In vitro production of Fab, Toxin, and Immunotoxin using PURE*frex*® *2.0* with increased productivity

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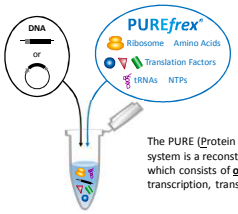
Abstract

PURE systemは大腸菌の転写・翻訳系の構成因子を個別に精製して再構 成した無細胞タンパク質合成系である。PURE systemはRNaseやプロテアーゼなどの混入がほとんど無い上、目的に応じて反応液の組成を自由に変更できるなど、他の合成系にはない特徴を有している。これまでに我々は、構成因子の精製法を最適化し、従来に比べてリボ多糖（LPS）混入量を1/1000に減少させた新規PURE system（**PURE*frex* *1.0***）を開発した。また、分子シャペロン等の添加により、単純タンパク質だけでなく、SS結合を有するタンパク質や不溶化しやすいタンパク質を活性型として合成可能であることも示した 。さらに反応液組成の見直しなどにより、PURE*frex*のタンパク質合成能力を飛躍的に向上させることに成功した（**PURE*frex* *2.0***）。本発表ではPURE*frex 2.0*の利用例として、抗HER2 抗体Fabや毒素タンパク質（Gelonin、PE38など）、およびそれらをリンカーでつないだImmunotoxinを合成し、機能評価を行った。PURE*frex 2.0*によるFabの合成量は約0.5 mg/mL で、大腸菌で生産したFabと同等の結合能力を示した（EC_{50}=0.5～0.6 nM）。毒素タンパク質は可溶性タンパク質として合成でき、真核細胞由来の無細胞タンパク質合成系を用いたアッセイではタンパク質合成を阻害する活性を示した。これらの融合タンパク質であるImmunotoxinは、Fabと毒素タンパク質の両方の機能を示した。さらに、合成産物をHER2高発現株のSK-BR-3に未精製の状態で添加したところ、Fabや毒素タンパク質単独では増殖抑制効果はなかったが、Immunotoxinでは添加量に応じた増殖抑制効果が確認された。これらの結果は、PURE*frex 2.0* を用いることでImmunotoxinのような複雑なタンパク質を簡便かつ機能評価に十分な量で合成できることを示している。

1. PURE*frex*®; based on the PURE system technology



The PURE (Protein synthesis Using Recombinant Elements) system is a reconstituted cell-free protein synthesis system, which consists of **only purified factors** necessary for transcription, translation and energy regeneration.

E. coli based cell-free protein synthesis systems

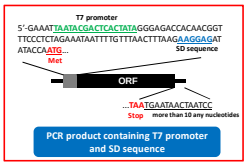
	Extract system	Reconstituted system		
	S30 system	PURE system (original*)	PURE*frex*® *1.0*	PURE*frex* *2.0* New
Typical Yield (μg/mL)	100-1,000	10-200	10-200	20-1,000
Contamination				
RNase	very High	Low	very Low	very Low
Endotoxin (LPS)	very High	High	very Low	very Low
Template DNA				
Plasmid DNA	OK	OK	OK	OK
PCR product	NG	OK	OK	OK
Customization of Reagent	Difficult	Easy	Easy	Easy
Purification of His-tagged product	OK	NG	OK	OK
In vitro display				
Ribosome display	△	○	◎	○
mRNA display	△	○	◎	○

*Reference; Shimizu Y. *et al.* (2001) *Nat. Biotechnol.*, vol. 19, p. 751

2. How to use PURE*frex*®

; for "**Easy**" and "**High throughput**" preparation of Proteins

① Prepare the template DNA.



PCR product containing T7 promoter and SD sequence

or

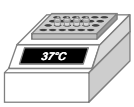
T7 promoter-driven protein expression vector for *E. coli*

② Mix the PURE*frex* reaction mixture in a tube as follows.

		PURE*frex* *1.0*	PURE*frex* *2.0*
Water		8-X μL	**7-X μL**
Solution I	(Amino acids, NTPs, tRNAs, etc.)	10 μL	**10 μL**
Solution II	(Translation factors)	1 μL	**1 μL**
Solution III	(Ribosome)	1 μL	**2 μL**
Template DNA	(1-3 ng/μL per 1 kb)	X μL	**X μL**
		20 μL	**20 μL**

・If necessary, add "**DS supplement**", "**DnaK Mix**", and/or "**GroE Mix**" to the reaction mixture.
・**"Co-synthesis of multiple protein"** is possible by using the mixed template DNA.

③ Incubate the tube at 37°C for >2 hours.



④ Analyze the synthesized product.

"Direct assay without purification"

3. Application of PURE*frex*® *2.0*

; can be applicable for synthesis/screening for scFv, Fab, Protein toxin and Immunotoxin

scFv and Fab

Protein synthesis

Reaction mix: PURE*frex* *2.0* + DS supplement (1 μM DsbC, 3 mM GSSG) + DnaK Mix (5 μM DnaK, 1 μM DnaJ, 1 μM GrpE)

Template DNA: **PCR product (final 10 nM)** with C terminal FLAG and His-tag

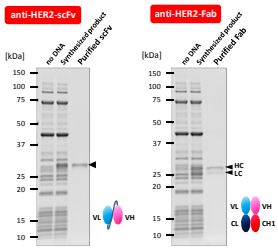
scFv (single template DNA): scFv DNA — 10 nM

Fab (Mixed template DNA): LC DNA — 2 nM + HC DNA — 8 nM

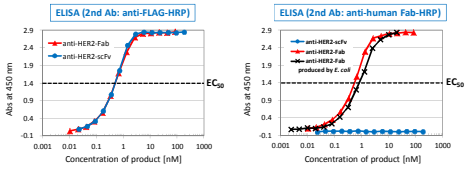
Incubation: **37°C for 6h**

Batch purification

Resin	TALON® Metal Affinity Resin (Clontech)
Binding buffer	50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20 mM MgOAc, 5 mM Imidazole
Wash Buffer1	Binding buffer + 5 mM ATP (Incubate at 30°C for 10 min, 3 times)
Wash buffer2	50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM Imidazole (Incubate at 4°C for 10 min, 3 times)
Elution buffer	50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 50 mM Imidazole



10-20% gradient SDS-PAGE gel, Oriole staining
"no DNA" and "Synthesized product": 0.5 μL of reaction/lane
"Purified scFv": 0.3 μg/lane, "Purified Fab": 0.2 μg/lane



Antigen: Extracellular domain of human ErbB2/HER2 (1 ng/well)

Summary (scFv and Fab)

		Template DNA [nM]	Protein MW [kDa]	Synthesized Protein [mg/mL]	Synthesized Protein [μM]	EC_{50} [nM]
anti-HER2-scFv		10	28.0	0.41	14.5	0.5
anti-HER2-Fab	LC	2	23.5	0.5	8.4	0.5-0.6
	HC	8	26.0			

EC_{50}: 50% effective concentration of target binding activity.

Protein toxin and Immunotoxin

Protein synthesis

Reaction mix: PURE*frex* *2.0* + DS supplement (1 μM DsbC, 3 mM GSSG) + DnaK Mix (5 μM DnaK, 1 μM DnaJ, 1 μM GrpE)

Template DNA: **PCR product (final 10 nM)**

Toxin (single template DNA): Toxin DNA — 10 nM

Immunotoxin (Mixed template DNA): LC DNA — 1 nM + HC-Toxin DNA — 9 nM

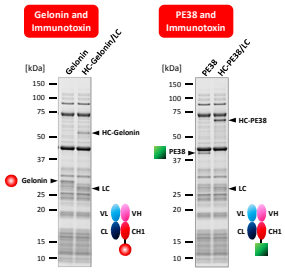
Incubation: **37°C for overnight**

Gelonin[*1]
; is a 30-kDa protein from plant *Gelonium multiflorum*.
; inactivates 28S rRNA of eukaryote ribosome via its N-glycosidase activity.

PE38[*2]
; is a 38-kDa truncated *Pseudomonas* exotoxin (PE) containing amino acids 253-364 and 381-613.
; inactivates eukaryote elongation factor 2 via its ADP-ribosylating activity.

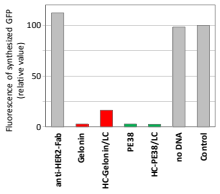
Immunotoxin
; is a fusion protein, in this study, fused "protein toxin" to "anti-HER2-Fab" via glycine-serine linker (G_4S).

*Reference;
1: Stirpe F. *et al.* (1980) *JBC.* vol. 255, p. 6947
2: Kreitman RJ. (2009) *BioDrugs.* vol. 23, p. 1



10-20% gradient SDS-PAGE gel, Oriole staining
Sample: 0.5 μL of reaction/lane

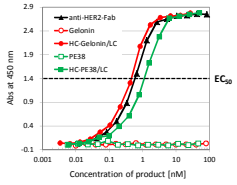
Translation Inhibition assay



Control + PURE*frex* *2.0* reaction products

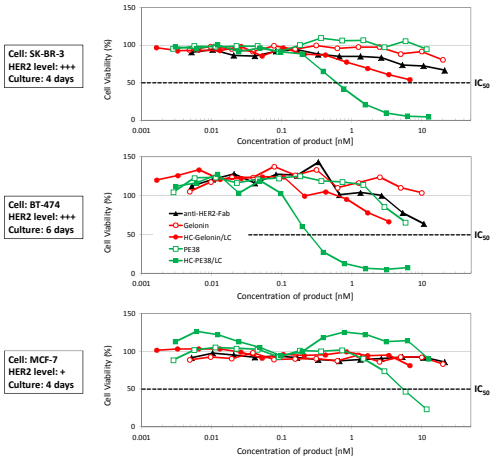
GFP synthesized by using HeLa cell-extract based cell-free protein synthesis system (1-Step Human Coupled IVT Kit-DNA, Thermo scientific) with PURE*frex 2.0* reaction products (without purification, 1:1000).

ELISA



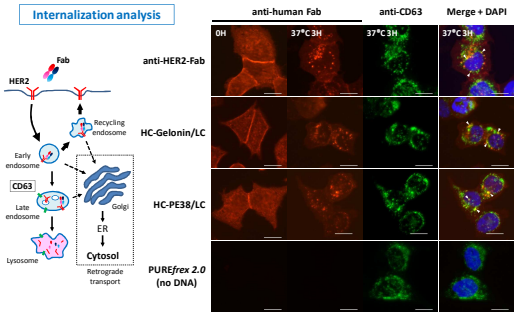
Antigen: Extracellular domain of human ErbB2/HER2 (1 ng/well)
2nd Ab: anti-human Fab-HRP 2nd antibody (Jackson Immuno Research)

Cell growth inhibition assay



HER2 positive breast cancer cell lines were cultured with the medium (DMEM, 10% FBS, Penicillin/Streptomycin) containing **PURE*frex* *2.0* reaction products (without purification, 1:400 to 1:1.64×10⁵)** at 37°C for 4-6 days.
Cell viability was determined by WST-8 method (Cell Counting Kit-8, Dojindo). Mean of triplicates is shown.

Internalization analysis



BT-474 cells were surface-labeled at 4°C for 60 min with the binding medium (DMEM, 3% BSA, 20 mM HEPES (pH 7.4)) containing **PURE*frex* *2.0* reaction products (without purification, 1:400)**. Cells were washed five times with the binding medium and incubated at 37°C for 3h. Cells were then fixed and processed for dual-label indirect immunofluorescence microscopy. CD63 (Lamp3) is marker of late endosomes and lysosomes. White arrows indicate partial co-localization of Fab or Immunotoxin with CD63. Bar indicates 20 μm.

Summary (Protein toxin and Immunotoxin)

		Template DNA [nM]	Protein MW [kDa]	Synthesized Protein [mg/mL]	Synthesized Protein [μM]	Translation inhibiting activity	EC_{50} [nM]	IC_{50} [nM] SK-BR-3	IC_{50} [nM] BT-474	IC_{50} [nM] MCF-7
Gelonin		10	29.3	0.23	8.0	○	ND	ND	ND	ND
HC-Gelonin/LC	LC	1	24.7	0.33	2.7	○	0.5	ND	ND	ND
	HC-Gelonin	9	53.2							
PE38		10	37.4	0.17	4.7	○	ND	ND	ND	6
HC-PE38/LC	LC	1	24.7	0.44	5.0	○	1.0	0.6	0.25	ND
	HC-PE38	9	62.5							

EC_{50}: 50% effective concentration of target binding activity. IC_{50}: 50% inhibitory concentration of cell growth. ND: Not detected.

Conclusion

- His-tagged anti-HER2 "-scFv" and "-Fab" protein were synthesized in the active form and purified using the affinity resin.
- LC and HC protein of anti-HER2-Fab were co-synthesized by using the mixed template DNA.
- "Protein toxin" and "Immunotoxin" were synthesized, showing the activity *in vitro*.
- PURE*frex 2.0* reaction products (without purification) were applied "**directly**" to living cells for the cytotoxicity assay of immunotoxin.

On-going Project

- Synthesis of "**full-length IgG**".
- Improvement of binding affinity and cytotoxicity of Immunotoxins.
- Development of a novel protein toxin.

Our Products

PURE*frex*® *1.0* / PURE*frex*® *2.0* New
a regular kit for the synthesis of proteins without disulfide bonds

DS supplement
a supplement for the synthesis of proteins containing disulfide bonds

DnaK Mix / GroE Mix
a supplement for the synthesis of aggregate-prone proteins