**Supplementary Information for:**

**Identification of the targets of T cell receptor therapeutic agents and cells by use of a high throughput genetic platform**

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**Data Availability**

The minigene counts and enrichment scores for each ESK1 and Pr20 library screen are available in the supplementary materials. The PresentER library sequencing reads are available at the following DOIs:

|  |  |
| --- | --- |
| **Description** | **DOI** |
| Minigene sequencing of sorted T2 cells expressing a library of HLA-A\*02:01 exomic peptides. Cells are sorted for high and low binding of the TCR mimic antibody ESK1. | DOI:10.5281/zenodo.1313110 |
| Minigene sequencing of sorted T2 cells expressing a library of HLA-A\*02:01 exomic peptides. Cells are sorted for high and low binding of the TCR mimic antibody Pr20. | DOI:10.5281/zenodo.1326544 |
| Minigene sequencing of T2 cells expressing a library of off targets (derived from A6 and B7 binding motifs in Hausmann 1999) after co-culture with A6, DMF5 or 1G4 expressing T cells. | DOI:10.5281/zenodo.1341943 |
| Minigene sequencing of T2 cells expressing a library of off targets (derived from A6 and B7 binding motifs in Hausmann 1999) are co-cultured with A6, B7, DMF5 or 1G4 expressing T cells. | DOI:10.5281/zenodo.1342624 |
| Minigene sequencing of T2 cells expressing a library of off targets (derived from A6 and B7 binding motifs in Hausmann 1999) are co-cultured with A6, B7 or 1G4 expressing T cells. | DOI:10.5281/zenodo.3554488 |
| Minigene sequencing of RMA/S cells expressing a library of wild-type mouse H-2Kb peptides (plus controls) after co-culture with activated B6 or OT-1 splenocytes. | DOI:10.5281/zenodo.1419780 |

**Supplemental Table S1: DNA and Protein sequences**

|  |  |  |  |
| --- | --- | --- | --- |
| **Name** | **Description** | **DNA (5’🡪3’)** | **Protein** |
| ENV\_MMTVC | MMTV ENV signal sequence (wild type) | ATGCCTAATCATCAGTCCGGGTCACCTACCGGCAGTTCAGACCTGCTCCTTGATGGCAAGAAACAACGAGCCCATCTGGCGCTGAGGAGAAAACGGCGACGGGAAATGCGCAAGATTAACCGAAAGGTGAGAAGAATGAATCTCGCACCCATTAAAGAAAAAACAGCCTGGCAGCACCTGCAAGCTCTGATCTTCGAGGCGGAAGAAGTGTTGAAGACTTCTCAAACTCCACAGACCTCCCTCACGCTGTTTCTGGCACTCTTGTCTGTACTCGGGCCCCCACCTGTGAGCGGG | MPNHQSGSPTGSSDLLLDGKKQRAHLALRRKRRREMRKINRKVRRMNLAPIKEKTAWQHLQALIFEAEEVLKTSQTPQTSLTLFLALLSVLGPPPVSG |
| PresentER signal sequence | PresentER signal sequence based on ENV\_MMTVC with SfiI sites FLALL[S>A]VL[G>A]PPP | ATGCCTAATCATCAGTCCGGGTCACCTACCGGCAGTTCAGACCTGCTCCTTGATGGCAAGAAACAACGAGCCCATCTGGCGCTGAGGAGAAAACGGCGACGGGAAATGCGCAAGATTAACCGAAAGGTGAGAAGAATGAATCTCGCACCCATTAAAGAAAAAACAGCCTGGCAGCACCTGCAAGCTCTGATCTTCGAGGCGGAAGAAGTGTTGAAGACTTCTCAAACTCCACAGACCTCCCTCACGCTGTTTCTAGCACTCTTGGCCGTATTGGCCCCGCCACCTGTGAGCGGG | MPNHQSGSPTGSSDLLLDGKKQRAHLALRRKRRREMRKINRKVRRMNLAPIKEKTAWQHLQALIFEAEEVLKTSQTPQTSLTLFLALLAVLAPPPVSG |
| PresentER Signal sequence scramble #1 |  | **ATGGAAGGGGCCAGAAGCCAACTGTTGCTGAATCACATCTTGACTCCTATGATTCTTAGACTGCGAGCACAACTAATTAGGGCGTGGCGCCCACATCCTAACCAGGATACTTCCACGAAAGGGAAACTGGTGCAGCTCTTTAAGGCCACCAAGATGCCAAAACTCCATCGAAAGAAGGAGAGACCTTCATTGGAAAGTGCGGGCTCTTCACAAGTGACACCGGTAGCCGCTTCCACCCTCCTCCGACTGGTGAAACGGCCCGAGTTCAATGGCCGGGCAGCCGAACAGGACCTG** | **MEGARSQLLLNHILTPMILRLRAQLIRAWRPHPNQDTSTKGKLVQLFKATKMPKLHRKKERPSLESAGSSQVTPVAASTLLRLVKRPEFNGRAAEQDL** |
| PresentER Signal sequence scramble #2 |  | **ATGCAGCTCTTGACTCCTACTAAGACAAAAAACGAATCAGCCATGGCTGCCGCGAAGGTGTCAGCAAAGTCTCGACTCTTGGCAAGAAGACTGACCGTACTCAAGTTCCGGCCAAGCCTGGACCTGGAACATAGTGTGGCCATTCGCCCTTCCAAACAACGATCCCAAATGCAGACGCGGCTTGATCTGGGCAATCCTGGCCAAGTGCAGAATCCACTAGGGGAAGGGATCATTTTTTGGCACGAAGCGCGAAAATTGCTCAAAAGACCCCATACCCTGCTGGCCAGGGAGCCG** | **MQLLTPTKTKNESAMAAAKVSAKSRLLARRLTVLKFRPSLDLEHSVAIRPSKQRSQMQTRLDLGNPGQVQNPLGEGIIFWHEARKLLKRPHTLLAREP** |
| PresentER antigen oligo | The region encoding the antigen is denoted by Xs. Th construct is amplified with PresentER-F and PresentER-R before digestion. | GGCCGTATTGGCCCCGCCACCTGTGAGCGGGXXX…XXXTAAGGCCAAACAGGCC |  |
| PresentER-F | Forward primer for amplifying antigen oligo | CGACTCACTATAGGGCCGTATTGGCC |  |
| PresentER-R | Reverse primer for amplifying antigen oligo | AGTGATTTCCGGCCTGTTTGGCC |  |
| P5 Primer | Forward primer for amplifying minigene for Illumina sequencing  -or-  Outer genomic DNA PCR Primer | AATGATACGGCGACCACCGAGATCT |  |
| P7Barcode Primer | Reverse, barcoded primer for amplifying minigene for Illumina sequencing. XXXXXX denotes the Illumina primer. | CAAGCAGAAGACGGCATACGAGATXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC |  |
| MMTV\_SP2\_R Primer | Outer genomic DNA PCR Primer | GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC |  |
| PresentER\_Short\_F Primer | Inner genomic DNA PCR Primer | GCCACCTGTGAGCGGG |  |
| PresentER\_Short\_R Primer | Inner genomic DNA PCR Primer | TCTTTGGCCTGTTTGGCCTTA |  |

**Supplemental Table S2: PresentER Constructs**

|  |  |  |
| --- | --- | --- |
| **Species / Protein / Position** | **DNA encoding peptide** | **Peptide** |
| Human WT1 126-134 | AGGATGTTTCCTAACGCGCCCTACCTG | RMFPNAPYL |
| Human PRAME 300-309 | GCTCTCTATGTGGACTCTTTATTTTTCCTT | ALYVDSLFFL |
| CMV pp65 495-503 | AACCTGGTGCCCATGGTGGCCACCGTG | NLVPMVATV |
| Human EW | CAGCTGCAGAACCCCAGCTACGACAAG | QLQNPSYDK |
| Influenza M peptide 58-66 | GGCATCCTGGGCTTCGTGTTCACCCTG | GILGFVFTL |
| Human WT1 239-247 | AACCAGATGAACCTGGGCGCCACCCTG | NQMNLGATL |
| Human MART-1 27-35 | GCGGCCGGAATAGGCATATTGACTGTA | AAGIGILTV |
| Human NY-ESO-1 157-165 | TCTCTGCTGATGTGGATCACTCAATGT | SLLMWITQC |
| HTLV-1 Tax 11-19 | CTGCTTTTTGGTTACCCTGTTTATGTT | LLFGYPVYV |
| Human ELAV-like protein 4 (HuD) 87–95 | CTTGGATATGGGTTTGTCAATTATATA | LGYGFVNYI |
| S. cerevisiae Tel1p 549-557 | ATGCTTTGGGGCTATCTGCAATACGTT | MLWGYLQYV |
| Human ADCY4 789-798 | AAACTCATGGGAGCAATCTCATTTTTTATA | KLMGAISFFI |
| Human EFNA1 186-194 | CGACTCTTTCCTCTCGCATGGACTGTC | RLFPLAWTV |
| Human SH3TC1 205-214 | CTCCTGATCCAAGAAGGGCCTTTCTTTGTT | LLIQEGPFFV |
| Human HARBI1 221-230 | TGGCTCCTCGGTGACTCTTCATTCTTCCTT | WLLGDSSFFL |
| Chicken ovalbumin 257-264 | AGCATCATCAACTTCGAGAAGCTG | SIINFEKL |
| Mouse PEDF 271-279 | ATGAGCATCATCTTCTTCCTGCCCCTG | MSIIFFLPL |

**Supplemental Table S3: A6 and B7 TCR sequences**

|  |  |  |
| --- | --- | --- |
| **Name** | **DNA (5’🡪3’)** | **Protein** |
| A6 TCR coding sequence for mammalian expression (with alpha and beta chain separated by P2A site) | ATGAAGTCTTTGCGCGTACTCTTGGTGATATTGTGGCTCCAATTGAGTTGGGTGTGGTCCCAGCAGAAGGAAGTGGAGCAGAACTCTGGACCCCTCAGTGTTCCAGAGGGAGCCATTGCCTCTCTCAACTGCACTTACAGTGACCGAGGTTCCCAGTCCTTCTTCTGGTACAGACAATATTCTGGGAAAAGCCCTGAGTTGATAATGTCCATATACTCCAATGGTGACAAAGAAGATGGAAGGTTTACAGCACAGCTCAATAAAGCCAGCCAGTATGTTTCTCTGCTCATCAGAGACTCCCAGCCCAGTGATTCAGCCACCTACCTCTGTGCCGTTACAACTGACAGCTGGGGGAAATTGCAGTTTGGAGCAGGGACCCAGGTTGTGGTCACCCCAGATATCCAGAACCCGGATCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAATGTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCTTCCCCAGCCCAGAAAGTTCCTGTGATGTCAAGCTGGTCGAGAAAAGCTTTGAAACAGATACGAACCTAAACTTTCAAAACCTGTCAGTGATTGGGTTCCGAATCCTCCTCCTGAAAGTGGCCGGGTTTAATCTGCTCATGACGCTGCGGCTGTGGTCCAGCCGGGCCAAGCGGTCCGGATCCGGAGCCACCAACTTCAGCCTGCTGAAGCAGGCCGGCGACGTGGAGGAGAACCCCGGCCCCATGAGCATCGGCCTCCTGTGCTGTGCAGCCTTGTCTCTCCTGTGGGCAGGTCCAGTGAATGCTGGTGTCACTCAGACCCCAAAATTCCAGGTCCTGAAGACAGGACAGAGCATGACACTGCAGTGTGCCCAGGATATGAACCATGAATACATGTCCTGGTATCGACAAGACCCAGGCATGGGGCTGAGGCTGATTCATTACTCAGTTGGTGCTGGTATCACTGACCAAGGAGAAGTCCCCAATGGCTACAATGTCTCCAGATCAACCACAGAGGATTTCCCGCTCAGGCTGCTGTCGGCTGCTCCCTCCCAGACATCTGTGTACTTCTGTGCCAGCCGGCCAGGTCTTGCCGGGGGACGACCAGAGCAGTATTTCGGGCCAGGGACGCGCCTTACGGTAACAGAAGACTTGAAGAATGTCTTTCCACCTGAGGTCGCCGTTTTTGAACCCTCCGAGGCCGAAATAAGTCATACTCAAAAGGCGACTCTGGTGTGCCTCGCCACCGGGTTTTACCCGGACCACGTAGAACTTAGCTGGTGGGTGAATGGTAAAGAGGTCCATAGCGGGGTGTGCACGGACCCACAGCCTCTCAAGGAACAACCCGCTCTGAATGATTCCAGGTATTGTCTTAGCTCACGGCTTCGAGTGTCAGCTACTTTTTGGCAAGATCCCCGCAACCACTTCCGCTGTCAAGTCCAGTTCTACGGGCTCTCGGAGAATGACGAGTGGACCCAGGATAGGGCCAAACCCGTCACCCAGATCGTCAGCGCCGAGGCCTGGGGTAGAGCAGACTGTGGCTTCACCTCCGAGTCTTACCAGCAAGGGGTCCTGTCTGCCACCATCCTCTATGAGATCTTGCTAGGGAAGGCCACCTTGTATGCCGTGCTGGTCAGTGCCCTCGTGCTGATGGCTATGGTCAAGAGAAAGGATTCCAGAGGCTAG | MKSLRVLLVILWLQLSWVWSQQKEVEQNSGPLSVPEGAIASLNCTYSDRGSQSFFWYRQYSGKSPELIMSIYSNGDKEDGRFTAQLNKASQYVSLLIRDSQPSDSATYLCAVTTDSWGKLQFGAGTQVVVTPDIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTNVSQSKDSDVYITDKCVLDMRSMDFKSNSAVAWSNKSDFACANAFNNSIIPEDTFFPSPESSCDVKLVEKSFETDTNLNFQNLSVIGFRILLLKVAGFNLLMTLRLWSSRAKRSGSGATNFSLLKQAGDVEENPGPMSIGLLCCAALSLLWAGPVNAGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVPNGYNVSRSTTEDFPLRLLSAAPSQTSVYFCASRPGLAGGRPEQYFGPGTRLTVTEDLKNVFPPEVAVFEPSEAEISHTQKATLVCLATGFYPDHVELSWWVNGKEVHSGVCTDPQPLKEQPALNDSRYCLSSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRADCGFTSESYQQGVLSATILYEILLGKATLYAVLVSALVLMAMVKRKDSRG |
| A6 TCR alpha chain coding sequence for bacterial expression | ATGCAGAAGGAAGTGGAGCAGAACTCTGGACCCCTCAGTGTTCCAGAGGGAGCCATTGCCTCTCTCAACTGCACTTACAGTGACCGAGGTTCCCAGTCCTTCTTCTGGTACAGACAATATTCTGGGAAAAGCCCTGAGTTGATAATGTCCATATACTCCAATGGTGACAAAGAAGATGGAAGGTTTACAGCACAGCTCAATAAAGCCAGCCAGTATGTTTCTCTGCTCATCAGAGACTCCCAGCCCAGTGATTCAGCCACCTACCTCTGTGCCGTTACAACTGACAGCTGGGGGAAATTGCAGTTTGGAGCAGGGACCCAGGTTGTGGTCACCCCAGATATCCAGAACCCGGATCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAATGTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCTTCCCCAGCCCAGAAAGTTCCTAA | MQKEVEQNSGPLSVPEGAIASLNCTYSDRGSQSFFWYRQYSGKSPELIMSIYSNGDKEDGRFTAQLNKASQYVSLLIRDSQPSDSATYLCAVTTDSWGKLQFGAGTQVVVTPDIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTNVSQSKDSDVYITDKCVLDMRSMDFKSNSAVAWSNKSDFACANAFNNSIIPEDTFFPSPESS |
| A6 TCR beta chain coding sequence for bacterial expression | ATGAACGCTGGTGTCACTCAGACCCCAAAATTCCAGGTCCTGAAGACAGGACAGAGCATGACACTGCAGTGTGCCCAGGATATGAACCATGAATACATGTCCTGGTATCGACAAGACCCAGGCATGGGGCTGAGGCTGATTCATTACTCAGTTGGTGCTGGTATCACTGACCAAGGAGAAGTCCCCAATGGCTACAATGTCTCCAGATCAACCACAGAGGATTTCCCGCTCAGGCTGCTGTCGGCTGCTCCCTCCCAGACATCTGTGTACTTCTGTGCCAGCAGGCCGGGACTAGCGGGAGGGCGACCAGAGCAGTACTTCGGGCCGGGCACCAGGCTCACGGTCACAGAGGACCTGAAAAACGTGTTCCCACCCGAGGTCGCTGTGTTTGAGCCATCAGAAGCAGAGATCTCCCACACCCAAAAGGCCACACTGGTGTGCCTGGCCACCGGTTTCTACCCCGACCACGTGGAGCTGAGCTGGTGGGTGAATGGGAAGGAGGTGCACAGTGGGGTCTGCACAGACCCGCAGCCCCTCAAGGAGCAGCCCGCCCTCAATGACTCCAGATACGCTCTGAGCAGCCGCCTGAGGGTCTCGGCCACCTTCTGGCAGGACCCCCGCAACCACTTCCGCTGTCAAGTCCAGTTCTACGGGCTCTCGGAGAATGACGAGTGGACCCAGGATAGGGCCAAACCCGTCACCCAGATCGTCAGCGCCGAGGCCTGGGGTAGAGCAGACTAA | MNAGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVPNGYNVSRSTTEDFPLRLLSAAPSQTSVYFCASRPGLAGGRPEQYFGPGTRLTVTEDLKNVFPPEVAVFEPSEAEISHTQKATLVCLATGFYPDHVELSWWVNGKEVHSGVCTDPQPLKEQPALNDSRYALSSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRAD |
| A6 TCR beta chain coding sequence for bacterial expression with C-terminal BirA biotinylation site | ATGAACGCTGGTGTCACTCAGACCCCAAAATTCCAGGTCCTGAAGACAGGACAGAGCATGACACTGCAGTGTGCCCAGGATATGAACCATGAATACATGTCCTGGTATCGACAAGACCCAGGCATGGGGCTGAGGCTGATTCATTACTCAGTTGGTGCTGGTATCACTGACCAAGGAGAAGTCCCCAATGGCTACAATGTCTCCAGATCAACCACAGAGGATTTCCCGCTCAGGCTGCTGTCGGCTGCTCCCTCCCAGACATCTGTGTACTTCTGTGCCAGCAGGCCGGGACTAGCGGGAGGGCGACCAGAGCAGTACTTCGGGCCGGGCACCAGGCTCACGGTCACAGAGGACCTGAAAAACGTGTTCCCACCCGAGGTCGCTGTGTTTGAGCCATCAGAAGCAGAGATCTCCCACACCCAAAAGGCCACACTGGTGTGCCTGGCCACCGGTTTCTACCCCGACCACGTGGAGCTGAGCTGGTGGGTGAATGGGAAGGAGGTGCACAGTGGGGTCTGCACAGACCCGCAGCCCCTCAAGGAGCAGCCCGCCCTCAATGACTCCAGATACGCTCTGAGCAGCCGCCTGAGGGTCTCGGCCACCTTCTGGCAGGACCCCCGCAACCACTTCCGCTGTCAAGTCCAGTTCTACGGGCTCTCGGAGAATGACGAGTGGACCCAGGATAGGGCCAAACCCGTCACCCAGATCGTCAGCGCCGAGGCCTGGGGTAGAGCAGACGGCCTGAACGATATTTTTGAAGCGCAGAAAATTGAATGGCATGAATAA | MNAGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVPNGYNVSRSTTEDFPLRLLSAAPSQTSVYFCASRPGLAGGRPEQYFGPGTRLTVTEDLKNVFPPEVAVFEPSEAEISHTQKATLVCLATGFYPDHVELSWWVNGKEVHSGVCTDPQPLKEQPALNDSRYALSSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRADGLNDIFEAQKIEWHE |
| B7 TCR coding sequence for mammalian expression (with alpha and beta chain separated by P2A site) | ATGAAGTCTTTGCGCGTACTCTTGGTGATATTGTGGCTCCAATTGAGTTGGGTGTGGTCCCAGCAACAGAAGAATGATGACCAGCAAGTTAAGCAAAATTCACCATCCCTGAGCGTCCAGGAAGGAAGAATTTCTATTCTGAACTGTGACTATACTAACAGCATGTTTGATTATTTCCTATGGTACAAAAAATACCCTGCTGAAGGTCCTACATTCCTGATATCTATAAGTTCCATTAAGGATAAAAATGAAGATGGAAGATTCACTGTCTTCTTAAACAAAAGTGCCAAGCACCTCTCTCTGCACATTGTGCCCTCCCAGCCTGGAGACTCTGCAGTGTACTTCTGTGCAGCAATGGAGGGAGCCCAGAAGCTGGTATTTGGCCAAGGAACCAGGCTGACTATCAACCCAAATATCCAGAATCCGGACCCGGCCGTTTATCAGCTGCGTGATTCAAAATCTTCGGATAAATCCGTGTGTCTGTTCACGGATTTTGATAGCCAGACCAATGTGTCCCAGTCAAAAGATAGTGATGTGTATATTACCGATAAATGCGTTCTGGACATGCGCAGTATGGATTTCAAAAGCAATTCGGCCGTGGCGTGGTCAAATAAATCGGATTTCGCATGTGCGAACGCGTTTAATAACAGCATCATCCCGGAAGATACGTTCTTTCCGAGCCCGGAAAGCTCTTGTGATGTCAAGCTGGTCGAGAAAAGCTTTGAAACAGATACGAACCTAAACTTTCAAAACCTGTCAGTGATTGGGTTCCGAATCCTCCTCCTGAAAGTGGCCGGGTTTAATCTGCTCATGACGCTGCGGCTGTGGTCCAGCCGGGCCAAGCGGTCCGGATCCGGAGCCACCAACTTCAGCCTGCTGAAGCAGGCCGGCGACGTGGAGGAGAACCCCGGCCCCATGAGCATCGGCCTCCTGTGCTGTGCAGCCTTGTCTCTCCTGTGGGCAGGTCCAGTGAATGCTGGTGTCACTCAGACCCCAAAATTCCAGGTCCTGAAGACAGGACAGAGCATGACACTGCAGTGTGCCCAGGATATGAACCATGAATACATGTCCTGGTATCGACAAGACCCAGGCATGGGGCTGAGGCTGATTCATTACTCAGTTGGTGCTGGTATCACTGACCAAGGAGAAGTCCCCAATGGCTACAATGTCTCCAGATCAACCACAGAGGATTTCCCGCTCAGGCTGCTGTCGGCTGCTCCCTCCCAGACATCTGTGTACTTCTGTGCCAGCAGTTACCCTGGAGGAGGCTTTTATGAGCAGTATTTCGGTCCTGGAACAAGGCTGACCGTGACGGAAGATTTGAAAAATGTCTTTCCCCCAGAGGTAGCAGTCTTCGAGCCGTCCGAGGCCGAGATATCCCATACCCAGAAGGCAACCCTTGTTTGCTTGGCAACGGGATTTTATCCAGATCATGTGGAATTGTCCTGGTGGGTCAACGGCAAAGAGGTTCACAGCGGCGTCTGCACAGATCCGCAACCACTCAAGGAACAGCCCGCTCTTAATGATTCTCGCTACTGTCTGAGTTCCAGGTTGCGGGTCAGCGCTACTTTCTGGCAGGATCCCCGCAACCACTTCCGCTGTCAAGTCCAGTTCTACGGGCTCTCGGAGAATGACGAGTGGACCCAGGATAGGGCCAAACCCGTCACCCAGATCGTCAGCGCCGAGGCCTGGGGTAGAGCAGACTGTGGCTTCACCTCCGAGTCTTACCAGCAAGGGGTCCTGTCTGCCACCATCCTCTATGAGATCTTGCTAGGGAAGGCCACCTTGTATGCCGTGCTGGTCAGTGCCCTCGTGCTGATGGCTATGGTCAAGAGAAAGGATTCCAGAGGCTAG | MKSLRVLLVILWLQLSWVWSQQQKNDDQQVKQNSPSLSVQEGRISILNCDYTNSMFDYFLWYKKYPAEGPTFLISISSIKDKNEDGRFTVFLNKSAKHLSLHIVPSQPGDSAVYFCAAMEGAQKLVFGQGTRLTINPNIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTNVSQSKDSDVYITDKCVLDMRSMDFKSNSAVAWSNKSDFACANAFNNSIIPEDTFFPSPESSCDVKLVEKSFETDTNLNFQNLSVIGFRILLLKVAGFNLLMTLRLWSSRAKRSGSGATNFSLLKQAGDVEENPGPMSIGLLCCAALSLLWAGPVNAGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVPNGYNVSRSTTEDFPLRLLSAAPSQTSVYFCASSYPGGGFYEQYFGPGTRLTVTEDLKNVFPPEVAVFEPSEAEISHTQKATLVCLATGFYPDHVELSWWVNGKEVHSGVCTDPQPLKEQPALNDSRYCLSSRLRVSATFWQDPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRADCGFTSESYQQGVLSATILYEILLGKATLYAVLVSALVLMAMVKRKDSRG |

**Supplemental Table S4: Protein sequences**

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| --- | --- | --- |
|  | **Alpha Variable Chain** | **Beta Variable Chain** |
| **1G4 TCR** | METLLGLLILWLQLQWVSSKQEVTQIPAALSVPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLIQSSQREQTSGRLNASLDKSSGRSTLYIAASQPGDSATYLCAVRPLYGGSYIPTFGRGTSLIVHP | MSIGLLCCAALSLLWAGPVNAGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQGEVPNGYNVSRSTTEDFPLRLLSAAPSQTSVYFCASSYVGNTGELFFGEGSRLTVL |
| **DMF5 TCR** | MMKSLRVLLVILWLQLSWVWSQQKEVEQNSGPLSVPEGAIASLNCTYSDRGSQSFFWYRQYSGKSPELIMFIYSNGDKEDGRFTAQLNKASQYVSLLIRDSQPSDSATYLCAVNFGG | MRIRLLCCVAFSLLWAGPVIAGITQAPTSQILAAGRRMTLRCTQDMRHNAMYWYRQDLGLGLRLIHYSNTAGTTGKGEVPDGYSVSRANTDDFPLTLASAVPSQTSVYFCASSLSFGTEAFFGQGTRLTVV |

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**Supplementary Figure 1:** Overview of the PresentER Method. **(A)** The PresentER method is based on an MSCV retroviral vector. The peptide antigen minigene is driven by the MSCV LTR and encodes an endoplasmic reticulum (ER) targeting sequencing followed by the precise peptide to be expressed. The vector contains a puromycin resistance gene and GFP driven by PGK. The endoplasmic reticulum sequence is the leader sequence from MMTV gp70 protein. A removable cassette is delimited by SfiI restriction sites. **(B)** A 75-78 oligonucleotide sequence (depending on the length of the peptide) encoding the antigen is amplified by PCR, digested with SfiI enzyme and ligated into the backbone. **(C)** A schematic of the cloned PresentER minigene construct is shown.

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**Supplementary Figure 2:** TCR and TCRm bind specifically to PresentER expressing cells.Representative contour plots and quantification of the TCR mimic antibodies ESK1 **(A-B)** and Pr20 **(C-D)** binding to T2 cells expressing their PresentER targets of irrelevant controls. Representative contour plots and quantification of two TCR multimers, one specific to HTLV-1 Tax peptide (**E,F**) and one specific to CMV pp65 peptide (**G,H**) binding specifically to T2 cells expressing their target PresentER minigenes or irrelevant minigenes.



**Supplementary Figure 3:** PresentER minigenes display peptides in TAP competent EL4 cells Representative histogram **(A)** and quantification **(B)** of the binding of the SIINFEKL/H-2Kb specific antibody (25-D1.16) to EL4 cells expressing PresentER-SIINFEKL or a control H-2Kb peptide MSIIFFLPL. All experiments performed in triplicate. Error bars indicate SEM.



**Supplementary Figure 4:** Mass spectrometry of PresentER peptide presentation and importance of signal peptide. **(A-B)** MHC-I ligands were isolated from wild-type T2 cells or T2 cells expressing PresentER-ALY or PresentER-RMF. Peptide sequences were identified by mass spectrometry and the intensity of ions corresponding to the ALY (**A**) or RMF (**B**) peptides in each sample are plotted. (**C**) ESK1 and Pr20 binding to T2 cells expressing PresentER-RMF and PresentER-ALY is compared with binding to T2 cells expressing RMF and ALY downstream of scrambled signal sequences. All experiments performed in triplicate. Error bars indicate SEM.



**Supplementary Figure 5:** Only a single copy of the PresentER minigene is required for antigen presentation. RMA/S cells were transduced with serially diluted PresentER-SIINFEKL or PresentER-MSIIFFLPL virus in biological replicates. The percent of GFP positive, SIINFEKL/H-2Kb positive cells after transduction is plotted as a function of fold dilution of viral supernatant **(A)** and percent of cells infected **(B)**. Low infection efficiency does not lead to loss H2-Kb SIINFEKL binding. All experiments performed in quadruplicate. Error bars indicate SEM.



**Supplementary Figure 6: (A)** ELISpot of genetically engineered T cells expressing the A6 (target: HTLV-1 Tax 11-19 LLFGYPVYV), DMF5 (target: MART-1 27-35 AAGIGILTV) or 1G4 (target: NY-ESO-1 157-165 SLLMWITQC) TCRs challenged with peptide-pulsed T2 cells or T2 cells expressing PresentER minigenes. **(B)** Results of *in vitro* co-culture killing assays where A6 or B7 (reactive with Tax peptide) expressing T cells were incubated with a mixture of T2 cells expressing PresentER-Tel1p (GFP)/PresentER MART-1 (mCherry), PresentER ELAVL4 (GFP)/PresentER MART-1 (mCherry) or without any T cells. The change in abundance of the T2 target cells are plotted relative to their abundance in the “No T cells” sample. All experiments performed in triplicate. Error bars indicate SEM.

A close up of text on a white background

Description automatically generated

**Supplementary Figure 7:** Enrichment of ESK1 and Pr20 control peptides in library screens. **(A)** The flow screen enrichment scores of control peptides known to be ESK1 binders or non-binders. **(B)** The flow screen enrichment scores of control peptides known to be Pr20 binders or non-binders.

A close up of a map

Description automatically generated

**Supplementary Figure 8:** Enrichment of Pr20 binding among library peptides.Each point is a unique peptide minigene with the x-axis indicating enrichment for Pr20 binding (with one set as no enrichment; average of 2 replicates) and y-axis indicating the peptide’s predicted ic50 (in nM) to HLA-A\*02:01. Lower ic50 indicates higher affinity. Marked control peptides and known Pr20 peptide targets are plotted as triangles; CR-ESK1 peptides, as circles and CR-Pr20 peptides, as squares. Peptides that validated as Pr20 binders by peptide pulsing are displayed in dark red. Peptides that did not validate by peptide pulsing are in dark blue.

**Supplementary References**

1. Borbulevych, O. Y. *et al.* T cell receptor cross-reactivity directed by antigen-dependent tuning of peptide-MHC molecular flexibility. *Immunity* **31,** 885–896 (2009).

2. Davis-Harrison, R. L., Armstrong, K. M. & Baker, B. M. Two different T cell receptors use different thermodynamic strategies to recognize the same peptide/MHC ligand. *J. Mol. Biol.* **346,** 533–550 (2005).

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