

# Supplementary Materials

**Table S1.** Oligonucleotides used in the selection.

| Oligonucleotides used for selection | Sequence (5' -3' )                                               |
|-------------------------------------|------------------------------------------------------------------|
| T7g10M.F48                          | TAATACGACTCACTATAGGGTTAACTTTAAGAAGGAGATATACATATG                 |
| NNK <sub>n</sub>                    | GCTGCCGCTGCCGCTGCCGCA(MNN) <sub>n</sub> CATATGTATATCTCCTTCTTAAAG |
| CGS3an13.R39                        | TTTCCGCCCCCGTCCTAGCTGCCGCTGCCGCTGCCGCA                           |
| puromycin linker                    | d(pCTCCCGCCCCCGTCC)-(SPC18)5-d(CC)-puromycin                     |

**Table S2.** X-ray data collection.

| Data Collection                                     | PfMATE + D8                                                                                                    |  |
|-----------------------------------------------------|----------------------------------------------------------------------------------------------------------------|--|
| Wavelength (Å)                                      | 0.91000                                                                                                        |  |
| Space group                                         | <i>H3</i>                                                                                                      |  |
| Unit cell parameters (Å, °)                         | <i>a</i> = 152.8, <i>b</i> = 152.8<br><i>c</i> = 163.0<br><i>α</i> = 90.0, <i>β</i> = 90.0<br><i>γ</i> = 120.0 |  |
| Resolution (Å)                                      | 50-3.22 (3.36-3.22)                                                                                            |  |
| Unique reflections                                  | 23064                                                                                                          |  |
| Total reflections                                   | 869988                                                                                                         |  |
| Completeness                                        | 100 (100)                                                                                                      |  |
| <i>I</i> / <i>σ</i> ( <i>I</i> )                    | 4.34 (1.95)                                                                                                    |  |
| Redundancy                                          | 12 (9.0)                                                                                                       |  |
| <i>R</i> <sub>sym</sub>                             | 0.390 (0.648)                                                                                                  |  |
| <b>Refinement</b>                                   |                                                                                                                |  |
| Resolution (Å)                                      | 47.8-3.22                                                                                                      |  |
| No. reflections                                     | 22937                                                                                                          |  |
| <i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> | 0.280/0.317<br>(0.391/0.393)                                                                                   |  |
| No. atoms                                           | 5857                                                                                                           |  |
| protein                                             | 5799                                                                                                           |  |
| cyclic peptide                                      | 58                                                                                                             |  |
| water                                               | 7                                                                                                              |  |
| <b>B-factors</b>                                    |                                                                                                                |  |
| protein                                             | 73.6                                                                                                           |  |
| cyclic peptide                                      | 226                                                                                                            |  |
| water                                               | 11.5                                                                                                           |  |
| <b>R.m.s deviations</b>                             |                                                                                                                |  |
| Bond length (Å)                                     | 0.003                                                                                                          |  |
| Bond angles (°)                                     | 0.833                                                                                                          |  |

The numbers in parentheses are for the highest resolution shell.  $*R_{\text{sym}} = \sum |I_{\text{avg}} - I_i| / \sum I_i$ .  $^{\dagger}R_{\text{cullis}} = \sum |E| / \sum |F_{\text{PH}}| - |F_{\text{P}}|$ , where  $F_{\text{PH}}$  is the amplitude of the protein plus the heavy atom and  $F_{\text{P}}$  is the amplitude of the protein.  $^{\ddagger}$ Phasing power = r.m.s. ( $|F_{\text{H}}|/E$ ), where  $|F_{\text{H}}|$  is the heavy atom structure-factor amplitude and  $E$  is the residual lack of closure error.  $^{\S}$ Figure of merit =  $\langle |\Sigma P(\alpha) \exp(i\alpha) / \Sigma P(\alpha)| \rangle$ , where  $\alpha$  is the phase and  $P(\alpha)$  is the phase probability distribution.

**Figure S1.** Genetic code reprogramming and the FIT system. **(A)** The reprogrammed genetic code used for the initiation of ribosomal peptide synthesis; **(B)** Charging of initiator  $\text{tRNA}^{\text{fMet}}_{\text{CAU}}$  with either *N*-(2-chloroacetyl)-L-phenylalanine or *N*-(2-chloroacetyl)-D-phenylalanine using flexizyme (eFx). Cyclization occurs spontaneously upon incorporation of a downstream cysteine; **(C)** Schematic representation of one round of *in vitro* selection from round 2 or a higher round.

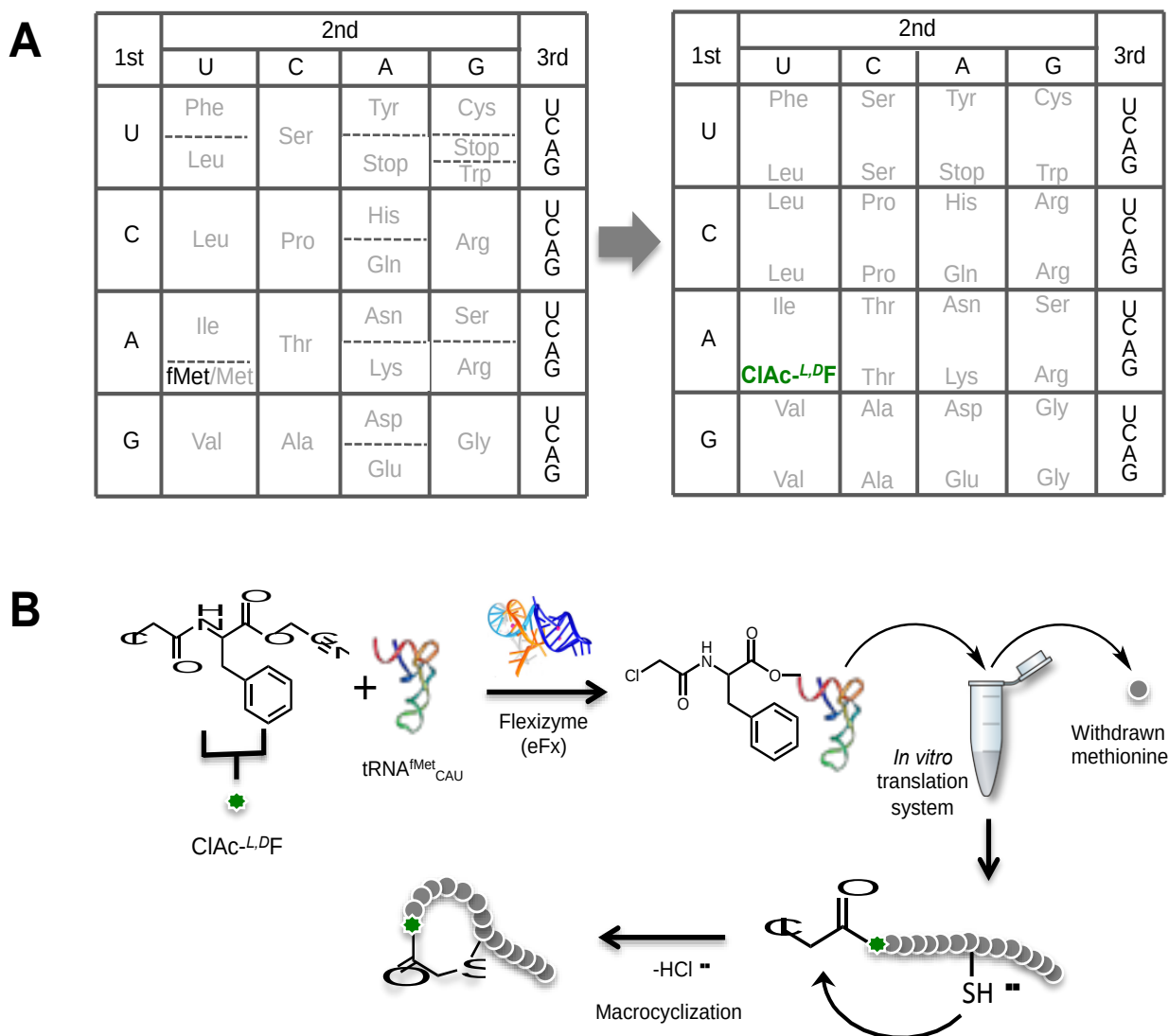
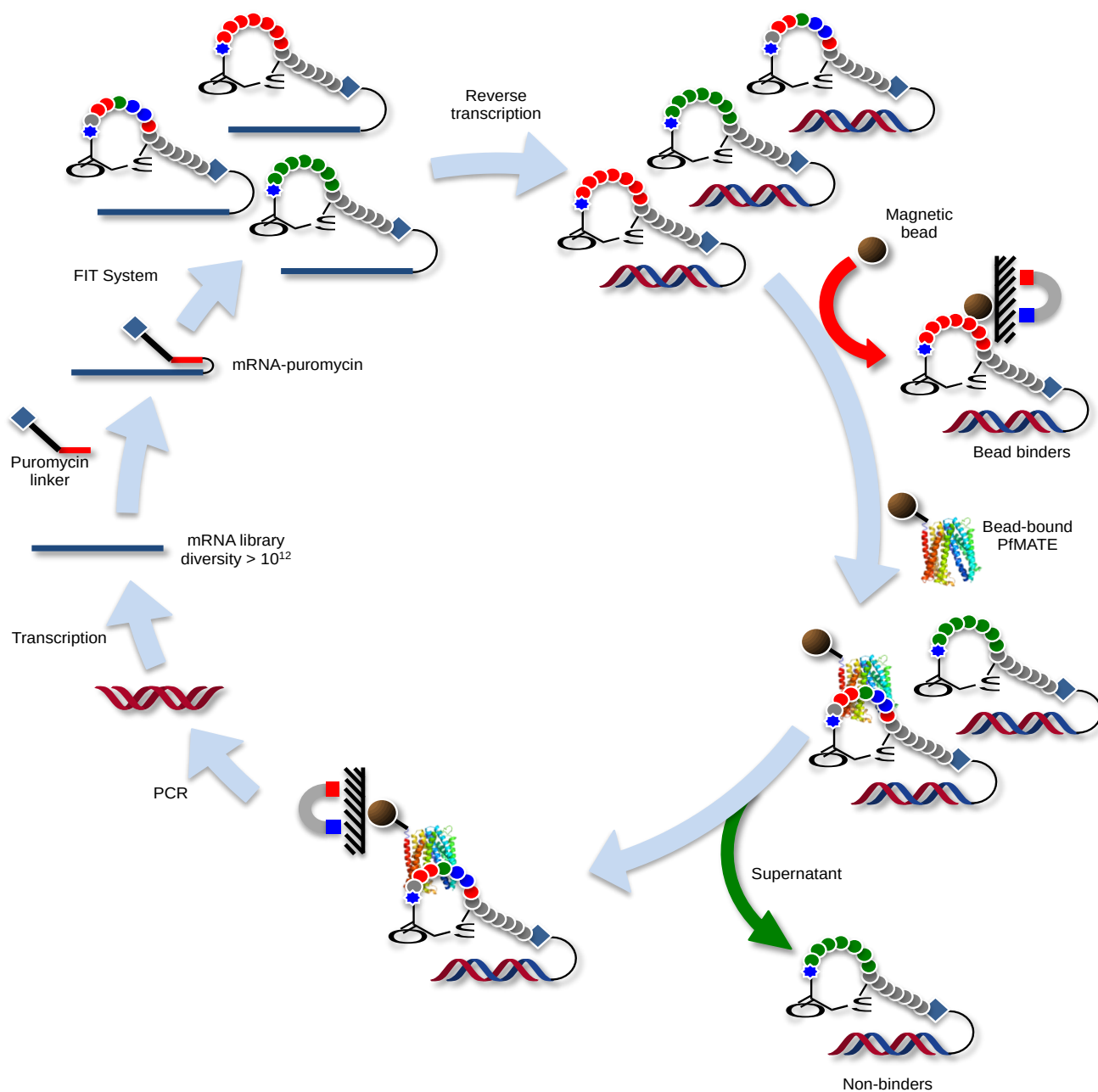
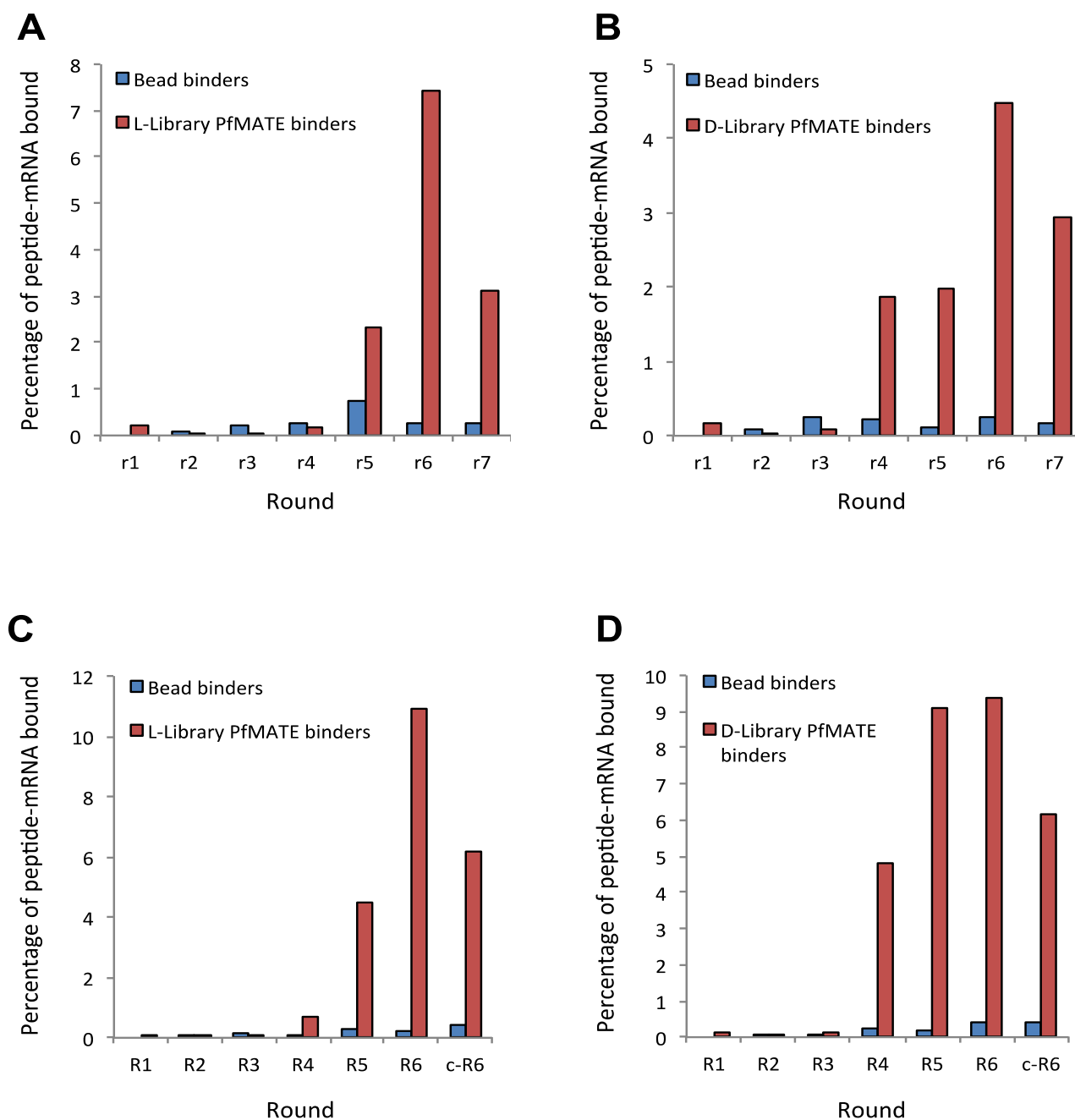


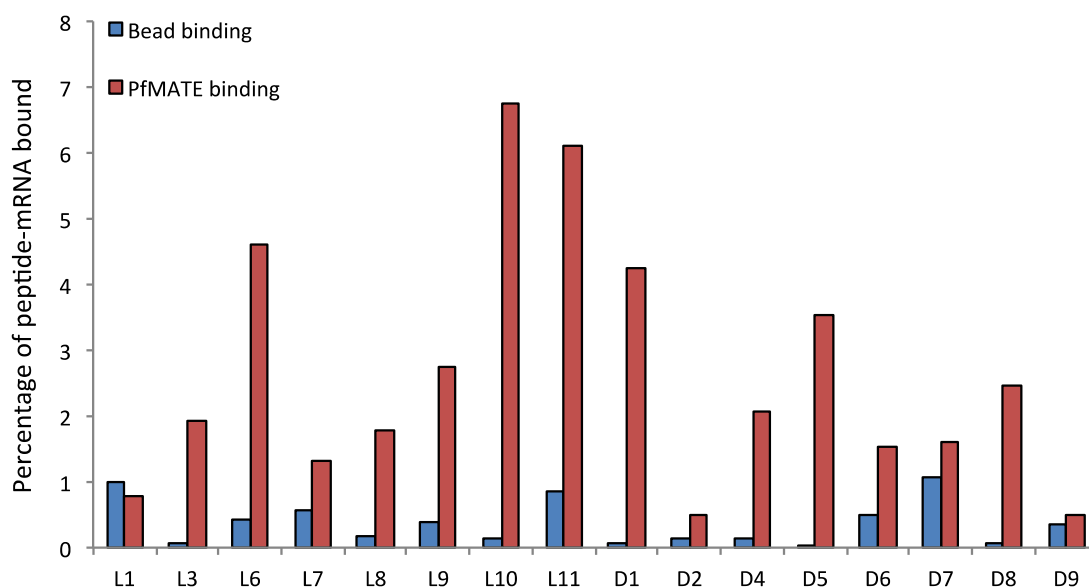
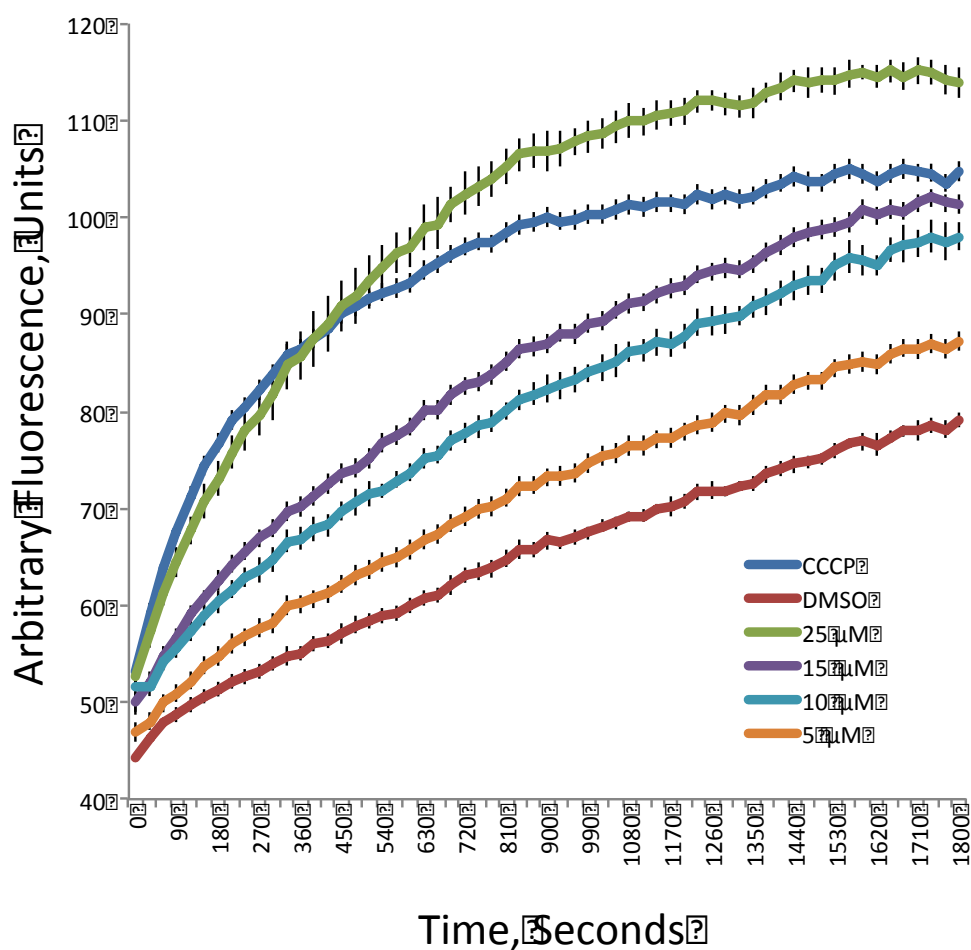
Figure S1. Cont.

C



**Figure S2.** Progress of the selections. The progress of the selections whose binding step was performed at 4 °C of the (A)  $^L$ F-Library and (B)  $^D$ F-Library. The progress of the selections whose binding step was performed at 37 °C of the (C)  $^L$ F-Library and (D)  $^D$ F-Library. The rounds represented farthest to the right are the competition rounds, c-Round 6. Percentages of peptides bound were determined by dividing the amount of recovered cDNA by the amount of input macrocyclic peptide-mRNA conjugate.



**Figure S3.** Percentages of peptides bound from single-clone display assays.**Figure S4.** MaD8 concentration-dependent increase in the rate of accumulation of intracellular EtBr. Errors bars were calculated from four separate trials.

**Figure S5.** Chemical structure of MaD8F.**MaD8F**