In vitro affinity maturation based on Ribosome Display System with PURE*frex*

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Abstract

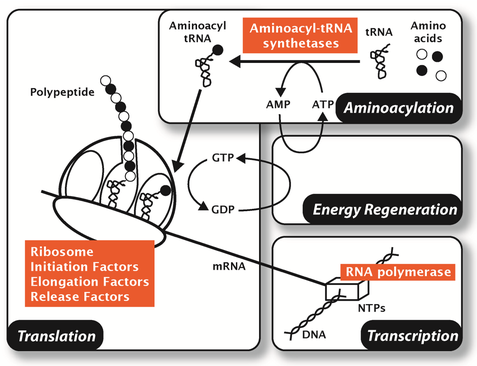
The affinity maturation is one of the key technologies to distinguish the binder from the others against the same target. For easy and feasible affinity maturation, ribosome display is well known to give great advantages. Especially, ribosome display on the PURE system was reported as a powerful method not only for *in vitro* selection of specific binders such as antibodies or designed proteins like scaffolds, but also for *in vitro* affinity maturation.

The PURE system is one of the cell-free protein synthesis system reconstituted from the purified components necessary for the translation in *E. coli*. Recently, we developed the upgraded PURE system (PURE*frex*), in which the contaminants such as RNases, lipopolysaccharides and other proteins unnecessary for the translation are extremely decreased. We also developed the ribosome display system utilizing PURE*frex* (PURE*frexRD*). The selection efficiency of PURE*frexRD* is raised approximately 100-fold or 1000-fold higher than ribosome display using the original PURE system or *E.coli* S-30 extract system.

In this conference last year, we reported *in vitro* selection of the binders to Erk2 protein from our own designed protein scaffold library based on FHA domain of RNF8 (human E3 ubiquitin ligase) by PURE*frexRD*. This year, we report the application of PURE*frexRD* to *in vitro* affinity maturation by off-rate selection. We prepared two randomized RNF8 libraries based on the specific binder to Erk2 by PCR-based DNA shuffling or grafting one of the loops of RNF8. After three rounds of off-rate selection over several days, we successfully improved the affinity approximately 35-fold more than the original binder in very simple steps.

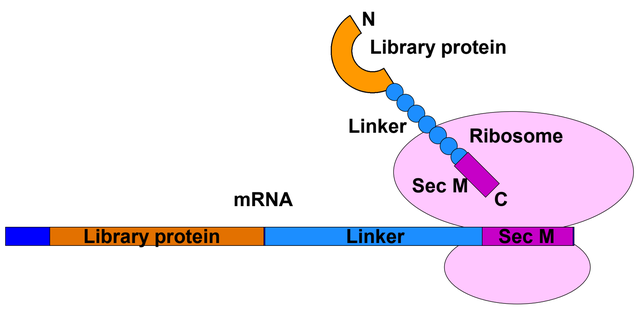
On the other hand, the affinity maturation of Fab will give great advantages in terms of seamless development for generating better antibody therapeutic. Therefore, we have applied the PURE*frexRD* to the affinity maturation of Fab fragments and have developed the protocols to successfully form Fab fragments on ribosome.

①PURE*frex*:Highly purified PURE system



PURE system is a reconstituted *in vitro* transcription and translation system which consists of purified 36 proteinous factors and *E. coli* ribosome 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 adding the template DNA to the reaction mixture. PURE*frex* is developed as " highly purified PURE system" which has been well optimized to ribosome display; Contamination of such as Lipopolysaccharide (LPS) in the system is very low.

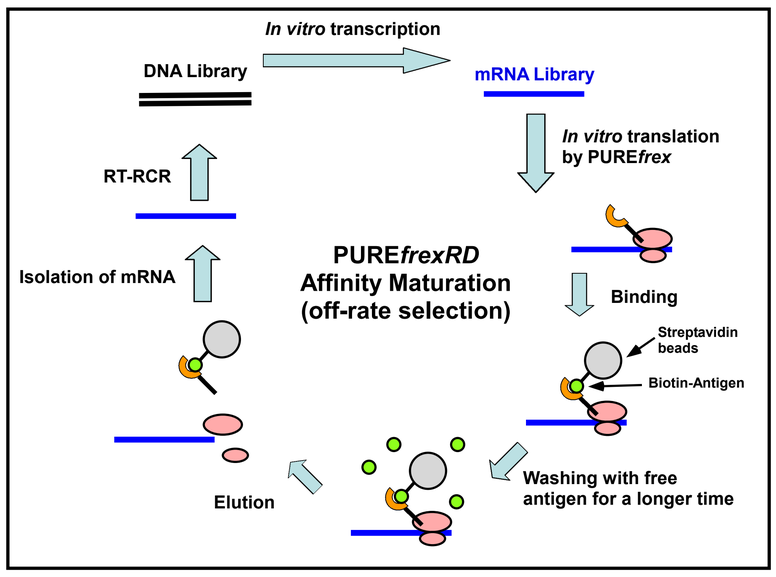
②mRNA-ribosome-protein complex of PURE*frexRD*



Advantages

1)mRNA-ribosome-protein complex is highly stable in the PURE*frex* because activies of nucleases and other Inhibitory factors are very low in this system.
2)The mRNA encoding a scaffold protein with translation arrest sequence of SecM remains stably attached to a ribosome in the PURE*frex*.
3)PURE*frexRD* have high selection efficiency than using normal PURE system RD.

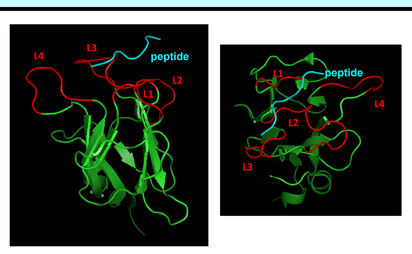
③*In vitro* affinity maturation with PURE*frexRD*



In off-rate selection, mRNA-ribosome-protein complexes bound to antigen-immobilized streptavidin beads are washed with a large excess of free antigen to prevent the rebinding of mRNA-ribosome-protein complexes to the beads. The higher-affinity binders can be retained on the beads for a longer washing time.

④Affinity maturation of RNF8 Erk2-binder

Feature of RNF8 Erk2-binder
- MW : 18kDa
- structure : β sandwich
- Loop: 4 loop
- Kd :2.9×10^{-8}
- antigen: Erk2 protein



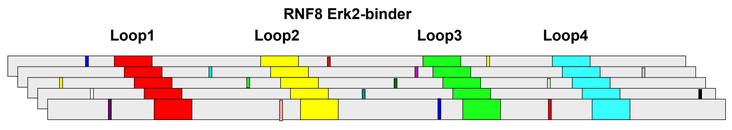
Native structure of RNF8-peptide complex (side-view & top-view)

Last year, we reported *in vitro* selection of the binders to Erk2 protein from the designed RNF8 scaffold library. The library was constructed by randomizing the 4 loops (red lines) to NNS. The Kd value of an obtained binder is 2.9×10^{-8}. This binder was used for affinity maturation.

RNF8 is a E3 ubiquitin ligase which plays a critical role in the early DNA-damage response via the ubiquitination of histone H2A and H2AX. The global fold of the FHA domain of RNF8 is an 11-stranded β-sandwich structure with the phosphopeptide-binding surface comprised of 4 loops.

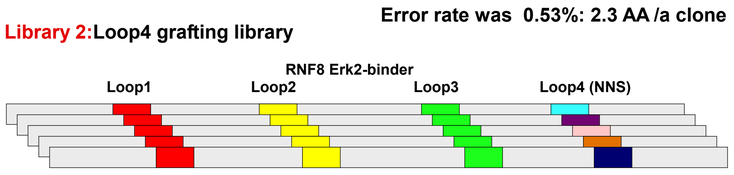
⑤Construction of mutated RNF8 Erk2-binder Library

Library 1:PCR-based DNA shuffling library (ref:Zhao et al.1998. Nat.Biotechnol., 16. 258)



Error rate was 0.53%: 2.3 AA /a clone

Library 2:Loop4 grafting library

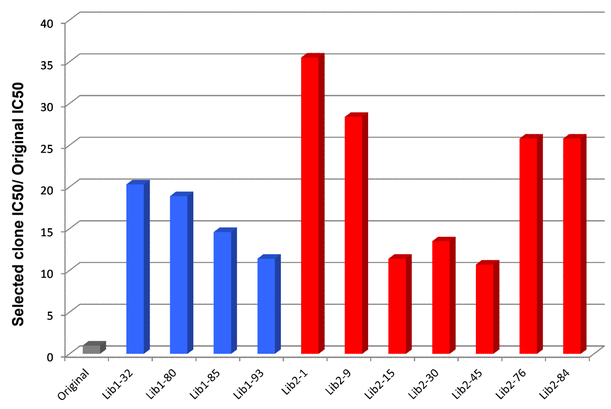


Library diversity: 1.2×10^{11}

⑥Protocol of off-rate selection with PURE*frexRD*

mRNA Libraries (1.2×10^{12} molecules)
↓
In vitro translation in 20 μL of PURE*frex*
↓4°C、30 min
Mixing with Erk2 protein (0.1 nM)-immobilized streptavidin beads in TBST 200 μL
↓4°C、120 min
Adding of free-Erk2 protein (final 1 μM)
↓4°C、incubation for long times
(1^{st} round:1 day, 2^{nd} round:4 days, 3^{rd} round:7 days)
Washing five times with 500 μL of TBST
↓
Eluting mRNA from mRNA-ribosome-protein complexes with 100 μL of 50 mM EDTA
↓
RT-PCR→Next round selection
↓
Subcloning
↓
Selecting unique clones by sequencing
↓
Screening higher affinity clones by competitive ELISA
↓
Purifying the higher affinity clones and determining IC_{50}

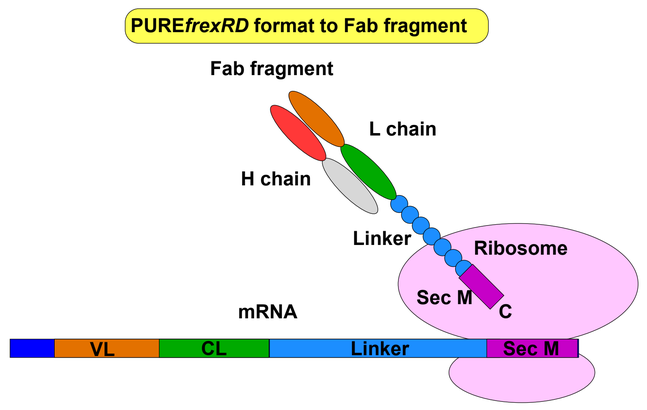
⑦Comparison of IC_{50} of selected higher affinity clones



IC_{50} of the each purified clone was determined, 4 clones from library 1, and 7 clones from library 2 showed more than 10-fold higher affinity than the original RNF8 Erk2-binder. The most improved clone was Lib2-1 from Library 2 (35-fold). As a whole, the higher-affinity mutants were easily obtained from Library 2 than library 1.

⑧Affinity maturation of Fab fragment with PURE*frexRD*

PURE*frexRD* format to Fab fragment

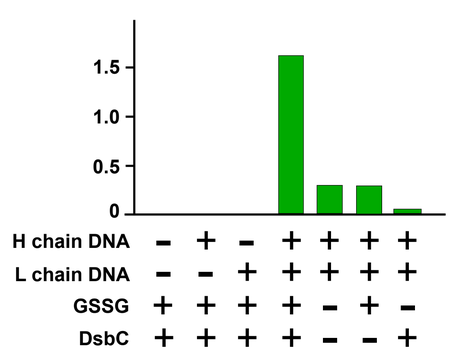


In almost the cases using cell-free display methods such as ribosome display, affinity maturation of antibody are only applicable to singe-chain Fvs (scFvs), but cannot applied to Fab fragment. On the other hand, it was reported that L chain shuffling method is highly effective on affinity maturation of Fab fragments with *in vitro* display method such as phage display and yeast display. Therefore, we have been developed the optimum representation of Fab fragments on ribosome. In PURE*frexRD* of Fab fragments, the randomized light chain (L chain) was presented on ribosome and the heavy chain (H chain) was translated as a free-protein.

⑨Optimization of PURE*frex* for Fab synthesis (PURE*frexSS*)

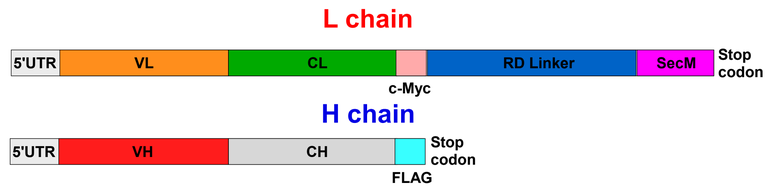
Synthesis of Fab fragment using PURE*frex* with oxidized glutathione (GSSG) and DsbC protein that catalyzed the formation of correct disulfide bounds.

PURE*frex*
↓+/- GSSG,DsbC
↓+/- H chain DNA (+FLAG)
+/- L chain DNA
↓incubation at 37°C for 4h
↓→Ag-coated well
↓+anti-FLAG/HRP
↓+TMB

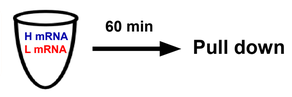


Fab fragment was synthesized using PURE*frex* supplemented with 3 mM GSSG and 23 μg/mL DsbC protein. When both of GSSG and DsbC were added in the system, functional Fab fragment with good binding activity was synthesized.

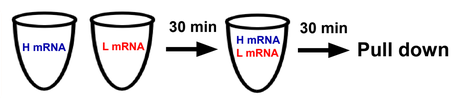
⑩Optimization of synthesis step of H chain and L chain fragment



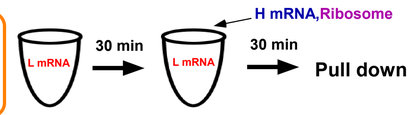
Condition 1 :H and L chain mRNA were translated in a tube at the same time



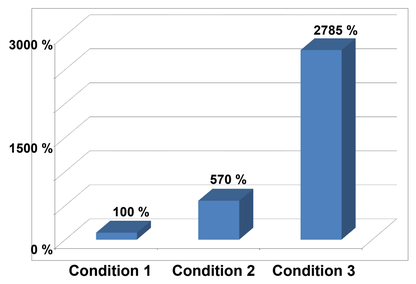
Condition 2 :H and L chain mRNA were separately translated in each tube, after 30 min, both tubes were mixed.



Condition 3:L chain mRNA was translated in a tube, after 30 min, H chain mRNA was added to L chain expressed tube.



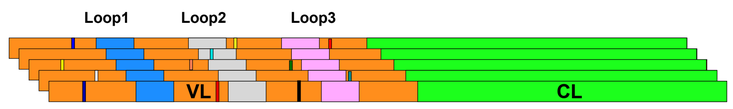
mRNA
↓PURE*frexSS*
↓translation (condition 1,2,3), 37°C
↓pull down (antigen ; TNF-α)
↓recovery mRNA
↓quantitative PCR



The recovered mRNA from condition 2 and 3 was increased at 5-fold and 25-fold than condition 1, respectively. These results indicate that H and L chain mRNA should be expressed in separated tube (condition 2) or in the same tube sequentially (condition 3). In translation of condition 1, because L chain mRNAs formed the L chain mRNA-ribosome-L chain protein complex, active ribosome was decreased, and it result in reduction of H chain protein. Also when H chain mRNA was independently translated, the synthesized H chain protein was easily caused to aggregation. Therefore, it seems that the recovered mRNA from condition 2 was lower than that from condition 3.

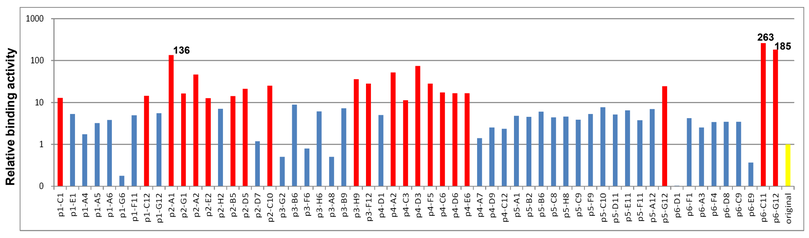
⑪Construction of mutated L chain Library

PCR-based DNA shuffling library (ref:Zhao et al.1998. Nat.Biotechnol., 16. 258)



↓ Off-rate selection by PURE*frex RD*

⑫Selection of Fab with higher affinity by ELISA



Library: L chain random mutagenesis library
Antigen: protein X (Soluble Domain of membrane protein)
Kd value of original antibody (IgG) :4.8×10^{-8}

Result

Relative specific binding activity (Original Fab = 1)	<1	1-10	1-100	100<
Number of clones	6	32	18	3

The figure shows the comparison of a specific binding activity of each clone. The specific binding activity is calculated by binding activity/protein. The activity and protein concentration were determined by ELISA and Western blotting, respectively, using cell extracts of *E.coli* expressed each clone after the three round selection. The higher activity clone with 10-fold more than the original clone (yellow) indicates in red bar.

Summary

1, We successfully demonstrated the affinity maturation with PURE*frexRD*. By means of the off-rate selection from DNA shuffling and loop grafting libraries, the affinity of mutated RNF8-binder was improved ~ 35-fold without affecting the specificity.

2, We developed the optimum display method of Fab fragments on ribosome based on PURE*frexRD*. In the optimum condition (Condition 3), functional Fab fragment was presented by 25-fold more better than in the conventional condition (Condition 1).

3, We were obtained a lot of the high affinity clones by PURE*frexRD* optimized Fab display, and three of clone showed >100-fold of specific activity than original.

In conclusion, PURE*frexRD* is expected to be applied to rapid and convenient affinity maturation for developing high-affinity binders of such as antibodies or scaffold proteins, especially for therapeutic and diagnostic applications.

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