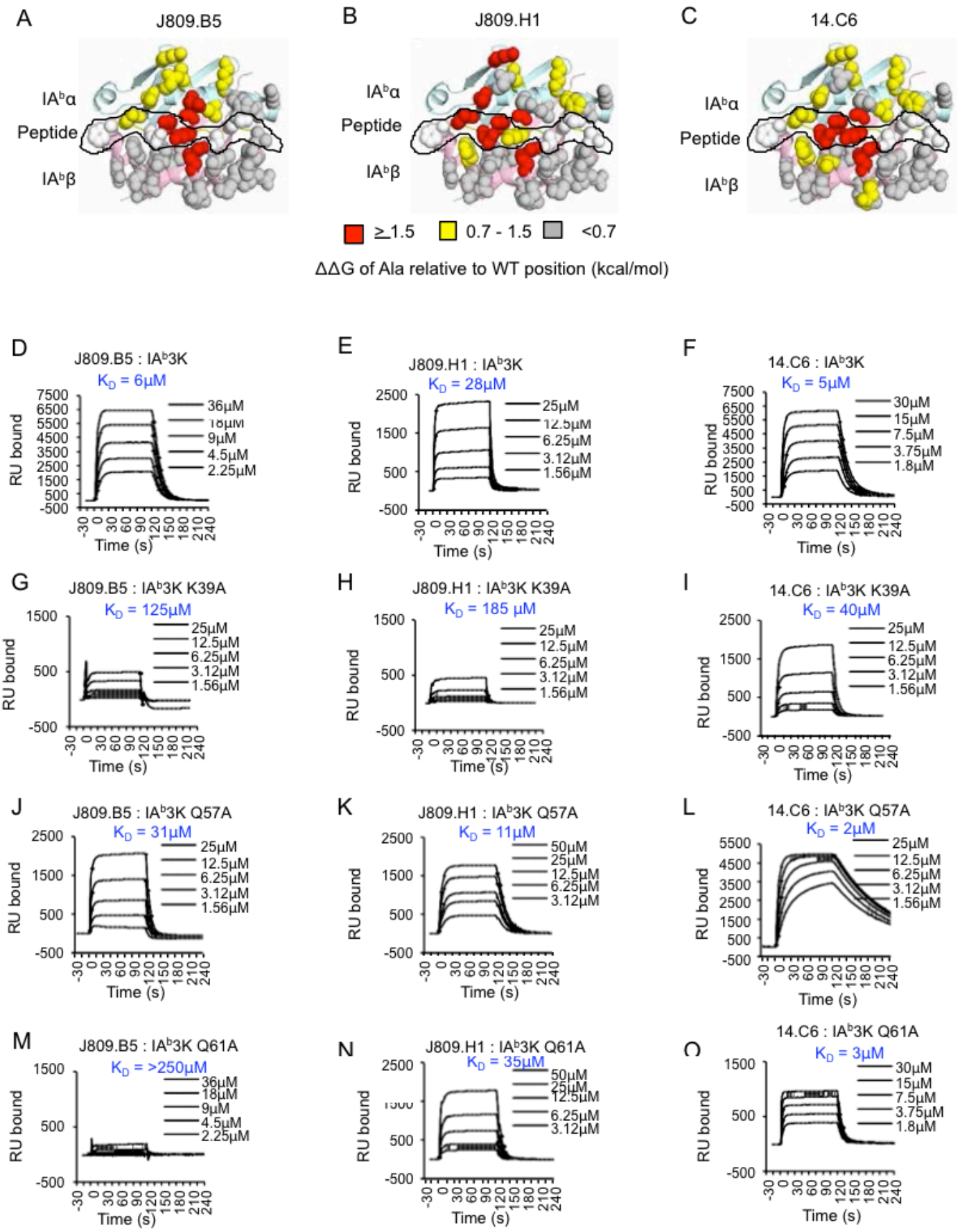
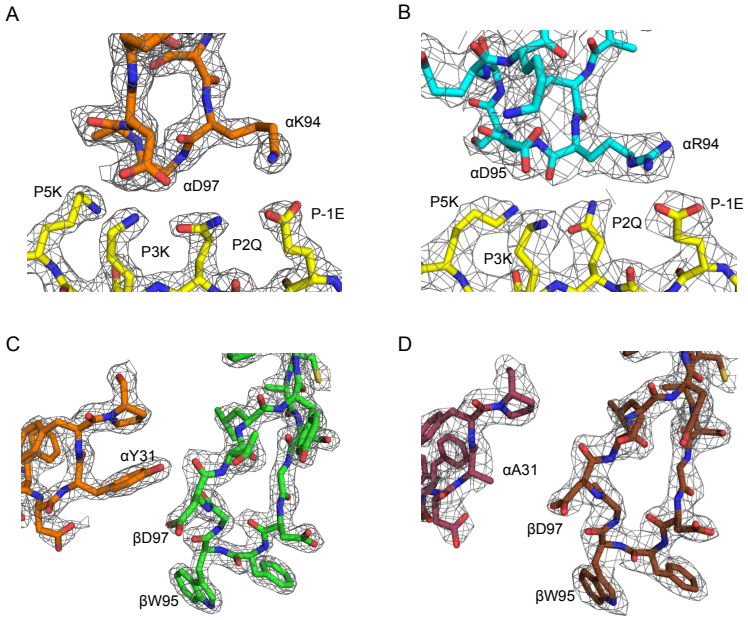
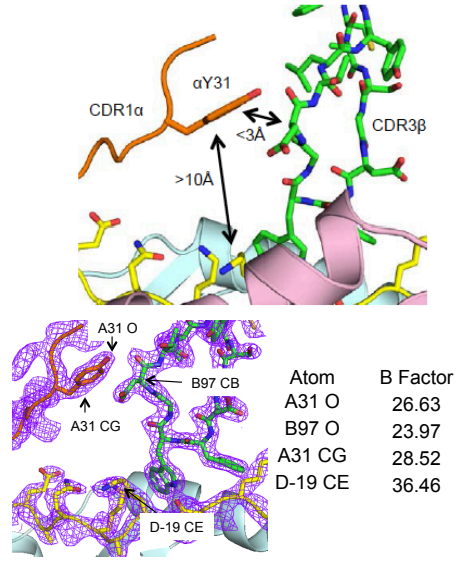

A
J809.B5
IA^{b}α
Peptide
IA^{b}β
B
J809.H1
IA^{b}α
Peptide
IA^{b}β
C
14.C6
IA^{b}α
Peptide
IA^{b}β
≥1.5
0.7 - 1.5
<0.7
ΔΔG of Ala relative to WT position (kcal/mol)
D
J809.B5 : IA^{b}3K
K_D = 6μM
36μM
18μM
9μM
4.5μM
2.25μM
E
J809.H1 : IA^{b}3K
K_D = 28μM
25μM
12.5μM
6.25μM
3.12μM
1.56μM
F
14.C6 : IA^{b}3K
K_D = 5μM
30μM
15μM
7.5μM
3.75μM
1.8μM
G
J809.B5 : IA^{b}3K K39A
K_D = 125μM
25μM
12.5μM
6.25μM
3.12μM
1.56μM
H
J809.H1 : IA^{b}3K K39A
K_D = 185 μM
25μM
12.5μM
6.25μM
3.12μM
1.56μM
I
14.C6 : IA^{b}3K K39A
K_D = 40μM
25μM
12.5μM
6.25μM
3.12μM
1.56μM
J
J809.B5 : IA^{b}3K Q57A
K_D = 31μM
25μM
12.5μM
6.25μM
3.12μM
1.56μM
K
J809.H1 : IA^{b}3K Q57A
K_D = 11μM
50μM
25μM
12.5μM
6.25μM
3.12μM
L
14.C6 : IA^{b}3K Q57A
K_D = 2μM
25μM
12.5μM
6.25μM
3.12μM
1.56μM
M
J809.B5 : IA^{b}3K Q61A
K_D = >250μM
36μM
18μM
9μM
4.5μM
2.25μM
N
J809.H1 : IA^{b}3K Q61A
K_D = 35μM
50μM
25μM
12.5μM
6.25μM
3.12μM
O
14.C6 : IA^{b}3K Q61A
K_D = 3μM
30μM
15μM
7.5μM
3.75μM
1.8μM
RU bound
Time (s)

FIGURE S1. CDR3α Sequences alone can alter the interaction of TCRβ residues with MHC. (**A-C**) The energetic contribution of peptide and MHC side chains for the Vα2.8^{+} Vβ8.2^{+} (**A**) J809.B5, (**B**) J809.H1 and (**C**) 14.C6 TCRs binding to IA^{b}-3K. Each TCR carries the identical TCRβ sequence. Data in (**A, B**) has been previous published (25) and is shown for comparison to the 14.C6 interaction with IA^{b}-3K. See Supplementary Table S1 for values for TCR interactions with individual pMHC residues. (**D-O**) Binding of Soluble (**D, G, J** and **M**) J809.B5 TCR, (**E, H, K, N**) J809.H1 or (**F, I, L, O**) 14.C6 TCRs were analyzed for equilibrium affinity binding to immobilized (**D-F**) IA^{b}-3K, (**G-I**) IA^{b}-3K which carries an alanine substitution at the IA^{b} α-chain residue K39 (K39A), (**J-L**) IA^{b}-3K which carries an alanine substitution at the IA^{b} α-chain residue Q57 (Q57A), or (**M-O**) IA^{b}-3K which carries an alanine substitution at the IA^{b} α-chain residue Q61 (Q61A) via SPR. Listed K_D are the average of three independent experiments, sensograms are representative analyses.

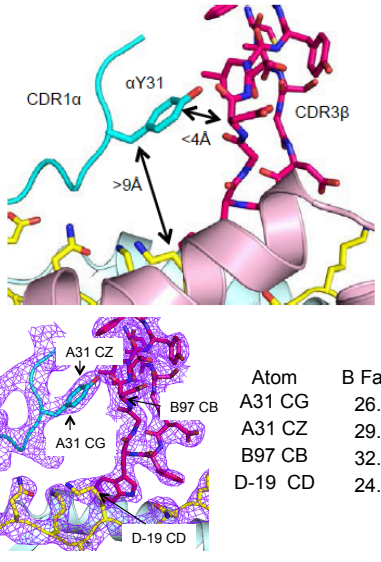


E. J809.B5:IA^b-3K PDB:4P23



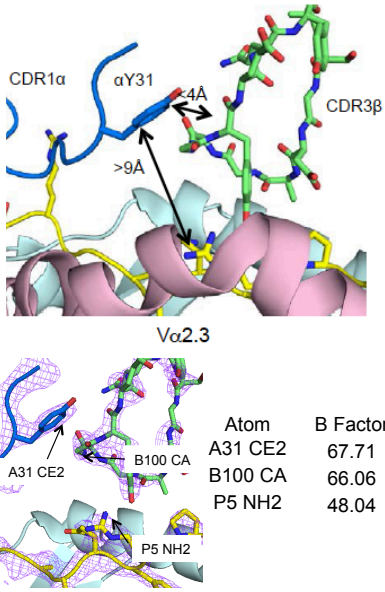
Atom	B Factor
A31 O	26.63
B97 O	23.97
A31 CG	28.52
D-19 CE	36.46

F. 14.C6:IA^b-3K PDB:4P5T



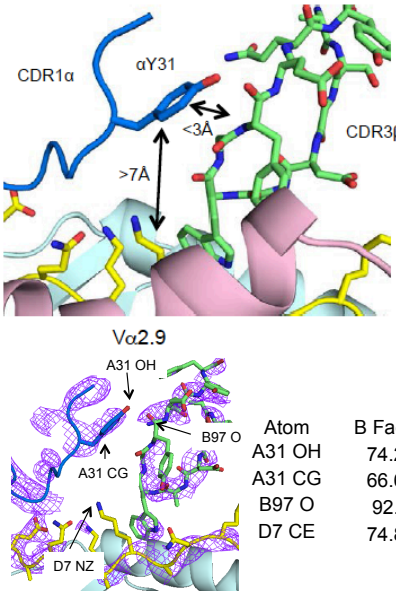
Atom	B Factor
A31 CG	26.38
A31 CZ	29.33
B97 CB	32.53
D-19 CD	24.43

G. 172.10:IA^u-MBP PDB:1UH3



Atom	B Factor
A31 CE2	67.71
B100 CA	66.06
P5 NH2	48.04

H. 2W20.4:IA^b-3K PDB:3C6L



Atom	B Factor
A31 OH	74.28
A31 CG	66.64
B97 O	92.4
D7 CE	74.81

FIGURE S2. Example of electron density for J809.B5, 14.C6 and J809.B5 Y31A TCRs bound to IA^b-3K, and the Relative Positioning of CDR1α residue Y31 to CDR3β loops in several crystal structure of $V\alpha2^+$ TCRs bound to pMHC ligands. Electron density was contoured at 1.5 sigma from $|2F_{obs}-F_{calc}|a_{calc}$ map (grey) calculated from portions of the models. (**A**) J809.B5 CDR3α loop and 3K peptide with IA^b αβ chains omitted. (**B**) J809.B5 CDR1α and CDR3β loops (**C**) 14.C6 CDR3α loop and 3K peptide with IA^b αβ chains omitted (**D**) J809.B5 Y31A CDR1α and CDR3β loops. J809.B5 TCRα chain in orange, TCRβ chain in green, 14.C6 TCRα chain in cyan, J809.B5 Y31A TCRα chain in purple, TCRβ chain in brown and the 3K peptide in yellow. (**E-H**) The TCR CDR1α residue Y31 is positioned within 4Å of the CDR3β loop in several co-crystal structures of $V\alpha2^+$ TCRs bound to pMHC ligands, while being at least 7Å away from the peptide (**E**) J809.B5 TCR bound to IA^b-3K, CDR1α loop is colored orange, CDR3β loop is colored green; PDB code 4P23. (**F**) 14.C6 TCR bound to IA^b-3K, CDR1α loop is colored cyan, CDR3β loop is colored magenta; PDB code 4P5T. (**G**) 172.10 TCR bound to IA^u-MBP, CDR1α loop is colored blue, CDR3β loop is colored mint green. PDB code 1U3H (**H**) 2W1S 20.4 TCR bound to IA^b-3K, CDR1α loop is colored blue, CDR3β loop is colored mint green; PDB code 3C6L. To assess the positioning of the atoms utilized for the distance measurements between the CDR1α Y31 residue and the CDR3β loop, as well as to the peptide, composite omit maps were generated with the CDR1α loop, the CDR3β loop and the peptide omitted from each of the models. Fo-Fc maps for the omitted regions are shown. Clear density is observed for all CDR1α Y31 side chains suggesting its position is defined by the data for all the models. Clear density is also observed for the other atoms used for the distance measurement, except for the D7 NZ atom in the peptide lysine in 36CL. However, possible alternate rotomers of the lysine side chains would still place the side chain >7Å away from the CDR1α Y31 side chain suggesting that no direct contacts are formed between the Y31 residue and the peptide. The B factors associated with each atom used for the distance measurements are listed.

Supplementary Table S1. Primary data for 14.C6 footprint analysis

	J809.B5				J809.H1				14.C6			
	K_d	SEM	ΔΔG	SEM	K_d	SEM	ΔΔG	SEM	K_d	SEM	ΔΔG	SEM
IA^b 3K	6	0.0	0	0	27	0	0	0	6	0.8	0.0	0.0
αK39A	45	0.2	1.2	0	218	13	1.3	0	41	2.2	1.2	0.0
αD55A	32	3.5	1	0.1	219	10	1.3	0	17	6.7	0.5	0.2
αQ57A	31	4.6	0.9	0.1	26	0.1	0	0	6	0.6	0.0	0.0
αL60A	30	5.1	0.9	0.1	137	22	0.9	0.1	33	5.1	1.0	0.1
αQ61A	151	0.0	1.9	0	35	3.1	0.1	0	6	0.8	0.0	0.0
αN62A	77	0.1	1.5	0	238	12	1.3	0	75	2.2	1.3	0.1
αV65A	43	1.7	1.1	0	73	13	0.6	0.1	26	6.2	0.8	0.2
αK67A	19	3.3	0.7	0.1	84	10	0.7	0.1	32	5.1	1.0	0.1
αH68A	17	3.1	0.6	0.1	85	8.4	0.7	0	33	5.0	1.0	0.1
αV72A	6	0.2	0	0	28	0.6	0	0	6	0.8	0.0	0.0
αK75A	8	0.5	0.2	0	34	2.2	0.1	0	11	3.7	0.3	0.2
P-2A	6	0.5	0	0	26	0.4	0	0	6	0.6	0.0	0.0
P-1A	43	0.7	1.1	0	284	10	1.4	0	36	4.4	1.0	0.1
P2A	19	4.2	0.6	0.1	249	13	1.3	0	98	2.9	1.8	0.0
P3A	259	0.0	2.2	0	240	13	1.3	0	173	3.4	2.1	0.0
P5A	458	0.0	2.5	0	177	16	1.1	0.1	82	2.5	1.7	0.0
P7A	6	0.1	0	0	27	0.5	0	0	6	0.7	0.0	0.0
P8A	7	0.6	0.1	0	31	1.7	0.1	0	9	2.3	0.2	0.1
P10A	6	0.2	0	0	28	0.4	0	0	7	1.0	0.0	0.0
P11A	8	0.7	0.1	0	28	1	0	0	6	0.7	0.0	0.0
βE59A	7	0.9	0.1	0.1	31	2.3	0.1	0	9	2.5	0.2	0.1
βY60A	9	1.3	0.2	0.1	31	1.3	0.1	0	8	1.3	0.1	0.1
βS63A	6	0.5	0	0	27	0.7	0	0	6	0.7	0.0	0.0
βQ64A	8	0.8	0.1	0.1	30	1.4	0.1	0	6	0.8	0.0	0.0
βP65A	7	1.0	0.1	0.1	28	7.8	0	0.1	6	0.6	0.0	0.0
βE66A	8	0.7	0.1	0.1	33	3.8	0.1	0.1	17	6.7	0.5	0.2
βI67A	8	1.3	0.2	0.1	44	7.4	0.3	0.1	7	0.9	0.0	0.0
βE69A	8	0.9	0.2	0.1	65	11	0.5	0.1	33	5.1	1.0	0.1
βR70A	198	0.0	2	0	294	12	1.4	0	199	3.5	2.2	0.1
βR72A	7	0.5	0	0	30	0.3	0.1	0	6	0.6	0.0	0.0
βD76A	7	0.7	0	0.1	27	1.1	0	0	6	0.6	0.0	0.0
βT77A	6	0.3	0	0	129	22	0.5	0.1	24	6.6	0.7	0.2
βR80A	7	0.3	0	0	28	0.5	0	0	6	0.7	0.0	0.0
βH81A	8	1.4	0.2	0.1	123	16	0.9	0.1	23	6.7	0.7	0.2
Eβ84A	7	0.5	0.1	0	32	2.5	0.1	0	6	0.8	0.0	0.0

Relative equilibrium affinities (K_d) and change in Gibbs free energy (ΔΔG) for J809.B5, J809.H1 and 14.C6 TCRs binding alanine substituted I-A^b 3K complexes. For each value the associated standard error of the mean (sem) is also reported. Data for the J809.B5 and J809.H1 TCRs has been reported previously in (Stadinski et al., 2011).

Table S2. EC_{50} values for T cells and peptides analyzed in this study

T cell	Peptide	MHC	EC_{50} ng/ml	SEM
J809.B5	3K	IA^b	2	0.4
J809.B5	FMRKA	IA^b	> 100,000	0
J809.B5 αY31A	3K	IA^b	312	121
J809.B5 αY31A	FMRKA	IA^b	1679	138
J809.B5 βN29A	3K	IA^b	58	19
J809.B5 βY46A	3K	IA^b	2107	120
J809.B5 βY848A	3K	IA^b	> 100,000	NA
J809.B5 βE54A	3K	IA^b	> 100,000	NA
J809.B5	3K	IA^b αK39A	> 100,000	NA
J809.B5	3K	IA^b αQ57A	376	164
J809.B5	3K	IA^b αQ61A	> 100,000	NA
J809.H1	3K	IA^b	12	5
J809.H1	RCKST	IA^b	3340	2577
J809.H1 αY31A	3K	IA^b	1	0
J809.H1 αY31A	RCKST	IA^b	4258	1487
J809.H1 βN29A	3K	IA^b	> 100,000	NA
J809.H1 βY46A	3K	IA^b	66	1
J809.H1 βY848A	3K	IA^b	> 100,000	NA
J809.H1 βE54A	3K	IA^b	2916	0
J809.H1	3K	IA^b αK39A	38,000	2,300
J809.H1	3K	IA^b αQ57A	0.3	0.2
J809.H1	3K	IA^b αQ61A	0.5	0.2
14.C6	3K	IA^b	1	1
14.C6	QKKLK	IA^b	70	15
14.C6 αY31A	3K	IA^b	0.8	0.5
14.C6 αY31A	QKKLK	IA^b	3	2
14.C6 βN29A	3K	IA^b	49	14
14.C6 βY46A	3K	IA^b	1	1
14.C6 βY848A	3K	IA^b	76	4
14.C6 βE54A	3K	IA^b	1	0
14.C6	3K	IA^b αK39A	7.9	1.4
14.C6	3K	IA^b αQ57A	0.2	0.004
14.C6	3K	IA^b αQ61A	0.5	0.15
J809.G3	3K	IA^b	1	0
J809.G3 αY31A	3K	IA^b	> 100,000	NA
J809.G3	YTRRT	IA^b	3532	1469
J809.G3 αY31A	YTRRT	IA^b	4254	2167
13.B1	3K	IA^b	12	5
13.B1 αY31A	3K	IA^b	964	346
13.B1	SKKRP	IA^b	40764	26032
13.B1 αY31A	SKKRP	IA^b	289	100
13.D5	3K	IA^b	153833	8268
13.D5 αY31A	3K	IA^b	20151	13348
13.D5	YTRRT	IA^b	5958	3577
13.D5 αY31A	YTRRT	IA^b	3637	1284
14.A6	3K	IA^b	10	4
14.A6 αY31A	3K	IA^b	9	7
14.A6	SKKRP	IA^b	217600	
14.A6 αY31A	SKKRP	IA^b	1222	452