# Selection and affinity maturation of cyclic peptide against CTLA-4 with PURE*frexRD*

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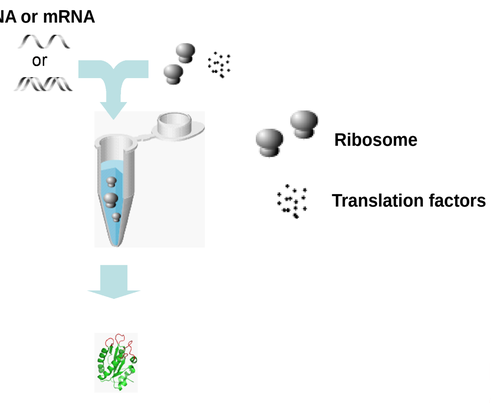
## Abstract

**[Background]**
Ribosome display (RD) is the simplest method among existing *in vitro* selection methods. We developed RD by optimizing the composition of PURE*frex*, for instance, removing release factors from the system, adjusting the concentration of metal ions and ribosome, etc. We named it as PURE*frexRD*. We have already reported that a lot of cyclic peptides and antibodies were successfully selected simply and effectively by PURE*frexRD*. This time, we carried out *in vitro* selection using PURE*frexRD* to select cyclic peptides binding to cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) with inhibitory activity to the interaction between CTLA-4 and CD80, which can be one of the new modality as an immune check point inhibitor.

**[Methods and Results]**
Cyclic peptide library having 12mer random sequences (Cys - X12 - Cys) was screened against a biotinylated CTLA-4-Fc fusion protein. After 3 rounds of selection, enriched mRNAs were subcloned, and a lot of enriched clones with variety were obtained from sequence analysis. The enriched clones were examined for their binding activity to CTLA-4 by RD pull down assay, and 3-02 clone showed specific binding activity to CTLA-4. To evaluate the affinity to CTLA-4 and the inhibitory activity to the interaction between CTLA-4 and CD80, 3-02 clone was expressed as MBP-fusion protein in *E.coli* and purified. Purified 3-02 clone showed very low affinity and very low inhibitory activity, so affinity maturation of 3-02 clone by off-rate selection using PURE*frexRD* was performed to improve its affinity and inhibitory activity. Mutated library against 3-02 clone was prepared by error prone PCR. After 3 rounds of off-rate selection over several hours (1st round; 2 hours, 2nd round; 19 hours, 3rd round; 67 hours), mutated clones were expressed in *E.coli*, and their binding activities were evaluated by ELISA. The purified clones showed the higher activity in ELISA, and each $EC_{50}$ was determined for comparison in specific activity of each clone to the original 3-02 clone. Finally, we have obtained several high affinity clones by approximately 50-fold more than the original 3-02 clone.

**[Conclusion]**
PURE*frexRD* will be applied to simple and effective *in vitro* selection of cyclic peptide and will contribute to the development of cyclic peptide based drugs.

## ①Best reconstituted cell-free protein synthesis system for *in vitro* selection having the lowest level of RNase contamination: PURE*frex*




**Points**
- Fully reconstituted.
- No cells, No bias.
- Fast, Easy, Pure.
- High throughput.
- Lower contaminants.

*Very low level of RNase, patented as JP4931135*

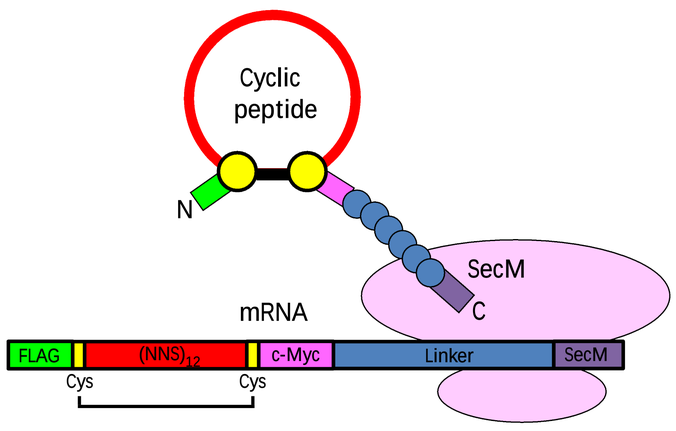
**Best suited for Ribosome display and mRNA display**

Shimizu Y et al., Nat.Biotechnol (2001) vol 19, p751-755

PURE*frex* is the reconstituted *in vitro* transcription and translation system which consists of purified 36 proteins and *E. coli* ribosomes necessary for transcription, translation and energy recycling. It also contains amino acids, NTPs and *E. coli* tRNA, so the target protein can be synthesized just by addition of the template DNA to the reaction mixture.

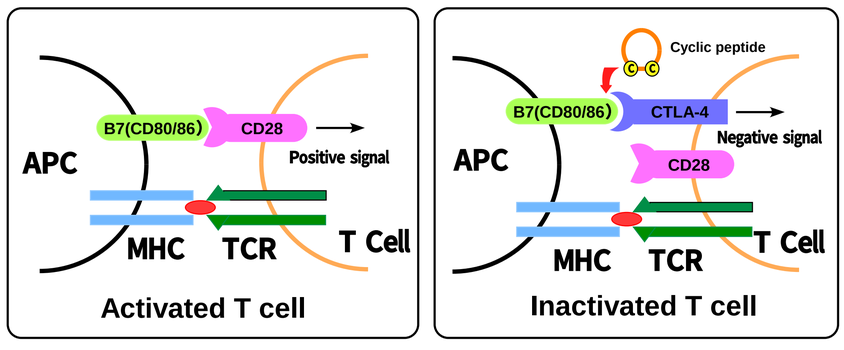
## ②Simple selection with PURE*frexRD* for cyclic peptide

**Ribosome Display complex with cyclic peptide**



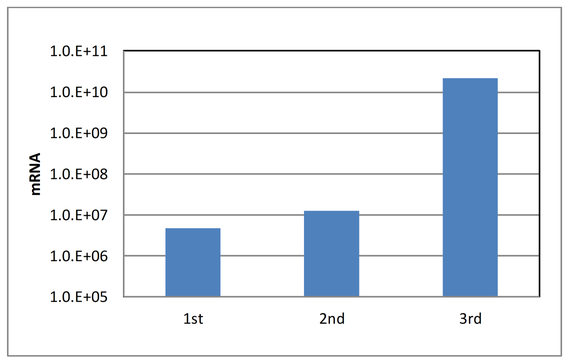

When the arrest sequence of SecM at 3'terminus is translated in RURE*frex*, a ribosome can be fixed strongly on mRNA. Also, release factors (RF1, RF2, RF3, RRF) were removed from PURE*frex*, and oxidized glutathione (GSSG) and disulfide isomerase from *E.coli* (DsbC) in optimized concentration to form disulfide bond were added into PURE*frex*. As a result, RD complex become highly stable, and cyclized peptide is displayed on ribosome with high efficiency.

## ③Immune checkpoint via CTLA-4




When CTLA-4 bind to B7, T-cells are led to anergy. Anergic T-cells have limited effector function. Anti-CTLA-4 neutralizing cyclic peptides inhibit CTLA-4 binding to B7 and promote T-cell activation.

## ④*In vitro* selection by PURE*frexRD*



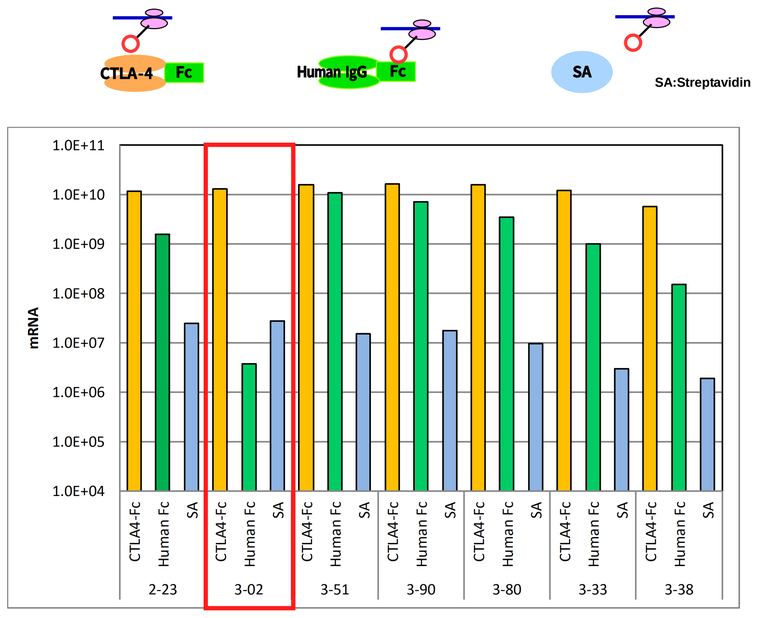

Cyclic peptide library having 12mer random sequences (Cys - X12 - Cys) was screened against biotinylated CTLA-4-Fc protein. Recovered mRNA increased along with the progress of selection round.

## ⑤Sequencing after 3rd round selection

| | | Squence | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Number of conc. | clone name | Cys | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Cys |
| 51 | 3-02 | | | | | | | | | | | | | | |
| 6 | 3-51 | | | | | | | | | | | | | | |
| 4 | 3-33 | | | | | | | | | | | | | | |
| 3 | 3-80 | | | | | | | | | | | | | | |
| 2 | 2-23 | | | | | | | | | | | | | | |
| 2 | 3-90 | | | | | | | | | | | | | | |
| 2 | 3-38 | | | | | | | | | | | | | | |

Some of enrichment were observed from the sequencing (94 clones). Each color represents specific amino acid.

## ⑥Confirmation of binding activity by RD pull down assay




Only 3-02 binder which was the most enriched clone bound to the CTLA-4 region in CTLA-4-Fc protein specifically (red-square), and another clones bound to the Fc region.

## ⑦Affinity measurement of 3-02 binder by BLItz system

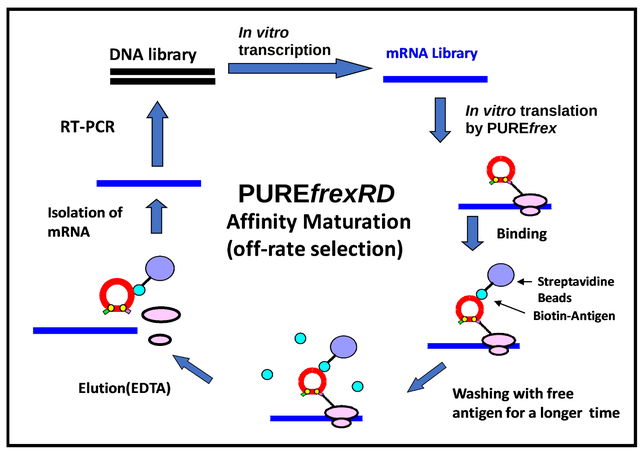
<Instrument for measurement>
- BLItz system (FORTE Bio)

<Condition>
- Sample : Cyclic peptide- MBP-fusion protien
- Antigen : Biotinylated CTLA-4-Fc protein
- Biosensor : SA

| | $k_a$ (1/Ms) | $k_d$ (1/s) | KD (M) |
|---|---|---|---|
| 3-02 | $3.18X10^{3}$ | $1.23X10^{-2}$ | $3.88X10^{-6}$ |

3-02 binder was expressed as MBP-fusion protein in *E.coli* and purified by affinity resin. Binding kinetics to CTLA-4-Fc was determined using BLItz system. Biotinylated CTLA-4-Fc was immobilized on a SA Biosensor according to the standard method.

## ⑧Affinity maturation by PURE*frexRD* (Off-rate selection)

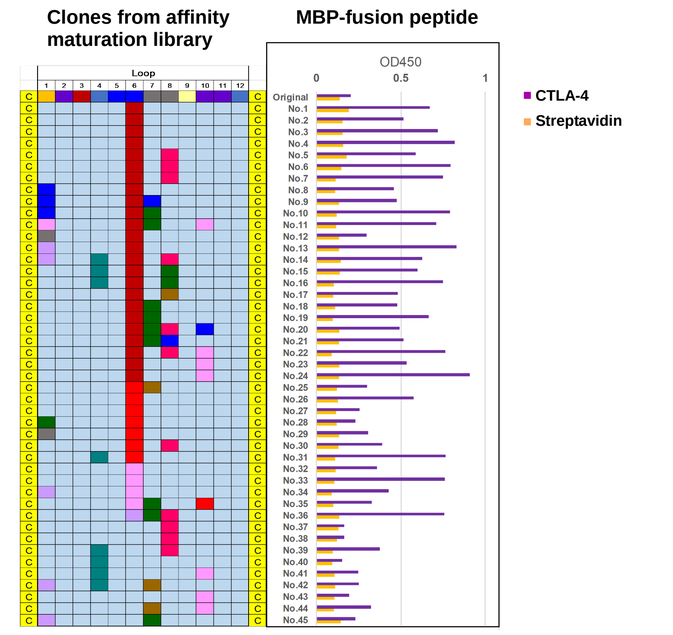



Washing time
1st round : 2h
2nd round : 19h
3rd round : 67h

Mutation :
Every round

In off-rate selection, RD complexes bound to CTLA-4-immobilized streptavidin beads were washed with large excess of free CTLA-4 to prevent the rebinding of RD complexes to the beads. The higher-affinity binders can be retained on the beads in a longer washing time. Library was prepared by error prone PCR using 3-02 clone as template DNA.

## ⑨Evaluation of amino acid sequence and ELISA in MBP-fusion format after affinity maturation by PURE*frexRD*

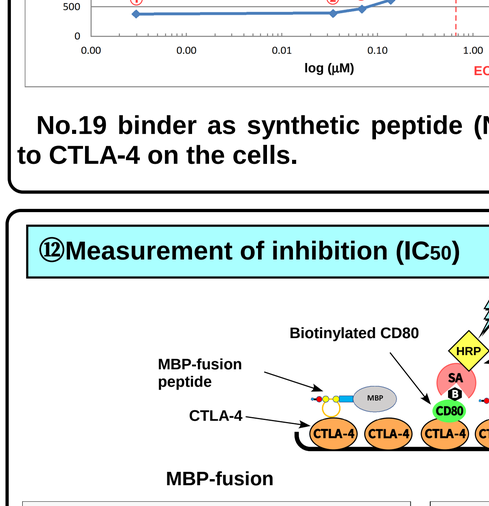


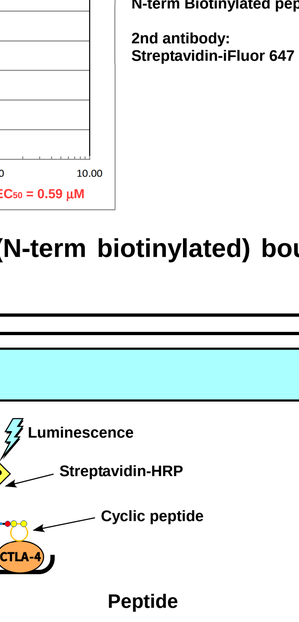

It seems that mutating of position 6 residue was very important to have the higher binding activity, and position 2,3,5,9,11,12 residues would be essential for specific binding to CTLA-4.

## ⑩Measurement of affinity ($EC_{50}$) as MBP-fusion peptide

*E.coli* expression host : BL21(DE3)
Culture condition : 30℃
Induction : final 0.1 mM IPTG

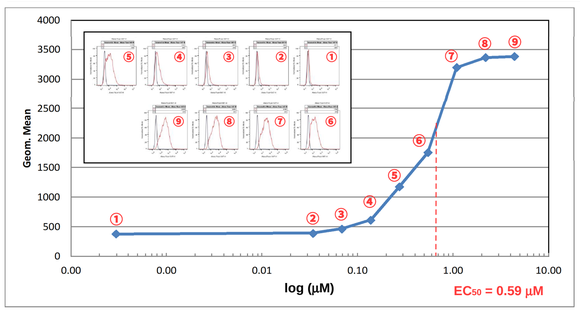
Purification : His tag purification




The high affinity clones (27 clones) from ELISA were purified, and $EC_{50}$ of the each were measured. As a result, all clones except No.27 showed the higher $EC_{50}$ than original. In particular, No.19, 10, 24 (red square) showed about 50 times higher affinities than the original.

## ⑪Cell based binding assay with the synthetic peptide (FACS)



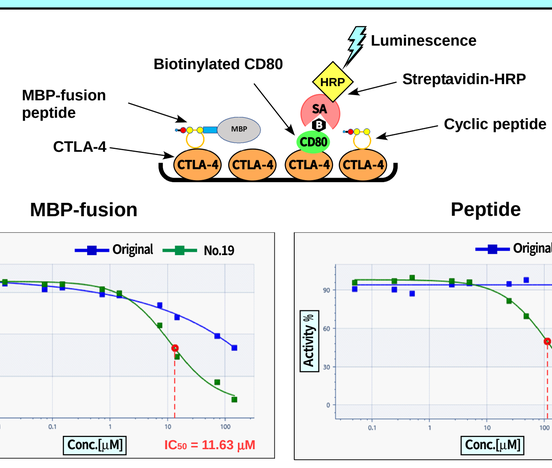

CTLA-4 Cell line:
1X $10^5$ cells/reaction, 100 μL

Synthetic peptide:
N-term Biotinylated peptide

2nd antibody:
Streptavidin-iFluor 647

No.19 binder as synthetic peptide (N-term biotinylated) bound to CTLA-4 on the cells.

## ⑫Measurement of inhibition ($IC_{50}$)




No.19 binder showed the inhibitory activity against interaction between CD80 and CTLA-4 in both MBP-fusion and fully synthetic peptide format, but original clone (3-02) showed very low activity. However, the inhibitory activity of the synthetic peptide was lower than MBP-fusion. It seemed that the activity of synthetic peptide was influenced by the characters of peptide such as solubility, isoelectric point and conformational fluctuation.

## Summary

1, Functional cyclic peptides against CTLA-4 were selected easily and rapidly by PURE*frexRD*.

2, In affinity maturation of CTLA-4 binders, we succeeded to select lead cyclic peptide with good $EC_{50}$/$IC_{50}$.

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