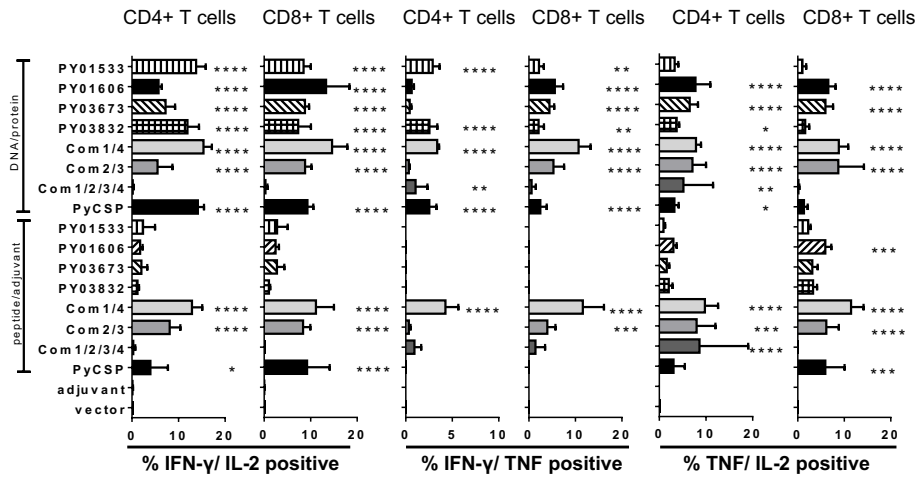


SUPPLEMENTARY INFORMATION:

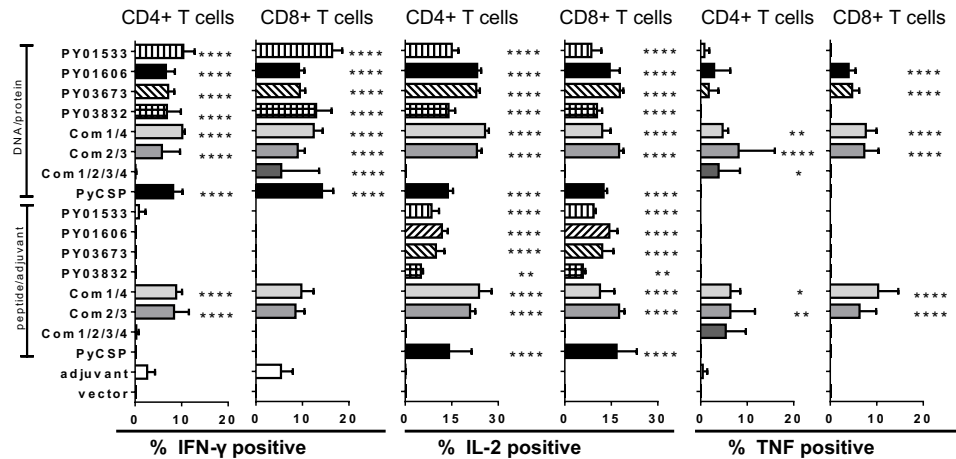
Sophie Schussek, Angela Trieu, Simon H Apte, John Sidney, Alessandro Sette, and Denise L Doolan

Novel Plasmodium antigens identified via genome-based antibody screen induce protection associated with polyfunctional T cell responses

A) double positive T cell responses



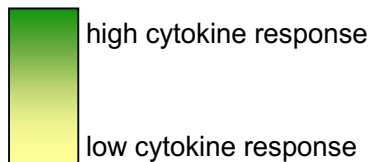
B) single positive T cell responses



Supplementary Figure S1: Double and single positive T cell populations. Splenocytes were harvested at 14 days post last immunisation and re-stimulated for 6h with A20 antigen presenting cells transfected with antigen-encoding plasmid DNA or incubated with synthetic peptide pools representing predicted CD8⁺ and CD4⁺ T cell epitopes for each antigen. Com1/4 = PY01533+PY03832; Com2/3 = PY01606+PY03673; Com1/2/3/4 = PY01533+PY03832+PY01606+PY03673. Multi-parameter flow cytometry was used to quantify **(A)** frequency of CD4⁺ or CD8⁺ T cell populations expressing any two of the three cytokines, IFN-γ, IL-2 or TNF **(B)** frequency of CD4⁺ or CD8⁺ T cell populations expressing only one of the cytokines IFN-γ, IL-2, and TNF, respectively. Error bars represent standard deviation (SD). Statistical comparison of immunised versus controls (empty vector or adjuvant only, for DNA/protein or peptide/adjuvant groups respectively) was performed using one-way ANOVA followed by Bonferroni's posthoc test, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Supplementary Figure S2: Association of vaccine-induced protective capacity and CD4⁺ or CD8⁺ Th1 cytokine production.

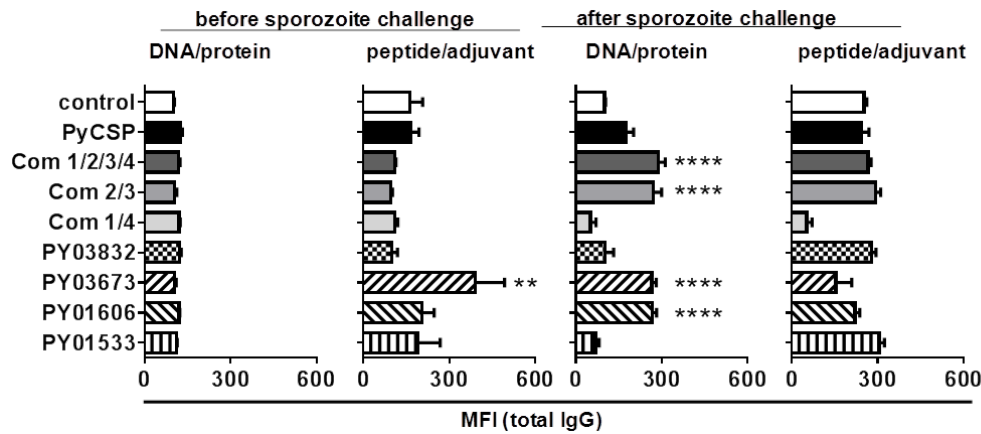
The protective capacity of each test group was calculated by determining the mean of the reduction of parasite burden in the liver and in the blood in reference to no-vaccine controls as presented in Table 3. The integrated median fluorescence intensity (iMFI) for each multifunctional CD8⁺ or CD4⁺ T cell population **(I)** IFN- γ iMFI; **(II)** IL-2 iMFI; **(III)** TNF iMFI) was calculated by multiplying the frequency of cells producing a given cytokine combination by the magnitude of the cytokine response (measured as median fluorescence intensity, MFI). Data are ranked according to the protective capacity (see protective index in Table 3) (top: highest protection, bottom: lowest protection).



protection	antigen	immunisation	IFN- γ	IL-1 β	IL-6	TNF	IL-4	IL-10	IL-12p70	IL-2	IL-13	IL-5
	Com1/4	peptide	873.9	6.0	8.5	28.6	1.4	4.0	11.2	1.9	n.d.	1.2
	PyCSP	DNA	531.6	22.0	252.5	332.3	1.3	4.7	10.4	4.2	15.4	1.8
	Com1/4	DNA	1111.1	33.6	49.4	78.4	7.1	160.6	88.9	10.0	9.4	18.1
	Com1/2/3/4	peptide	676.7	n.d.	170.3	115.2	2.2	176.3	n.d.	224.3	433.9	96.1
	PyCSP	peptide	17.2	5.6	9.6	14.7	2.3	5.1	17.2	38.5	5.8	1.6
	Com1/2/3/4	DNA	301.9	3.1	16.2	53.7	0.3	17.8	30.1	1.5	3.2	0.1
	Com2/3	DNA	301.9	10.6	176.9	623.1	0.6	39.6	n.d.	3.5	11.2	n.d.
	PY03832	DNA	39.3	0.2	n.d.	51.0	0.4	n.d.	28.9	24.1	77.9	0.5
	PY01533	peptide	245.5	2.5	29.7	49.5	0.3	31.6	4.1	1.0	7.8	0.5
	PY03832	peptide	36.7	5.2	3.8	38.0	1.1	312.8	2.2	17.1	472.2	31.4
	PY01606	peptide	245.5	2.5	29.7	49.5	0.3	31.6	4.1	1.0	7.8	0.5
	PY01606	DNA	9.2	18.5	5.3	5.5	1.6	1.4	2.5	10.5	45.2	0.3
	PY01533	DNA	410.1	0.4	39.9	15.3	0.5	n.d.	0.4	1.6	4.0	n.d.
	Com2/3	peptide	707.1	2.6	133.9	1.2	5.0	196.6	192.2	132.1	908.9	354.2
	PY03673	peptide	224.3	0.5	7.4	10.2	n.d.	2.3	5.6	0.8	0.6	0.8
		adjuvant	n.d.	9.8	n.d.	142.8	n.d.	7.4	17.4	n.d.	11.9	1.4
	PY03673	DNA	56.7	10.2	1.4	14.1	0.8	5.3	3.8	0.6	59.3	2.6
		vector	0.1	0.5	0.6	21.9	n.d.	16.2	n.d.	1.1	n.d.	n.d.
		non-immunised	0.3	n.d.	1.4	17.9	0.5	24.1	n.d.	1.8	1.9	1.3

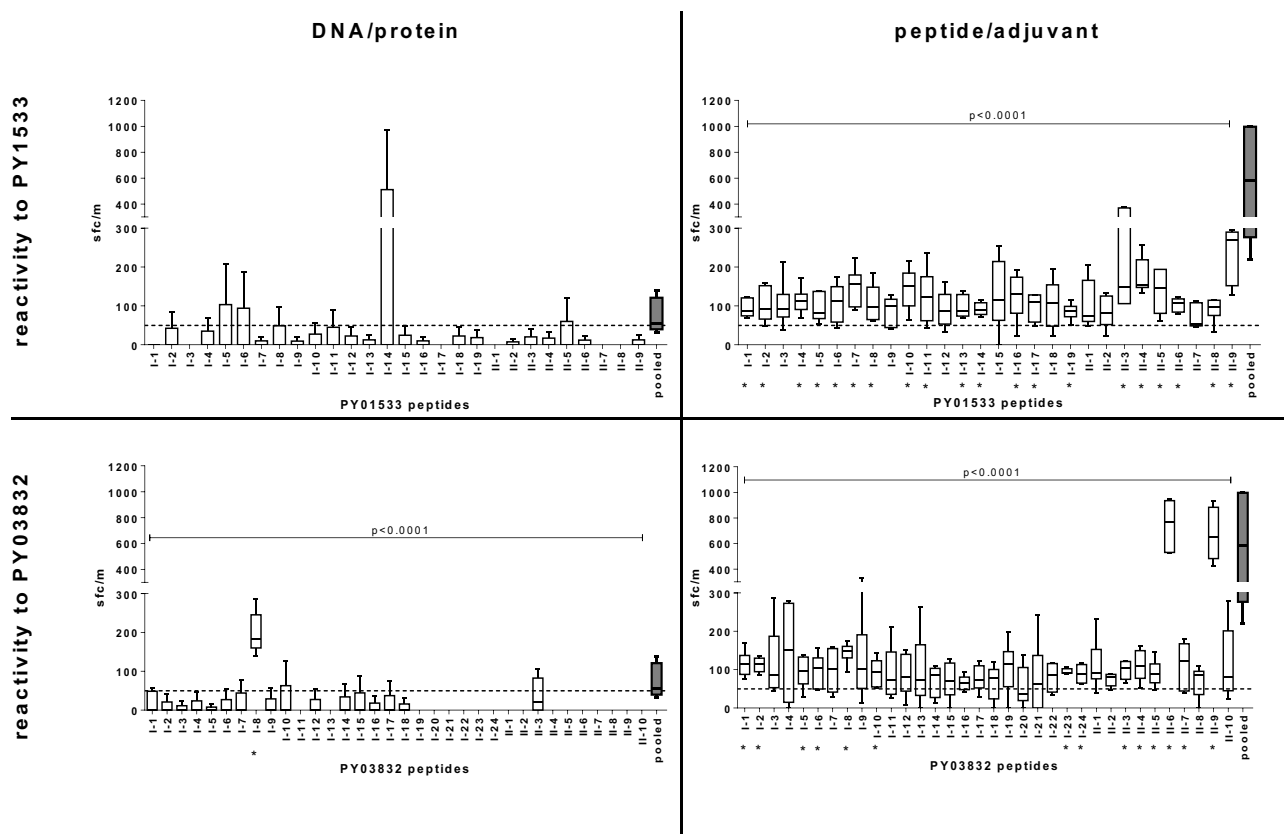
Supplementary Figure S3: Association of vaccine-induced protective capacity and antigen-specific and combination-specific cytokine responses by Cytokine Bead Array analysis.

Splenocytes were harvested at 14 days post last immunisation, re-stimulated for 48h with A20 antigen presenting cells transfected with antigen-encoding plasmid DNA or by incubation with peptide pools representing each antigen, and the amount of secreted cytokines was analysed. The protective capacity was calculated and correlated to cytokine responses as described in the legend to Figure 6. Cytokine data for significantly correlated cytokine species are presented in pg/ml (mean of data from one n=5 or two n=10 independent experiments) after correction for background cytokine production in mock-stimulated cells, and arranged according to the protective capacity (top: highest protection, bottom: lowest protection).



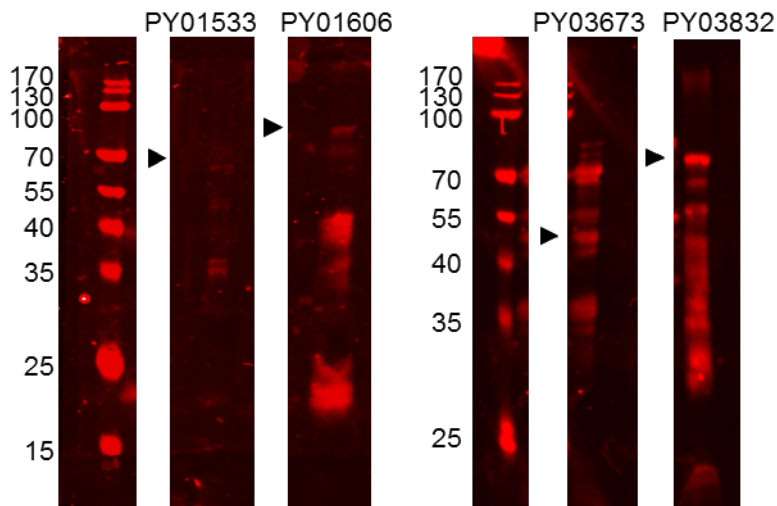
Supplementary Figure S4: Antigen-specific and combination-specific antibody responses.

Flow cytometric analysis of total IgG levels binding to *P. yoelii* blood stage extract in the serum of DNA/protein or peptide/adjuvant immunised mice before or 23 days after sporozoite challenge. Com1/4 = PY01533+PY03832; Com2/3 = PY01606+PY03673; Com1/2/3/4 = PY01533+PY03832+PY01606+PY03673. Control= empty vector only or adjuvant only for DNA/protein or peptide/adjuvant groups respectively. Data are presented as Median Fluorescence Index (MFI) with standard deviation (SD), correlating to the antibody titre, normalised against non-immunised controls (n=5 mice/group). Statistical comparison of immunised versus non-immunised controls was performed using one-way ANOVA followed by Bonferroni's posthoc test, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.



Supplementary Figure S5: Identification of novel T cell epitopes.

IFN- γ production of splenocytes harvested from mice immunised with Com1/4 DNA/protein (**left**) or Com1/4 peptide/adjuvant (**right**) at 14 days post last immunisation and re-stimulation for 48h *in vitro* with individual synthetic peptides representing predicted CD8⁺ and CD4⁺ T cell epitopes for antigens PY03832 and PY01533 or the corresponding peptide pool for each antigen. Data are presented as mean spot forming cells (SFCs)/million splenocytes for individual mice (n=5/group) with error bars representing the total range of values. The dotted line represents the mean background value of IFN- γ spot forming cells induced in adjuvant only and empty vector control group. Statistical significance across all peptide stimulations was determined by one-way ANOVA.



Supplementary Figure S6: SDS-PAGE of recombinant proteins. Proteins were expressed from a custom pIVEX-HIS/HA vector using the cell-free protein expression system RTS 500 HY *E. coli*. Recombinantly expressed proteins were HIS purified using Cobalt Talon Resin and endotoxin was removed with wash buffer containing 0.05% Triton X-114. After elution and buffer exchange with PBS, proteins were loaded onto a 4ml 3K Amicon Ultra Filtration tube (Merck Millipore, Billerica, MA) and concentrated in three alternating spinning and washing steps. Purified samples were separated by 10% SDS-PAGE and HIS-tagged proteins were detected by Western Blot in PVDF-FL membranes using IRDye-680 conjugated anti-HIS antibodies. As expected, full-length as well as partial length protein of varying sizes was produced in the cell-free system, covering all possible epitopes and effectively boosting DNA primed responses.

Supplementary Table 1: Top 1%: MHC class I and top 2% MHC class II binding epitope predicted for each antigen: Predicted for mouse MHC class I alleles H-2K^d and H-2D^d and for mouse MHC class II alleles IA^d and IE^d using algorithms available on the Immune Epitope Database (www.IEDB.org).

Antigen	MHC-peptide	Sequence	Length	Position	H-2 Kd percentile	H-2 Dd percentile
Py01533	I-1	SYEPSRRSL	9	640	0.09	49.52
Py01533	I-2	SYEYVSDKL	9	730	0.14	72.69
Py01533	I-3	AYSRTIASI	9	176	0.18	27.79
Py01533	I-4	KYDGERAQI	9	476	0.82	58.97
Py01533	I-5	LSPVHTAAI	9	759	13.88	0.14
Py01533	I-6	SKPNLFFNI	9	366	13.14	0.33
Py01533	I-7	EGDTRLLEPI	9	377	35.33	0.40
Py01533	I-8	ELPNIEIMI	9	416	50.92	0.41
Py01533	I-9	YGAFVLAAY	9	683	24.02	0.44
Py01533	I-10	IMPQVTESI	9	517	13.17	0.47
Py01533	I-11	YEPSRRSLN	9	641	18.56	0.54
Py01533	I-12	FKDSLLETF	9	93	50.26	0.55
Py01533	I-13	FDAHYYWEI	9	744	24.35	0.63
Py01533	I-14	GDTRLLEPI	9	378	66.63	0.70
Py01533	I-15	GIGLRFPRF	9	775	85.04	0.74
Py01533	I-16	GVPVKEPL	9	575	35.20	0.75
Py01533	I-17	GKGKRSQVY	9	675	83.71	0.78
Py01533	I-18	ILPFQILTT	9	540	75.22	0.87
Py01533	I-19	IFLPLRLTI	9	208	10.13	0.95

Py01606	I-1	NYLNGPSSI	9	722	0.13	40.05
Py01606	I-2	NYDTSNNEI	9	801	0.21	35.53
Py01606	I-3	SGLGGVQAI	9	136	0.34	4.32
Py01606	I-4	NYEESDEYI	9	975	0.36	47.12
Py01606	I-5	SSLFVREDPI	9	608	0.37	23.75
Py01606	I-6	IYSYNREFI	9	452	0.41	9.90
Py01606	I-7	QITMSFQTI	9	490	0.50	16.60
Py01606	I-8	GFLKIGTPL	9	2	0.67	10.11
Py01606	I-9	SPIKINKTI	9	187	0.75	50.23
Py01606	I-10	SPHSRYVTL	9	343	0.84	2.05
Py01606	I-11	NGPSSIGWR	9	725	33.35	0.01
Py01606	I-12	CTPYLGGFL	9	521	29.33	0.07
Py01606	I-13	TGASFLRKX	9	867	73.79	0.16
Py01606	I-14	GTPSVPFKF	9	104	90.83	0.17
Py01606	I-15	ISPHSRYYT	9	342	42.33	0.23
Py01606	I-16	PPPILDNNY	9	715	91.00	0.26
Py01606	I-17	SSPINLSPI	9	181	1.68	0.51
Py01606	I-18	WTPEYSSFT	9	93	68.48	0.60
Py01606	I-19	KNPIKNYYV	9	468	97.53	0.80
Py01606	I-20	ELPLKKNYY	9	569	84.80	0.84
Py01606	I-21	VDPHKERIK	9	918	99.00	0.85
Py01606	I-22	TPEYSSFTI	9	94	4.88	0.91
Py01606	I-23	TLPCYPNVF	9	145	78.15	0.94

Py03673	I-1	FYIGISDNI	9	1350	0.04	19.31
Py03673	I-2	FYNHTINSI	9	798	0.06	8.82
Py03673	I-3	KYTNDSNV	9	1130	0.11	96.77
Py03673	I-4	IYIQGNIL	9	313	0.24	74.53
Py03673	I-5	CYENRRNFI	9	465	0.26	7.37
Py03673	I-6	SYKLRDDDI	9	61	0.27	18.69
Py03673	I-7	LYFASINRK	9	755	0.28	88.79
Py03673	I-8	VYLKNTNFI	9	346	0.30	19.33
Py03673	I-9	MYVRMEDDI	9	1313	0.43	27.48
Py03673	I-10	SYMIEKNNL	9	1250	0.55	25.25
Py03673	I-11	KYYESEQIV	9	1291	0.64	90.09
Py03673	I-12	KYEKQLTEF	9	1409	0.70	13.40
Py03673	I-13	NYQEIQENM	9	1000	0.71	53.11
Py03673	I-14	IYSNKRRLI	9	16	0.94	21.72
Py03673	I-15	YIQKNVDTL	9	519	0.95	31.35
Py03673	I-16	VRPFKNNII	9	532	35.68	0.11
Py03673	I-17	EMPIYLNII	9	269	34.03	0.21
Py03673	I-18	LFPFNDFVL	9	624	29.13	0.48
Py03673	I-19	KKPYFYIGI	9	1346	34.97	0.50
Py03673	I-20	TPLDNAVY	9	1052	37.90	0.58
Py03673	I-21	VTPEMPYYL	9	266	38.02	0.73
Py03673	I-22	YAPCDKRSI	9	978	11.93	0.81
Py03673	I-23	CTPLNIFTS	9	446	78.89	0.88
Py03673	I-24	VGKRFREFYS	9	987	67.19	0.92

Py03832	I-1	YYQTPLSEI	9	1799	0.03	29.16
Py03832	I-2	LYQLTIESI	9	431	0.10	19.70
Py03832	I-3	IYKQNFDSI	9	2897	0.16	14.04
Py03832	I-4	CFLKVRNTI	9	1929	0.17	11.25
Py03832	I-5	KYRIVQNKI	9	2668	0.20	49.20
Py03832	I-6	LYYFDSSNI	9	1191	0.23	22.39
Py03832	I-7	QNHTTFSTI	9	2931	0.31	15.19
Py03832	I-8	FFINSINNI	9	1914	0.33	9.40
Py03832	I-9	SILSAENSI	9	1392	0.38	16.33
Py03832	I-10	AFQTRLDOI	9	1351	0.40	3.48
Py03832	I-11	LRLFMETDI	9	1105	0.44	43.82
Py03832	I-12	TINNSNTYI	9	1556	0.46	26.10
Py03832	I-13	FYFOFFYDI	9	1175	0.47	19.49
Py03832	I-14	SYLKEHKYL	9	630	0.48	84.01
Py03832	I-15	NYPNKDFTF	9	2482	36.72	0.04
Py03832	I-16	IGPSKSDQK	9	1307	75.41	0.06
Py03832	I-17	NGPQMVFSQ	9	795	41.36	0.13
Py03832	I-18	NNPNLSNTI	9	1738	2.97	0.18
Py03832	I-19	GDPTCAICA	9	91	96.89	0.28
Py03832	I-20	TGAKIDFLI	9	218	8.46	0.30
Py03832	I-21	HSPLVRFFM	9	517	34.39	0.31
Py03832	I-22	NIPRKTSYI	9	1198	10.89	0.38
Py03832	I-23	VDPKFKQRF	9	249	72.76	0.43
Py03832	I-24	AFPCKHPSI	9	2071	29.81	0.46

PyCSP	I-1	SYVPSAEQI	9	304	0.07	64.51
PyCSP	I-2	QGPGAPQGP	9	139	74.58	0.24
PyCSP	I-3	QGPGAPQEP	9	247	72.32	0.36
PyCSP	I-4	EPPQQPPQQ	9	254	97.28	0.37
PyCSP	I-5	QPPQQPPQQ	9	258	88.69	0.65
PyCSP	I-6	QPPQQPRPQ	9	278	74.44	0.77
PyCSP	I-7	SYVPSAEQIL	10	304	0.26	67.36

Summary

Class I 9mers	H-2 Kd	H-2 Dd	Total
Py01533	4	15	19
Py01606	10	13	23
Py03673	15	9	24
Py03832	14	10	24
PyCSP	2	5	7

Antigen	MHC-peptide	Sequence	Length	Position	H-2 IAd percentile	H-2 IEd percentile
Py01533	II-1	YIVRFLQQLRIGVN	15	256	0.04	63.90
Py01533	II-2	TSEAKYIVRFLQQLR	15	251	0.40	67.74
Py01533	II-3	LMKKCTVKTGVVPVQP	15	436	0.65	60.29
Py01533	II-4	TVKTGVVPQPMIAKP	15	441	1.19	97.50
Py01533	II-5	AHYVWEIKAADLSLS	15	746	1.71	35.30
Py01533	II-6	QIVKQIMPGVTESI	15	511	1.89	92.55
Py01533	II-7	LQQLRLRIGVNSATVL	15	261	1.95	75.83
Py01533	II-8	RSLNWLKVKKDIYEG	15	646	46.88	1.45
Py01533	II-9	RRKLLYSLLRCKEGV	15	586	32.58	1.62

Py01606	II-1	FFGMSMCCQQTMSF	15	481	0.38	85.41
Py01606	II-2	LVTDMVISPHSRYYT	15	336	0.54	74.89
Py01606	II-3	HISSLFVRDPVVF	15	606	0.67	78.09
Py01606	II-4	DQLAVIAPFLAITA	15	506	1.02	84.56
Py01606	II-5	GWRVEFRTPDIQITD	15	731	1.55	75.32
Py01606	II-6	SRYYTLTKNIRKRRG	15	346	22.65	0.54
Py01606	II-7	LEENLFRSSKRDAIL	15	776	28.74	0.82
Py01606	II-8	NEYIQFVKAACKGEI	15	851	44.33	1.22
Py01606	II-9	VHSYKCNKDRKDEHI	15	31	80.68	1.34
Py01606	II-10	SMTNKKKINEKKKI	15	411	85.70	1.88

Py03673	II-1	IKSSINKALSILTNN	15	226	1.98	46.85
Py03673	II-2	KKIKLYFASINRKKK	15	751	35.69	0.14
Py03673	II-3	YFASINRKKKKINEI	15	756	63.50	0.26
Py03673	II-4	YKFLKYLKNNKKENKI	15	1161	76.55	0.47
Py03673	II-5	VTKYNVLLKKKFKIL	15	936	56.14	0.90
Py03673	II-6	NKGLLKFLNDEKKKY	15	171	91.45	0.92
Py03673	II-7	SNTLYYKFLKYLKNN	15	1156	65.39	0.98
Py03673	II-8	PIYWIKLEKLKNIN	15	136	38.59	1.25
Py03673	II-9	AQHFFHPLNKKGDIM	15	726	91.74	1.72

Py03832	II-1	TYFKWINMGAPTAA	15	641	0.26	67.62
Py03832	II-2	IINMGAPTAASSTA	15	646	0.27	98.46
Py03832	II-3	FDMKALRQSIILGG	15	2906	0.85	53.36
Py03832	II-4	YWVRNGPQMVFQSNE	15	791	0.98	84.23
Py03832	II-5	EIMRICDLKYLLY	15	336	1.00	66.42
Py03832	II-6	YWYKYVYLLKIKKKK	15	541	46.62	0.04
Py03832	II-7	RKTSYIKYKRGKYQ	15	1201	64.70	0.17
Py03832	II-8	DIADIFYFYKYKSHHK	15	2281	82.55	0.33
Py03832	II-9	CIDDLWYKYVYLLK	15	536	36.98	0.37
Py03832	II-10	IIEKRRKGKNNKKLE	15	1876	73.62	0.58

PyCSP	II-1	QILEFVKQISSOLTE	15	311	0.31	92.98
PyCSP	II-2	MKKCTILVASLLLV	15	1	0.47	94.84
PyCSP	II-3	DSLPLPGYGQNKSVQA	15	16	0.90	34.94
PyCSP	II-4	GYGQNKSVQAQRNLN	15	21	1.15	54.46

Class II 15mers	H-2 IAd	H-2 IEd	Total
Py01533	7	2	9
Py01606	5	5	10
Py03673	1	8	9
Py03832	5	5	10
PyCSP	4	0	4