For more information

Rebuilding Expression System & its Applications for R&D of Biologics.

Reconstituted cell-free protein synthesis kit
PURE*frex*®

Takashi (Ebi) Ebihara, Ph.D.
COO
GeneFrontier Corporation

GeneFrontier

PepTalk
13-16 of Jan, 2025

Corporate Summary

GeneFrontier

Founded :	***Oct 13th, 2010*** (renewed)
Shareholder :	***KANEKA Corporation*** (100%)
People :	***14*** (Ph.D. 8, MS 1)
Place :	***Chiba, Japan***

Mission : ***Rebuilding and Manipulating Biological system for Inspiring the world!***

Reconstituted cell-free protein synthesis kit

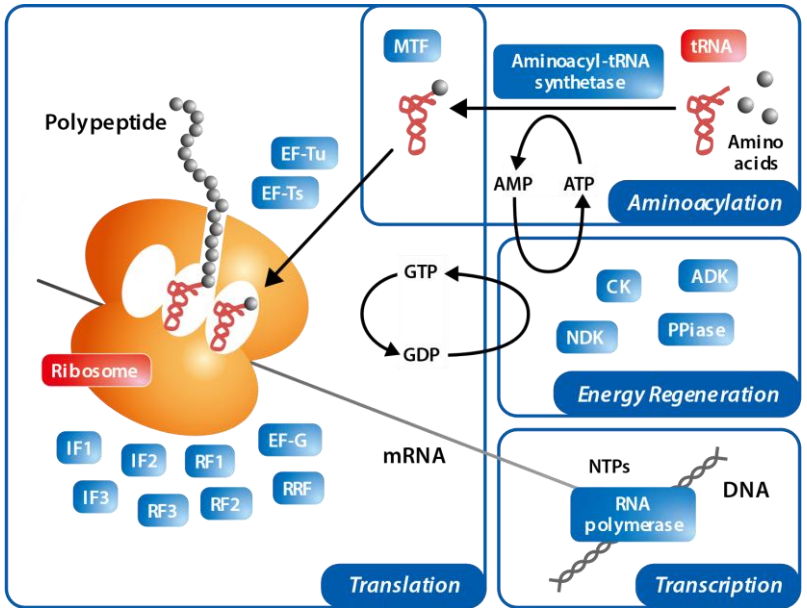
PURE*frex*®

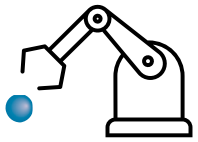
-Customize expression toolbox for your research-

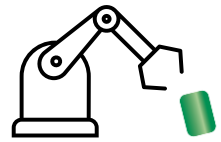
For more information

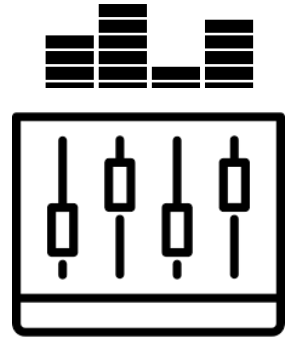
GeneFrontier

Totally constructive, molecular based system

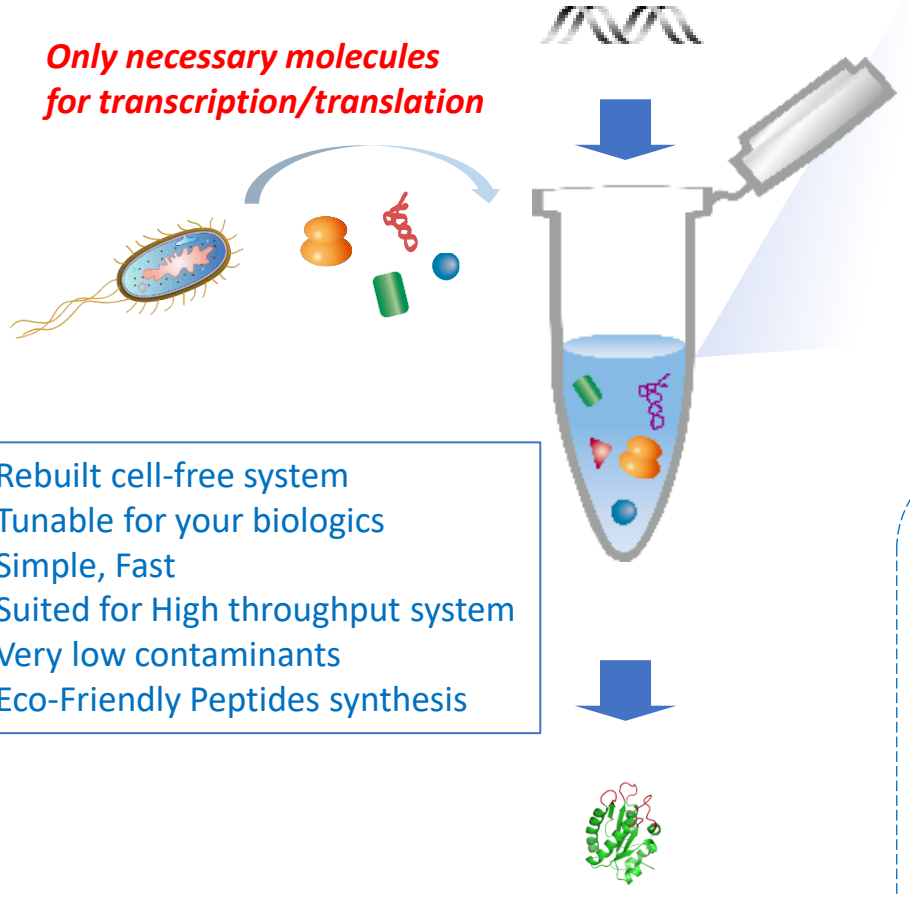








Only necessary molecules for transcription/translation



- ✓ Rebuilt cell-free system
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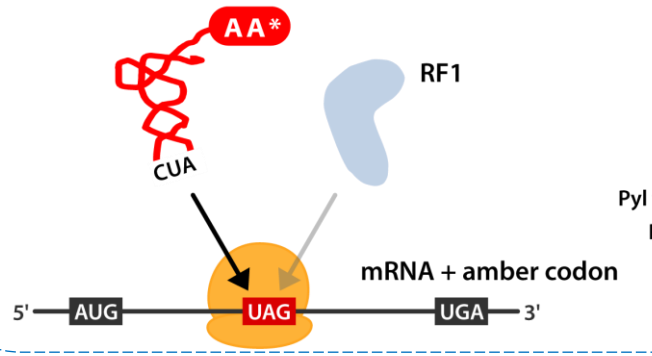
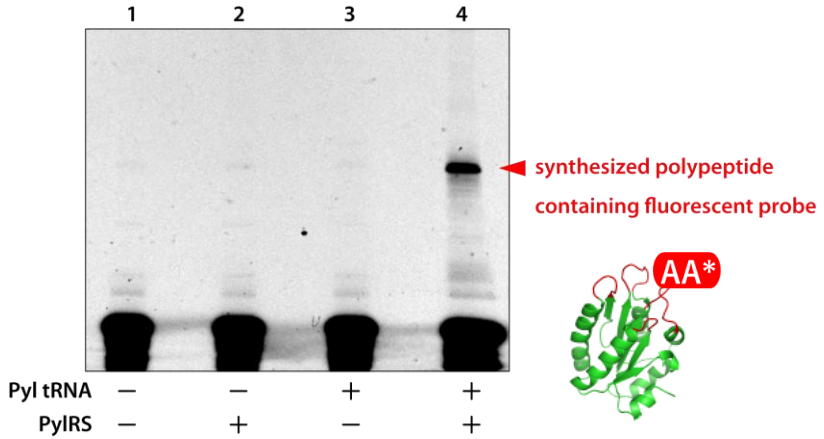
<Ex, Non-natural AA introduction>

Translation - RF1

suppressor tRNA + non-natural amino acid





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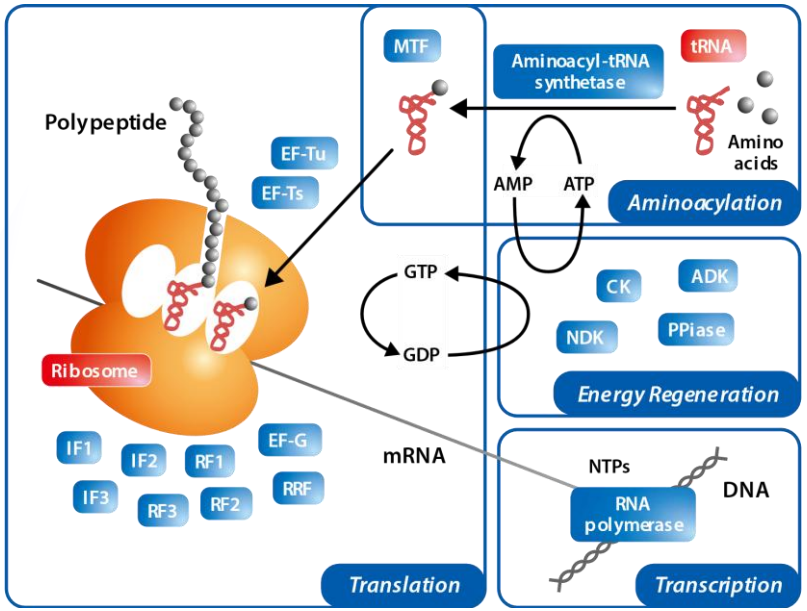

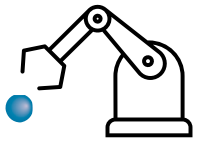

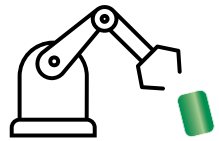

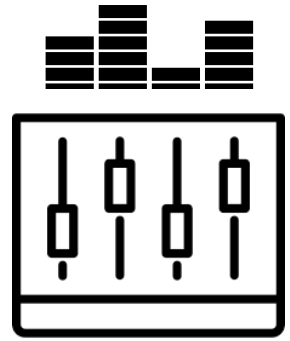

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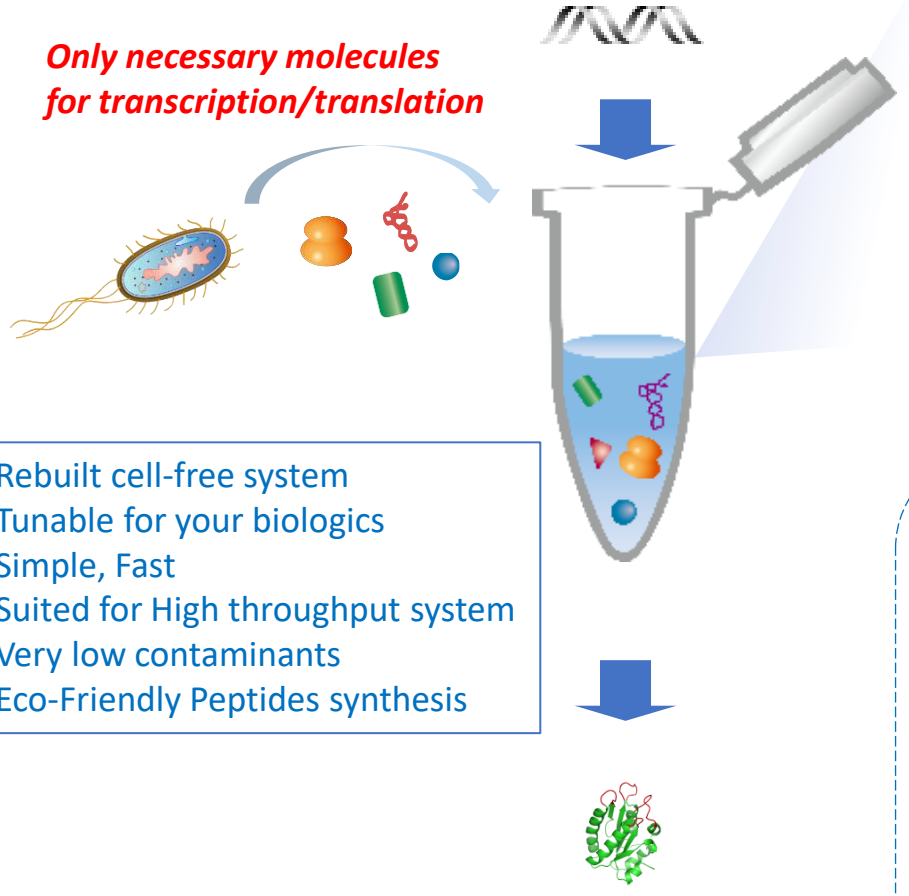

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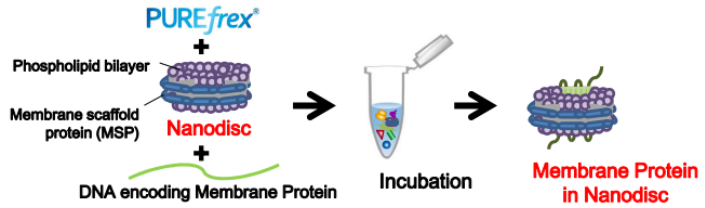
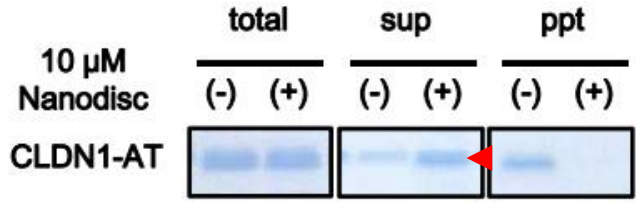
<Ex, Membrane protein with Nanodisc; artificial membrane-like structure>





The condition of membrane protein synthesis

Reaction mix	Template DNA	Incubation
PURE*frex*® *2.0* +Nanodisc (MSP1E3D1-His POPC*, final 10 μM)	PCR product	37°C, 4 h

*Ref: Denisov *et al.* (2007) *J.Biol.Chem.*, vol. 282, p. 7066.

Solubilized hCLDN1 was synthesized using PURE*frex*® and Nanodisc.

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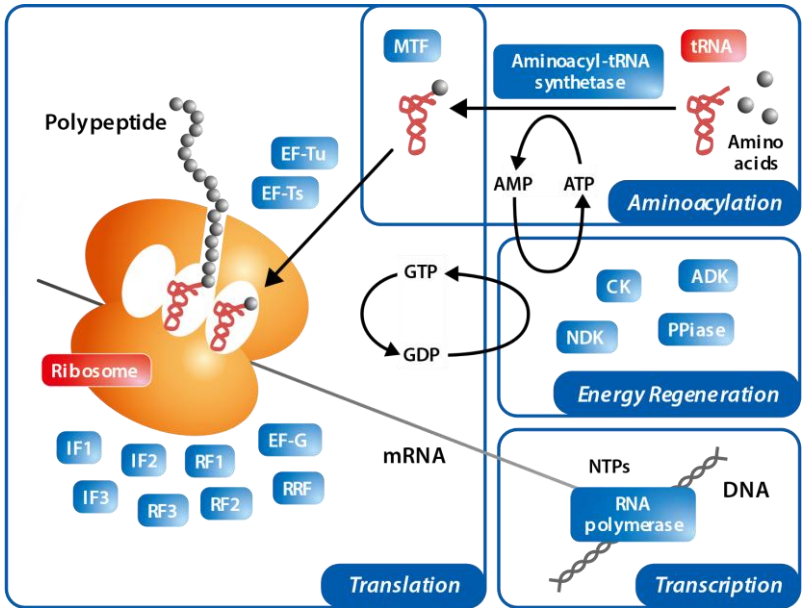

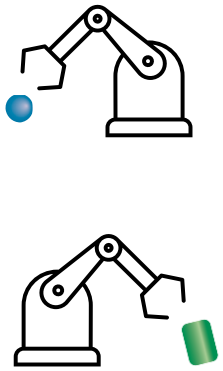

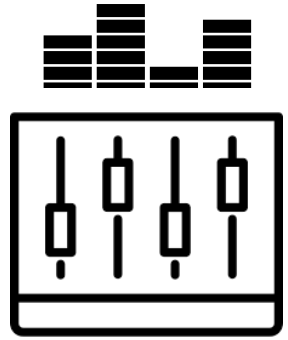

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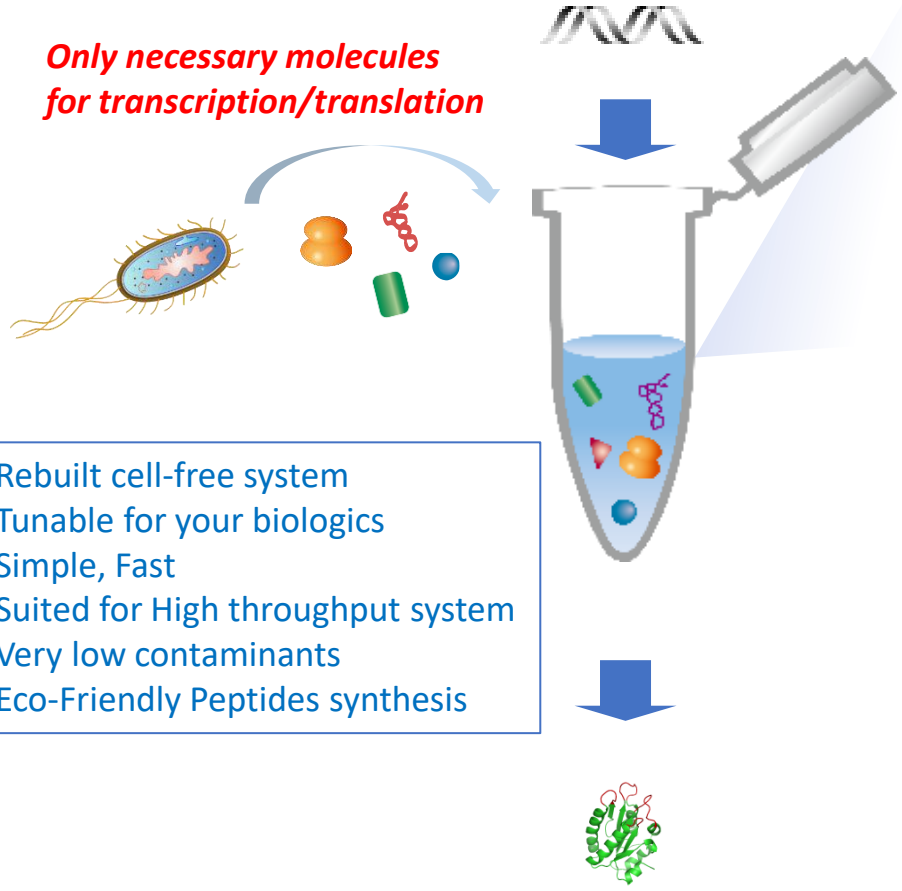

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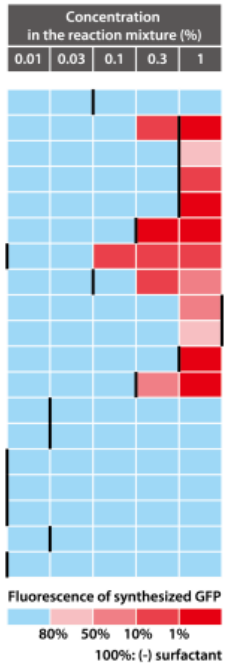
Experimental conditions for protein synthesis

Reaction mixture	Incubation	Template DNA
PURE*frex*®2.1 (4 mM GSH) + Sufractants	37°C 4 h	sfGFP PCR product (1 ng/μL)

→ **Measurement of GFP fluorescence**

- Most surfactants did not inhibit the protein synthesis reaction by PURE*frex*® below the CMC.
- Some surfactants such as Triton X-100 and Tween 20 could be used even above the CMC.

Surfactant Name	CMC (%)
Digitonin	0.061
Sodium cholate	0.628
CHAPS	0.492
CHAPSO	0.505
n-Octyl-β-D-glucoside	0.731
n-Octyl-β-D-thioglucoside	0.278
n-Dodecyl-β-D-maltoside	0.009
n-Decyl-β-D-maltoside	0.087
n-Octyl-β-D-maltoside	1.064
Mega-8	1.864
Mega-9	0.839
Mega-10	0.245
Triton X-100	0.016
Triton X-114	0.011
NP-40	0.009
Tween 20	0.007
Tween 80	0.002
Brij 35	0.011
Brij 58	0.009



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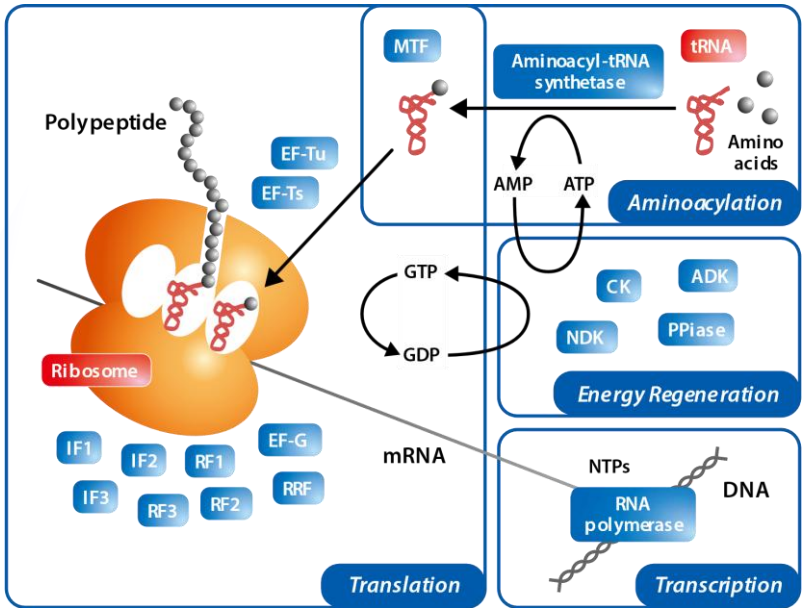

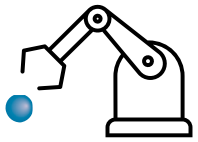

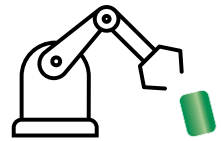

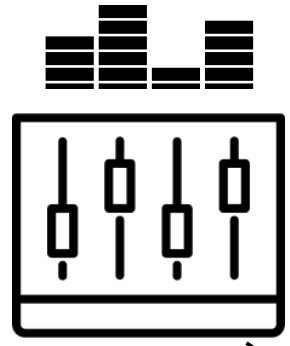

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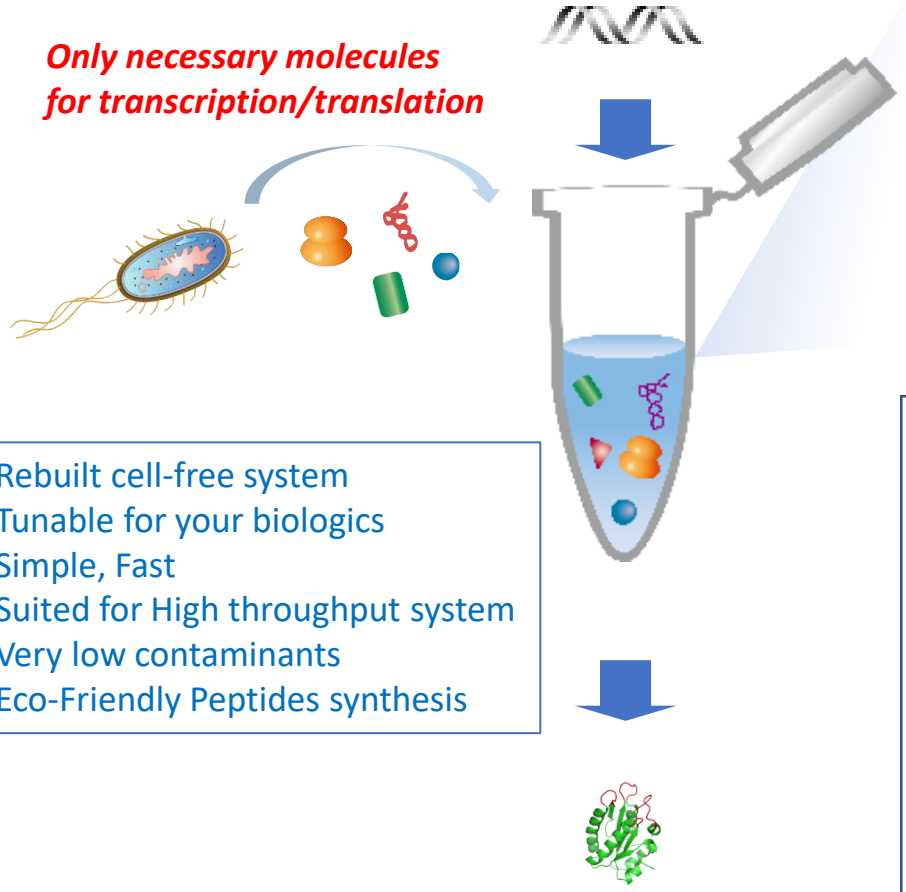

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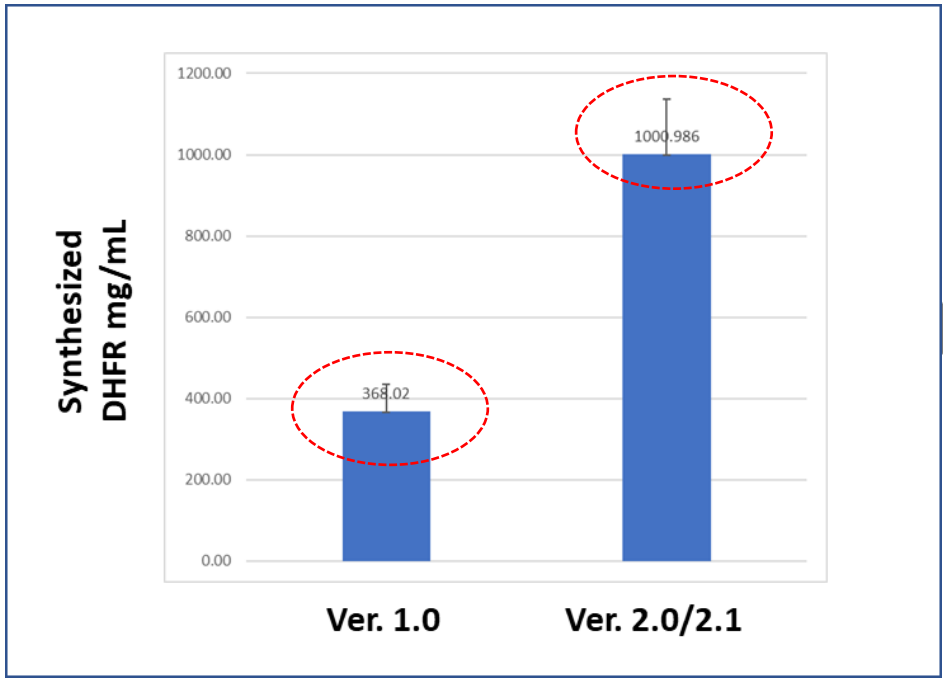

Versatile and Robust Platform for protein synthesis

Huge potential as New platform in Biotech industry

東京大学 THE UNIVERSITY OF TOKYO
東京工業大学 Tokyo Institute of Technology
Keio University 1858
JAMSTEC
TU Delft Delft University of Technology
tbi Toulouse Biotechnology Institute Bio & Chemical Engineering
JAIST Japan Advanced Institute of Science and Technology 1990

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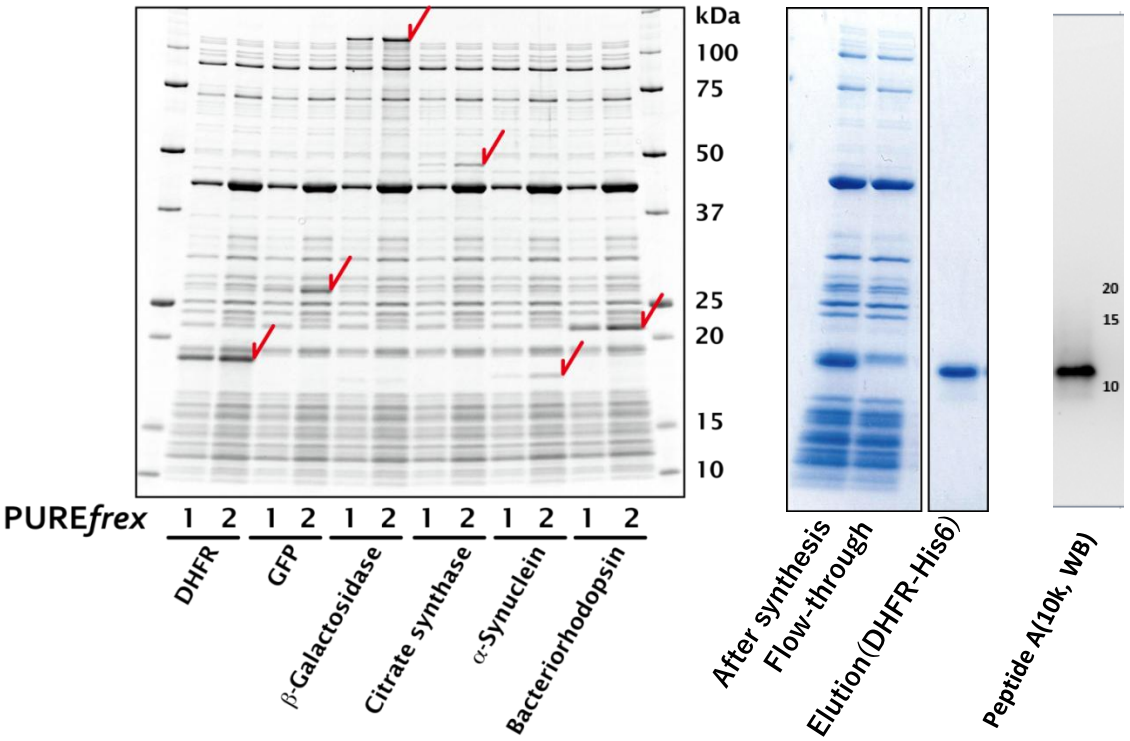
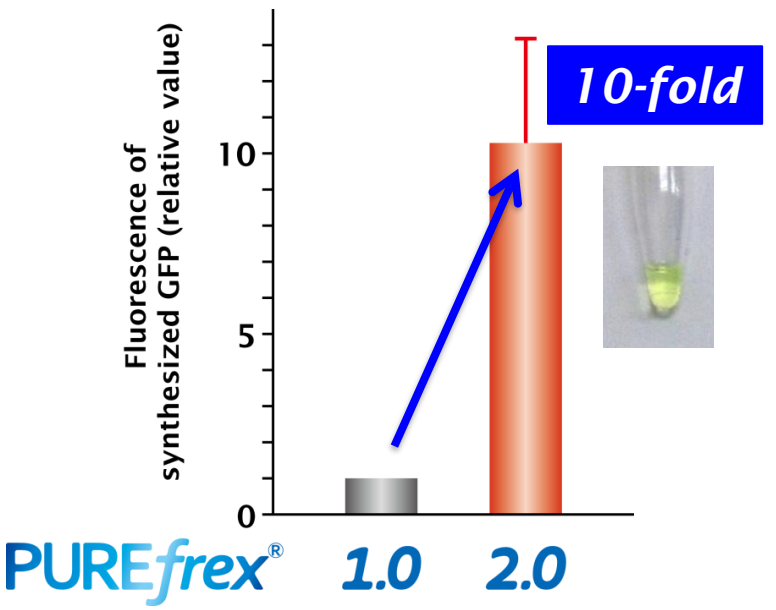
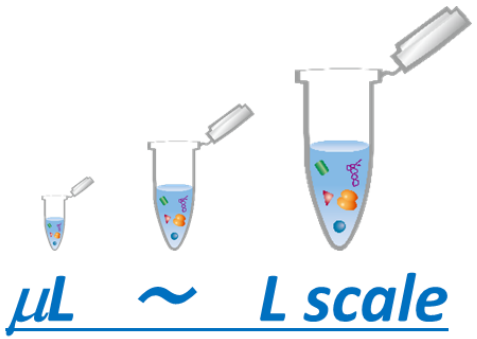
-Having good productivity-







Great Flexibility from basic research to industrial applications

- Reaction at 37ºC for 4 h
- 0.5 μL of reaction mix/ lane
- stained with Oriole (Bio-Rad) and analyzed with an image analyzer (LAS)

✓ Good expression for many proteins, small to large.
✓ Good purity with simple purification.
✓ Good productivity, ~g/L.

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-KSF; AT rich codon on N-term-

Fab Heavy Chain (Herceptin)

Herceptin Fab HC

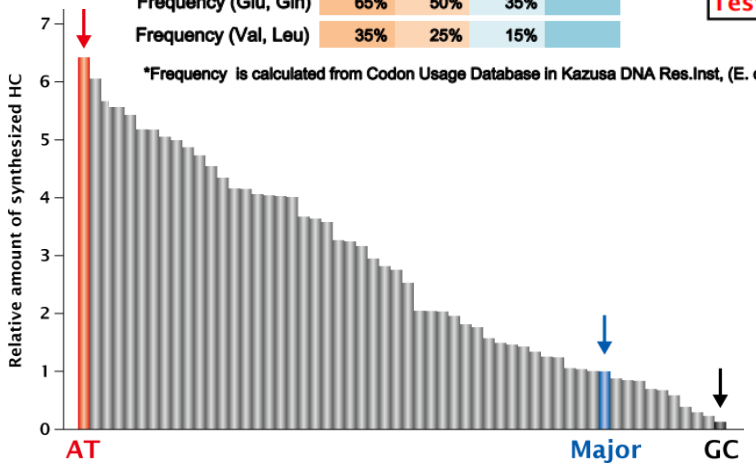
Met- Glu-Val-Gln-Leu-Val- FLAG

2		3		4		5		6	
Glu		Val		Gln		Leu		Val	
codon	freq (%)	codon	freq (%)	codon	freq (%)	codon	freq (%)	codon	freq (%)
gaa	70	gtt	25	caa	30	ttg	15	gtt	25
gag	30	gtc	18	cag	70	tta	12	gtc	18
		gta	17			ctt	12	gta	17
		gtg	40			ctc	10	gtg	40
						cta	5		
						ctg	46		

All clones; 384
Tested clones; 56

Frequency (Glu, Gln)	65%	50%	35%
Frequency (Val, Leu)	35%	25%	15%

*Frequency is calculated from Codon Usage Database in Kazusa DNA Res.Inst, (E. coli K-12 strain)

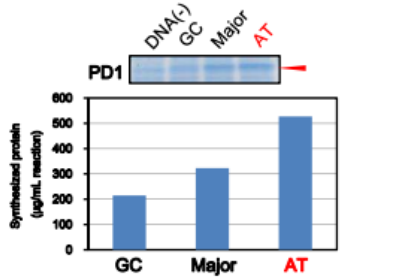


Design of DNA template is important.
Manual is Free to download from our Web site here.

PD1

Organism	*Homo sapiens*
Synthesized region	36Thr-150Glu-(His*8)
Length	124 a.a.
Molecular weight	14,148 Da

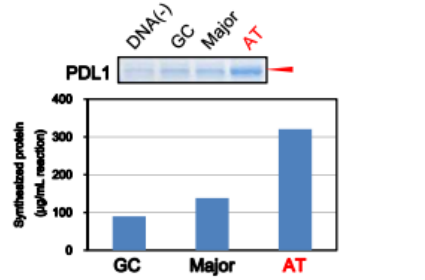
N-term type	1	2(36)	3(37)	4(38)	5(39)	6(40)	GC(%)
	Met	Thr	Phe	Ser	Pro	Ala	1-6 a.a.
GC	atg	acc	ttc	tcc	ccg	gcg	67%
Major	atg	acc	ttt	tct	ccg	gcg	56%
AT	atg	act	ttt	tca	cca	gct	39%



PDL1

Organism	*Homo sapiens*
Synthesized region	18Ala-239Thr-(His*8)
Length	231 a.a.
Molecular weight	26,593 Da

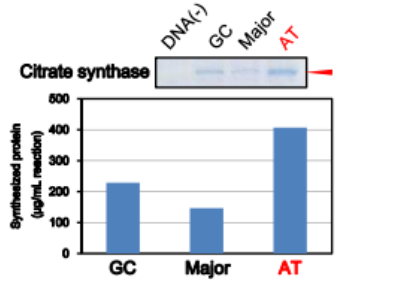
N-term type	1	2(18)	3(19)	4(20)	5(21)	6(22)	GC(%)
	Met	Ala	Phe	Thr	Val	Thr	1-6 a.a.
GC	atg	gcg	ttc	acc	gtg	acc	61%
major	atg	gcg	ttt	acc	gtg	acc	56%
AT	atg	gct	ttt	act	gta	aca	33%



Citrate Synthase

Organism	*Saccharomyces cerevisiae*
Synthesized region	38Ser-479Asn
Length	443 a.a.
Molecular weight	49,346 Da

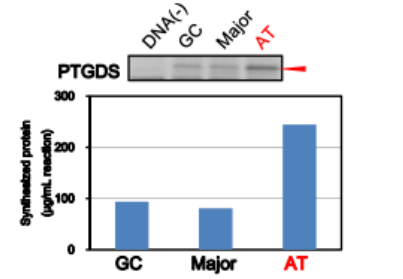
N-term type	1	2(38)	3(39)	4(40)	5(41)	6(42)	GC(%)
	Met	Ser	Ser	Ala	Ser	Glu	1-6 a.a.
GC	atg	tcc	tcc	gcg	tcc	gag	67%
major	atg	tct	tct	gcg	tct	gaa	44%
AT	atg	tca	tca	gct	tca	gaa	39%



PTGDS

Organism	*Homo sapiens*
Synthesized region	23Ala-190Gln
Length	169 a.a.
Molecular weight	18,829 Da

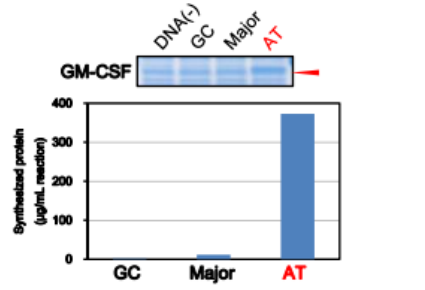
N-term type	1	2(23)	3(24)	4(25)	5(26)	6(27)	GC(%)
	Met	Ala	Pro	Glu	Ala	Gln	1-6 a.a.
GC	atg	gca	ccg	gaa	gca	cag	61%
major	atg	gcg	ccg	gaa	gcg	cag	72%
AT	atg	gca	cct	gaa	gct	caa	50%



GM-CSF

Organism	*Homo sapiens*
Synthesized region	18Ala-144Glu
Length	128 a.a.
Molecular weight	14,608 Da

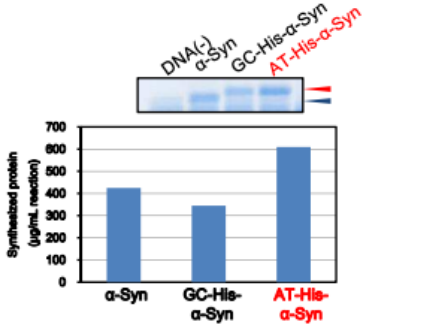
N-term type	1	2(18)	3(19)	4(20)	5(21)	6(22)	GC(%)
	Met	Ala	Pro	Ala	Arg	Ser	1-6 a.a.
GC	atg	gcg	ccg	gcg	cgc	tcc	83%
major	atg	gcg	ccg	gcg	cgc	tct	78%
AT	atg	gca	cct	gct	aga	tca	50%



His-α-Synuclein

Organism	*Homo sapiens*
Synthesized region	(Hisx6)-(Gly-Ser)-2(10)Asp-140(148)Ala
Length	148 a.a.
Molecular weight	15,427 Da

Tag type	1	2	3	4	5	6	7	8	9	GC(%)
	Met	His	His	His	His	His	His	Gly	Ser	1-9 a.a.
GC	atg	cac	cac	cac	cac	cac	cac	ggt	tct	59%
AT	atg	cat	cat	cat	cat	cat	cat	ggt	tct	37%



-KSF; AT rich codon on N-term-

Fab Heavy Chain (Herceptin)

Herceptin Fab HC

Met- Glu-Val-Gln-Leu-Val- FLAG

2		3		4		5		6	
Glu		Val		Gln		Leu		Val	
codon	freq (%)	codon	freq (%)	codon	freq (%)	codon	freq (%)	codon	freq (%)
gaa	70	gtt	25	caa	30	ttg	15	gtt	25
gag	30	gtc	18	cag	70	tta	12	gtc	18
		gta	17			ctt	12	gta	17
		gtg	40			ctc	10	gtg	40
						cta	5		
						ctg	46		

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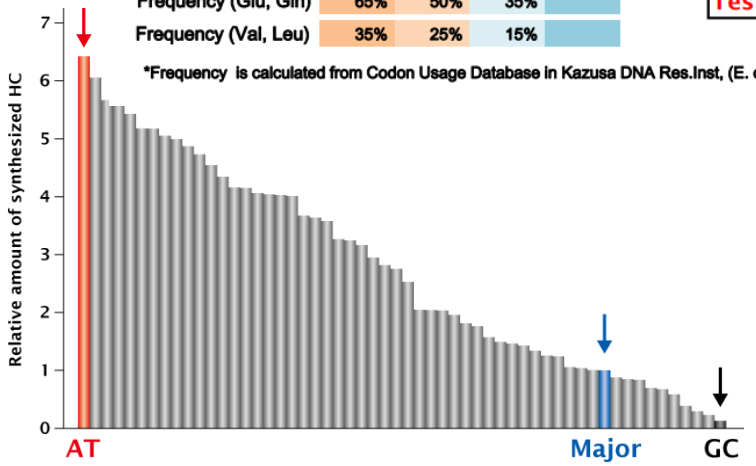

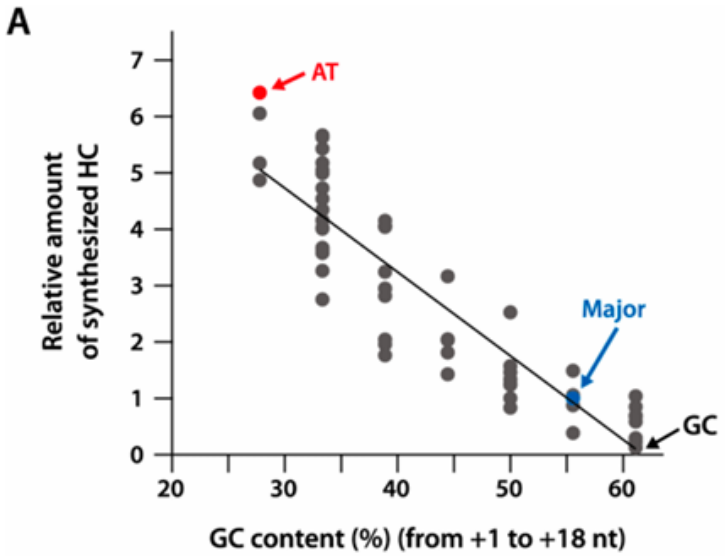

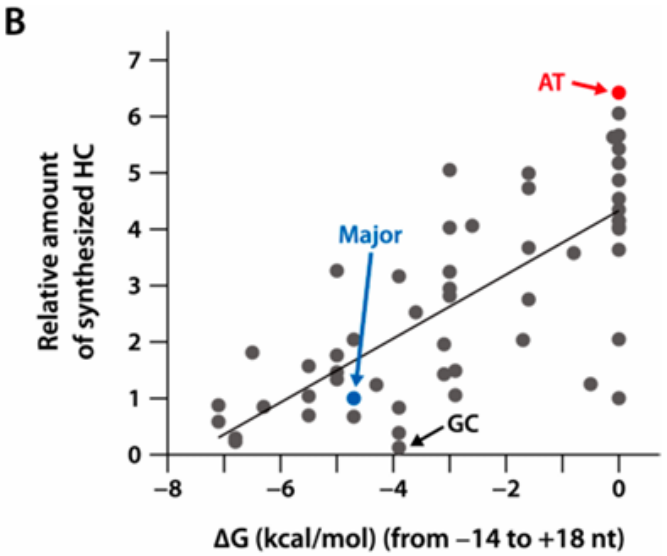

C

	1	2	3	4	5	6		
Name	Met	Glu	Val	Gln	Leu	Val	GC (%)	ΔG (kcal/mol)
AT	atg	gaa	gta	caa	tta	gtt	28	0.0
Major	atg	gaa	gtg	cag	ctg	gtg	56	−4.7
GC	atg	gag	gtg	cag	ctg	gtc	61	−3.9

Murakami et al. (2024) Int. J. Mol. Sci. 2024, 25(10), 5264

Design of DNA template is important.
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For more information

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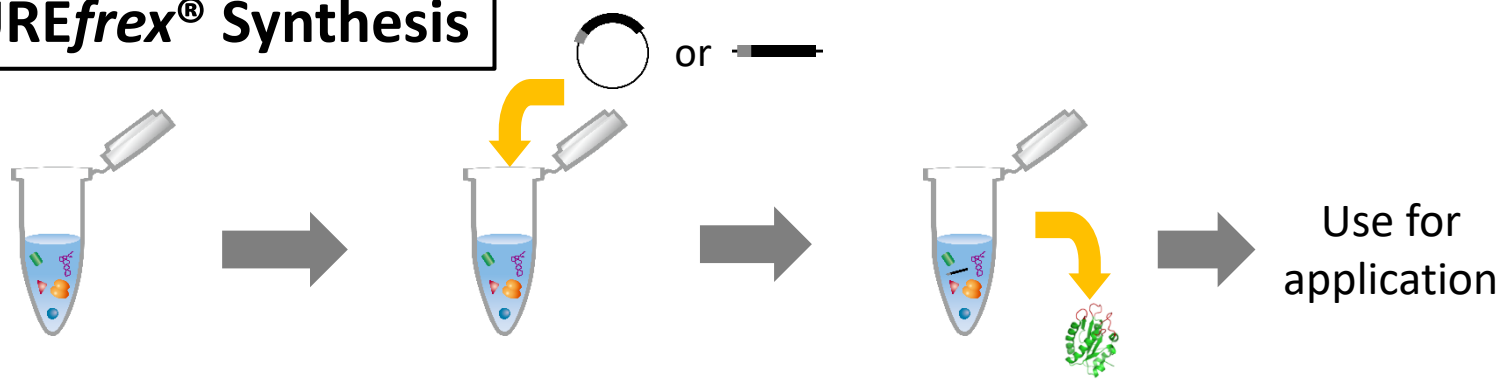
-Improve Expression from Days to Hours-

PURE*frex*® Synthesis

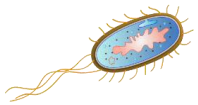


Mix solution I, II and III → Addition of template DNA → Incubation for 2-4 hours (Purification, if necessary) → Use for application

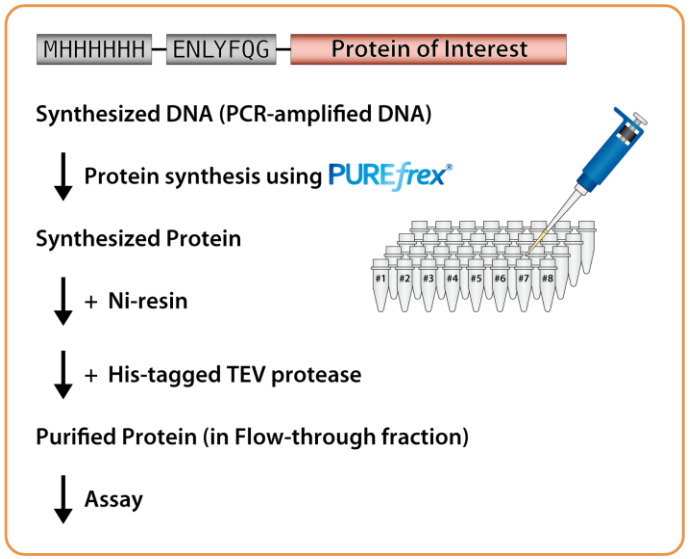
Total: 2-4 hours

E. coli Expression

Transformation and overnight cultivation → Induction and expression → Purification → Use for application



Total: 3-4 days



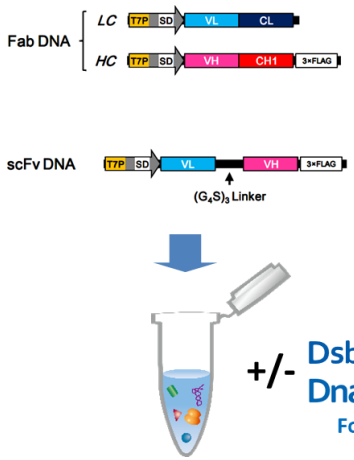
Reconstituted cell-free protein synthesis kit

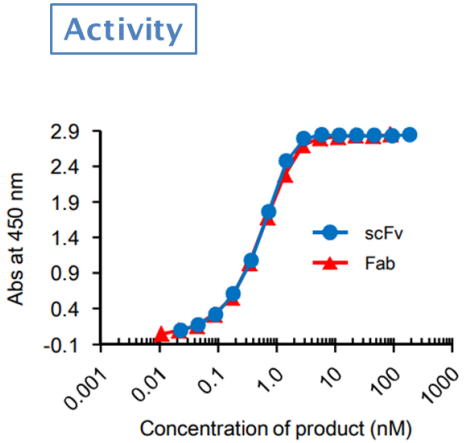
PURE*frex*®

For more information

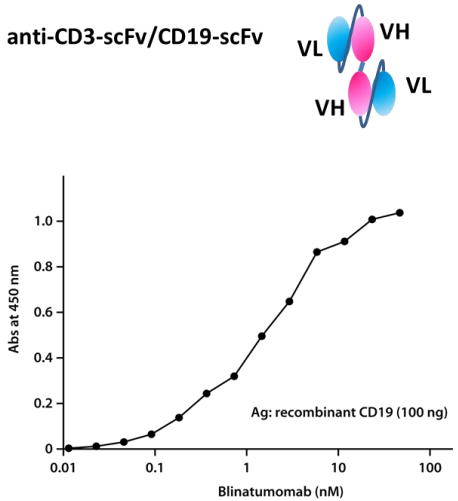
GeneFrontier

-Expression of scFv, Fab and more-



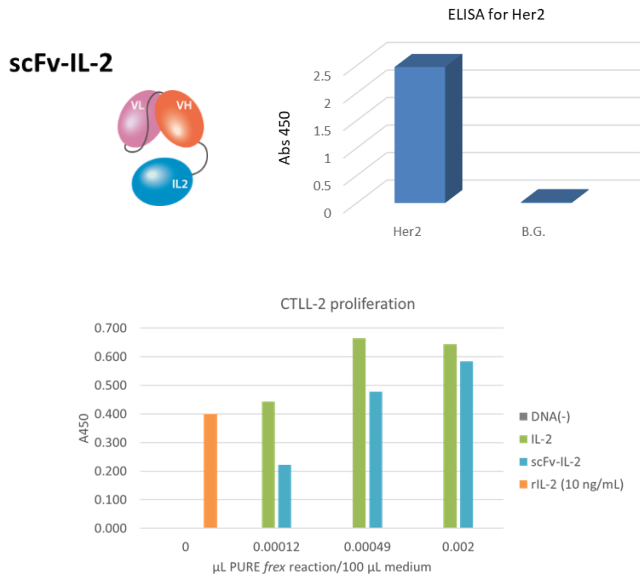


Murakami et al. (2019) Sci. Rep. vol.9, p.671. (Supplementary Information)

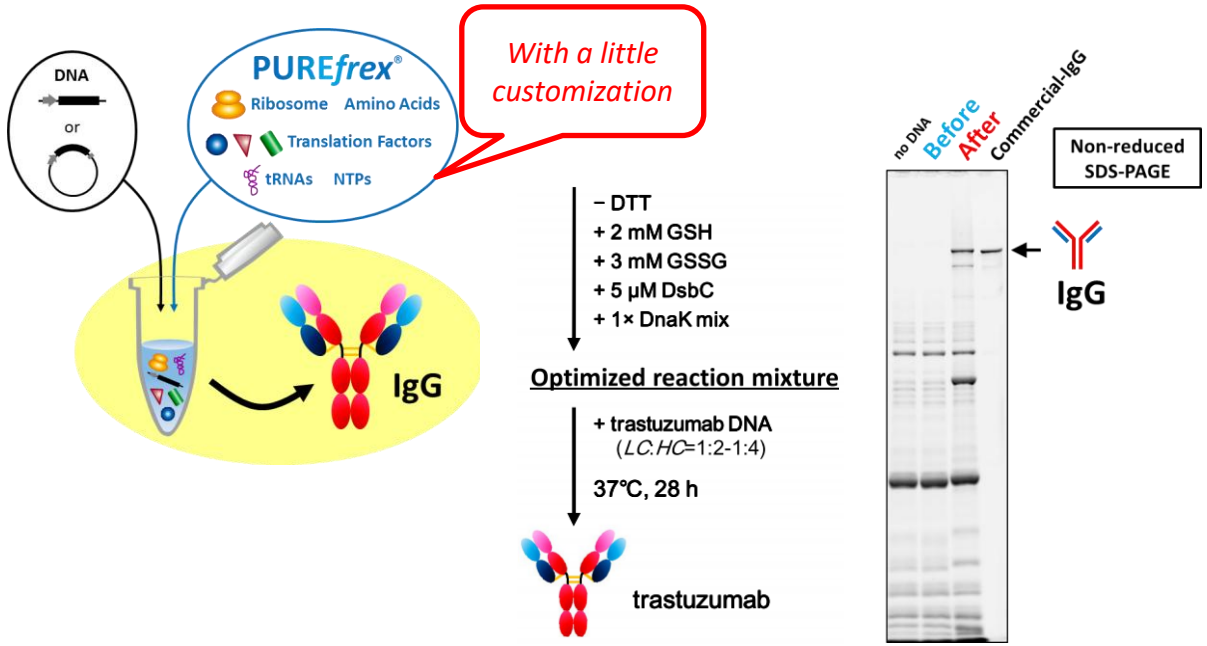


	1	2	3	4	5	6	7	8	9	10
	Proinsulin Aspart	Proinsulin Lispro	Proinsulin Glargine	Regular Proinsulin	Insulin A Chain	Insulin B Chain	Insulin A Chain Heterodimer	Insulin B Chain Heterodimer	Oxytocin	Glucagon
PURE	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Clm24	X	X	X	X	✓	X	✓	✓	✓	✓
BL21	X	X	X	X	✓	X	✓	✓	✓	X
759	✓	✓	✓	✓	✓	✓	✓	✓	✓	X
	11	12	13	14	15	16	17	18	19	20
	Glucagon Like Peptide 1 mutant (GLP-1 mut)	Glucagon Like Peptide 1 (GLP-1)	Insulin Like Growth Factor	Growth Hormone (GH)	Leptin	Vaso-pressin	Angiotensin II	Parathyroid Hormone (PTH)	Somato-statin	Leuprolide
PURE	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Clm24	✓	✓	X	✓	✓	✓	✓	✓	✓	✓
BL21	X	X	X	X	X	X	✓	✓	✓	✓
759	✓	✓	X	✓	✓	✓	✓	X	✓	✓

DeWinter et al. (2023) ACS Synth. Biol. vol.12, 4, p1216. (Supplementary Information)

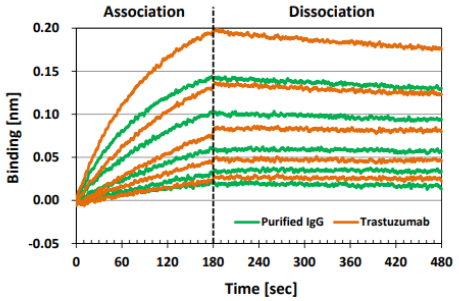


-Application for IgG-



Only 2 days for IgG!!

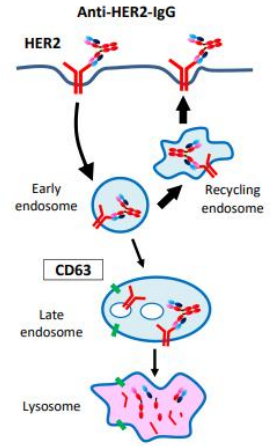
Binding kinetics analysis



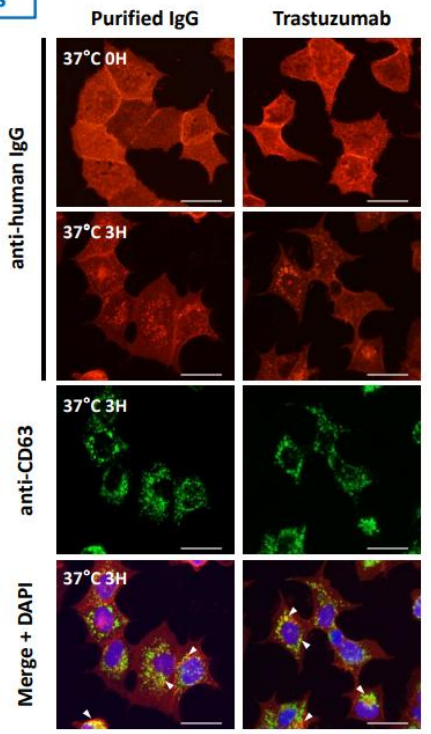
	K_D [M]	k_{on} [1/Ms]	k_{off} [1/s]
Purified IgG	4.24E-10	6.38E+05	2.70E-04
Trastuzumab	4.03E-10	6.29E+05	2.53E-04

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



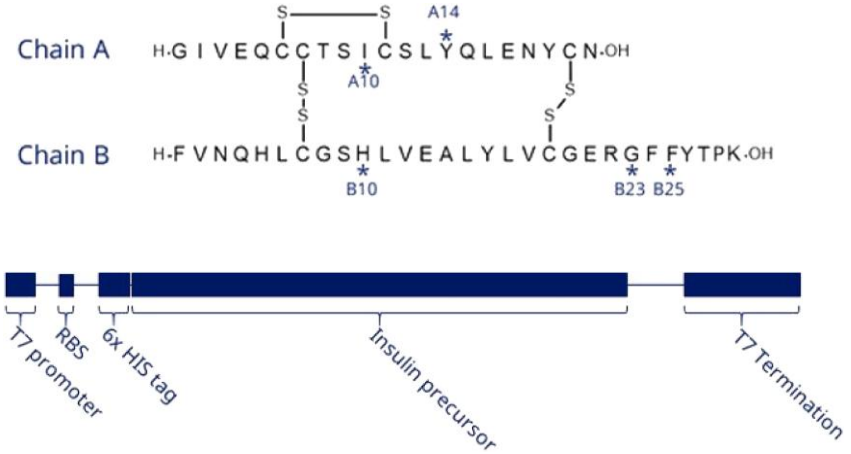
BT-474 cells were surface-labeled at 4°C for 60 min with the binding medium (DMEM, 3% BSA, 20 mM HEPES (pH 7.4)) containing 10 nM of "purified IgG" or "Trastuzumab". Cells were washed five times with the binding medium and incubated at 37°C for 3h. Cells were then fixed and processed for dual-label indirect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

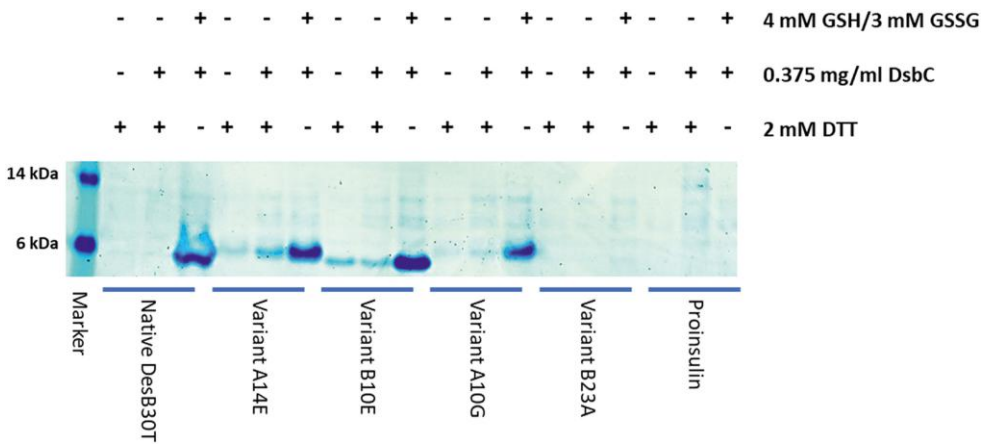


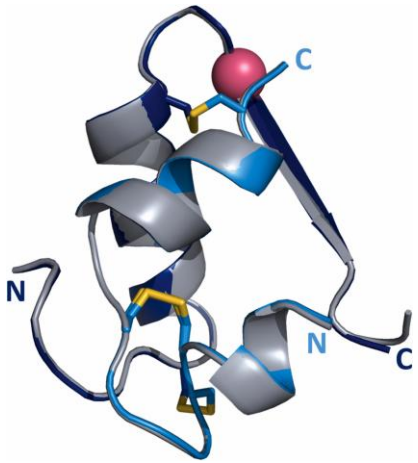
Murakami et al. (2019) Sci. Rep. vol.9, p.671.
Poster_PSSJ 2017

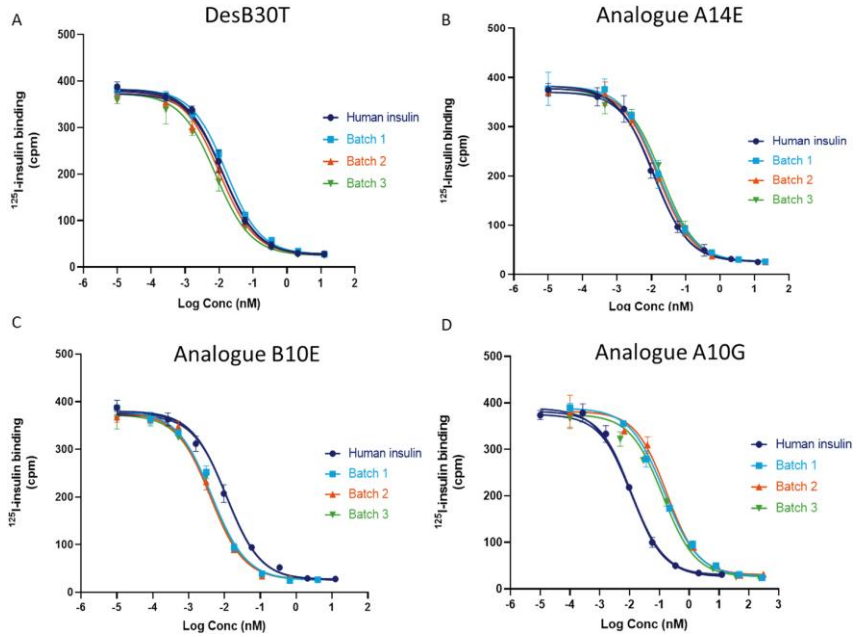
✓ **Full size IgG can be synthesized.**

-Application for complex molecule-









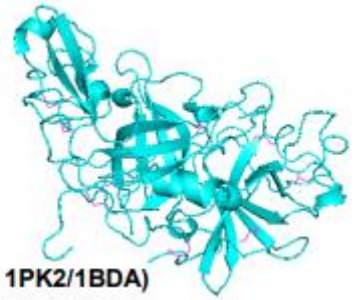
Jensen et al. (2021) Protein Expr. Purif., 186, 105910.

✓ **Functional insulin analogues can be synthesized.**

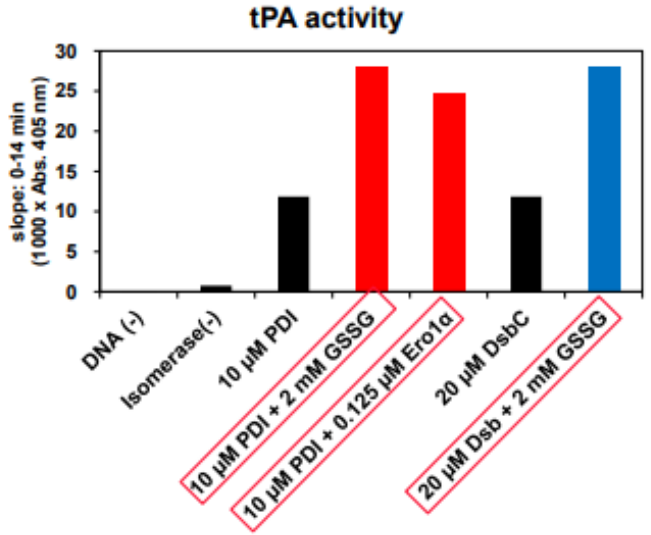
-Application for complex molecule-

truncated version of tissue plasminogen activator (tPA)

Organism	*Homo sapiens*
Synthesized region	36Ser-40Ile/211Gly-562Pro (+FLAG)
Length	368 a.a.
Molecular weight	41,072 Da
No. of disulfide bonds	9

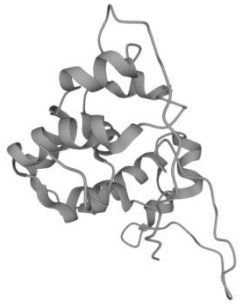


(PDB ID: 1PK2/1BDA)

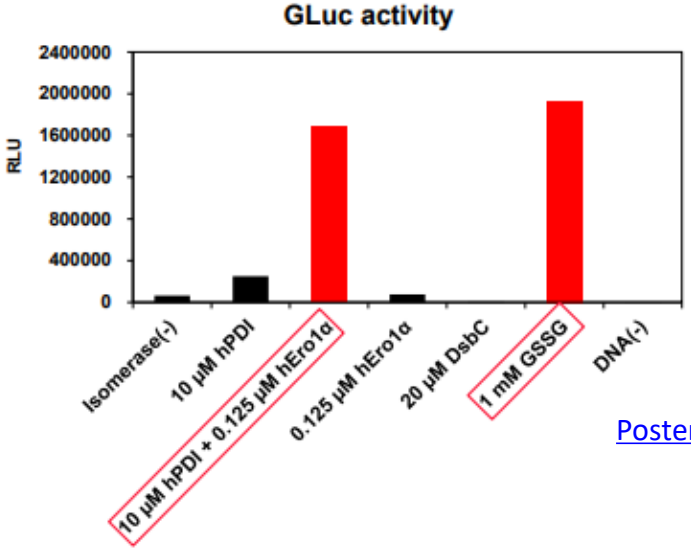


Gaussia Luciferase (GLuc)

Organism	*Gaussia princeps*
Synthesized region	18Lys-185Asp (+FLAG-His6)
Length	187 a.a.
Molecular weight	20,407 Da
No. of disulfide bonds	5



Luciferase - Gaussia princeps (uniprot.org)



Poster_PSSJ 2018

Reconstituted cell-free protein synthesis kit

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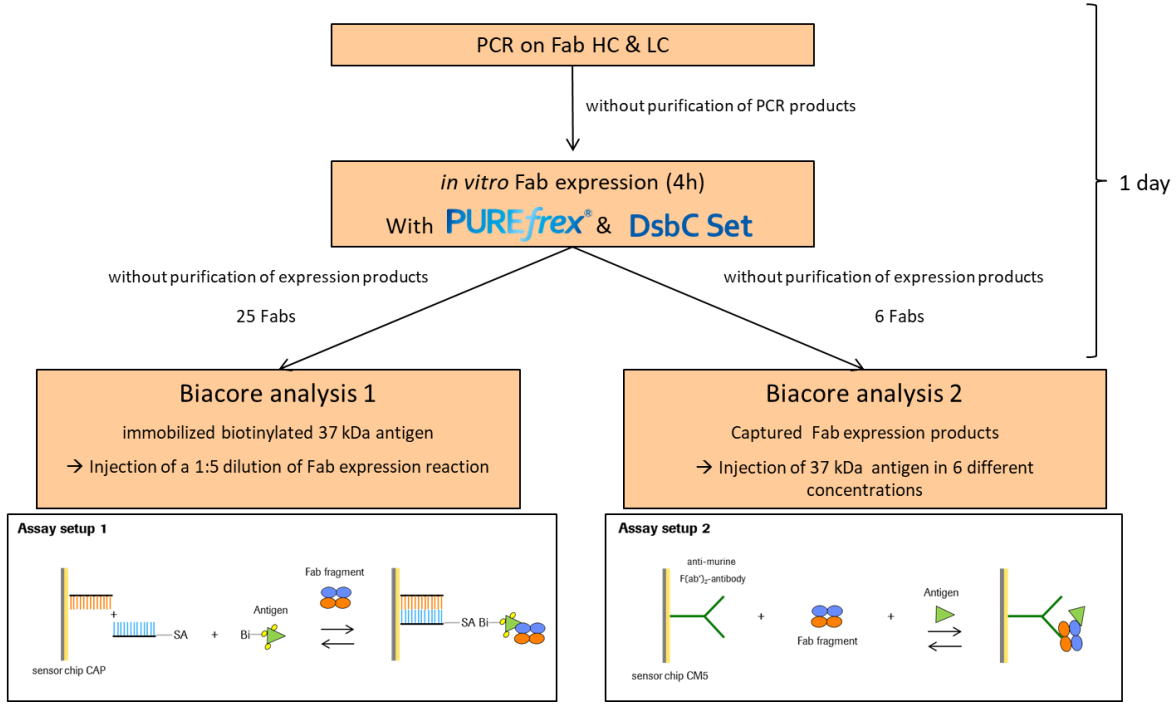
-For HTS of mAb-

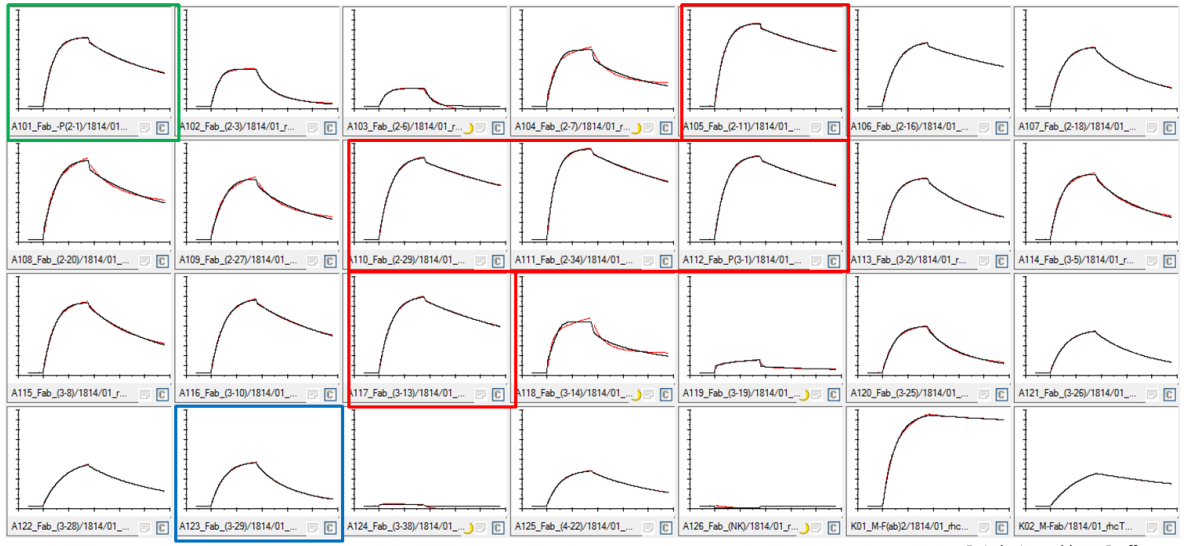
For more information

GeneFrontier

In vitro expression and Biacore analysis of Fab fragments



Kinetic analysis of 25 Fab binders

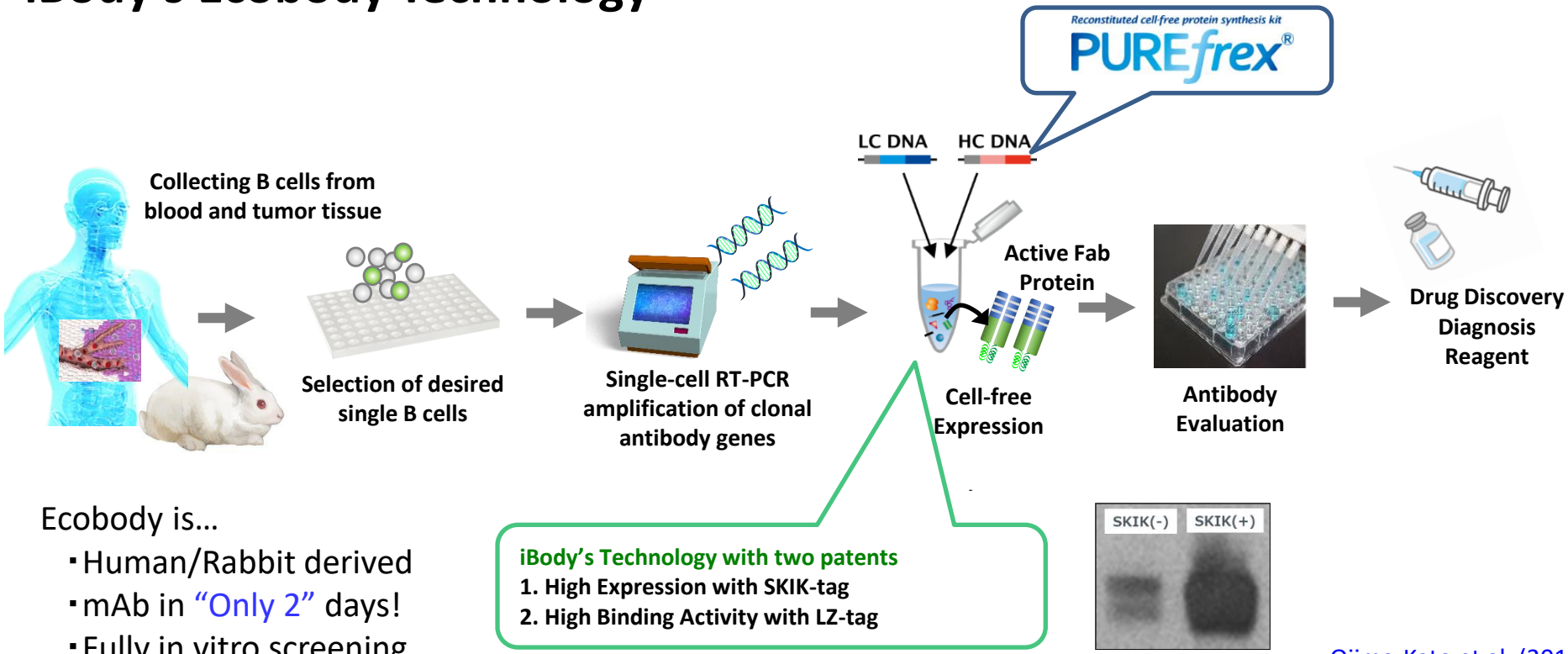


Data by Leopold von Proff

→ Selection of Fabs for further kinetic analysis

✓ **Active Fabs are expressed/screened in HT manner.**

iBody's Ecobody Technology



Ecobody is…

- Human/Rabbit derived
- mAb in "Only 2" days!
- Fully in vitro screening
- No culture

Ojima-Kato et al. (2017) Sci. Rep., 7, 13979.

https://www.ibody.co.jp/en/

✓ **Active Fab is expressed/screened in HT manner.**

Reconstituted cell-free protein synthesis kit

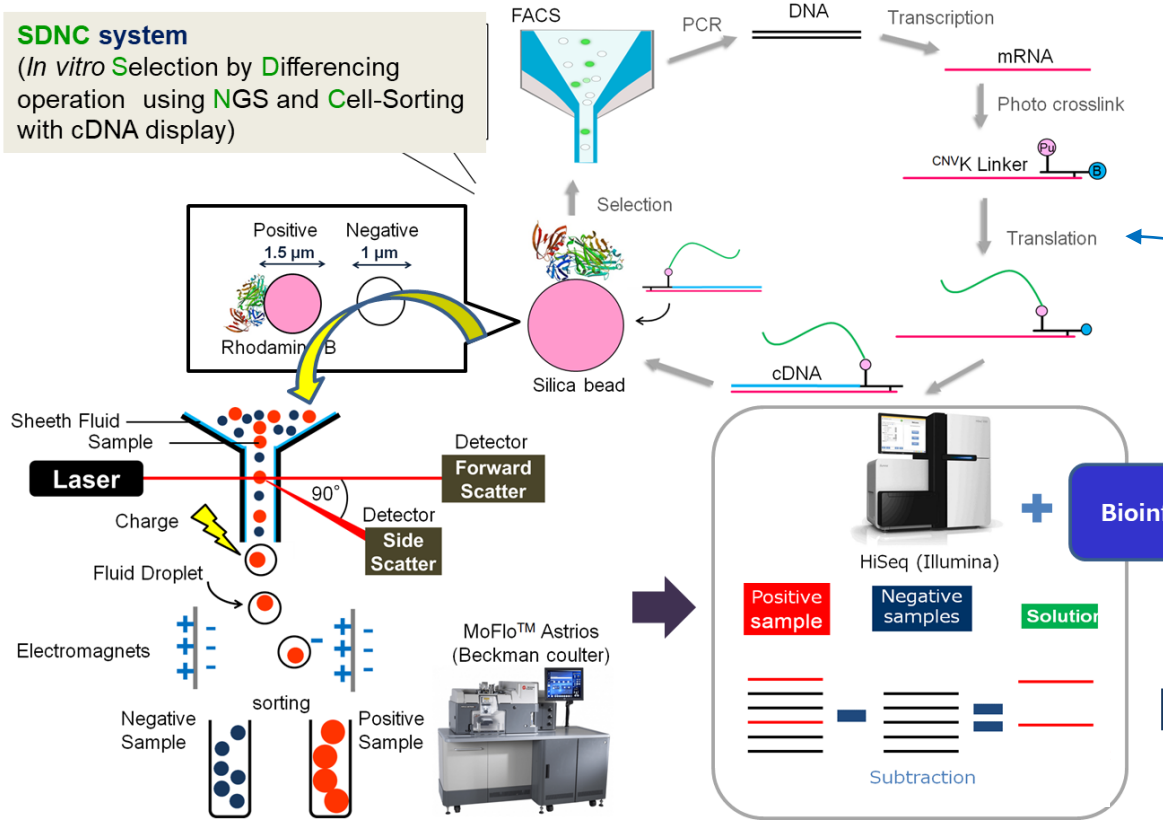
PURE*frex*® -For display technology-

For more information

GeneFrontier

Epsilon Molecular Engineering
Molecular Design for Human Life

SDNC system
(*In vitro* Selection by Differencing operation using NGS and Cell-Sorting with cDNA display)



SDNC system is…
- for VHH/cyclic peptide
- with High diversity
- Fully in vitro screening

Reconstituted cell-free protein synthesis kit PURE*frex*®

Evaluation
- ELISA
- SPR(BIAcore)
- BLI(Octet)
- Cell based assay

https://www.epsilon-mol.co.jp/eng/

✓ PURE*frex* is applied for cDNA display based screening.

Reconstituted cell-free protein synthesis kit

PURE*frex*® -For display & HTS-

For more information

GeneFrontier

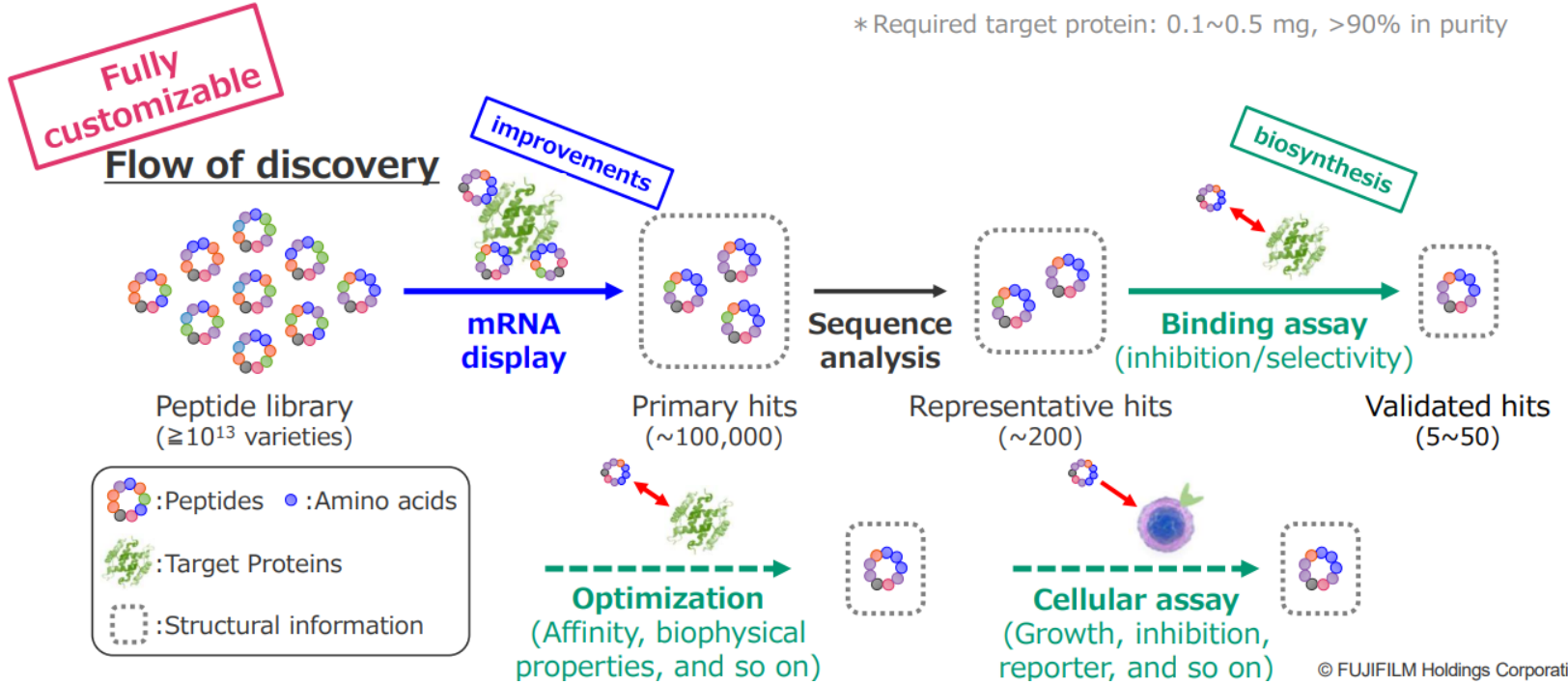
FUJIFILM peptide discovery services collaborated with PURE*frex*®

- ✓ **Innovative improvements** in **mRNA display** enable screening from >10^{13} peptides
- ✓ **Practical biosynthesis & assays** enable rapid selection and activity explorations.

→ Peptides hits with **wide varieties** and **high-affinities** can be obtained.

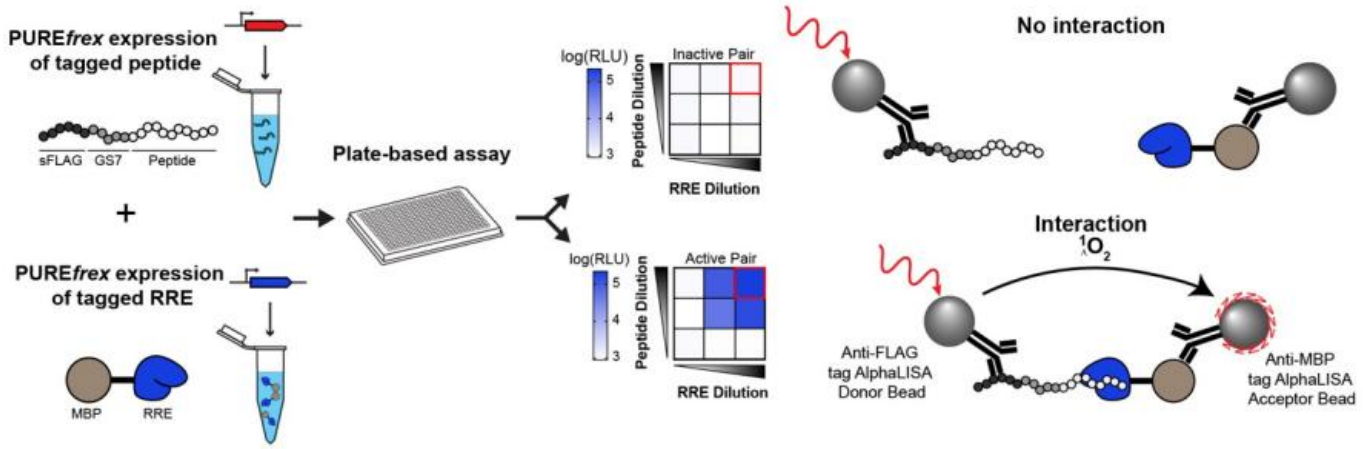
We provide a CRO service, in which we receive target ( ) from the customer* and return the structural information of the acquired peptides ( ).

＊Required target protein: 0.1~0.5 mg, >90% in purity

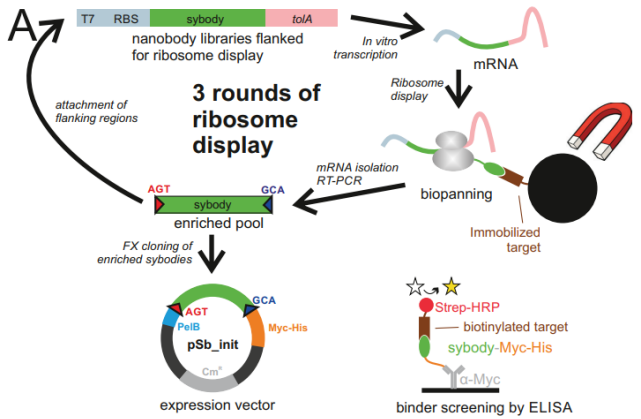


https://labchem-wako.fujifilm.com/europe/category/95372.html

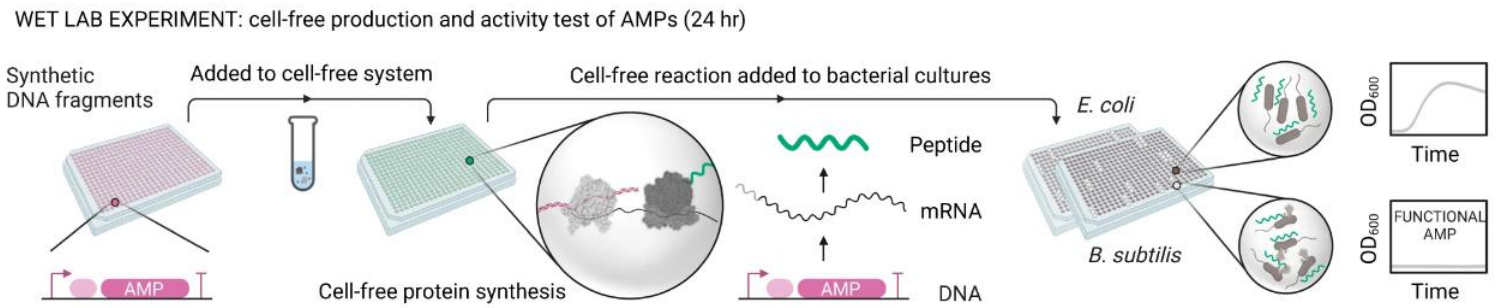
-Broad applications, yet to come!-



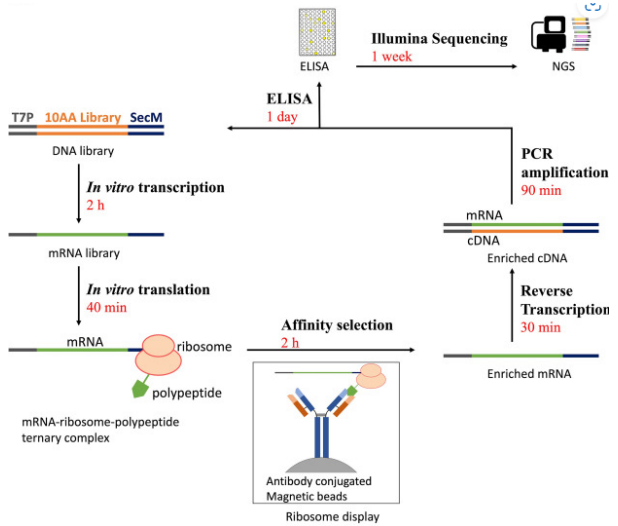
Wong et al. (2024) bioRxiv https://doi.org/10.1101/2024.03.25.586624.



Zimmermann I. et al. (2018) eLife, 7, e34317.



Pandi et al. (2023) Nat Communications. vol.14(7197).



Jia B. et al. (2024) J Biosci Bioeng, 137(4):321-328.

Reconstituted cell-free protein synthesis kit

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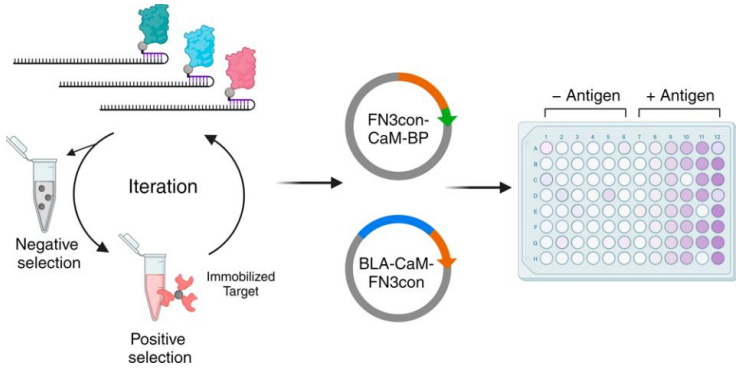
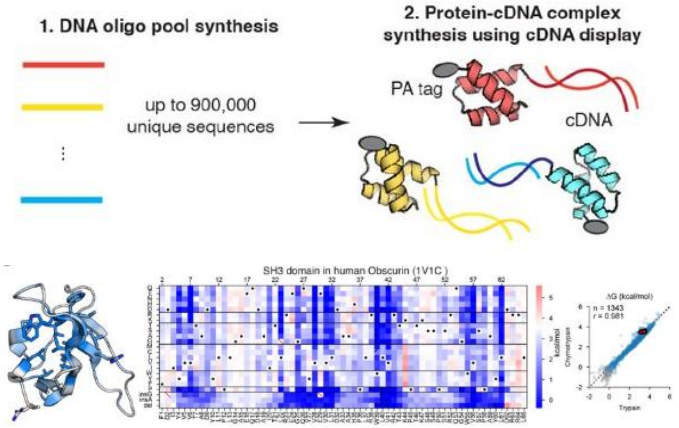
For more information

GeneFrontier

-Broad applications, yet to come!-



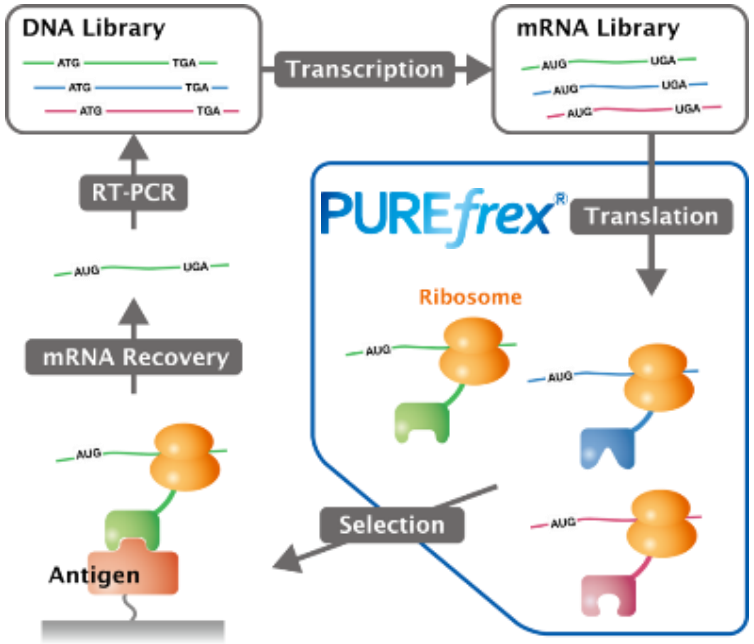
Chui Z. et al. (2024) ACS Sens, 9(6):2846-2857.



Tsuboyama et al. (2023) Nature, 620, p434.

in vitro protein selection technology

PURE*frex*® RD



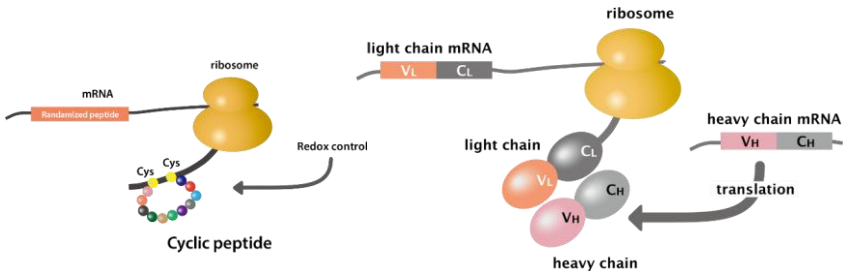
Licensed technology under JP4931135 etc.

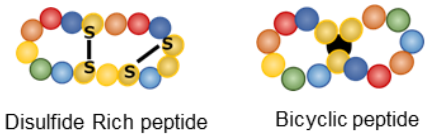
◆ **Advanced screening system for Biologics**

- **mAb (scFv / Fab)**
- **VHH**
- **Cyclic peptide**

◆ **High Selection Efficiency**

- **Completely molecular based system**
- **>10^{12} diversity**

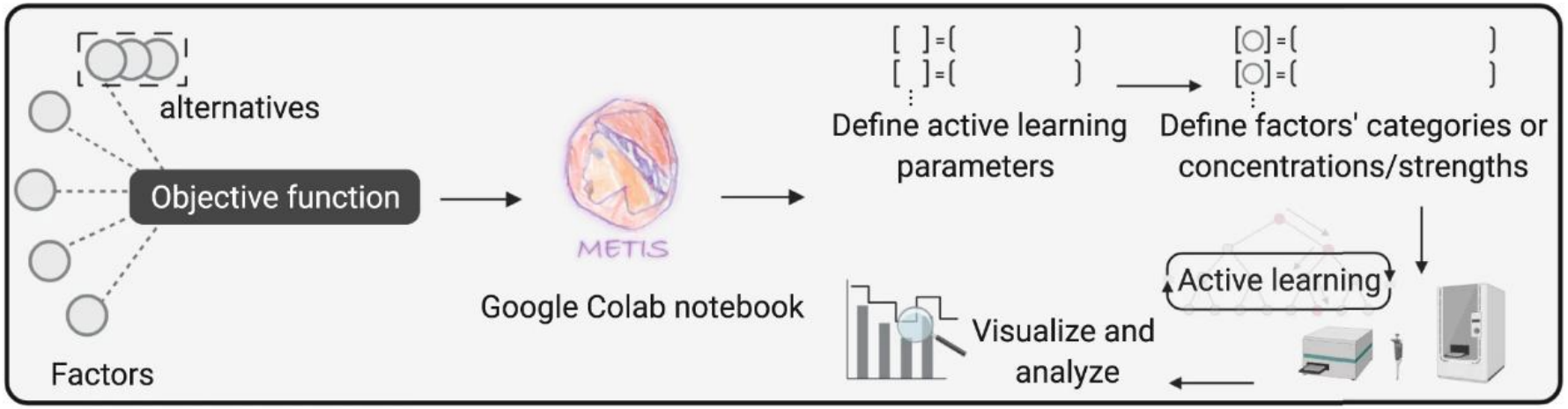




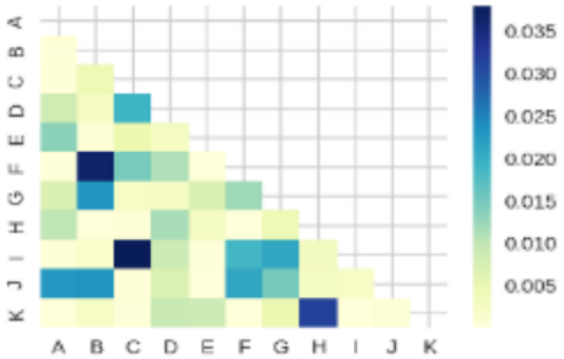
Licensed to

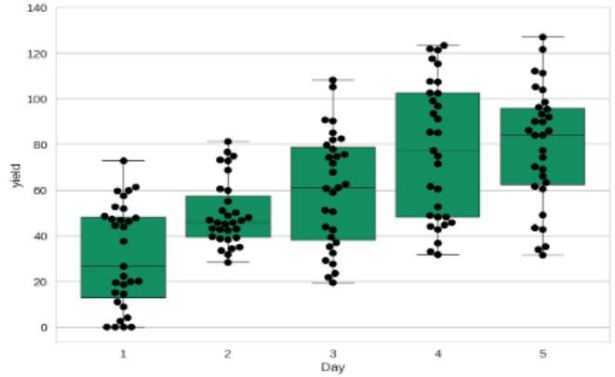
SUTRO BIOPHARMA

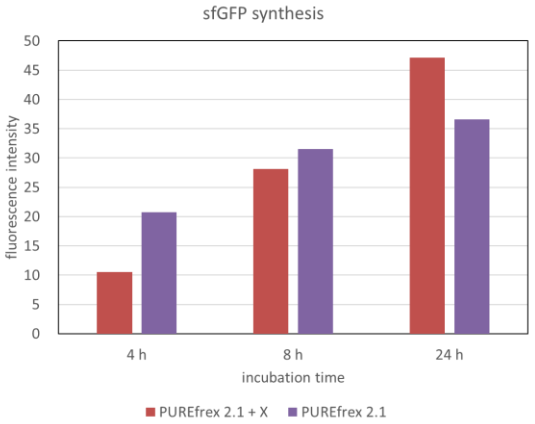
-Broad applications, yet to come!-



Pandi A et al. (2022) Nature Communications, 13, 3876.







- ✓ **Perfect fit to AI/ML approach with great controllability & reproducibility.**
- ✓ **Unique expression platform will give you great advantage in R&D.**

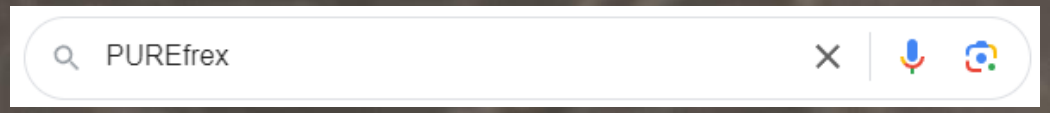
Contact information

Reconstituted cell-free protein synthesis kit

PURE*frex*®

For reagent use for expression / screening of biologics

https://purefrex.genefrontier.com/



in vitro protein selection technology

PURE*frex*® RD

For screening service / collaboration / technology transfer for generation of new biologics

Takashi Ebihara, Ph.D., COO, GeneFrontier

E-mail: ebihara@genefrontier.com