

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



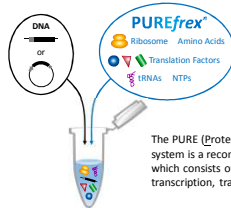
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Abstract

PURE system is a bacterial transcription-translation system that is highly efficient and easy to use. PURE system is a reconstituted cell-free protein synthesis system, which consists of only purified factors necessary for transcription, translation and energy regeneration. PURE system is a bacterial transcription-translation system that is highly efficient and easy to use. PURE system is a reconstituted cell-free protein synthesis system, which consists of only purified factors necessary for transcription, translation and energy regeneration.

1. PURE[®] 2.0; 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

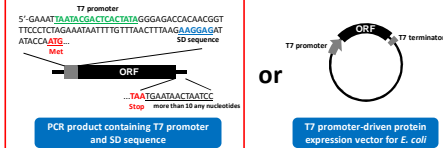
	Extract system		Reconstituted system	
	S30 system	PURE system (original)	PURE [®] 1.0	PURE [®] 2.0
Typical Yield (μg/mL)	100-1,000	10-200	10-1,000	20-1,000
Contamination				
RNase	very High	Low	very Low	very Low
Endotoxin (LPS)	very High	High	very Low	very Low
Template DNA				
Plasmid DNA	OK	OK	OK	OK
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	Δ	○	⊙	○
mRNA display	Δ	○	⊙	○

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

2. How to use PURE[®] 2.0

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

① Prepare the template DNA.



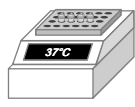
② Mix the PURE[®] 2.0 reaction mixture in a tube as follows.



	PURE [®] 2.0	PURE [®] 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[®] 2.0

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

scFv and Fab

Protein synthesis

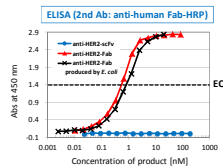
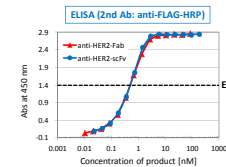
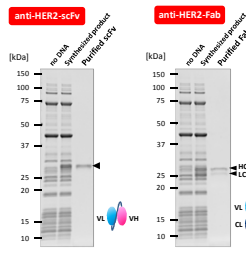
Reaction mix: PURE[®] 2.0 + DS supplement + DnaK Mix
(1 μM DnaK, 3 mM GSSE) (5 μM DnaK, 1 μM DnaI, 1 μM GroE)

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

Incubation: 37°C for 6h

Batch purification

	anti-HER2-scFv	anti-HER2-Fab
Resin	TALON [®] Metal Affinity Resin (Clontech)	TALON [®] Metal Affinity Resin (Clontech)
Binding buffer	50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20 mM MgCl ₂ , 5 mM Imidazole	50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20 mM MgCl ₂ , 5 mM Imidazole
Wash buffer 1	Binding buffer + 5 mM ATP (Incubate at 30°C for 10 min, 3 times)	Binding buffer + 5 mM ATP (Incubate at 30°C for 10 min, 3 times)
Wash buffer 2	50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM Imidazole (Incubate at 4°C for 10 min, 3 times)	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	50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 50 mM Imidazole



Summary (scFv and Fab)

	Template DNA (nM)	Protein MW (kDa)	Synthesized Protein (mg/mL)	IC ₅₀ (nM)
anti-HER2-scFv	10	28.0	0.41	14.5
anti-HER2-Fab	LC 2	23.5	0.5	8.4
	HC 8	26.0		0.5-0.6

EC₅₀: 50% effective concentration of target binding activity.

Protein toxin and Immunotoxin

Protein synthesis

Reaction mix: PURE[®] 2.0 + DS supplement + DnaK Mix
(1 μM DnaK, 3 mM GSSE) (5 μM DnaK, 1 μM DnaI, 1 μM GroE)

Template DNA: PCR product (final 10 nM)

Incubation: 37°C for overnight

Gelolin⁺

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

PE38⁺

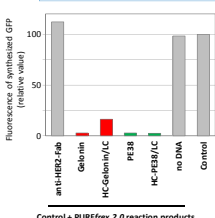
; is a 38-kDa truncated Pseudomonas exotoxin (PE) containing amino acids 253-364 and 381-613.
; inactivates eukaryote elongation factor 2 via its ADP-ribosylating activity.

Immunotoxin

; is a fusion protein, in this study, fused "protein toxin" to "anti-HER2-Fab" via glycine-serine linker (C₂S).

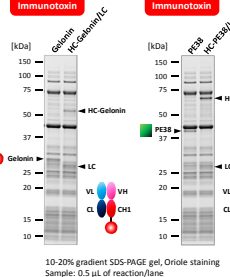
*Reference: 1: Shirpe F. et al. (1980) JBC, vol. 255, p. 6947
2: Kretzmar R. (2009) Biologics, vol. 23, p. 1

Translation inhibition assay

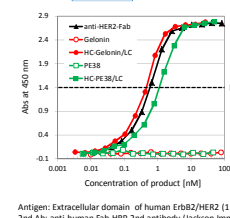


GFP synthesized by using HeLa cell-extract based cell-free protein synthesis system (1-Step Human Coupled WT kit-DNA, Thermo scientific) with PURE[®] 2.0 reaction products (without purification, 1:1000).

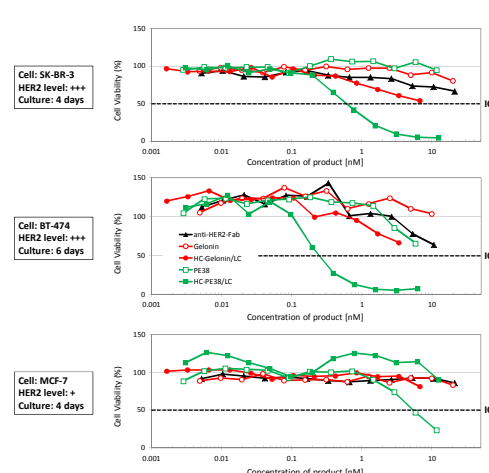
Gelolin and Immunotoxin



ELISA

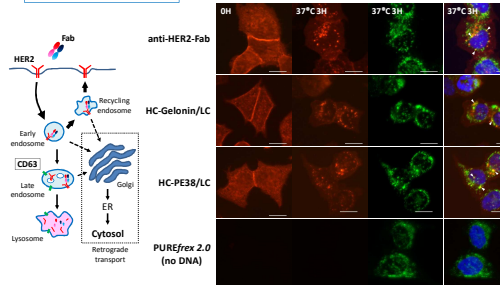


Cell growth inhibition assay



HER2 positive breast cancer cell lines were cultured with the medium (DMEM, 10% FBS, Penicillin/Streptomycin) containing PURE[®] 2.0 reaction products (without purification, 1:400 to 1:1.64x10⁵) 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[®] 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)	Translocation inhibitory activity	EC ₅₀ (nM)	IC ₅₀ (nM)	SK-BR-3	BT-474	MCF-7
Gelolin	10	29.3	0.23	8.0	○	ND	ND	ND	ND
HC-Gelolin/LC	LC 1	24.7	0.33	2.7	○	0.5	ND	ND	ND
	HC-Gelolin	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						

EC₅₀: 50% effective concentration of target binding activity. IC₅₀: 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[®] 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[®] 1.0 / PURE[®] 2.0

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