Video Article

# Generating *De Novo* Antigen-specific Human T Cell Receptors by Retroviral Transduction of Centric Hemichain

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## Abstract

T cell receptors (TCRs) are used clinically to direct the specificity of T cells to target tumors as a promising modality of immunotherapy. Therefore, cloning TCRs specific for various tumor-associated antigens has been the goal of many studies. To elicit an effective T cell response, the TCR must recognize the target antigen with optimal affinity. However, cloning such TCRs has been a challenge and many available TCRs possess sub-optimal affinity for the cognate antigen. In this protocol, we describe a method of cloning *de novo* high affinity antigen-specific TCRs using existing TCRs by exploiting hemichain centricity. It is known that for some TCRs, each TCRα or TCRβ hemichain do not contribute equally to antigen recognition, and the dominant hemichain is referred to as the centric hemichain. We have shown that by pairing the centric hemichain with counter-chains differing from the original counter-chain, we are able to maintain the antigen specificity, while modulating its interaction strength for the cognate antigen. Thus, the therapeutic potential of a given TCR can be improved by optimizing the pairing between the centric and counter hemichains.

## Video Link

The video component of this article can be found at https://www.jove.com/video/54697/

## Introduction

T cell receptors (TCRs) are heterodimeric adaptive immune receptors expressed by T lymphocytes, composed of a TCRα and TCRβ chain. They are generated via somatic rearrangement of V(D)J gene segments, which produces a highly diverse repertoire capable of recognizing virtually unlimited configurations of HLA/peptide complexes. Clinically, T cells engineered to express clonotypic TCRs specific for tumor-associated antigens have demonstrated efficacy in a variety of cancers[1]. However, many TCRs cloned for this purpose lack sufficient affinity for the antigen of interest, which limit their therapeutic application.

Here, we describe a method to overcome this limitation for existing TCRs by exploiting chain-centricity. It has been reported that one TCR hemichain could play a more dominant role in recognition of the target antigen[2], here termed centricity. Crystal structural analyses have shown that one centric hemichain of a TCR could account for the majority of the footprint on the MHC/peptide complex[3,4]. Using this concept, we have previously demonstrated that the SIG35α TCRα can pair with a diverse repertoire of TCRβ chains and maintain reactivity against the $MART1_{27-35}$ peptide presented by HLA-A2[5]. Similar results were obtained with the TAK1 TCR, where the centric TCRβ hemichain paired with various TCRα chains and maintained reactivity for the $WT1_{235-243}$ peptide presented by HLA-A24[6]. Both MART1 and WT1 are tumor-associated antigens. Chain-centricity was also applied to study antigen recognition of CD1d-restricted invariant natural killer (iNKT) TCRs, by pairing the invariant Vα24-Jα18 (Vα24i) TCRα chain of human iNKT TCRs with different Vβ11 TCRβ chains[7].

In all cases, we were able to generate a *de novo* repertoire of TCRs by transducing the centric TCR hemichain to peripheral blood T cells, where the introduced hemichain paired with the endogenous TCRα or TCRβ counter-chains. In essence, the centric hemichain serves as a bait that can be used to identify the appropriate counter-chains, which when paired together form TCRs that maintain the antigen specificity of interest, yet varying in affinity. From these novel repertoires, we were able to isolate clonotypic TCRs with improved interaction strength against the target antigen compared to pre-existing TCRs. Therefore, we believe this method will accelerate the pipeline of identifying optimal TCRs for clinical application.

 

## Protocol

# 1. Preparing Retroviral Construct Encoding TCR Hemichain of Interest

1. Linearize pMX vector to allow the insertion of a TCR gene in subsequent steps. Digest the plasmid DNA with EcoRI and NotI restriction enzymes at 37 °C for 3 hr (**Table 1**)[8].
2. Carry out electrophoresis of the digested plasmid on 1.2% agarose gel. Excise band of approximately 4,500 base pairs (bps), and elute in 30 µl sterile water using commercially available gel extraction kits[9].
3. Design 5' and 3' primers for the TCR gene of interest that also encode 15-20 bps overlapping the EcoRI and NotI digestion site of pMX vector, respectively[8].
4. Amplify TCR gene with primers. Carry out electrophoresis of the PCR product and elute band of approximately 1,000 bps as described in step 1.2.
5. Clone TCR gene into digested vector by combining each linear fragment with commercially available plasmid assembly master mix reagent and incubating at 50 °C for 1 hr.
   NOTE: Refer to manufacturer's protocol for relative volumes of each component. This assembly method is based on the technique originally described by Gibson *et al.*[10].
6. (Optional) Tag TCR gene to mark transduced cells with the truncated form of human nerve growth factor receptor (ΔNGFR, amino acids 1-277)[11] separated by furin cleavage site, serine-glycine linker, and 2A sequences[12,13]. Design primers encoding sequences overlapping the fragment ends and assemble plasmid as described in steps 1.3-1.5.
   NOTE: These sequences can be found in references[11-13].
7. Transform chemically competent *E. coli* with assembled plasmid following manufacturer's protocol[14]. Seed transformed bacteria on agar plates (20 mg/ml lysogeny broth (LB), 15 mg/ml agar, and 1 µg/ml ampicillin) and incubate at 37 °C for 18 hr.
8. Culture a single bacterium colony in 50 ml of LB medium (20 mg/ml LB and 1 µg/ml ampicillin) for 16 hr in a shaking incubator at 37 °C and 200 rounds per min.
9. Purify plasmids using commercially available midiprep kits following manufacturer's protocol. Dilute plasmid to concentration of around 1 µg/µl.
   NOTE: The plasmid purification protocol is based on the alkaline extraction method[15].

# 2. Generating Stable Packaging Cell Line

NOTE: Both 293GPG and PG13 cells are adherent. Culture cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 50 µg/ml gentamicin. Culture 293GPG cells with 1 µg/ml tetracycline before transfection. Incubate cells at 37 °C with 5% $CO_2$ between all steps.

1. Transfect 293GPG packaging cells[16] cultured in T75 flask with the hemichain-encoding vector obtained in step 1.9, using commercially available transfection reagent following manufacturer's protocol[17]. NOTE: Transfect 293GPG cells at 50-60% confluency.
2. Aspirate medium for 293GPG cells and add 10 ml of fresh DMEM medium 1 day post transfection.
3. Harvest transiently produced virus from transfected 293GPG cells 2 days after step 2.2 by transferring culture medium to syringe and passing through 0.45 µm filter.
   NOTE: Virus can be used immediately or stored at -80 °C for future use.
4. Culture 1 x $10^5$ PG13 cells in T25 flask. Count cells using a hemocytometer. After one day, aspirate culture medium and add 1.5 ml of 293GPG-derived virus from step 2.3 and 1.5 ml DMEM medium, along with 8 µg/ml of polybrene.
5. Change the medium as described in step 2.4 once per day for 4 days, to establish stable PG13 packaging cell line producing retrovirus encoding the TCR hemichain of interest.
6. Aspirate medium and replace with fresh DMEM medium for PG13 cell lines 24 hr after last infection for further culture.
   NOTE: Freeze or sub-culture cells by detaching with 0.05% trypsin-EDTA solution.
7. To produce virus from transduced PG13 cell lines, culture 2 x $10^6$ cells in T75 flask with 10 ml DMEM medium, and harvest virus as described in step 2.3 three days after seeding the cells.
   NOTE: Virus is best used immediately but can be stored at -80 °C for up to two months.

# 3. Activation and Transduction of Human T cells

NOTE: Human samples are obtained and used in accordance with the institutional ethics committee approved protocols. Culture primary human cells in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% human serum instead of FCS and 50 µg/ml gentamicin. Incubate cells at 37 °C with 5% $CO_2$ between all steps.

1. Isolate human peripheral blood mononuclear cells (PBMC) by density gradient separation following manufacturer's protocol[18].
2. Activate T cells to induce proliferation required for retroviral infection. Culture 2 x $10^7$ fresh or thawed PBMC per well in 6-well plate, in 5 ml of RPMI medium with 100 IU/ml of recombinant human interleukin-2 (rhIL-2) and 50 ng/ml of anti-CD3 monoclonal antibody (clone OKT3).
3. Three days post stimulation, collect T cells by pipetting and centrifuge at 350 x g for 4 min. Discard supernatant and seed 0.5-1 x $10^6$ T cells per well in 24-well plate resuspended in 1 ml of PG13 virus from step 2.7, and 1 ml of RPMI medium supplemented with 200 IU/ml of rhIL-2. Centrifuge plate at 1,000 x g and 32 °C for 1 hr.
4. After 24 hr, collect T cells by pipetting and centrifuge at 350 x g for 4 min. Discard supernatant and resuspend cells in 1 ml of PG13 virus from step 2.7, and 1 ml of RPMI medium supplemented with 200 IU/ml of rhIL-2. Repeat this step for a total of 6 infections.
   NOTE: Number of infections should be optimized depending on titer of virus produced by packaging cell line. If necessary, passage T cells by removing 20-30% of the cells each day to prevent overgrowth.

5. 24 hr after the last infection, collect T cells by pipetting and centrifuge at 350 x g for 4 min. Discard supernatant and resuspend cells in RPMI medium with 100 IU/ml of rhIL-2 for further culture.
6. 2-3 days after step 3.5, stain T cells with HLA multimer at 4 °C for 30 min, then anti-human CD3 and co-receptor monoclonal antibodies (mAbs) at 4 °C for 15 min. Analyze by flow cytometry. Use irrelevant multimer and/or untransduced cells as negative controls (**Figure 1**-**3**)[19].
7. If the introduced centric hemichain is a TCRα chain, analyze Vβ usage of the *de novo* multimer positive cells using commercially available Vβ-specific antibody panel by flow cytometry[20].

## 4. Cloning *De Novo* TCR Counter-hemichains

1. Sort the *de novo* multimer positive population in step 3.6 by flow-assisted cell sorting.
2. Isolate RNA from sorted T cells using the acid guanidinium thiocyanate-phenol-chloroform extraction method[21,22].
   NOTE: RNA can be stored at -80 °C, but should be used to generate cDNA as soon as possible.
3. Synthesize cDNA library from extracted RNA using commercially available reverse transcriptase-PCR kits following manufacturer's protocol[23,24].
4. If cloning TCRβ counter-chains, design Vβ gene and TCRβ constant region specific primers, as determined in step 3.7, and clone full-length TCRβ genes as described in steps 1.3-1.9. See step 4.5 if counter-chain is TCRα, otherwise continue to step 5.1.
5. Commercially available Vα-specific antibodies are limited, thus Vα gene usage cannot be determined by flow cytometry. Clone TCRα counter-chains via 5'-rapid amplification of cDNA ends (RACE)[25], using commercially available 5' RACE kits[6].
   1. Synthesize 5' RACE compatible cDNA from RNA extracted in step 4.2 following manufacturer's protocol.
   2. Perform 1$^{st}$ round PCR as described in **Table 2**.
   3. Carry out electrophoresis of the PCR product and elute band of approximately 1,100 bps, as described in step 1.2.
   4. Perform 2$^{nd}$ round PCR as described in **Table 3**, using 1$^{st}$ round PCR product as template.
      NOTE: Primer sequences shown under **Table 3** were designed for cloning into EcoRI and NotI digested pMX vector.
   5. Carry out electrophoresis of the PCR product and elute band of approximately 1,000 bps, as described in step 1.2.
   6. Clone TCRα genes into EcoRI and NotI digested pMX vector and purify plasmids as described in steps 1.5-1.9.

## 5. Reconstituting Novel Antigen-specific TCR Clones

NOTE: Culture Jurkat 76 cells and subsequent cell lines in RPMI medium supplemented with 10% FCS and 50 µg/ml gentamicin. Incubate cells at 37 °C with 5% $CO_2$ between all steps.

1. Transduce Jurkat 76 cells[26] (or equivalent TCR$^{-/-}$ human T cell line) with centric TCR hemichain using 293GPG virus produced in step 2.3. For Jurkat 76, seed 5 x 10$^4$ cells per well in 24-well plate with 1 ml of virus and 1 ml of RPMI medium. Centrifuge plate at 1,000 x g and 32 °C for 1 hr.
2. 24 hr after infection, collect hemichain transduced Jurkat 76 cells by pipetting and centrifuge at 350 x g for 4 min. Discard supernatant and resuspend in fresh RPMI medium for further culture.
3. Purify transduced cells if the hemichain is molecularly tagged 2-3 days after step 5.2, by staining with fluorophore conjugated anti-NGFR mAb followed by flow-assisted or magnetic-assisted cell sorting (optional)[27].
4. Following steps 2.1 to 2.3, produce 293GPG virus encoding a TCR counter-chain cloned from steps 4.4 or 4.5.
5. To fully reconstitute the TCR, transduce T cell line stably expressing the centric TCR hemichain generated in steps 5.1-5.3 using virus from step 5.4. Perform transduction as described in steps 5.1-5.2.
6. Purify CD3$^+$ transfectants using anti-CD3 magnetic-assisted cell sorting following manufacturer's protocol, 2-3 days after step 5.5[27].
7. To validate antigen specificity, stain the transfectants expressing clonotypic TCRs composed of the centric TCR hemichain paired with various counter-chains, with anti-CD3 and/or co-receptor mAbs, and HLA multimer, 3-5 days post CD3 purification. Analyze cells by flow cytometry (**Figure 4**-**5**)[19].

## Representative Results

Without prior knowledge of which hemichain is chain-centric, the TCRα and TCRβ chain should be separately cloned and transduced to peripheral blood T cells, which was done in the case of HLA-A24/WT1 reactive TAK1 TCR (**Figure 1**). Transduction of TAK1β yielded a noticeably higher frequency of antigen-specific T cells. Conversely, transduction of a non-centric hemichain would not yield *de novo* multimer positive T cells, as seen with TAK1α chain (**Figure 1**). During analysis, gate on NGFR$^+$ cells if the TCR gene was tagged to specifically analyze transduced cells (**Figure 1**-**2**). On the other hand, the single centric hemichain can be transduced if it is known to be dominant, for example the SIG35α and invariant Vα24 TCRα chains (**Figure 2** and **3**). T cells specific for the respective cognate antigen increased in frequency upon transduction of a centric TCRα hemichain. (**Figure 2**-**3**). Together, these data demonstrate that introduction of a centric TCRα or TCRβ hemichain to peripheral blood T cells can generate *de novo* TCRs with the antigen-specificity of interest.

Antigen-specific population can be sorted by flow-assisted cell sorting and TCR hemichains can be cloned as described in the Protocol section 4. Clonotypic TCR counter-chains can be individually reconstituted on a T cell line stably expressing the centric hemichain. Jurkat 76 transfectants, expressing newly cloned unique TCRs from TAK1β or SIG35α centric hemichain transduced T cells, possessed varying avidity for the respective cognate antigen, as determined by HLA/peptide multimer staining[5,6]. Specifically, we were able to isolate *de novo* TCRα counter-chains that when paired with TAK1β were stained with either lower or higher intensity by the WT1 antigen complex than the parental TAK1αβ pairing (**Figure 4**). Similarly, SIG35α paired with unique counter-chains recognized HLA-A2/MART1 with moderate to high avidity (**Figure 5**). SIG35α was cloned independent of a TCRαβ heterodimer[5], therefore a parental pairing for this hemichain was unavailable for comparison. Importantly, these data indicate that it is possible to modulate the affinity for the target antigen by pairing the centric hemichain with various counter-chains.

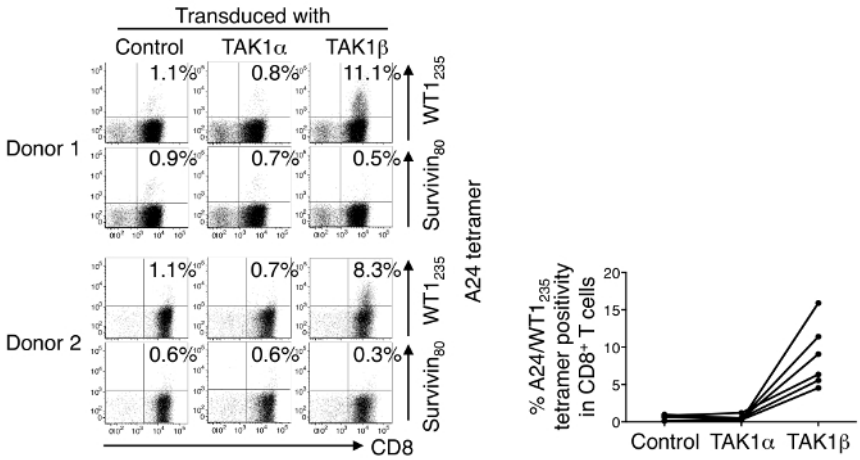


**Figure 1: TAK1β as an example of HLA-restricted TCRβ centric hemichain.** TCRα or TCRβ hemichains of the HLA-A24/WT1 reactive TAK1 TCR was tagged with truncated nerve growth factor receptor (ΔNGFR), and individually transduced to peripheral blood T cells. Transfectants were stained with PC5-conjugated anti-CD8 (133x diluted) and FITC-conjugated anti-NGFR (20x diluted) monoclonal antibodies (mAbs), and indicated HLA-A24 multimer (5 μg/ml), following two times antigen-specific stimulation. Total of 6 donors were tested. All data were gated on NGFR$^+$ cells. Figure re-printed with permission from reference[6]. Please click here to view a larger version of this figure.

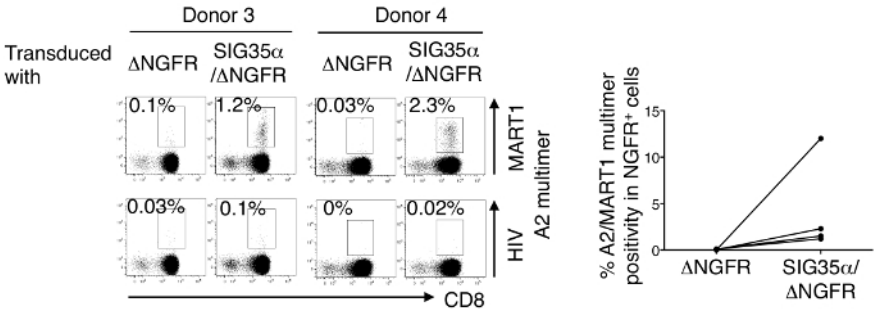


**Figure 2: SIG35α as an example of HLA-restricted TCRα centric hemichain.** SIG35α TCRα chain tagged with ΔNGFR, or the tag alone, was transduced to peripheral blood T cells. Transfectants were stained with PC5-conjugated anti-CD8 (133x diluted) and FITC-conjugated anti-NGFR (20x diluted) mAbs, and indicated HLA-A2 multimer (8 μg/ml). Total of 4 donors were tested. All data were gated on NGFR$^+$ cells. Figure re-printed with permission from reference[5] (*Copyright 2015. The American Association of Immunologists, Inc.*). Please click here to view a larger version of this figure.

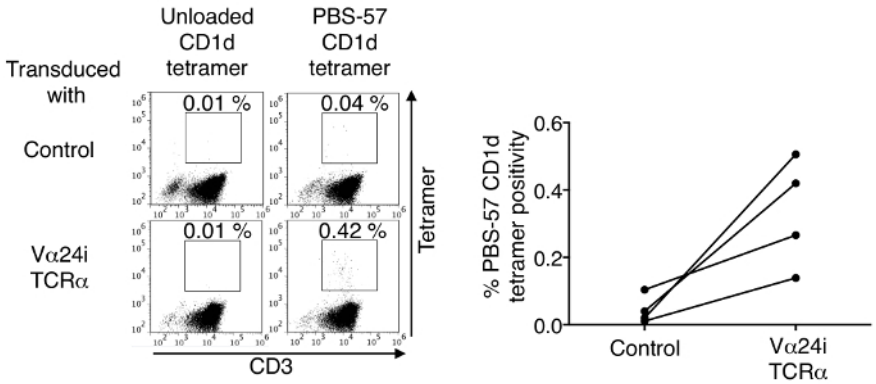


**Figure 3: Invariant natural killer T (iNKT) cell receptor Vα24 (Vα24i) as an example of CD1d-restricted TCRα centric hemichain.** Vα24i TCRα chain was transduced to peripheral blood T cells. Transfectants were stained with FITC-conjugated anti-CD3 (20x diluted) mAb and indicated CD1d multimer (5 μg/ml). Unloaded CD1d molecules were produced from HEK293 cells and present endogenous unknown self-lipids. iNKT TCR is defined by recognition of CD1d presenting α-GalCer or its analog PBS-57. Total of 4 donors were tested. Figure re-printed with permission from reference[7]. Please click here to view a larger version of this figure.

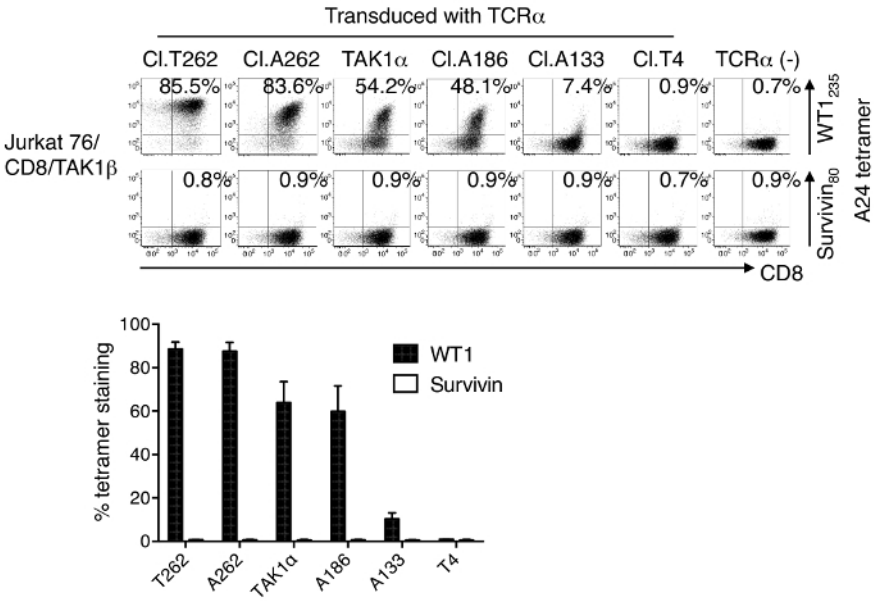


**Figure 4: Representative TCRα counter-chains cloned from TAK1β transduced T cells.** TCRα clones T262, A262, A186, A133, and T4 were cloned from HLA-A24/WT1 multimer positive TAK1β transduced peripheral T cells, and individually reconstituted on Jurkat 76 cells expressing CD8, along with the TAK1β chain. Transfectants were stained with PC5-conjugated anti-CD8 mAb (133x diluted) and indicated HLA-A24 multimer (5 μg/ml). Data are representative two independent experiments. Error bars indicate range. Figure re-printed with permission from reference[6]. Please click here to view a larger version of this figure.

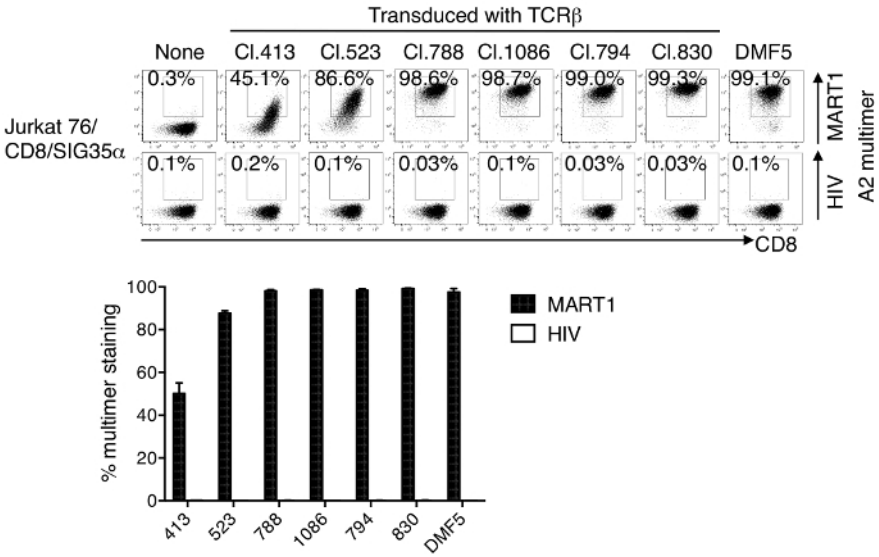


**Figure 5: Representative TCRβ counter-chains cloned from SIG35α transduced T cells.** TCRβ clones 413, 523, 788, 1086, 794, and 830 were cloned from HLA-A2/MART1 multimer positive SIG35α transduced peripheral T cells, and individually reconstituted on Jurkat 76 cells expressing CD8, along with the SIG35α chain. DMF5 was a previously cloned high affinity TCR recognizing HLA-A2/MART1. Transfectants were stained with PC5-conjugated anti-CD8 mAb (133x diluted) and indicated HLA-A2 multimer (2 μg/ml). Data are representative of two independent experiments. Error bars indicate range. Figure re-printed with permission from reference[5] (*Copyright 2015. The American Association of Immunologists, Inc.*). Please click here to view a larger version of this figure.

| Component | volume (μL) |
|---|---|
| pMX vector (1 μg/μL) | 1 |
| EcoRI (20,000 units/mL) | 1 |
| NotI (10,000 units/mL) | 1 |
| Buffer | 3 |
| sterile water | 24 |
| total | 30 |

**Table 1: Setup for pMX vector digestion.** Reagents and respective volumes are shown. Please click here to view a larger version of this table.

| Component | volume (µL) |
|---|---|
| RACE-ready cDNA | 1 |
| 5' Universal Primer Mix | 5 |
| 3' TCRα primer (10 µM) | 1 |
| 5X polymerase buffer | 10 |
| 10 mM dNTP mix | 1 |
| DNA polymerase (2,000 units/mL) | 0.5 |
| sterile water | 31.5 |
| Total | 50 |

3' primer sequence is 5'-GGAGAGTTCCCTCTGTTTGGAGAG-3'
5' universal primers are supplied by kit

| cycles | time (sec) | temperature (°C) |
|---|---|---|
| 1 | 30 | 98 |
| 35 | 10 | 98 |
| | 30 | 60 |
| | 45 | 72 |
| 1 | 600 | 72 |

**Table 2: Setup for 1st round 5' RACE PCR.** Reagents and respective volumes, PCR settings, and primer sequence are shown. Please click here to view a larger version of this table.

| Component | volume (µL) |
|---|---|
| 1st round PCR product | 1 |
| 5' primer (10 µM) | 1 |
| 3' primer (10 µM) | 1 |
| 5X polymerase buffer | 10 |
| 10 mM dNTP mix | 1 |
| DNA polymerase (2,000 units/mL) | 0.5 |
| sterile water | 35.5 |
| Total | 50 |

5' primer sequence is 5'-GTGTGGTGGTACGGGAATTCAAGCAGTGGTATCAACGCAGAGT-3'
3' primer sequence is 5'-ACCACTGTGCTGGCGGCCGCTCAGCTGGACCACAGCCGCAGCG-3'

| cycles | time (sec) | temperature (°C) |
|---|---|---|
| 1 | 30 | 98 |
| 35 | 10 | 98 |
| | 30 | 61 |
| | 45 | 72 |
| 1 | 600 | 72 |

**Table 3: Setup for 2nd round 5' RACE PCR.** Reagents and respective volumes, PCR settings, and primer sequences are shown. Please click here to view a larger version of this table.

## Discussion

The first requirement for successful application of this method is achieving sufficient transduction efficiency of primary T cells with the hemichain of interest. In our experience, the combination of using PG13 as packaging cell line and pMX as retroviral vector results in stable and efficient expression of the introduced gene in human primary T cells. PG13 packaging cells can be single-cell cloned to select for high-titer packaging cells to improve transduction efficiency. Furthermore, proliferation of T cells is also required for high transduction efficiency by retrovirus.
In the described protocol, stimulation with soluble anti-CD3 mAb OKT3, along with high concentration of rhIL-2, provides the necessary activation signals to induce cell division. Monocytes are required for the stimulatory capacity of soluble OKT3, likely through binding with Fc receptors. Therefore, bulk PBMC, and not purified T cells, is required for this particular stimulation protocol. T cells should be cultured in RPMI supplemented with human serum for optimal experimental results. All other cells described here can be cultured in the presence of fetal calf serum.

Second, *de novo* generated antigen-specific TCRs expressing cells must be detectable. For certain TCR hemichains, such as TAK1β, *de novo* TCR expressing cells are only detectable after stimulation with antigen-pulsed antigen-presenting cells (APCs). Thus, if antigen-specific TCRs are not detected immediately after transduction of centric hemichain, they could be detectable after expansion by APCs. Note that transduction of a non-centric hemichain would not yield *de novo* antigen-specific T cells even after antigen-specific expansion with APCs, as seen with the TAK1α hemichain (**Figure 1**). In our studies, HLA multimers are used for detection, however, this might not be available for some TCRs, especially HLA class II restricted TCRs. An alternative would be to detect T cell functional responses. Hemichain transduced T cells can be stimulated with APCs pulsed with the antigen of interest. Vehicle-pulsed APCs and untransduced T cells can be used as controls. Responding T cells can be detected by upregulation of surface activation markers such as CD107a, CD154, or CD137.

Lastly, it is important to validate that the cloned counter-chains do in fact recognize the antigen of interest when paired with the centric hemichain. This is particularly important when the centric hemichain is TCRβ and counter-chains are TCRα, since allelic exclusion for TCRα

genes is leaky and it is estimated that a significant portion of peripheral αβ T cells express more than one TCRα chain[28]. Reactivity can be confirmed with multimer staining or measuring functional response after stimulation with APCs presenting the cognate antigen.

One limitation of this technique is that not every TCR will compose a centric hemichain, since both TCRα and TCRβ contribute comparably to antigen recognition in some cases[3]. Therefore, peripheral T cells transduced with such non-centric hemichains may not yield *de novo* antigen-specific TCRs.

Nevertheless, this method represents a conceptual advancement upon the conventional approach to cloning TCRs, where both TCRα and TCRβ genes must be cloned and correct pairing must be ensured[29,30]. In our technique, pairing between hemichains is no longer a main concern and only one of the TCR chains needs to be cloned, given that the other is fixed. In addition to HLA class I or CD1d-restricted TCRs, the described method can also be applied to the isolation of HLA class II-restricted TCRs for TCR gene therapy.

## Disclosures

The University Health Network has filed a patent related to this methodology on which N.H., M.N., and T.O. are named as inventors. The other authors have no financial conflicts of interest.

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