# Utilize a Soluble T-cell Receptor TCR Targeting Tumor Intracellular Antigen PRAME to Develop Therapeutic and Radioactive Diagnostic Agents for PRAME-positive Tumors

**#7193**

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## Abstract

**Background:** T-cell receptor (TCR)-based therapeutics and diagnostics hold great promise for targeting intracellular tumor antigens presented by HLA molecules. The preferentially expressed antigen in melanoma (PRAME) is expressed in many cancers, but is highly restricted in normal tissues, making it a good target for TCR-based therapy. We have established a proprietary high-efficiency TCR discovery platform, enabling rapid identification and screening of antigen-specific TCRs from healthy donors and cancer patients. We are developing PRAME$_{425-433}$/ HLA-A*02:01-specific TCR based radioactive diagnostic agents and therapeutic drugs.

**Methods and Results:** TCRs specifically targeting PRAME425-433 peptide/ HLA-A*02:01 complex were cloned from healthy donor's PBMC. T cells transduced with lentivirus encoding PRAME-specific TCRs exhibited robust IFN-γ production and cytolytic function in an antigen-specific manner. By engineering the constant regions of both TCR α and β chains, We constructed and produced multiple formats of monovalent and bivalent native PRAME-specific TCRs. Using our exclusive cell-based TCR binding assay and flow cytometry, we were able to screen soluble TCRs for antigen-specificity and binding affinities without TCR affinity enhancement. The soluble TCR HP-002 with a higher binding affinity was selected for further development. Surface plasmon resonance (SPR) analysis confirmed that the bivalent native TCR protein HP-002 had a higher affinity and it binds to PRAME425-433/HLA-A*02:01 monomer with a KD of 1.08 × $10^{-7}$ M. Without TCR affinity enhancement, this bivalent native TCR was used to construct TCR-CD3 T cell engagers and radioactive diagnostic agents for in vivo imaging. Our bivalent native TCR-CD3 T cells engagers were able to fully activate T cells in vitro and induced potent tumor cell killing by T cells. Iodine-125 ($^{125}$I)-labeled bivalent native TCR protein HP-002 exhibited in vivo antigen-dependent tumor accumulation in xenograft tumor models by SPECT/CT imaging.

**Conclusions:** Our TCR discovery and molecular engineering platform enables rapid generation of soluble, functional, tumor antigen-specific TCRs with high affinity. HP002 shows specific recognition of PRAME$_{425-433}$/HLA-A02:01 and tumor-targeting capability in vivo, supporting its further development as a radioactive diagnostic agent for HLA-A02:01 PRAME-positive tumors. We are optimizing TCR affinity and molecular structure to enhance tumor accumulation for future diagnostic and therapeutic applications.

## Summary

- We have established an efficient 4-week TCR discovery program to identify and clone antigen-specific TCRs using PBMCs/TILs from healthy donors or cancer patients.
- We have successfully built the TCR engineering technology for productive expression of monovalent and multi-valent sTCRs and TCR-CD3 T cell engagers.
- We have invented a proprietary cell-based screening platform for binding affinity of soluble TCRs. We have solved the big challenge in characterizing native soluble TCRs in preclinical research. Native TCRs with high affinity can be identified for the further development of TCR therapy and diagnostic agents.
- Our native TCRs with high affinity has the potential for TCR-CD3 TCE therapy without affinity enhancement.
- $^{125}$I-labeled bivalent PRAME$_{425-433}$/HLA-A02:01 TCR protein HP-002 exhibited in vivo antigen-dependent tumor accumulation in PRAME-positive tumors by SPECT/CT imaging, .

## Native TCR Discovery and Validation Protocol

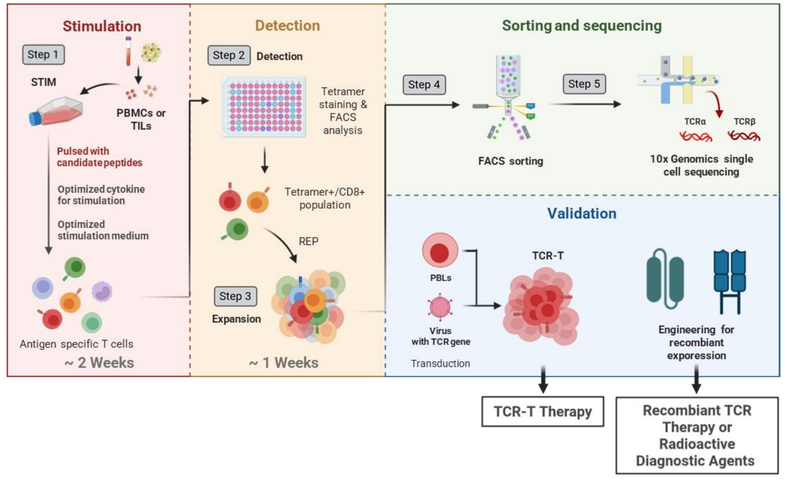


**Figure 1:** PBMC or TILs are stimulated from HLA-A*02:01 positive healthy donors or cancer patients. PRAME$_{425-433}$-specific T cells are identified by flow cytometry analysis with tetramer staining and intracellular IFN-γ staining. T cell culture wells with PRAME$_{425-433}$-specific T cells are further expanded for one week. PRAME$_{425-433}$/HLA-A*02:01 tetramer positive CD8 T cells were sorted by FACS and used for 10X genomics single cell TCR sequencing. Lentivirus encoding PRAME-specific TCRs are used for validation of TCR specificity and development of TCR-T therapy. Monovalent and bivalent native PRAME-specific TCR are constructed and soluble TCR production are achieved and optimized through molecular engineering. TCRs with high affinities are used to develop TCR-CD3 T cell engagers and radioactive diagnostic agents.

## PRAME$_{425-433}$–specific TCRs Cloned from HLA-A*02:01-positive Healthy Donor

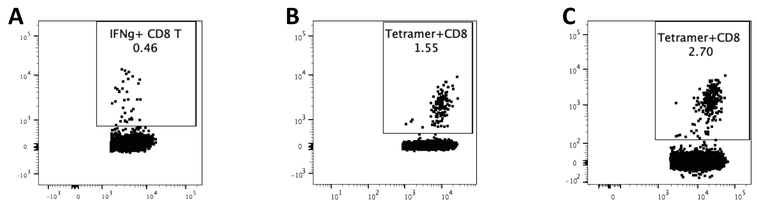


**Figure 2:** HLA-A*02:01 positive healthy donor PBMCs were stimulated with PRAME$_{425-433}$ peptide in 96-well plates. The wells were screened for IFNg producing CD8 T cells by intracellular staining (**Panel A**). The cells from IFNg-positive wells were confirmed by PRAME$_{425-433}$/HLA-A0201tetramer staining (**Panel B**) . The pooled tetramer positive CD8 T cells were sorted by FACS (**Panel C**) for single cell TCR sequencing.

## TCR-T Cells: Antigen-specificity of PRAME$_{425-433}$-specific TCRs

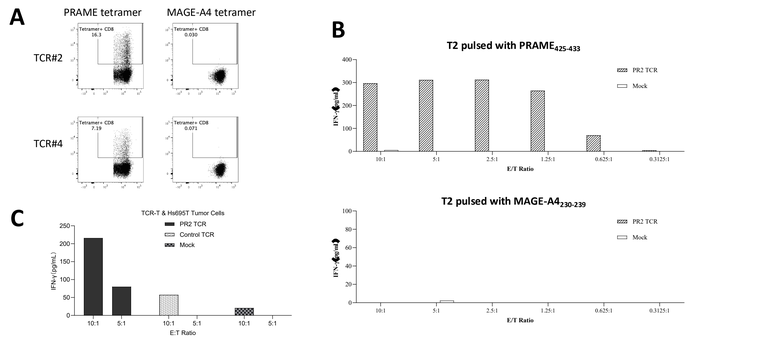


**Figure 3:** Antigen-specificity of TCRs were confirmed by TCR-T cells. **A.** HLA-A*02:01 positive PBMC cells were transduced with lentivirus encoding PRAME$_{425-432}$-specific TCR. TCR-T cells were positive for PRAME$_{425-433}$/HLA-A *02:01 tetramer staining by flow cytometry. **B.** TCR-transduced PBMCs secreted robust IFN-γ upon co-culture with PRAME$_{425-433}$ peptide-pulsed T2 cells and PRAME IFN-γ secretion were not detectable when co-cultured with T2 cells pulsed with MAGE-A4$_{230-239}$ peptide. C. IFN-γ secretion when co-cultured with PRAME-positive Hs695T tumor cells.

## Molecule Engineering and Production of Soluble Native TCRs

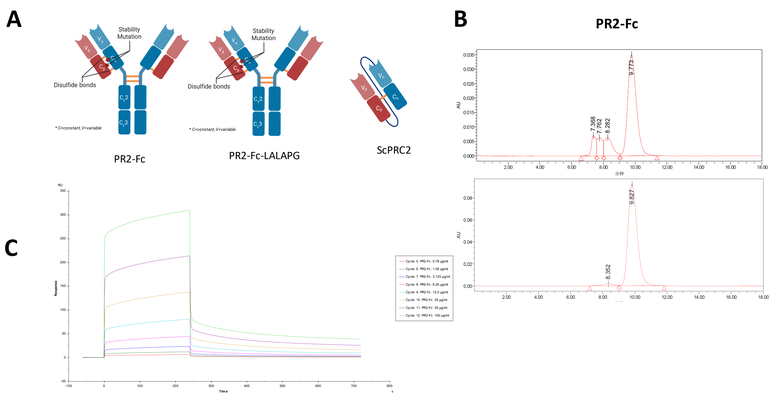


**Figure 4:** Soluble TCR (sTCR) engineering, expression and binding affinity measurement. **A.** Illustration of multiple formats of sTCR for distinct applications and experiments: To generate the bivalent PR2-Fc, the constant region of PRAME$_{425-433}$-specific TCR was engineered to contain an additional pair of disulfide bond and stabilizing mutations, and fused to the N-terminus of an Fc fragment (wild-type human IgG1). The version of PR2-Fc-LALAPG was engineered by introducing LALAPG mutations in the Fc region of the PR2-Fc protein. This is used for in vivo imaging study. To generate a monovalent version of sTCR, PRAME$_{425-433}$-specific TCR was engineered to generate a single-chain TCR (scPRC2) protein. **B.** The purity of soluble TCR (bivalent PR2-FC) reached 99.15% (SEC) after two-step purifications. **C.** Binding affinity of the bivalent PR2-Fc protein was measured by surface plasmon resonance (SPR). The KD of soluble bivalent TCR (PR2-Fc) is 1.084 X $10^{-7}$ M, with $K_a$= 1.65 × $10^4$ $M^{-1}s^{-1}$ and $K_d$ = 0.001788 $s^{-1}$.

## A Proprietary Screening Platform for TCR Ag-specificity and Binding Affinity

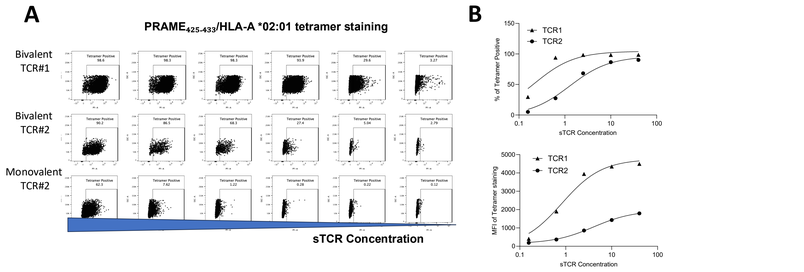


**Figure 5:** A unique cell based TCR screening platform is built to screen soluble TCRs for antigen specificity and binding affinity. **A.** By engineering the binding capability between the screening cell lines and sTCR, we linked the sTCR to the cell line. Then we stained the cell-bound TCRs with peptide-specific tetramers. The bivalent TCR1, TCR2, and monovalent scTCR2 were measured for binding to the tetramer by flow cytometry. Tetramer staining demonstrated TCR1 had a higher binding to the tetramer than TCR2; and bivalent TCR2 had a higher binding to the tetramer than monovalent scTCR2. **B.** The % of tetramer positive cells (Top) and the MFI of tetramer staining of the cells were shown at different concentrations of soluble TCRs. It demonstrated TCR1 had a stronger binding to the tetramer than TCR 2, which was confirmed by SPR measurement of TCR affinity.

## *Engineering of* native TCR-CD3 TCEs and *in vitro* function

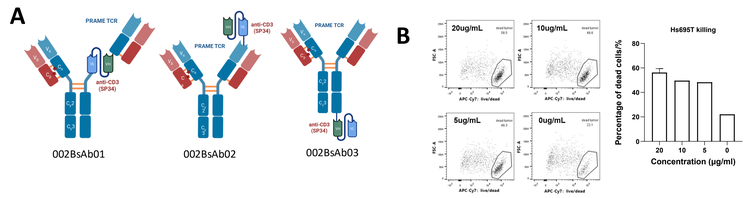


**Figure 6:** Engineering of native TCR-CD3 T cell engager (TCE) and *in vitro* function analysis. **A.** Schematic representation of PRAME$_{425-433}$/HLA-A *02:01-specific TCR-CD3 bispecific formats with distinct molecular architectures. The anti-CD3 fragment was derived from the SP34 clone of anti-CD3 through in-house humanization and was further engineered into an scFv format. Three formats of CD3-TCR TCE were designed to evaluate the impact of CD3 engagement geometry and valency on T cell activation and redirection. **(Left)** An asymmetric heterodimeric Fc-based format, comprising two PRAME-specific TCR arm and one anti-CD3 scFv fused between one PRAME-specific TCR arm and the Fc, enabling monovalent CD3 engagement. **(Middle)** A Fc-based format displaying two PRAME TCR arms with one anti-CD3 scFv fused to the N terminal of one TCR arm, allowing bivalent TCR binding and independent CD3 recruitment. **(Right)** A bivalent TCR-Fc fusion in which two PRAME TCRs are fused to the Fc backbone, with one anti-CD3 scFv appended at the C-terminus, creating a spatially distal CD3-binding configuration. **B.** Purified CD3 T cells were co-cultured with PRAME-expressing Hs695T tumor cells in the presence of various concentration of 002BsAb03. Tumor cells were labeled for cell tracking dye CTV. Live versus dead cells were quantified by flow cytometry after 3-day co-culture. The 002BsAb03 led to efficient killing of tumor cells through activated T cells. Our data demonstrate, through TCR affinity screening and molecule engineering, native TCR-CD3 TCEs have the potential for TCE therapy without TCR affinity enhancement. The use of native TCR with high affinity can reduce the cross-reactivity and off-target killing.

## $^{125}$I-labeled Soluble TCR Exhibited the Accumulation in Tumor in Hs695T Xenograft Model

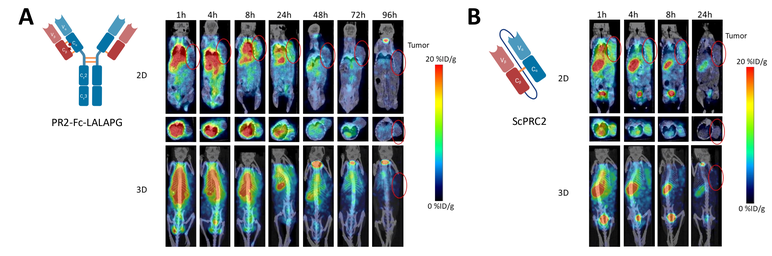


**Figure 7:** $^{125}$I-labeled native PRAME$_{425-433}$-specific TCR protein exhibited tumor accumulation in Hs695T xenograft model. **A.** Bivalent PR2-Fc-LALAPG protein was radiolabeled with iodine-125 using the Iodogen method and purified by ultrafiltration to achieve a radiochemical purity ≥95% prior to *in vivo* studies. Tumor-bearing mice (Hs695T xenografts) received a single intravenous administration of $^{125}$I-PR2-Fc-LALAPG (~200 μCi per mouse). Whole-body SPECT/CT imaging was performed at multiple time points post-injection (1, 4, 8, 24, 48, 72, and 96 hours). Following intravenous administration, $^{125}$I-PR2-Fc-LALAPG showed predominant uptake in the circulation and highly perfused organs (heart, liver, kidney) at early time points, with rapid systemic clearance over time. Tumor uptake increased gradually, peaking at ~ 24 hours post-injection, followed by a slow decline. **B.** Monovalent scPRC2,, a single-chain TCR protein, exhibited the highest tumor accumulation at ~ 4 hours post-injection, followed by a faster decline than bivalent PR2-Fc-LALAPG protein .

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