Efficient generation of transgene-free human and mouse iPS cells using a cell-permeant Tat-Cre protein

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Abstract

Reprogramming of somatic cells using viral transduction of defined transcription factors remains a widely used and efficient method to obtain induced pluripotent stem cells (iPSCs). However, the presence of viral transgenes in iPSC is undesirable, as it raises the possibility of insertional mutagenesis leading to malignant transformation and has also been shown to affect differentiation potential. Various strategies have been employed to address this issue, including non-integrating viruses, RNA transfection, protein transduction and site specific recombinases to excise the transgenes after reprogramming. Here, we show efficient generation of transgene-free mouse and human iPSCs through the use of a Cre-excisable polycistronic lentiviral vector expressing the "stem cell cassette" (STEMCCA™) comprised of all four transcription factors (OKSM) followed by exposure of the full reprogrammed iPSC to cell permeable TAT-Cre recombinant protein. Notably highly efficient excision (100% for mouse iPSCs and up to 60% for human iPSCs) could be demonstrated following exposure of iPSCs to 4 – 6 mM TAT-Cre for 1 – 2 hours. The high degree of efficiency obtained with protein transduction is in marked contrast to results obtained with electroporation of a plasmid expressing Cre-recombinase (<10%) and also for adenovirus expressing Cre recombinase which has been shown to be effective for mouse iPSCs but not for human iPSCs. Additionally, we present a simple and robust PCR strategy that enables fast identification of deleted clones directly from primary iPSC colonies. Establishment of transgene-free iPSCs required approximately two weeks from the time of addition of the cell-permeant TAT-Cre protein. Factor-free human and mouse iPSCs expressed appropriate morphological and immunochemical staining characteristics of pluripotent cells. Factor-free human iPSCs possessed a normal karyotype and were capable of differentiating into derivatives of all three germ layers *in vivo*. In summary, we have established a robust system for highly efficient excision of viral vectors from iPSCs using cell permeant TAT-Cre protein. Efficient delivery of an active recombinant Cre protein to mammalian cells has broad applications not only for somatic cell reprogramming, but also for controlled genetic modification of mammalian genomes.

Materials and Methods

TAT-Cre fusion protein :

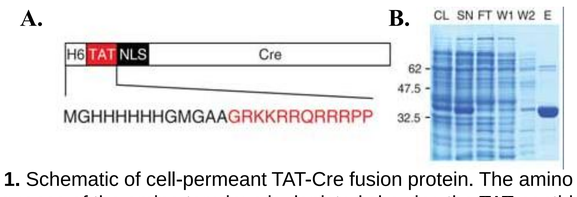
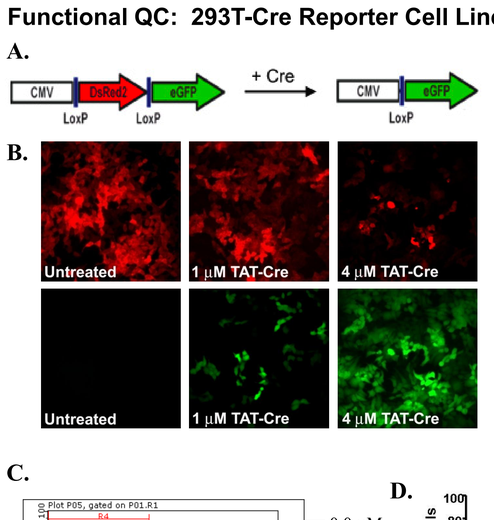


Figure 1. Schematic of cell-permeant TAT-Cre fusion protein. The amino acid sequence of the amino terminus is depicted showing the TAT peptide sequence in red (**A**). Purification of recombinant TAT-Cre from bacteria, as analyzed by Coomassie® blue staining of an SDS-PAGE (**B**). CL, cleared lysate; SN, supernatant; FL, flow through; W1, W2, wash fractions 1 and 2; E, eluted fraction. Numbers on the left indicate molecular weight (kDa) of marker proteins, Cre protein is approximately 41 kDa. (Figure from F. Edenhofer, Nature Methods, 2006 Jun;3(6):461-7)

Functional QC: 293T-Cre Reporter Cell Line



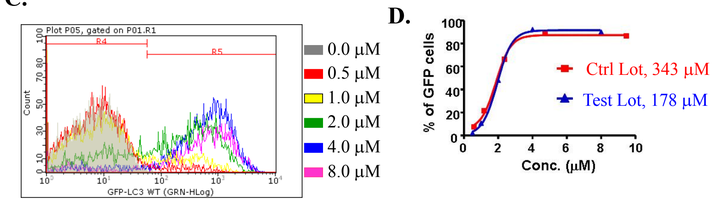


Figure 2. A rapid and reliable assay to validate TAT-Cre transduction and recombination activities. A 293T cell line stably expressing a double fluorescent reporter construct was used to monitor Cre recombination (**A**). Cells express RFP before Cre-recombination. Cre-mediated recombination induces the expression of the GFP, by deleting the LoxP-flanking RFP gene. Maximal GFP expression was achieved when 4 mM TAT-Cre was used to treat the cells overnight. (**B, C**). Dose dependent increases of GFP expressing cells were quantified using flow cytometry analysis (**C**). Percentage of GFP positive cells were plotted against the dosage of TAT-Cre used (**D**). Consistent lot-to-lot performance was observed.

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Human iPSC excision with TAT-Cre

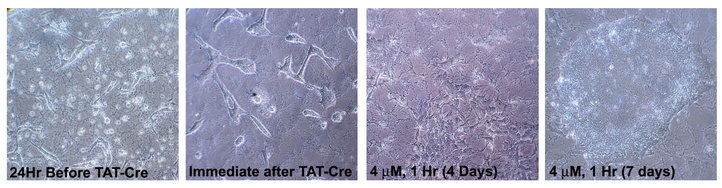
HUMAN PROTOCOL

A.

- Transition hiPSC to feeder-free conditions.
- One day before passaging, add ROCK inhibitor.
- Dissociate into single cell suspension.
- Plate 50K – 100K cells per well of 12-well plate.
- Incubate O/N to allow cells to attach.
- Incubate cells with 2 – 5 mM TAT-Cre.
- After 7 – 9 days, colonies will start to re-emerge and can be expanded.
- Extract genomic DNA for real-time quantitative PCR analysis.

TIME COURSE OF TAT-CRE TREATMENT

B.



REAL-TIME qPCR ANALYSIS OF GENOMIC DNA

C.

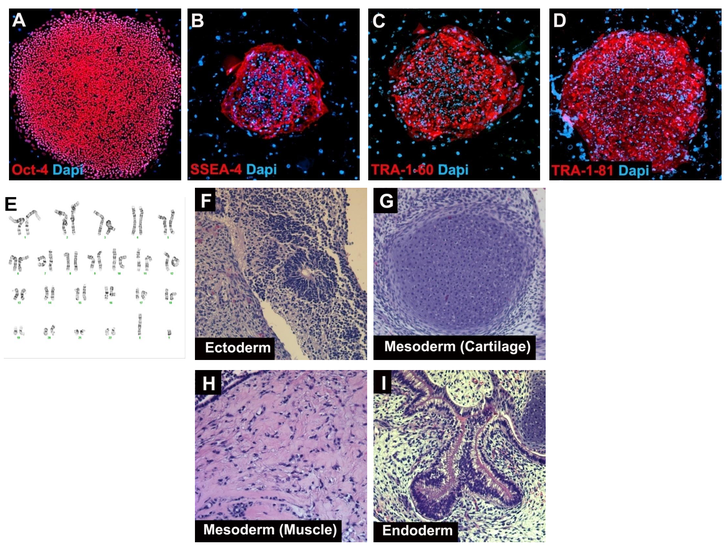
	Ct hGAPDH	Ct WPRE	Delta Ct WPRE-hGAPDH	TAT-CRE Treatment	Excision Efficiency
Human iPS Clones	24.70	29.50	4.80	>16X less than ctrl	1/4 clones
	25.30	27.00	1.70	5 mM for 1 hr	25% Efficiency
	25.40	26.10	0.60		
	28.10	29.40	1.30		
	25.50	30.30	4.80		
	23.50	29.50	6.00		
	25.00	30.10	5.00	5 mM for 1 hr	7/12 clones
	25.10	30.50	5.40		58% Efficiency
	26.00	30.20	4.20		
	23.50	29.20	5.60	>128X less than ctrl	
	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 ΔCT values indicate absence of virus.

Low ΔCT inversely correlates with relative abundance of viral transgene.

Figure 3. Protocol for human excision (**A**). Time course of TAT-Cre treatment (**B**). Individual colonies were picked at 9-14 days post-treatment and added to directly to Lysis Buffer for real time quantitative PCR analysis (**C**). The Ct value of WPRE in the excised samples should correlate with the negative controls, untreated hiPSCs and no template control (**C**).

CHARACTERIZATION



J

	Ct mGAPDH	Ct WPRE	Delta Ct WPRE-hGAPDH	Post-Excised, p15 Human iPS SubClones
	25.40	31.60	6.20	SubClone 1, Matrigel
	24.60	31.30	6.70	SubClone 2, Matrigel
	24.70	31.30	6.60	SubClone 3, Matrigel
	22.20	31.30	9.10	Pooled, Matrigel
	24.60	32.60	8.10	Pooled, cultured on MEF
Controls	24.60	23.50	-1.10	Pre-Excised, p15 Human iPS Pooled, Matrigel
	35.10	32.00	-3.20	No template control

Figure 4. In vitro and in vivo characterization of post-excised human iPSC clones. Post-excised clones (p17) expressed the appropriate pluripotent markers (**A-D**), alkaline phosphatase (data not shown) and possessed normal karyotype (**E**). Teratoma analysis (**F-I**). Individual subclones were selected along with pooled subclones and analyzed for presence of transgene. All subclones and pooled demonstrate complete excision after 15 passages and similar results could be observed when expansion was conducted in feeder-based culture (**J**).

Mouse iPSC excision with TAT-Cre

MOUSE PROTOCOLS

A.

FEEDER-BASED CULTURE

- Mouse iPSCs are grown on pMEF feeder layer in mESC media.
- Dissociate cells (miPSCs and pMEF) to SINGLE cell suspension with Accutase® solution.
- Treat 10K cells with 4 mM TAT-CRE in 200 mL mESC media for 2- 4 HR in a 96-well plate at 37ºC.
- Transfer cells to fresh 6-well plate coated with pMEF feeders.
- After 5 – 6 days, colonies will start to re-emerge and can be selectively expanded.
- Extract genomic DNA for real-time quantitative PCR analysis.

FEEDER-FREE CULTURE

- Culture mouse iPSCs in ESGRO®-2i medium for 2-3 passages.
- Dissociate miPSCs to SINGLE cell suspension with Accutase® solution.
- Plate 100K cells onto gelatin-coated 6-well plates.
- Incubate cells with 4 mM TAT-CRE O/N in ESGRO®-2i medium.
- After 9-10 days, colonies will start to re-emerge and can be selectively expanded.
- Extract genomic DNA for real-time quantitative PCR analysis.

REAL-TIME qPCR ANALYSIS OF GENOMIC DNA

B.

	Ct mGAPDH	Ct WPRE	Delta Ct WPRE-mGAPDH	TAT-CRE Treatment	Excision Efficiency
Mouse iPS Clones	24.68	34.66	9.98	2 mM for 2 hr	6/7 clones
	24.85	34.08	9.23		
	26.24	36.30	10.06		86% Efficiency
	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 mM for 2 hr	7/7 clones
	26.16	37.02	10.86		
	27.21	36.38	9.17		100% Efficiency
	27.39	35.67	8.28		
	27.41	34.81	7.40		
	25.06	35.89	10.83		
	25.75	34.08	8.33		
Controls	21.84	22.15	0.31	Untreated miPSCs	
	21.61	32.36	10.75	mESC	
	N/A	41.18	N/A	No template control	

Figure 5. Excision efficiency of mouse STEMCCA™ Cre-excisable polycistronic (OKSM) iPSCs. Feeder-based and serum-free, feeder-free protocols for mouse excision (**A**). Real-time qPCR analysis of genomic DNA (**B**). In the two experiments shown, ΔCt >5 was considered a significant difference of DNA expression levels and indicated a successful excision. The Ct value of WPRE in the excised samples should correlate with the negative controls, mESC and no template control. Similar results were obtained when mouse iPSCs were cultured in serum-free, feeder-free condition (ESGRO®-2i medium, Merck Millipore Cat# SF016, data not shown).

Conclusions

- **Simple Protocol**: Simple addition of cell permeant TAT-Cre protein enables robust excision and establishes transgene-free iPSCs in 5-6 days for mouse and less than two weeks for human iPSCs.
- **High Efficient Excision**: Up to 100% excision efficiency could be obtained for mouse iPSCs and >60% excision efficiency for human iPSCs.
- **Fast Screening Assay**: A quick and easy qPCR strategy to identify deleted clones.
- **Broad Applications**: The ability to deliver recombinant Cre protein to cells serves as a powerful tool for rapid genetic manipulation of the mammalian genomes.