PURE*frex RD*: the unique antibody engineering method based on Ribosome Display with PURE*frex*®

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Abstract

Recently, *in vitro* screening methods such as phage display or yeast display are commonly used for engineering antibodies, while they have Pros/Cons. Standing on this point, the development of other screening methods will contribute to engineer and make it better therapeutic antibodies. Ribosome display (RD) is expected to have several advantages over existing *in vitro* screening methods, so we developed our own RD (PURE*frex RD*) using very effective fully reconstituted cell-free expression system (PURE*frex*). We showed that a scaffold, a scFab format and a Fab format without conversion to scFv could be used on PURE*frex RD*. That was the first report of a direct Fab fragment selection on RD (PEGS 2012).

Here we report another application of PURE*frex RD* that can be utilized for further antibody engineering such as affinity maturation on the Fab format. We applied PURE*frex RD* to the lead therapeutic antibody for *in vitro* affinity maturation. We investigated the process for forming Fab displaying RD complex, which greatly influenced on the screening efficiency. For instance, we found that several conditions, such as the amount of ribosome to add into the system, or synthesizing H and L chain at different timing, had huge impact on the screening efficiency. We prepared two kinds of randomly mutated L chain libraries for whole region or only for CDRs of the L chain by PCR-based DNA shuffling. After three rounds of off-rate selection over several days, the Fabs were expressed in *E. coli* and their binding affinities were evaluated by ELISA assay. Finally, EC_{50} was determined for comparison in specific activity of each Fabs to the original Fab. We have successfully improved the EC_{50} approximately 60-fold better than the original Fab.

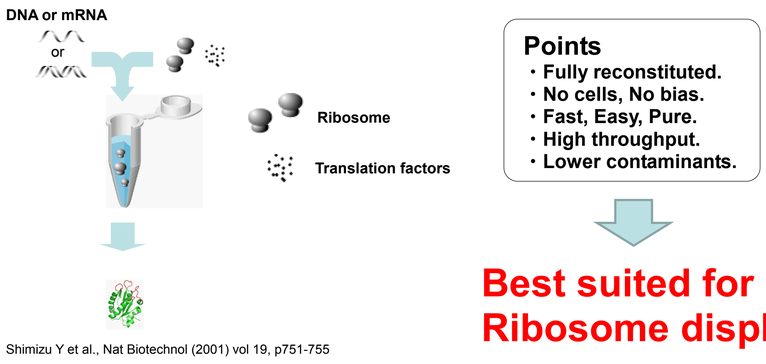
As such, PURE*frex RD* is the unique screening method, and it could be customized for various use. We would like to discuss the potential applications of PURE*frex RD* here.

① Our unique solutions for protein based biologics

PURE*frex*® — Fully reconstituted cell-free protein expression system

PURE*frex*®*RD* — Rapid and Easy in vitro screening method based on ribosome display for scFv, scaffold or Fab

② Our cell-free protein synthesis system: PURE*frex*



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

Best suited for Ribosome display

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

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. PURE*frex* is developed as "highly purified PURE system", and it is clearly more suitable for Ribosome Display.

③ Advantage of affinity maturation using Ribosome Display

Ribosome Display

Preparation of Library
- Library with high diversity can be prepared easily and quickly by only PCR.
- Preparation for multiple-clones are doable in parallel.

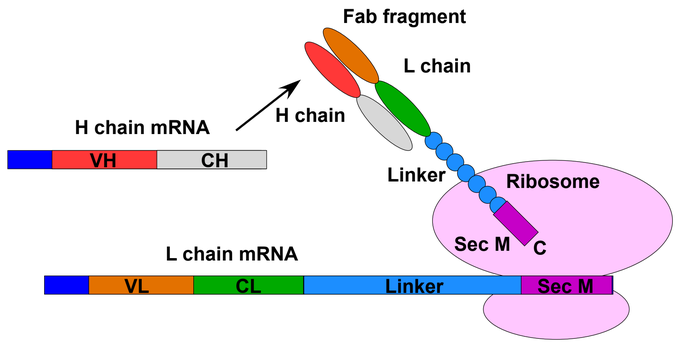
Panning
- Introducing mutation at every selection round is easy.

Phage Display

Preparation of Library
- Library with high diversity can not be prepared because it is limited by the efficiency of transformation.
- Preparation of multiple-clones are very difficult.

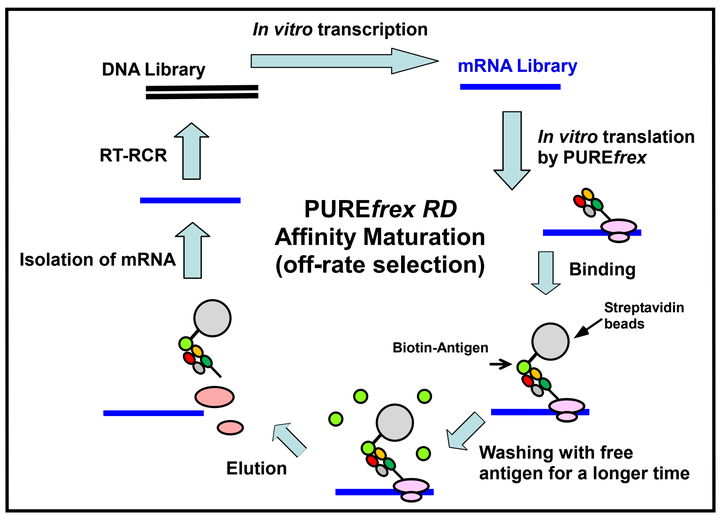
Panning
- Introducing mutations at every selection round is very difficult.

④ Affinity maturation of Fab antibody using PURE*frex RD*



In most cases of ribosome display, affinity maturation of antibody were applicable only to singe-chain Fvs (scFvs), and could not be applied to Fab format. On the other hand, it was reported that L chain shuffling method was highly effective on affinity maturation of Fab fragments with *in vitro* display such as phage display or yeast display. In 2012, we succeeded the efficient presentation of Fab fragments on ribosome by using PURE*frex RD* (PEGS 2012). PURE*frex RD* of Fab fragments is constituted from mutated L chain on ribosome and a H chain expressed as a free-protein.

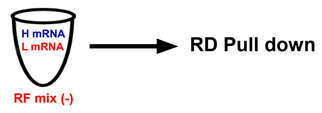
⑤ *In vitro* affinity maturation in PURE*frex RD*



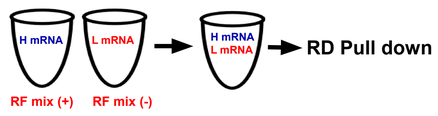
In off-rate selection, RD complexes bound to antigen-immobilized streptavidin beads were washed with large excess of free antigen to prevent the rebinding of RD complexes to the beads. The higher-affinity binders can be retained on the beads for a longer washing time.

⑥ Optimization of synthesis step of H chain and L chain

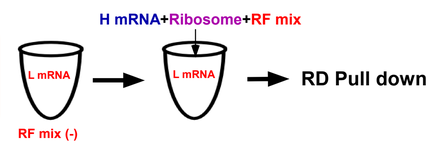
Condition 1 :H and L chain mRNA were translated in a tube at the same time



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



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

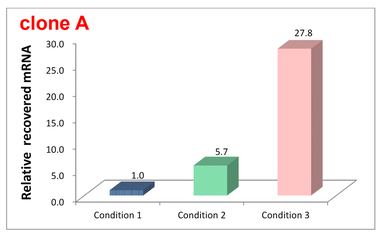


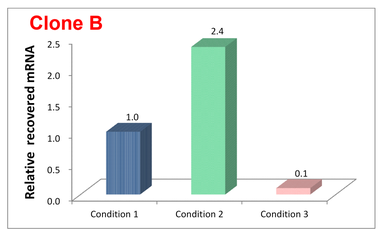
RF mix(Release factor 1, 2, 3, and ribosome recycling factor)

Because removal of RF mix from PURE*frex* is effective for sticking ribosome on mRNA, the efficiency of constructed L chain displaying RD complex has been increased in Condition 1 above. However, a part of H chain mRNA also constructed H chain displaying RD complex in this condition, and it resulted in the reduction of whole Fab displaying RD complex. Therefore, we investigated the suitable process for forming Fab displaying RD complex, which greatly influenced on the screening efficiency. Accordingly, we found that several conditions, such as the amount of ribosome to add into the system, or synthesizing H and L chain at different timing, have huge impact on the screening efficiency, so we optimized these conditions.

RD pull down
PURE*frex* (customized)
↓ translation (Condition 1, 2, 3), volume;20 μL
↓ binding , washing
↓ recovery of mRNA
↓ quantitative RT-PCR

Fab sample
Clone A
affinity; KD = 10^{-10} M
Clone B
affinity; KD = 10^{-9} M

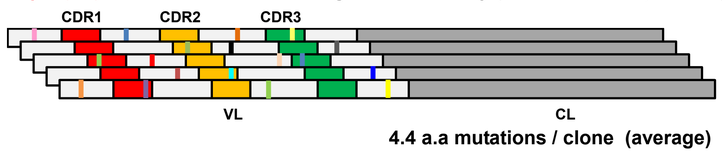




In the case of Clone A, condition 3 showed the highest recovery of mRNA among 3 conditions. In contrast, the best condition for Clone B was Condition 2, while Condition 3 was the worst. These results indicated the suitable condition for forming Fab displaying RD complex are different in each clone along with the characters such as expression level, stability, and solubility of Fab. Therefore, it is important to optimize expressing conditions of Fab to fully utilize the effective Fab displaying RD complex in the screening.

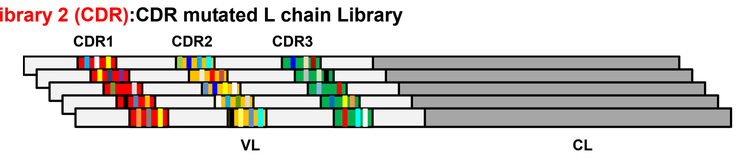
⑦ Construction of mutated L chain Library

Library 1 (EP):PCR-based DNA shuffling L chain Library (Nat.Biotechnol., 16. 258)

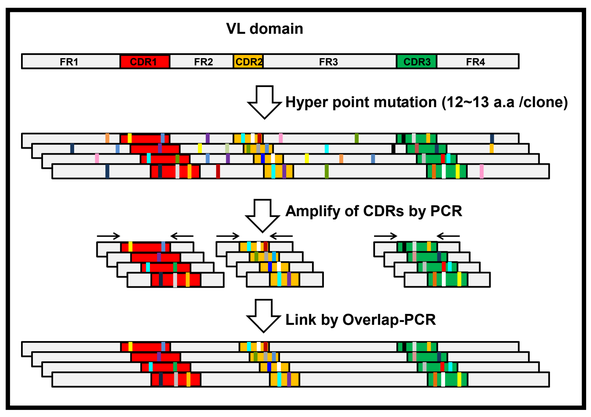


4.4 a.a mutations / clone (average)

Library 2 (CDR):CDR mutated L chain Library



	CDR1	CDR2	CDR3
Total residues	11	7	9
Mutations (average)	1.57	1.00	1.07

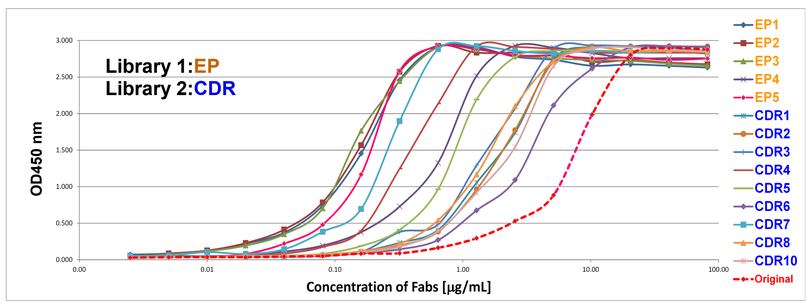


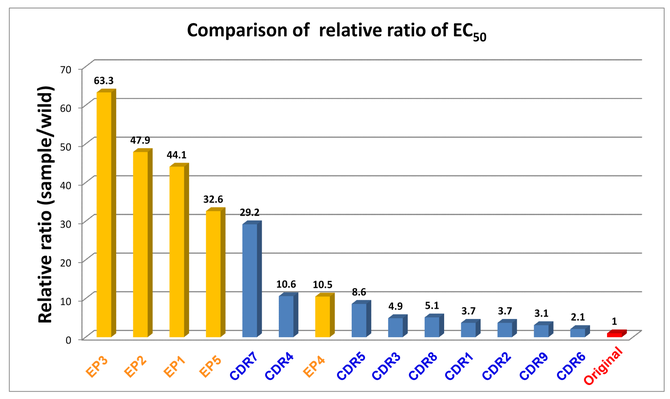
⑧ Protocol of off-rate selection with PURE*frex RD*

<Material>
Target : membrane protein
Original clone: KD=10^{-8} M (humanized antibody, epitope identified)
Panning antigen: Biotinylated Peptide (epitope peptide)
Free-antigen: Peptide (epitope peptide)

mRNA Libraries （diversity:1.2×10^{12})
↓
In vitro translation with PURE*frex* (customized, 200 μL)
↓
Antigen peptide-immobilized streptavidin beads in TBST 200 μL
↓4 ℃, 60 min
Washing five times with 500 μL TBST X 5 times
↓
Addition of free-antigen peptide (final 1 μM)
↓4 ℃, incubation for long time (1st round:1 day, 2nd round:3 days, 3rd round:7 days)
Washing five times with 500 μL TBST X 10 times
↓
Elution of mRNA from RD complexes with 100 μL EDTA (50 mM)
↓
RT-PCR→next round selection
↓
Sub-cloning
↓
Expression in *E.coli* and 1st screening ELISA with cell extract (380 clones), and pick up the higher affinity binders with 3-fold more than the original. (ELISA antigen; peptide or antigen expressing cell)
↓
Screening unique clones by sequencing.
↓
Expression and Purification of unique clones in small scale (*E.coli*. 5 mL culture).
↓
2nd Screening ELISA of higher affinity clones than the original clone in 1st screening ELISA.
↓
Purification of higher affinity clones in large scale (*E.coli* 200 mL culture), and measurement of EC_{50} and KD values.

⑨ Determination of EC_{50} by ELISA for peptide antigen



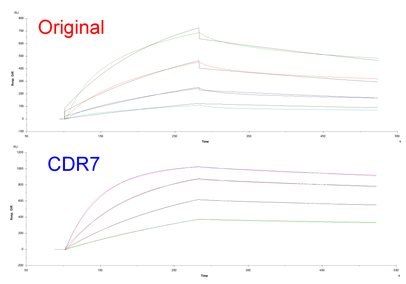


The high affinity clones from 2nd screening ELISA were purified, and EC_{50} of each was measured for peptide antigen. EC_{50} of each clone was shown as relative ratio to original clone. Clones from Library 1 (EP series, orange bars) tended to have higher affinity. EC_{50} of the original clone was 7.49 μg/mL, and that of EP3 was the lowest with 0.12 μg/mL.

⑩ Determination of KD by BIACORE

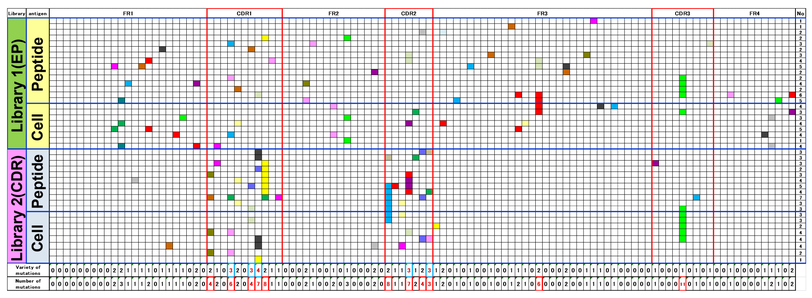
Instrument; BIACORE 3000
Sensor Tip; SA Tip
Immobilized antigen(biotinylated-peptide); 126 RU
Flow Rate; 30 μL/min
Injection time; 3 min
Dissociation time;5 min
Concentration of sample;100 nM, 50 nM, 25 nM, 12.5 nM

Sample	ka (1/Ms)	kd (1/s)	KD (M)
Original	6.52E+04	1.32E−03	2.02E−08
CDR7	2.79E+05	4.63E−04	1.66E−09
CDR4	2.65E+05	4.75E−04	1.79E−09
EP5	2.64E+05	5.44E−04	2.06E−09
CDR5	2.42E+05	6.05E−04	2.50E−09
CDR3	2.34E+05	6.14E−04	2.65E−09
CDR1	2.17E+05	6.08E−04	2.80E−09
CDR2	2.03E+05	6.50E−04	3.21E−09
CDR6	2.02E+05	7.98E−04	3.95E−09
CDR9	2.06E+05	8.10E−04	3.95E−09
EP3	1.05E+05	5.04E−04	4.83E−09
CDR8	1.42E+05	7.34E−04	5.18E−09
EP1	6.58E+04	4.25E−04	6.47E−09
EP2	6.19E+04	4.80E−04	7.77E−09
EP4	6.99E+04	1.04E−03	1.49E−08



KD of the high affinity clones from 2nd screening ELISA were determined. Approximately, a lot of clones showed more than 10-fold lower KD than that of the original clone. EP1-EP4 clones indicated low EC_{50}, however, they showed the same degree of KD of the original clone. From these results, Library 2 (CDR) seemed better than Library 1 (EP) to obtain higher affinity binders.

⑪ Alignment of amino acid sequence of binders



This table shows multiple alignment of amino acid sequences of selected binders with higher affinity than the original clone. These binders were screened against peptide antigen or antigen expressing cell. Rightmost column shows number of mutations of each clone. Each color in table shows species of amino acids. The average of mutations for all clones was 3.0 mutations/clone. The frequently mutated region is indicated in red, and the region which has diversity in mutations is indicated in blue at the two bottom lanes. These positions are candidates to explore more mutations for creating higher affinity clones, and combination of those mutations could result better affinity. This comprehensive information can be a good resource for generating more qualified Library for further affinity maturation.

Summary

1. It is important to optimize expressing conditions of Fab to fully utilize the Fab displaying RD complex as much as possible in the screening, for example, the amount of ribosome to add into the system, and synthesizing H and L chain at different timing.

2. KD of the higher affinity clones were determined, and a lot of clones showed more than 10-fold lower KD than that of the original clone. Library 2 (CDR) seemed better than Library 1 (EP) to obtain higher affinity binders.

3. Alignment of amino acid sequences of selected binders with higher affinity than the original clone indicated the region of frequently mutated or mutated in common, which suggests important region to improve affinity, and exploration of those mutations will lead to better affinity.

GFC pipline

We are engineering these antibodies with PURE*frex RD*.

	Target	Indication	Stage
GFC101	CD69	Inflammatory diseases (Asthma, COPD, Colitis, RA, etc.)	Pre-clinical
GFC201 GFC202	ADAM28	Oncology (Breast, Lung cancers)	Pre-clinical
GFC301	ADAMTS4	Arthritis (RA, OA)	R&D

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