using a mix of 3' and 5' fragment of the GOI as template and external primers only.
6. Digest pPB 4xPylT/mCherry-TAG-EGFP vector (e.g. using *Nhe*I, *Not*I), and purify backbone (8 kb) using agarose gel electrophoresis.
7. Use restriction cloning and ligation (or homology-based cloning, e.g. Gibson, In-Fusion) to insert PCR products into the digested vector.
8. Transform ligation products into Stbl3 or NEB Stable cells and plate on LB/Amp agar plates (see **Note 5**).
9. Pick colonies, mini-prep and validate GOI by restriction digest and Sanger sequencing (using EF1_up and IRES_down sequencing primers)
10. Maxi-prep validated clones for transfection into cell lines. Plasmid should be eluted from column using sterile Elution Buffer or ddH_2O, under sterile or semi-sterile conditions (hood or benchtop).

3.2 Transfection of adherent cell lines

PiggyBac vectors for integration and pPBase vector are introduced into the cells using lipofection. The ratio of the three vectors has been optimized to 1ug pPB 4xPylT/PylRS, 1 ug pPB 4xPylT/GOI, 0.5 ug pPBase (2:2:1 ratio). In addition to your GOI, perform a control transfection with 2:2:1 pPB 4xPylT/PylRS:pPB 4xPylT/mCherry-TAG-EGFP:pPBase to allow easy assessment of amber suppression efficiency by fluorescent or western blot readout.

1. For each transfection, seed 2.5x10^5 cells in a 6-well plate 24 hours before transfection in medium without antibiotics. Grow in an incubator at 37˚C with 5% CO_2 atmosphere.

11. Prepare plasmid mix, 2.5ug total DNA, in 250uL OPTI-MEM.
12. Vortex gently to mix.
13. Add 7.5uL 1mg/mL PEI.
14. Vortex gently to mix.
15. Incubate for 20-30 min to form transfection complex.
16. Add transfection mix drop wise to entire area of the well.
17. Incubate 12-24 hours before changing medium to fresh medium (may contain Pen/Strep).
18. Split cells and start selection 48 hours after transfection.

3.2a Optimized protocol for transfection of mouse embryonic stem cells

Mouse ESC and other primary cells are difficult-to-transfect and PEI can be toxic to those cells. Thus, an optimized protocol for transfection of mouse ESC is described here. 150 000 cells are transfected with 1ug plasmid and 3uL TransIT-X2 (Mirus) in suspension, yielding an excellent transfection efficiency of 50-70% and low toxicity. The ratio of vectors is 0.4 ug 4xPylT/PylRS, 0.4 ug 4xPylT/GOI, 0.2 ug pPBase (2:2:1 ratio).

2. Add 2mL ES Medium to wells of a gelatin-coated 12-well plate and let equilibrate in an incubator at 37˚C with 5% CO_2 atmosphere.
3. Prepare plasmid mix, 1ug total DNA, in 100uL OPTI-MEM.
4. Vortex gently to mix.
5. Add 3uL TransIT-X2.
6. Vortex gently to mix.
7. Incubate for 15-30 min before step 10.
8. Trypsinize cells, resuspend in ESC medium, count and dilute to $0.6x10^6$ cells/mL in ESC medium.
9. Take 12-well plate out of incubator.

10. Add 250 uL cell suspension to each 100 uL transfection mix and for each transfection continue to steps 11 and 12 within one minute.
11. Mix well by pipetting up and down three times.
12. Transfer entire volume to a well of the preequilibrated 12-well plate, distributing cells evenly.
13. Incubate cells in an incubator at 37˚C with 5% CO_2 atmosphere. Exchange medium after 24 hours (may contain Pen/Strep) and start selection 48h after transfection.

3.3. Selection of polyclonal pool.

1. Split cells 48 hours after transfection into 6 wells of the same size. Make a series of Puromycin and G418 concentrations to find the highest feasible selection condition (See **Note 6**). For example:

Puro G418	0.5 ug/mL 500 ug/mL	1 ug/mL 1000 ug/mL	1 ug/mL 1500 ug/mL
Puro G418	1 ug/mL 2000 ug/mL	2 ug/mL 2000 ug/mL	5 ug/mL 2000 ug/mL

2. Maintain cells under selection for at least 7 days to ensure stable integration, replacing medium every 2-4 days (see **Note 7**).
3. Collect wells that contain surviving cells at the highest drug concentration and expand.
4. Single clones can be derived from polyclonal pool by single cell sorting or serial dilution.

3.4. Selection of individual clones

1. Split cells 48 hours after transfection, split each well into 3x 10 cm dishes. Add three different concentrations of antibiotic selection markers.

2. Maintain cells under selection for at least 7 days to ensure stable intergration, changing medium every 2-3 days.
3. Collect single colonies at the highest antibiotic concentration and expand.

3.5. Functional test of amber suppression

1. Expand polyclonal pool after selection or monoclonal cell line from a single colony and make frozen stocks as soon as sufficient cells are available (see **Note 8**).
2. Seed $2.5x10^5$ cells per well in several wells of a 6-well plate and let attach.
3. After 24 hours replace medium with fresh medium, supplementing one well with unnatural amino acid (typical range 0.2-1mM for most unnatural amino acids). Additional wells can be used to test a range of concentrations for optimization (e.g. 0.1, 0.25, 0.5, 1 mM).
4. Harvest cells 24 to 48 hours after addition of unnatural amino acid for downstream analysis such as western blotting, immunofluorescent staining or FACS analysis.

Figure 1 – Vectors used for PiggyBac transposase-mediated integration of amber suppression machinery. Two variants with complementary selection markers are used to integrate both Pyrrolysyol-tRNA syntethase (PylRS) as well as the gene of interest (here the double fluorescent reporter mCherry-TAG-EGFP). Note the C-terminal FLAG and HA tags for detection. The vector backbone contains inverted repeats for recognized by PiggyBac transposase (pink triangles), insulator elements ("Ins", gray box), a tandem cassette of four PylT genes driven by a U6 Pol III promoter, and strong EF1-α Pol II promoter and IRES driving a Puro or Neo resistance gene.

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1 Note that PEI length and branching may vary, and using the specific product from Polyscience (Polyethylenimine 25kD linear, cat# 23966-2) is important for high transfection efficiency. To prepare PEI stock solution:

- Heat 50mL ddH2O to ~80C (e.g. by briefly boiling in a microwave)
- Dissolve 50mg PEI powder
- Let cool to room temperature
- Neutralize to pH 7.0, filter sterilize (0.22um), aliquot and store at -20°C; a working stock (e.g. 1mL aliquot) can be kept at 4°C for extended periods.

2 Vectors can be requested from the Centre for Chemical & Synthetic Biology (CCSB) at MRC Laboratory of Molecular Biology

3 A C-terminal tag will detect full-length protein only, and can identify secondary translation initiation events after the amber codon that lead to production a protein fragment lacking the N-terminus. An N-terminal tag is useful to assess the stability of the N-terminal fragment terminated by the amber codon in the absence of unnatural amino acid.

4 Placement of the amber codon requires careful consideration: The position may be predetermined if a specific functional residue is intended to be replaced with an unnatural amino acid (e.g. for genetically encoding a site-specific post-translational modification [1]). In other cases, the placement may be more flexible, and in such cases it is beneficial to test three or more positions to find one that is efficiently suppressed. Several factors may be helpful to consider: First, the position of the amber codon within the open reading frame may crucially affect the production of desired full-length protein product and unwanted byproducts. Placing the amber codon immediately after or close to the ATG start codon will favor secondary initiation, producing an unwanted N-terminally truncated protein product [5]. Placing the amber codon

close to the C-terminus of the POI may lead to the production of a stable truncation product in the absence of unnatural amino acid. Thus as a general rule, inserting unnatural amino acids in or before the first folded domain of the POI, but not within the first ten residues will be most useful. Second, the local context of the amber codon may affect efficiency. Thus moving the amber codon even by one residue may have an impact. In our hands, no particular consensus has been found for upstream or downstream bases or motifs that enhance amber suppression efficiency.

5 Recombination-deficient E. coli strains are used to maintain the 4x U6PylT tandem repeats.

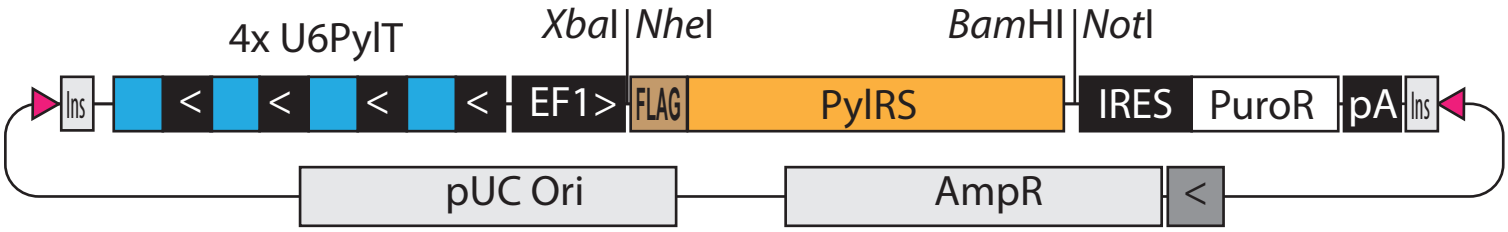
6 For a new cell line it is highly recommended to perform 'kill curves' with Puromycin and G418 prior to this selection experiment and/or to include a control transfection without pPBase plasmid to establish antibiotic concentrations that effectively kill cells without stable integrants.

7 Cells at lower antibiotic concentrations may expand rapidly and need to be split once or twice within the 7-day selection period. At higher antibiotic concentrations, cells may grow considerably slower but eventually recover. Amber suppression efficiency crucially depends on high expression levels of PylT and GOI, thus it is important to check growth in the wells with higher antibiotic concentrations.

8 Some cell lines such as HEK293 cells have a highly unstable chromosome composition and may lose transgenes spontaneously. Thus, periodic reselection with the original antibiotic concentrations ensure the maintenance of the integrated amber suppression machinery.

Figure 1

pPiggyBac 4xPylT/PylRS



pPiggyBac 4xPylT/mCherry-TAG-EGFP