Development of a method for the synthesis of aglycosylated full-length IgG using PURE*frex*

再構成型無細胞タンパク質合成システムPURE*frex*を用いた全長IgG抗体の合成法開発

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Abstract

Aglycosylated IgG (including IgG1, IgG2 and IgG4 subclasses) were synthesized using PURE*frex*. 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 fragment antibodies such as Fab and scFv by last year. Here, we report the further application using PURE*frex 2.0* for production of IgG. We chose Trastuzumab as a model protein and optimized the composition of PURE*frex 2.0* reaction mixture and the reaction conditions as below; 1) adding disulfide bond isomerase (DsbC); 2) choosing GSH as an effective reductant; 3) adjusting GSH/GSSG ratio; 4) adding molecular chaperone (DnaK mix); 5) long-time incubation for 28h. We also suggested that synthesis temperature and template DNA ratio (light chain/heavy chain) should be optimized for individual IgGs for the best yield. At the best mode of synthesis, the productivity of Trastuzumab reached to 124 μg/mL. Moreover, the 68 μg of purified Trastuzumab was obtained from 1 mL of the reaction mixture after the purification by protein A resin and the following gel filtration. The purified Trastuzumab exhibited high binding affinity to recombinant HER2 protein (KD=4.24E-10 M) and internalized into HER2 expressing BT-474 cells. Furthermore, other IgGs including IgG1, IgG2 and IgG4 were also synthesized and confirmed their binding activity in ELISA. These results indicate that PURE*frex* will be useful tool for high-throughput production/screening of functional antibodies (scFv, Fab, and IgG).

What's PURE*frex*® ?

PURE*frex*® is a **reconstituted cell-free protein synthesis system** based on the PURE system* technology.
PURE*frex*® consists of **only purified factors** necessary for transcription, translation and energy regeneration.

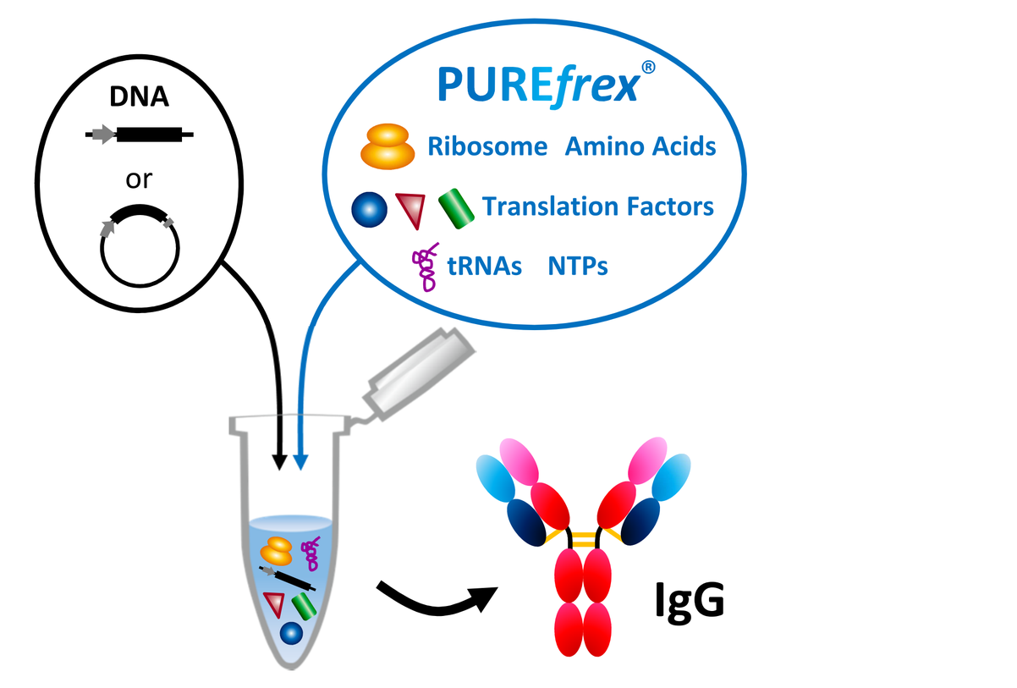
Advantage

- Low level of contamination
- Easy adjustment of the reagent composition
- PCR products usable as a template DNA

Application

- High-throughput preparation of proteins (including **Fab**, **scFv**, protein toxin etc.)
- Protein science research
- *In vitro* display (Ribosome display, mRNA display etc.)

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



Our Products (Coming soon)

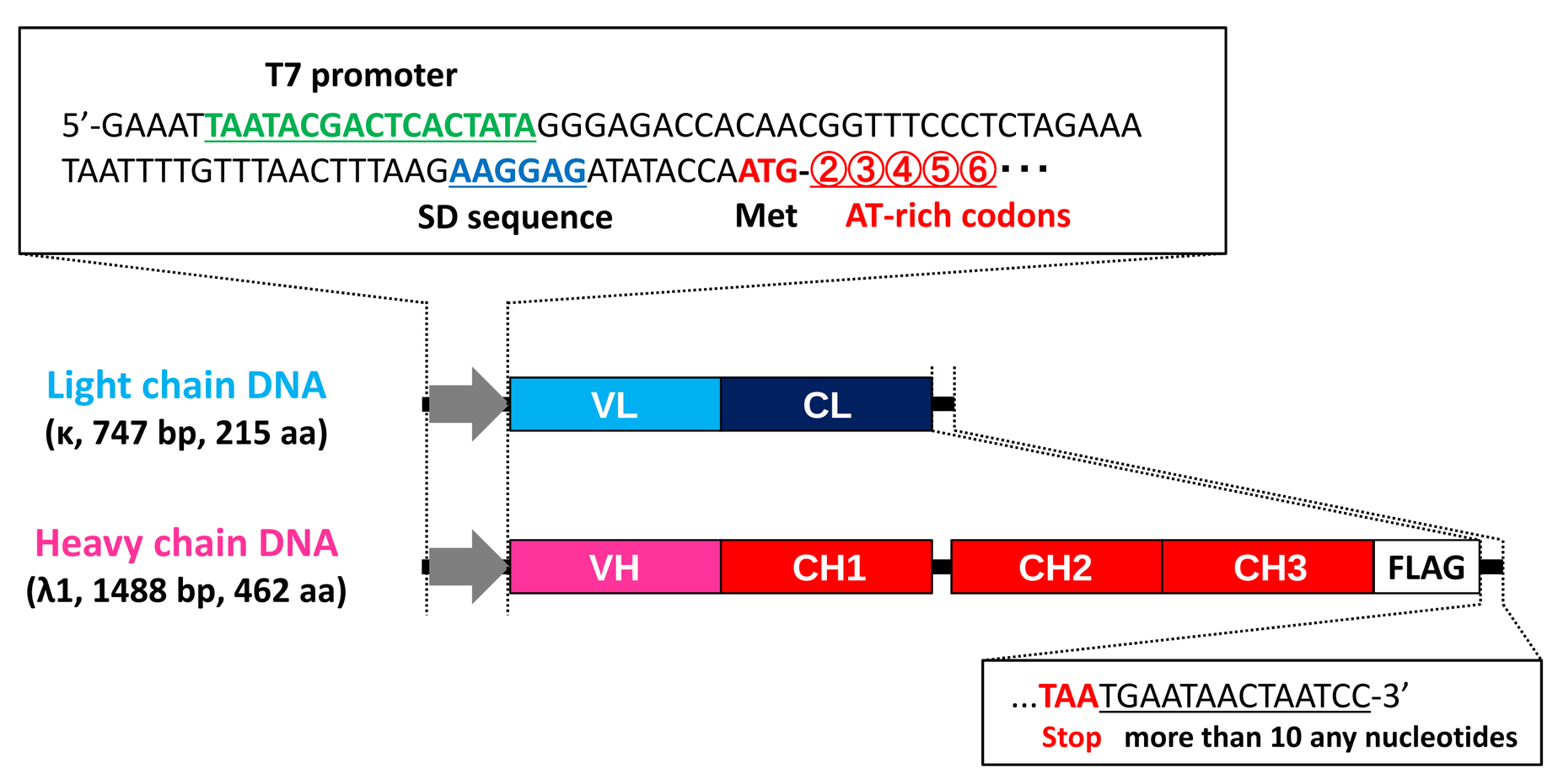
PURE*frex*® 1.0 / PURE*frex*® 2.0 / PURE*frex*® 2.1
a regular kit for the synthesis of proteins without disulfide bonds

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

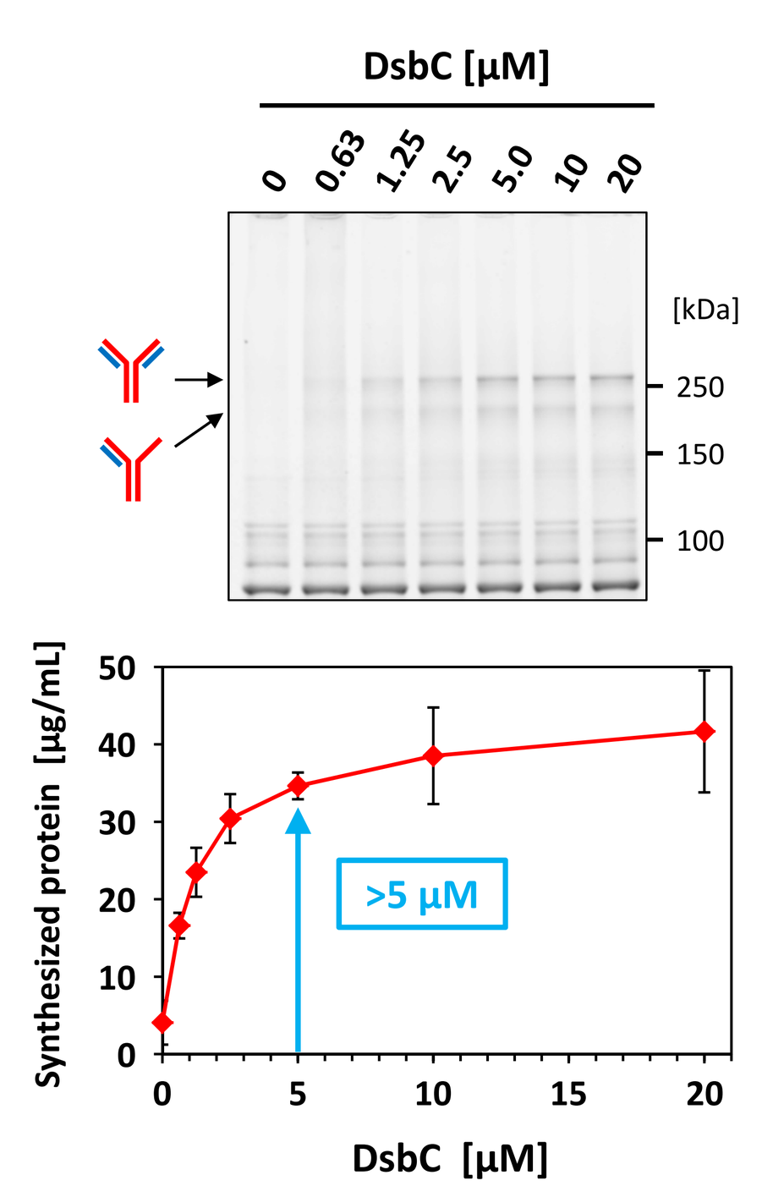
1. Optimization of the synthesis of Trastuzumab using PURE*frex*®

Template DNA design of Trastuzumab (PCR product)

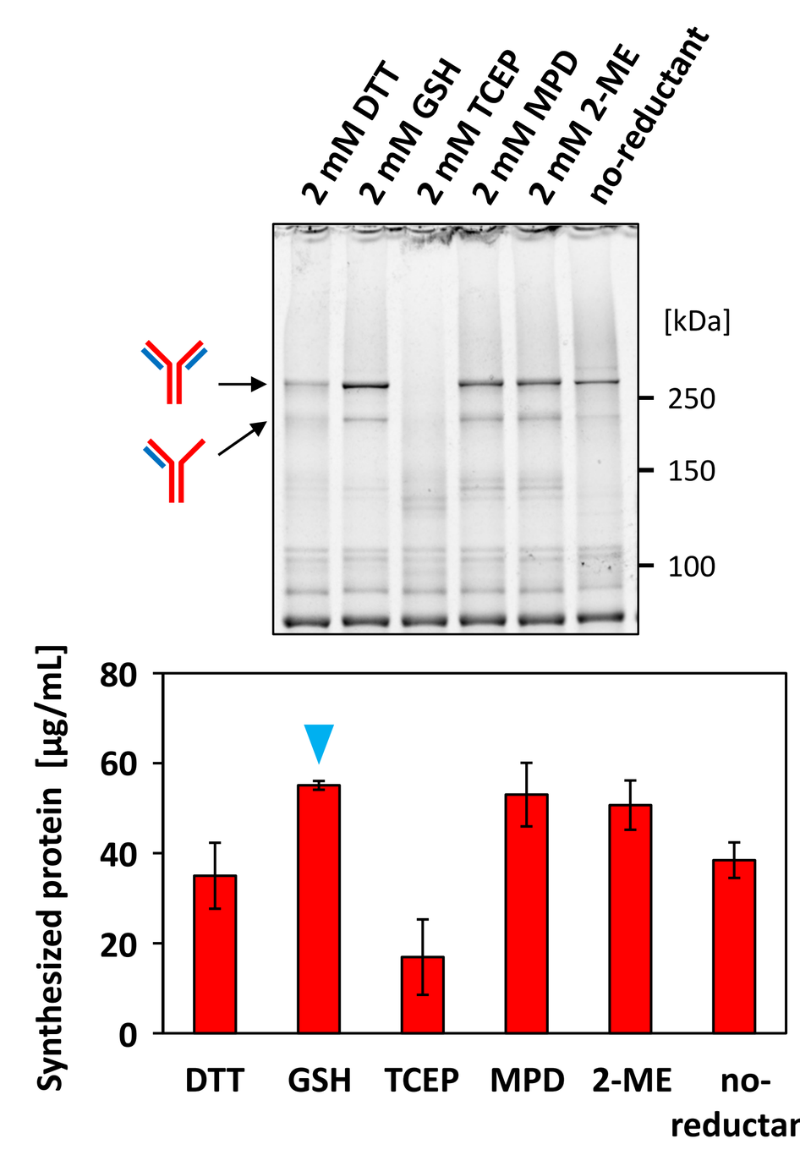


The DNAs of *E. coli* codon-optimized Trastuzumab were designed from amino acid sequences registered in public database (Drug Bank, https://www.drugbank.ca). To enhance the protein productivity, we changed **the codons of N-terminal 2nd to 6th amino acid to AT-rich codons** (not "most frequently used codons"). The synthetic genes were amplified with PCR, then purified and used to protein synthesis. The **final 10 nM of mixed template DNAs (light chain + heavy chain)** were added to PURE*frex 2.0* reaction mixture.

#2: DsbC concentration

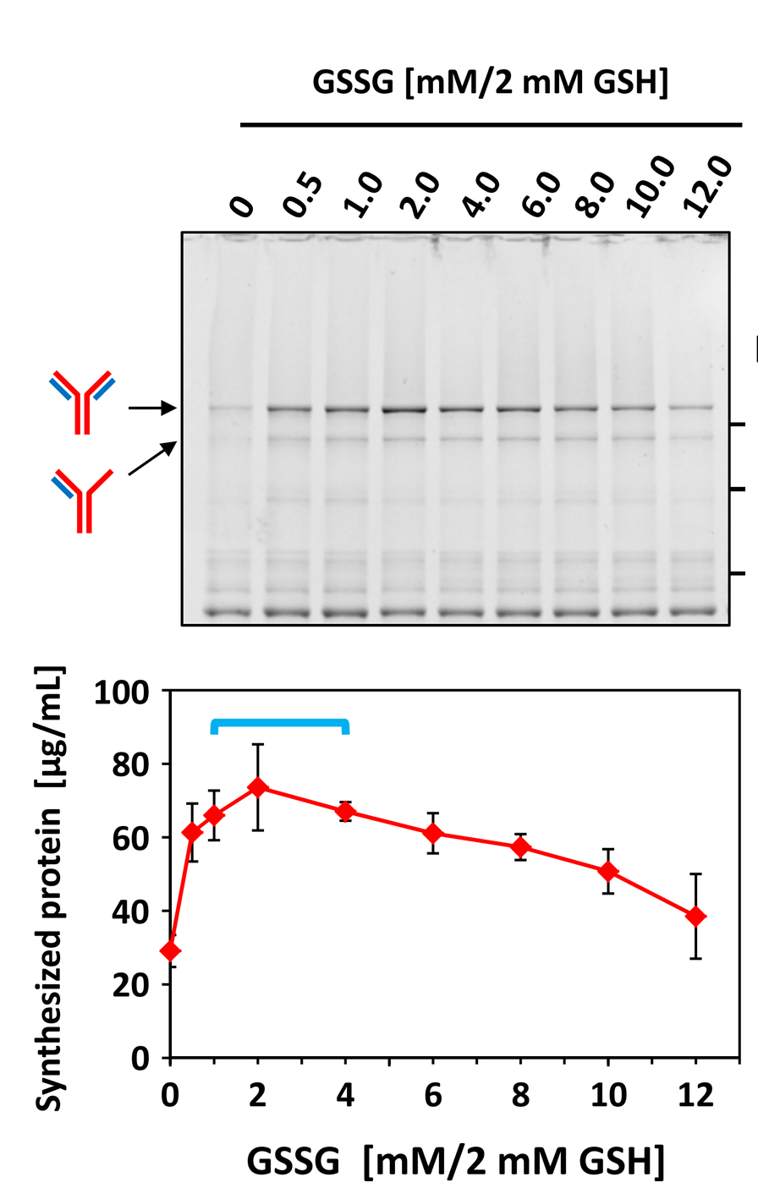


#3: Reducing agent

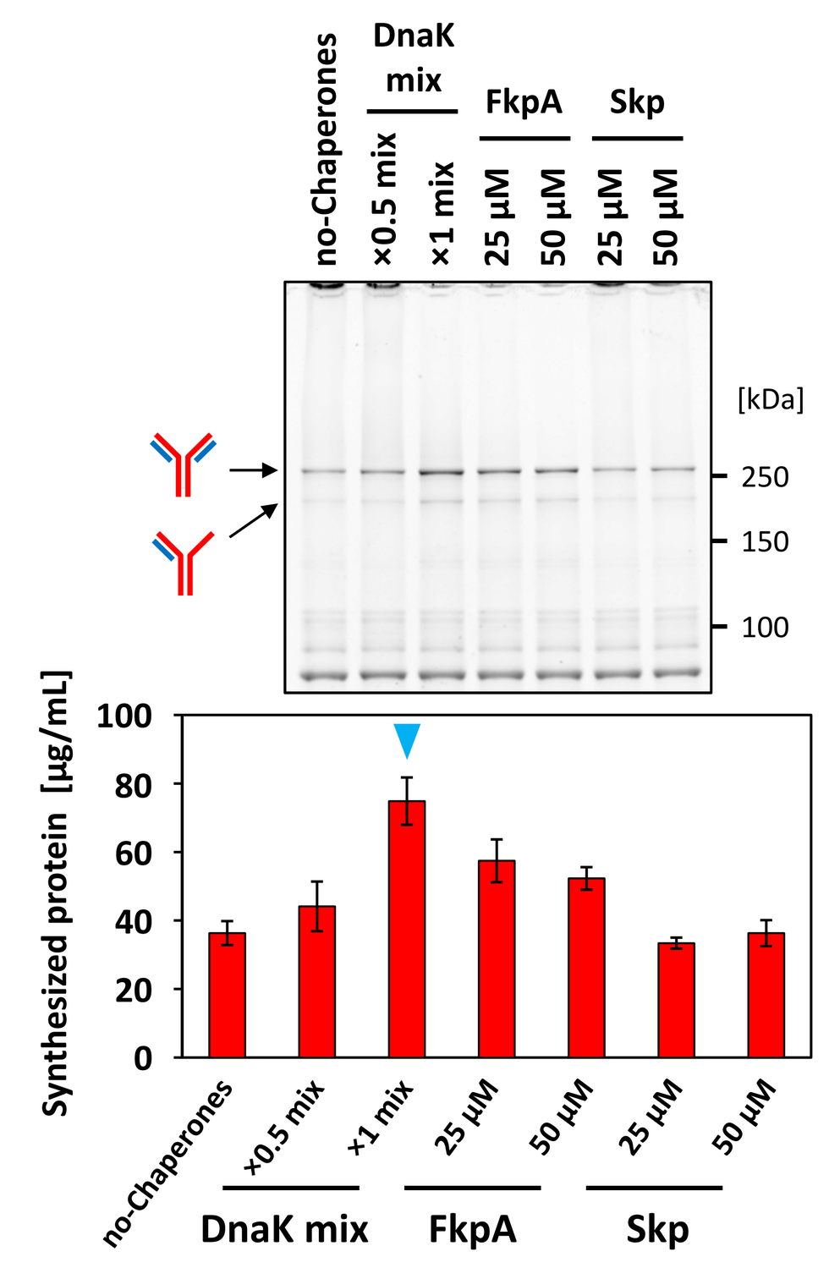


TCEP: Tris(2-carboxyethyl) phosphine, 2-ME: 2-mercaptoethanol, MPD: 3-Mercapto-1,2-propanediol

#4: GSH/GSSG ratio



#5: Molecular chaperone and chaperone-like proteins



DsbC: Periplasmic disulfide bond isomerase from *E. coli*, which promotes the correct disulfide bond formation.*1

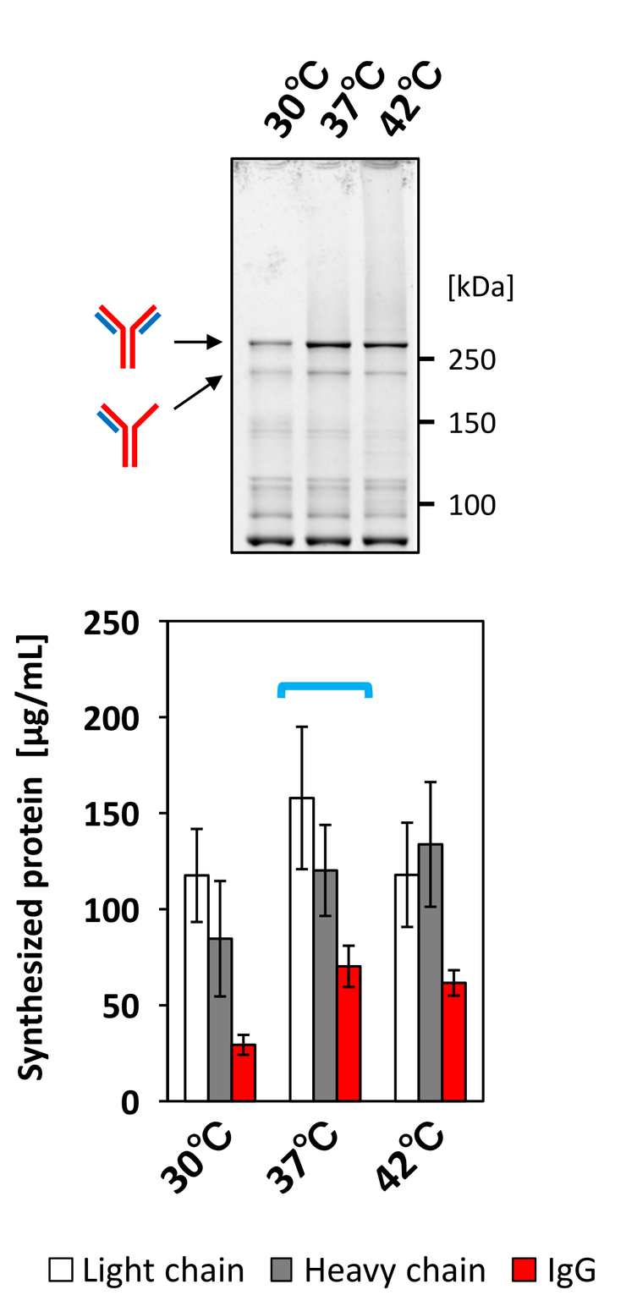
DnaK mix: Mixture of cytoplasmic molecular chaperones from *E. coli*, which contains 5 μM DnaK, 1 μM DnaJ, 1 μM GrpE.*2

FkpA: Periplasmic peptidyl-prolyl *cis-trans* isomerase (PPIase) from *E. coli*, which has a chaperone-like function independent of PPIase activity.*1

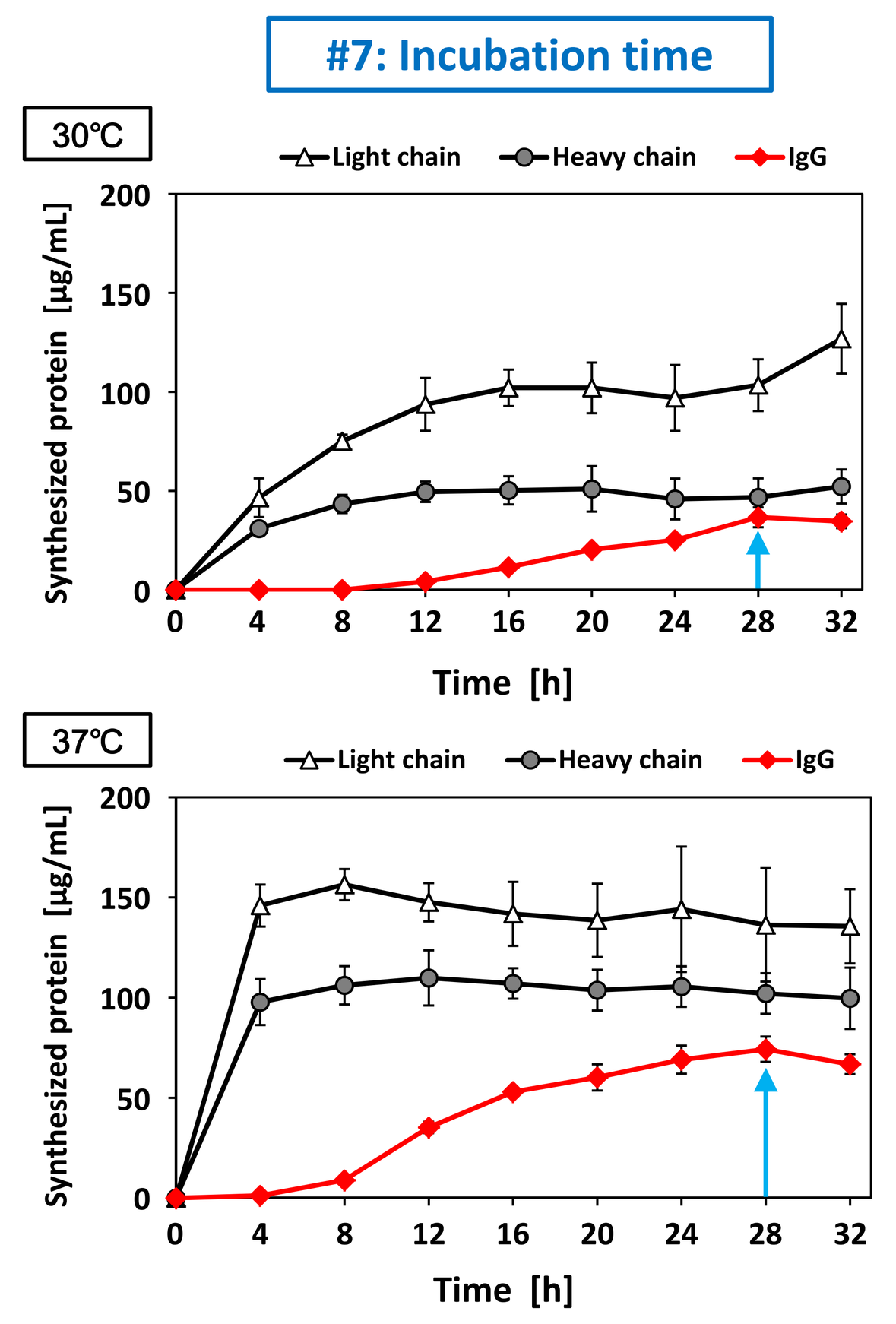
Skp: Periplasmic protein from *E. coli*, which shows chaperone-like activity to β-barrel protein.*1

【Reference】
*1: Goemans C. *et al*. (2014) *BBA*. vol. 1843, p. 1517
*2: Castanié-Cornet MP. *et al*. (2014) *BBA*. vol 1843, p1442

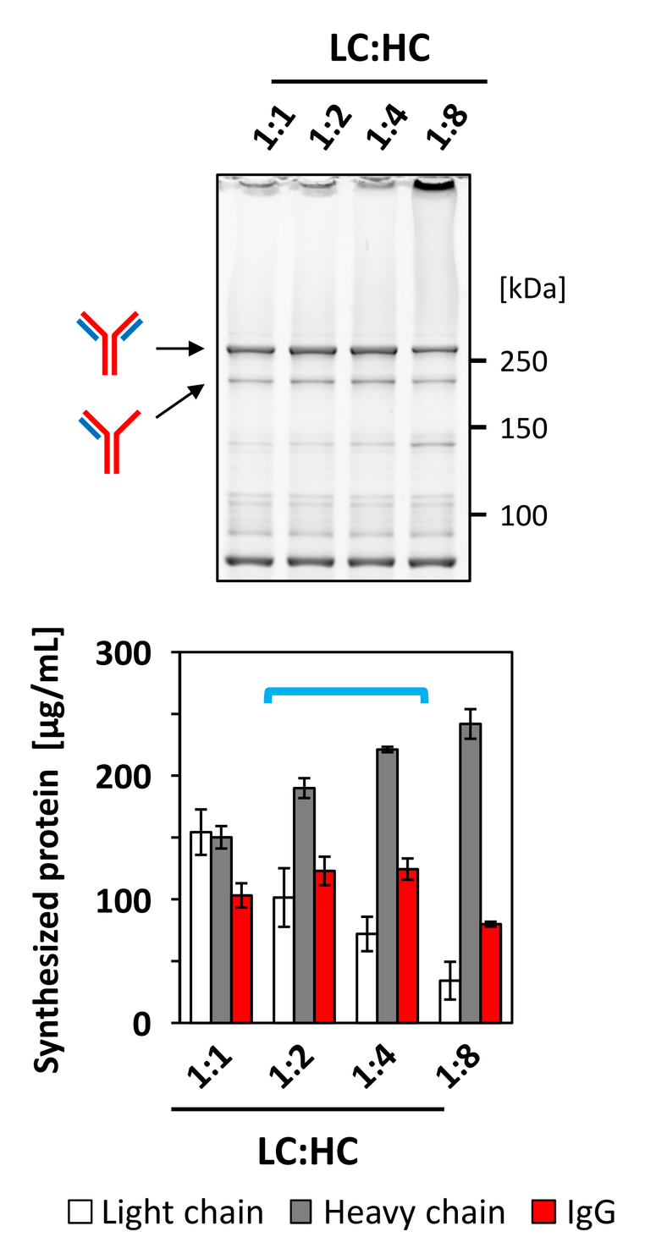
#6: Synthesis temperature



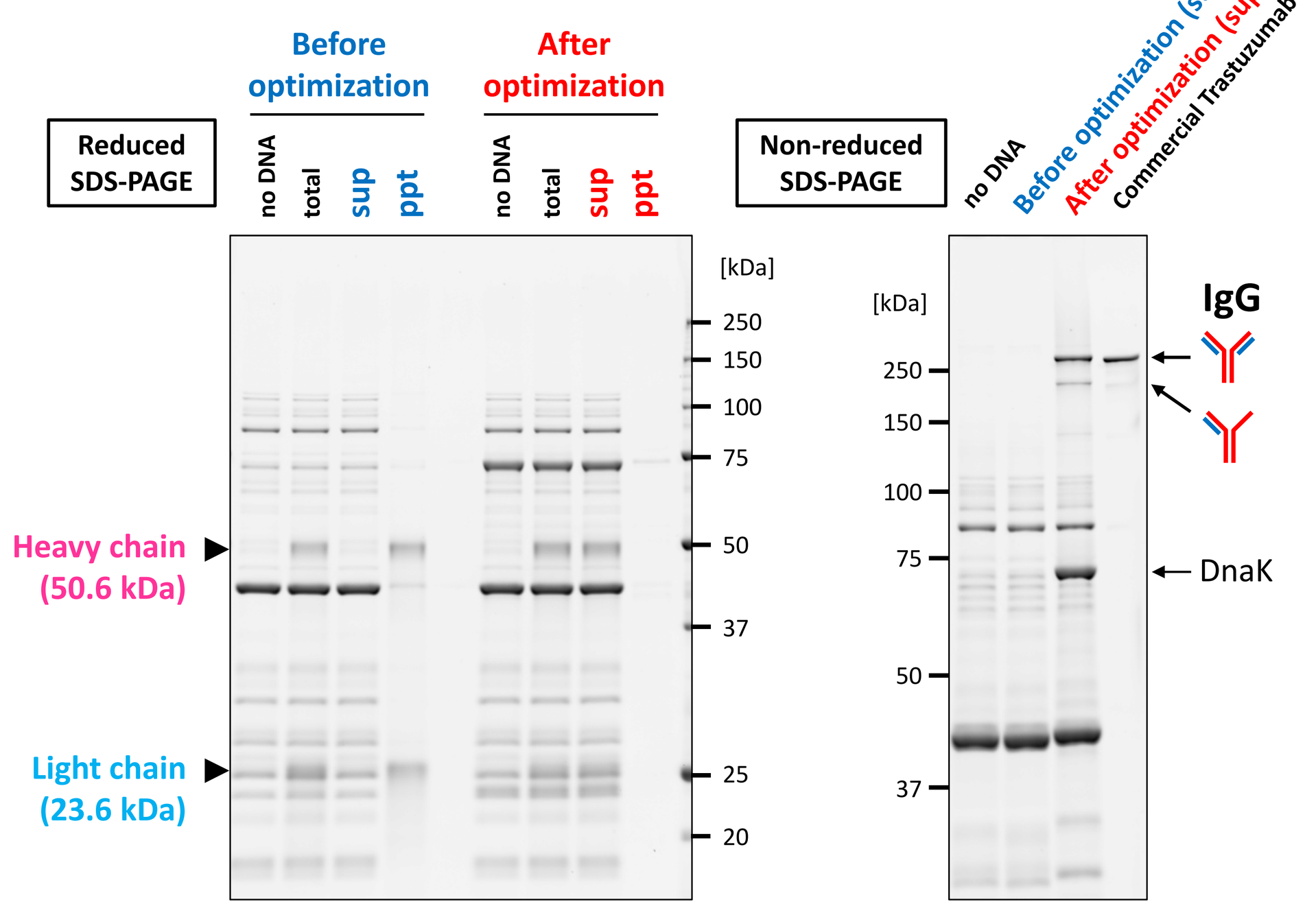
#7: Incubation time



#8: Template DNA ratio (Light chain/Heavy chain)



#1 and #9: Before and After optimization



*The amount of both light chain and heavy chain were **almost the same in before and after optimization**. Therefore, the improvements for making whole IgG were "**solubilization**" and "**tetramer formation**".

Summary of the optimization

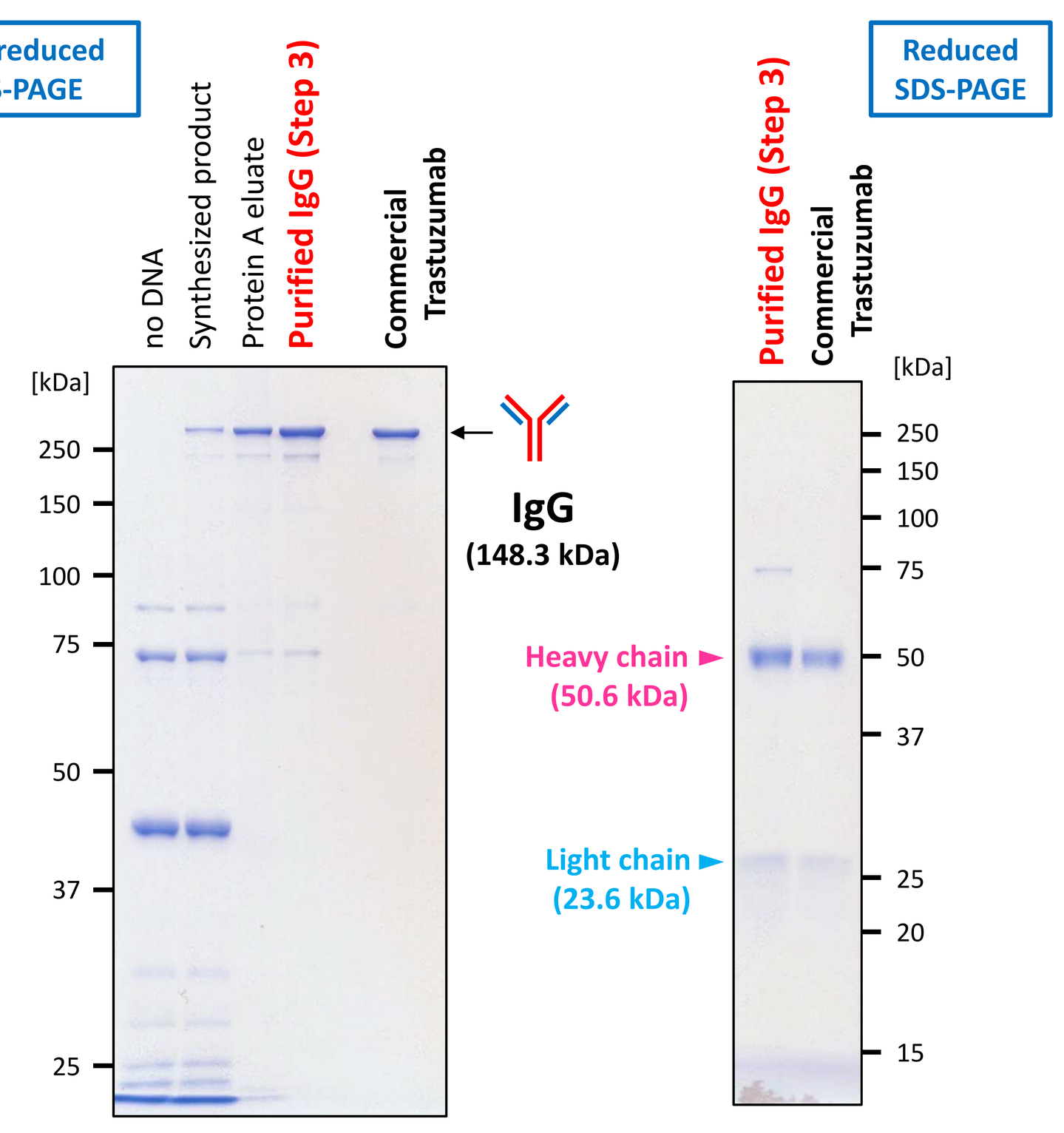
No	Experimental title	Composition of the reaction mixture	Incubation	Template DNA ratio (LC:HC, total 10 nM)	IgG Yield [μg/mL]
1	**Before optimization**	**PURE*frex 2.0* (normal version, 2 mM DTT)**	**37°C for 16h**	**1:1**	**ND**
2	DsbC concentration	PURE*frex 2.0* (normal version, 2 mM DTT) with 3 mM GSSG, **0-20 μM DsbC** and DnaK mix (x1)	37°C for 16h	1:1	**35±2**
3	Reducing agent	**PURE*frex 2.0* (without DTT)** with **2 mM reducing agent**, 3 mM GSSG, 5 μM DsbC and DnaK mix (x1)	37°C for 16h	1:1	**55±1**
4	GSH/GSSG ratio	PURE*frex 2.0* (without DTT) with **2 mM GSH**, **0-12 mM GSSG**, 5 μM DsbC and DnaK mix (x1)	37°C for 16h	1:1	**74±12**
5	Molecular chaperone and chaperone-like proteins	PURE*frex 2.0* (without DTT) with 2 mM GSH, 3 mM GSSG, 5 μM DsbC and **chaperones**	37°C for 16h	1:1	**75±7**
6	Synthesis temperature	Optimized reaction mixture (See No. 9)	**30, 37 or 42°C** for 16h	1:1	**70±11**
7	Incubation time	Optimized reaction mixture (See No. 9)	**30 or 37°C for 0 - 32 h**	1:1	**74±6**
8	Template DNA ratio	Optimized reaction mixture (See No. 9)	37°C for 28 h	**1:1, 1:2, 1:4, 1:8**	**124±9**
9	**After optimization**	**PURE*frex 2.0* (without DTT) with 2 mM GSH, 3 mM GSSG, 5 μM DsbC and DnaK mix (x1)**	**37°C for 28 h**	**1:2 - 1:4**	

Aglycosylated IgG (Trastuzumab) 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 (12.5% 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). Yields are expressed as means ± SD (n=3). ND, not detectable.

2. Evaluation of the synthesized Trastuzumab

Purification method

1 mL of optimized PURE*frex 2.0* reaction mixture
(Synthesized product)
↓
Step 1: **Protein A purification**
(KANEKA KanCapA™, Kaneka)
↓
Step 2: **Gel filtration**
(Superdex-200 10/300 GL, GE healthcare)
↓
Step 3: **Protein enrichment**
(AmiconUltra-0.5, MWCO: 30 kDa, Merck Millipore)



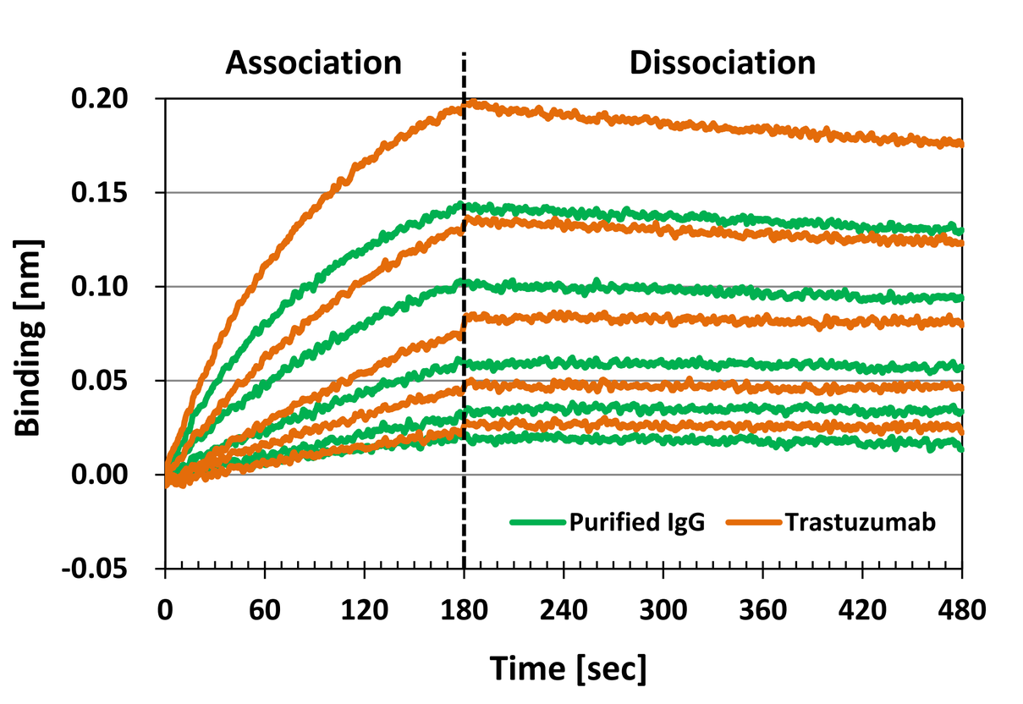
Non-reduced SDS-PAGE on 10% gel. CBB staining. Samples: 1.0 μL/lane. Trastuzumab: 500 ng/lane.

Reduced SDS-PAGE on 12.5% gel. CBB staining. Purified IgG: 1.0 μL/lane. Trastuzumab: 500 ng/lane.

Purification summary

	IgG concentration		Volume	Total protein	Yield
	[μg/mL]	[μM]	[mL]	[μg]	[%]
Synthesized product	131	0.88	**1.0**	**131**	100
Protein A eluate (Step 1)	330	2.22	0.3	99	75.4
Purified IgG (Step 3)	**678**	**4.57**	**0.1**	**68**	**51.7**

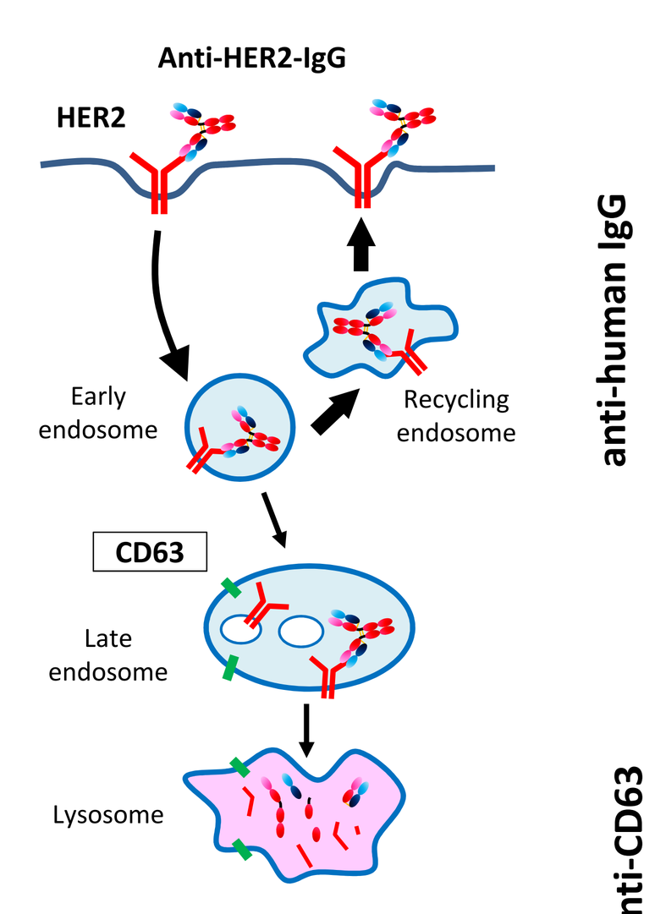
Binding kinetics analysis

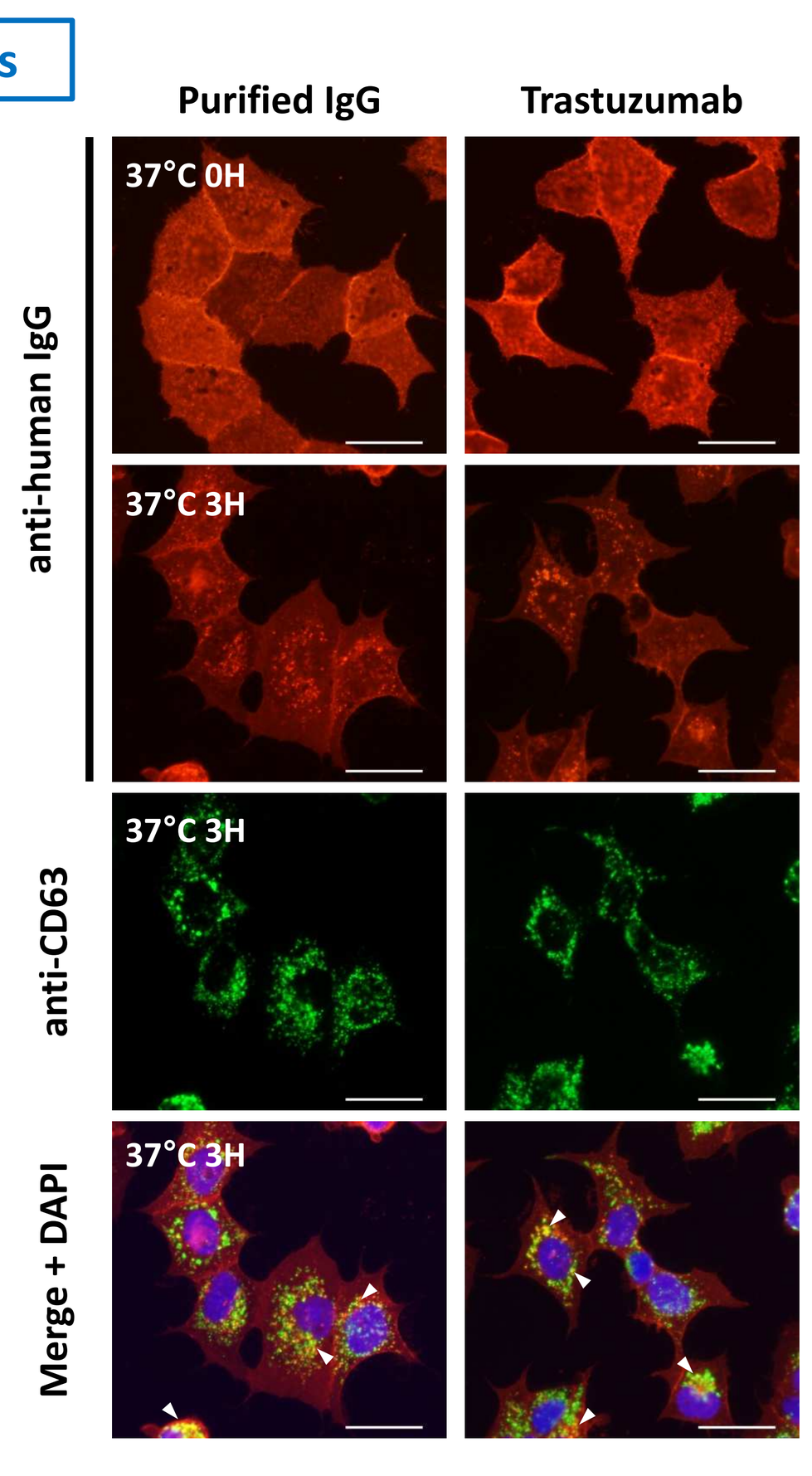


	K_D [M]	k_{on} [1/Ms]	k_{off} [1/s]
Purified IgG	**4.24E-10**	**6.38E+05**	**2.70E-04**
Trastuzumab	**4.03E-10**	**6.29E+05**	**2.53E-04**

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.

Internalization analysis





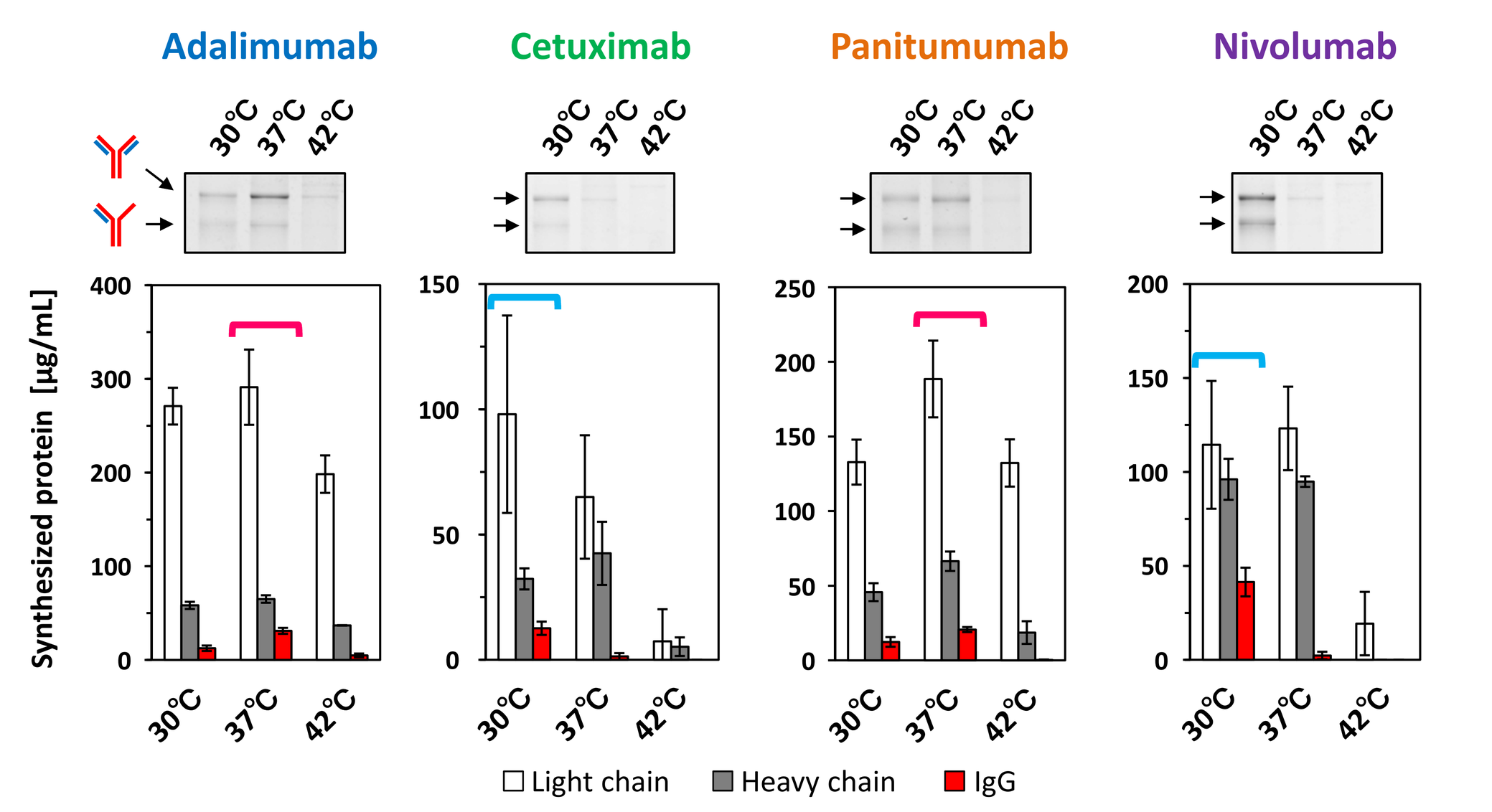
BT-474 cells were surface-labeled at 4°C for 60 min with the binding medium (DMEM, 3% BSA, 20 mM HEPES (pH 7.4)) containing 10 nM of "purified IgG" or "Trastuzumab". Cells were washed five times with the binding medium and incubated at 37°C for 3h. Cells were then fixed and processed for dual-label indirect 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.

The performance of purified IgG was similar to the commercial Trastuzumab!!!

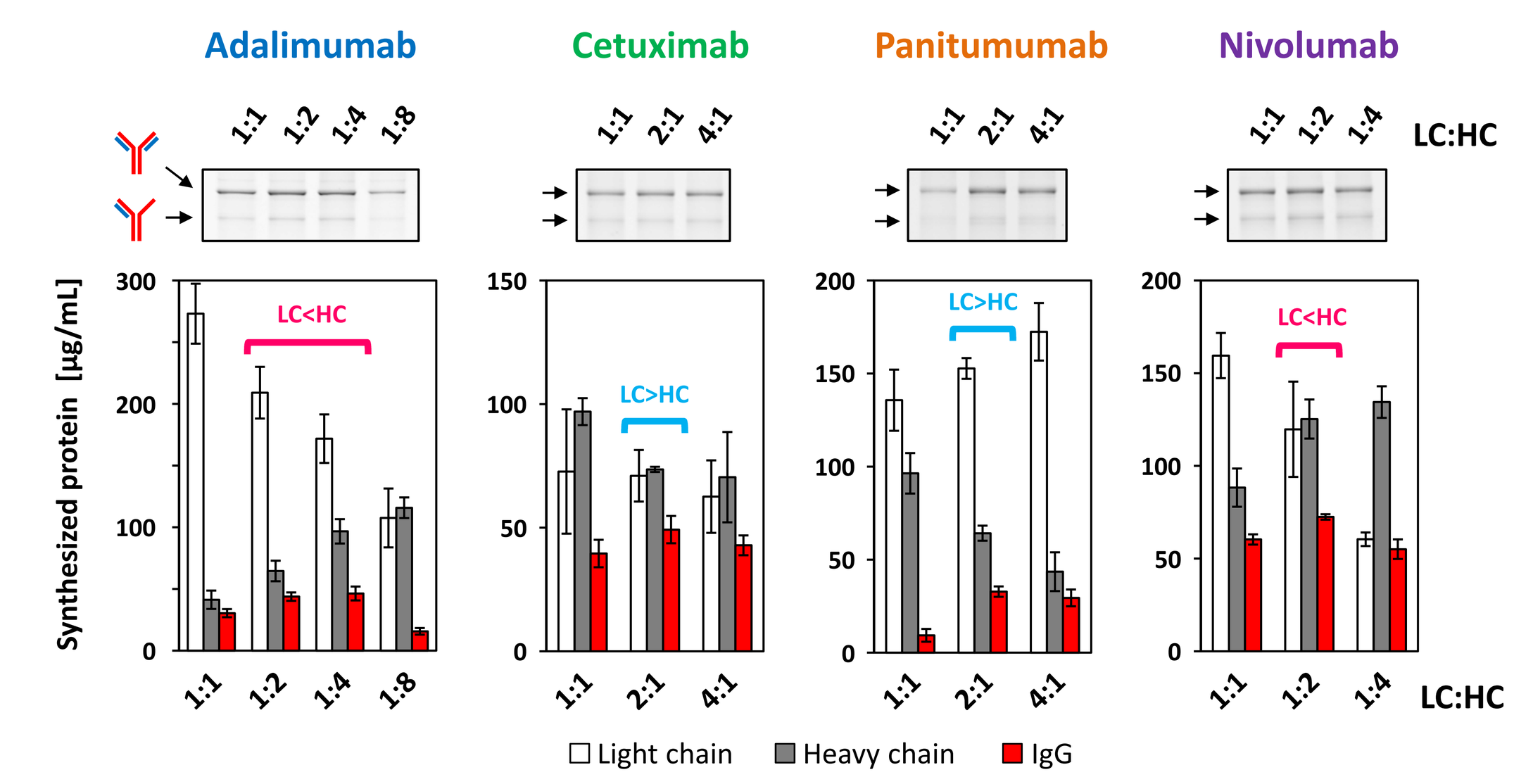
【Acknowledgements】
We thank Dr. Yosuke Uchinashi (Nihon Pall Ltd.) and Keita Iguchi (KANEKA Corporation) for technical support and experimental advice.

3. Synthesis of other IgGs using PURE*frex*®

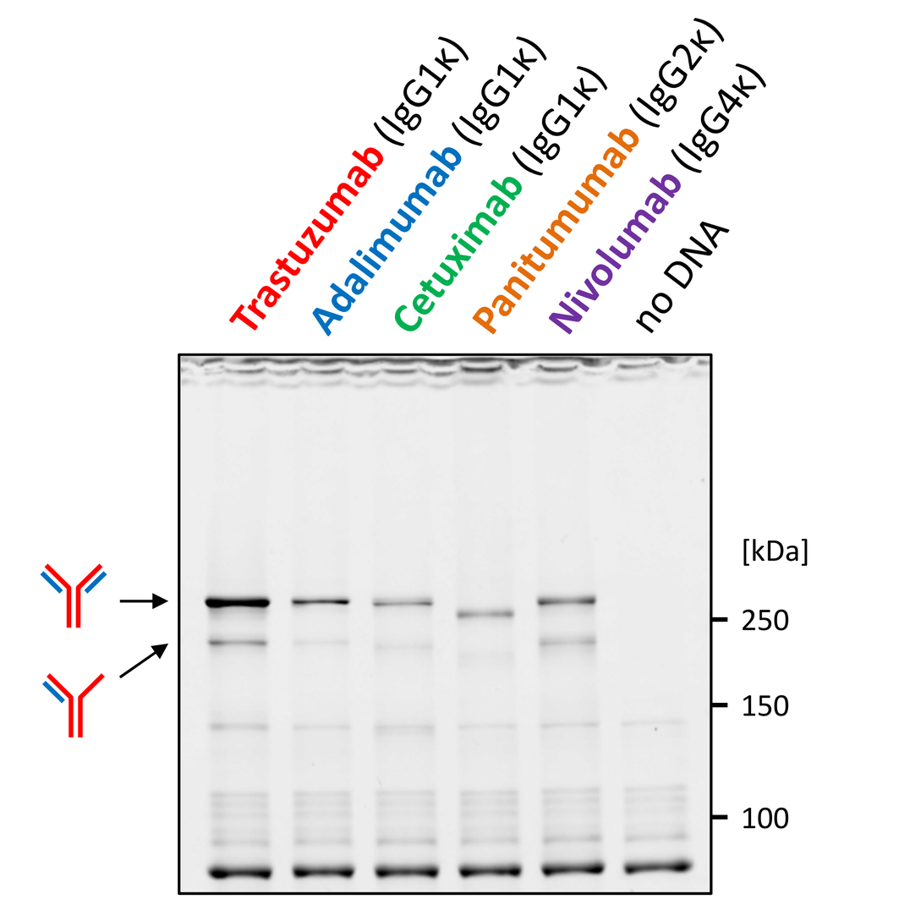
Synthesis temperature



Template DNA ratio (Light chain/Heavy chain)

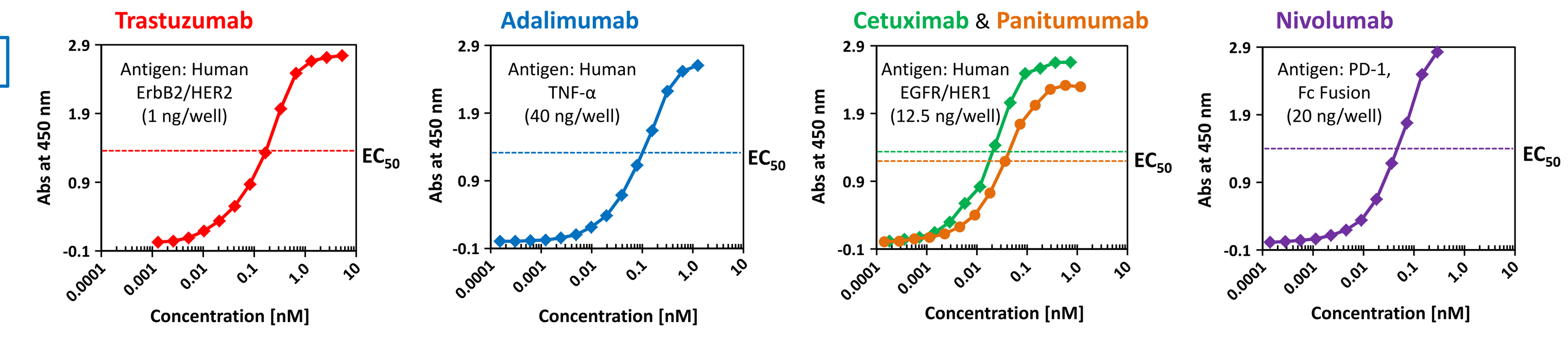


Non-reduced SDS-PAGE



Non-reduced SDS-PAGE on 10% gel, Oriole staining.
Samples: 1.0 μL of reaction/lane.

ELISA



2nd Ab: anti-human Fab 2nd antibody (Jackson Immuno Research), EC_{50}: 50% effective concentration of target binding activity in ELISA.

Summary of the synthesis of several IgGs using PURE*frex*®

IgG name	Subclass	Antigen	MW [kDa]	Synthesis temperature	Template DNA ratio (LC:HC, total 10 nM)	IgG Yield [μg/mL]	EC_{50} [nM]
Trastuzumab	IgG1κ	HER2	148.3	37°C	1:2-1:4	124±9	0.16
Adalimumab	IgG1κ	TNF-α	147.4	37°C	1:2-1:4	46±6	0.1
Cetuximab	IgG1κ	EGFR	148.3	30°C	2:1	49±6	0.02
Panitumumab	IgG2κ	EGFR	144.9	37°C	2:1	33±3	0.036
Nivolumab	IgG4κ	PD-1	144.2	30°C	1:2	73±2	0.05

Yields are expressed as means ± SD (n=3). EC_{50}: 50% effective concentration of target binding activity in ELISA.

Method for the synthesis of IgG using PURE*frex*

Reaction mix:
- **PURE*frex*® 2.0 Customized solution** (without DTT and with **2 mM GSH**) → (Coming soon) **PURE*frex*® 2.1**
- + **DS supplement** (3 mM GSSG, 5 μM (117 μg/mL) DsbC)
- + **DnaK Mix** (5 μM DnaK, 1 μM DnaJ, 1 μM GrpE)

Incubation : **30 - 37°C for 28h**

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

Synthesis temperature and **template DNA ratio (light chain/heavy chain)** should be optimized for individual IgGs for the best yield.

*If you want to synthesize **multiple IgGs** at the same time (e.g. **IgG screening**), we recommend "**30°C** and **LC:HC=1:1**" as a first step.

Conclusion

- Aglycosylated IgG (including IgG1, IgG2 and IgG4 subclasses) was synthesized in the active form using PURE*frex 2.0*.
- Disulfide bond isomerase (DsbC), redox state (reducing agent/oxidizing agent ratio) and long-time incubation were important to form a correct hetero tetramer of two heavy chains and two light chains.
- IgG yield, optimum synthesis temperature and optimum template DNA ratio were different between individual IgGs, which may reflect the difference of CDR sequence and structural stability.
- **PURE*frex*®** will be "**useful**" and "**powerful**" tool for high-throughput production/screening of functional antibodies.