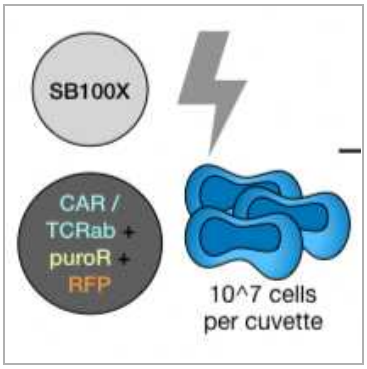


MAR 20, 2023

Generation of long-term genetically modified human T cells using Sleeping Beauty

Zaki Molvi[1]

[1]Memorial Sloan Kettering Cancer Center

Zaki Molvi
Memorial Sloan Kettering Cancer Center

DISCLAIMER

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.j8nlkw64xl5r/v1

Protocol Citation: Zaki Molvi 2023. Generation of long-term genetically modified human T cells using Sleeping Beauty. **protocols.io** https://dx.doi.org/10.17504/protocols.io.j8nlkw64xl5r/v1

Protocol status: Working
We use this protocol and it's working

Created: Feb 09, 2023

Last Modified: Mar 20, 2023

PROTOCOL integer ID:
76701

Keywords: t cell modification, CAR T cells, TCR T cells, Sleeping Beauty, Non-viral gene transfer, Stable gene transfection, T cell transduction, primary human t cells, pooled screens, T cell activation, neoantigen TCR, WT1 T cells

ABSTRACT

Genetic modification of T cells is an important step in T cell engineering for adoptive cellular therapy. Long-term T cell lines expressing a therapeutic antigen receptor, such as a chimeric antigen receptor (CAR) or exogenous T cell receptor (TCR), are useful for in vitro and in vivo studies of antigen recognition. The *Sleeping Beauty* (SB) system is a non-viral method for stable gene transfection, which can be used to genetically modify T cells to redirect their specificity. Previous studies employing SB to modify T cells have utilized expansion methods tailored to the genetically introduced surface receptors. The protocol presented herein is agnostic to surface markers, instead utilizing antibiotic resistance to enforce transgene expression, and thus is applicable to a wide range of use cases. Using this protocol, we routinely insert and express 1.5-1.8kb transgenes encoding CARs or TCRs to generate highly pure populations of engineered T cells, which we have expanded and maintained in culture for least 2 months. We find that we can generate 25-90% TCR or CAR modified T cells in ~2 weeks, which maintain expression and effector function over multiple restimulation cycles. This generalizable protocol can be used for the functional study of transgenes, such as antigen receptors, transcription factors, or gRNA, in primary human T cells.

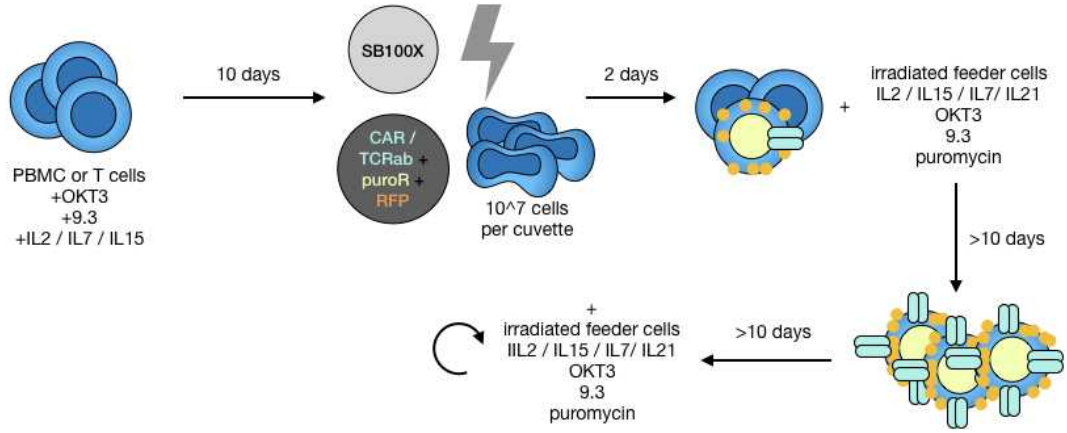
Introduction

1 Genetic modification of T cells is an important step in T cell engineering for adoptive cellular therapy. Long-term T cell lines expressing a therapeutic antigen receptor, such as a chimeric antigen receptor (CAR) or exogenous T cell receptor (TCR), are useful for in vitro and in vivo studies of antigen recognition. The *Sleeping Beauty* (SB) system is a non-viral method for stable gene transfection, which we and others have used to genetically modify T cells to redirect their specificity.

Previous studies employing SB to modify T cells have utilized antigen-positive feeder cells to specifically expand engineered T cells following transfection with a CAR (Singh et al.; Deniger et al.). Other studies have used magnetic enrichment using antibodies to surface markers introduced in the transgene cassette, such as the TCRβ constant regions (Deniger et al.). Our protocol is agnostic to surface markers, instead utilizing antibiotic resistance to enforce transgene expression, and thus is applicable to a wide range of use cases. Using this protocol, we routinely insert and express 1.5-1.8kb transgenes encoding CARs or TCRs to generate highly pure populations of engineered T cells which we have expanded and maintained in culture for least 2 months.

This protocol comprises three phases. The first comprises initial activation of T cells (OKT3 blast generation) for at least 10 days. In the second phase, cells are electroporated to co-transfect the SB100X transposase and transposon encoding the gene-of-interest (GOI) and rested for 2 days. In the third phase, modified T cells are expanded with feeder cells in antibiotic-

containing media for 10-14 days, after which, cells are analyzed for transgene expression and can be frozen or continuously propagated using feeder cells.



Schematic overview of protocol.

Materials

2

- Electroporation device (e.g. Lonza Nucleofector IIb)
- Generic electroporation cuvettes with 2mm gap
- SB100X transposase mammalian expression plasmid (e.g. pCMV(CAT)T7-SB100X; Addgene plasmid #34879)
- GOI in SB transposon plasmid with antibiotic resistance (e.g. pSBbi-RP; Addgene plasmid #60513)
- Electroporation media (e.g. Chicabuffer 1M)
- Irradiator
- Human PBMC
- EBV-BLCL as feeders, such as JY, or T2 cell hybridoma
- IL2
- IL15
- Optional: IL21, IL7
- T cell medium: Either Xvivo-15 (preferred) or RPMI+2mM L-Glutamine+NEAA, supplemented with 5-10% human AB serum
- Recovery medium: RPMI+2mM L-Glutamine+NEAA+20%FBS without antibiotics
- PBS without Ca or Mg
- Appropriate selection antibiotic (e.g. puromycin if using SBbi-RP plasmid)
- anti-CD3: OKT3 mAb
- anti-CD28: 28.2 or 9.3 mAb
- Adequate culture flasks: T25, T75, T225

Protocol

3 Initial T cell activation (OKT3 blast generation)

1. Culture fresh or thawed PBMC at 1 - 2x10^6 cells/mL in T cell medium containing 300 IU/mL IL2, 50ng/mL anti-CD3, and 1ug/mL anti-CD28. Optionally add 5ng/mL IL15 or IL7 to preserve younger, less differentiated CD8 T cell subsets.
2. Every 2-3 days, or when cell density exceeds 1x10^6 cells/mL, split cells in fresh T cell medium, optionally supplemented with cytokines.
3. On day 10-13 following CD3/28 activation, freeze cells at up to 100x10^6 cells/mL in 90%FBS/10%DMSO or proceed to electroporation.

Electroporation

1. If continuing from fresh culture, harvest activated T cells into conical. If resuming a frozen culture, thaw and resuspend cells overnight in recovery medium with cytokines.
2. Count T cells. Aim to use 10x10^6 cells per electroporation cuvette.
3. Wash harvested T cells twice in PBS (without Ca or Mg). Centrifuge at 200xg for 10 min.
4. Prepare a 6-well plate with 3-5mL per well of recovery media supplemented with 300 IU/mL IL2 and 5ng/mL IL15. Keep at 37C
5. Resuspend cells in electroporation media at density of 50x10^6 cells/mL
6. Per electroporation cuvette, add 10x10^6 cells, 5ug SB100X plasmid, and 15ug SB transposon plasmid containing antibiotic resistance (e.g. puromycin).
7. Electroporate, e.g. nucleofect using Lonza Nucleofector IIb on program U-014
8. Immediately after electroporation, transfer cells to single well of 6-well containing recovery medium. Minimize pipetting to reduce shear force experienced by the newly electroporated cells.
9. Repeat steps 6-8 as needed depending on number of experimental groups.
10. Incubate cells for 2 days.

Antibiotic-driven Expansion

1. Prepare feeder cells by pooling allogeneic PBMC and BLCL/T2 into a conical and administering 90Gy radiation. Per electroporated sample, aim for 20-30x10^6 feeder cells containing an allogeneic PBMC:BLCL ratio of >10:1.
2. Harvest electroporated cells from a single well and transfer to a T25 flask in 25mL T cell medium containing 30M irradiated feeders, 100ng/mL anti-CD3, 100ng/mL anti-CD28, 300 IU/mL IL2, 10ng/mL IL15, and appropriate antibiotic based on plasmid (e.g. 1ug/mL puromycin). Optionally add IL21 30ng/mL, or IL7 and IL12 at 10ng/mL each, for this initial stimulation step only.
3. Incubate upright for 3 days.
4. On day 3 post-stimulation, remove top 10mL without disturbing lymphocyte carpet, and replace with fresh T cell medium containing a final concentration of IL2 300 IU/mL and IL15 10ng/mL. Continue to incubate upright.
5. Every 2-3 days thereafter split cells 1:2 in fresh T cell media and continue to add IL2/15 as in the previous step. Note that despite the initially low density of live cells, splitting the cells provides room for T cell growth by diluting feeder cells.

6. On day 10-17 post-stimulation, measure transgene expression e.g. by FACS or functional assays. If higher cell numbers are needed, or cells need to be maintained, restimulate cells as in step 2 no later than day 18 post-stimulation.

Results

4 We have tested this protocol for the generation of both TCR- and CAR-modified human T cells. We find that we can generate >40% TCR modified T cells in ~2 weeks when starting from activated bulk T cells and 29% modified T cells when using activated naive T cells (Fig. 1B). When compared to antibody-based murine TCR constant region enrichment using the H57-597 antibody followed by CD3/28 stimulation in the presence of feeders without antibiotics, we find that culture of unselected electroporated cells using CD3/28 stimulation and feeders in the presence of selective antibiotics is superior in promoting outgrowth of TCR-modified T cells bearing murine constant regions (Fig. 1B). Furthermore, TCR and RFP expression are maintained over subsequent stimulation steps (Fig. 1C). Using this protocol, we have also generated CAR-modified T cells expressing 25-94% CAR in 8 days following electroporation (Fig. 2). We have also confirmed that TCR-modified T cells display antigen-specific cytotoxic activity (Fig. 3), which is maintained throughout restimulation cycles.

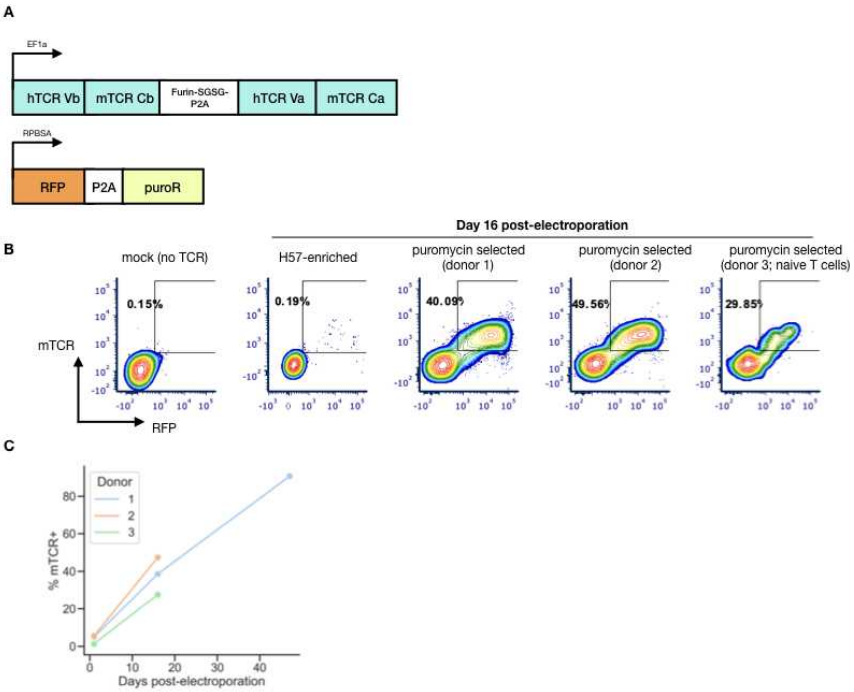


Fig. 1: Generation of TCR transgenic T cells. (A) Topologies of TCR and fluorescent protein (RFP) / antibiotic resistance (puromycin) transgenes. (B) Detection of modified T cells co-expressing RFP and murine TCR (mTCR) constant region 16 days following electroporation. Compared to anti-TCR antibody enrichment and expansion, a significantly more pure gene-modified population is seen following puromycin-based expansion. (C) % mTCR expression over culture days in 3 donors.

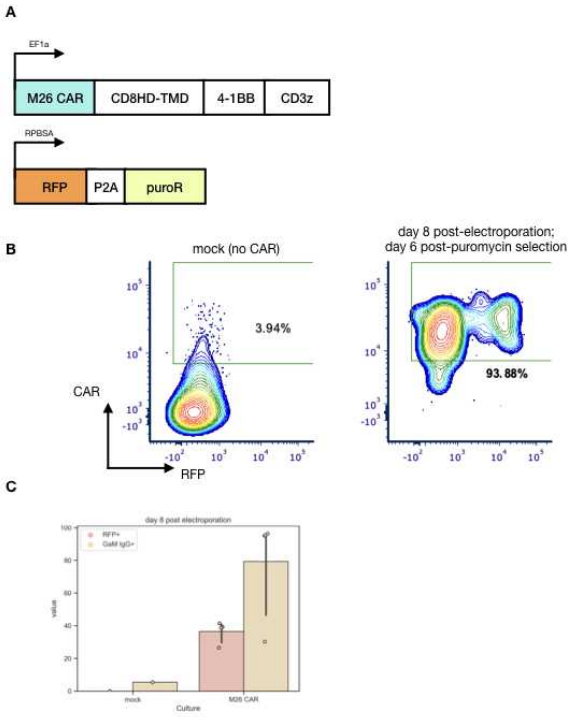


Fig. 2: Generation of CAR-modified T cells. (A) CAR and associated RFP/puromycin resistance transgene topologies. (B) Co-expression of CAR, detected by Goat anti-mouse (GaM) IgG, and RFP 8 days following electroporation. (C) Expression RFP and CAR (detected by GaM) 8 days post-electroporation in 4 independent cultures.

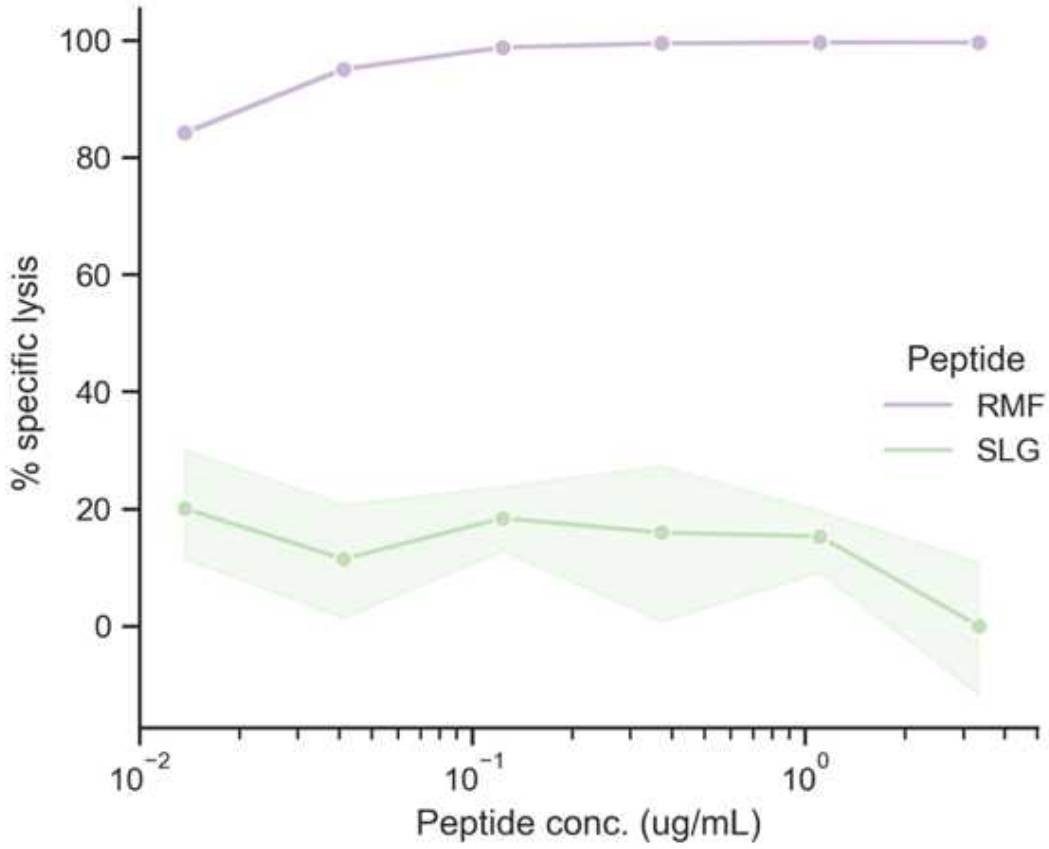


Fig. 3: Functionality of TCR-modified T cells. T cells modified to express an HLA-A2-restricted WT1 RMF peptide-specific TCR were co-cultured overnight with A2+ targets pulsed with graded doses of RMF peptide, or irrelevant (SLG) peptide. >80% target killing is seen at nM concentrations of cognate antigen.