Efficient *in vitro* expression of aglycosylated full-length IgG using a reconstituted cell-free protein synthesis system, PURE*frex*® *2.0*

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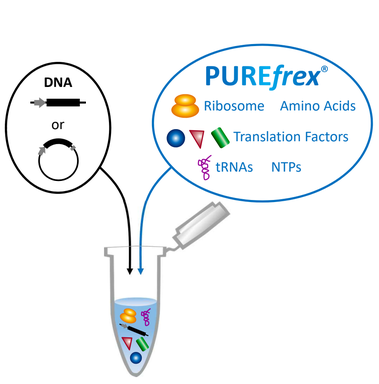
Abstract: Aglycosylated full-length IgG (anti-HER2 monoclonal antibody) was synthesized using PURE*frex 2.0*. The PURE (Protein synthesis Using Recombinant Elements) system is a reconstituted cell-free protein synthesis system, which consists of only purified factors necessary for transcription, translation and energy regeneration. Recently, we developed an updated PURE system with higher productivity, which was launched as "PURE*frex 2.0*" in 2015. We reported that PURE*frex 2.0* could be used for production of functional proteins such as Fab, scFv, a protein based toxin and an immunotoxin last year. Here, we report the further application using PURE*frex 2.0* for production of IgG. To synthesize IgG, we optimized the composition of the reaction mixture and the reaction conditions as below; 1) adding molecular chaperone (DnaK mix) to increase the solubility of the product; 2) adding DsbC to form disulfide bonds between the correct cysteine residues; 3) adjusting GSH/GSSG ratio for optimum redox state; 4) long-time incubation (over 20h) to assemble the hetero tetramer of two heavy chains and two light chains. At the best mode of synthesis, the productivity of IgG reached to 0.042 mg/mL. The synthesized IgG was detected as single band on non-reduced SDS-PAGE after the purification by protein A resin and the following gel filtration. The purified IgG exhibited high binding affinity to recombinant HER2 protein. Fifty percent effective concentration of target binding activity (EC50) in ELISA was 0.16 nM, which is similar to the trastuzumab. This result indicates that PURE*frex 2.0* will be useful tool for high-throughput expression/screening of functional antibodies (scFv, Fab, and IgG).

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.

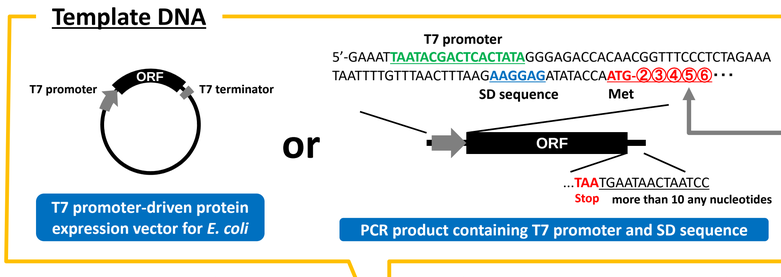
***E. coli* based cell-free protein synthesis systems**

	Extract system	Reconstituted system		
	S30 system	PURE system (original*)	PURE*frex*® *1.0*	PURE*frex*® *2.0*
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	△	○	◎	**○**

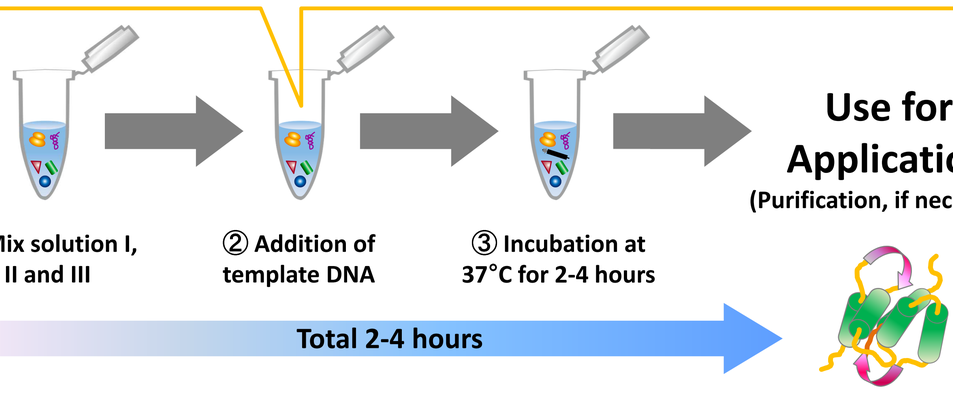
【Reference】*: Shimizu Y. *et al*. (2001) *Nat. Biotechnol.*, vol. 19, p. 751

2. How to use PURE*frex*®; "**Easy**" and "**High throughput**" preparation of Proteins

Template DNA

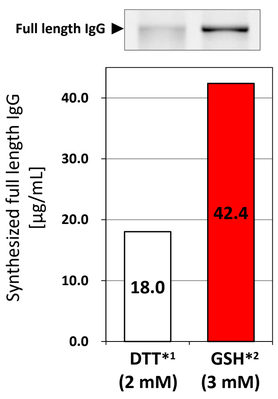


⚠ To enhance the protein productivity, we recommend to change the codons of N-terminal 2-6 amino acids to **AT-rich codons** (not "most frequently used codons").

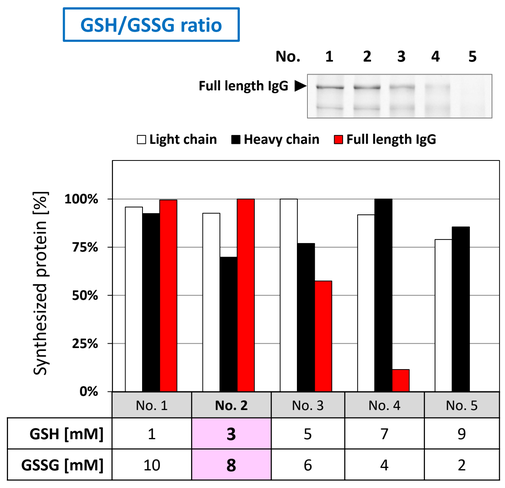


3. Optimization of the synthesis of full length IgG

Reducing agent (DTT or GSH)

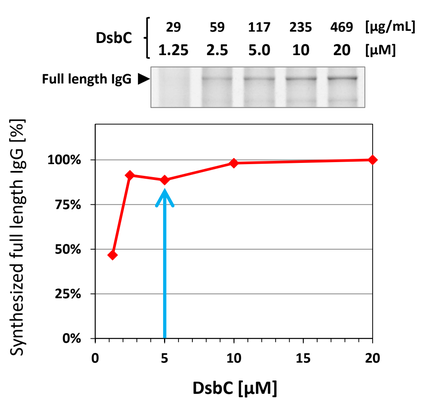


GSH/GSSG ratio

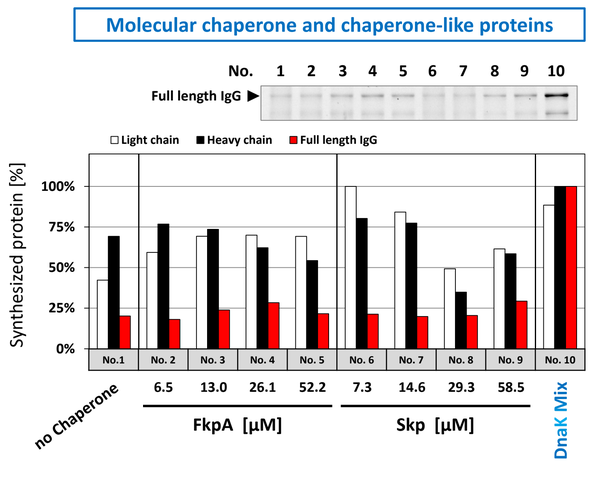


	No. 1	No. 2	No. 3	No. 4	No. 5
GSH [mM]	1	**3**	5	7	9
GSSG [mM]	10	**8**	6	4	2

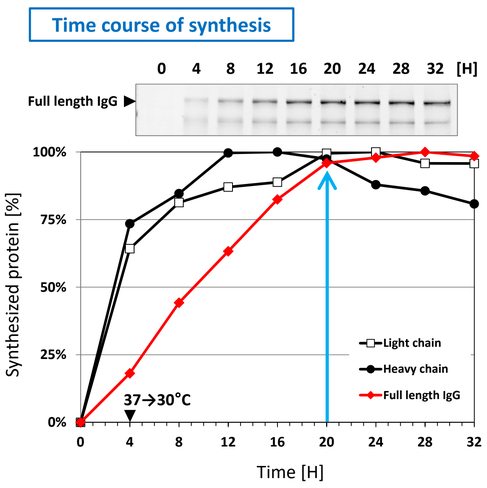
DsbC concentration



Molecular chaperone and chaperone-like proteins



Time course of synthesis



Aglycosylated full-length IgG (anti-HER2 monoclonal antibody) was synthesized using PURE*frex 2.0* under various conditions. After synthesis, all samples were centrifuged at 9,100 xg for 10 min and 1.0 μL of supernatant was applied to non-reduced (10% gel) or reduced (10-20% gel) SDS-PAGE. The gels were stained with Oriole fluorescent gel stain (Bio-Rad) and the protein bands were quantitated using LAS-4000 system (GE Healthcare).
*1: Initial condition of the optimization (PURE*frex* 2.0 (normal version, 2 mM DTT) with 3 mM GSSG, 5 μM DsbC and DnaK mix; Reported at PepTalk 2016, San Diego, CA, USA).
*2: Final condition of the optimization. The yield of full length IgG was reached to 0.042 mg/mL.

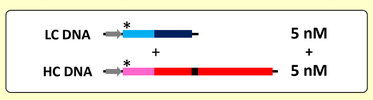
FkpA: Periplasmic peptidyl-prolyl *cis-trans* isomerase (PPIase) from *E. coli*, which has a chaperone-like function independent of PPIase activity.*1
Skp: Periplasmic protein from *E. coli*, which shows chaperone-like activity to β-barrel protein.*1
DnaK mix: Mixture of cytoplasmic molecular chaperones from *E. coli*, which contains 5 μM DnaK, 1 μM DnaJ, 1 μM GrpE.*2
DsbC: Periplasmic disulfide bond isomerase from *E. coli*, which promotes the correct disulfide bond formation.*1
【Reference】*1: Goemans C. *et al*. (2014) *BBA*. vol. 1843, p. 1517 *2: Castanié-Cornet MP. *et al*. (2014) *BBA*. vol 1843, p1442

4. Synthesis and purification of full length IgG using PURE*frex*® *2.0*

Optimized condition of IgG synthesis

Reaction mix:
- **PURE*frex*® *2.0* Customized solution** (without DTT and with 3 mM GSH)
- + **DS supplement** (8 mM GSSG, 5 μM (117 μg/mL) DsbC)
- + **DnaK Mix** (5 μM DnaK, 1 μM DnaJ, 1 μM GrpE)

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



*We recommend to change the codons of N-terminal 2-6 amino acids to AT-rich codons (not "most frequently used codons") to enhance the protein productivity.

Incubation: **37°C for 4h to 30°C for 16h (total: 20 h)**

Purification method

Step 1: Protein A purification

Resin	**Protein A cellulose (KANEKA KanCapA™, Kaneka)** ・Equilibrate with "wash buffer 2" and apply 25 μL of resin to 1 mL of PURE*frex* reaction mixture. ・Rotate at 4°C for 1h.
Wash buffer 1	**20 mM Na-Phosphate buffer (pH 7.0), 20 mM MgOAc, 0.05% Tween 20** ・Add 500 μL to resin. Incubate at 4°C for 2 min.
Wash buffer 2	**20 mM Na-Phosphate buffer (pH 7.0), 0.05% Tween 20** ・Add 500 μL to resin. Incubate at 4°C for 2 min. 3 times.
Elution buffer	**50 mM Glycine-HCl (pH 2.5), 0.05% Tween 20** ・Add 100 μL to resin. Incubate at 25°C for 3 min. 3 times. ・Add 5 μL of 1 M Tris-HCl (pH 8.8) to the eluate for adjusting to neutral pH.

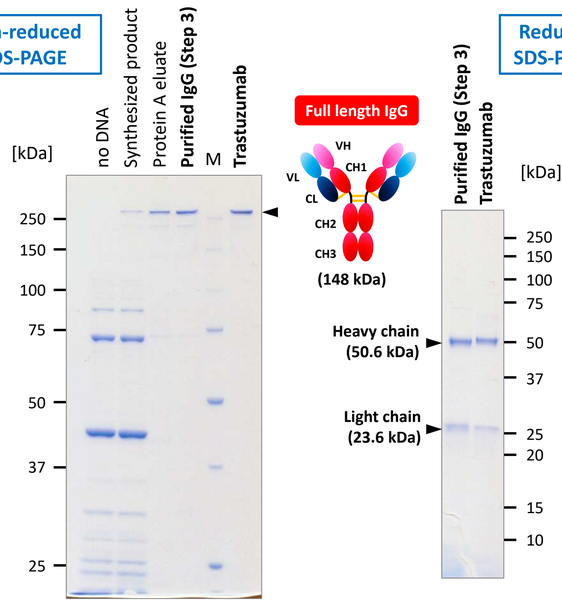
Step 2: Gel filtration

Column	Superdex-200 10/300 GL (GE healthcare)
Buffer	PBS-T (0.05% Tween 20)
Flow rate	0.5 mL/min (using ÄKTA system)
Elution position	Full length IgG: 21-25 min (total: 2 mL)

Step 3: Protein enrichment

Centrifugal filter	**AmiconUltra-0.5 (MWCO: 30 kDa, Merck Millipore)** Concentrate 2 mL of the eluate to 100 μL.

Non-reduced SDS-PAGE



Non-reduced SDS-PAGE on 10% gel (CBB staining).
Samples: 1.0 μL/lane.
Trastuzumab: 200 ng/lane. M: marker.

Reduced SDS-PAGE

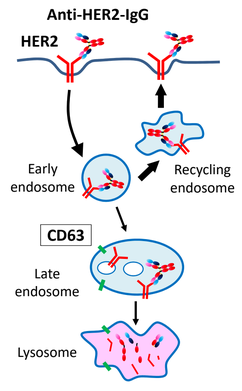
Reduced SDS-PAGE on 10-20% gel (CBB staining).
Purified IgG: 3.0 μL/lane.
Trastuzumab: 450 ng/lane.

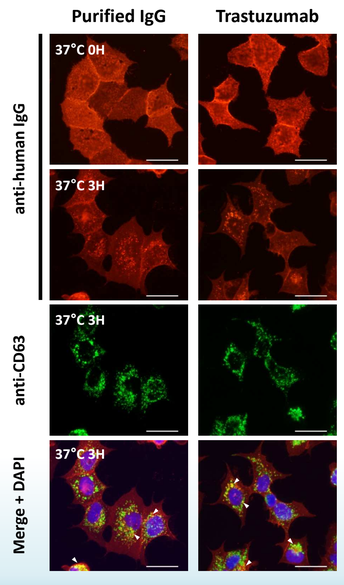
Purification summary

	IgG concentration [μg/mL]	IgG concentration [μM]	Volume [mL]	Total protein [μg]	Yield [%]
Synthesized product	**34.3**	0.23	1.0	**34.3**	100
Protein A eluate (Step 1)	103.6	0.70	0.3	31.1	90.6
Purified IgG (Step 3)	**157.0**	**1.06**	**0.1**	**15.7**	**45.7**

5. Evaluation of purified full length IgG

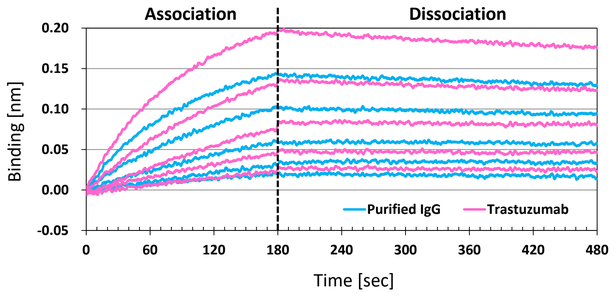
Internalization analysis





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 10 nM of "purified IgG" or "Trastuzumab". 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 anti-HER2-IgG with CD63. Bar indicates 20 μm.

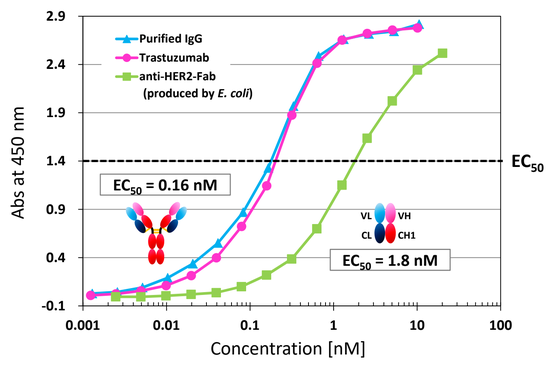
Binding kinetics analysis



	K_D [M]	k_{on} [1/Ms]	k_{off} [1/s]
Purified IgG	**4.03E-10** ±1.0E-11	**6.29E+05** ±4.7E+03	**2.53E-04** ±6.0E-06
Trastuzumab	**4.24E-10** ±8.8E-12	**6.38E+05** ±4.2E+03	**2.70E-04** ±5.3E-06

System: Octet RED96 System (Pall ForteBio)
Biosensor: Anti-Human IgG Fc Capture (AHC) biosensor (Pall ForteBio)
Buffer: Kinetics Buffer 10X (Pall ForteBio)
Ligand: "Purified IgG" or "Trastuzumab"
Analyte: 17.6, 8.8, 4.4, 2.2 and 1.1 nM of Extracellular domain of Human ErbB2/HER2 (Sino Biological)
K_D: Measured affinity of interaction; affinity constant in Molar.
k_{on}: Association rate constant. k_{off}: Dissociation rate constant.

ELISA



Antigen: Extracellular domain of Human ErbB2/HER2 (1 ng/well)
2nd Ab: anti-human Fab 2nd antibody (Jackson Immuno Research)
EC_{50}: 50% effective concentration of target binding activity.

The performance of purified IgG was similar to the trastuzumab!!!

Conclusion

- Aglycosylated full-length IgG was synthesized in the active form using PURE*frex 2.0*.
- Redox state (GSH/GSSG ratio) and long-time incubation (over 20h) were important to form a correct hetero tetramer of two heavy chains and two light chains.
- The 15.7 μg of synthesized IgG was purified from 1.0 mL of PURE*frex 2.0* reaction mixture.

On-going Project

- Synthesis of other IgGs. (e.g. anti-TNF-α, EGFR, VEGF, etc.)
- Synthesis of bispecific IgG antibody.

【Acknowledgements】
We thank Dr. Yosuke Uchinashi (Nihon Pall Ltd.) and Keita Iguchi (KANEKA Corporation) for technical support and experimental advice.

GeneFrontier

Our Products:

PURE*frex*® *1.0* / PURE*frex*® *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