Preparation of labeled cyclic peptides with PURE*frex*.

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[Background]
We reported that various cyclic peptides were selected simply and effectively by Ribosome display with PURE*frex* (PURE*frexRD*). But for preparation of those peptides for further investigation, it takes a lot of cost and time with chemical synthesis. Therefore, we have developed a new preparation method of labeled cyclic peptides in high throughput manner with PURE*frex*.

[Methods]
- Cyclic peptides were expressed as the N-term Maltose Binding Protein (MBP) fused cyclic peptide.
- Cyclic peptides were released by digesting with Enterokinase.
- Labeled puromycin was added to PURE*frex* to generate the C-term labeled cyclic peptides.
- Labeling efficiency of puromycin were investigated with FITC-puromycin under several conditions. (+/- Release factor mix (RF mix;RF1 ,2 ,3 and RRF), Removing the stop codon from DNA template, PURE*frex* Ver.1.0/2.0)
- Optimized conditions were applied for actual screening of cyclic peptides against CTLA4.

[Results]
PURE*frex* RFmix(-) showed the best efficiency of FITC-puromycin uptaking at C-terminal of full length proteins. In contrast, in the case of using PURE*frex* RFmix(+), the translation reaction stopped in the middle of the protein by adding FITC-puromycin. But the absence of stop codon in DNA template improved the uptaking efficiency of FITC-puromycin even under using PURE*frex* RFmix(+) in some cases.
The application of Biotinylated-puromycin instead of FITC-puromycin led to higher sensitive ELISA with Streptavidin HRP even in free peptide format.
In the screening of CTLA4 binders with this method, we were able to identify the best cyclic peptide with good EC_{50}/IC_{50}.

[Conclusion]
This new method for preparation of cyclic peptides with PURE*frex* will contribute for simple and effective high throughput functional screening of cyclic peptides, and will be applied to the development of peptide based drugs.

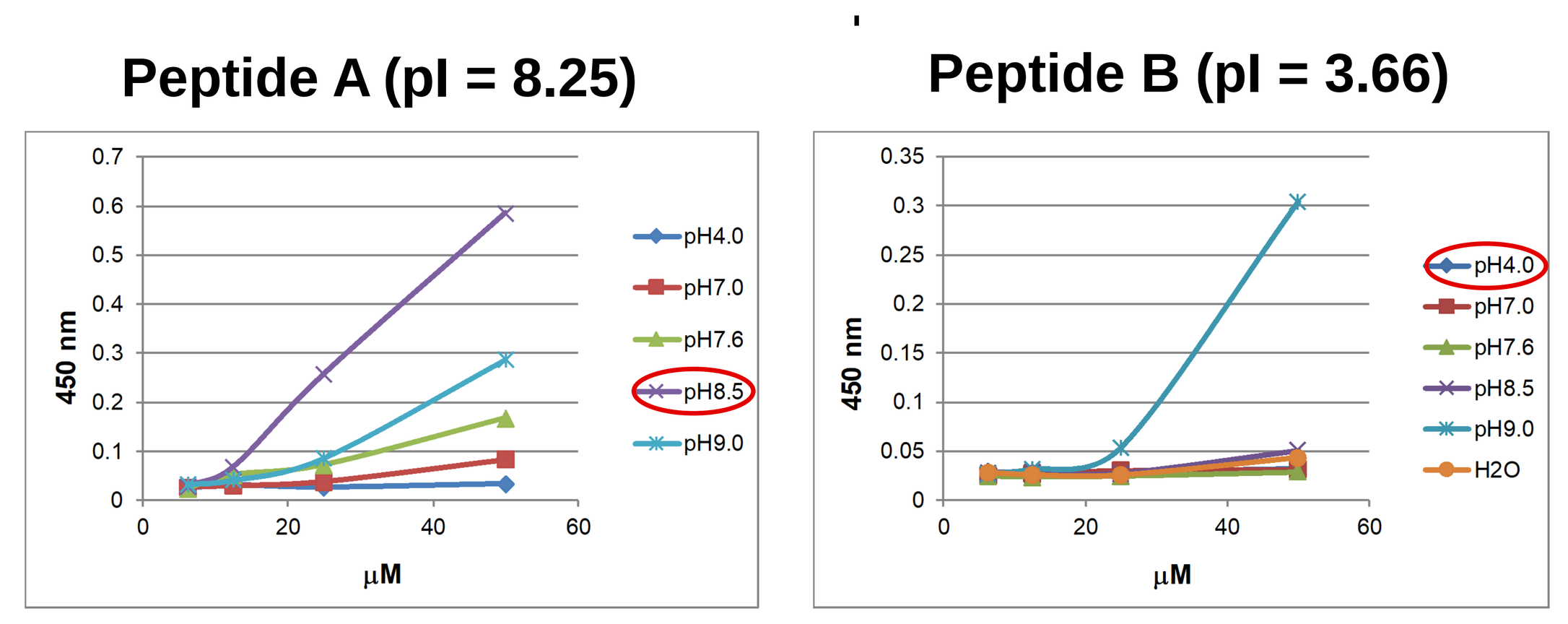
(1) Problems for developing cyclic peptide

The physical properties of cyclic peptide displayed by *in vitro* selection technologies such as ribosome display or phage display, and differed from those in synthesized free peptide, for example, followings are major points for the difference.

- Isoelectric Point
- Fluctuation
- Solubility

(1)-1, Effect of Isoelectric Point

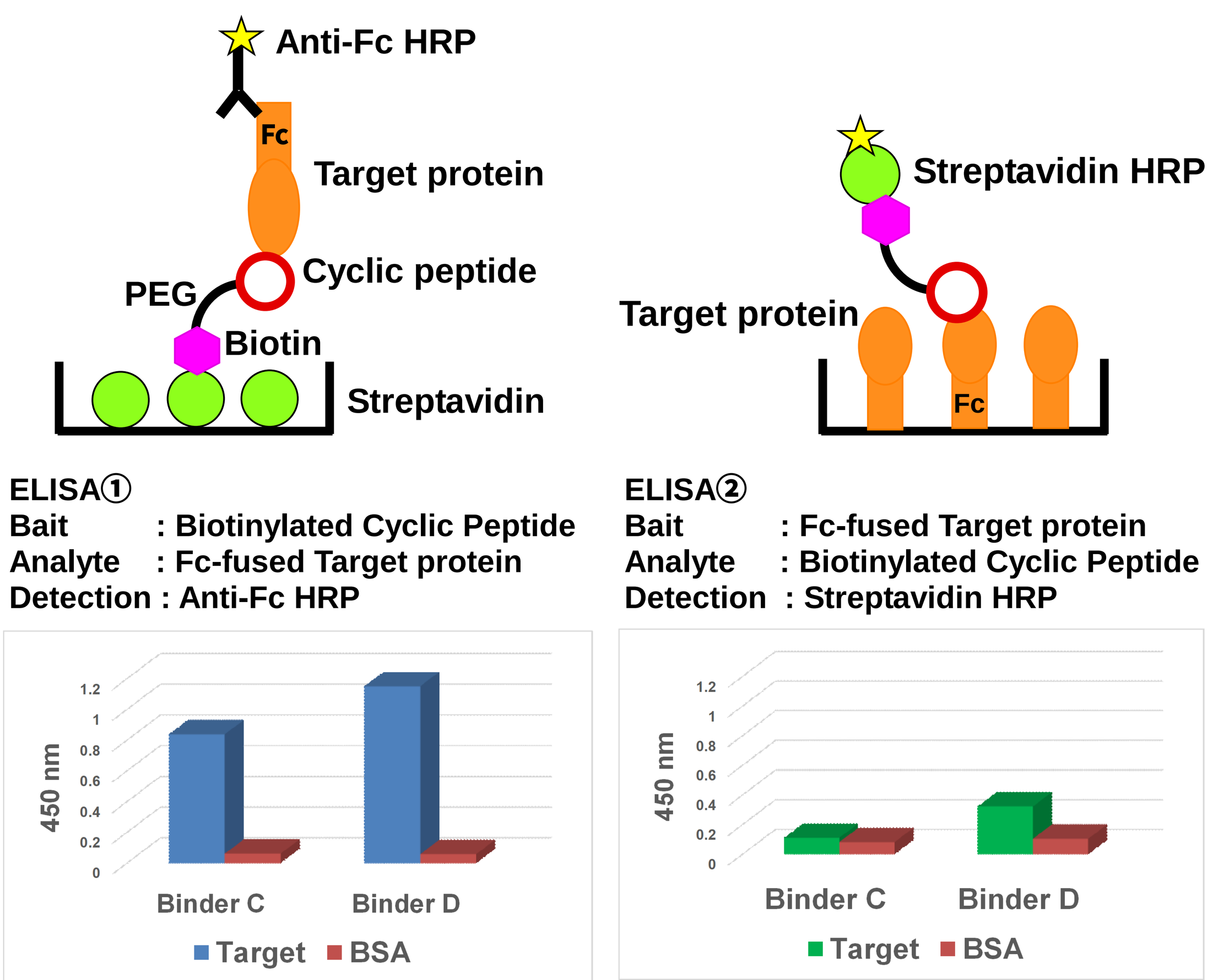
Selection of cyclic peptides by *in vitro* display technologies → Peptide A, Peptide B, Peptide C …



Detection: anti-FLAG M2 HRP

The binding activities of both cyclic peptides in ELISA showed that Isoelectric Points of free cyclic peptides and pH of buffer solutions have great impact on its activity, although both had same binding activity on *in vitro* display technology.

(1)-2, Effect of Peptide Fluctuation



The binding activity depended on immobilized format, which suggests molecular fluctuation have impact on its activity.

(1)-3, Effect of Solubility

- *In vitro* display technologies generated various binders independent from hydrophobicity/hydrophilicity.
- Highly hydrophobic peptide was insoluble or slightly soluble in water, and was hard to synthesize, or in some cases, not to be synthesized at all.

Screening in free peptide format is necessary.

But Chemical synthesis takes high cost and a long time.

Solutions

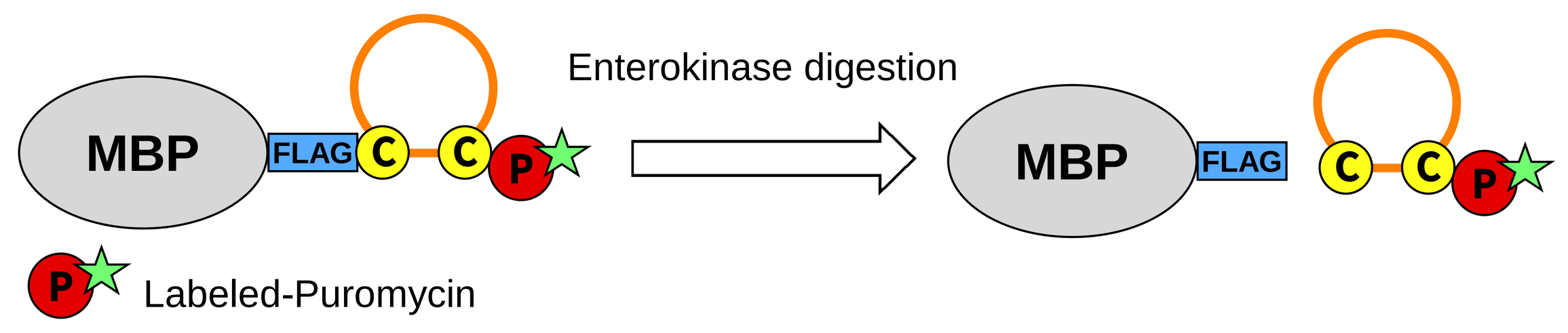
Preparation of free peptides with PUREfrex

- Possible to prepare various peptides with low cost in a short time.
- Possible to label the synthesized peptide easily.

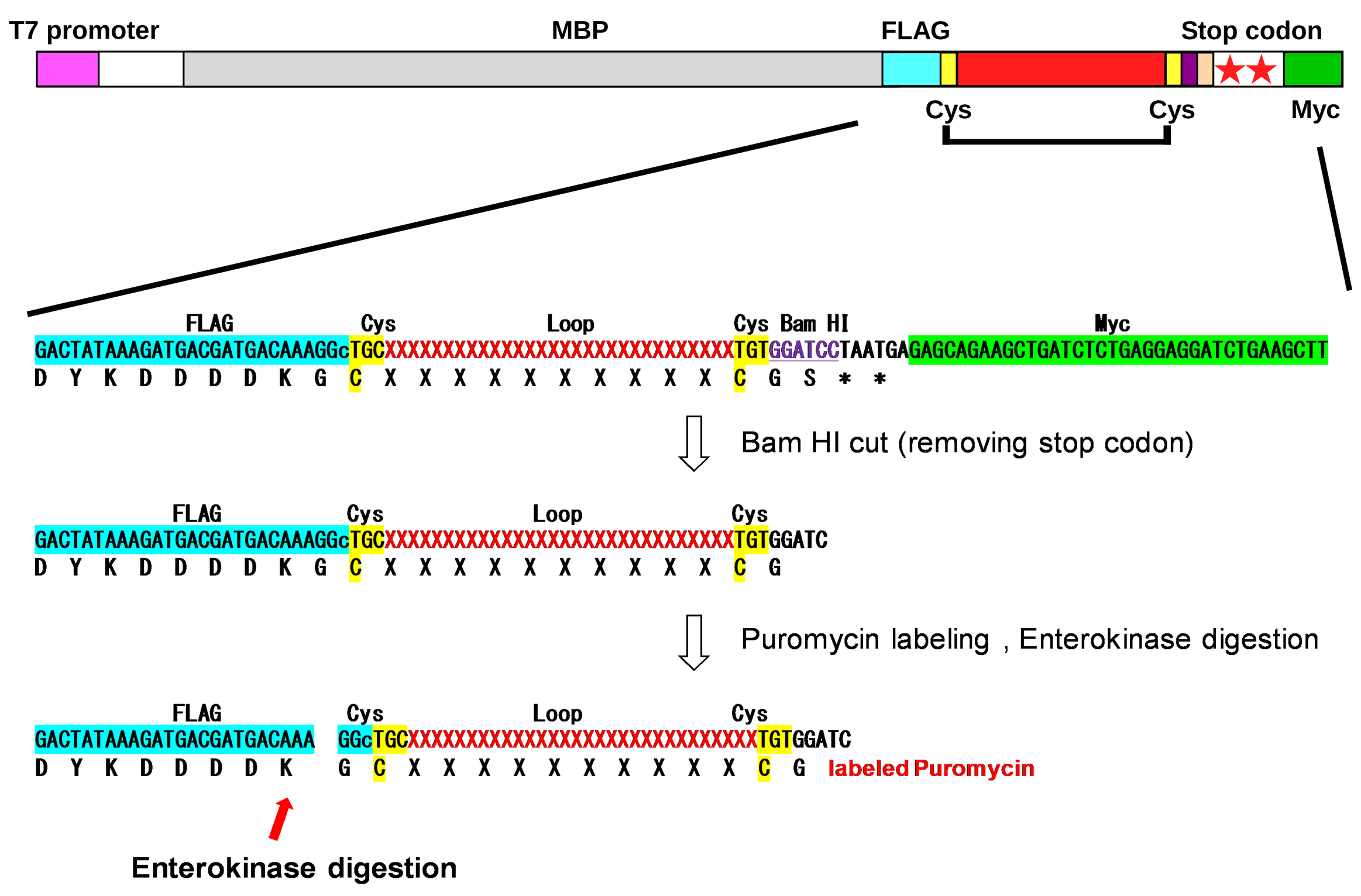
↓

It enables high throuhghput functional screening of various binders in free peptide format

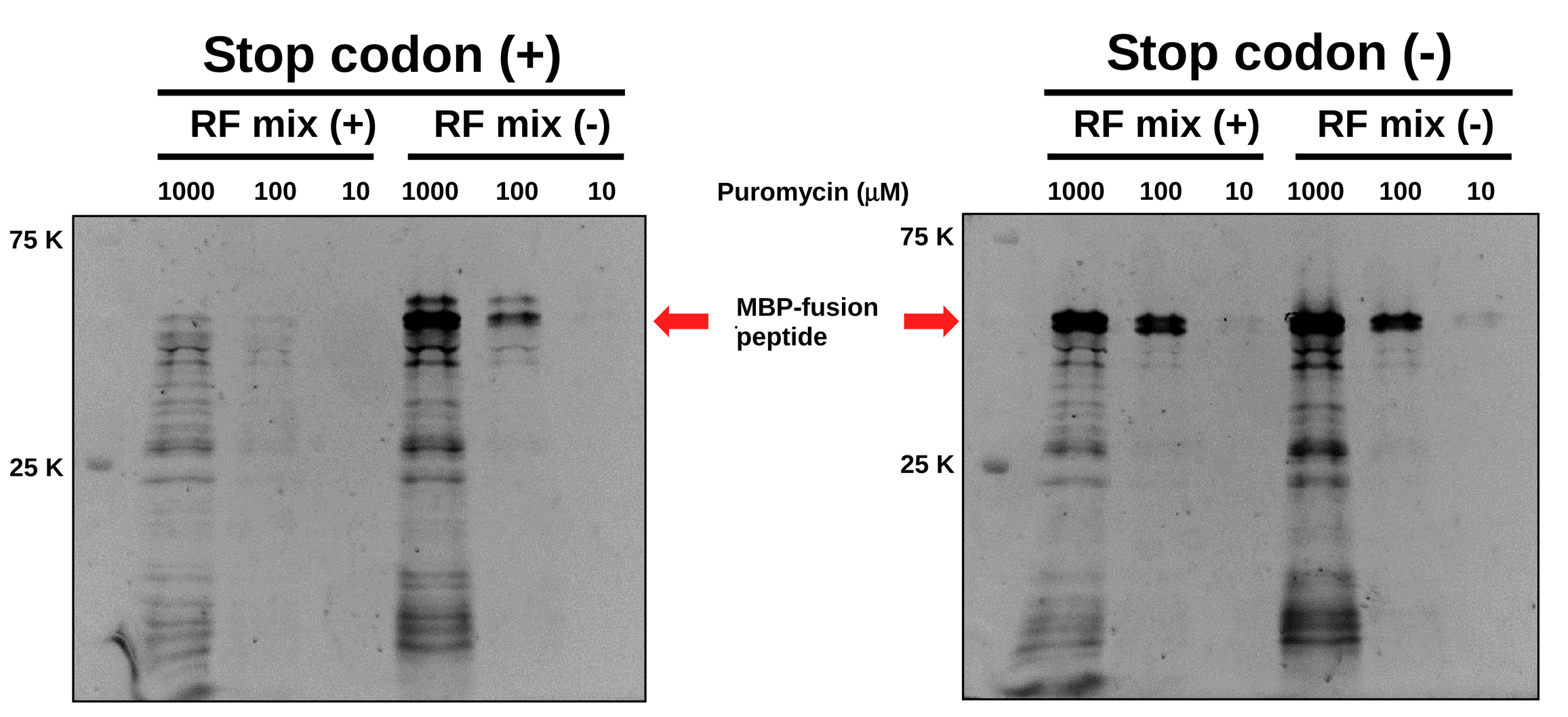
(2) Preparation of labeled cyclic peptides with PURE*frex*



(Expression Construct)



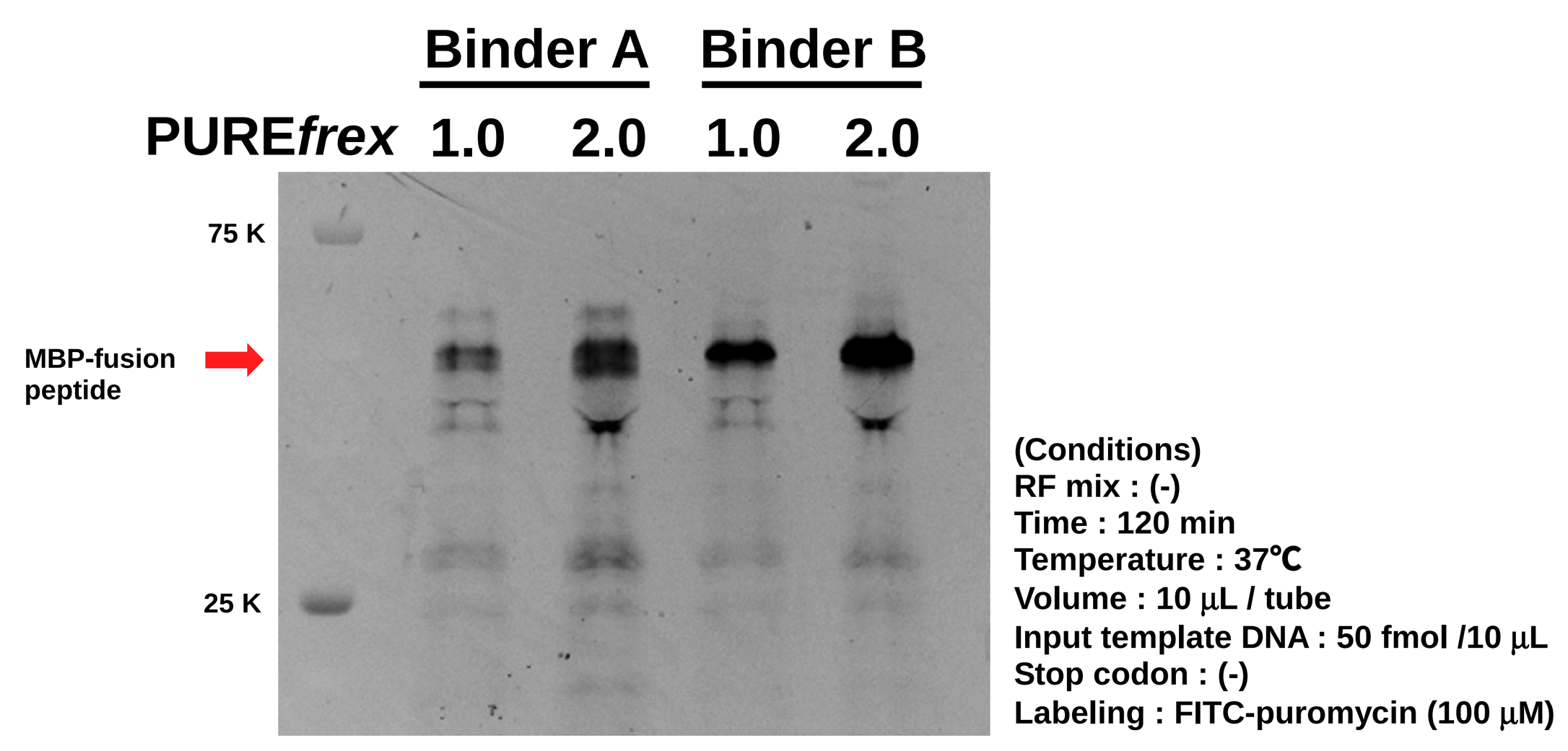
(2)-1, Effect of RF mix, Stop codon, and Concentration of labeled-puromycin



(Conditions)
Translation system : PURE*frex* 1.0 DS supplement, RF mix (-)/(+)
Time : 60 min
Temperature : 37℃
Volume : 10 μL / tube
Input template DNA : 50 fmol /10 μL
Labeling : FITC-puromycin

RF mix(-), Stop codon(-), and 100 μM labeled puromycin was the best.

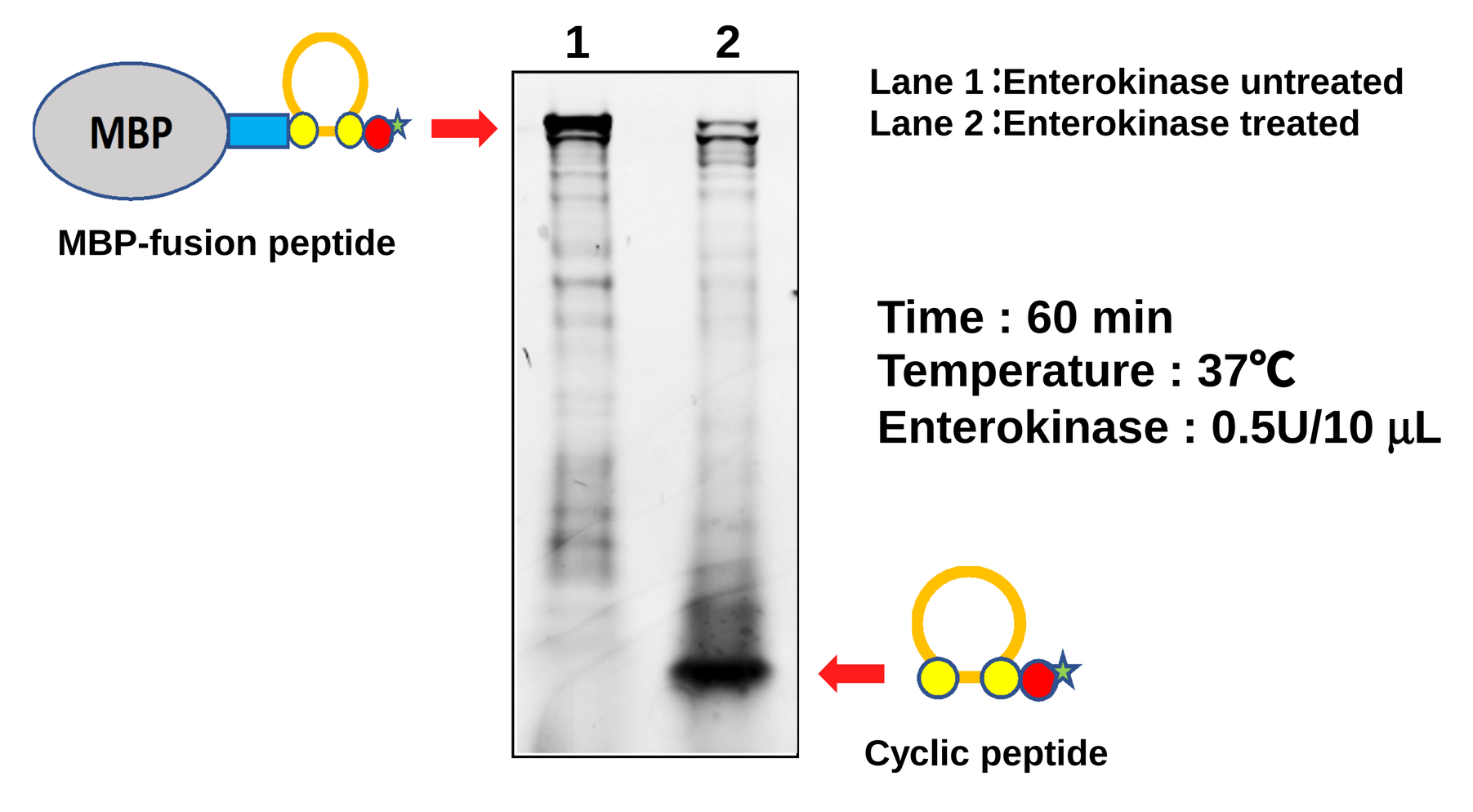
(2)-2, Comparison of expression between PURE*frex* 1.0 and 2.0



(Conditions)
RF mix : (-)
Time : 120 min
Temperature : 37℃
Volume : 10 μL / tube
Input template DNA : 50 fmol /10 μL
Stop codon : (-)
Labeling : FITC-puromycin (100 μM)

PURE*frex* 2.0 was better than PURE*frex* 1.0.

(2)-3, Release of cyclic peptide by Enterokinase treatment

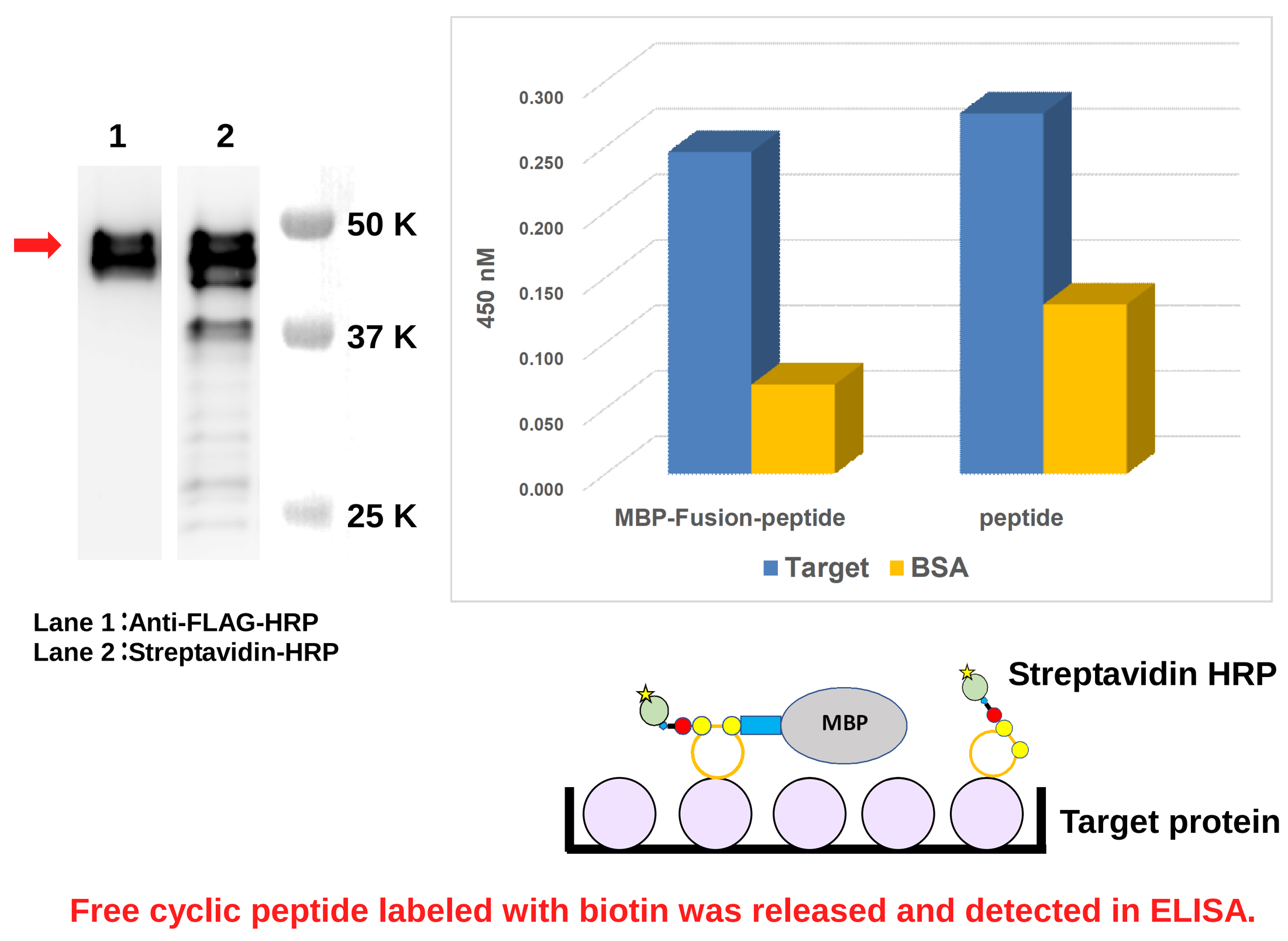


Lane 1 :Enterokinase untreated
Lane 2 :Enterokinase treated

Time : 60 min
Temperature : 37℃
Enterokinase : 0.5U/10 μL

Labeled cyclic peptide was effectively released by Enterokinase.

(2)-4, ELISA in different format, MBP fusion peptide and free peptide labeled with Biotin-Puromycin



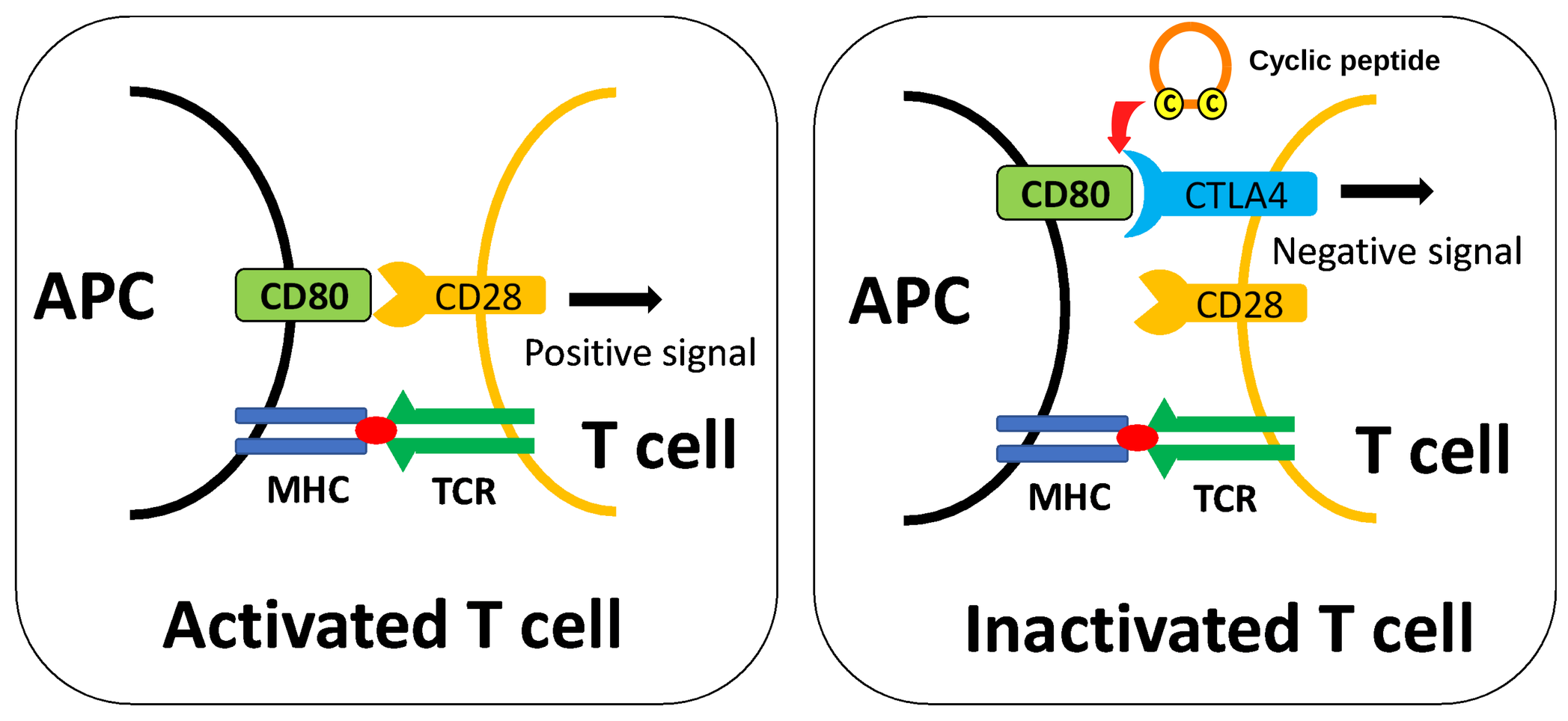
Lane 1 :Anti-FLAG-HRP
Lane 2 :Streptavidin-HRP

Free cyclic peptide labeled with biotin was released and detected in ELISA.

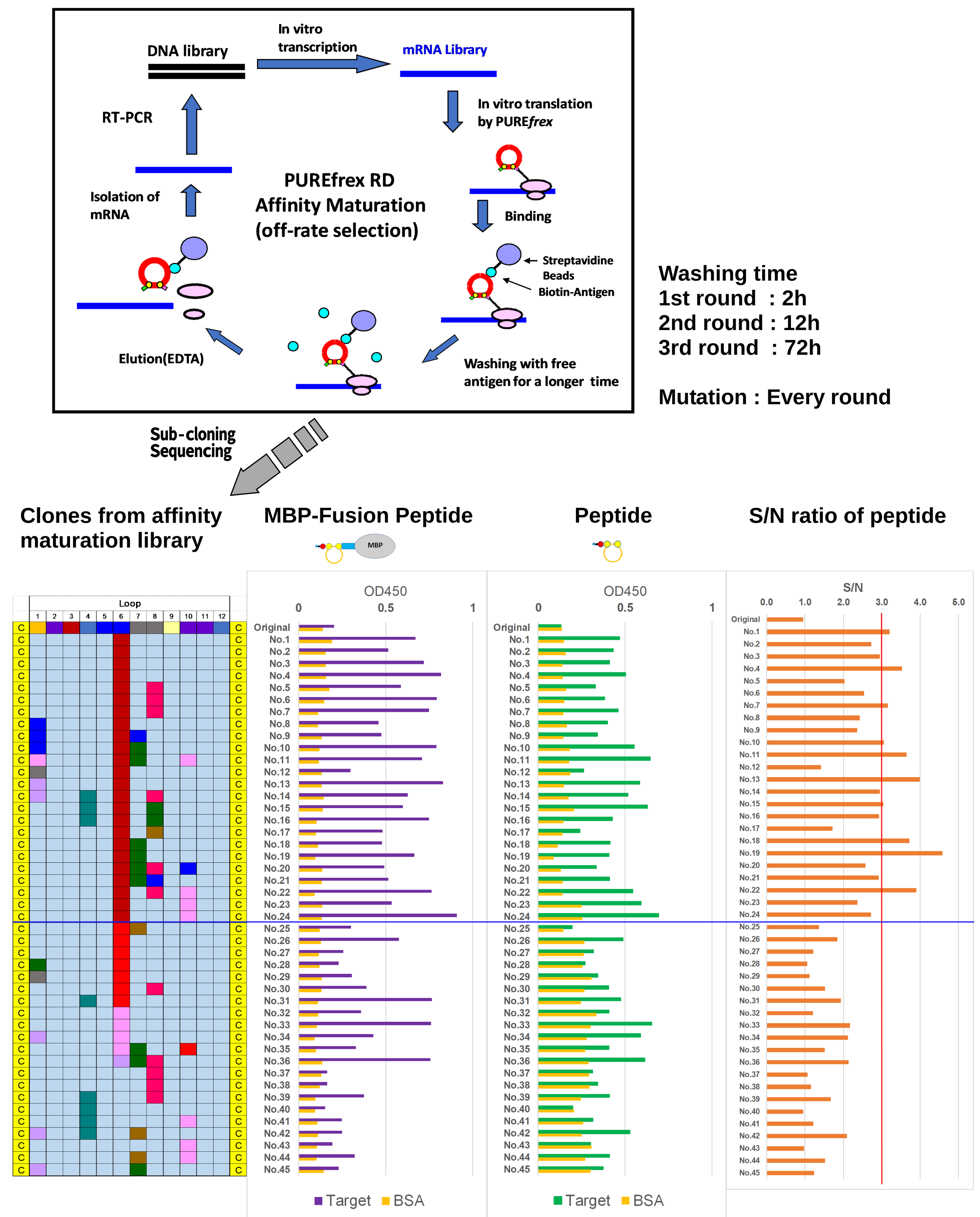
Applicable for high-throughput screening in free peptide format.

(3) Example; Peptide screening for cyclic peptide to CTLA4, derived from affinity maturation library

(3)-1, Immune checkpoint via CTLA4



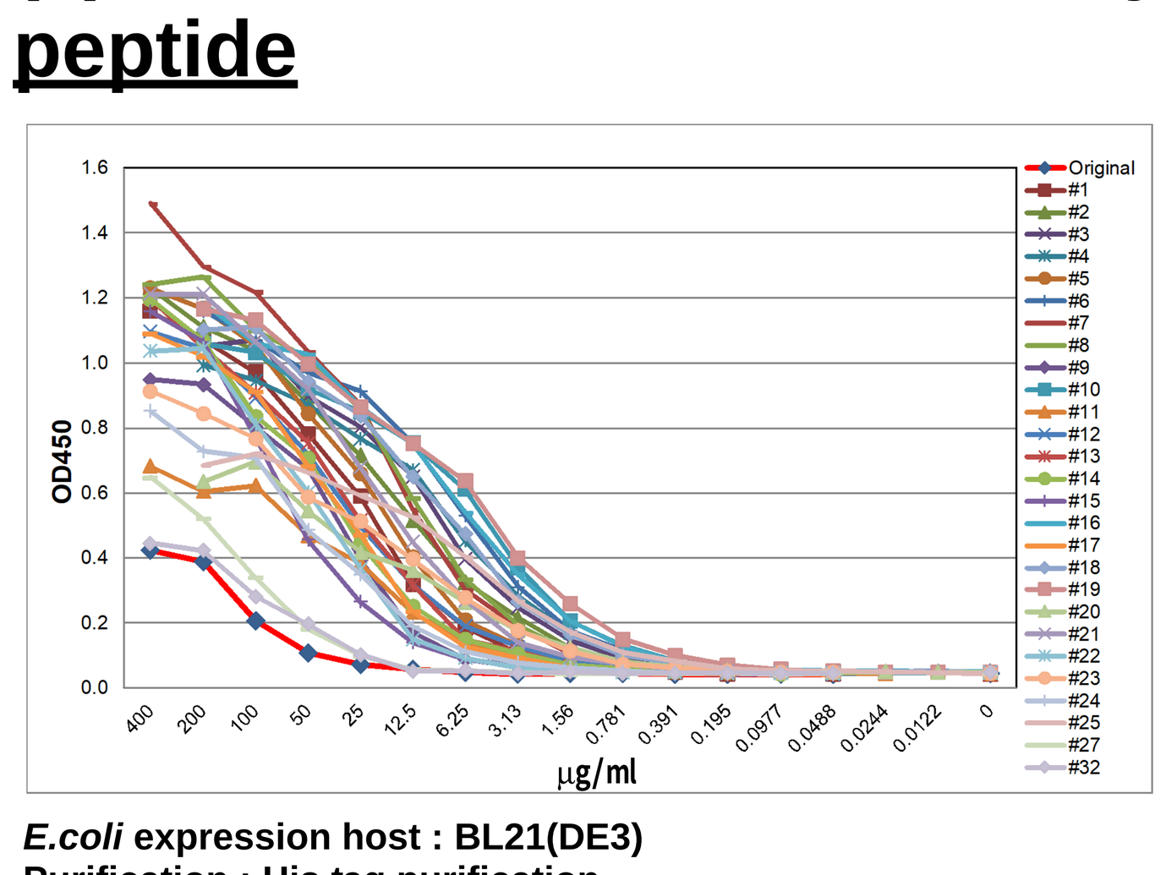
(3)-2, Evaluation of amino acid sequence and ELISA in MBP fusion or free peptide format after affinity maturation by PURE*frexRD*



Binders with good binding activity were selected in free peptide format.

Key AAs and their positions were identified.

(3)-3, Measurements of affinity (EC_{50}) as MBP-fusion peptide



E.coli expression host : BL21(DE3)
Purification : His tag purification

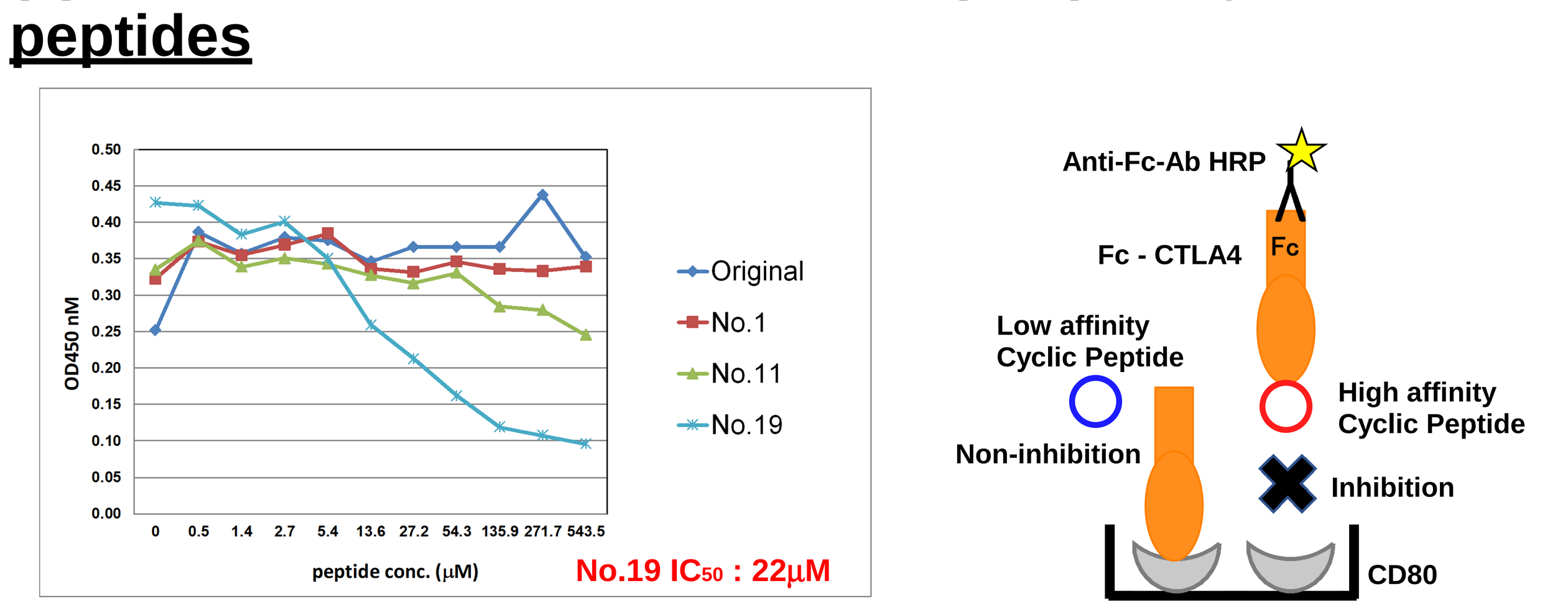
Affinity matured binders were identified.

The affinities of the binder No. 19, 10 and 24 were improved about 50 times higher than that of the original binder.

Asterisk (★) indicate that binders were performed to chemical synthesis.

Clone	EC50 (uM)	Fusion S/N	Peptide S/N	
Original	3.921	1.5	1.0	★
No.19	0.075	6.8	4.6	★
No.10	0.082	6.6	3.1	
No.24	0.085	6.7	2.7	
No.20	0.160	3.6	2.6	
No.3	0.187	4.6	3.0	
No.18	0.189	4.3	3.7	
No.16	0.217	7.2	2.9	
No.6	0.222	5.4	2.5	
No.4	0.225	5.1	3.5	
No.5	0.288	3.3	2.0	
No.11	0.300	6.0	3.6	★
No.21	0.322	3.9	2.9	
No.22	0.342	8.4	3.9	
No.7	0.352	6.5	3.2	
No.2	0.369	3.3	2.7	
No.8	0.474	4.1	2.4	
No.25	0.668	2.5	1.4	
No.23	0.674	4.0	2.4	
No.14	0.703	4.3	2.9	
No.17	0.826	4.8	1.7	
No.13	0.832	6.2	4.0	
No.9	0.867	3.6	2.4	
No.12	0.889	2.2	1.4	
No.1	1.035	3.5	3.2	★
No.32	1.493	3.2	1.2	
No.15	1.637	4.3	3.0	
No.27	4.181	2.2	1.2	

(3)-4, Measurements of inhibition (IC_{50}) as synthetic peptides



Binder No.19 showed good inhibitory activity against interaction between CD80 and CTLA4 in fully synthetic peptide format.

Summary

1, We established new preparation method for labeled cyclic peptide with puromycin and optimized PURE*frex*.

2, In the screening of CTLA4 binders by this method, we succeeded to select lead cyclic peptides with good EC_{50}/IC_{50} as synthetic peptide format.