



# **TAT-CRE Recombinase**

Product Manual for Catalog No.  
SCR508

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USA & Canada Phone: +1(800) 645-5476  
In Europe, please contact Customer Service:  
France: 0825.045.645;  
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Germany: 01805.045.645  
Italy: 848.845.645  
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## Introduction

Cre-Recombinase has wide utility for genetic manipulation of DNA in vitro and in vivo, however one limitation has been the difficulty expressing and delivering a consistently active enzyme to mammalian cells. Various strategies have been employed to address this issue. Transient transfection of DNA vectors expressing Cre-Recombinase suffers from low transfection efficiencies resulting in variable and low recombination efficiencies. Viral transduction methods are more efficient, but carry the risk of insertional mutagenesis.

EMD Millipore has developed a cell-permeant TAT-CRE Recombinase<sup>1,2</sup> fusion protein which can be directly delivered to mammalian cells and results in high recombination efficiencies (75 – 100% in mouse and ~ 60% in human cells). TAT-CRE readily translocates to mammalian cells and can catalyze highly efficient recombination. The dose and timing of Cre exposure can be precisely controlled thus allowing for the careful titration of Cre activity.

**Packaging:** 1 vial containing 150 µL of 10,000 Units/mL TAT-Cre Recombinase protein.

**Activity:** A standard of 100 Units is defined as the amount of TAT-CRE (µg) in 1.0 mL of tissue culture medium that is required to induce 50% GFP expression in a HEK293T reporter cell line assay.

**Quality Control:** Each lot of TAT-CRE Recombinase protein is rigorously quality control tested for the following parameters:

- Purity: Single band around 41 kDa with >70% protein purity on an SDS-PAGE gel
- Functional activity: mediates recombination of LoxP-modified alleles in a HEK293T- Cre reporter cell line
- Endotoxin levels: < 1 EU/µg protein
- Mycoplasma negative

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## Background

Cre Recombinase is an enzyme from bacteriophage P1 that catalyzes the site-specific recombination between two DNA recognition sites termed loxP sites. The LoxP recognition site consists of two 13 bp inverted repeats flanking a 8 bp spacer region. Because the Cre gene and loxP sites are not native to the genomes of most species, LoxP sites can be engineered and introduced into target cells and thus used as a means to precisely control the expression of genes in vitro (i.e. cultured cells) and in vivo (i.e. animal models)<sup>3,4</sup>.

|               |              |               |                     |
|---------------|--------------|---------------|---------------------|
| <b>13 bp</b>  | <b>8 bp</b>  | <b>13 bp</b>  | where 'N' indicates |
| ATAACTTCGTATA | - NNNTANNN - | TATACGAAGTTAT | bases that may vary |

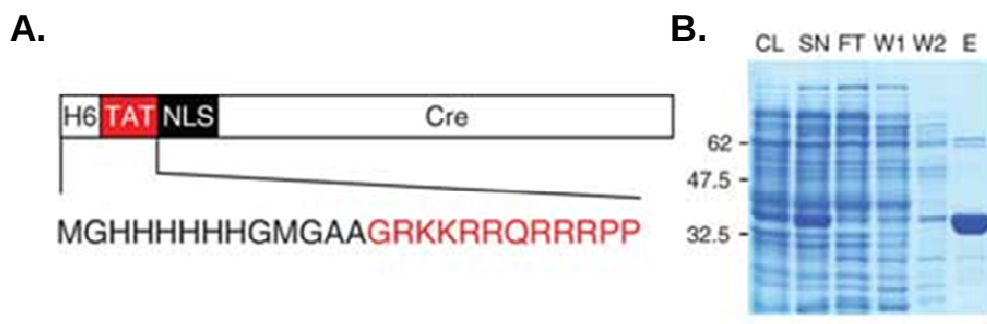
DNA sequences located between 2 loxP sites are said to be “floxed”. Depending upon the location and directional orientation of the loxP sites, Cre-mediated recombination will have different consequences for the floxed segment.

- If LoxP sites are located on different chromosomes, Cre recombinase will mediate a chromosomal translocation.
- If LoxP sites are oriented in the opposite direction, Cre recombinase will mediate an inversion of the floxed segment.
- If LoxP sites are oriented in the same direction, Cre recombinase will mediate a deletion of the floxed segment.

In this way, placement of the LoxP sites allows genes to be activated, repressed or exchanged for other genes.

## Product Description

EMD Millipore's TAT-CRE Recombinase is a recombinant cell-permeant fusion protein consisting of a basic protein translocation peptide derived from HIV-TAT (TAT), a nuclear localization sequence (NLS), the Cre protein and an N-terminal histidine tag (H6) for efficient purification of the protein from *E. coli*.<sup>1,2</sup>



**Figure 1.** Schematic of cell-permeant TAT-CRE fusion protein. H6, 6-His tag. The amino acid sequence of the amino terminus is depicted showing the TAT peptide sequence in red (A). Purification of recombinant TAT-CRE from bacteria, as analyzed by Coomassie blue staining of an SDS-PAGE gel (B). CL, cleared lysate; SN, supernatant; FT, flow through; W1, W2, wash fractions 1 and 2; E, eluted fraction. Numbers on the left indicate molecular weight (kDa) of marker proteins. (Figure from Nolan, L et al. *Nature Methods*, 2006 3(6): 461-7).

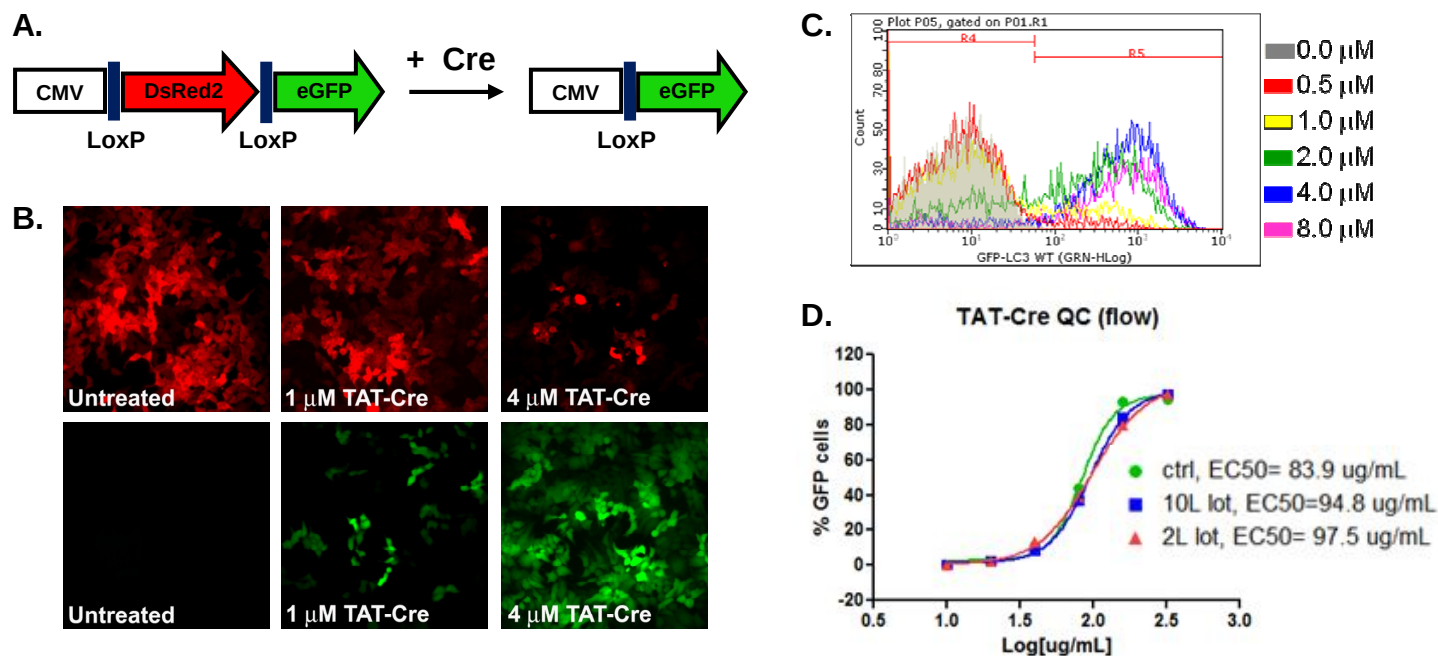
**Each lot of TAT-CRE Recombinase protein is rigorously quality control tested for protein purity and functional activity to induce recombination of LoxP-modified alleles in a HEK293T-Cre reporter cell line.**

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## Functional QC: HEK293T-Cre Reporter Cell Line



**Figure 2.** Each lot of TAT-CRE is quality control tested for protein purity and for transduction and recombination activities. A HEK293T cell line stably expressing a double fluorescent reporter construct was used to monitor Cre recombination (A). Cells express dsRed2 before Cre-recombination. Addition of TAT-CRE mediates recombination and results in expression of the eGFP, by deleting the LoxP-flanking dsRed2 gene. Maximal eGFP expression was achieved when 4  $\mu\text{M}$  TAT-Cre was used to treat the cells overnight (B, C). Dose dependent increases of eGFP expressing cells were quantified using flow cytometry analysis (C). Percentages of eGFP positive cells were plotted against the amount of TAT-CRE applied. Consistent lot to lot performance was observed (D)

**Enzymatic activity for each lot of TAT-CRE (C)** was determined by incubating 100,000 HEK293T-CRE reporter cells per well of a 6-well plate with varying concentrations of TAT-CRE (320  $\mu\text{g/mL}$ , 160  $\mu\text{g/mL}$ , 80  $\mu\text{g/mL}$ , 40  $\mu\text{g/mL}$ , 20  $\mu\text{g/mL}$  and 0  $\mu\text{g/mL}$ ) for 16-18 hours. Cells were then trypsinized & the percentage of GFP expression quantified by flow cytometry.

**100U is the amount of TAT-Cre protein ( $\mu\text{g}$ ) required to induce 50% excision (i.e 50% GFP expression) in 1 mL of solution.**

For example: If  $\text{EC}_{50} = 50\% \text{ eGFP} = 94.8 \mu\text{g/mL}$  TAT-CRE

$$\begin{aligned} 100 \text{ Units} &= 94.8 \mu\text{g/mL TAT-CRE} \\ 1 \text{ Unit} &= 94.8 \mu\text{g}/100 = 0.948 \mu\text{g} \end{aligned}$$

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## Storage and Handling

Each vial contains 150  $\mu$ L of 10,000 Units/mL TAT-CRE Recombinase protein. Stable for 3 months from date of receipt when stored at -20°C or -80°C. Upon first thaw, centrifuge the vial and gently mix the solution. Aliquot into smaller working volumes and freeze at -20°C or -80°C. Before use, dilute TAT-CRE to the appropriate concentration with culture medium and filter through a 0.2  $\mu$ m low – protein binding syringe filter (Millipore Cat. No. SLGV 033RS or SLGV013 SL).

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## General Protocol

1. Plate cell type of interest in the appropriate expansion medium and incubate in a 37°C incubator until the cells are ready to be passage (approximately 80% confluent).
2. Wash cells with 1X PBS. Aspirate.
3. Dissociate the cells into a single cell suspension with Accutase (Cat. No. SCR005). Incubate in a 37°C incubator for 3 – 5 minutes.

**Note:** *Different cell lines may require different incubation time. It is important to monitor the cell dissociation. Accutase treatment should be stopped when the cells start to dissociate.*

4. Inspect the plate and ensure the complete detachment of the cells by gently tapping the side of the plate with the palm of your hand. Confirm under the microscope that the cells are in single cell suspension. Pipet the cells gently to break into a single cell suspension.
5. Collect the dissociated cells to a 15 mL conical tube. Rinse the wells with an additional 2 mL of expansion medium to collect any remaining cells. Add the rinse to the 15 mL conical tube.
6. Centrifuge the 15 mL conical tube containing the cell suspension at 300 x g for 5 minutes at room temperature (15 - 25°C).
7. Aspirate the supernatant. Resuspend in a small volume of fresh expansion medium for cell counting.
8. Count the number of cells using a hemacytometer. Ensure that the cells are in a single cell suspension. Determine the cell viability using Trypan Blue exclusion.
9. Determine the optimal seeding density for the cell type of interest. For example, plate 50K, 75K, 100K cells /well in a 12-well plate in the appropriate expansion medium. Incubate at 37°C overnight to allow the cells to attach.
10. The next day, replace with fresh expansion medium containing a range of TAT-CRE concentrations ranging from 1  $\mu$ M, 2  $\mu$ M, 3  $\mu$ M, 4  $\mu$ M, 5  $\mu$ M). Dilute the required amount of TAT-CRE protein from the glycerol stock into expansion medium and filter sterilize with a 0.2  $\mu$ m low protein binding syringe filter (Cat. No. SLGV 033RS or SLGV013SL). Volume = 0.5 – 1 mL per well of a 12-well plate.

11. Monitor every 30 minutes – 1 hour after the addition of TAT-CRE to the culture. TAT-CRE treatment should be stopped when you observe significant cell death. Because high concentrations of TAT-CRE may be toxic to the cells, cell death is used as an indicator of TAT-CRE penetration. Ideally you want to have the highest concentration of TAT-CRE as measured by the most cell death while still retaining sufficient viable live cells.

**Note:** *TAT-CRE sensitivity may vary in different cell lines and will need to be determined empirically. It is recommended to set up a range of conditions such that TAT-CRE treatment is halted when you observe approximately 50 – 80% cell death.*

12. To halt TAT-CRE treatment, wash the cell twice with 1X PBS. Aspirate after each wash.
13. Add fresh expansion medium to each treated sample and change the medium daily or every other day until colonies start to emerge and expand.
14. When colonies are of sufficient size, but are not touching or merging with each other, pick individual clones for expansion and genomic DNA extraction and real-time quantitative PCR analysis.

**Note:** *We recommend picking at least 10 colonies per experiment.*

15. For each clone, isolate genomic DNA and set up real-time quantitative PCR reactions. Follow the protocol as outlined in the section titled “qRT-PCR analysis of TAT-CRE treated clones”.

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## **TAT-CRE Application 1: Excision of viral transgenes from human iPS cells**

Cre recombinase has broad applications for controlled genetic modification of the mammalian genome. The following protocol was developed for human induced pluripotent stem (iPS) cells generated using the Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (Cat. No. SCR545). The viral transgenes were removed using cell permeable TAT-CRE with excision confirmed by quantitative PCR of genomic DNA extracted from treated human iPS clones. Please follow the specific manufacturer’s protocol for reprogramming. The following protocol should only be used as a **reference** to begin optimizing conditions that will enable transduction of other cell lines.

### **Protocol:**

1. If they are not already in a feeder-free culture system, transition pluripotent human iPS cells to feeder-free cultures and culture for at least two passages before proceeding to the next step. Feeders competitively take up the TAT-CRE and hence may deplete the amount of protein available for the target hiPS cells. We recommend the use of PluriSTEM™ (Cat. No. SCM130), but other commercially available feeder-free medium would also work.
2. Coat 12-well plates with 1:20 dilution of ice-cold Matrigel (1.0 mL per well). Swirl the culture plates to spread the Matrigel evenly across the surface of the plate. Incubate 2 – 8°C overnight or at room temperature for 1 – 2 hours before use.

3. On the day of passage, acclimate matrigel coated plates for 1 hour at room temperature. After 1 hour, remove the matrigel coating. Add 1 mL PluriSTEM™ or other feeder-free medium to each well. Set plate aside until cells are ready to be passaged.
4. Aliquot sufficient PluriSTEM™ or other feeder-free medium, DMEM/F12 (Cat. No. DF-041-B), and Alfazyme (Cat. No. L11-012, PAA Laboratories) to passage the cells. Warm reagents at room temperature (15 – 25°C) for 5 – 10 minutes.
5. One hour before passaging, add the ROCK Inhibitor, Y-27632 (Cat. No. SCM075) to each well of the 6-well plate for a final concentration of 10 µM. ROCK Inhibitor, Y-27632 is added to prevent apoptosis of human iPS cells in single cell suspension.
6. After 1 hour, use a dissection microscope to visually inspect the plate containing human pluripotent cells to be passaged. Inspect the colonies for areas of spontaneous differentiation.
7. Use a sterile p200 pipette tip attached to a p200 pipetman to scrape away areas of spontaneous differentiation. Be discriminating and scrape away any areas that harbor a hint of differentiation.
8. Aspirate the medium containing the scrapped areas from the well. Rinse with 2 mL per well with 1X PBS (Cat. No. BSS-1006-B).
9. Aspirate and replace with 1 mL of Alfazyme (Cat. No. L11-012, PAA Laboratories) per well of the 6-well-plate containing the human iPS cells. Incubate at 37°C for 20 – 30 minutes. Inspect the plate and ensure the complete detachment of the cells by pipeting up and down. Be careful to not create bubbles.

**Note:** *Different cell lines may require different incubation time. It is thus important to monitor the cell dissociation. Alfazyme treatment should be stopped when the cells start to dissociate.*

10. Quench the Alfazyme reaction by adding 1 mL PluriSTEM™ or other feeder-free medium for each mL of Alfazyme used. Gently pipet up and down to break any remaining cell clumps into a single cell suspension.
11. Collect the singly dissociated cells to a 15 mL conical tube. Rinse the wells with an additional 2 mL of PluriSTEM™ or other feeder-free medium to collect any remaining cells. Add the rinse to the 15 mL conical tube.
12. Centrifuge the 15 mL conical tube containing the cell suspension at 300 x g for 5 minutes at room temperature (15 – 25°C).
13. Aspirate the supernatant. Resuspend the cells in a small volume of fresh PluriSTEM™ or other feeder-free medium containing 10 µM ROCK Inhibitor, Y-27632 to allow cell counting.
14. Count the number of cells using a hemacytometer. Ensure that the cells are in a single cell suspension. Determine the cell viability using Trypan Blue exclusion.
15. Plate 50,000 to 100,000 cells per well into a matrigel-coated 12-well plate (prepared from step 2) in PluriSTEM™ or other feeder-free medium containing 10 µM ROCK Inhibitor, Y-27632. Incubate at 37°C overnight to allow the cells time to attach.

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**Note:** 100,000 cells per well was empirically determined to be the optimal seeding density for our in-house generated human iPS cells. A careful titration of seeding density may be required for other hiPS cell lines.

16. The next day, wash two times with 1X PBS. Aspirate after each wash.
17. Prepare PluriSTEM™ or other feeder-free medium containing a range of TAT-CRE concentrations (2  $\mu$ M, 3  $\mu$ M, 4  $\mu$ M, and 5  $\mu$ M). ROCK Inhibitor, Y27632 is no longer required from this step forward. Dilute the required amount of TAT-CRE protein from the glycerol stock into PluriSTEM™ or other feeder-free medium and filter-sterilize with a 0.2  $\mu$ m low protein binding syringe filter (Cat. No. SLGV033RS or SLGV013SL) into falcon tubes. Volume = 0.5 – 1 mL per well of a 12-well plate.
18. Monitor every 30 minutes after the addition of TAT-CRE to the culture. TAT-CRE treatment should be stopped when you observe significant cell death. Because high concentrations of TAT-CRE are toxic to the cells, cell death is used as an indicator of TAT-CRE penetration. Ideally you want to have the highest concentration of TAT-CRE as measured by the most cell death while still retaining sufficient viable live cells.

**Note:** TAT-CRE sensitivity may vary in different cell lines and will need to be determined empirically. For our in-house iPS clones, a 5  $\mu$ M TAT-CRE treatment was halted at 2 hours when 50 – 70% cell death was observed.

19. To halt TAT-CRE treatment, wash the cells twice with 1X PBS. Aspirate after each wash.
20. Add 1 mL fresh PluriSTEM™ or other feeder-free medium to each treated sample and change with fresh PluriSTEM™ or other feeder-free medium every day. Colonies will emerge and can be expanded 9 – 12 days after treatment.

**Note:** After treatment, there may be significant cell death. Do not throw out the culture as excised hiPS single cells may not be immediately visible, but are present and will proliferate and form distinct colonies after 2 weeks.

21. When colonies are of sufficient size, but are not touching or merging with each other, pick individual colonies for expansion and genomic DNA extraction and real-time quantitative PCR analysis.

**Note:** We recommend picking at least 10 colonies per experiment. Approximately 60% excision efficiency was obtained when using TAT-CRE on our in-house human iPS clones.

## Expansion of TAT-CRE treated hiPS clones

Once TAT-CRE treated colonies have emerged, it is essential to identify clones that have successfully excised the viral transgenes. The following protocol is recommended.

22. Coat 4-well plates with 1:20 dilution of ice-cold Matrigel (0.5 mL per well). Swirl the culture plates to spread the Matrigel evenly across the surface of the plate. Incubate 2 – 8°C overnight or at room temperature for 1-2 hours before use.



23. On the day that colonies are to be picked, acclimate matrigel coated plates for 1 hour at room temperature. After 1 hour, remove the matrigel coating. Add 0.5 mL PluriSTEM™ or other feeder-free medium to each well. Set plate aside until cells are ready to be passaged.
24. Transfer the 12-well plate containing iPS cell colonies to a tissue culture hood containing a dissecting microscope. Use a 21 gauge needle attached to a 3 mL syringe to cut each iPS colony into 4 – 9 pieces, depending upon the colony size. One half of each colony will be used for continued colony expansion while the other half will be used for qRT-PCR analysis to determine excision status of the clone.
25. Using a sterile p200 pipette tip attached to a p200 pipetman, nudge and transfer **half** of the pieces from each colony into a new well of a matrigel-coated 4-well-plate containing 0.5 mL PluriSTEM™ media or other feeder-free medium. This step establishes and expands individual potentially post-excised iPS clones. Agitate the plates gently from side to side and forward and backwards to ensure that iPS clumps are evenly distributed. Place the plate in a 37°C, 5% CO<sub>2</sub> incubator.

**Note:** It is critical to keep track of individual colony pieces and transfer pieces from one colony to 1 well of a 4- well plate. Avoid cross-colony contamination as this may compromise subsequent qPCR analysis.

## Quantitative RT-PCR analysis of TAT-CRE treated clones

26. Transfer the other **half** of the pieces of the isolated colony to an eppendorf containing 100 µL of 1X PBS. Centrifuge at 3000 rpm for 5 minutes. Aspirate the supernatant and add 20 – 30 µL of EpiCentre's quick DNA extraction buffer (Cat. No. QE0905T, EpiCentre). Mix and heat at 65°C for 6 minutes and 98°C for 2 minutes. The resultant cell lysates can be used for qPCR directly.
27. As a negative control, also isolate genomic DNA from iPS colonies that have not undergone the excision process.
28. Use the following primers (not provided) to set up PCR reactions for each TAT-CRE treated human iPS clone.

**Note:** The WPRE primer set is specific to the viral genome while the GAPDH-pro primer set detects the GAPDH promoter region and is used to normalize for cDNA template between PCR reactions.

| Marker          | Primer Sequence                                   |
|-----------------|---------------------------------------------------|
| WPRE            | Forward 5' – TCT TTA TGA GGA GTT GTG GCC CGT - 3' |
|                 | Reverse 5' – AAA GCG AAA GTC CCG GAA AGG A - 3'   |
| Human GAPDH-pro | Forward 5' - TAC TAG CGG TTT TAC GGG CG- 3'       |
|                 | Reverse 5' - TCG AAC AGG AGG AGC AGA GAG CGA-3'   |
| Mouse GAPDH-pro | Forward 5' – CAA AGG CGG AGT TAC CAG AG- 3'       |
|                 | Reverse 5' – CTG CAG TAC TGT GGG GAG GT-3'        |

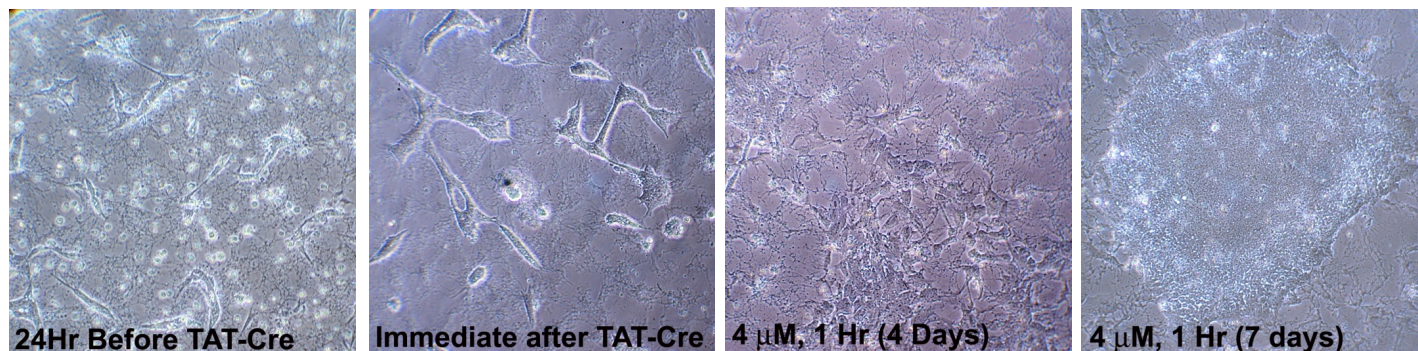
29. For each iPS clone, prepare the following PCR reaction mix for each primer set (WPRE and GAPDH-pro) in separate, clean DNase-free PCR tubes.

| Component                                    | Per Reaction |
|----------------------------------------------|--------------|
| DNA Lysate                                   | 2.0 $\mu$ L  |
| 2xSupermix (BioRad)                          | 12.5 $\mu$ L |
| 5 $\mu$ M forward primer (WPRE or GAPDH-pro) | 1.0 $\mu$ L  |
| 5 $\mu$ M reverse primer (WPRE or GAPDH-pro) | 1.0 $\mu$ L  |
| Sterile distilled water                      | 8.5 $\mu$ L  |

**Total Volume** = ~25  $\mu$ L

30. Briefly centrifuge the PCR tubes. Transfer the reactions to a BioRad CFX qPCR machine and perform PCR using the following optimized cycling parameters:
- Initial denaturation at 95°C for 3 minutes.
  - 44 cycles of:
    - 95°C 30 seconds
    - 60°C 30 seconds
  - Melt Curve 65°C to 95°C of 0.5°C increments, 5 seconds each.
  - Plate read
31. **Excision Criteria:** Human iPS clones that passed **all** four of the following criteria are deemed excised and transgene-free.
- 1) CT value of hGAPDH-pro ideally needs to be less than 25 to ensure sufficient DNA has been loaded for each sample.
  - 2) The CT value of WPRE for the non-template control (NTC) sample has to be larger than 30 (This primer set formed self-dimers).
  - 3) CT value of WPRE has to be equal or greater than those from the NTC or negative control (i.e. H9 cells).
  - 4)  $\Delta$ CT (WPRE-hGAPDH-pro) value has to be equal or greater than 5.

## A. Time Course of TAT-CRE treatment



## B. Real-time qPCR analysis of genomic DNA

|                     | Ct<br>hGAPDH | Ct<br>WPRE | Delta Ct<br>WPRE-hGAPDH | TAT-CRE<br>Treatment | Excision<br>Efficiency                   |
|---------------------|--------------|------------|-------------------------|----------------------|------------------------------------------|
| Human<br>iPS Clones | 24.70        | 29.50      | 4.80                    | >16X less than ctrl  | 1/4 clones                               |
|                     | 25.30        | 27.00      | 1.70                    | 5 $\mu$ M for 1 hr   | <b>25%<br/>Efficiency</b>                |
|                     | 25.40        | 26.10      | 0.60                    |                      |                                          |
|                     | 28.10        | 29.40      | 1.30                    |                      |                                          |
|                     | 25.50        | 30.30      | 4.80                    | 5 $\mu$ M for 1 hr   | 7/12 clones<br><b>58%<br/>Efficiency</b> |
|                     | 23.50        | 29.50      | 6.00                    |                      |                                          |
|                     | 25.00        | 30.10      | 5.00                    |                      |                                          |
|                     | 25.10        | 30.50      | 5.40                    |                      |                                          |
|                     | 26.00        | 30.20      | 4.20                    |                      |                                          |
|                     | 23.50        | 29.20      | 5.60                    |                      |                                          |
|                     | 23.90        | 31.00      | 7.20                    |                      |                                          |
|                     | 25.40        | 26.00      | 0.60                    |                      |                                          |
|                     | 28.50        | 30.10      | 1.60                    |                      |                                          |
|                     | 25.30        | 28.30      | 3.10                    |                      |                                          |
|                     | 23.20        | 25.80      | 2.60                    |                      |                                          |
|                     | 25.50        | 28.00      | 2.60                    |                      |                                          |
| Controls            | 27.50        | 28.00      | 0.50                    | Untreated hiPSCs     |                                          |
|                     | 26.20        | 26.80      | 0.70                    | Untreated hiPSCs     |                                          |
|                     | 40.70        | 31.10      | -9.70                   | No template control  |                                          |

High  $\Delta$ CT values indicate absence of virus.

Low  $\Delta$ CT inversely correlates with relative abundance of viral transgene.

**Figure 3.** Time course of TAT-CRE treatment (A). Human iPS cells were dissociated into single cells using Alfasyme and allowed to attach overnight before treatment with 4 -5  $\mu$ M TAT-CRE for 1-2 hours. Individual colonies were picked at 9-14 days post-treatment and added directly to quick DNA extraction buffer (Cat. No. QE0905T, EpiCentre) for real time quantitative PCR analysis. The CT values of WPRE in the excised samples should correlate with the no template control (B).

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## TAT-CRE Application 2: Excision of viral transgenes from mouse iPS cells

TAT-CRE treatment may be performed on mouse iPS cells maintained on inactivated mouse embryonic fibroblasts (pMEFs) feeder layer or in serum-free, feeder-free culture conditions. Protocols for each culture conditions are provided.

### Mouse iPS cells maintained on inactivated feeder-layer

The following protocol has been optimized for mouse iPS cells maintained on inactivated MEF feeder layers (Millipore Cat. No. PMEF-CF) in mouse ESC medium.

**Mouse ESC Medium.** Sterile filter with a 0.22  $\mu$ m filter.

| Component                               | Quantity  | Final Conc. | Vendor            | Cat. No.  |
|-----------------------------------------|-----------|-------------|-------------------|-----------|
| DMEM High-Glucose Medium                | 44 mL     | 1X          | EMD Millipore     | SLM-021-B |
| Knockout™ Serum Replacement             | 7.5 mL    | 15%         | LIFE Technologies | 10828-028 |
| L-Glutamine (100X) 200 mM               | 0.5 mL    | 2 mM        | EMD Millipore     | TMS-002-C |
| 100X Non-essential amino acids          | 0.5 mL    | 1X          | EMD Millipore     | TMS-001-C |
| $\beta$ -mercaptoethanol (100X)         | 0.5 mL    | 1X          | EMD Millipore     | ES-007-E  |
| ESGRO LIF, $10^7$ units (10,000X)       | 5 $\mu$ L | 1X          | EMD Millipore     | ESG1107   |
| Penicillin Streptomycin Solution (100X) | 0.5 mL    | 1X          | EMD Millipore     | TMS-AB2-C |

### One Day Prior to Excision Analysis: Prepare a plate of Inactivated MEFs

1. One day prior to passaging the mouse iPS colonies, prepare a fresh 6-well plate containing inactivated MEFs to support the expansion of the potentially excised mouse iPS colonies as follows:
  - a. Coat each well of a fresh sterile 6-well plate with 2 mL of 0.1% gelatin solution (Cat. No. ES-006-B). Incubate for 30 minutes at 37°C. Set a side until ready to receive inactivated MEFs.
  - b. Aspirate the 0.1% gelatin coating solution from each well before seeding the inactivated MEFs. Thaw inactivated MEFs (Cat. No. PMEF-CF). Count the number of thawed MEFs and seed  $4 \times 10^5$  cells per well of a 6-well dish. Use MEF medium to culture the cells (see below). Total volume per well should be 3 mL. Incubate overnight in a 37°C, 5% CO<sub>2</sub> incubator.

**MEF Medium.** Sterile filter with a 0.22  $\mu\text{m}$  filter.

| Component                               | Quantity | Final Conc. | EMD Millipore Cat. No. |
|-----------------------------------------|----------|-------------|------------------------|
| DMEM High-Glucose Medium                | 44 mL    | 1X          | SLM-021-B              |
| Fetal Bovine Serum                      | 5.0 mL   | 10%         | ES-009-B               |
| L-Glutamine (200 mM)                    | 0.5 mL   | 2 mM        | TMS-002-C              |
| Penicillin Streptomycin Solution (100X) | 0.5 mL   | 1X          | TMS-AB2-C              |

**Day of Excision Analysis:**

2. Aliquot sufficient mouse ESC medium, Accutase (Cat. No. SCR005) and warm the reagents at room temperature (15 – 25°C) for 5 – 10 minutes.
3. To each well containing approximately 80% confluent mouse iPS clones, add 1 mL of Accutase and incubate in a 37°C incubator for 3 – 5 minutes.
4. Inspect the wells and ensure the complete detachment of the cells by gently tapping the side of the plate with the palm of your hand. Confirm under the microscope that the cells are in single-cell suspension.
5. Apply 1 mL mouse ESC medium to each well. Pipette up and down several times to ensure a single cell suspension and collect the cell suspension to a 15 mL conical tube.
6. Centrifuge for 5 minutes at 300 g to pellet the cells. Remove the supernatant and resuspend with 1 mL fresh mouse ESC Media.
7. Count the number of cells using a hemocytometer. Ensure that the cells are in a single cell suspension. Determine the cell viability using trypan blue exclusion.
8. Treat 10,000 singly dissociated cells with a range of TAT-CRE concentrations ranging from 2  $\mu\text{M}$  to 4  $\mu\text{M}$  (filtered) in 200  $\mu\text{L}$  mouse ESC medium in a low adhesion 96-well plate. Incubate in a 37°C incubator for 2 – 4 hours.

**Note:** TAT-CRE protein may be diluted from the glycerol stock with mouse ESC medium and sterile filter with a 0.2  $\mu\text{m}$  low protein binding syringe filter (Cat. No. SLGV033RS or SLGV013SL).

9. Monitor every 30 minutes after the addition of TAT-CRE to the culture. TAT-CRE treatment should be stopped when you observe significant cell death. Because high concentrations of TAT-CRE are toxic to the cells, cell death is used as an indicator of TAT-CRE penetration. Ideally you want to have the highest concentration of TAT-CRE as measured by the most cell death while still retaining sufficient viable live cells.

**Note:** TAT-CRE sensitivity may vary in different cell lines and will need to be determined empirically. For our in-house iPS clones, a 4  $\mu\text{M}$  TAT-CRE treatment was halted at 2 – 4 hours when approximately 50% cell death was observed.

10. Meanwhile, aspirate the MEF medium from the 6-well-plate containing inactivated pMEFs prepared from the day before (Step 1). Wash each well with 1X PBS. Aspirate and add 4 mL per well of mouse ESC medium. Set plate aside until ready to plate TAT-CRE-treated hiPS clones.
11. After the 2 – 4 hour TAT-CRE treatment, add the entire 200  $\mu$ L cell suspension to the 6 well plate containing inactivated MEFs (from Step 10 above). This step establishes and expands individual potentially excised iPS clones. Agitate the plates gently from side to side and forward and backwards to ensure that iPS cells are evenly distributed. Place the plate in a 37°C, 5% CO<sub>2</sub> incubator.
12. Replace the media daily with fresh mouse ESC medium until colonies start to appear.
13. When TAT-CRE treated colonies are of sufficient size (approximately 5 – 6 days), but are not touching or merging with each other, pick individual colonies for expansion and genomic DNA extraction and real-time quantitative PCR analysis.

**Note:** We recommend picking at least 10 colonies per experiment. Approximately 90-100% excision efficiency was obtained when using TAT-CRE on our in-house mouse iPS clones.

## Expansion of TAT-CRE treated Mouse iPS clones

Once TAT-CRE treated colonies have emerged, it is essential to expand individual clones. The following protocol is recommended.

14. Transfer the 6-well plate containing TAT-CRE treated colonies to a tissue culture hood containing a dissecting microscope. Scrape and pipette up one TAT-CRE treated mouse iPS colony and deposit it into a labeled 15 mL conical tube containing 0.25% trypsin-EDTA. Repeat this step for each iPS colony to be clonally expanded, being careful to keep each iPS colony in separate conical tubes.
15. Incubate the 15 mL conical tubes at room temperature for 5-10 minutes to allow the trypsin to dissociate the cell colonies.
16. Add 5 mL fresh mouse ESC medium to each 15 mL conical tube containing the dissociated cell colonies.
17. Using a 5 or 10 mL pipette, slowly pipette up and down to break apart any remaining cell clumps.
18. Discard the media from the plate of inactivated MEFs prepared beforehand, and add the dissociated mouse iPS colony from each 15 mL conical tube (approximately 5 mL volume) into separate wells of the 6-well dish containing inactivated MEFs. Incubate the 6-well dish in a 37°C, 5% CO<sub>2</sub> incubator.

**Note:** It is important to avoid cross-colony contamination between mouse iPS clones. Therefore, each well of the 6-well dish should only receive dissociated cells from one mouse iPS colony.

19. Replace with fresh mouse ESC medium daily or every other day until the mouse iPS colonies are 80% confluent.
20. When mouse iPS clones are 80% confluent, they can be further expanded and frozen.
21. For each clone, isolate the genomic DNA and set up real-time quantitative PCR reactions to identify fully excised clones. Follow the protocol as outlined in the section titled “qRT-PCR Analysis of TAT-CRE treated clones” on page 8, steps 26-31.

### **Mouse iPSC in serum-free, feeder-free condition (ESGRO-2i)**

1. Directly transition mouse iPS cells that were originally cultured on pMEF feeder layer to serum-free, feeder-free condition using ESGRO-2i (Cat. No.SF016, please follow the manufacturer's instructions).
2. Grow mouse iPS cells in ESGRO-2i for 2-3 passages. After the 3rd passage, cells should have homogeneous round morphologies.
3. Aliquot sufficient ESGRO-2i Medium, Accutase (Cat. No. SCR005) and warm reagents at room temperature (15 – 25°C) for 5 – 10 minutes.
4. Add 1 mL of Accutase to each well and incubate in a 37°C incubator for 3-5 minutes.
5. Inspect the wells and ensure the complete detachment of cells by gently tapping the side of the plate with the palm of your hand. Confirm under the microscope that the cells are in single-cell suspension.
6. Apply 1 mL ESGRO-2i medium to each well. Pipette up and down several times to ensure a single cell suspension and collect the cell suspension to a 15 mL conical tube.
7. Centrifuge for 5 minutes at 300 g to pellet the cells. Remove the supernatant and resuspend with 1 mL fresh ESGRO-2i Medium.
8. Count the number of cells using a hemocytometer. Ensure that the cells are in a single cell suspension. Determine the cell viability using trypan blue exclusion.
9. Treat 100,000 cells singly dissociated cells with a range of TAT-CRE concentrations ranging from 2  $\mu$ M to 4  $\mu$ M (filtered) in 3 mL ESGRO-2i medium in a 0.1% gelatin coated 6-well plate. Incubate in a 37°C incubator overnight.

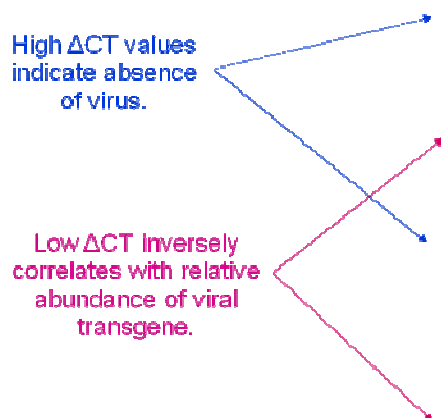
**Note:** TAT-CRE protein may be diluted from the glycerol stock with ESGRO-2i medium and sterile filter with a 0.2  $\mu$ m low protein binding syringe filter (Cat. No. SLGV033RS or SLGV013SL).

10. Monitor every 30 minutes – 1 hour after the addition of TAT-CRE to the culture. TAT-CRE treatment should be stopped when you observe significant cell death. Because high concentrations of TAT-CRE are toxic to the cells, cell death is used as an indicator of TAT-CRE penetration. Ideally you want to have the highest concentration of TAT-CRE as measured by the most cell death while still retaining sufficient viable live cells.

**Note:** TAT-CRE sensitivity may vary in different cell lines and will need to be determined empirically.

11. To halt the TAT-CRE treatment, remove the medium and wash once with 1X PBS. Aspirate.
12. Exchange with fresh ESGRO-2i medium and replace the medium daily or every other day until colonies start to emerge.
13. When TAT-CRE treated colonies are of sufficient size (approximately 5-6 days), but are not touching or merging with each other, pick individual clones for expansion and subsequent genomic DNA extraction and real-time quantitative PCR analysis. Follow the protocol as outlined in the section titled “qRT-PCR analysis of TAT-CRE treated clones” on page 8, steps 26-31.

**Note:** We recommend picking at least 10 colonies per experiment. Approximately 90-100% excision efficiency was obtained when using TAT-CRE on our in-house mouse iPS clones.



| Ct<br>mGAPDH | Ct<br>WPRE | Delta Ct<br>WPRE-mGAPDH | TAT-CRE<br>Treatment | Excision<br>Efficiency               |                     |
|--------------|------------|-------------------------|----------------------|--------------------------------------|---------------------|
| 24.68        | 34.66      | 9.98                    | 2 $\mu$ M for 2 hr   | 6/7 clones<br><b>86% Efficiency</b>  | Mouse<br>iPS Clones |
| 24.85        | 34.08      | 9.23                    |                      |                                      |                     |
| 26.24        | 36.30      | 10.06                   |                      |                                      |                     |
| 26.47        | 34.29      | 7.82                    |                      |                                      |                     |
| 26.06        | 34.01      | 7.95                    |                      |                                      |                     |
| 25.19        | 34.93      | 9.74                    |                      |                                      |                     |
| 24.56        | 24.73      | 0.17                    |                      |                                      |                     |
| 26.11        | 36.25      | 10.14                   | 8 $\mu$ M for 2 hr   | 7/7 clones<br><b>100% Efficiency</b> | Mouse<br>iPS Clones |
| 26.16        | 37.02      | 10.86                   |                      |                                      |                     |
| 27.21        | 36.38      | 9.17                    |                      |                                      |                     |
| 27.39        | 35.67      | 8.28                    |                      |                                      |                     |
| 27.41        | 34.81      | 7.40                    |                      |                                      |                     |
| 25.06        | 35.89      | 10.83                   |                      |                                      |                     |
| 25.75        | 34.08      | 8.33                    |                      |                                      |                     |
| 21.84        | 22.15      | 0.31                    | Untreated miPSCs     |                                      | Controls            |
| 21.61        | 32.36      | 10.75                   | mESC                 |                                      |                     |
| N/A          | 41.18      | N/A                     | No template control  |                                      |                     |

**Figure 5.** Quantitative RT-PCR analysis of mouse iPS clones cultured on pMEF feeder layers to identify excised clones. Mouse iPS cells cultured on inactivated pMEF feeder layer were dissociated into single cells using Accutase and allowed to attach overnight before treatment with 2  $\mu$ M – 8  $\mu$ M TAT-CRE for 2 hrs at 37°C in two separate experiments. Individual colonies were picked at 5 – 6 days post treatment and were added directly to quick DNA extraction buffer (Cat. No. QE0905T, EpiCentre) for real-time quantitative PCR analysis. In the two experiments shown,  $\Delta$ CT > 5 was considered a significant difference of DNA expression levels and indicated a successful excision. The CT value of WPRE in the excised samples correlated with the negative controls, mESC and the no template control. Similar results were obtained when mouse iPS cells were cultured in serum-free, feeder-free condition (ESGRO-2i, Millipore Cat. No. SF016, data not shown).



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## References

1. Peitz, M., Pfannkuche, K., Rajewsky, K., and Edenhofer, F. (2002) Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: A tool for efficient genetic engineering of mammalian genomes. *PNAS* **99(7)**: 4489-94.
2. Nolden, L., Edenhofer, F., Haupt, S., Koch, P., Wunderlich, F. T., Siemen, H., and Brüstle, O. (2006) Site-specific recombination in human embryonic stem cells induced by cell-permeant Cre recombinase. *Nat Methods* **3 (6)**: 461-67.
3. Nagy, A. (2000) Cre recombinase: the universal reagent for genome tailoring. *Genesis* **26(2)**: 99-109.
4. Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewsky, K. (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265(5168)**: 103-6.

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