Chapter 13

Gene Engineering T Cells with T-Cell Receptor for Adoptive Therapy

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Abstract

Prior to clinical testing of adoptive T-cell therapy with T-cell receptor (TCR)-engineered T cells, TCRs need to be retrieved, annotated, gene-transferred, and extensively tested in vitro to accurately assess specificity and sensitivity of target recognition. Here, we present a fundamental series of protocols that cover critical preclinical parameters, thereby enabling the selection of candidate TCRs for clinical testing.

Key words T-cell receptor, T-cell engineering, TCR cloning, TCR annotation, Gene transfer, In vitro assays, Specificity, Sensitivity

1 Introduction

Adoptive therapy with T-cell receptor (TCR)-engineered T cells is based on the insertion of genes into the patient's T cells that encode for a TCR directed against a predefined tumor antigen and are re-infused back into the patient. Once transferred to the patient, TCR-engineered T cells specifically migrate toward and kill tumor cells that express this antigen. The promises and challenges of this form of immunotherapy are reviewed elsewhere [1, 2]. Here we provide an overview of steps and details of laboratory protocols necessary to obtain and test TCRs, thereby providing a platform for the identification and selection of those TCRs amenable for further preclinical studies and, when successful, clinical studies.

Epitope-specific T cells and their corresponding TCRs are generally retrieved from tumor-infiltrating lymphocytes (TILs) or peripheral blood mononuclear cells (PBMCs) derived from either patients or healthy donors. In some cases, frequencies of epitope-specific T cells can be amplified in co-culture systems with antigen-presenting cells (*not* part of this chapter, but well be described in Theaker et al. and Wölfl et al. [3, 4]). Epitope-specific T cells can be

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detected and isolated by fluorescent-activated cell sorting (FACS) using peptide-major histocompatibility complexes (pMHC). Then, the RNA of these sorted T cells can be isolated. In Subheadings 2.1 and 3.1, we present materials and protocols to obtain and sequence and identify TCRα and β chains from RNA isolated from pMHC-sorted T cells. TCRα and β sequences are identified with the SMARTer RACE cDNA Amplification Kit (Takara Bio) and Sanger sequencing, after which sequences are annotated using the IMGT database and the HighV-QUEST tool.

Depending on the presence and frequency of T-cell clones, a variable number of TCRα and β chains are identified, and single α and β chains can be co-introduced into T cells to test TCRαβ heterodimers. In Subheadings 2.2 and 3.2, we present materials and protocols to introduce TCRαβ genes into T cells. TCRα and β chains that are molecularly connected with a 2A linker are cloned into an expression vector and retrovirally transduced into T cells. To this end, packaging cells are transfected with the TCRα and β genes as well as retroviral helper constructs, which will enable the secretion of virus particles with RNA encoding the TCR gene construct. PBMCs from healthy donors are activated with stimulatory antibodies and/or cytokines and incubated with the virus particles, leading to a stable integration of TCR genes.

TCR-transduced T cells can be validated in vitro. In Subheadings 2.3 and 3.3, we present materials and protocols to assess TCR surface expression and sensitivity as well as the specificity of T cells expressing an epitope-specific TCR. The surface expression of the TCR transgene is measured using pMHC multimers at the single cell level via flow cytometry. Functional avidity of T cells expressing such TCR transgenes can be determined by measuring IFNγ secretion upon co-culture of these T cells with antigen-presenting cells (APCs) loaded with different concentrations of the cognate epitope. Additionally, the specificity of TCR transgene-expressing T cells is determined by identifying the recognition motif of the TCR, i.e., those amino acids and their positions in the cognate epitope that are critical for recognition by this particular TCR. The more stringent the TCR recognition motif (i.e., the more amino acid residues critically contribute to the epitope's recognition), the lesser the chance that the TCR is cross-reactive. Finally, tumor cell recognition assays can be performed to test if the TCR can recognize epitopes that are the product of endogenous antigen processing and presentation by tumor cells. Extensive in vitro testing of the TCR using sensitivity and specificity assays is crucial to assess its potential clinical value [5]. Collectively, the below protocols provide a stepwise approach to identify TCRαβ sequences, introduce the TCR into T cells, and characterize the TCR in vitro (*see* Fig. 1).

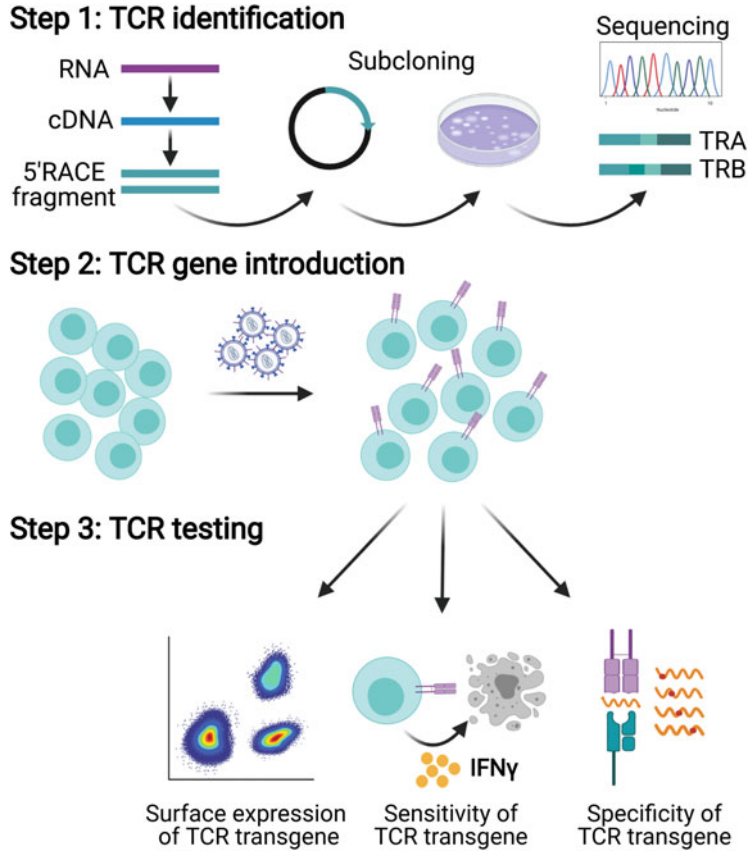

Fig. 1 A stepwise methodological approach to gene-engineered T cells with T-cell receptors for adoptive therapy. The steps are threefold: identification, gene introduction into T cells, and in vitro characterization of TCRαβ sequences (The illustration is created with BioRender.com)

2 Materials

2.1 Identification of TCR from RNA Isolated from pMHC-Positive T Cells

1. SMARTer RACE cDNA Amplification kit (*see* **Notes 1** and **2**).
2. Plasmid isolation kit (*see* **Note 3**).
3. GSP1 primers: prepare a 10 μM stock in sterile dH_2O (*see* **Note 4**). Store at −20 °C.

   GSP1α: GATTACGCCAAGCTTGTTTTGTCTGTGATATACACA.

   GSP1β: GATTACGCCAAGCTTTGCACCTCCTTCCCATTCACCC-ACCAGCTCAGCTC.
4. Nested primers: prepare a 10 μM stock in sterile dH_2O. Store at −20 °C.

NP1α: GATTACGCCAAGCTTGTGACACATTTGTTTGAGAAT.

NP1β: GATTACGCCAAGCTTGGCTCAAACACAGCGACCTC.

5. M13 primers: prepare a 10 μM stock in sterile dH_2O. Store at −20 °C.

   Forward M13: GTAAAACGACGGCCAGT.

   Reverse M13: CAGGAAACAGCTATGAC.

6. 2× Q5 Master Mix (*see* **Note 5**).
7. DreamTaq DNA polymerase and DreamTaq buffer.
8. Deoxynucleotide triphosphates (dNTP) mix (10 mM).
9. 1% agarose gel in modified Tris Acetate-EDTA (TAE) buffer. Dilute TAE buffer in dH_2O to a 1× concentrated solution. Add 1% agarose; heat up the solution in the microwave to dissolve the agarose; and after cooling down to 50–60 °C, pour the agarose in a gel casting tray, with the appropriate well comb in place.
10. 5× DNA loading buffer: dissolve 10 g sucrose in 20 mL H_2O. Add 50 mg Orange G to the solution. Add H_2O up to 50 mL and store at 4 °C.
11. GelRed can be used as a fluorescent nucleic acid dye for visualization of the DNA in the gel (*see* **Note 6**). Add 2.5 μL GelRed to 500 μL 5× DNA loading buffer. Mix 5 μL GelRed/DNA loading buffer, and mix with 20 μL sample before adding onto the gel.
12. LB medium: add 20 g LB to 1 L of dH_2O in a glass bottle. Autoclave the bottle, and let the medium cool down. Store at RT or 4 °C.
13. LB agar plates: add 15 g agar to 1 L LB medium. Autoclave the bottle and let it cool down to approximately 40 °C. Add 100 μg/mL ampicillin, mix gently by shaking, and pour LB/Agar + Amp in 10 cm petri dishes. Let petri dishes cool down at RT until agar is solid before dishes are stored at 4 °C.
14. PCR tubes.
15. 10 cm petri dishes.
16. 1.5 mL and 2 mL Eppendorf tubes.
17. PCR thermocycler.
18. Heating block.
19. Incubator with rotation at 37 °C.
20. Spectrophotometer for nucleic acid quantification.

2.2 Gene Transfer of TCR into T Cells

1. The adherent cell lines 293T and Phoenix-Amp (*see* **Note 7**) should be cultured twice a week using 0.5–1.0 × 10^6 cells per T75 culture flask in 10 mL $DMEM^{++++}$ medium. Additionally, the Phoenix Amp cells need to undergo a 1-week selection procedure with 300 μg/mL Hygromycin B and 1 μg/mL diphtheria toxin, which is repeated after 15 passages of culturing. Cells are cultured for a maximum of 30 passages.
2. Peripheral blood mononuclear cells (PBMCs) (*see* **Note 8**).
3. $DMEM^{++++}$: DMEM medium supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, 200 mM L-glutamine, and 1% penicillin-streptomycin (PS).
4. $RPMI\ Hepes^{HuS++}$: RPMI medium supplemented with 25 mM Hepes, 6% human serum (*see* **Note 9**), 200 mM L-glutamine, and 1% PS.
5. $RPMI\ Hepes^{FBS++}$: RPMI medium supplemented with 25 mM Hepes, 10% FBS, 200 mM L-glutamine, and 1% PS.
6. PBS.
7. PBS/1% FBS: add 5 mL FBS to 500 mL PBS. Store at 4 °C.
8. PBS/0.1% gelatin: add 25 mL 2% gelatin solution to 500 mL PBS.
9. Trypsin/EDTA.
10. Hygromycin B and diphtheria toxin.
11. Promega Calcium Phosphate Transfection Kit.
12. TCR construct in expression vector (e.g., the pMP71 vector).
13. pHIT60 and pColtGalV helper constructs.
14. Ficoll-Paque plus (density: 1.077 g/mL).
15. 10 μg/mL OKT-3 (anti-CD3 MoAb) in PBS stored at −80 °C.
16. Retronectin: 12 μg/mL in dH_2O stored at −20 °C or −80 °C (*see* **Note 10**).
17. 100 IU/mL IL-2 (during transduction) and 360 IU/mL IL-2 (during culture).
18. Trypan blue (TB) for cell counting.
19. Hemacytometer counter and cover slips.
20. Light microscope.
21. T75 culture flasks.
22. 0.45 μm filter.
23. 10 mL syringes.
24. 50 mL tubes.
25. 50 mL Leucosep tubes.
26. Non-tissue culture (NTC) 24-well plate.
27. Parafilm.

2.3 In Vitro Validation of TCR

1. Peptide-MHC-Dextramer with PE label (pMHC-PE) (ProImmune) (*see* **Note 11**).
2. Flow cytometry antibodies (*see* **Note 12**).
   Anti-CD3 FITC (Clone SK7, BD).
   Anti-CD8 APC (Clone SK1 eBioScience): make 1/80 pre-dilution in PBS.
3. FACS buffer: PBS/1% FBS. Add 5 mL FBS to 500 mL PBS. Store at 4 °C.
4. 1% paraformaldehyde in PBS (PFA).
5. RPMI Hepes^{FBS++}: RPMI medium supplemented with 25 mM Hepes, 10% FBS, 200 mM L-glutamine, and 1% PS.
6. Recombinant interferon gamma (IFNγ): dissolve in PBS to a final concentration of 50 ng/mL.
7. Epitope dissolved according to manufacturer (*see* **Note 13**).
8. Epitopes containing individual alanines as replacements at every single position of the cognate epitope (*see* **Note 14**).
9. T2 or BSM cells (*see* **Note 15**).
10. Cell lines expressing target antigen and human leukocyte antigen (HLA) allele of interest (*see* **Notes 16** and **17**).
11. Human IFNγ ELISA Kit (*see* **Note 18**).
12. 5 mL round bottom polystyrene FACS tubes.
13. 96-well tissue culture treated (TCT) round bottom plates.
14. Flow cytometer.

3 Methods

3.1 Identification of TCR from RNA Isolated from pMHC-Positive T Cells

3.1.1 RACE-Ready cDNA, PCR, Cloning, and TCR Sequencing

1. Isolate RNA from epitope-specific T cells according to manufacturer's protocol, and elute the RNA in 10 μL.
2. Measure RNA concentration with a spectrophotometer (*see* **Note 19**).
3. Prepare buffer mix for 5′RACE-ready cDNA synthesis by pipetting 4 μL 5× first strand buffer with 0.5 μL DTT (100 mM) and 1 μL dNTPs (20 mM) in Eppendorf tube 1.
4. Prepare 5′RACE-ready cDNA reaction in Eppendorf tube 2 by mixing 9 μL RNA with 1 μL 5′CDS primer A and 1 μL sterile dH_2O. Incubate the mix 3 min at 72 °C and 2 min at 42 °C, and spin down briefly at 14,000 × *g*.
5. Add 1 μL SMARTer II A oligonucleotide per reaction to Eppendorf tube 2.
6. Add 0.5 μL RNase inhibitor (40 U/μL) and 2.0 μL SMARTScribe reverse transcriptase (100 U) to Eppendorf tube 1. Add

to Eppendorf tube 2, mix by pipetting up and down, and briefly spin down. Incubate Eppendorf tube 2 for 90 min at 42 °C followed by 10 min at 70 °C.

7. Dilute the reaction: add 10 μL of Tricine-EDTA buffer if you started with <200 ng of total RNA, or add 90 μL if you started with >200 ng RNA (*see* **Note 20**).
8. Perform RACE PCR with GSP1 primers. Per condition, two reactions will be performed to separately identify TCRα and β chains. Prepare a master mix containing 15.5 μL PCR-grade H_2O, 25 μL 2× SeqAmp buffer, and 1 μL SeqAmp DNA polymerase.
9. Mix the master mix with 2.5 μL 5′RACE-ready cDNA, 5 μL 10× UPM, and 1 μL GSP1α or β primer, and perform PCR according to the following settings (after putting tubes in PCR thermocycler):

   Five cycles: 94 °C, 30 s; 72 °C, 1.5 min.

   Five cycles: 94 °C, 30 s; 68 °C, 30 s; 72 °C, 1.5 min.

   20 cycles: 94 °C, 30 s; 65 °C, 30 s; 72 °C, 1.5 min
10. Perform nested PCR on the RACE PCR products from **step 9** of Subheading 3.1.1 (*see* **Note 19**). Mix 1 μL RACE PCR product with 1 μL nested universal primer, 1 μL NP1α- or β-primer, 22 μL dH_2O, and 25 μL 2× Q5 master mix in a PCR tube, and perform nested PCR according to the following settings:

    25 cycles: 94 °C, 30 s; 65 °C, 30 s; 72 °C, 1.5 min
11. Load 20 μL of nested PCR product with a fluorescent nucleic acid dye onto a 1% agarose gel.
12. Cut out bands at the correct size of around 800 bp (*see* Fig. 2).
13. Transfer the cut out bands to an Eppendorf tube and add 200 μL NTI buffer. Let the gel dissolve for 5–10 min at 50 °C, while vortexing every 3 min.
14. Place the NucleoSpin column into an Eppendorf tube, and transfer 700 μL of the dissolved sample onto the column. Spin down the Eppendorf tube for 30 s at 11,000 × *g*, and discard the flow-through.
15. Add 700 μL NT3 buffer to the column, and spin down for 30 s at 11,000 × *g*. Discard the flow-through and centrifuge the tube again at 11,000 × *g* for 1 min. Place the column into a new Eppendorf tube. Elute the DNA in 15 μL NE buffer, incubate for 1 min at RT, and centrifuge at 11,000 × *g* for 1 min.
16. Following elution, In-Fusion cloning can be performed according to manufacturer's protocol: transfer 7 μL of eluted

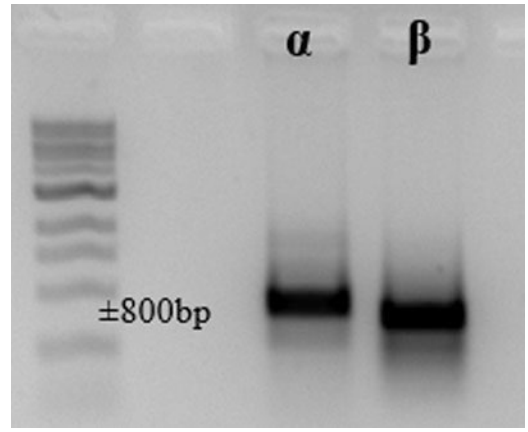

Fig. 2 Correct size of amplified TCRα and β products after nested PCR. Correct size is around 800 bp. Intrinsically the TCRβ chain is larger than the TCRα chain; however, due to the design of RACE primers, the TCRα chain fragment is slightly larger after nested PCR

DNA to an Eppendorf tube. Add 1 μL linearized pRACE vector and 2 μL In-Fusion HD premix to the Eppendorf tube, and mix by vortexing. As a negative control, prepare an empty vector, replacing the eluted DNA with 7 μL dH_2O. The reaction of the positive control provided by the manufacturer consists of 1 μL pUC19 vector, 2 μL 2 kb control insert, 2 μL In-Fusion HD premix, and 5 μL dH_2O. Incubate the reactions for 15 min at 50 °C, and transfer to ice (*see* **Note 21**).

17. Use 2.5 μL cloning reaction from **step 16** of Subheading 3.1.1 for transformation of 25 μL SOC bacteria (*see* **Note 22**). Mix gently by pipetting up and down, after which the reaction should be incubated on ice for 30 min. Perform a heat-shock at 42 °C for 45 s, and put on ice for 1–2 min.
18. Add 225 μL warm super optimal broth (SOC) medium (37 °C) to bacteria, and shake suspension at 200 × *g* for 1 h at 37 °C.
19. Plate out the reaction from **step 18** of Subheading 3.1.1 over 3 LB + Amp plates:
    (a) 1/10: 25 μL of culture +50 μL SOC medium
    (b) 1/100: 2.5 μL of culture +50 μL SOC medium
    (c) Left over.
20. Incubate the plates upside down O/N at 37 °C.
21. Screen for colonies with inserts of expected size. Perform a PCR reaction with M13 primers to amplify DNA inserts of about 20 colonies, and visualize inserts in an agarose gel. The size of the colonies should be around 800–1000 bp for TCRα and β chains. Prepare a PCR premix with following reagents per sample:

(a) 32.5 μL dH_2O

(b) 4 μL 10× DreamTaq buffer

(c) 0.5 μL dNTPs

(d) 1 μL M13 forward primer

(e) 1 μL M13 reverse primer

(f) 0.5 μL DreamTaq DNA polymerase.

22. Add 40 μL premix to each PCR tube (one tube per colony).
23. Pick the colony using a pipette and a small tip, inoculate another LB Agar plate by putting a stripe on the plate with the tip to store the colony, and dip the tip into the PCR mix in the PCR tube. Pipette up and down to mix the DNA with the premix. PCR settings are as followed:

    1 cycle: 95 °C, 5 min

    25 cycles: 95 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min

    1 cycle: 72 °C, 5 min.
24. Load 20 μL sample with a fluorescent nucleic acid dye onto a 1% agarose gel. Select the colonies with the correct size for further analysis.
25. Incubate the LB agar plates at 37 °C overnight.
26. Pick colonies with the correct size the next day with a small pipette tip, and put the tip in a 10–15 mL tube containing 2 mL LB + Amp. Incubate tubes using a 200 × *g* shaker overnight at 37 °C.
27. Transfer bacterial culture to a 2 mL Eppendorf tube, and centrifuge these tubes at 11,000 × *g* for 30 s.
28. Remove supernatant.
29. Continue with plasmid isolation (*see* **Note 3**): add 250 μL buffer A1. Resuspend the pellet by vortexing.
30. Add 250 μL buffer A2. Mix gently by flipping the tube 6–8 times. Incubate for 5 min at RT.
31. Add 300 μL buffer A3. Mix by flipping the tube 6–8 times. Blue color should disappear completely. Centrifuge for 5 min at 11,000 × *g*.
32. Place the NucleoSpin Plasmid Column in a collection tube provided by the kit, and add 750 μL supernatant onto the column. Centrifuge for 1 min at 11,000 × *g*.
33. Discard the flow-through, and place the column back onto the same (now empty) collection tube. Add 500 μL AW buffer, and centrifuge for 1 min at 11,000 × *g*.
34. Discard the flow-through, and place the column back onto the empty collection tube. Add 600 μL buffer A4 and centrifuge for 1 min at 11,000 × *g*.

35. Discard the flow-through, and place the column back onto the empty collection tube. Centrifuge for 2 min at 11,000 × *g* to dry the membrane.
36. Place the NucleoSpin Plasmid Column in a 1.5 mL Eppendorf tube, and add 50 μL buffer AE. Incubate for 1 min at RT and centrifuge for 1 min at 11,000 × *g*.
37. After elution, measure DNA concentration with spectrophotometer.
38. Send samples for Sanger sequencing with M13 primers (*see* **Note 23**).

3.1.2 TCR Sequence Annotation

1. Process TCRα and β chain sequences with alignment software such as Chromas. The software translates the chromatogram file to a sequence.
2. Copy the sequence in plain text or FASTA format.
3. Classify the TCR-V, D, and J genes with the IMGT database and the HighV-QUEST tool (http://www.imgt.org/IMGT_vquest/vquest). Submit the sequence by copy/paste, select *Homo sapiens* in the species section, and select the α (TRA) or β (TRB) sequence in the type of receptor/locus section. The TCR-V, D, and J genes are classified according to the most recent Lefranc nomenclature (*see* **Note 24**).
4. Determine whether the constant region of the β chain is TCRβ constant 1 (Cβ1) or 2 (Cβ2). Align TCR-Cβ of interest with Cβ1 or Cβ2 sequences as reported in https://www.ncbi.nlm.nih.gov/nuccore.
5. Determine the reading frame using the Expasy tool (https://web.expasy.org/translate/). Use Verbose as output format, and determine the in-frame sequence. In the case the sequence has multiple start codons that are in-frame, choose the start codon that is at the exact 5′ end of the leader sequence according to SignalP (http://www.cbs.dtu.dk/services/SignalP/).
6. Design the TCRαβ sequence according to scheme below (*see* **Note 25**).

   *Not*I—GCCACC (Kozak sequence) TCRVβ—Cβ1 or 2 without stop codon—T2A linker—TCRVα—Cα—stop codon—*Eco*RI
7. Order TCRαβ sequences as part of an expression vector (*see* **Note 26**).

3.2 Gene Transfer of TCR into T Cells

3.2.1 Packaging TCR Viruses

1. Precoat T75 flask per condition (*see* **Note 27**) with 5 mL PBS/0.1% gelatin for 10 min at RT.
2. Wash the adherent 293T and Phoenix-Amp cell line with PBS, and loosen the cells with 2 mL trypsin/EDTA at 37 °C.

3. Add 8 mL DMEM^{++++} medium, centrifuge, dissolve in 10 mL fresh DMEM^{++++} medium, and count the cells with TB.
4. Transfer 1.5×10^6 cells of each cell line together in one coated T75 flask in 10 mL DMEM^{++++}.
5. Incubate the cells overnight at 37 °C/5% CO_2.
6. The next day, refresh medium of the packaging cells with 10 mL DMEM^{++++} 3 h prior to transfection.
7. Use the Promega Calcium Phosphate Transfection Kit to transfect the packaging cells. Per T75 flask, prepare in Eppendorf tube 1 the following:
   (a) 10–15 μg TCR construct.
   (b) 5 μg of each helper construct pHIT60 and pColtGalV.
   (c) Add dH_2O to a volume of 500 μL.
   (d) Add 62 μL $CaCl_2$
8. Prepare in Eppendorf tube 2 a 500 μL 2× HBS buffer.
9. Gently vortex the 2× HBS. Slowly add the DNA solution from Eppendorf tube 1 dropwise to the HBS in Eppendorf tube 2 while vortexing. Incubate the mixture at RT for 30 min. Vortex again, and then immediately add the solution to the packaging cells in the T75 flask.
10. Incubate overnight at 37 °C/5% CO_2.
11. The next day, refresh medium of the transfected packaging cells with 10 mL RPMI Hepes^{FBS++}.

3.2.2 Activation of Peripheral Blood Mononuclear Cells (PBMCs)

1. Thaw PBMCs or use freshly collected blood.
2. When using freshly collected blood, isolate PBMCs from healthy donor buffy coats with Leucosep tubes (*see* **Note 8** and steps below).
3. Pipette 15 mL Ficoll Paque in Leucosep tubes.
4. Centrifuge the tube for 10 s at 1000 × *g*.
5. Dilute the buffy coat with PBS/1% FBS in a 1:1 volume ratio.
6. Divide the buffy coat over five Leucosep tubes.
7. Centrifuge the tubes for 10 min at 1000 × *g* with slow deceleration settings.
8. Harvest the cells from the interphase. First, aspirate most of the upper layer (serum), and pour the PBMC (within interphase) in three 50 mL tubes.
9. Add PBS/1%FBS up to 50 mL per tube, and centrifuge for 5 min at 450 × *g*. Aspirate the supernatant, and repeat the washing step three times.
10. Count PBMCs with TB (*see* **Note 28**), and resuspend in RPMI Hepes^{HuS++} at a cell density of 1×10^6/mL.

11. Add 10 ng/mL OKT-3 (*see* **Note 29**).
12. Transfer the PBMCs to a T75 flask, and incubate horizontally for 2 days at 37 °C/5% CO_2.
13. Also coat a non-tissue culture (NTC) 24-well plate with Retronectin. Thaw the Retronectin and add 500 μL to each well; use two wells for each transduction-condition. Seal the plate with parafilm and store overnight at 4 °C.

3.2.3 Transduction of PBMCs

1. Remove Retronectin from the wells (**step 13** of Subheading 3.2.2).
2. Block the wells with 1 mL PBS/2% FBS for 30 min at 37 °C.
3. Harvest virus supernatant from the transfected packaging cells (**step 11** of Subheading 3.2.1), and filter through a 0.45 μM filter using a 10 mL syringe into a 50 mL tube.
4. Add 100 IU/mL IL-2 to the filtered virus supernatant.
5. Add 10 mL fresh RPMI $Hepes^{FBS++}$ to the packaging cells to start a second production round of TCR-encoding virus particles.
6. Aspirate the PBS/2% FBS from the wells of the 24-well plate (**step 2** of Subheading 3.2.3), and add 0.3 mL virus supernatant to each well.
7. Centrifuge for 15 min at 1000 × *g* with slow deceleration settings.
8. Harvest the activated PBMCs (**step 12** of Subheading 3.2.2) by pipetting the cells up and down and using a cell scraper to scrape the cells loose. Transfer the cells to a 50 mL tube.
9. Centrifuge and add RPMI $Hepes^{HuS++}$ to the cells.
10. Count the cells with TB, and use 1×10^6 activated PBMCs per well (*see* **step 11** of Subheading 3.2.3); two wells per condition are used (i.e., use 2×10^6 cells for two wells) (*see* **Note 27**). Transfer the cells to a tube, and centrifuge, aspirate, and resuspend the cells in 0.6 mL virus supernatant.
11. Add 0.3 mL of the PBMC/virus-sup suspension from **step 10** of Subheading 3.2.3 per well (*see* **Note 30**).
12. Seal the plate with parafilm (handle carefully), and centrifuge for 1 h at 1000 × *g* with slow deceleration settings.
13. Remove the parafilm and incubate 5 h (37 °C/5% CO_2).
14. Add 0.8 mL RPMI $Hepes^{HuS++}$ supplemented with 100 IU/ml IL-2.
15. Incubate overnight at 37 °C/5% CO_2.
16. The next day, harvest supernatant from the transfected packaging cells from **step 11** of Subheading 3.2.1, and filter through a 0.45 μM filter using a 10 mL syringe into a 50 mL tube.

17. Add 100 IU/ml IL-2 to the filtered virus supernatant.
18. Carefully remove 1.2 mL from each well, and add 0.6 mL of freshly harvested virus supernatant to each well.
19. Seal the plate with parafilm (handle carefully), and centrifuge for 1 h at 1000 × *g* with slow deceleration settings.
20. Remove the parafilm and incubate 5 h (37 °C/5% CO_2).
21. Harvest the cells, resuspend in RPMI Hepes^{HuS++} supplemented with 360 IU/mL IL-2 at a final concentration of 0.25×10^6/mL. Incubate for 72 h at 37 °C/5% CO_2.
22. TCR- or mock-transduced T cells can be expanded in RPMI Hepes^{HuS++} supplemented with 360 IU/mL IL-2 at a final concentration of 1×10^6/mL for up to 3 weeks (*see* **Note 29**). Culture medium should be refreshed weekly. After these 3 weeks, cells need to be co-cultured with feeder cells (*see* **Note 31**). TCR-engineered T cells can be used for further in vitro validation (*see* **Note 32**).

3.3 In Vitro Validation of TCR

3.3.1 Surface Expression of TCR Transgene

1. Transfer $0.5–1.0 \times 10^6$ TCR- or mock-transduced T cells to FACS tubes.
2. Wash the cells twice with FACS buffer.
3. Aspirate the FACS buffer, and incubate the cells with 5–10 μL pMHC-PE for 10 min at RT in the dark (*see* **Note 11**).
4. Add the following antibodies, and incubate for 20 min at 2–8 °C in the dark.
   (a) 1 μL anti-CD3 FITC
   (b) 2.5 μL 1/80 diluted anti-CD8 APC
   (c) FACS buffer to make a total volume of 10 μL.
5. After incubation, wash the cells twice with FACS buffer.
6. Aspirate and dissolve pellet in 200 μL 1% PFA.
7. Measure surface expression of TCR transgene with flow cytometer (*see* **Note 32**).

3.3.2 Sensitivity of TCR Transgene

1. Harvest TCR- and mock-transduced T cells.
2. Centrifuge and resuspend T cells in RPMI Hepes^{FBS++} at a final concentration of 0.6×10^6 cells/mL.
3. Harvest BSM or T2 cells.
4. Centrifuge and resuspend BSM cells in CTX medium at a final concentration of 0.2×10^6 cells/mL.
5. Transfer 1 mL of BSM cells to each tube.
6. Incubate BSM cells with the cognate epitope for 15 min at 37 °C. Different concentrations per epitope can range from 1 pM to 1 μM (*see* **Note 13**). Also include an irrelevant epitope

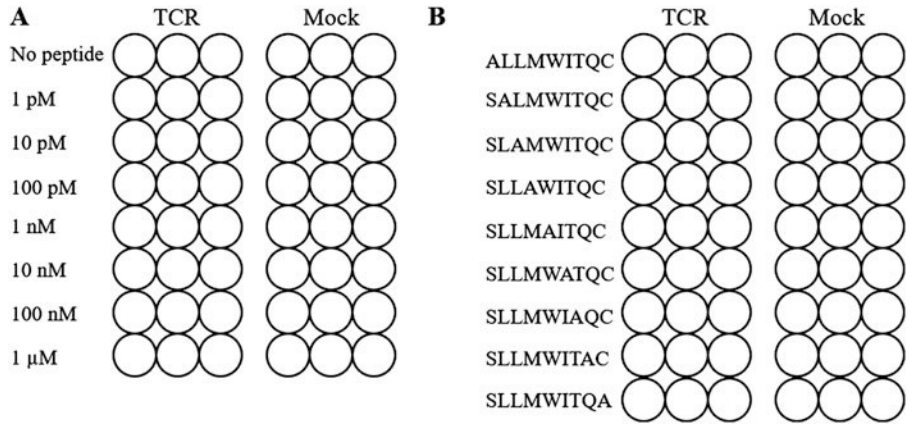

Fig. 3 Pipetting scheme to assess the TCR transgene's sensitivity (**a**) and specificity (**b**) for its cognate epitope. Different concentrations of the cognate epitope (**a**) or different single amino acid mutants of the cognate epitope (illustrated with an example sequence) (**b**) are incubated with BSM or T2 cells prior to co-culture with TCR- or mock-transduced T cells

that should not be recognized by the TCR-transduced T cells as a negative or background control. An example of the layout for the 96-well plate with different epitope concentrations and controls is shown in Fig. 3a.

7. Add 100 μL of T cells from **step 2** of Subheading 3.3.2 to each well (in triplicates) of a 96-well TCT round bottom plate.
8. Add 100 μL of epitope-loaded BSM cells to each well (in triplicates), making a total volume of 200 μL.
9. Centrifuge the plate for 2 min at 220 × *g* with slow deceleration settings.
10. Incubate the plate for 16–24 h at 37 °C/5% CO_2.
11. Centrifuge the plate for 2 min at 220 × *g* with slow deceleration settings.
12. Harvest 150 μL supernatant which can be used to measure IFNγ levels with ELISA-based methods as a readout for sensitivity of TCR transgene-mediated T-cell responses (*see* **Notes 33** and **34**).

3.3.3 Specificity of TCR Transgene

1. Harvest TCR- and mock-transduced T cells.
2. Centrifuge and resuspend T cells in CTX medium at a final concentration of 0.6 × 10^6 cells/mL.
3. Harvest BSM or T2 cells.
4. Centrifuge and resuspend BSM cells in CTX medium at a final concentration of 0.2 × 10^6 cells/mL.
5. Transfer 1 mL of BSM cells to each FACS tube.

6. Incubate BSM cells with different single amino acid mutants of the cognate epitope (10 μM) (*see* **Notes 13** and **14**) for 15 min at 37 °C. Add 1 μL of 10 mM epitope to 1 mL of BSM cells. An example of the layout for the 96-well plate with different epitope mutants is shown in Fig. 3b.
7. Add 100 μL of T cells to each well (in triplicates) of a 96-well TCT round bottom plate.
8. Add 100 μL of epitope-loaded BSM cells to each well (in triplicates), making a total volume of 200 μL.
9. Centrifuge the plate for 2 min at 220 × *g* with slow deceleration settings.
10. Incubate the co-culture for 16–24 h at 37 °C/5% CO_2.
11. Centrifuge the plate for 2 min at 220 × *g* with slow deceleration settings.
12. Harvest supernatant which can be used to measure IFNγ levels with ELISA-based methods as a readout for specificity of TCR transgene-mediated T-cell responses (*see* **Note 35**).

3.3.4 Target Cell Recognition

1. Pre-treat target cells (*see* **Notes 16** and **17**) with 50 pg/mL IFNγ (1:1000 dilution) 48 h prior to co-culture with TCR or mock-transduced T cells.
2. Harvest TCR- and mock-transduced T cells.
3. Centrifuge and resuspend T cells in CTX medium at a final concentration of 0.6 × 10^6 cells/mL.
4. Harvest target cells from **step 1** of Subheading 3.3.4.
5. Centrifuge and resuspend target cells in CTX medium at a final concentration of 0.2 × 10^6 cells/mL.
6. Add 100 μL of T cells to each well (in triplicates) of a 96-well TCT round bottom plate.
7. Add 100 μL of target cells to each well (in triplicates), making a total volume of 200 μL.
8. Centrifuge the plate for 2 min at 220 × *g* with slow deceleration settings.
9. Incubate the plate at for 16–24 h at 37 °C/5% CO_2.
10. Centrifuge the plate for 2 min at 220 × *g* with slow deceleration settings.
11. Harvest supernatant which can be used to measure IFNγ levels with ELISA-based methods as a readout for TCR transgene-mediated recognition of target cells (*see* **Note 36**).

4 Notes

1. The SMARTer RACE cDNA Amplification Kit contains the reagents needed to identify TCRαβ sequences. Components should be stored at different temperatures upon arrival.
2. Other options to identify sequences of the α and β chains of the TCR can be (single cell) RNA sequencing [6].
3. Plasmid isolation can be done with kits from different manufacturers. It is recommended to follow the instructions of the manufacturer.
4. The GSP primers are designed to hybridize within the constant region of the α or β chains of the TCR with a 15 bp PiggyBac vector overhang.
5. 2× Q5 Master Mix can be used as ready-to-use mixture to perform PCR. Also, individual PCR reagents can be used to make a mixture for the PCR reaction.
6. Other fluorescent nucleic acid dyes can also be used to stain DNA or RNA in agarose gels.
7. Use both packaging cell lines for optimal production of virus particles [7, 8].
8. PBMCs can be isolated using Ficoll density gradient centrifugation from healthy donors first described by Böyum in 1968 [9]. They can be freshly used or frozen and thawed on the day of activation of the PBMCs.
9. Human serum used in the culture media is an equal volumetric mixture from five different donors.
10. Retronectin significantly enhances retrovirus-mediated gene transduction into mammalian cells [10, 11].
11. The optimal protocol for pMHC stainings can be different per manufacturer of pMHCs; it is recommended to follow the instructions of the manufacturer.
12. Anti-CD3 and anti-CD8 antibodies with different fluorochromes can be used, as long as spectral overlap between fluorochromes is limited or adequately compensated for.
13. Epitopes (i.e., peptides) should be dissolved in 50–100% DMSO with a final concentration of 10 mM. Dissolvement may very per epitope according to its hydrophobicity profile.
14. To determine the recognition motif of a TCR, an alanine scan is performed. For nine amino acid epitopes, every single position is replaced with an alanine, resulting in nine different epitope mutants.
15. T2 and BSM cells should be cultured twice a week in $RPMI^{+++}$ medium at 0.2×10^6 cells/mL. Depending on the HLA allele

of interest (in these examples, epitopes bound by HLA-A2 are considered), also other cell lines with other HLA alleles can be used.

16. It is important to include positive and negative controls to this assay. Positive controls can include PHA (phytohemagglutinin)/PMA (phorbol myristate acetate), or enterotoxin B (for TCR-independent T-cell stimulation) can be used. Negative controls can include T cells only (without targets), target cells with a different HLA allele, or target cells with the HLA allele under study and no antigen expression. Also blocking antibodies to HLA allele can be used to verify HLA restriction.
17. To test whether the TCR can recognize and kill target cells (i.e., tumor cell lines), it is important to have identified cell or cell lines that express target antigen as well as HLA allele of interest. Expression of target antigen can be determined at RNA (via RT-PCR) and protein level (via immunocytochemistry or Western blot), and expression of HLA allele can be done via flow cytometry.
18. The production of IFNγ by T cells can be used as a readout for TCR-mediated T-cell responses. The enzyme-linked immunosorbent assay (ELISA) is a widely used method to measure levels of IFNγ. The ELISA protocol is generally based on the capturing of the analyte (i.e., IFNγ or other cytokine molecules) by antibodies, which enables the quantification of the analyte present in the sample. Every particular ELISA can have minor adaptations to the standard protocol; therefore, we recommend to follow the instructions of the manufacturer. More detailed information on ELISA-based methods can be found elsewhere [12].
19. Low frequency of antigen-specific T cells in, for instance, PBMC, and difficulties to enrich for these populations can give low RNA quantity and quality. Therefore, a nested PCR on RACE PCR products is recommended for further amplification. If nested PCR products don't show clear bands on agarose gel, the amount of cDNA input can be optimized.
20. cDNA samples can be stored at −80 °C. It is recommended to continue with the RACE and nested PCRs within a week; a decrease in quality can be observed after prolonged storage at −80 °C.
21. The cloning reaction product can be stored at −20 °C. Always take along the positive control in the transformation of bacteria.
22. SOC bacteria are provided as part of the SMARTer RACE cDNA Amplification Kit. Thaw the bacteria on ice and mix by gently pipetting up and down. Store the bacteria at −80 °C and prevent multiple freezing-thawing cycles.

23. Sanger sequencing can be done by several sequencing companies. Generally, DNA and primers have to be provided, but concentrations can differ per company.
24. The IMGT database provides the nucleotide sequences of the TCRα and β chains. It is important to translate this sequence to the amino acid sequence using, for example, the Expasy tool (https://web.expasy.org/translate/), and determine the correct open reading frame.
25. TCRαβ sequences should include the α and β chain sequence connected with a T2A linker [13] and surrounded by restriction sites (*Not*I and *Eco*RI) and a Kozak region prior to the start codon of the variable β-region.
26. TCRαβ sequences can be designed and ordered via an online vector design program. The restriction sites, Kozak region, and 2A linker should be protected, and the rest of the TCRαβ sequences should be codon-optimized for the species *Homo sapiens*.
27. For the transduction of the TCR transgene and further validation of the TCR with in vitro assays, it is important to take mock-transduced T cells along as a negative control. Mock-transduced T cells are created similarly as the TCR-transduced T cells, except that the TCR transgene itself is replaced by an empty vector.
28. Trypan blue (TB) labels death cells with a blue color and is used to facilitate counting of viable cells via a light microscope. Add TB to a cell suspension in a 1:1 ratio (10 μL TB and 10 μL cell suspension, dilution factor is 2) in a 96-well TCT round bottom plate. Mix by pipetting up and down, transfer 10 μL of the mixture to a chamber of a hemacytometer counter, and cover chamber with a slip. Count all cells that are not stained blue in 25 squares, and calculate the concentration of live cells in the cell suspension with the following formula:

$$\text{Concentration/mL} = (\text{counted cells} \times \text{dilution factor} \times 10^3)/(\text{number of squares counted} \times \text{surface area per square in mm}^2 \times \text{depth in mm}).$$

29. Besides OKT-3 and also other antibodies and/or cytokines can be used to activate T cells, such as CD28 mAbs, IL-7, IL-15, and/or IL-21. In fact, the activation of T cells with soluble anti-CD3/CD28 mAbs in the presence of IL-15 and IL-21 resulted in a younger T-cell phenotype and enhanced pMHC binding [14]. The cytokines IL-7, IL-15, and/or IL-21 can also be used to improve T-cell expansion [14–17].
30. TCR gene transfer can also be combined with gene editing using the CRISPR-Cas9 principle [18–21].

31. When necessary to maintain TCR-engineered T cells in culture for longer than 3 weeks, it is required to make use of a feeder cell system as described by Griend et al. This system enables T-cell expansion for up to 2 months while retaining target specificity and cytolytic capacity [22].
32. A TCR transgene is considered to be surface expressed when minimally 5% of $CD3^+$ T cells show an expression of the transgene. Please note that transduction efficiency varies per donor and TCR transgene; therefore, one cannot formally exclude TCR with a surface expression <5% of $CD3^+$ T cells. We therefore recommend to evaluate expression for multiple donors and include other TCR transgenes as controls and use the 5% cutoff as a guideline. In these analyses, mock-transduced T cells can be used as background. Gating strategy for analysis: (1) lymphocytes; (2) $CD3^+$; and (3) $CD8^+$, pMHC multimer$^+$. More detailed information on flow cytometry is well described elsewhere [23]. In the case the TCR genes are only expressed in a low percentage (<10%), cells can be enriched using FACS or magnetic-activated cell sorting (MACS) techniques to increase the percentage of cells expressing the TCR transgene. Again, the need for sorting varies per donor and TCR transgene, for which reason we recommend to use the 10% cutoff as a guideline.
33. Supernatant can be directly used to measure cytokine levels, or the supernatant can be frozen down (−20 °C) and tested at later moments. Avoid repeated freezing-thawing cycles, since this can drastically decrease cytokine levels in supernatants [24].
34. Titrations of cognate epitope and the measurement of IFNγ production by T cells enable the retrieval of half-maximal effective concentrations and thus a measure for functional avidity of the TCR-engineered T cell. A detailed description on how to calculate EC50 can be found elsewhere [25].
35. The recognition motif of a cognate epitope for a particular TCR transgene is the amino acid sequence that is found critical for TCR binding. To determine whether an amino acid is critical for the TCR's recognition, the T-cell response (IFNγ production) to the sequence variant with an alanine replacement is measured. The variant sequence in which a critical amino acid is replaced by an alanine will induce a reduced T-cell response, i.e., at least a twofold drop in IFNγ levels compared to the levels induced by the cognate peptide. The recognition motif, i.e., the sequence of critical amino acids, can be used to search for self-peptides that contain the particular recognition motif (and constitute a potential source for cross-reactive T-cell responses), thereby assessing the risk of off-target toxicity [5].

36. To determine whether a T-cell response against target cell lines is meaningful, IFNγ levels between TCR T cells and mock T cells should be compared. Significant differences in IFNγ levels can be tested with the Mann-Whitney test.

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