JBC Papers in Press. Published on November 18, 2011 as Manuscript M111.289488
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M111.289488

A SINGLE AUTOIMMUNE T-CELL RECEPTOR RECOGNIZES OVER A MILLION DIFFERENT PEPTIDES*

Linda Wooldridge[1#], Julia Ekeruche-Makinde[1#], Hugo A. van den Berg[2#], Anna Skowera[3,4], John J. Miles[1], Mai Ping Tan[1], Garry Dolton[1], Mathew Clement[1], Sian Llewellyn-Lacey[1], David A. Price[1], Mark Peakman[3,4] and Andrew K. Sewell[1¶]

[1] Institute of Infection and Immunity, Cardiff University School of Medicine, Henry Wellcome Building, Heath Park, Cardiff CF14 4XN, UK. [2] Mathematics Institute, University of Warwick, Coventry CV4 7AL, UK. [3] Department of Immunobiology, King's College London, London SE1 9RT, UK. [4] NIHR Biomedical Research Centre at Guy's & St Thomas' NHS Foundation Trust and King's College London, UK.

Running Title: The promiscuity of an autoimmune TCR
To whom correspondence should be addressed: Andrew K. Sewell, Cardiff University School of Medicine, Henry Wellcome Building, Heath Park, Cardiff CF14 4XN, UK. Tel.: 44-29-206-87055; Fax: 44-29-206-87007; E-mail: sewellak@cardiff.ac.uk.

Keywords: T-cell receptor; crossreactivity; degeneracy; promiscuity; T-cell activation.

Background: How does a limited pool of $<10^8$ T-cell receptors (TCRs) provide immunity to $>10^{15}$ antigens?
Result: A single TCR can respond to over one million different decamer peptides.
Conclusion: This unprecedented level of receptor promiscuity explains how the naive TCR repertoire achieves effective immunity.
Significance: The enormous potential of TCR degeneracy to be the root cause of autoimmune disease.

SUMMARY

The T-cell receptor (TCR) orchestrates immune responses by binding to foreign peptides presented at the cell surface in the context of major histocompatibility complex (MHC) molecules. Effective immunity requires that all possible foreign peptide-MHC (pMHC) molecules are recognized, or risks leaving holes in immune coverage that pathogens could quickly evolve to exploit. It is unclear how a limited pool of $<10^8$ human TCRs can successfully provide immunity to the vast array of possible different peptides that could be produced from 20 proteogenic amino acids and presented by self MHC molecules ($>10^{15}$ distinct pMHCs). One possibility is that T-cell immunity incorporates an extremely high level of receptor degeneracy, enabling each TCR to recognize multiple peptides. However, the extent of such TCR degeneracy has never been fully quantified. Here, we perform a comprehensive experimental and mathematical analysis to reveal that a single patient-derived autoimmune $CD8^+$ T-cell clone of pathogenic relevance in human Type I diabetes recognizes over a million distinct decamer peptides in the context of a single MHC class I molecule. A large number of peptides that acted as substantially better agonists than the wildtype 'index' preproinsulin-derived peptide (ALWGPDPAAA) were identified. The RQFGPDFPTI peptide (sampled from $>10^8$ peptides) was >100-fold more potent than the index peptide despite differing from this sequence at 7 of 10 positions. Quantification of this previously unappreciated high level of T-cell crossreactivity represents an important step towards understanding the system requirements for adaptive immunity and highlights the enormous potential of TCR degeneracy as a determinant of as a causative factor in autoimmune disease.

The mammalian T-cell receptor (TCR) orchestrates immune responses by binding to foreign peptides presented at the cell surface in the context of major histocompatibility complex (MHC) molecules. Recognition is mediated by the highly variable complementarity determining regions of the αβ TCR (1,2). *A priori*, the TCR

repertoire must be broad enough to respond to all foreign peptides that can bind to self MHC molecules (3). If this were not the case, then pathogens could rapidly evolve to exploit such deficiencies in immune coverage. Current estimates of human αβ TCR diversity suggest that there are $<10^8$ different antigen receptors in the naïve T-cell pool (4), a number that is dwarfed by the potential number of antigenic peptide-MHC (pMHC) molecules that could be encountered. Although next generation sequencing technologies may lead to an increased estimate of TCR diversity, such future revisions are unlikely to alter the fact that a relatively small number of TCRs must, and do, provide effective immune recognition of all peptides that can be generated from 20 proteogenic amino acids and that also bind self MHC molecules ($>10^{15}$ distinct pMHCs). This represents a particular biochemical challenge to the immune system because the TCR, unlike the B cell receptor, cannot undergo affinity maturation in the form of somatic hypermutation.

It is unclear how the limited naive T-cell pool responds to a multitude of ligands that it has never encountered before and cannot adapt to at the protein sequence level. One possibility is that T-cell immunity inherently features an extremely high level of receptor degeneracy, enabling each TCR to recognize multiple peptides. However, clonal selection theory suggests that individual T-cells are specific for a single pMHC molecule with recognition of alternative ligands unlikely. In contrast, studies published in the 1990s demonstrated that T-cells can recognize several peptides (5-11). Since then, observations of TCR degeneracy have continued to accumulate in the literature (12-19). In addition, other studies have shown that TCRs can recognize distinct peptides in the context of non-self MHC, a phenomenon known as alloreactivity (20-25). The majority of previous studies of TCR degeneracy have examined sets of between 2 and 200 peptides, with one recent study examining ~4000 peptides (19). Given that the entire universe of decamer peptides alone comprises $>10^{13}$ distinct amino acid sequences, the proportion of the peptide universe at this length that has been examined in the most comprehensive study to date (19) remains extremely small (<0.000000045%).

The aim of this study was to probe the entire decamer peptide universe systematically in order to quantify how many peptides a single TCR can recognize in the context of a single MHC molecule. We demonstrate an unprecedented level of receptor degeneracy that allows a single monoclonal T-cell to respond to over one million distinct peptides. As such, the TCR represents one of the most remarkable biological receptors and by far the most promiscuous known.

EXPERIMENTAL PROCEDURES

Generation and maintenance of an autoimmune CD8$^+$ T-cell clone - The 1E6 CD8$^+$ T-cell clone specific for the HLA A*0201-restricted autoantigen preproinsulin peptide ALWGPDPAAA (PPI_{15-24}) was generated as described previously (26).

Decamer combinatorial peptide library (CPL) scan - The decamer combinatorial peptide library (Pepscan) contains a total of 9.36×10^{12} ($=(10+19)\times19^9$) different decamer peptides and is divided into 200 different peptide mixtures (see Figure 1). In every peptide mixture, one position has a fixed L-amino acid residue and all other positions are degenerate, with the possibility of any one of 19 natural L-amino acids being incorporated in each individual position (cysteine is excluded). Each library mixture consists of $3.2\text{x}10^{11}$ (19^9) different decamer peptides in approximately equimolar concentrations. For CPL screening, 1E6 CD8$^+$ T-cells were washed and rested overnight in RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM L-glutamine and 2% heat inactivated fetal calf serum (all Life Technologies). In 96-well U-bottom plates, $6\text{x}10^4$ C1R-A2 cells were incubated with various peptide library mixtures (at 100 μg/ml) in duplicate for 2 hours at 37°C. Following peptide pulsing, $3\text{x}10^4$ 1E6 CD8$^+$ T-cells were added and the assay was incubated overnight at 37°C. Subsequently, the supernatant was harvested and assayed for MIP1β by ELISA according to the manufacturer's instructions (R&D Systems).

CD8$^+$ T-cell effector function assays: MIP1β ELISA - Individual peptides were assayed for agonist activity by MIP1β ELISA as described above. Functional sensitivity is expressed by the $p\text{EC}_{50}$ of each peptide with respect to the TCR. This is defined as minus 1 times the base-10 logarithm (p) of the 50% efficacy concentration

(EC_{50}); a greater functional sensitivity is indicated by a larger pEC_{50} value, which was estimated as described in the supplemental equations.

Overview of sampling approaches used to quantify TCR degeneracy - Although the TCR has an appreciable degeneracy, it is still specific enough that sampling peptides at random will most likely result in less than ~10 strong agonists for every ~10,000 peptides sampled. For this reason, we employed conditioned sampling. In one approach, we sampled from motif-restricted peptide sets. This results in lower bound estimates of the actual number of agonist ligands, in that any agonist not fitting the prescribed motif is excluded from the sample. In general, more stringent motifs exclude more agonists but provide better resolution at the high pEC_{50} end of the curve, and *vice versa*; for this reason, a range of motifs of varying stringency was used. In a second approach (CPL-based importance sampling), we sampled from the entire peptide universe with bias towards peptides that were likely to elicit a response, then estimated a true distribution by applying a correction weighting to the observations (i.e. dividing back by the bias).

Sampling equations: Motif-restricted sampling - A sampling motif specifies, for each of the m positions in an m-mer peptide, one or more amino acid residues that may occur at that position. If n_p alternative residues have been specified at position p, the probability that a sampled peptide has a given residue at position p equals $1/n_p$ if the given residue is one of the n_p given alternatives, and zero otherwise. Consider a sample of n peptides, consisting of peptides $P^{[1]}$, $P^{[2]}$, ..., $P^{[n]}$, with measured functional sensitivities $pEC_{50}^{[1]}$, $pEC_{50}^{[2]}$, ..., $pEC_{50}^{[n]}$. The crossreactivity of the TCR is expressed by the number of peptides whose pEC_{50} exceeds a given value ω. The degeneracy of the TCR is then represented by determining this number for a range of ω values, estimated as follows:

$$\mathcal{N}[pEC_{50} > \omega] \geq \frac{\sum_{j=1}^{n} \mathcal{I}(pEC_{50}^{[j]} > \omega)}{n} \prod_{p=1}^{m} n_p \qquad (1)$$

Here, $\mathcal{N}[pEC_{50}>\omega]$ is the number of peptides that have a pEC_{50} for the TCR that exceeds ω, and

$$\mathcal{I}(pEC_{50}^{[j]} > \omega) = 1$$

if sample peptide j has a pEC_{50} that exceeds ω;

$$\mathcal{I}(pEC_{50}^{[j]} > \omega) = 0$$

otherwise. The sampling motif typically excludes a number of agonists (unless $n_p = 20$ at every position, i.e. the "universal motif" that was not used). This exclusion means that the quantity on the right in eqn (1) always underestimates the true $\mathcal{N}[pEC_{50}>\omega]$. Thus, this means that the motif-based method provides a lower bound to TCR degeneracy, i.e. a conservative estimate.

Sampling equations: CPL-based importance sampling - The idea of sampling motifs can be generalized to the well known strategy of importance sampling by specifying, for all positions and amino acids, the probability that a given amino acid occurs at a given position. Then, the probability of drawing a peptide P that has amino acid residue $P(p)$ at position p is given by:

$$\mathbb{P}[P] = \mathbb{P}[P(1)P(2)\cdots P(m)] = \prod_{p=1}^{m} \mathbb{P}[P(p)] \qquad (2)$$

where $\mathbb{P}[P(p)]$ is the probability of drawing amino acid P at position p. Standards rules of probability stipulate that for each fixed p, the $\mathbb{P}[P(p)]$ sum to unity over the 20 amino acids. The CPL was used to generate distributions that could be expected to bias the sample towards good agonists. However, to correct for the bias in the estimate, observations must be weighted by the reciprocal of the bias. Accordingly, degeneracy was estimated as follows:

$$\mathcal{N}[pEC_{50} > \omega] \approx n \exp\{H\} \frac{\sum_{j=1}^{n} \mathbb{P}[P^{[j]}]^{-1} \mathcal{I}(pEC_{50}^{[j]} > \omega)}{\sum_{j=1}^{n} \mathbb{P}[P^{[j]}]^{-1}} \qquad (3)$$

where H is the entropy of the sampling distribution, defined by:

$$H = -\sum_{p=1}^{m}\sum_{P=1}^{20} \mathbb{P}\left[P(p)\right] \ln \mathbb{P}\left[P(p)\right] . \quad (4)$$

The factor $n\exp[H]$ in eqn (3) is an estimate of the effective sample size, based on a standard result for the probability of sampling a fixed set of n peptides. According to this well-known theorem (27) this probability is close to $\exp\{-n\,H\}$. On the other hand, in unbiased sampling from a set of size N, the probability of obtaining any given n-element sample is exactly equal to N^{-n}. Together, these observations indicate that $\exp\{H\}$ can be interpreted as the "diameter" of the population from which the peptide is drawn. Consequently, an n-element sample of m-mer peptides probes an effective set size of

$$20^m \left(1 - \left(1 - \exp\{H\}20^{-m}\right)^n\right) \approx n\exp\{H\}; \quad (5)$$

the approximation is accurate when n is tiny compared to $\exp\{-H\}$, as is the case in the experiments reported here.

RESULTS

CPL screening reveals the potential for TCR degeneracy - To determine the extent of T-cell crossreactivity, we probed the peptide recognition degeneracy of the autoimmune $CD8^+$ T-cell clone IE6 using a CPL comprising 9.36×10^{12} different decamer peptides (Figure 1). Using this approach, we were able to scan every amino acid at every position of the peptide within a random residue 'backbone' and build a detailed picture of the molecular landscape preferred by the 1E6 TCR. The 1E6 clone was generated from a patient with Type 1 diabetes and is the only documented example of an autoreactive $CD8^+$ T-cell that can kill human pancreatic islet β-cells (26). Killing is mediated via recognition of residues 15-24 (ALWGPDPAAA) of the autoantigen preproinsulin bound to human leukocyte antigen (HLA) A*0201 on the β-cell surface. The majority of HLA $A*0201^+$ patients with Type I diabetes recognize the preproinsulin 15-24 epitope (26), and HLA A*0201 is known to confer an increased risk of disease (28,29). The number of amino acids that were recognized by the 1E6 clone was restricted in the central region of the peptide (residues 4-6), suggesting that this TCR makes the majority of its peptide contacts with these residues. In contrast, recognition was highly degenerate at the remaining positions. This degeneracy was confirmed by the ability of 1E6 T-cells to recognize robustly a panel of peptides with any of the 20 natural proteogenic L-amino acids at peptide position 8 (Figure S1), with half of the substitutions leading to increased levels of functional sensitivity. Similar results were obtained with corresponding scans at other degenerate positions (data not shown). The CPL scan results also revealed that the index peptide is suboptimal in all positions outside the central region (residues 4-6). Thus, positional peptide degeneracy is extreme at 7/10 positions, hinting at the potential for a single TCR to recognize a multitude of different amino acid combinations.

Quantifying the number of decamer peptides that can be recognized by a single TCR - We next sought to quantify the number of decamer peptides that can be recognized by the 1E6 clone. The total number of decamers that can be made by combinations of the 20 natural proteogenic L-amino acids is 20^{10} (1.02×10^{13}). Only a small proportion (~1-3%) of all peptides are predicted to bind any given MHC (3,19), although our own experiments predict that this percentage could be far greater for HLA A*0201 (data not shown). Even with the most conservative estimates of MHC binding (19), the number of potential antigenic HLA A*0201-restricted decamer peptides is still extremely large (1.02×10^{11}) and precludes screening all possibilities in T-cell recognition assays. To overcome this problem, we screened sets of 30 peptides sampled (Mathematica®; Figure S5) from larger motif-restricted or CPL-based importance-sampled sets differing in total size from 225 to 1.66×10^8 individual peptides as described below.

Quantifying T-cell crossreactivity using motif-restricted sampling - Motif-restricted peptide sets were designed based on CPL evidence for amino acid preference at each position of the peptide. First, we screened 30 peptides sampled at random from a total set size of 225 (Motif I: RQWGPDP{A/C/D/F/H/I/K/L/M/N/P/R/S/V/Y}{A/C/G/H/I/K/L/M/N/P/Q/R/S/T/V}A; Figure 2 & S2). Values of pEC_{50} (minus 1 times the base-10 logarithm of the 50% efficacy concentration) as a measure of functional sensitivity were estimated

for all peptides using simultaneous curve fitting as described in the supplementary equations (eqn S1; Figure S6 & Table S3). Accordingly, increases in functional sensitivity translate into increases in the pEC_{50} value. The 1E6 clone recognized all peptides within this subset efficiently, with 24 of 30 peptides eliciting greater levels of functional sensitivity than the index peptide. A further 30 peptides were sampled at random from a total set size of 5776 (Motif II: RQWGP{D/F}{P/F}xx{A/I/L/V}, where x denotes any one of the amino acids excluding cysteine; Figure 3 & S2, Table S3). One peptide from this subset was recognized poorly; 16 were recognized with pEC_{50} >7, with a total of 8 peptides recognized more efficiently than the index peptide (Figure 3 & S2). A further two motif-restricted sets of increasing degeneracy were screened (Motif III: RQxGPDxxxA, total set size 19^4, and Motif IV: xQxGPDxxxV, total set size 19^5; x denotes any one of the amino acids excluding cysteine; Figure 4, 5 & S3, Table S3). The extent of peptide recognition when peptides are sampled at random from large subsets demonstrates the considerable degree of degeneracy exhibited by the clonal 1E6 TCR.

Quantifying T-cell crossreactivity using CPL-based importance sampling - A limitation of sampling from motif-restricted sets is that strong ligands will invariably be excluded, resulting in an under-estimate of the true number of agonists (i.e. a lower bound estimate of the number of different peptides that a single TCR can recognize). In order to obtain a more accurate estimate of true T-cell crossreactivity, we employed CPL-based importance sampling, which makes no assumptions about TCR contact or MHCI binding residues. Importance sampling ensures that every peptide has a chance of being sampled (although cysteine was excluded to avoid the potential for oxidation), but incorporates bias towards strong agonists predicted using CPL scan data. This bias is adjusted for to yield unbiased estimates of agonist numbers (see sampling equations). Raw data from the primary CPL scan were modified as described in Table S1 and subsequently normalized (Table S2) to provide a peptide sampling distribution biasing the sample towards good agonists. The chance of picking a peptide is the product of the normalized weights assigned to each amino acid residue at each given peptide position. Two sets of 30 peptides were drawn from an effective set size of 1.66×10^8 (calculated from the sampling entropy; see sampling equations; eqn 5). Out of a total of 60 peptides, 34 were recognized efficiently with a pEC_{50} >7 and 22 were better agonists than the index peptide (Figure 6, 7 & S4, Table S3). Interestingly, just this set of 60 peptides identified four peptides with functional sensitivities 100-fold better than the index peptide, despite differing from the index peptide sequence (ALWGPDPAAA) at 6 (YQFGPDFPIA, KQFGPDFPTA) or 7 (RQFGPDFPKL, RQFGPDFPTI) positions (Figure 6, 7 & S4, Table S3). Thus, CPL-based importance sampling demonstrates that a large proportion of peptides from a biased set of 1.66×10^8 peptides would be recognized and that these recognized peptides can differ considerably from the index peptide.

A single TCR can recognize over one million different peptides - The pEC_{50} expresses the potency of a ligand, often referred to as 'functional avidity' (Figure S6). Crossreactivity can be quantified precisely by specifying the number of ligands that the TCR recognizes with a pEC_{50} of at least a given value ω. This number decreases as ω increases; an insight into the nature of TCR degeneracy is afforded by plotting this agonist number as a function of ω. Estimation of this number was performed using eqns 1 & 3 (see sampling equations), resulting in Figure 8. The motif-restricted estimate (solid lines in Figure 8) is a lower bound, which becomes tighter as the degeneracy of the motif increases; however, this advantage is offset by the decreasing chances of finding good agonists in a sample of 30 peptides. The estimates based on the biased sampling (dashed lines in Figure 8) indicate that in the order of one million agonists exist for 1E6 that are as least as good as the index peptide. For comparison, the curve derived from TCR activation theory (30,31) is also shown (grey dashed line in Figure 8; eqn S2).

DISCUSSION

Despite the huge potential importance of TCR degeneracy to human health, there has never been a comprehensive attempt to quantify the number of peptides that can be recognized by a

single TCR. To address this issue, we examined the extent of crossreactivity exhibited by a single autoimmune T-cell clone with pathogenic relevance in human Type I diabetes (1E6). Our analysis demonstrates that the 1E6 TCR can recognize ~500 peptides within a factor 2 of the optimal agonist (i.e. peptides that have a functional sensitivity that is at least 50% of the functional sensitivity of the optimal agonist). An estimated 60,000 peptides have a functional sensitivity within a factor 10 of the optimal agonist, and ~1.3×10^6 peptides are within a factor 100 of the optimal agonist. These considerations are especially significant given that the functional sensitivity of 1E6 $CD8^+$ T-cells for the index peptide is at least 100-fold lower than the optimal agonist; this is illustrated by RQFGPDFPTI, the functional sensitivity of which is 100-fold better than the index. Almost 10 million peptides are within a factor 1000 of the optimal agonist, but such weak agonists will not generally be physiologically significant unless presented at very high copy numbers. Taken together, these results indicate that the 1E6 TCR has over a million significant peptide agonists at concentrations with the potential to be physiologically relevant.

When putting TCR degeneracy into perspective, it is important to realize that individual TCRs capable of recognizing 10^6 decamer peptides still only have a less than 1 in 10^7 chance of crossreacting with any peptide chosen at random from the entire decamer peptide universe (~10^{13}). A high level of crossreactivity is therefore amply compatible with the degree of specificity required for self/non-self determination. Furthermore, the number of decamer peptides that it is possible to make from the entire human proteome (excluding post-translational amino acid modifications) is only one millionth of the possible peptide universe at this length. Functional recognition of ~10^6 decamer peptides by a single TCR translates into a frequency of crossreactivity of 1:100,000 (assuming that 1% of peptides bind to MHC), which is likely to be the most accurate estimate of this parameter to date due to the comprehensive nature of our approach. The probability of crossreactivity with any individual peptide is an important consideration in terms of viral escape, bystander activation and autoimmune side effects, and the results presented here fit well with theoretical considerations of T-cell immunity (3). It should be noted, however, that the degeneracy curves are estimates based on samples that constitute a small fraction of the number of possible peptide ligands and, hence, should be considered as depicting an order-of-magnitude estimate that is supported by the lower bounds inferred from motif-based samples.

The 1E6 $CD8^+$ T-cell clone was chosen for these studies to highlight the huge potential for T-cell crossreactivity as a possible cause of autoimmunity. In support of the generality of our findings, we have also observed high levels of degeneracy at some positions in a 9 amino acid residue non-autoimmune epitope (32). Furthermore, the recognition of longer, MHC class II-restricted peptides by $CD4^+$ T-cells with a 'TCR footprint' of similar size could ensure that these cells incorporate the capacity to recognize tens, or possibly even hundreds, of millions of peptides at physiologically relevant surface densities. The reality of T-cell crossreactivity, as quantified here, has far-reaching implications. It provides an explanation for how a limited pool of TCRs can provide the broad antigenic coverage that is required for effective immunity. In addition, the extent of TCR degeneracy suggests that almost any peptide antigen can be improved for any given cognate TCR, in the sense of there being at least one stronger agonist than the original index peptide, thereby providing scope for rational therapeutic interventions based on the directed manipulation of T-cell immunity.

References

1. Rudolph, M. G., and Wilson, I. A. (2002) *Curr Opin Immunol* **14**, 52-65.
2. Rudolph, M. G., Stanfield, R. L., and Wilson, I. A. (2006) *Annu Rev Immunol* **24**, 419-466
3. Mason, D. (1998) *Immunol Today* **19**, 395-404
4. Arstila, T. P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J., and Kourilsky, P. (1999) *Science* **286**, 958-961
5. Wraith, D. C., Bruun, B., and Fairchild, P. J. (1992) *J Immunol* **149**, 3765-3770
6. Bhardwaj, V., Kumar, V., Geysen, H. M., and Sercarz, E. E. (1993) *J Immunol* **151**, 5000-5010
7. Reay, P. A., Kantor, R. M., and Davis, M. M. (1994) *J Immunol* **152**, 3946-3957
8. Evavold, B. D., Sloan-Lancaster, J., Wilson, K. J., Rothbard, J. B., and Allen, P. M. (1995) *Immunity* **2**, 655-663
9. Wucherpfennig, K. W., and Strominger, J. L. (1995) *Cell* **80**, 695-705
10. Hemmer, B., Fleckenstein, B. T., Vergelli, M., Jung, G., McFarland, H., Martin, R., and Wiesmuller, K. H. (1997) *J Exp Med* **185**, 1651-1659
11. Kersh, E. N., Shaw, A. S., and Allen, P. M. (1998) *Science* **281**, 572-575.
12. Kissler, S., Anderton, S. M., and Wraith, D. C. (2002) *J Autoimmun* **19**, 183-193
13. Nino-Vasquez, J. J., Allicotti, G., Borras, E., Wilson, D. B., Valmori, D., Simon, R., Martin, R., and Pinilla, C. (2004) *Mol Immunol* **40**, 1063-1074
14. Crawford, F., Huseby, E., White, J., Marrack, P., and Kappler, J. W. (2004) *PLoS Biol* **2**, E90
15. Lee, J. K., Stewart-Jones, G., Dong, T., Harlos, K., Di Gleria, K., Dorrell, L., Douek, D. C., van der Merwe, P. A., Jones, E. Y., and McMichael, A. J. (2004) *J Exp Med* **200**, 1455-1466
16. Kan-Mitchell, J., Bajcz, M., Schaubert, K. L., Price, D. A., Brenchley, J. M., Asher, T. E., Douek, D. C., Ng, H. L., Yang, O. O., Rinaldo, C. R., Jr., Benito, J. M., Bisikirska, B., Hegde, R., Marincola, F. M., Boggiano, C., Wilson, D., Abrams, J., Blondelle, S. E., and Wilson, D. B. (2006) *J Immunol* **176**, 6690-6701
17. Hausmann, S., Martin, M., Gauthier, L., and Wucherpfennig, K. W. (1999) *J Immunol* **162**, 338-344
18. Dai, S., Huseby, E. S., Rubtsova, K., Scott-Browne, J., Crawford, F., Macdonald, W. A., Marrack, P., and Kappler, J. W. (2008) *Immunity* **28**, 324-334
19. Ishizuka, J., Grebe, K., Shenderov, E., Peters, B., Chen, Q., Peng, Y., Wang, L., Dong, T., Pasquetto, V., Oseroff, C., Sidney, J., Hickman, H., Cerundolo, V., Sette, A., Bennink, J. R., McMichael, A., and Yewdell, J. W. (2009) *J Immunol* **183**, 4337-4345
20. Udaka, K., Tsomides, T. J., and Eisen, H. N. (1992) *Cell* **69**, 989-998
21. Reiser, J. B., Darnault, C., Guimezanes, A., Gregoire, C., Mosser, T., Schmitt-Verhulst, A. M., Fontecilla-Camps, J. C., Malissen, B., Housset, D., and Mazza, G. (2000) *Nat Immunol* **1**, 291-297
22. Archbold, J. K., Macdonald, W. A., Miles, J. J., Brennan, R. M., Kjer-Nielsen, L., McCluskey, J., Burrows, S. R., and Rossjohn, J. (2006) *J Biol Chem* **281**, 34324-34332
23. Felix, N. J., and Allen, P. M. (2007) *Nat Rev Immunol* **7**, 942-953
24. Archbold, J. K., Ely, L. K., Kjer-Nielsen, L., Burrows, S. R., Rossjohn, J., McCluskey, J., and Macdonald, W. A. (2008) *Mol Immunol* **45**, 583-598
25. Macdonald, W. A., Chen, Z., Gras, S., Archbold, J. K., Tynan, F. E., Clements, C. S., Bharadwaj, M., Kjer-Nielsen, L., Saunders, P. M., Wilce, M. C., Crawford, F., Stadinsky, B., Jackson, D., Brooks, A. G., Purcell, A. W., Kappler, J. W., Burrows, S. R., Rossjohn, J., and McCluskey, J. (2009) *Immunity* **31**, 897-908
26. Skowera, A., Ellis, R. J., Varela-Calvino, R., Arif, S., Huang, G. C., Van-Krinks, C., Zaremba, A., Rackham, C., Allen, J. S., Tree, T. I., Zhao, M., Dayan, C. M., Sewell, A. K., Unger, W., Drijfhout, J. W., Ossendorp, F., Roep, B. O., and Peakman, M. (2008) *J Clin Invest* **118**, 3390-3402
27. Karlin, S., and Taylor, H. M. (1974) *Academic Press, New York*, pp. 495ff

28. Nejentsev, S., Howson, J. M., Walker, N. M., Szeszko, J., Field, S. F., Stevens, H. E., Reynolds, P., Hardy, M., King, E., Masters, J., Hulme, J., Maier, L. M., Smyth, D., Bailey, R., Cooper, J. D., Ribas, G., Campbell, R. D., Clayton, D. G., and Todd, J. A. (2007) *Nature* **450**, 887-892
29. Todd, J. A., Walker, N. M., Cooper, J. D., Smyth, D. J., Downes, K., Plagnol, V., Bailey, R., Nejentsev, S., Field, S. F., Payne, F., Lowe, C. E., Szeszko, J. S., Hafler, J. P., Zeitels, L., Yang, J. H., Vella, A., Nutland, S., Stevens, H. E., Schuilenburg, H., Coleman, G., Maisuria, M., Meadows, W., Smink, L. J., Healy, B., Burren, O. S., Lam, A. A., Ovington, N. R., Allen, J., Adlem, E., Leung, H. T., Wallace, C., Howson, J. M., Guja, C., Ionescu-Tirgoviste, C., Simmonds, M. J., Heward, J. M., Gough, S. C., Dunger, D. B., Wicker, L. S., and Clayton, D. G. (2007) *Nat Genet* **39**, 857-864
30. van den Berg, H. A., Rand, D. A., and Burroughs, N. J. (2001) *J Theor Biol* **209**, 465-486
31. van den Berg, H. A., and Rand, D. A. (2007) *Immunol Rev* **216**, 81-92
32. Wooldridge, L., Laugel, B., Ekeruche, J., Clement, M., van den Berg, H. A., Price, D. A., and Sewell, A. K. (2010) *J Immunol* **185**, 4625-4632

Acknowledgements - We thank Don Mason and Bent Jakobsen for critical reading of the manuscript and David Rand for careful scrutiny of the mathematics.

FOOTNOTES

[*]This work was supported by the Biotechnology and Biological Sciences Research Council (Grant BB/H001085/1) and the UK Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London (AS and MP). LW is a Wellcome Trust Clinical Intermediate Fellow (Grant WT079848MA). AS, GD, DAP, MP and AKS are funded by the Juvenile Diabetes Research Foundation (Grants 7-2005-877, 1-2007-1803 and 17-2009-806). JJM is supported by a Welsh Office of Research and Development (WORD) Translational Fellowship.

[#]LW, JE-M and HvdB contributed equally to this manuscript.

[¶]To whom correspondence should be addressed. Tel.: 44-29-206-87055; Fax: 44-29-206-87007; E-mail: sewellak@cardiff.ac.uk.

FIGURE LEGENDS

Figure 1: Decamer combinatorial peptide library (CPL) scan of the 1E6 $CD8^+$ T-cell clone. **A**. 6×10^4 C1R-A2 B-cells were pulsed in duplicate with each mixture from a decamer CPL (100 µg/ml) at 37°C. After 2 hours, 3×10^4 1E6 $CD8^+$ T-cells were added and incubated overnight. Supernatant was harvested and assayed for MIP1β. **B**. Data from panel A displayed as a box plot summary.

Figure 2: Recognition of 30 peptides sampled at random from a large peptide set (motif: RQWGPDP{A/C/D/F/H/I/K/L/M/N/P/R/S/V/Y}{A/C/G/H/I/K/L/M/N/P/Q/R/S/T/V}A; total set size = 225). 6×10^4 C1R-A2 B-cells were pulsed with peptides at various concentrations. Assays were performed as in Figure S1. Each panel displays titrations of 5 different peptides relative to index. Panel A shows titrations of peptides with the highest functional sensitivities; panel F shows titrations of peptides with the lowest functional sensitivities. Standard deviation from the mean of two replicates is shown. $p\mathrm{EC}_{50}$ values for each peptide are displayed in Table S3.

Figure 3: Recognition of 30 peptides sampled at random from a large peptide set (motif: RQWGP{D/F}{P/F}xx{A/I/L/V}; total set size = 5776). Details as described for Figure 2.

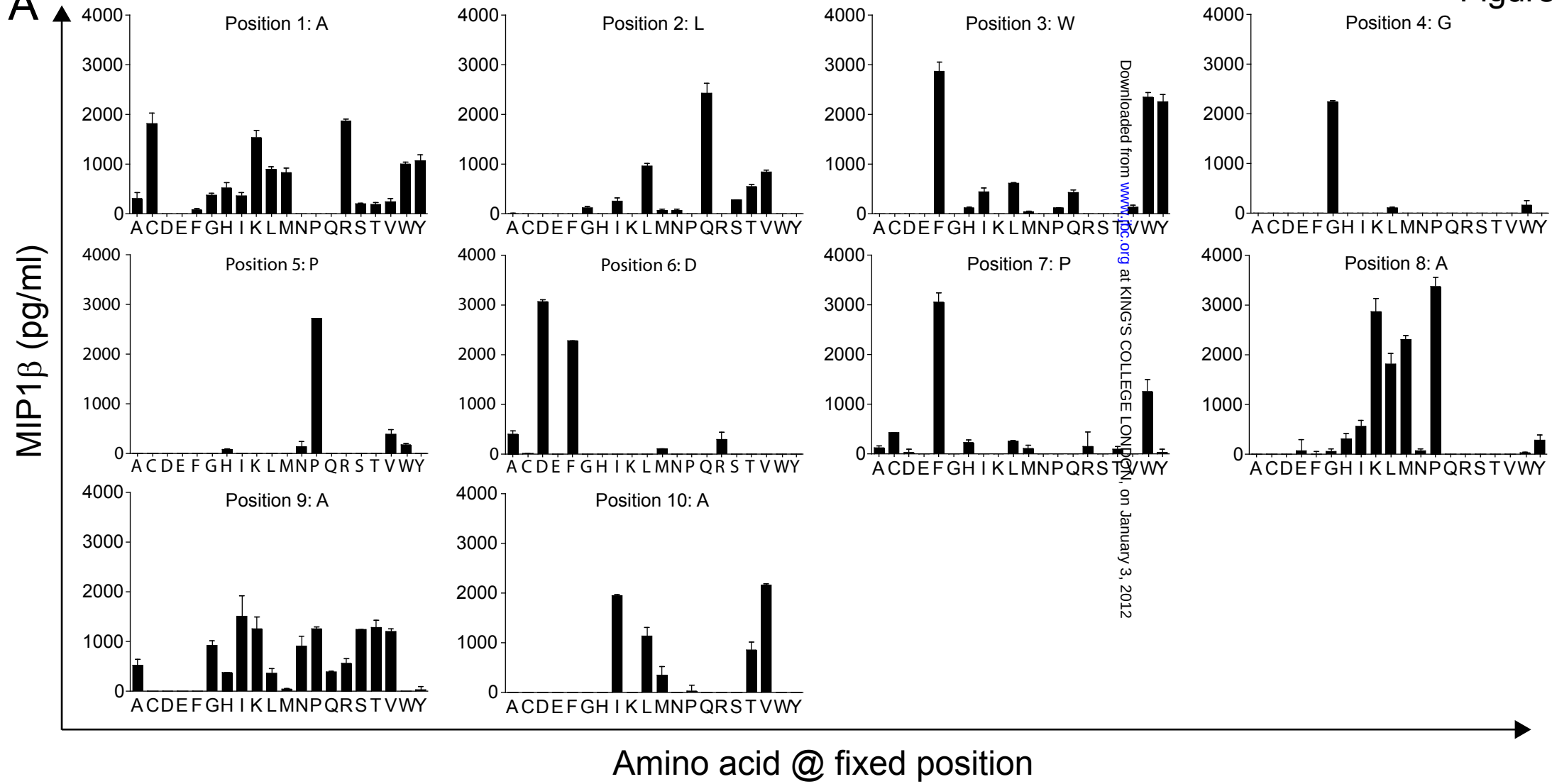
Figure 4: Recognition of 30 peptides sampled at random from a large peptide set (motif: RQxGPDxxxA; total set size = 19^4). Details as described for Figure 2.

Figure 5: Recognition of 30 peptides sampled at random from a large peptide set (motif: xQxGPDxxxV; total set size = 19^5). Details as described for Figure 2.

Figure 6: Recognition of 30 peptides drawn from a CPL-based importance sampling set with effective size = 1.66×10^8 (calculated from the sampling entropy) (1st set). Details as described for Figure 2.

Figure 7: Recognition of 30 peptides drawn from a CPL-based importance sampling set with effective size = 1.66×10^8 (calculated from the sampling entropy) (2nd set). Details as described for Figure 2.

Figure 8: 1E6 $CD8^+$ T-cells can recognize more than a million different decamer peptides. I: RQWGPDP{A/C/D/F/H/I/K/L/M/N/P/R/S/V/Y}{A/C/G/H/I/K/L/M/N/P/Q/R/S/T/V}A (set size 225; 30 peptides sampled at random); II: RQWGP{D/F}{P/F}xx{A/I/L/V} (set size 5776; 30 peptides sampled at random); III: RQxGPDxxxA (set size 19^4; 30 peptides sampled at random); IV: xQxGPDxxxV (set size 19^5; 30 peptides sampled at random). In the motifs, x denotes any one of the 19 amino acids excluding cysteine. Va,b: two replicates of a biased sampling set (effective set size 1.66×10^8, calculated from the sampling entropy); each set of 30 peptides was sampled with bias towards strong agonists, where the bias weights were based on the primary CPL scan. Relative functional sensitivities ($p\mathrm{EC}_{50} - p\mathrm{EC}_{50}$ index) are plotted as survivor curves. Grey dashed line: theoretical curve (eqn S2, with $\alpha = 4.5$, $\beta = 10$, $\gamma = 2$, $N_0 = 20^8$ anchorable peptides). The biased samples (black dashed and dotted lines) estimate the TCR degeneracy spectrum, whereas the motif-based samples (black solid lines) provide a lower bound to the TCR degeneracy spectrum.



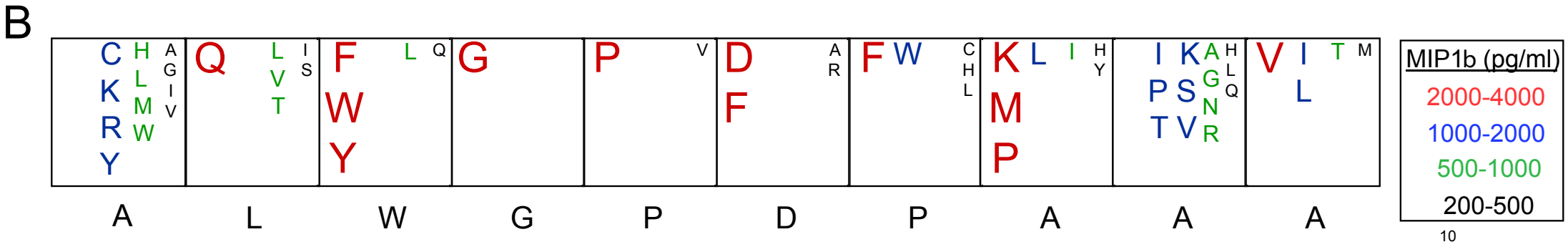
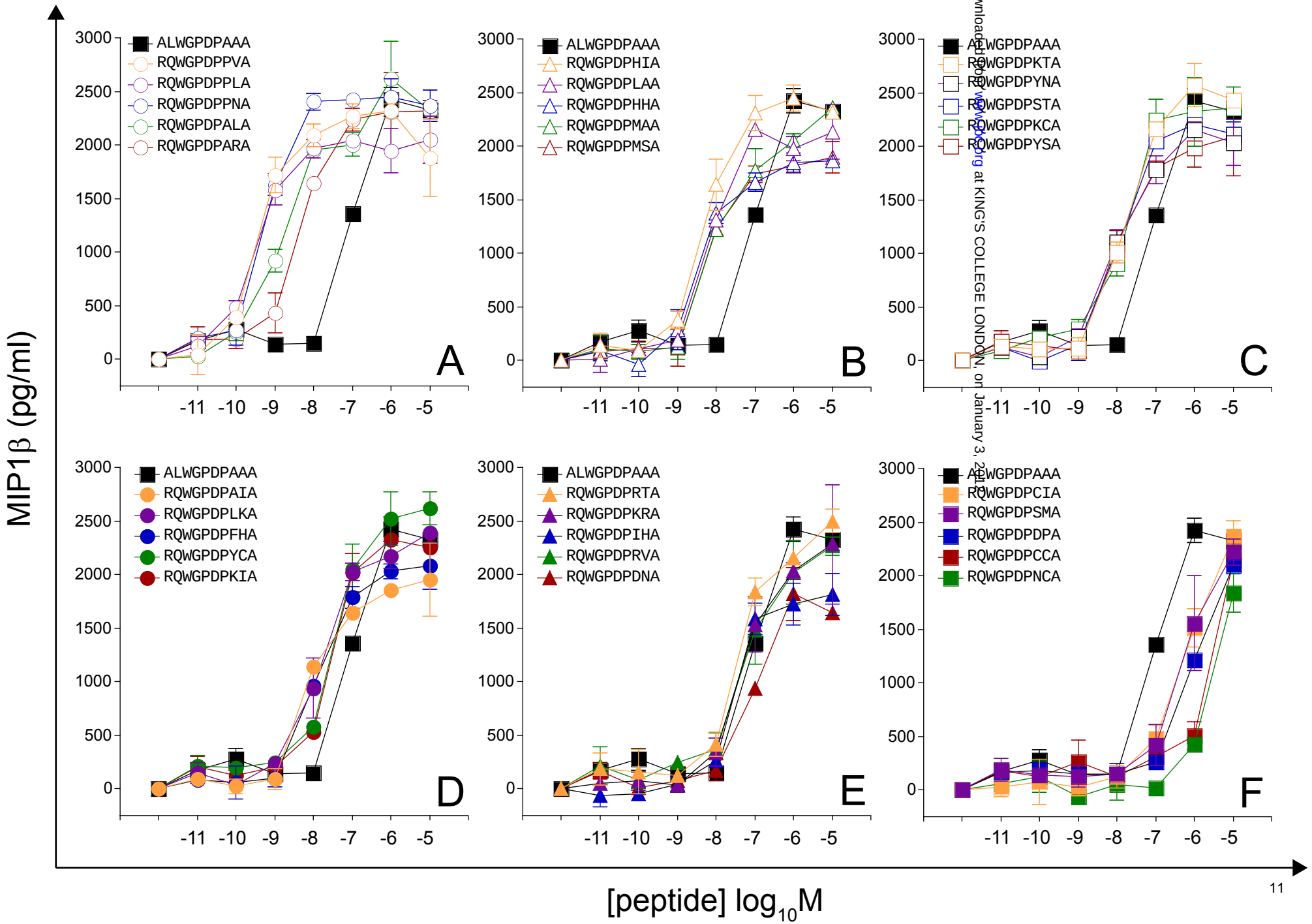


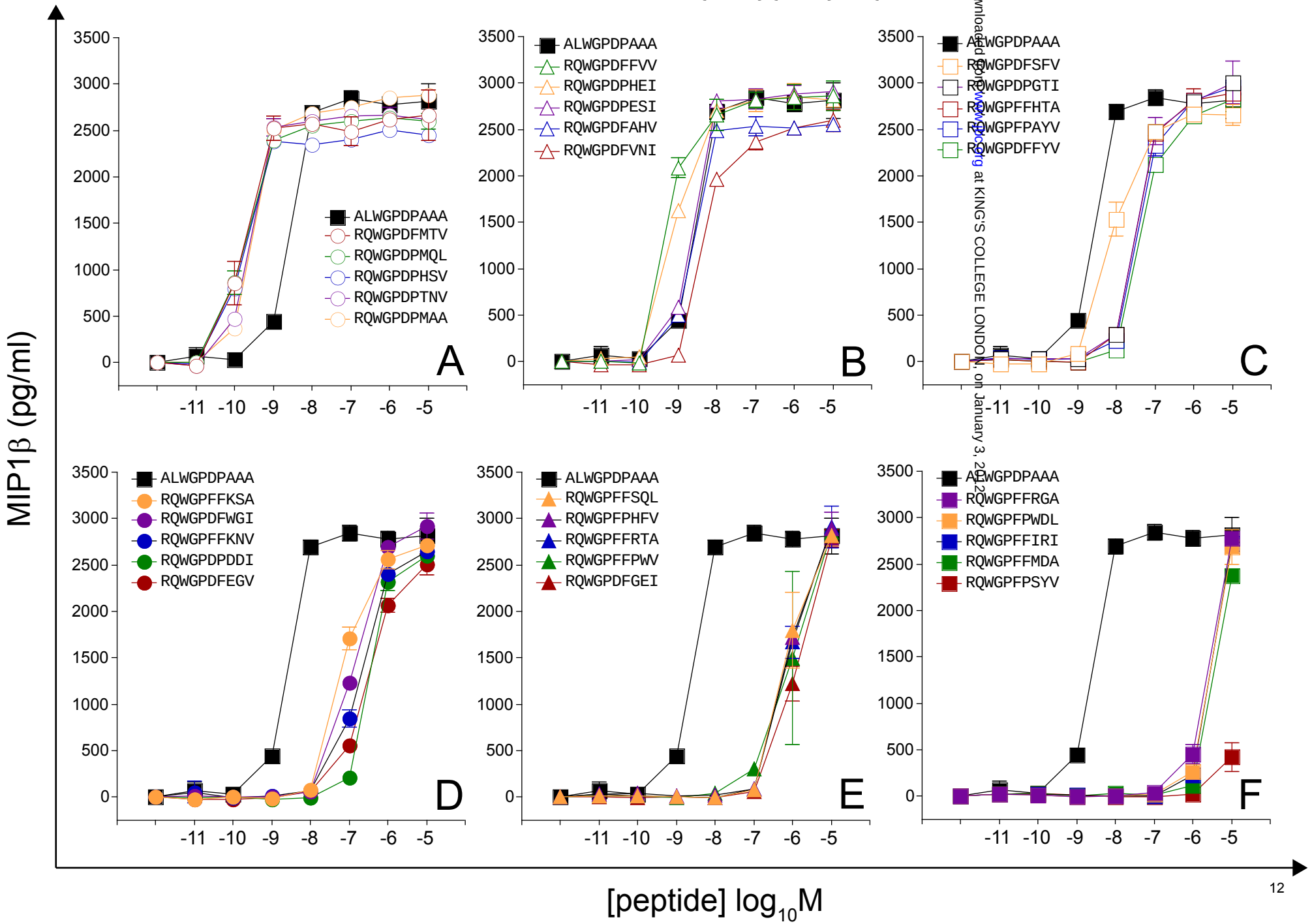
Figure 2

Recognition of 30 peptides sampled at random from a total set size of 225

Motif: RQWGPDP{A/C/D/F/H/I/K/L/M/N/P/R/S/V/Y}{A/C/G/H/I/K/L/M/N/P/Q/R/S/T/V}A



Recognition of 30 peptides sampled at random from a total set size of 5776
Motif: RQWGP{D/F}{P/F}xx{A/I/L/V}



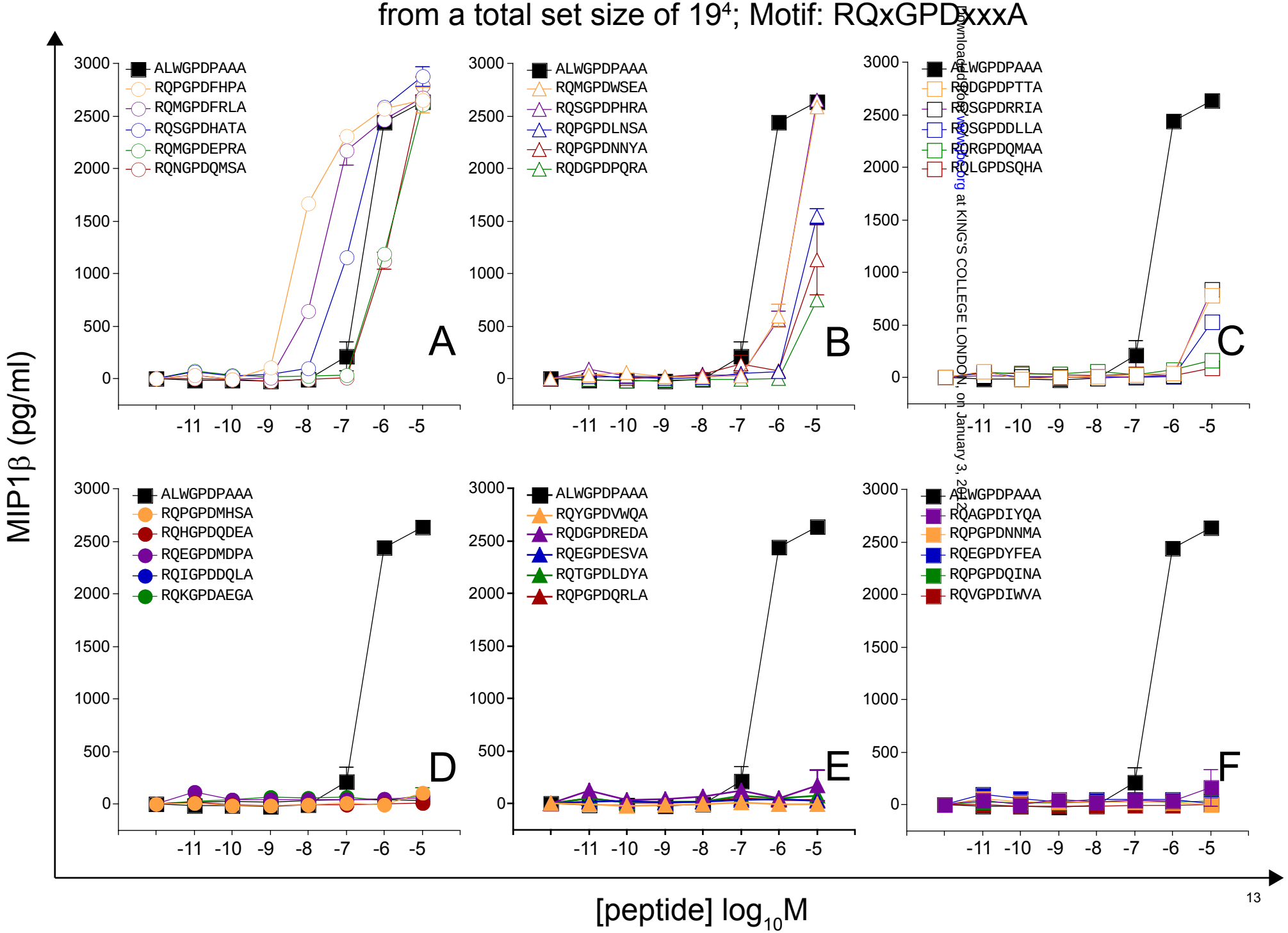
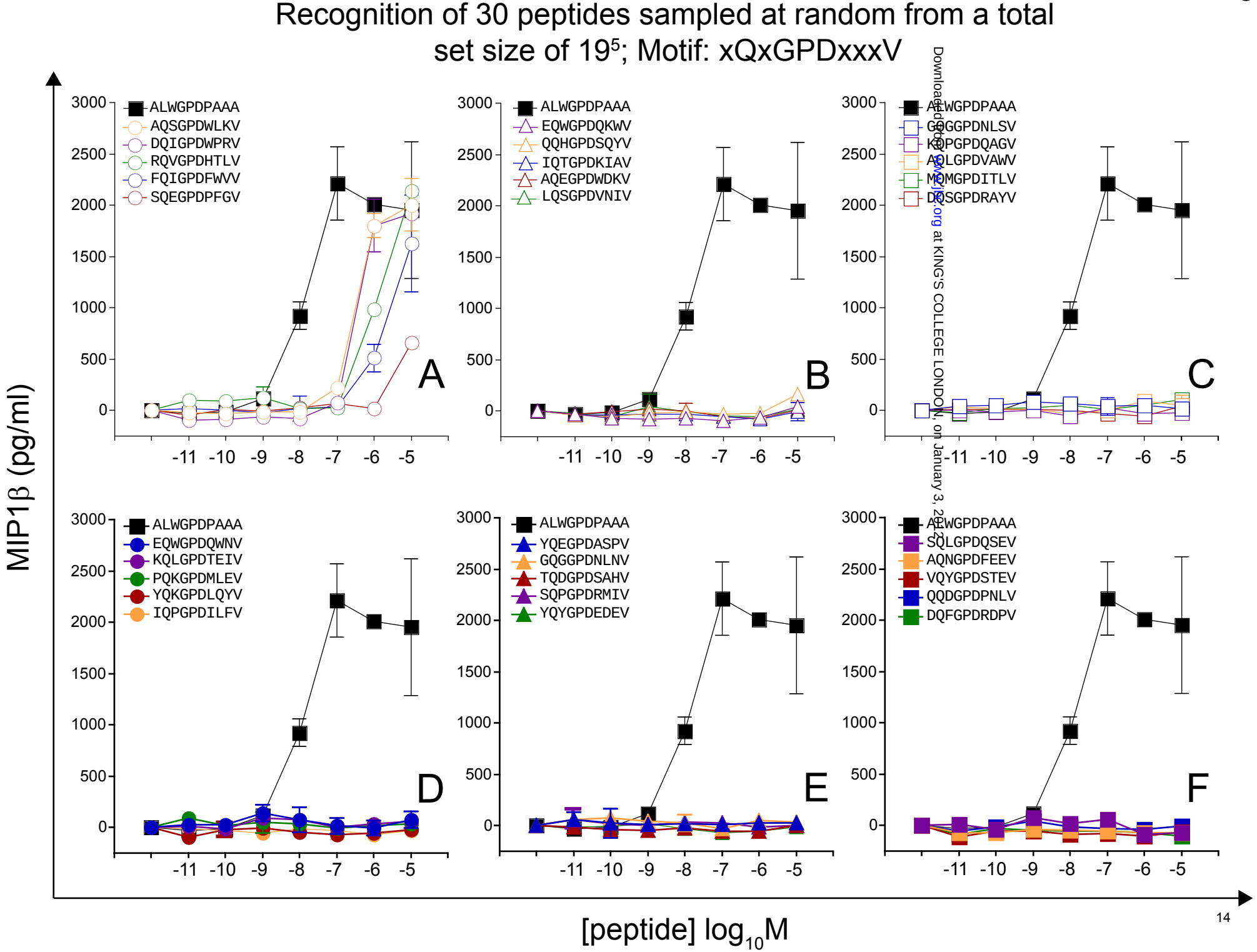
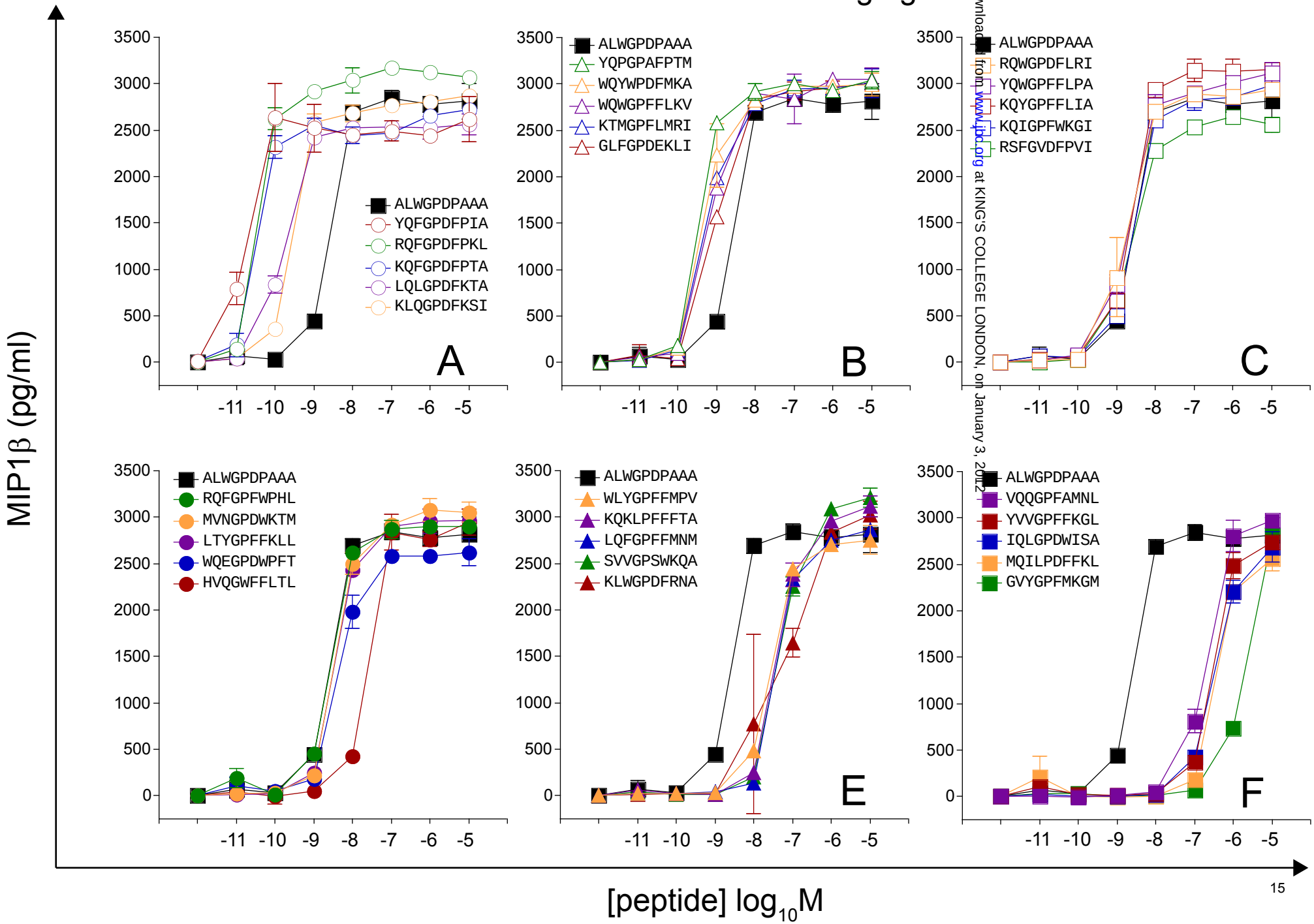


Figure 5



Recognition of 30 peptides sampled from an effective set size of 1.66×10^8 biased towards strong agonists



Recognition of 30 peptides sampled from an effective set size of 1.66×10^8 biased towards strong agonists

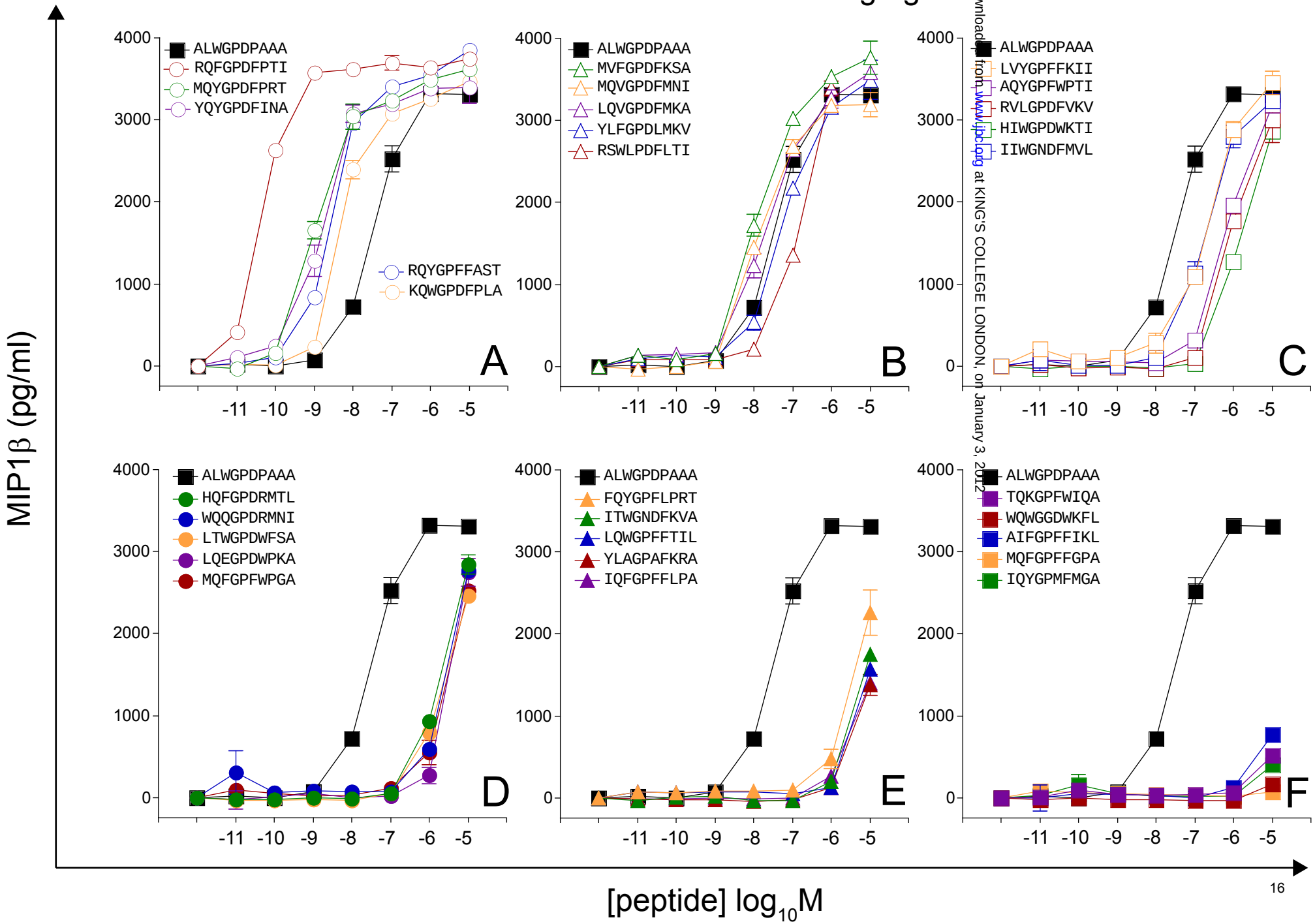


Figure 8