**Supplementary Table S1. Different methods of fluorescence data analysis using OriginPro 2017.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **HUGO** | **Sequence** | **NetMHC 4.0** | **Tm (oC) Sigmoidal Mid** | **Tm (oC) Sigmoidal Derivative** | **Tm (oC) Bi-Gaussian** |
| MART-1 10mer | ELAGIGILTV | 254 | 58.9 ± 0.3 | 58.9 ± 0.3 | 59.3 ± 0.5 |
| MART-1 9mer | AAGIGILTV | 2254 | 42.3 ± 0.7 | 42.3 ± 0.7 | 43.7 ± 0.8 |
| Tyrosinase | YMDGTMSQV | 7 | 65.8 ± 1.8 | 65.8 ± 1.8 | 65.5 ± 0.2 |
| WT-1\* | RMFPNAPYL | 7 | 56.2 ± 0.2 | 56.2 ± 0.2 | 56.9 ± 0.2 |
| WT-1-M2A | RAFPNAPYL | 563 | 46.9 ± 0.3 | 46.9 ± 0.3 | 48.0 ± 0.2 |
| SMARCD3 | KLFEFLVYGV | 3 | 60.7 ± 1.9 | 60.7 ± 1.9 | 61.4 ± 1.0 |
| CDK4 | ALDPHSGHFV | 57 | 64.9 ± 1.4 | 64.9 ± 1.3 | 64.1 ± 0.4 |
| GCN1L1 | ALLETPSLLL | 54 | 61.2 ± 0.5 | 61.2 ± 0.5 | 61.4 ± 0.3 |

 \*Refolded complex

Triplicate data points, from one or three experiments, for each pep/HLA-A2 complex were analyzed using OriginPro 2017 software. The sigmoidal denaturation curve was fit non-linearly to a Boltzmann Equation and the Tm was obtained by finding the midpoint of the unfolding transition, or the peak of the first derivative curve. Alternatively, the first derivative of the curve obtained from bi-Gaussian fitting was used to calculate the precise midpoint (Tm).

**Supplementary Table S2. Tm analysis of WT-1/HLA-A2 complexes prepared by protein refolding or UV-peptide exchange.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Complex** | **Experiment 1** | **Experiment 2** | **Ave Tm** | **SD** |
| UV-Exchanged WT-1/A2 | 58.1 | 57.9 | 57.6 | 57.7 | 57.1 | 58.1 | 57.8 | 0.4 |
| Refolded WT-1/A2 | 56.6 | 57.1 | 57.2 | 56.8 | 57.1 | 56.7 | 56.9 | 0.2 |
| Refolded UV/A2 | 53.4 | 53.4 | 53.6 | 53.3 | 53.2 | 53.5 | 53.5 | 0.3 |

UV peptide/HLA-A2 and WT-1/HLA-A2 complexes were refolded and purified from heavy chain and 2m inclusion bodies expressed in *E. coli*. A sample from the UV preparation was then added, with excess WT-1 peptide, to a UV-mediated ligand exchange reaction (UV-exchanged WT-1/HLA-A2). To analyze each of the complexes, two separate DSF experiments were performed, with three replicates each, to obtain average Tm values and standard deviations (SD).

**Supplementary Table S3. Well-Studied Self-Peptides, Single Peptide Variants, and Viral Antigens.**

|  |  |  |  |
| --- | --- | --- | --- |
| **HUGO** | **Sequence** | **NetMHC 4.0 (nM)** | **Tm (oC)** |
| Tyrosinase | YMDGTMSQV | 7 | 65.5 ± 0.2 |
| NYESO-1 | SLLMWITNC | 458 | 53.3 ± 0.1 |
| NYESO-1 C9V | SLLMWITNV | 5 | 56.2 ± 0.6 |
| HER2 | KIFGSLAFL | 14 | 53.5 ± 0.4 |
| HER2 | RLLQETELV | 38 | 55.1 ± 0.1 |
| Survivin T2M | LMLGEFLKL | 25 | 54.2 ± 0.1 |
| PAP | TLMSAMTNL | 9 | 57.9 ± 0.4 |
| Glypican-3 | FVGEFFTDV | 35 | 51.0 ± 0.5 |
| TAX | LLFGYPVYV | 3 | 61.9 ± 0.2 |
| MART-1 | AAGIGILTV | 2254 | 43.7 ± 0.8 |
| MART-1 10-mer | ELAGIGILTV | 254 | 59.3 ± 0.5 |
| MART-1 10-mer-E1A | ALAGIGILTV | 18 | 64.9 ± 0.4 |
| MART-1 10-mer-L2A | EAAGIGILTV | 5323 | 46.2 ± 1.0 |
| MART-1 10-mer-A3G | ELGGIGILTV | 733 | 45.0 ± 0.1 |
| MART-1 10-mer-G4A | ELAAIGILTV | 274 | 56.4 ± 0.4 |
| MART-1 10-mer-I5A | ELAGAGILTV | 361 | 58.2 ± 0.1 |
| MART-1 10-mer-G6A | ELAGIAILTV | 258 | 48.4 ± 0.2 |
| MART-1 10-mer-I7A | ELAGIGALTV | 890 | 45.2 ± 0.8 |
| MART-1 10-mer-L8A | ELAGIGIATV | 312 | 56.8 ± 0.3 |
| MART-1 10-mer-T9A | ELAGIGILAV | 300 | 55.4 ± 0.2 |
| MART-1 10-mer-V10A | ELAGIGILTA | 1597 | 50.1 ± 0.8 |
| MART-1 10-mer-L2I | EIAGIGILTV | 1929 | 52.9 ± 0.4 |
| MART-1 10-mer-G6S | ELAGISILTV | 256 | 45.4 ± 0.3 |
| MART-1 10-mer-I7L | ELAGIGLLTV | 276 | 50.4 ± 0.3 |
| MART-1 10-mer-V10I | ELAGIGILTI | 906 | 51.0 ± 0.2 |
| WT-1 | RMFPNAPYL | 7 | 57.8 ± 0.4 |
| WT-1-R1A | AMFPNAPYL | 6 | 56.4 ± 0.1 |
| WT-1-M2A | RAFPNAPYL | 563 | 48.0 ± 0.2 |
| WT-1-F3A | RMAPNAPYL | 21 | 55.6 ± 0.1 |
| WT-1-P4A | RMFANAPYL | 7 | 54.9 ± 0.2 |
| WT-1-N5A | RMFPAAPYL | 6 | 54.6 ± 0.2 |
| WT-1-A6G | RMFPNGPYL | 11 | 49.2 ± 0.2 |
| WT-1-P7A | RMFPNAAYL | 12 | 52.6 ± 0.3 |
| WT-1-Y8A | RMFPNAPAL | 19 | 54.4 ± 0.7 |
| WT-1-L9A | RMFPNAPYA | 14 | 54.8 ± 0.3 |
| WT-1-M2L | RLFPNAPYL | 7 | 55.1 ± 0.3 |
| WT-1-L9I | RMFPNAPYI | 8 | 55.0 ± 0.3 |
| WT-1-M2A-L9A | RAFPNAPYA | 1726 | 43.8 ± 0.2 |

A collection of well-studied self-peptides, including WT-1, MART-1 and their single peptide variants, were UV-exchanged to form complexes with HLA-A2. To analyze each of the complexes, one to three DSF experiments were performed, with three replicates each, to obtain average Tm values and standard deviations (SD).

**Supplementary Table S4. Half-life measurements of pep/HLA-A2 Complexes measured in this report.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **HUGO** | **Sequence** | **NetMHC 4.0** | **t1/2 (hours)** | **Tm (oC)** |
| MART-1 10-mer | ELAGIGILTV | 254 | 12.6 ± 1.8 | 59.3 ± 0.5 |
| MART-1 10-mer E1A | ALAGIGILTV | 18 | 40.3 ± 6.8 | 64.9 ± 0.4 |
| MART-1 9-mer | AAGIGILTV | 2254 | 0.6 ± 0.2 | 43.7 ± 0.8 |
| WT-1 | RMFPNAPYL | 7 | 15.1 ± 0.9 | 57.8 ± 0.4 |
| WT-1-M2A | RAFPNAPYL | 563 | 1.2 ± 0.2 | 48.0 ± 0.2 |
| WT-1-A6G | RMFPNGPYL | 11 | 1.8 ± 1.4 | 49.2 ± 0.2 |
| Tyrosinase | YMDGTMSQV | 7 | 82.2 ± 8.6 | 65.5 ± 0.2 |
| CDK4 | ALDPHSGHFV | 57 | 45.0 ± 3.8 | 64.1 ± 0.4 |
| GCN1L1 | ALLETPSLLL | 54 | 42.1 ± 4.4 | 61.4 ± 0.3 |

Peptide/HLA-A2 complex half-lives were measured using a bead-based flow cytometry assay to measure pep/HLA-A2 dissociation rates with an antibody to 2m or soluble, biotinylated high-affinity TCRs (WT-1 and WT-1-M2A). Each half-life value and standard deviation represents the mean of two or more experiments.

**Supplementary Table S5. Tm analysis of T cell epitopes with known binding affinity and half-life data.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Immunogenic** | **Sequence** | **Predicted IC50 (nM)1** | **IC50 (nM)2** | **t1/2 (hours)2** | **Tm (oC)** |
| Yes | FLTSVINRV | 6 | 7 | 22.3 | 58.1 ± 0.2 |
| No | NQNDNEETV | 3527 | 7 | 1.3 | 46.6 ± 0.3 |
| Yes | ILDDNLYKV | 4 | 1 | 28 | 58.7 ± 0.5 |
| Yes | GLFDFVNFV | 3 | 2 | 23 | 57.1 ± 0.1 |
| Yes | SLSAYIIRV | 5 | 2 | 23 | 58.1 ± 0.1 |
| Yes | YLPEVISTI | 6 | 1 | 19 | 58.0 ± 0.4 |
| Yes | FLIVSLCPT | 31 | 1 | 17 | 58.7 ± 0.4 |
| Yes | RLYDYFTRV | 4 | 4 | 14 | 57.0 ± 0.7 |
| Yes | LMYDIINSV | 3 | 1 | 13 | 57.8 ± 0.1 |
| Yes | VLYDEFVTI | 9 | 1 | 12 | 56.9 ± 0.3 |
| Yes | RVYEALYYV | 4 | 1 | 11 | 58.1 ± 0.5 |
| Yes | IIIPFIAYFV | 15 | 51 | 7 | 53.4 ± 0.2 |
| Yes | HVDGKILFV | 138 | 41 | 3 | 52.8 ± 0.1 |
| No | ALFGIKLPAL | 18 | 1 | 12 | 55.9 ± 0.1 |
| No | YLYQPCDLL | 37 | 1 | 7 | 55.3 ± 0.4 |
| No | YLIHDNIMYT | 9 | 2 | 3 | 48.9 ± 0.1 |
| No | FTFSNVCESV | 64 | 9 | 3 | 52.9 ± 0.2 |
| No | FVDTMSIYI | 14 | 1 | 3 | 53.0 ± 0.1 |
| No | FTSSFYNYV | 10 | 5 | 2 | 51.4 ± 0.3 |
| No | VLPFDIKYI | 335 | 20 | 1 | 48.0 ± 0.5 |
| No | RTCSNWVPL | 2393 | 329 | 0.5 | 44.2 ± 0.1 |
| No | ETDDYMFFV | 52 | 38 | 0.3 | 44.7 ± 0.8 |
| No | FQYLLPGFVL | 278 | 219 | 0.3 | 42.8 ± 0.7 |

1Predicted with NetMHC 4.0. 2Values from Harndahl et al (ref 9)

Peptides with previously measured half-life and binding affinity data were UV-exchanged with HLA-A2 and analyzed in one DSF experiments, with three replicates each, to obtain average Tm values and standard deviations (SD).

**Supplementary Table S6. Neoantigens from** Stronen (ref 10)

**Neoantigens from Melanoma Patient 1**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **HUGO** | **Sequence (wt)** | **NetMHC 4.0** | **t1/2 (hours)1** | **Tm (oC)** |
| AKAP6 | WLIDM**K**SLV(E) | 14 | 12.3 | 58.1 ± 0.1 |
| GDAP1 | LLDSLPMD**V**(A) | 32 | 6.7 | 53.8 ± 0.2 |
| GNL3L | NLN**C**CSVPV(R) | 54 | 23.5 | 59.9 ± 0.3 |
| KIF3B | SL**F**ALGNVI(S) | 195 | 3.6 | 48.7 ± 0.1 |
| LAMA1 | STA**F**DFLAV(S) | 342 | 3.7 | 52 ± 0.6 |
| MAP2K3 | YLVDSVAK**M**(T) | 14 | 17.9 | 59.7 ± 0.9 |
| MLL2 | ALSPVIP**H**I(L) | 11 | 47.7 | 64.4 ± 0.4 |
| SIVA1 | ALCGQCVR**I**(T) | 484 | 5 | 59.0 ± 0.9 |
| ASTN1 | K**L**YGLDWAEL(P) | 14 | 8.4 | 54.3 ± 0.2 |
| CDK4 | A**L**DPHSGHFV(R) | 57 | 47.5 | 64.1 ± 0.4 |
| GCN1L1 | ALLET**P**SLLL (L) | 54 | 39.9 | 61.4 ± 0.3 |
| WDR59 | HMM**G**FRTQEV (E) | 24 | 7.2 | 53.7 ± 0.2 |
| DNAH8 | FMTKIN**D**LEV(G) | 23 | 15.4 | 57.8 ± 0.2 |
| KIF3B | **F**ALGNVISAL(S) | 79 | 4.6 | 46.9 ± 0.4 |
| PRAMEF11 | Y**L**APQESYGA(P) | 95 | 5 | 51.8 ± 0.4 |
| SLC26A9 | GLLPVLSWL**L**(P) | 17 | 6.4 | 57.4 ± 0.3 |
| SMARCD3 | KLFEFLV**Y**GV(H) | 3 | 83.7 | 61.4 ± 1.0 |
| SMARCD3 | FLV**Y**GVRPGM(H) | 50 | 3.2 | 47.6 ± 0.7 |

**Neoantigens from Melanoma Patient 2**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **HUGO** | **Sequence (wt)** | **NetMHC 4.0** | **t1/2 (hours)1** | **Tm (oC)** |
| AGFG2 | FLQ**F**RGNEV(S) | 48 | 3.8 | 51.1 ± 0.5 |
| ENTPD7 | FL**S**QRVAFL(R) | 12 | 23.5 | 60.9 ± 0.3 |
| GIGYF1 | GSLDV**L**MAV(P) | 74 | 5 | 53.0 ± 0.3 |
| MRM1 | LLFGM**P**PCL(T) | 12 | 14.3 | 59.1 ± 0.2 |
| MRM1 | GM**P**PCLLAL(T) | 34 | 5.4 | 50.1 ± 0.4 |
| PGM2 | QQFAVGS**Y**V(H) | 174 | 5.4 | 52.6 ± 0.2 |
| PGM2 | AVGS**Y**VYSV(H) | 29 | 12.4 | 58.4 ± 0.5 |
| SLC38A1 | I**L**AALFLGL(W) | 18 | 6.9 | 53.2 ± 0.2 |
| SNX24 | KLSHQ**L**VLL(P) | 26 | 24.2 | 59.5 ± 0.6 |
| SSPN | FLMASISS**F**(S) | 32 | 4.6 | 51.7 ± 0.5 |
| SSPN | LMASISS**F**L(S) | 11 | 19.2 | 58.8 ± 0.2 |
| TARBP1 | **F**IGPLMDAL(I) | 175 | 2.9 | 48.7 ± 0.2 |
| TRPM8 | AQ**F**KGAWIL(S) | 206 | 2.9 | 47.1 ± 0.1 |
| USP28 | LIIP**F**IHLI(C) | 25 | 10 | 55.1 ± 0.1 |
| ZNF169 | FQPSF**S**HLV(P) | 11 | 10 | 57.6 ± 0.1 |
| ARHGEF12 | AI**L**PFTPEFL(S) | 71 | 4.8 | 50.7 ± 0.4 |
| ARHGEF12 | I**L**PFTPEFLV(S) | 26 | 3.4 | 51.4 ± 0.2 |
| BRWD3 | SLSSSGAPS**L**(P) | 473 | 2.8 | 48.8 ± 0.4 |
| CSGP4 | GTAWLEW**W**HV(R) | 441 | 4.3 | 46.5 ± 0.2 |
| GIGYF1 | **L**MAVAILKEV(P) | 48 | 11.7 | 56.4 ± 0.3 |
| MEF2D | GLFR**P**YGSTV(R) | 92 | 4.1 | 47.2 ± 0.3 |
| SSPN | FLMASISS**F**L(S) | 11 | 17.8 | 58.2 ± 0.5 |
| SSPN | LMASISS**F**LL(S) | 28 | 6.7 | 56.2 ± 0.1 |
| TGIF1 | LLGSSCAPP**L**(P) | 79 | 3.2 | 51.8 ± 0.7 |
| TNC | KLPVGSQC**L**V(S) | 337 | 4.1 | 56.8 ± 0.1 |

**Neoantigens from Melanoma Patient 3**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **HUGO** | **Sequence (wt)** | **NetMHC 4.0** | **t1/2 (hours)1** | **Tm (oC)** |
| ALS2CR4 | TLAY**S**FQSL(P) | 79 | 3.4 | 47.9 ± 0.5 |
| BCS1L | ALA**Q**KGVQL(R) | 1160 | 4.4 | 52.7 ± 0.3 |
| BCS1L | ALALA**Q**KGV(R) | 642 | 9.2 | 56.2 ± 0.6 |
| EBP41 | IL**D**VCSSGL(G) | 120 | 3.9 | 50.6 ± 0.4 |
| EIF2AK3 | KVDIF**F**LGL(S) | 701 | 3.9 | 45.2 ± 0.2 |
| GOLGA3 | SLD**L**TTSPV(P) | 19 | 13.7 | 59.5 ± 0.5 |
| GPC4 | VMD**S**IDVKI(P) | 115 | 5.1 | 51.3 ± 0.5 |
| HELLS | VT**Y**SGKFLI(N) | 417 | 3.7 | 51.4 ± 0.1 |
| PAFAH2 | TQF**W**CAVAL(R) | 400 | 4.3 | 42 ± 0.8 |
| DCI | VMKFKNP**L**V(P) | 356 | 3.4 | 49.7 ± 0.3 |

The Schumacher group previously published measured half-life and immunogenicity data for a collection of HLA-A2-restricted neoantigens found in melanoma patients. These peptides were UV-exchanged with HLA-A2 and analyzed in one DSF experiments, with three replicates each, to obtain average Tm values and standard deviations (SD).

**Supplementary Table S7. Sequences of Inflamed Neoantigens from The Cancer Genome Atlas**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| RIAPPEPKV | ILDEPEVPV | LLHLGALYV | SICEVVKCL | LLALLILGL | FLIKGPNKI |
| GLTGDLCEL | AISPPSASL | KLWNPEGTV | SLWAVPAIV | RLVPELDTV | CLGEFRHEV |
| PLPENVVLL | PLGGFILGL | GIIRGSMKV | SICGDIPEL | SLAGHKSSV | NIKDQLNKL |
| FLLKKKYLV | MLDALVLDL | GLGVGTSEV | IINGFVLPL | LLSPASNSL | SLRCRTYYV |
| LLSPSILKL | ILYPKREFL | LLVGRYLEV | ALPTALPGL | IVLGDLSSL | ALAPPVWHL |
| SLLGSAAEL | ILFQSIHFI | RLRAEVAQV | SLQLTLRSL | KILRDEVSL | LLLVYVTEV |
| SLSSPIAAL | SLVKGKGKV | LLLLGPPSV | KLNGFFNKL | DLNGVEHKV | SIAELIYAV |
| LLLRDLETL | SLCTFAAAV | SLRQLVYRV | ALTVGLAYI | ALMGSGASV | ILRPGLWVL |
| GLAPDTDTL | LLYGPEVDI | LLPPMMASL | MLGELLTPV | LLAASLGPV | SILDDVVMV |
| ILHNFVLAL | ALGEEFFSV | LLESFLSSL | TILEEVKEL | LLDMPLLYL | SIIPLLEPL |
| NLDGMVALL | HLYPNTQAL | LLPETTENV | YLVYHLVQL | LLIIRLRYL | GMADGVNLL |
| LLEKEVNEV | GLGKGQESL | SIIPTSGSV | RIVEKTWKL | ALMSELEVL |  |
| ALNGLRYGL | LLGVGLPSA | SLFPEPLHV | KLAAVLQFI | ILSQFTDSL |  |
| CLKPSIESL | YLKDLWTTL | SINGKSFSL | KMAADLECL | QLEDKITTL |  |
| SICIPIVPL | NLFKRLMIV | VLNSLRCEL | YLALGVLAL | AALEDLRTL |  |

**Supplementary Table S8. Sequences of Non-Inflamed Neoantigens from The Cancer Genome Atlas**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| LILPFLGKL | LLCDTVLKI | SLEGEKLSL | ALDFDISEI | LLDVIIHKL | CLRPRVLEI |
| HLAPREGKV | RLFRSLPEL | SLILTSFEL | LVASELHTL | SLVPSKVSV | KILKQIMTL |
| ALQSVSLEV | ALPSWLDTL | KLTGSVRQL | SLGESEHLL | LLQLNLLQL | ALNKMLCQL |
| SLLSPTVEL | LLPPLSAPV | ILAPVKSNL | GLPTDVQLV | LLPEDLAEA | RLSAKLGEL |
| YLIGLLFDL | ILYRTLEPL | RLPLPVSGL | FLGESIKAI | SIGTSVISV | SLVPQLSAI |
| FLGEYIPTV | WLSLNVLSV | LLARASPAL | ILIRGDPHI | LLPLTLQHL | TMSEGLKKL |
| SLAAGILDL | ALGPPSVVV | ILFKNLKRV | KLLCAKHGL | SLFAGNPQL | GIPGGLQAI |
| FLISYVEKL | GLFYDLCEL | ALSSSVMCL | LIPALIPTL | LLQPLPKSL | ALGLDLCLL |
| ALMPMTVGV | ALYVVTGSV | FLHPEPVVV | NIESGVPSL | LLEQEIFNL | KLLQFSVIV |
| LLLKVLRKL | KLAVCTREL | MLALHTLNL | AMLDLLKQI | SMAVALKPV | YLEGSVCVI |
| LLFLDIAPL | ALQYNIRGV | TLHGLSCEV | LLNCCVAGV | SLSSPGFSL | NILGYVVST |
| SLDCGLRGL | AVFKGIAAV | FLLDTEDAV | ALQEPVKLL | ALEEEIAKV |  |
| RLLPVVRAL | VLPPGQPLL | KLIVYPPPL | GIAAQLLNV | VLKAFVVDI |  |
| HLLRGISIL | TLAKGWGEL | YLKFCLPEI | LLCGRLPDL | KIDASLSEV |  |
| SLSCRLPMV | SLPDLTGVV | SIAATIIYV | TLREPLFTL | SMQGDLKTI |  |
| GLYSQVAKL | GLAGKLEVA | YLLKDVGSL | ALKDTVEEV | ALLSVLLEL |  |