In vitro selection from designed protein scaffold library with Ribosome Display on PURE system

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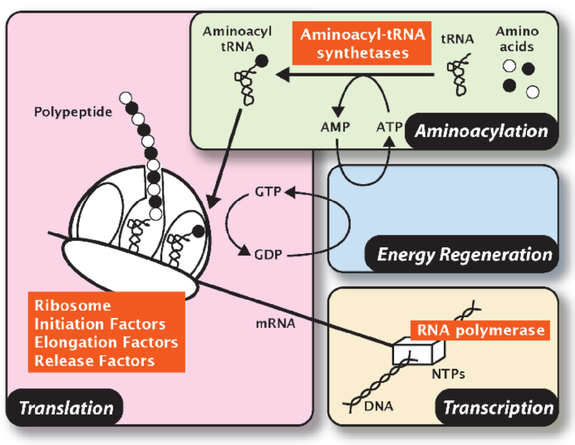
Abstract

Ribosome Display (RD) on the Protein synthesis Using Recombinant Elements (PURE) system (PURE RD) was reported as a powerful method for *in vitro* selection of specific binders such as antibodies or antibody fragments. The PURE system is a novel cell-free protein synthesis system reconstituted from the purified components (translation factors and enzymes) necessary for the translation in *E.coli*. The PURE system shows very little activities of nucleases and other inhibitory factors. Therefore, mRNA/protein/ribosome ternary complex (RD complex) in the PURE system is highly stable and the mRNA recovery rate is greatly increased as compared with the conventional cell extract-based RD. Our current researches on the PURE system indicate that PURE RD has high potential to be a new standard method to screen the specific binders from the peptide and protein library such as scaffold library.

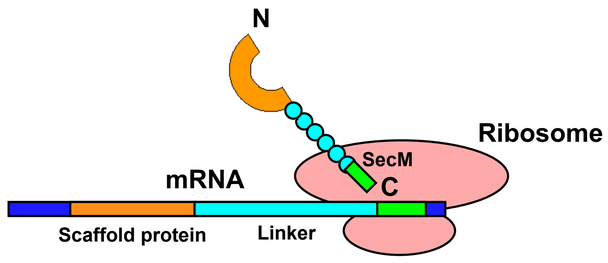
Here we showed results of PURE RD applying to our own designed protein scaffold library based on the substrate recognition domain of human ubiquitin ligase E3 protein. After 4th round *in vitro* selection of the binders to Erk2(extracellular signal-regulated kinase 2) from this library, several binders were obtained. These Erk2 binders showed specific binding activity to be used in general applications *in vitro*. In addition to that, the Erk2 binders showed intracellular binding activity for Erk2 in the cytoplasm and inhibited the phosphorylation activity of Erk2 for nuclear protein p90RSK significantly in HEK293T. Also, the Erk2 binder with a Nuclear Localization Signal (NLS) resulted in the nuclear translocation of Erk2. These results indicate that the Erk2 binders could work not only *in vitro* but also in the cells as an intrabody.

PURE system: Protein synthesis Using Recombinant Elements system

PURE system is a novel reconstituted *in vitro* transcription and translation system which consist of purified 36 enzymes 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.



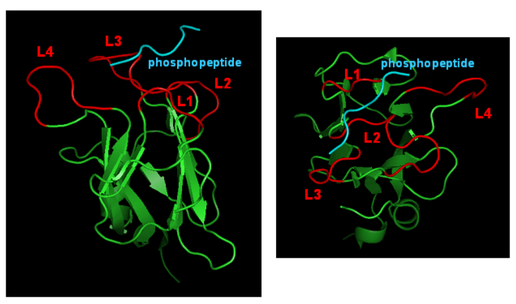
Ribosome Display complex in the PURE system (PURE RD)



Advantages:
1) Ribosome Display complex is highly stable in the PURE system because activities 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 ribosomes in the PURE system.

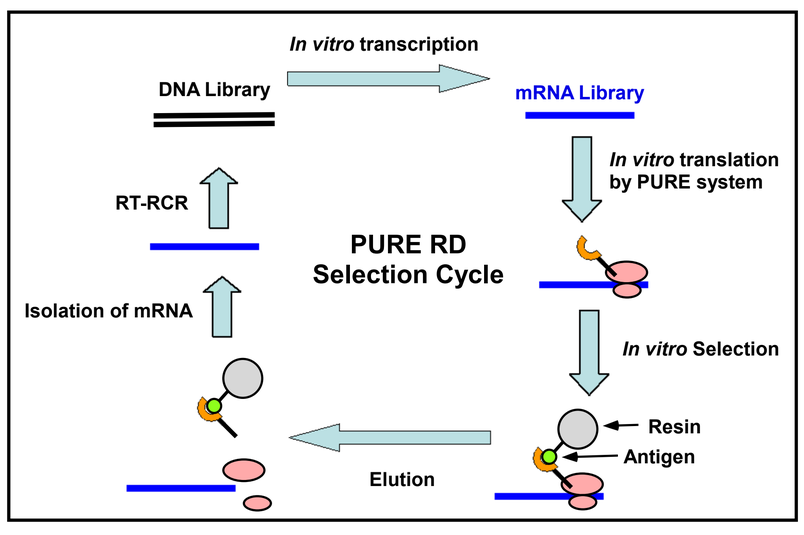
RNF8 (E3 Ubiquitin Ligase) FHA Domain scaffold Library

Feature
- **MW:18KDa**
- **Localization:**
  Nucleus and Cytoplasm
- **Structure: β sandwich**
- **Loop:4 loops (NNS)**



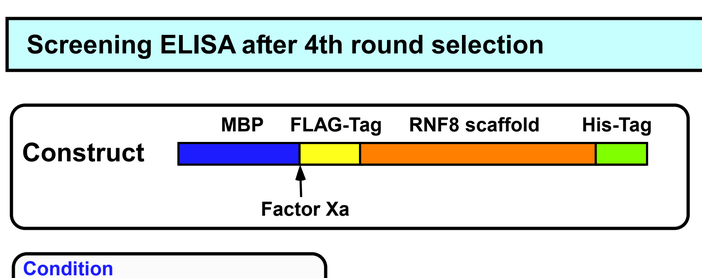
RNF8 is a E3 ubiquitin ligase which plays a critical role in the early DNA-damage response via the ubiquitination of histone H2A and H2AX. The global fold of the RNF8 FHA (Forkhead associated) domain in an 11-stranded β sandwich structure with the phosphopeptide-binding surface comprised of 4 loops. We have randomized the nucleotide sequences encoding those 4 loops to NNS (red lines). Construction of library was carried out by overlap-extension PCR of synthetic DNA fragments.

In vitro selection of binders for Erk2 protein using PURE RD



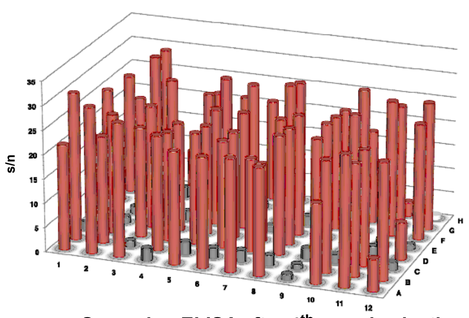
Condition
- **Initial library size:$(1\times10^{10})\times1000$ moleclues**
- **Antigen:Biotinylated Erk2 protein**
- **Resin :Streptavidine Magnetic Beads**
- **Selection cycles:4 round**

Screening ELISA after 4th round selection



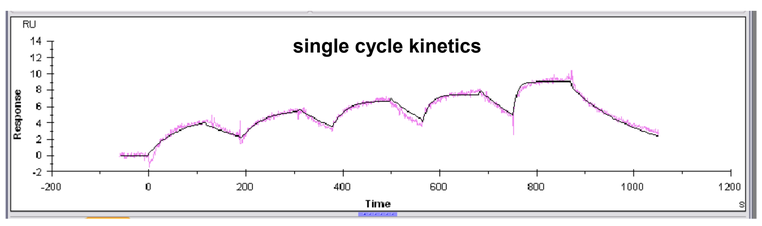
Condition
- **Expression format:**
  MBP-fused RNF8 scaffold
- **Host:**
  BL21(DE3)
- **2nd Antibody:**
  anti-FLAG(M2)-HRP

Results
- **Hit:64/95 (67.4%)**
- **Uniqueness: total 14 clones**
  No.44 (80%), No.33 (10 %),
  single clones (10%)



Screening ELISA after 4th round selection

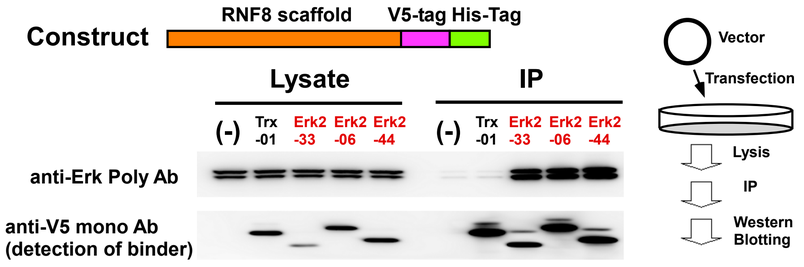
Determination of affinity by Biacore experiment



	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (M)
Erk2 binder No.33	2.3×10^{5}	7.0×10^{-3}	2.9×10^{-8}

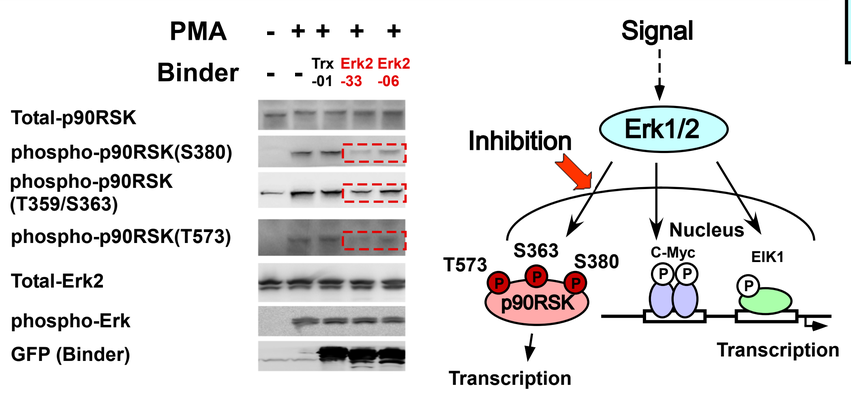
Erk2 binder (No.33) was purified by amylose resin. Final yield of the purified binder (fused MBP) was 200-250 mg from 1L of culture. Binding kinetics of Erk2 binder to Erk2 was determined using surface plasmon resonance with Biacore Biosensors T100. Biotinylated Erk2 was immobilized on a SA sensor chip according to standard method. At a flow rate of 30 μl/min, six concentrations from 500 nM to 30 nM were used to record sensorgram and k_{on}, k_{off} and K_D were determined by evaluation software.

Immunoprecipitation of Endogenous Erk2 by Erk2 binder (pull down assay)



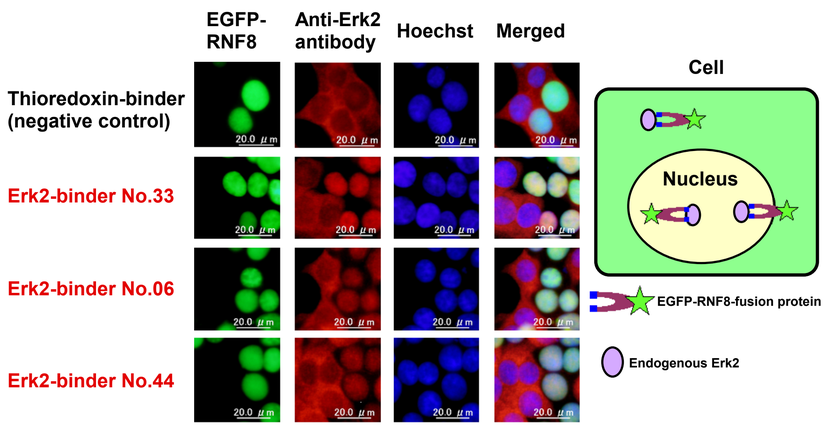
After transfection of expression vectors with Erk2 binder gene, HEK293T cells were incubated for 24 hours, and were lysed by detergent. The Erk2 binder-Erk2 protein complex was isolated by Co^{2+} affinity beads, and were examined by western blotting using anti-Erk polyclonal antibody and anti-V5 monoclonal antibody. As a result the expressed Erk2 binders could specifically bind to endogenous Erk2 protein in HEK293T cells. This result indicates that RNF8 scaffold could properly work as intrabody in cell.

Inhibition of Erk2 phosphorylation activity by Erk2 binders



After RNF scaffold expression vector was transfected in HEK293T. The expressed EGFP-Erk2 binders obviously inhibited the phosphorylation activity of Erk2 for nuclear protein p90RSK in phorbol 12-myristate-13-acetate(PMA)-induced HEK293T cell.

Constitutive nuclear localization of endogenous Erk2 by the Erk2 binder containing NLS

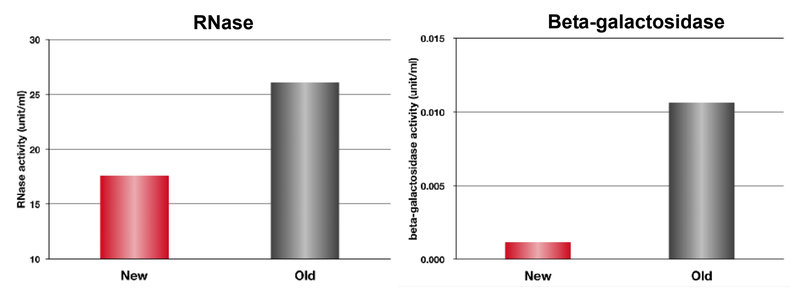


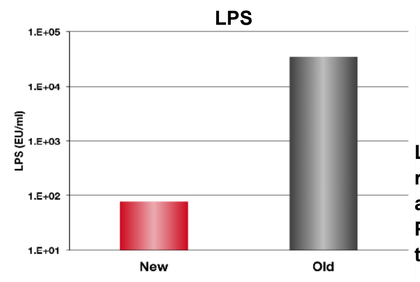
After transfection of RNF8 scaffold expression vector, the EGFP-Erk2 binders with NLS (nuclear localization signal) translocated the endogenous Erk2 protein from cytosol to nucleus. This result indicates that RNF8 scaffold may be able to regulate the localization of intracellular target protein in a strategic way by addition of NLS or NES (nuclear exporting signal) to RNF8 scaffold.

Development of "highly purified PURE system" which has been more optimized to Ribosome Display

Improvements
1, Highly purified protein factors by addition of futher column work.
2, Removal of Lipopolysaccharide (LPS) by treatment of detergent.

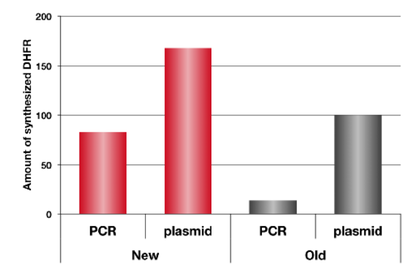
The comparison of the amount of contaminants in New and Old PURE system





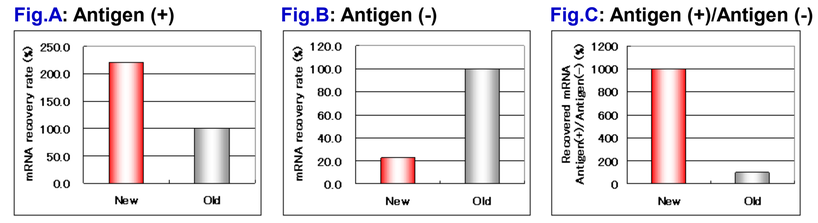
LPS,RNase and beta-galactosidase in the reaction mixture was examined. The amount of all contaminants in the New PURE system was significantly less than that in the Old PURE system.

The comparison of activity for protein-synthesis between the New and Old PURE system



The amount of synthesized DHFR in the New PURE system increases more than that in the Old PURE system, especially, with PCR products as a template DNA.

The comparison of mRNA recovery rate for ribosome display between the New and Old PURE system



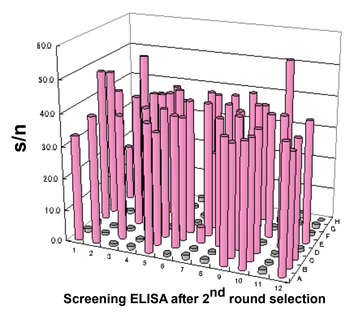
Erk2 binder No.33 was used as positive control in ribosome display model selection with or without antigen (Erk2 protein). The amount of mRNA was determined by real-time PCR. As shown in Fig.A, the recovered antigen specific mRNA from New PURE system was increased at 2.2-fold. Also, the recovered non-specific mRNA as a background from New PURE system was decreased to 1/5 (Fig.B). As a result, the selection efficiency of ribosome display using New PURE system raised approximately 10-fold higher than that using Old PURE system.

Model experiment of PURE RD selection with New and Old PURE system

Condition
- **Initial library size:Trx binder 10^{13} moleclues + Erk2 binder No.33 10^{3} moleclues**
- **Antigen:Biotinylated Erk2 protein**
- **Resin :Streptavidine Magnetic Beads**
- **Selection cycles: 3 round**

Results of screening ELISA

	New	Old
2nd round	53.7 % (51/95)	ND (0/95)
3rd round	80.0 % (76/95)	29.5 % (28/95)



Screening ELISA after 2nd round selection

More than 50 % of selected clones could bind antigen after second round selection using New PURE system-based RD, but using Old PURE system could not select Erk2 binders at second round selection.

Summary

1, We developed a new designed protein scaffold library based on the RNF8 FHA domain, and a number of Erk2 binders were selected from this library using ribosome display with PURE system (PURE RD). It is noteworthy that selected RNF8 scaffold could work properly as intrabody in cell. The current intrabodies are developed by an antibody-based technology, which is emerging as novel therapeutic molecules for the treatment of Alzheimer's, Parkinson's and Huntington's diseases. We believe that RNF8 scaffold also have a potential of new therapeutic molecules for these diseases.

2, Ribosome Display using Highly purified PURE system (New PURE system) had high selection efficiency than that using normal PURE system (Old PURE system). As a result, we will be able to select a lot of binders to antigens with high diversity in a shorter time than ever.

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