

Treg and Th17 T cell Immunology Tools Cat. #'s TCLxxx series

User Manual

**Store Cell lines at -80°C immediately upon receipt
Store Vectors at -20°C immediately upon receipt**

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I. Introduction and Background

A. Overview

Regulatory T cells (Treg), known as suppressor T cells maintain tolerance to self-antigens, and downregulate autoimmune responses and diseases such as transplant graft rejection, and reduce inflammatory bowel disease, autoimmune diseases like rheumatoid arthritis and allergy conditions. Human clinical trials have been attempted to treat autoimmune disease such as type I diabetes, and facilitate organ transplantation using Treg cell infusion therapy. Although high activity of Treg cells can prevent autoimmune disease, prevalence of Treg cells contributes to tumor growth. Foxp3, a member of the forkhead/winged-helix family of transcription factors, is a master regulator of Treg development and suppressive function. Foxp3 expression can reprograms normal T cells into Treg-like cells by positively and negatively regulate downstream gene expression. The sustained high level of Foxp3 expression is required for suppressive function of Tregs. So called “Ex-Tregs” that have lost both Foxp3 expression and suppressive function are detected in inflamed tissues. Therefore Foxp3 expression level is often used for the index of suppressive function of Tregs.

Th17 cells are a subset of T helper cells producing interleukin 17 (IL-17). Th17 cells serve a very important function in anti-microbial immunity at epithelial / mucosal barriers to clear out certain types of microbe (such as *Candida* and *Staphylococcus*). Thus, a severe lack of Th17 cells may leave the host susceptible to opportunistic infections. However excessive amounts of the Th17 cell can lead to the development of autoimmune disease. Similar to Treg cells, Th17 cells express a master regulator of transcription factor, ROR γ t for mouse and RORC for human, members of the retinoic acid receptor-related orphan nuclear hormone. ROR γ t positively regulate IL-17 gene expression upon activation of Th17 cells.

Immunosuppressive Tregs and pro-inflammatory Th17 cells functionally antagonize each other. Interestingly, Treg cells can be re-differentiated into Th17 cells under pro-inflammatory environment. Because Treg and Th17 cells' immune function and differentiation programs are interconnected, it is emerging concept to study both Treg cells and Th17 cells at any given experimental model.

Because of clinical implications and the promising therapeutic potential, Treg and Th17 cell fields are rapidly growing and expanding among immunology-related research. SBI has developed immunological tools for Treg and Th17 cells to facilitate these research fields.

This manual provides details and information necessary to use the overexpression constructs, reporters and cell lines to study T cell dynamics. To ensure optimal results, please read the entire manual before using the reagents and material supplied with this kit.

B. Lentivectors expressing Foxp3 and RORC/ROR γ t

Overexpression of Foxp3 and ROR γ t in cells can be achieved by transduction of cells by lentivirus. We cloned cDNAs for Foxp3 and ROR γ t (mouse and human) into lentivector (mouse Foxp3, TCL100A-1; human FOXP3, TCL200A-1; mouse ROR γ t, TCL300A-1; human RORC, TCL400A-1). Foxp3 and ROR γ t expression is driven by MSCV promoter. EF1 α promoter controls GFP and puromycin resistant gene (**Figure 1**). Packaged virus can be used to transduce difficult-transfected cells such as Jurkat T cells and primary cells. Using this system, Foxp3 and RORC/ROR γ t expression in targeted cells can be monitored either by microscopy and flow cytometry of GFP in live cells or by western blotting analysis of cell lysates.

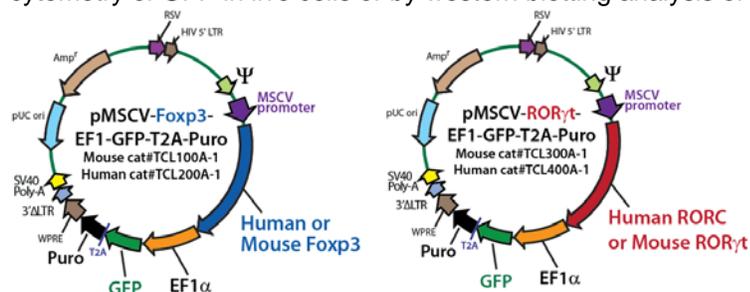


Figure 1: Plasmid maps for lentivectors expressing Human or Mouse Foxp3 and Human RORC or Mouse ROR γ t.

To test lentivectors expressing Foxp3 and ROR γ t, virus was packaged by transfection of TCLxxxA-vectors along with pPACK (Cat# LV500A-1) in 293 FT cells followed by instructions of SBI's lentivector expression system manual. 72 h after transfection, viral supernatant was collected and used for spinoculation of Jurkat T cells (detailed protocol for spinoculation, please see page 11). Infected cells were selected with puromycin (2.5 μ g/ml) for 7 days. Flow cytometric analysis showed that over 99% (for Foxp3) and 97% (ROR γ t and RORC) stable cells express GFP. Foxp3 and ROR γ t protein were detected from cell lysate of stable cells by western blotting analysis. Data are shown in **Figure 2** below.

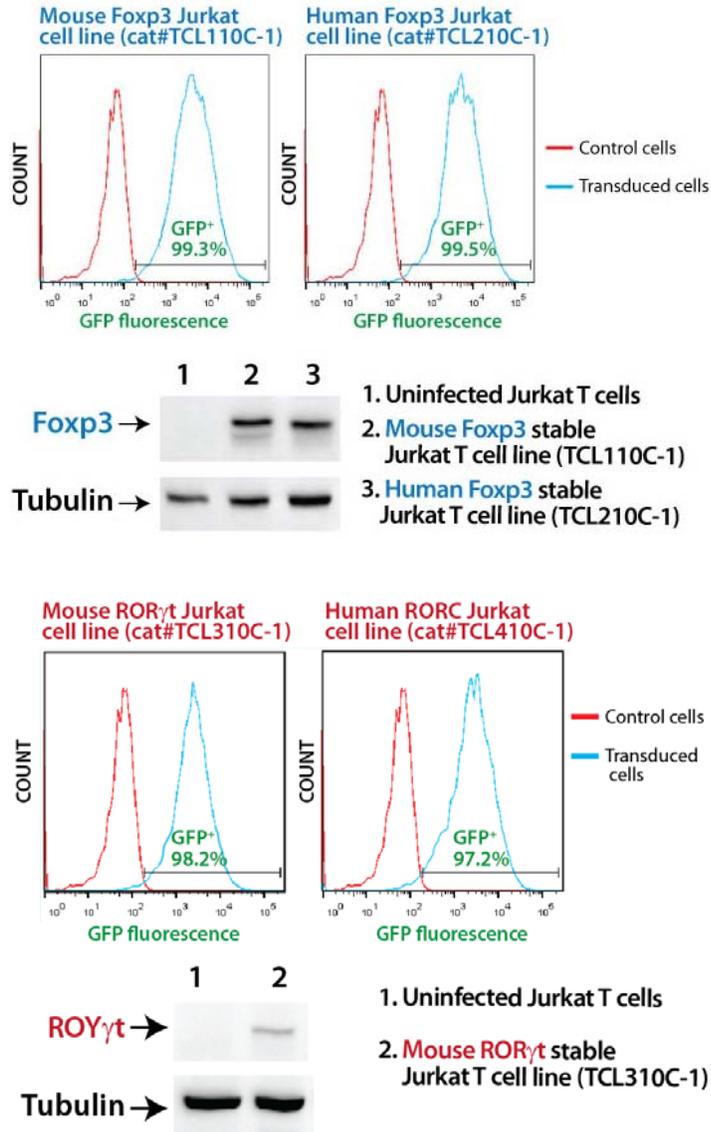
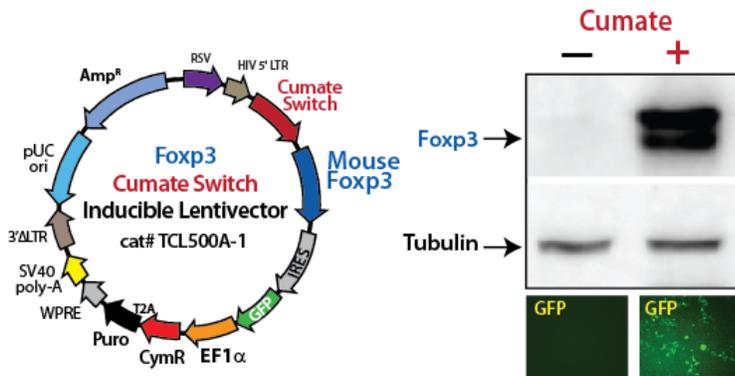


Figure 2: Flow cytometric analysis (the upper panel) and western blotting analysis (the lower panel) of Jurkat stable cell line expressing Foxp3 and RORC/ROR γ t.

Inducible Foxp3 cumate switch lentivector

The all-in-one cumate switch lentivector can be used to establish stable cell lines that can be induced to overexpress Foxp3 using cumate. The mouse Foxp3 was cloned upstream of the IRES GFP cassette and was placed under the control of the upstream cumate switch promoter. The EF1 alpha promoter drives the expression of the CymR repressor-T2A-Puro cassette. The TCL500A-1 CuO-Mouse Foxp3-IRES-GFP-EF1-CymR-T2A-Puro lentivector plasmid was packaged into lentivirus and transduced into 293FT cells. A stable cell

line was established using puromycin (2.5ug/ml) selection for three days. To induce the over expression of mFoxp3, 300ug/ml cumate solution was added to the media and cells imaged after 7 days. The GFP marker serves as a positive response marker to cumate induction. Cellular proteins were harvested after seven days and western blot analysis performed to test for mFoxp3 protein induction using an anti-Foxp3 antibody and also probed with an anti- tubulin antibody for protein loading controls. The GFP co-induction marker cell images and the Western blot data are depicted below.



Applications: Lentivectors expressing human and mouse Foxp3, ROR γ t, and RORC can be used for studying dynamics of Foxp3 protein in cultured cells and/or animal model. Packaged virus can be infected into cell line or primary cells (mainly activated CD4⁺ T cells). For example, Foxp3 transduced mouse CD4⁺ T cells can be injected into mice and the stability of Foxp3 protein can be monitored in vivo by measuring GFP⁺Foxp3⁺ cells using FACS analysis. Jurkat stable cell line expressing Foxp3, ROR γ t, and RORC are useful to study kinetics of its protein stability as well as post-translational modifications of protein. Extracellular stimuli, small molecule activators and/or inhibitors, drug can be treated in stable cell lines and post-translational modification of protein such as acetylation, ubiquitination, phosphorylation, and methylation can be monitored by either western blotting analysis using modification specific antibodies or FACS analysis.

C. PiggyBac vectors expressing Foxp3 and ROR γ t

Foxp3 and ROR γ t can be overexpressed in cells by transient transfection of PiggyBac vectors (mouse Foxp3, TCL100PA-1; human FOXP3, TCL200PA-1; mouse ROR γ t, TCL300PA-1; human RORC, TCL400PA-1). Similar to lentivector constructs, Foxp3 and ROR γ t expression is driven by MSCV promoter and GFP and puromycin resistant gene by EF1 α promoter. Stable cell lines can be established by co-transfection with Super PiggyBac Transposase expression vector (PB200PA-1) followed by selection with puromycin. Foxp3 and ROR γ t expression in targeted cells can be monitored either by microscopy and flow cytometry of GFP in live cells or by western blotting analysis of cell lysate.

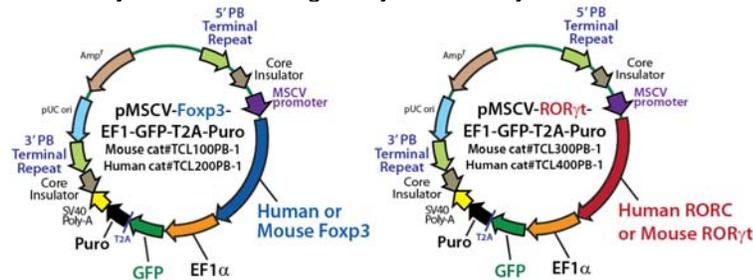


Figure 3: Plasmid maps for PiggyBac vectors expressing Foxp3 and ROR γ t.

We have tested TCL100PA-1 in 293FT cells. TCL100PA-1 was transfected into 293FT cells along with PB200PA-1. 24 hour after transfection, cells were treated with puromycin (2.5 mg/ml) and further selected for 10 days (**Figure 4**). Stable GFP and Foxp3 expression was observed while TCL100PA-1 alone transfected cells failed to grow after 10 days culture in the presence of puromycin due to the loss of TCL100PA-1 plasmid.

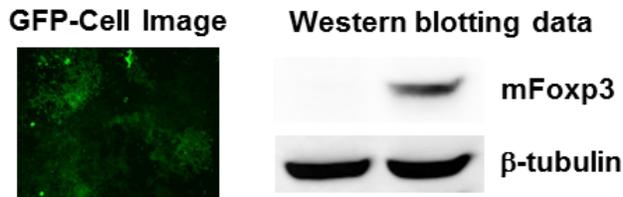


Figure 4: 293FT stable cells established by cotransfection of TCL100PA-1 and PB200PA-1 and puromycin selection. GFP expression was visualized by microscopy and Foxp3 expression from total cell lysate was detected by western blotting analysis with anti-mFoxp3 and anti-β-Tubulin antibodies.

Applications: PiggyBac vectors expressing human and mouse Foxp3, ROR γ t, and RORC can be used for studying dynamics of its protein in cultured cells and/or animal models. PiggyBac vectors can be introduced into cell lines or primary cells (mainly activated CD4+ T cells) by highly efficient transfection method such as nucleofection or electroporation. 293FT stable cell line expressing Foxp3, ROR γ t, and RORC are useful to study kinetics of its protein stability as well as post-translational modifications of protein. Extracellular stimuli, small molecule activators and/or inhibitors, drug can be treated in stable cell lines and post-translational modification of protein such as acetylation, ubiquitinylation, phosphorylation, and methylation can be monitored by either western blotting using modification specific antibodies or FACS analysis.

D. Reporter constructs for Foxp3, IL-17, ROR γ t promoters

In addition to naturally occurring Thymus-derived Treg (nTreg), induced Treg (iTregs) can be developed in the peripheral tissues. Th17 cells are differentiated during inflammatory response in the periphery. Since expression levels of Foxp3, IL-17, ROR γ t are direct indicators of numbers and activities of Tregs and Th17 cells, mechanisms responsible for regulating these genes are actively searched. Differentiation of Tregs and Th17 cells are elaborated processes coupled with extracellular stimuli (TCR mediated activation) and cytokine signaling which negatively or positively regulate downstream transcription factors. Differentiation of iTregs can be recapitulated in vitro by culturing naïve CD4+ T cells in the presence of suboptimal costimulation of TCR-mediated signals (anti-CD3/CD28 antibodies) and TGF- β . Small molecules such as TSA, class I HDAC inhibitor and Ex-527, SIRT1 inhibitor have been tested to modulate Treg differentiation using in vitro system. Th17 cells can be differentiated in vitro as well by culturing naïve CD4+ T cells with TCR-mediated signals, TGF- β and IL-6 cytokines.

Several important transcription factors such as ROR γ t, Runx1, Stat3, Stat4, Samd3, and HIF1 have identified as key factors for Treg and Th17 cell differentiation. Detailed mapping of promoter regions, cross interactions, and searching for new transcription factors are actively investigated in the field. We have built reporter constructs for mouse Foxp3, IL-17, ROR γ t promoter in which promoter activity can be measured both GFP expression and luciferase activity. The promoter sequences are carefully designed based on the recent literatures.

Promoter sequences and structure designs are based on the following publications:

Foxp3 promoter: Tone M, Greene MI, 2011, Zheng Y, et al., 2011 and Burgler S, et al., 2010.

ROR γ t promoter: Dang EV, et al., 2011, Lazarevic V, et al., 2011 and Ruan Q, et al., 2011.

IL-17 promoter: Zhang F, Meng G, Strober W, 2008.

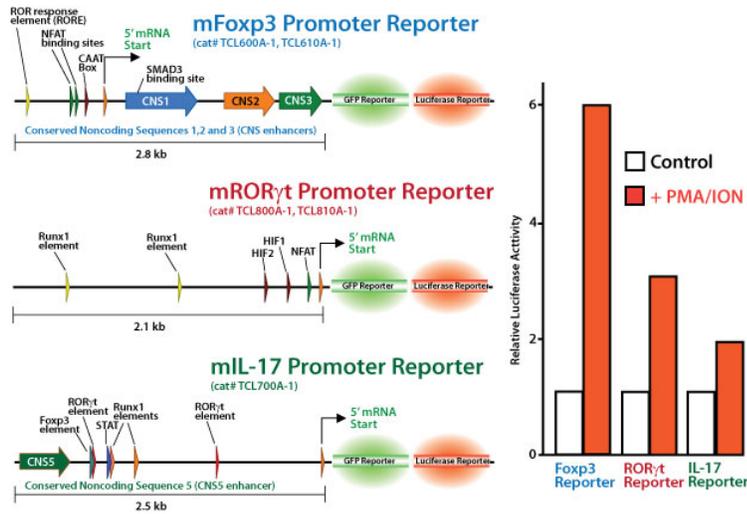


Figure 5: The schematic structures of mouse Foxp3, ROR γ t, and IL-17 promoter are shown. Upon PMA/Ionomycin stimulation, luciferase activity is increased in transduced Jurkat T cells with lentivirus containing the Foxp3, ROR γ t, and IL-17 promoter reporters.

Applications: The specific transcription factor activity to regulate mouse Foxp3, IL-17, ROR γ t gene expression can be tested using the reporter constructs of Foxp3, IL-17, ROR γ t promoter. For example, 293T cells or Jurkat T cells can be transduced with lentivirus carrying reporter constructs. Expression vectors for various transcription factors can be transfected for activity on the promoters. GFP expression and luciferase activity can be measured. Reporter constructs for mouse Foxp3, ROR γ t, and IL-17 promoter can be used to monitor Treg and Th17 cell differentiation from homogenous such as CD4⁺ T cells or heterogeneous population such as PBMC (peripheral blood mononuclear cells). Transduced cells can be treated with extracellular stimuli (like TCR activation by anti-CD3/CD28) with combination of cytokines (TGF- β , IL-6) to promote differentiation of Tregs and Th17 cells. Double positive populations (Foxp3+GFP+, IL17+GFP+, ROR γ t+GFP+) can be monitored as differentiated Tregs and Th17 cells using FACS analysis. Small molecule activators and/or inhibitors, drug can be screened for regulating Treg and Th17 cell differentiation.

E. Product Handling Guidelines

Bacterial culture of plasmids

All lentivector and PiggyBac plasmids for expressing Foxp3, ROR γ t, and RORC are recommended to transform into Stbl-2 bacterial strain (Invitrogen) that minimize the chance for recombination within HIV LTR region and PiggyBac ITR region.

Packaging Lentivirus

The detailed procedure of packing of lentivectors (TCL100A-1, TCL200A-1, TCL300A-1, and TCL400A-1) can be found in the manual of SBI's lentivector expression system.

Propagating Cell lines

Jurkat stable cells should culture in RPMI1650 with 10% FBS and 1% penicillin/streptomycin. Please maintain the cells at a density of about 250,000-2,000,000 cells/ml. For long term storage of Jurkat stable cell line, cells can be stored in liquid nitrogen in freezing media (90% FBS and 10% DMSO) followed by standard cell freezing method.

II. Protocols

A. Packaging lentivectors into virus

SBI has all the reagents needed to produce high titer, concentrated lentivirus.

See this PDF file for details on the protocol.

[Guide to Lentivector Packaging and Transduction of Target Cells \(PDF\) »](#)

http://www.systembio.com/downloads/web_manual_lentivector_exp_sys_071510.pdf

B. Spinoculation of virus and Jurkat T cells

[Spinoculation Protocol \(PDF\) »](#)

http://www.systembio.com/downloads/Spinoculation_protocol.pdf

C. Transfecting PiggyBac transposons

SBI has all the reagents needed to stably transpose your target cells using the piggyBac transposon system.

See this PDF file for details on the protocol.

[PiggyBac Transposon Vector System User Manual \(PDF\) »](#)

http://www.systembio.com/downloads/Manual_PiggyBac_Web.pdf

III. T cell Technical References (selected)

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