Simple and Effective Selection of cyclic peptides by Ribosome display with PURE*frex*®

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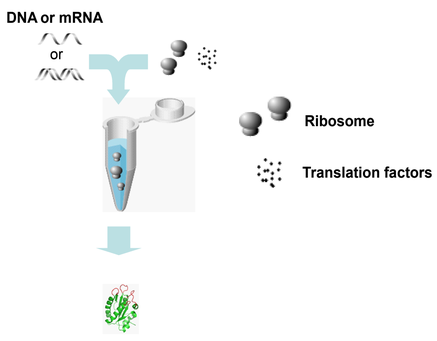
Abstract

Ribosome Display (RD) has many advantages over existing in vitro selection methods in some ways, so we developed RD utilizing PURE*frex*, which is a fully reconstituted cell-free expression system, and named it as PURE*frex RD*. We have already reported that the PURE*frex RD* showed higher selection efficacy than that using other reconstituted cell-free expression systems or using S30 extract systems, approximately with 100-fold or 1000-fold, respectively. We also showed that selection of a scaffold protein or even a Fab format without converting to scFv could be applied on PURE*frex RD*. This year, we report the result from the selection of cyclic peptides using PURE*frex RD*. Cyclic peptides are the peptides having a disulfide bond between two cysteine residues. The natural examples are such as endothelin and atrial natriuretic peptide, and those cyclic peptides show preferable drug like property than linear peptides. There are several methods reported for the development of cyclic peptides, but those methods need many steps and techniques (non-natural amino acids, enzymes, chemicals, etc.), and in most cases, it needs extra work for the screening.

Here we report very simple and effective approach for the development of cyclic peptides based on PURE*frex RD*. A constrained 10 mer random peptide library FLAG-Cys-X10-Cys-Myc was screened against biotinylated Erk2 protein (MAP kinase family protein relating to cell signaling) as a target antigen. The nascent polypeptide-ribosome-mRNA complex (RD complex) was formed using the customized PURE*frex* supplemented with oxidized glutathione and *E. coli* disulfide isomerase. After ~4 round selections, mRNA was recovered with greatly increased outputs. Following cloning and sequencing resulted in good variety of the binders with some enrichment. Then, we established screening process following the expression of those peptides in periplasm of *E. coli* as a fusion protein. These fusion proteins had the binding activities for antigen under the oxidized condition only without DTT. This result indicates that the fusion proteins hold ring structure with disulfide bond, and that the formation of a disulfide bond is necessary for its binding activity.

As such, we show that PURE*frex RD* works for the selection of functional cyclic peptides simply and effectively, and it could be applied for the development of peptide based drugs, such as cyclic peptides or more constrained peptides such as Disulfide Rich Peptide (DRP).

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)

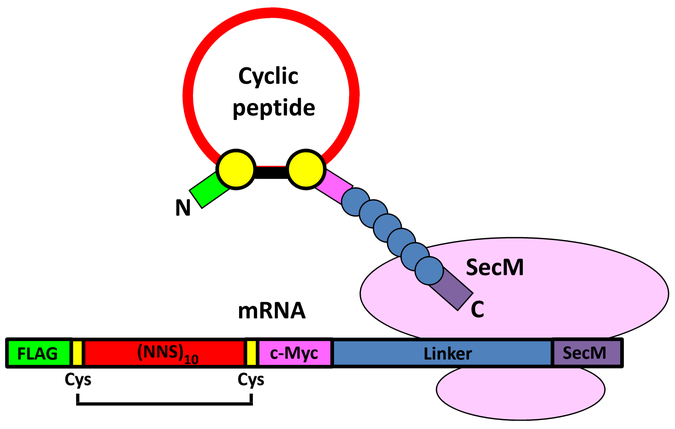
Best suited for Ribosome display

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

PURE*frex* was developed as "highly purified PURE system", and it is clearly more suitable for Ribosome Display. PURE system is a well-known 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*frex RD* for cyclic peptide

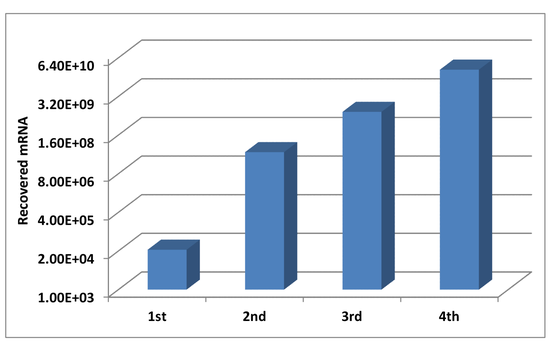
Ribosome Display complex with cyclic peptide



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.

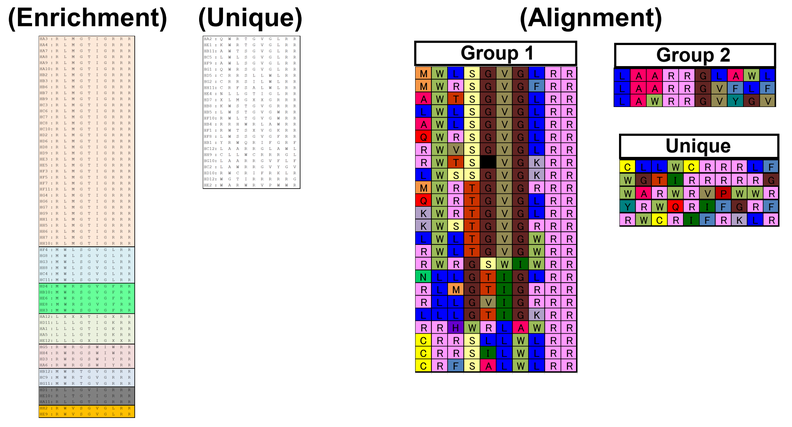
③*In vitro* selection of binders for the target, Erk2

<Condition>
- Target antigen : Biotinylated Erk2 protein



The peptide library with variety of 3 X 10^{11} was used. Recovered mRNA increased along with the progress of selection round. Final recovery rate against input mRNA after 4th round was about 2.0 %.

④Sequencing after 4th round selection



Some of enrichment were observed and many unique clones were identified from the sequencing (94 clones). In addition, alignment analysis showed that some of the clones were categorized into two groups as in the above. As an overview, arginine and hydrophobic amino acids such as leucine or tryptophan appeared with high frequency.

⑤Simple expression of cyclic peptide as MBP-fusion protein

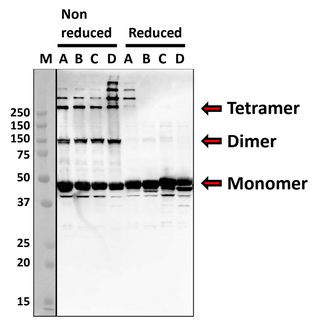
<Construct>

MW:51KDa

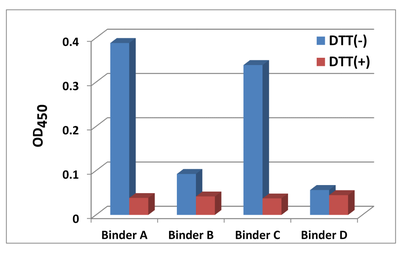
Expression by *E.coli*

<Host>
- *E.coli* (BL21DE3, Periplasm)

<Culture conditon>
- Temperature : 30 ℃
- Induction : final 0.1 mM IPTG



Loading : Culture volume 10 μL /lane
2nd antibody : anti-FLAG HRP
Detection : ECL prime (GE)



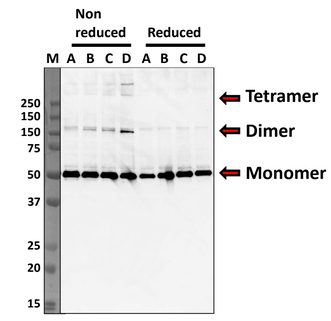
Loading : Culture volume 10 μL /well
2nd antibody : anti-FLAG HRP

Some of the enriched clones in sequencing were expressed in periplasm of *E.coli* in very simple construct (FLAG - Cys - 10aa - Cys - c-Myc - His8) , but they could not be detected in western blotting and ELISA (not shown). Therefore, we tried to express it as cyclic peptide-MBP fusion protein as in the above. The data of western blotting (left above) indicated that the expressed fusion proteins were polymerized under oxidized condition in periplasm of *E.coli*. That polymerizations seemed to occur through the SS-bond because polymerization disappeared under reducing condition. In addition, these fusion proteins had the binding activities for the target under the oxidized condition only without DTT (as shown in ELISA data, right above). This result indicates that the fusion proteins hold ring structure with disulfide bond, and that the formation of a disulfide bond is necessary for its binding activity. On the other hand, binder B and D showed very weak signal, which suggest that those clones might be dropped in the screening with this expression & ELISA.

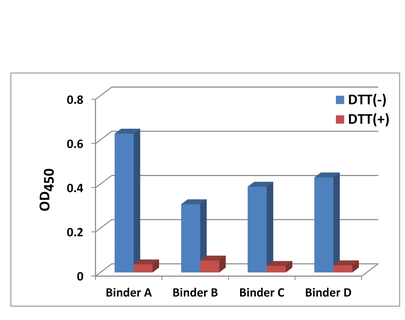
Expression by PURE*frex*

<Expression reagent>
- PURE*frex* (customized PURE*frex*)
- DTT : final 2 mM
- Oxidized Glutathione (GSSG) : final 3 mM
- Disulfide isomerase (DsbC) : final 16 μM

<Reaction>
- Temperature : 37 ℃
- Time : 2 hour



Loading : Reaction mix 1 μL /lane
2nd antibody : anti-FLAG HRP
Detection : ECL prime (GE)



Loading : Reaction mix 2 μL /well
2nd antibody : anti-FLAG HRP

Cyclic peptide-MBP fusion proteins were expressed in PURE*frex*. In this case, the polymerizations were remarkably suppressed than that in *E.coli* expression (as shown in western blotting, left above), and all the binders showed good binding activity (as shown in ELISA, right above). Those results suggest that PURE*frex* is suitable for the expression of those binders.

⑥Comparison of 1st screening ELISA between PURE*frex* and *E. coli* expression

(PURE*frex*)

(Condition)
Expression : 5 μL (reaction volume) /well
ELISA input : 2 μL /well

S/N>3	1	2	3	4	5	6	7	8	9	10	11	12
A	9.3	14.1	4.4	10.6	7.3	11.4	13.2	12.9	14.7	3.7	12.4	8.1
B	15.6	11.0	9.7	12.4	11.3	11.0	11.8	1.1	11.3	6.9	13.3	10.2
C	9.7	11.2	14.5	8.9	13.1	18.0	11.5	10.8	13.9	13.1	5.5	8.3
D	5.5	11.3	10.0	1.1	9.2	10.5	4.7	2.9	12.9	14.1	7.1	6.3
E	11.1	13.0	7.6	12.8	9.6	10.8	10.8	8.2	7.4	9.5	12.7	8.2
F	12.5	15.3	10.2	8.0	7.8	7.5	7.9	9.7	11.8	11.6	0.9	8.3
G	11.1	6.0	8.6	6.8	7.2	10.7	4.2	9.5	9.7	8.7	7.0	8.2
H	11.1	10.4	8.0	5.4	6.9	8.9	8.2	9.1	7.0	7.1	1.0	0.9

(Positive: 95 %)

(*E.coli*)

(Condition)
Expression : 100 μL (culture volume)/well
ELISA input : 1 μL (culture lysis) /well

S/N>3	1	2	3	4	5	6	7	8	9	10	11	12
A	7.4	7.3	6.0	9.7	1.0	1.2	1.7	1.3	6.4	0.6	2.7	0.9
B	3.9	2.4	0.9	5.4	1.6	6.1	1.5	1.0	1.8	0.9	1.4	6.1
C	1.6	1.9	5.6	0.7	1.2	3.7	6.8	17.7	6.3	1.2	1.0	2.5
D	3.0	3.0	4.3	1.2	1.1	7.3	1.4	0.5	1.6	1.4	0.6	6.8
E	3.4	1.9	1.8	1.2	1.8	1.1	1.2	0.9	1.4	1.2	19.8	2.0
F	1.5	1.5	8.7	1.3	1.5	1.0	1.6	2.9	1.1	1.2	1.0	7.8
G	1.3	0.6	1.7	2.7	1.3	1.1	1.1	4.8	0.8	0.6	0.4	1.2
H	2.7	1.9	1.8	2.3	2.1	5.5	1.4	1.4	4.8	14.8	1.0	0.9

(Positive: 26 %)

Due to the good expression property with PURE*frex*, it showed remarkably better hit rate than that with *E. coli*. The data suggests that PURE*frex* is more suitable for the expression and the screening at the initial process, not to miss the potential binders.

⑦Affinity measurement of selected binders by BIACORE

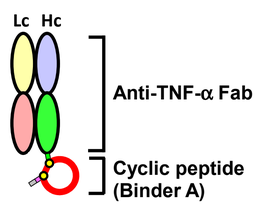
<Condition>
- Sample : Cyclic peptide- MBP-fusion protien (Binder A and C)
- Antigen : Biotinylated Erk2 protein (immobilized RU=162)
- Sensor chip : SA

	k_a (1/Ms)	k_d (1/s)	KD (M)
Binder A	1.48X10^4	5.41X10^{-4}	3.66X10^{-8}
Binder C	1.51X10^4	4.43X10^{-4}	2.93X10^{-8}

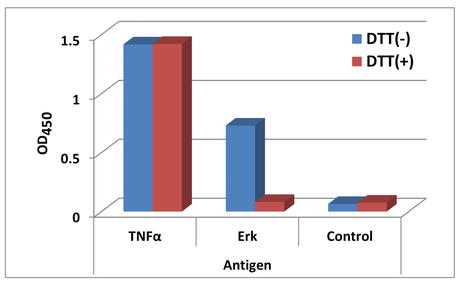
Binders were purified by Ni resin. Binding kinetics of binders to Erk2 were determined using surface plasmon resonance with BIACORE. Biotinylated Erk2 was immobilized on a SA sensor chip according to the standard method. At a flow rate of 20 μL/min, five concentrations from 0.3 μM to 4.2 μM were used to record sensorgram, and k_a, k_d and KD were determined by evaluation software.

⑧Application I; For bispecifics

<Construct>



<Expression system>
- Customized PURE*frex*



<Condition>
- Temperature : 37 ℃
- Time : 4 hour

Loading : Reaction mix 1 μL /well
2nd antibody : anti-FLAG HRP

As an application of cyclic peptide, we looked into the combination with antibody to make it bispecific. Selected cyclic peptide binding to Erk2 was simply fused to C-terminal of a heavy chain of anti-TNF-α Fab, and the each binding activity against TNF-α or Erk2 protein were checked up by ELISA. As a result, this fusion protein bound to the each antigen under non-reducing condition as designed, while only the specific binding activity to Erk2 disappeared under reducing condition.

⑨Application II; For epitope mapping/mimotope development, etc.

Target antigen : Herceptin (anti-HER2 antibody)

<Supposed application of binder>
- Epitope mapping
- Anti-idiotype binder (like anti idiotype antibody)
- Mimotope vaccination

(1st screening ELISA)

Herceptin: IgG1 Positive 68.4%

S/N>3	1	2	3	4	5	6	7	8	9	10	11	12
A	2.15	8.51	9.82	5.83	5.89	0.87	11.6	11.3	7.5	50.8	0.77	5.9
B	2.03	5.8	9.7	4.45	1	1.53	1.91	0.81	6.54	3.99	1.75	2.93
C	1.75	4.86	1.86	10.4	13.7	4.58	2.89	3.3	3.83	5.66	29.9	2.95
D	6.58	4.48	1.53	1.19	1.87	2.18	2.65	5.03	0.98	3.66	7.92	7.72
E	11.1	3.96	0.96	4.51	7.26	6.61	5.32	5.49	6.95	3.05	3.87	31.5
F	0.88	15.7	6.29	9.09	9.56	7.59	2.24	3.62	3.75	1.4	4.29	3.6
G	5.15	3.78	11.2	30.1	10.1	0.8	5.6	5.12	5.58	3.9	5.51	4.43
H	5.75	1.1	4.76	5.34	4.05	7.28	5.33	1.05	3.83	1.92	2.77	1

Sequencing

Control Antibody A: IgG1

S/N>3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.77	0.91	0.8	0.78	0.78	0.64	0.85	0.75	0.72	0.84	0.79	0.94
B	0.92	0.93	0.86	0.92	0.86	0.81	0.87	0.78	0.81	0.86	0.85	0.84
C	0.86	0.84	0.84	0.9	0.88	0.66	0.84	0.98	0.8	0.77	0.9	0.94
D	1.16	1.06	1.03	1.07	1.07	0.93	1.05	1.01	0.96	0.98	1.07	0.91
E	0.89	0.92	0.78	0.88	0.89	0.83	0.91	1.01	1	0.93	0.98	1.19
F	0.92	1.03	0.92	1	0.98	0.94	0.98	0.91	1	1	0.98	1.06
G	0.98	0.88	0.9	0.98	0.99	0.87	1	0.99	1.08	1.03	0.93	1.13
H	1	0.9	0.93	1.06	1.01	0.93	0.99	0.93	1.02	0.89	0.99	1

Control Antibody B: IgG4

S/N>3	1	2	3	4	5	6	7	8	9	10	11	12
A	0.74	0.72	0.78	0.65	0.82	0.64	0.79	2.01	0.95	0.81	0.84	0.77
B	0.73	1.01	0.87	0.92	0.87	0.94	0.89	0.73	0.83	0.89	1.01	0.86
C	0.79	0.8	0.71	0.84	1.05	0.96	0.87	0.78	0.79	0.73	0.86	0.82
D	0.79	0.99	0.92	1.31	1.16	1.18	1.09	0.98	0.84	0.97	1.01	0.96
E	0.82	0.91	0.85	0.84	0.98	0.99	0.97	1.01	1.02	0.94	0.93	1.01
F	0.78	0.9	0.84	0.86	0.86	0.83	0.83	0.97	0.89	0.94	0.97	1.04
G	0.82	0.77	0.83	0.82	0.85	0.88	0.88	1	0.97	1.04	0.91	0.91
H	0.78	0.83	1.01	0.85	0.9	0.96	0.92	0.79	0.97	1.02	1.04	0.99

(Enrichment of binders)

Sequence	Frequency
Binder A	56/89
Binder B	5/89
Binder C	5/89
Binder D	5/89
Binder E	4/89
Binder F	4/89
Binder F	1/89

Cyclic peptides were selected against anti-Her2 monoclonal antibody. A lot of binders were obtained from 1st screening ELISA, and some of the enrichments were observed as shown above. The binders didn't bind to control antibody A or B classified into IgG1 or IgG4, respectively. This result shows that those binders bind only to the CDR regions of Herceptin specifically, not to the constant or Fc region.

Summary

1, Simple and effective selection system for the development of cyclic peptide were reported.
- PURE*frex* (fully reconstituted, customized, the lowest RNase contamination) is a key to success.

2, Actual example of the selection of cyclic peptide against Erk2 as target was performed.
- Many, various, unique binders were obtained in just 4 round panning.
- 10^{-8} KD binders were selected without affinity maturation.

3, Simple screening is possible with PURE*frex*.
- Only with natural amino acids, expression is easy.
- Many clones can be expressed for the screening in parallel.
- PURE*frex* makes it more effective comparing to *E. coli.*

4, Unique applications were proposed.
- Cyclic peptide can add another binding activity to the existing biologics to make it bispecific in very simple manner.
- Targeting existing antibodies, epitope mapping, mimotope development, anti-idiotypic binder (like anti-idiotypic antibody) development can be done with PURE*frex RD*.

(On-going)

- **Unique cyclic peptide for PD-1 (and for other therapeutic targets) are under validation.**
- **DRP (Disulfide Rich Peptide) screening is on-going aiming for the development of orally available biologics.**

<Ackowledments>
We thank Mr. Keita Iguchi in KANEKA corporation for BIACORE analysis.

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