For more information

Rebuilt Cell-Free System (PUREfrex®);

From basic research to industrial application

Reconstituted cell-free protein synthesis kit

PURE*frex*®

Takashi Ebihara, Ph.D.
COO
GeneFrontier Corporation

GeneFrontier

SEED 2023
30 May - 2 June 2023

Reconstituted cell-free protein synthesis kit

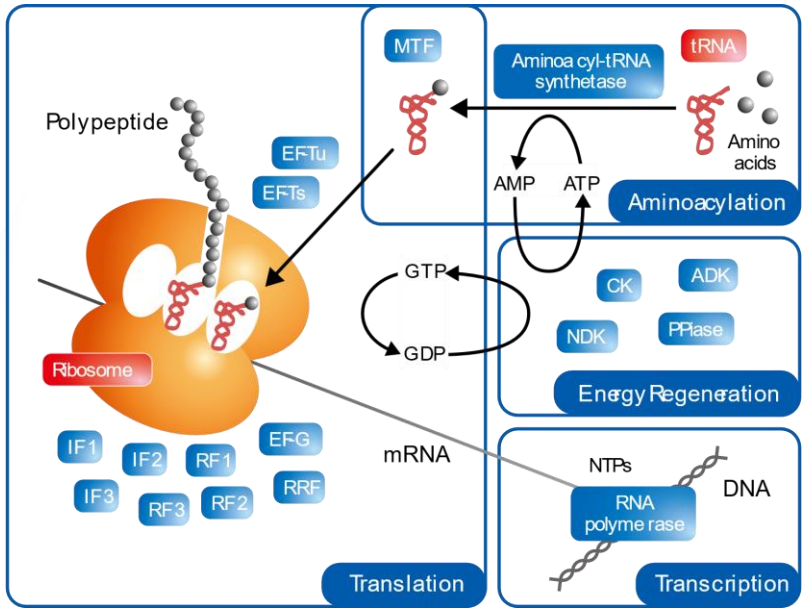
PURE*frex*®

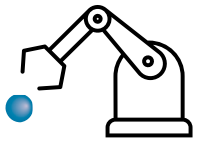
-Customize expression toolbox for your research-

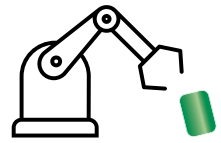
For more information

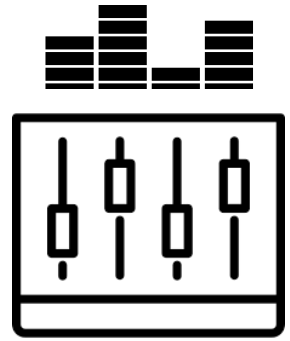
GeneFrontier

Totally constructive, molecular based system

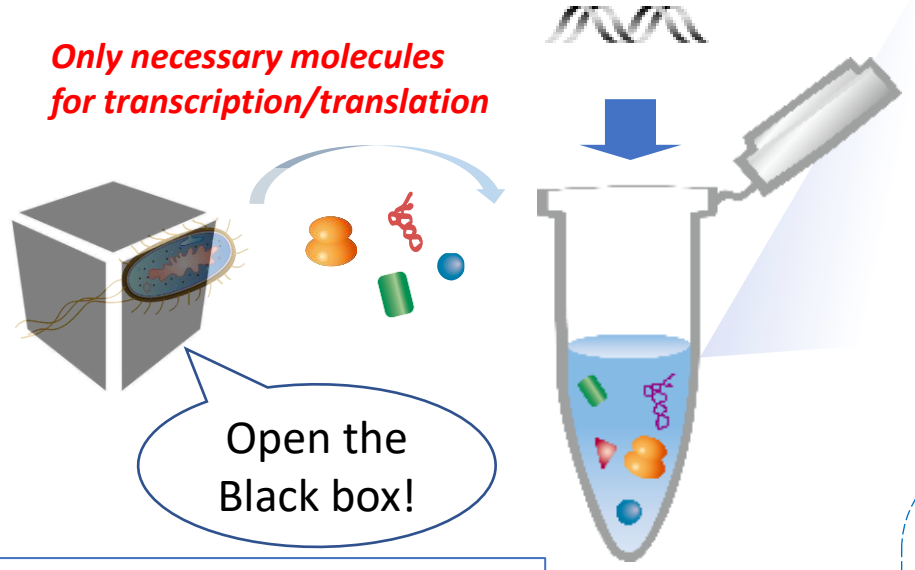








Only necessary molecules for transcription/translation



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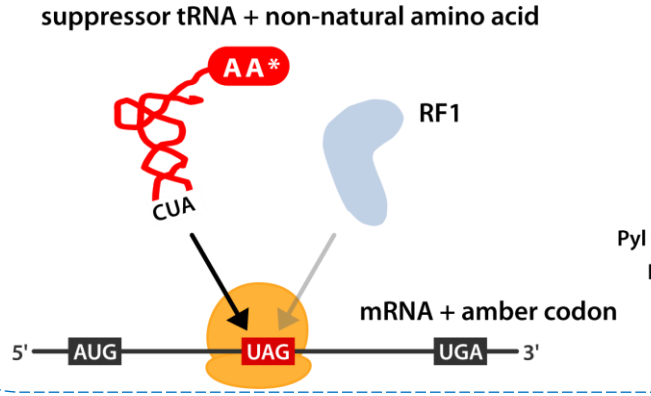
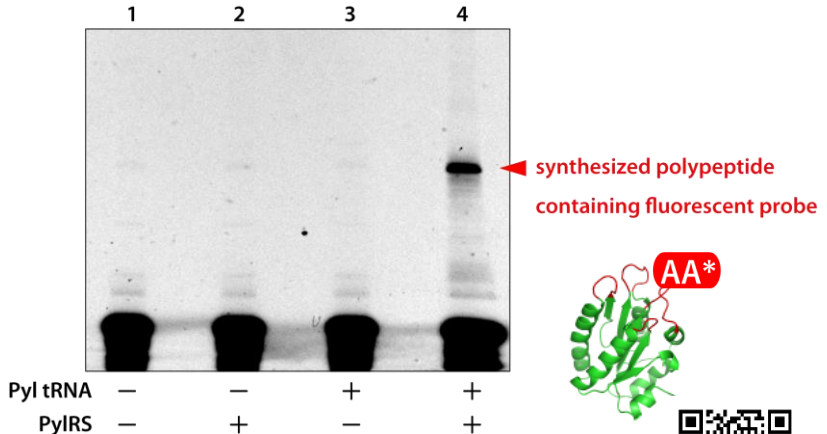
- ✓ Very low contaminants

<Ex, Non-natural AA introduction>

Translation - RF1





Ready-made kit is coming soon!

Reconstituted cell-free protein synthesis kit

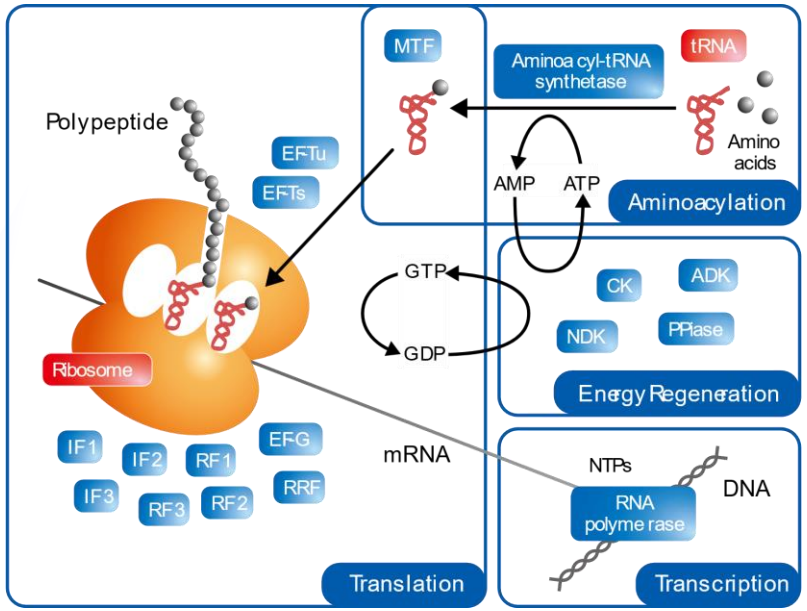
PURE*frex*®

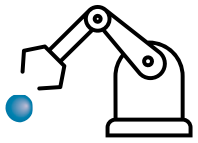
-Customize expression toolbox for your research-

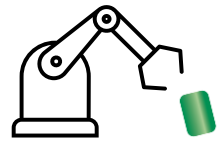
For more information

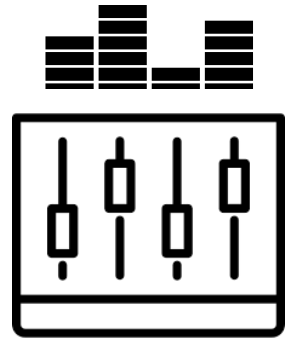
GeneFrontier

Totally constructive, molecular based system

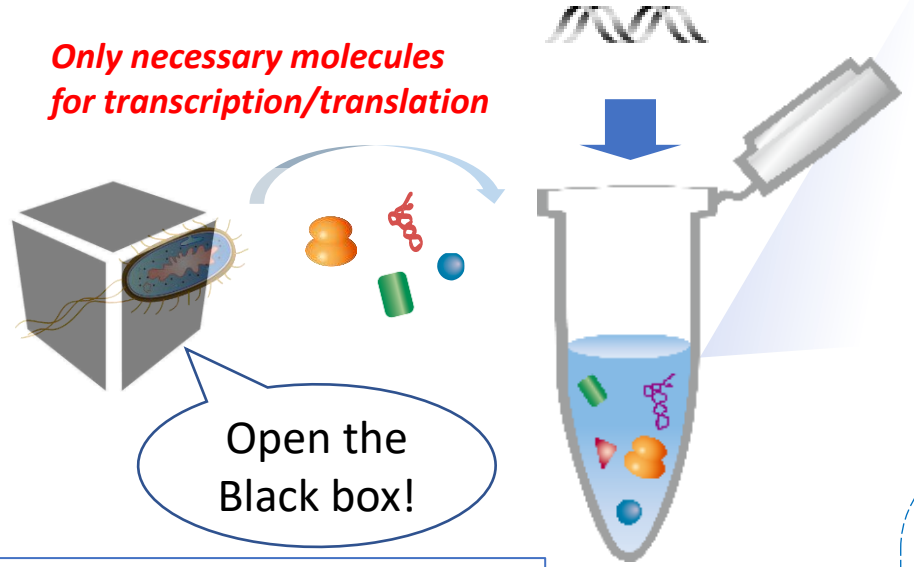








Only necessary molecules for transcription/translation



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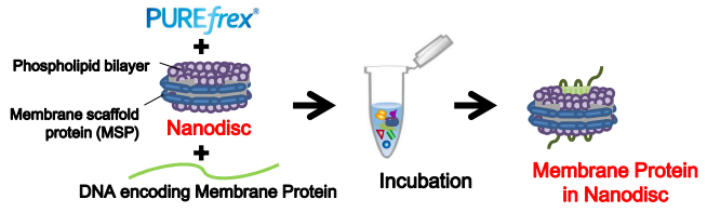
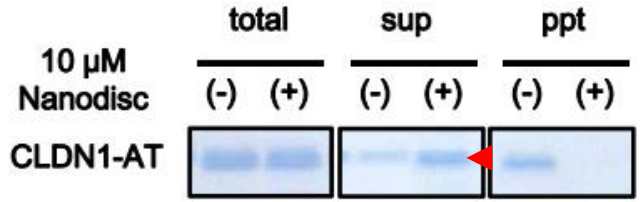
<Ex, Membrane protein with Nanodisc; artificial membrane-like structure>





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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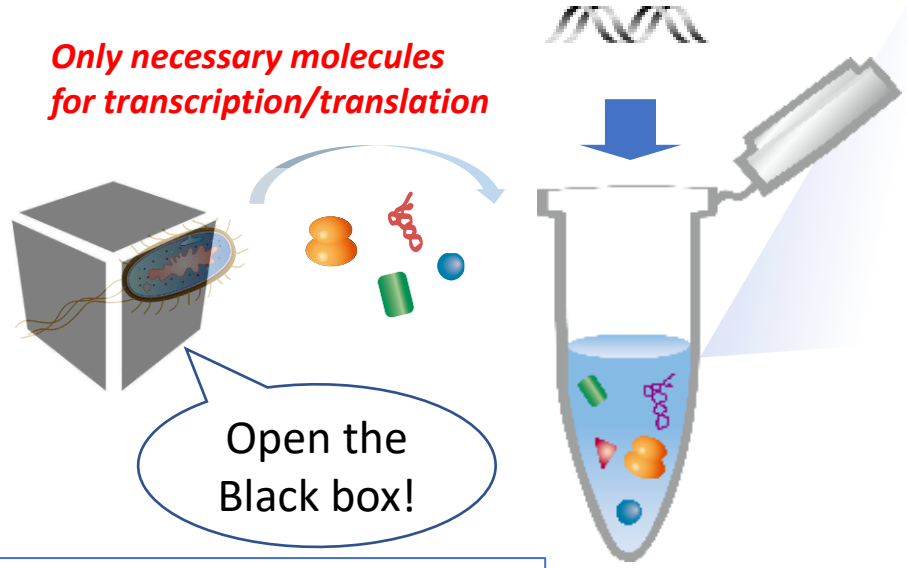

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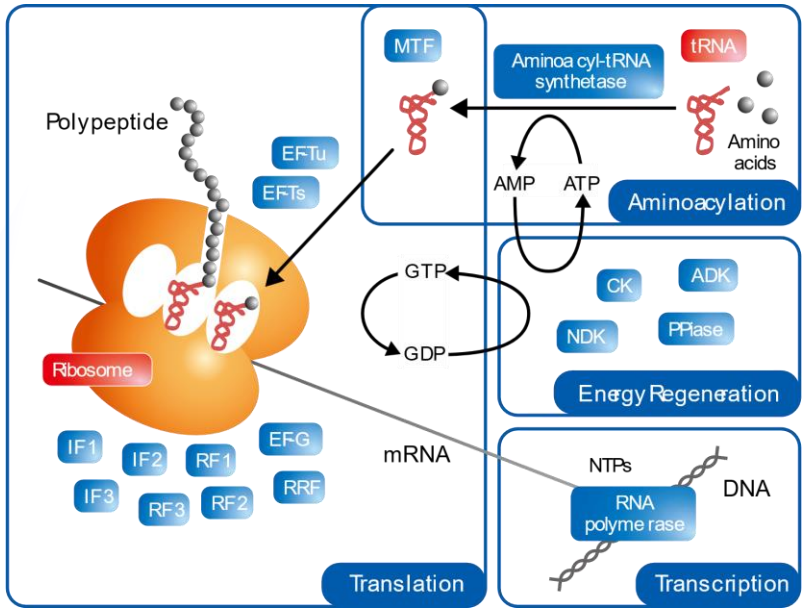
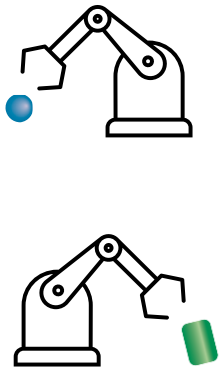
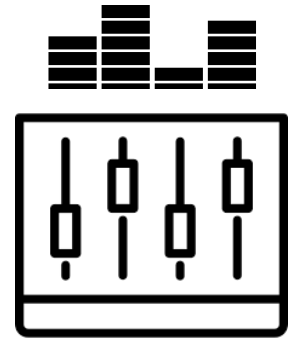






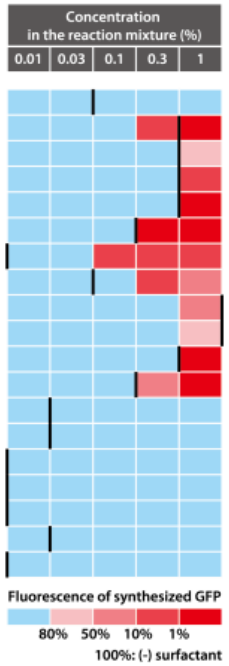
Experimental conditions for protein synthesis

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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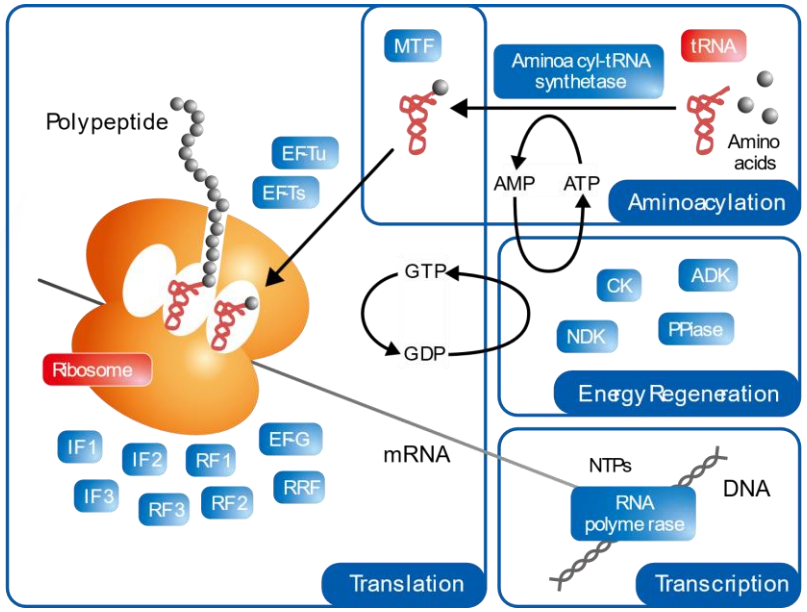

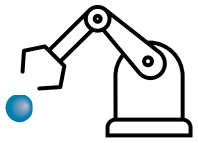

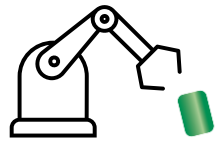

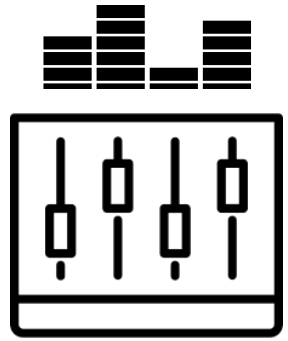

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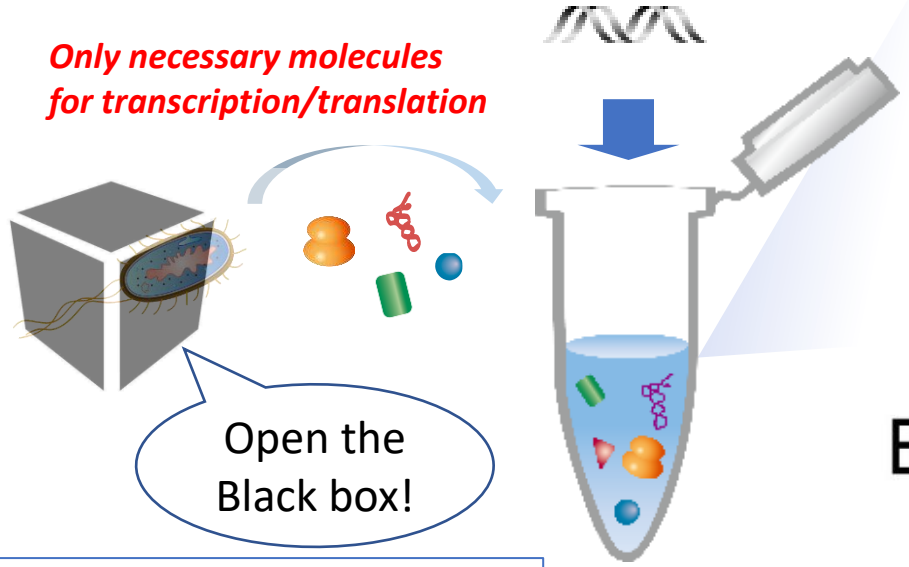

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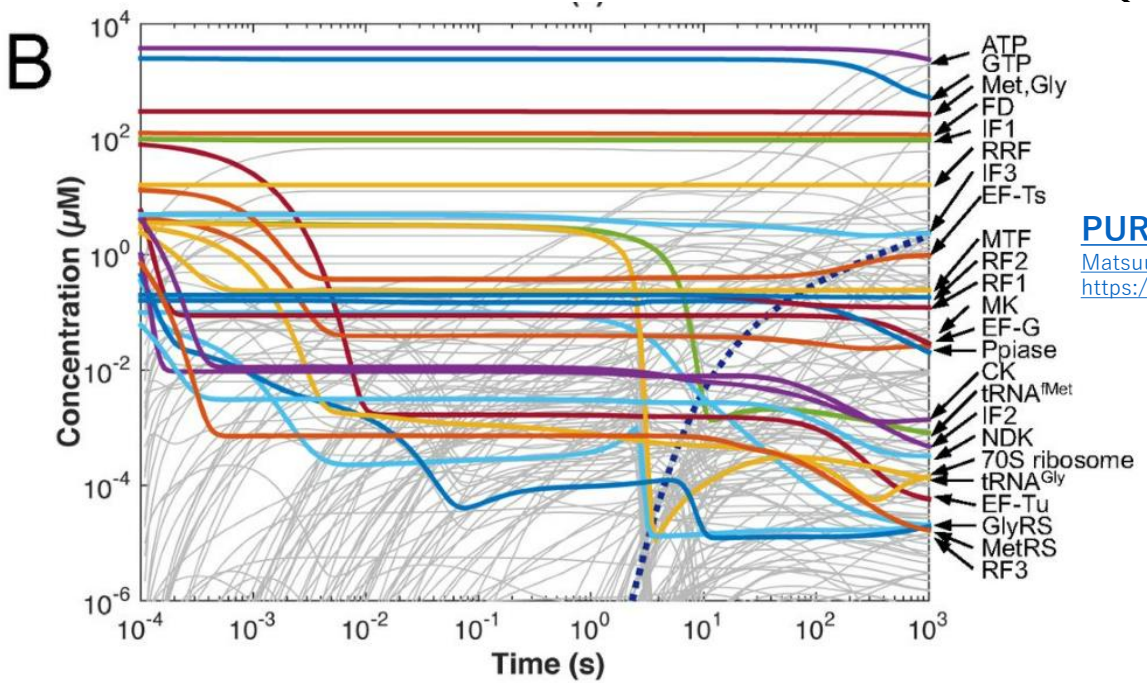

PURE system simulator

Matsuura T. et al. PNAS (2017) 114 (8) E1336
https://doi.org/10.1073/pnas.1615351114

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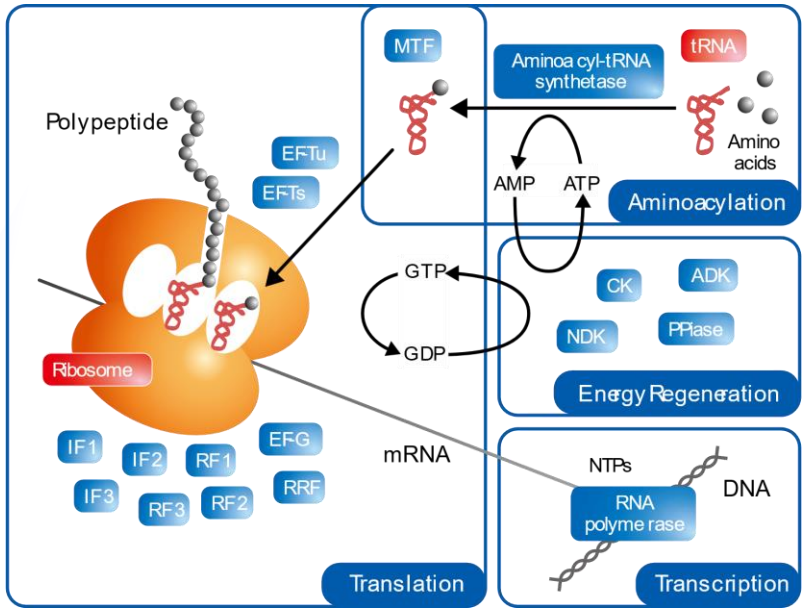
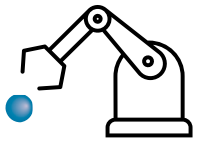
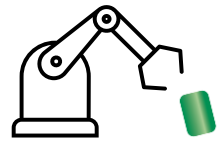
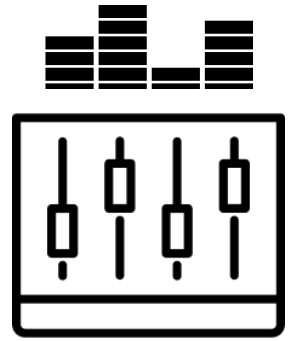


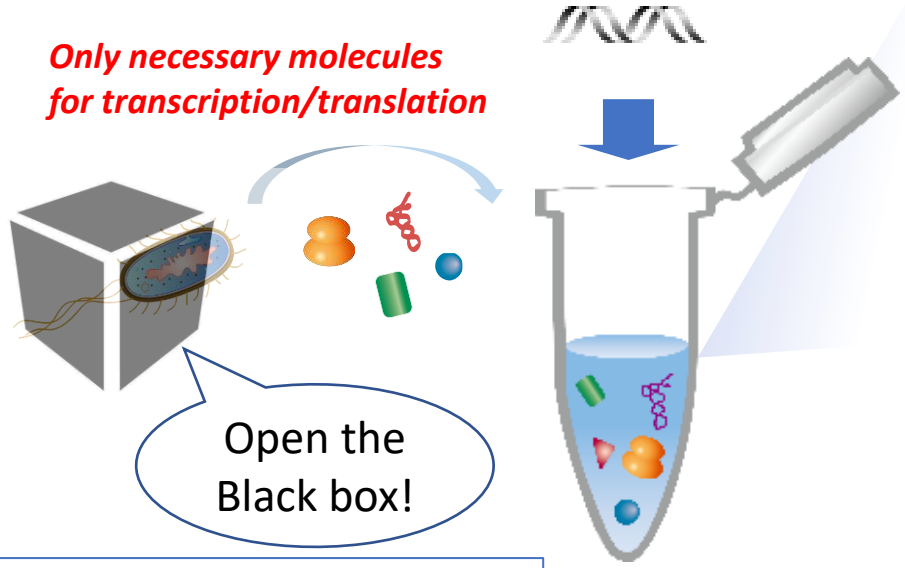






Translation factors
Chaperones
Detergent
Temperature/pH

Only necessary molecules for transcription/translation



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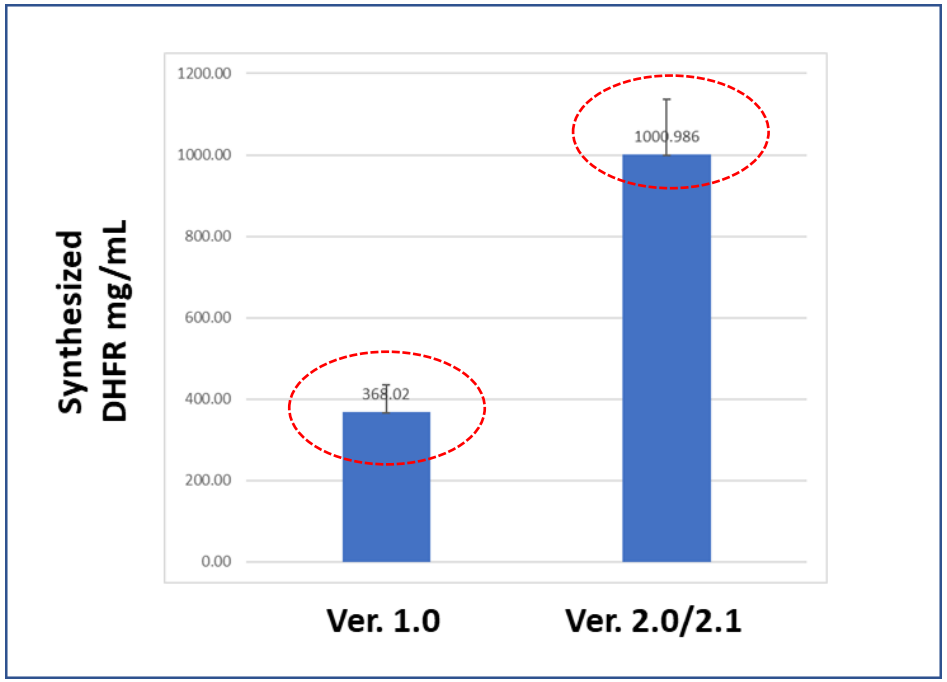


Versatile and Robust Platform for protein synthesis

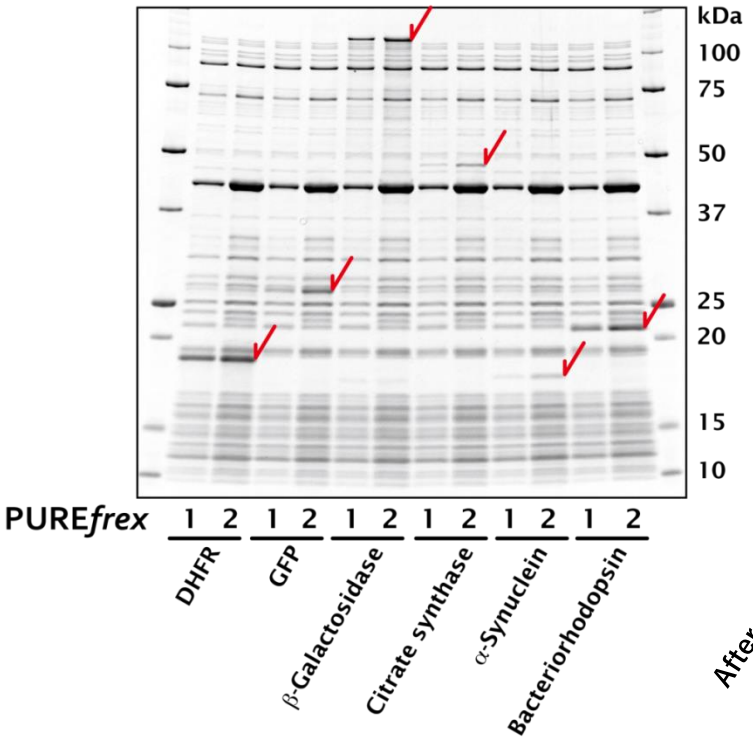
Huge potential as New platform in Biotech industry

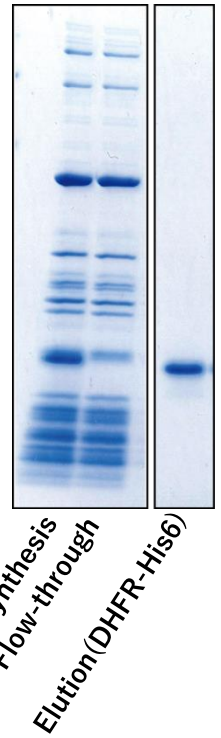
Reconstituted cell-free protein synthesis kit

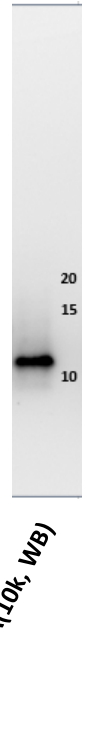
PURE*frex*®

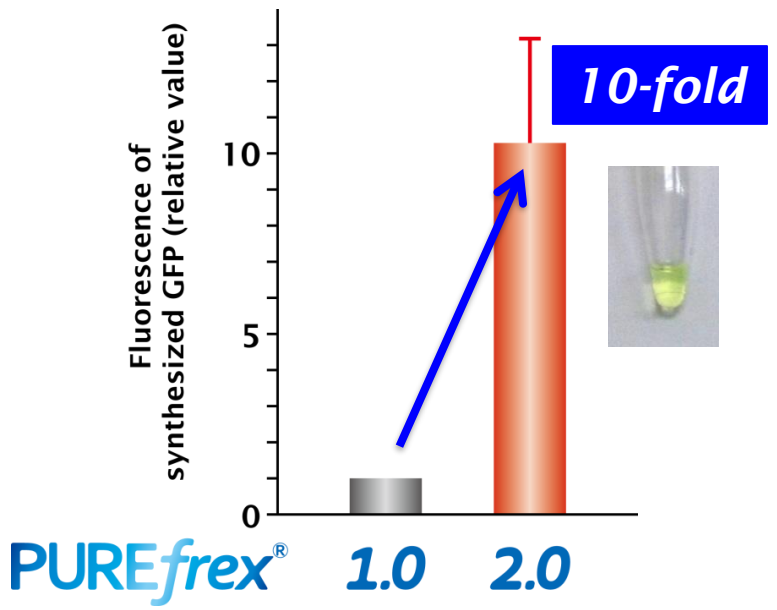
-Having good productivity-

GeneFrontier









- Reaction at 37°C 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.

Reconstituted cell-free protein synthesis kit

PURE*frex*®

-KSF; AT rich codon on N-term-

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Fab Heavy Chain (Herceptin)

Herceptin Fab HC

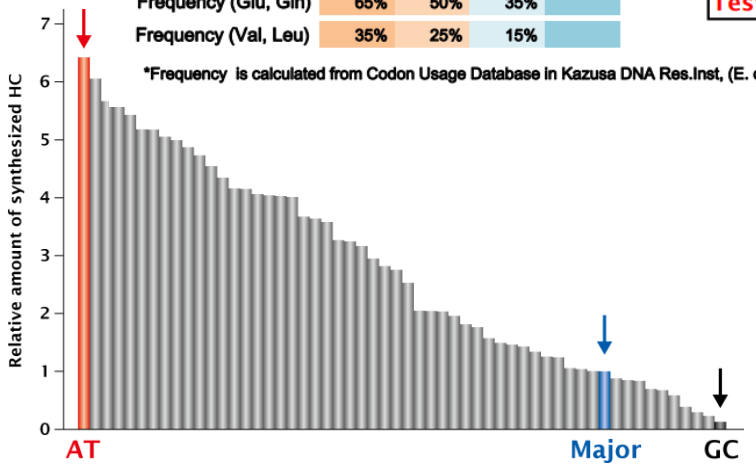
Met- Glu-Val-Gln-Leu-Val- FLAG

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)

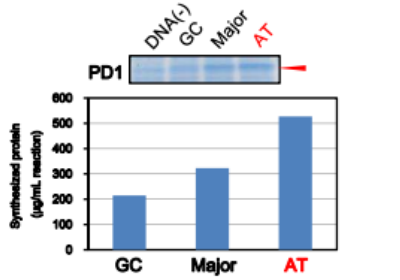


[Design of DNA template is important.](#)
[Manual is Free to download from our Web site here.](#)

PD1

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

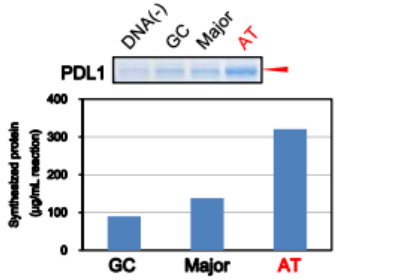
N-term type	1	2(36)	3(37)	4(38)	5(39)	6(40)	GC(%)
	Met	Thr	Phe	Ser	Pro	Ala	1-6 a.a.
GC	atg	acc	ttc	tcc	ccg	gcg	67%
Major	atg	acc	ttt	tct	ccg	gcg	56%
AT	atg	act	ttt	tca	cca	gct	39%



PDL1

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

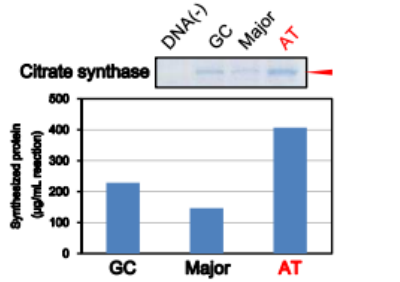
N-term type	1	2(18)	3(19)	4(20)	5(21)	6(22)	GC(%)
	Met	Ala	Phe	Thr	Val	Thr	1-6 a.a.
GC	atg	gcg	ttc	acc	gtg	acc	61%
major	atg	gcg	ttt	acc	gtg	acc	56%
AT	atg	gct	ttt	act	gta	aca	33%



Citrate Synthase

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

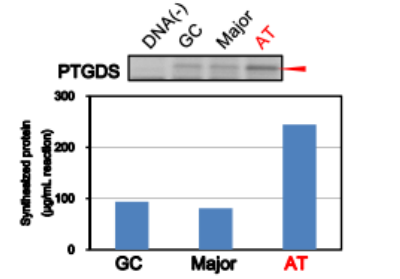
N-term type	1	2(38)	3(39)	4(40)	5(41)	6(42)	GC(%)
	Met	Ser	Ser	Ala	Ser	Glu	1-6 a.a.
GC	atg	tcc	tcc	gcg	tcc	gag	67%
major	atg	tct	tct	gcg	tct	gaa	44%
AT	atg	tca	tca	gct	tca	gaa	39%



PTGDS

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

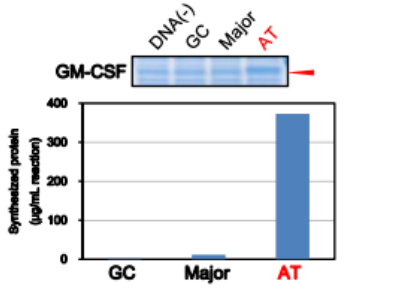
N-term type	1	2(23)	3(24)	4(25)	5(26)	6(27)	GC(%)
	Met	Ala	Pro	Glu	Ala	Gln	1-6 a.a.
GC	atg	gca	ccg	gaa	gca	cag	61%
major	atg	gcg	ccg	gaa	gcg	cag	72%
AT	atg	gca	cct	gaa	gct	caa	50%



GM-CSF

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

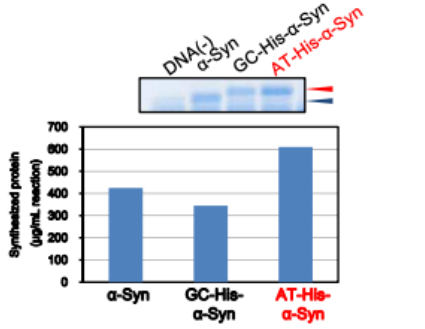
N-term type	1	2(18)	3(19)	4(20)	5(21)	6(22)	GC(%)
	Met	Ala	Pro	Ala	Arg	Ser	1-6 a.a.
GC	atg	gcg	ccg	gcg	cgc	tcc	83%
major	atg	gcg	ccg	gcg	cgc	tct	78%
AT	atg	gca	cct	gct	aga	tca	50%



His-α-Synuclein

Organism	*Homo sapiens*
Synthesized region	(His×6)-(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%



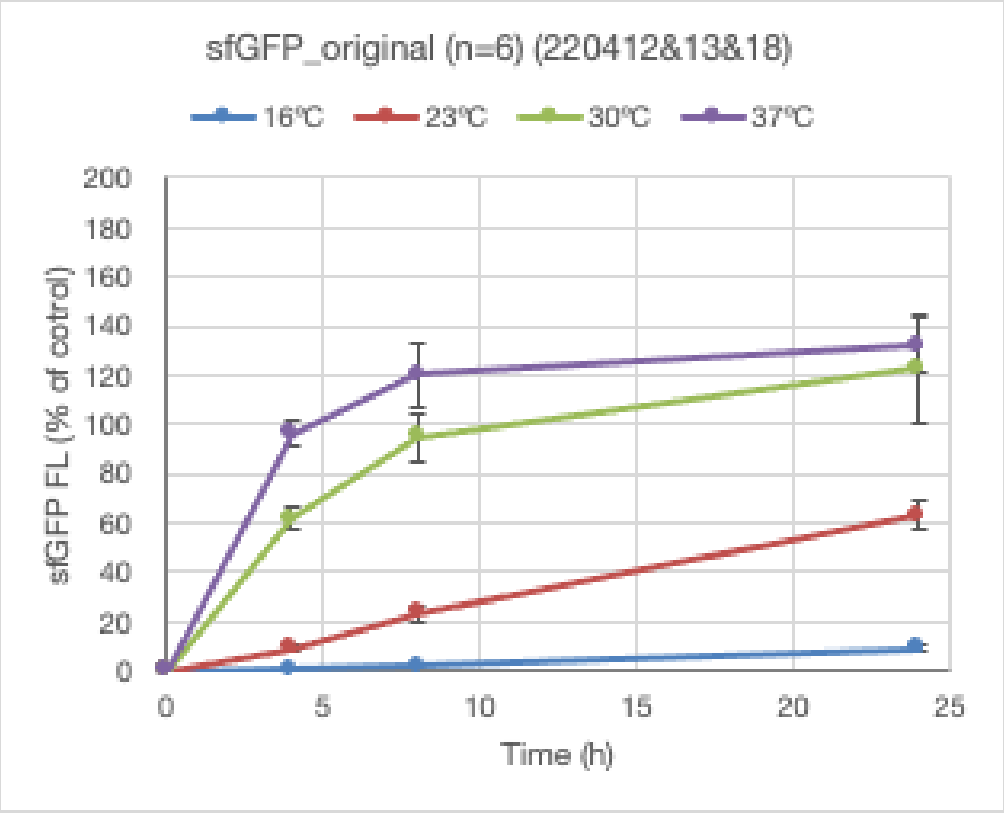
-Template, Temperature, Time-

ver. 2 (4 mM GSH), 1 ng/μL DNA
reaction at 16/23/30/37ºC for 4/8/24 hours

sfGFP

M S K G E E L F T G

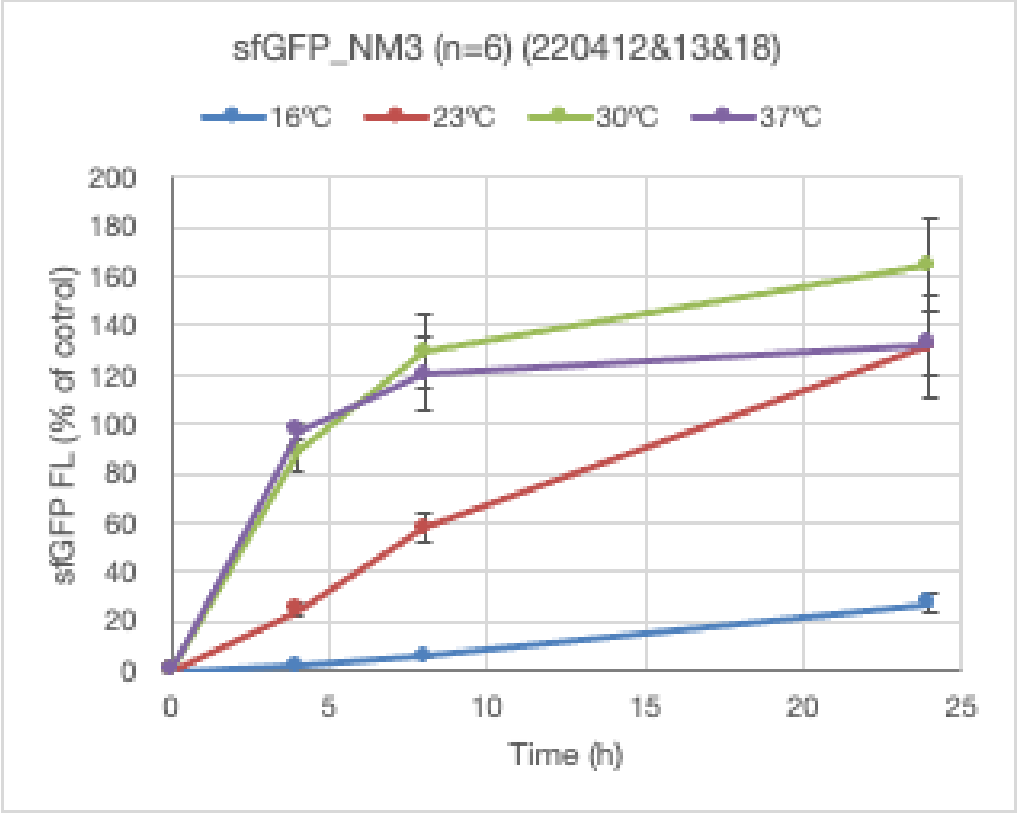
ATGAGTAAAGGAGAAGAACTTTTCACTGGA



sfGFP (ST) (NM3)

M S K S T E L F T G

ATGAGTAAATCTACTGAACTTTTCACTGGA



Reconstituted cell-free protein synthesis kit

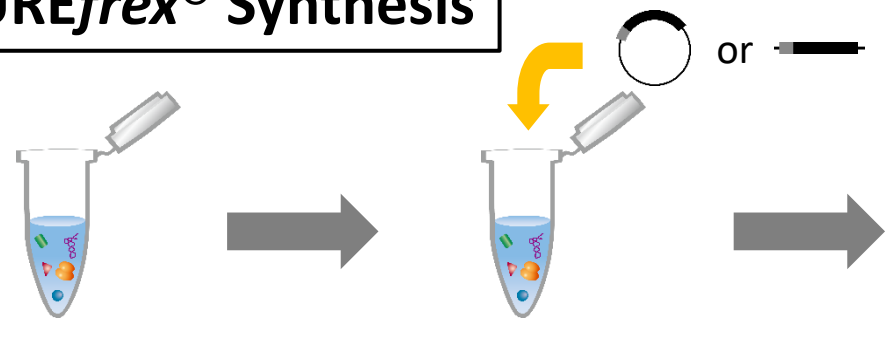
PURE*frex*®

For more information

GeneFrontier

-Improve process from Days to Hours-

PURE*frex*® Synthesis

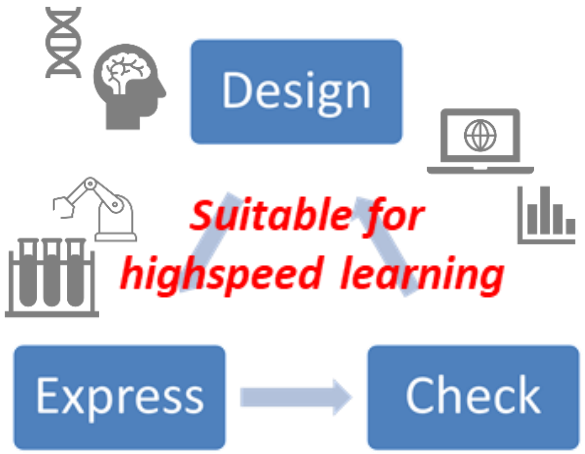


Mix solution I, II and III

Addition of template DNA

Incubation for 2-4 hours (Purification, if necessary)

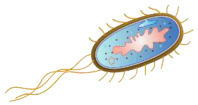
Use for application



Total: 2-4 hours

E. coli Expression

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



Total: 3-4 days

100mL package will be launched soon!

µL ～ L scale

Great Flexibility from basic research to industrial applications

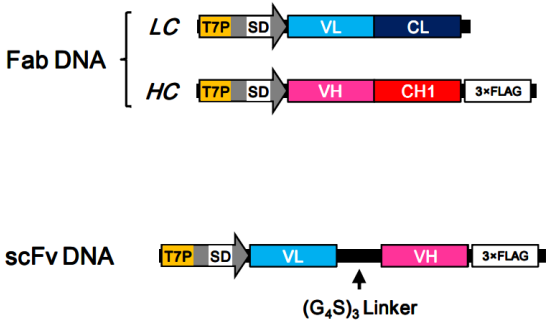
Reconstituted cell-free protein synthesis kit

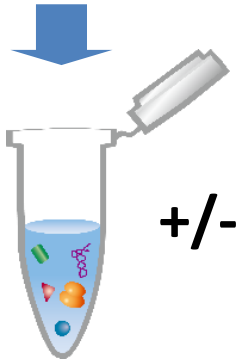
PURE*frex*®

For more information

GeneFrontier

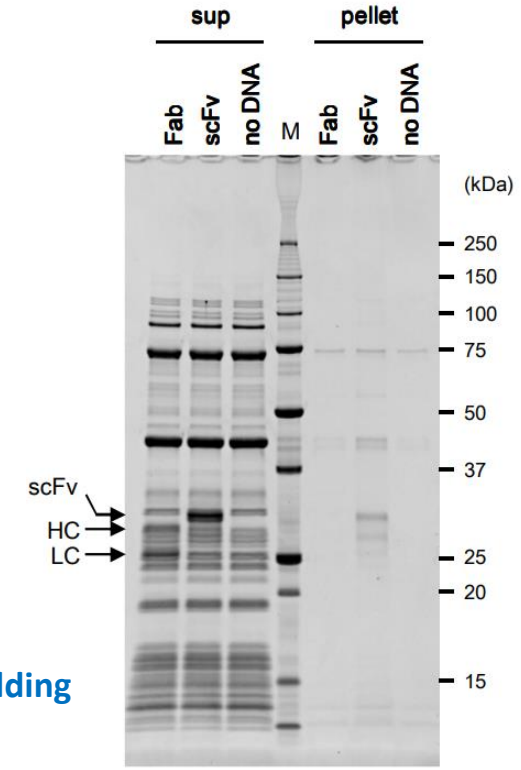
-Expression of scFv and Fab-



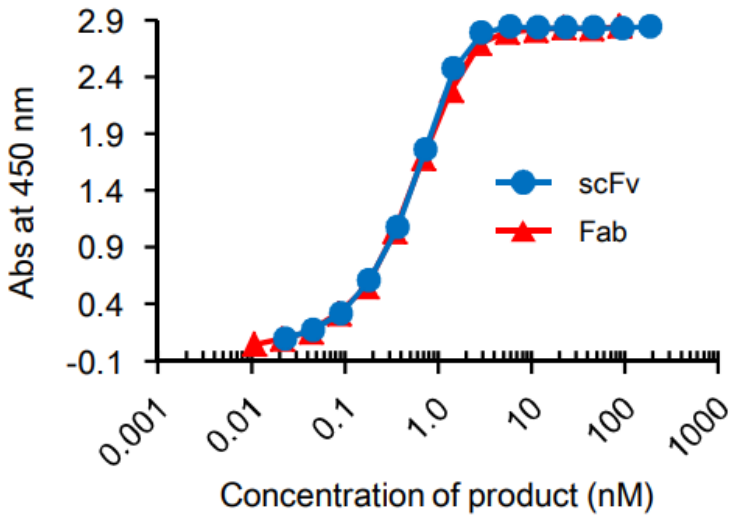


+/- **DsbC Set**
DnaK Mix
For correct folding

SDS-PAGE



Activity



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

✓ **Active scFv and Fab (derived from Herceptin) can be synthesized.**

Reconstituted cell-free protein synthesis kit

PURE*frex*®

-For HTS of mAb-

For more information

GeneFrontier

In vitro expression and Biacore analysis of Fab fragments

PCR on Fab HC & LC

without purification of PCR products

in vitro Fab expression (4h)

With PURE*frex*® & DsbC Set

1 day

without purification of expression products

25 Fabs

without purification of expression products

6 Fabs

Biacore analysis 1

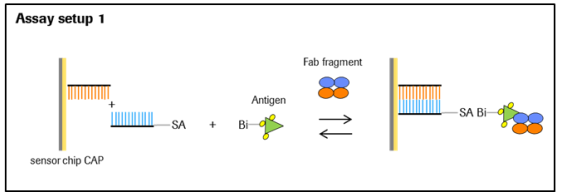
immobilized biotinylated 37 kDa antigen

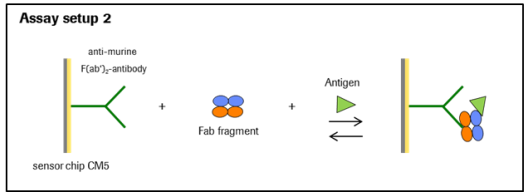
→ Injection of a 1:5 dilution of Fab expression reaction

Biacore analysis 2

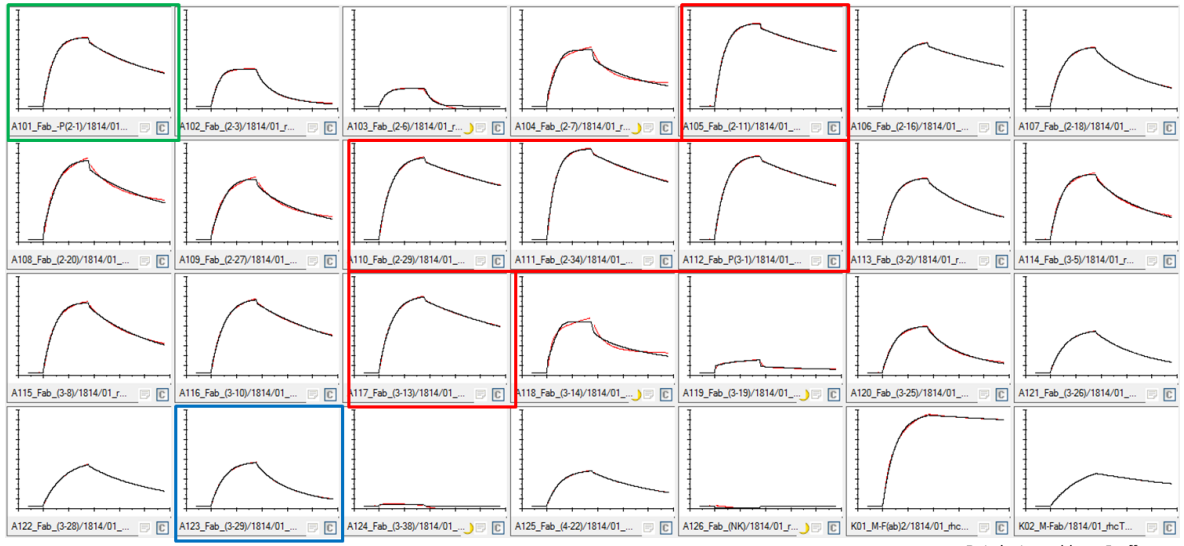
Captured Fab expression products

→ Injection of 37 kDa antigen in 6 different concentrations





Kinetic analysis of 25 Fab binders

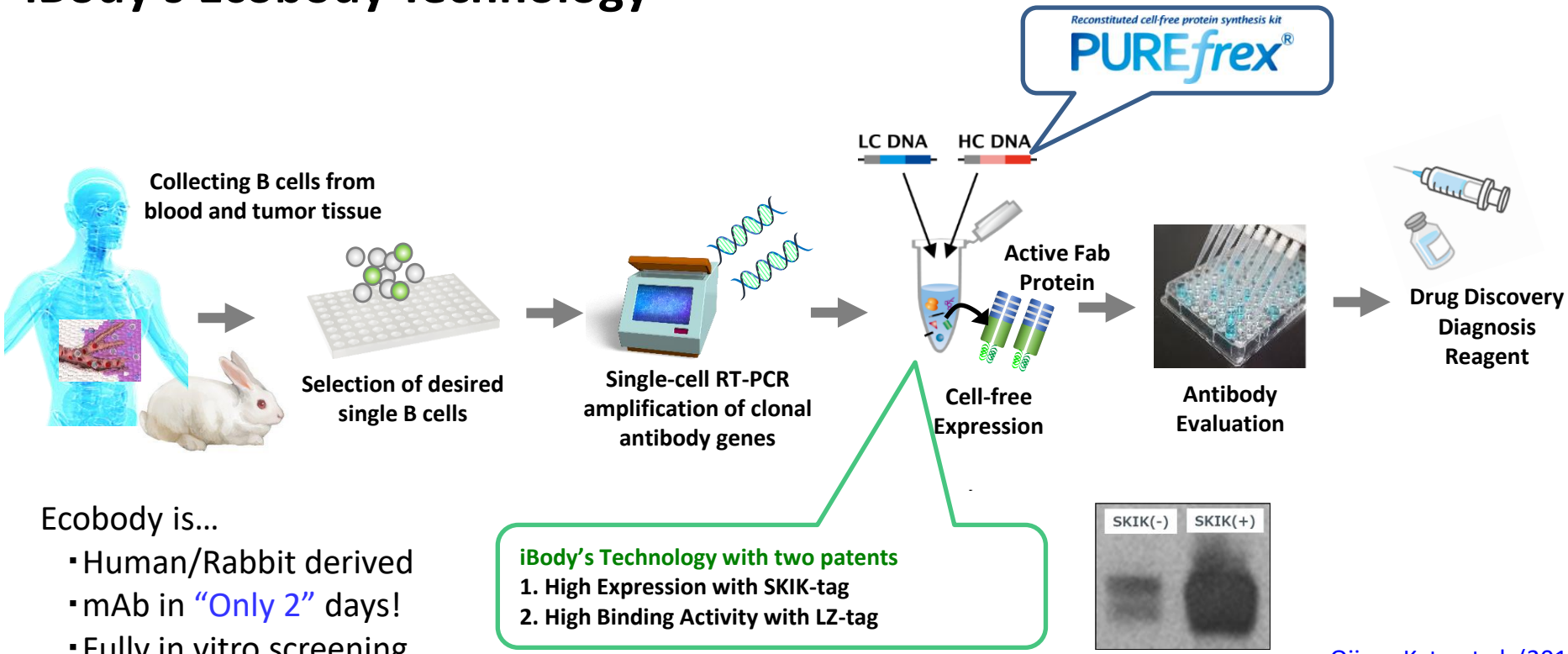


Data by Leopold von Proff

→ Selection of Fabs for further kinetic analysis

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

iBody's Ecobody Technology



Ecobody is…

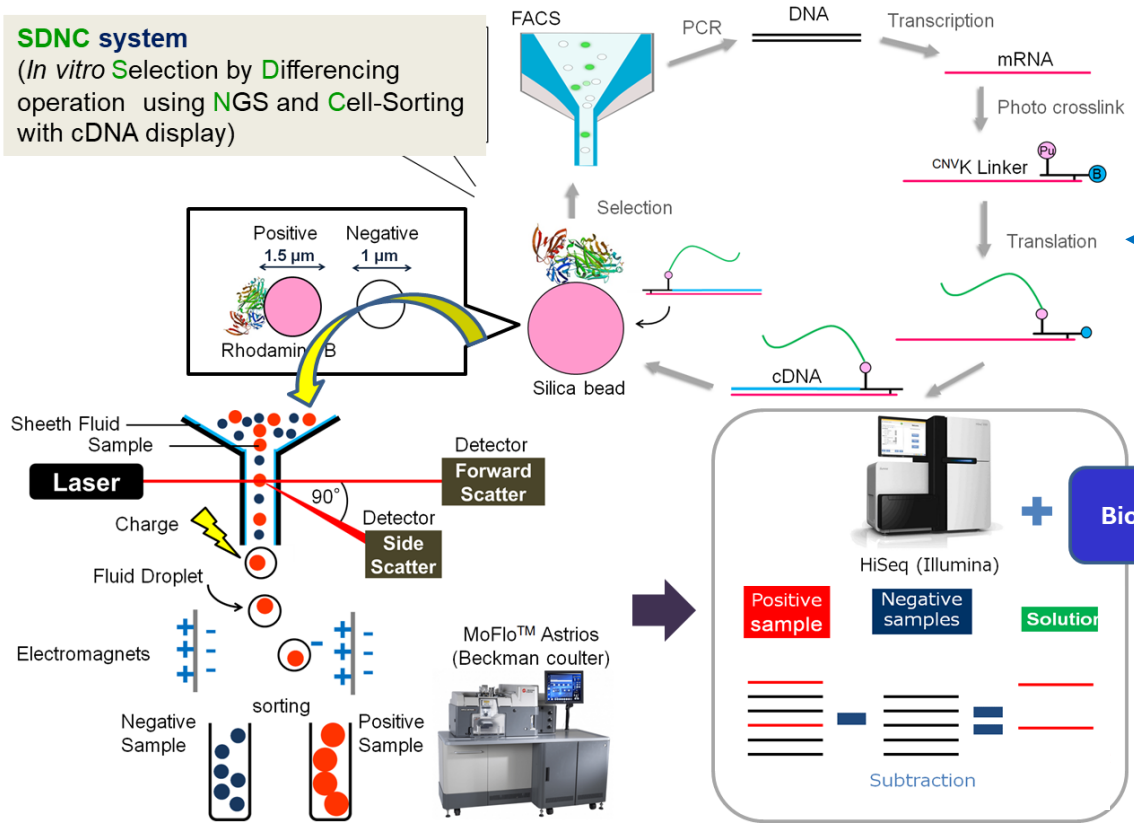
- Human/Rabbit derived
- mAb in "Only 2" days!
- Fully in vitro screening
- No culture

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

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

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

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

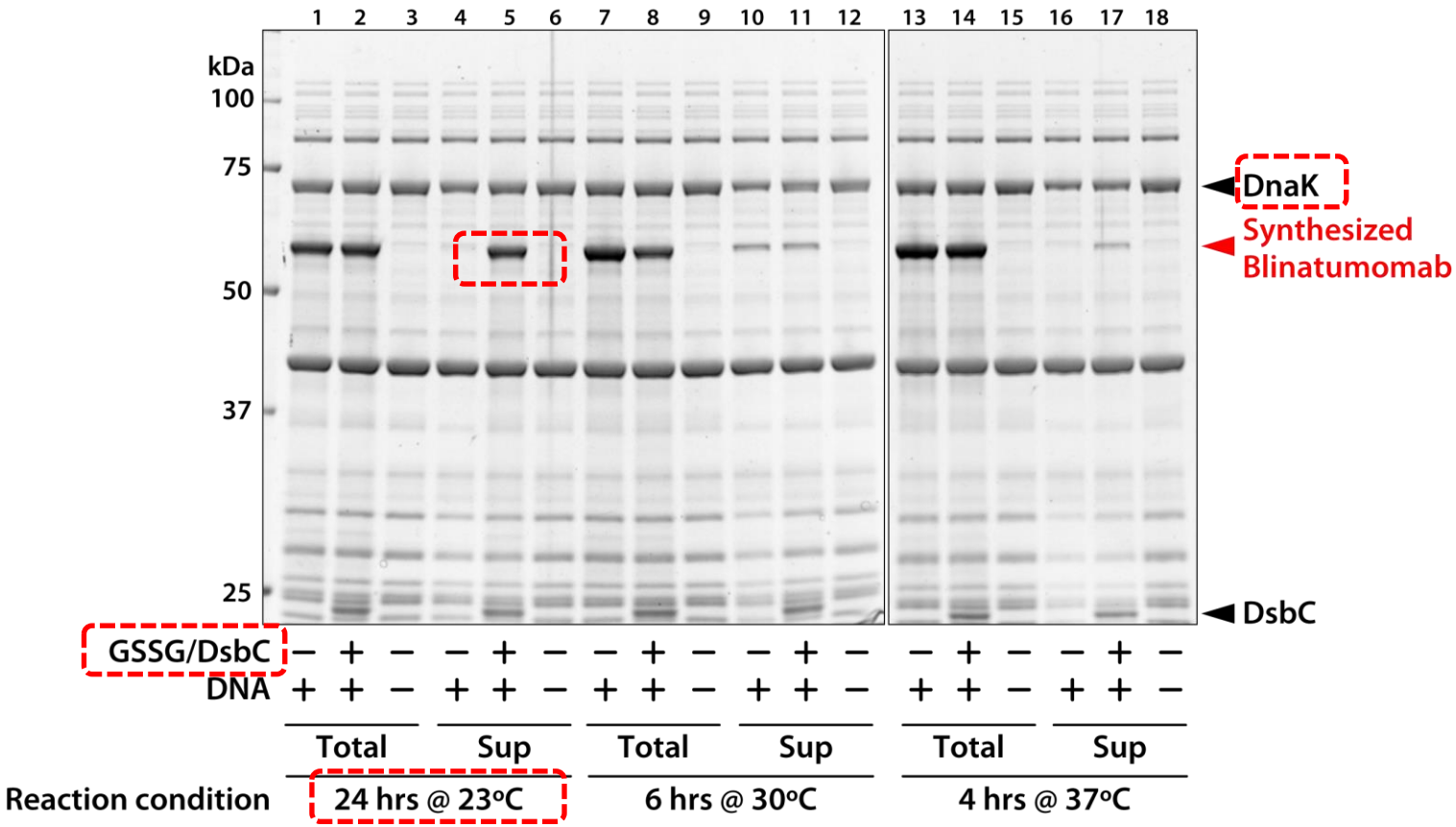
Reconstituted cell-free protein synthesis kit
PURE*frex*®

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

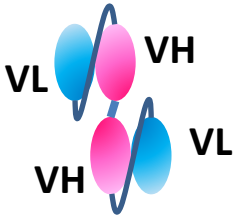
https://www.epsilon-mol.co.jp/eng/

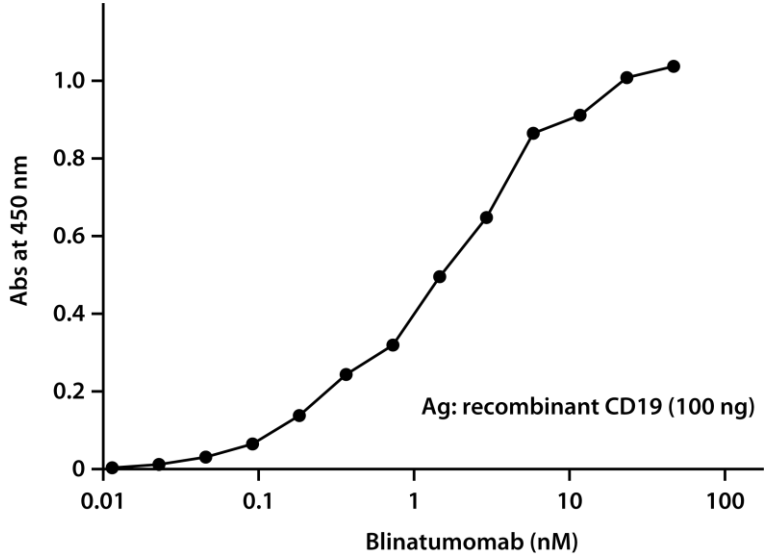
✓ PURE*frex* is applied for cDNA display based screening.

-Application for BiTE-



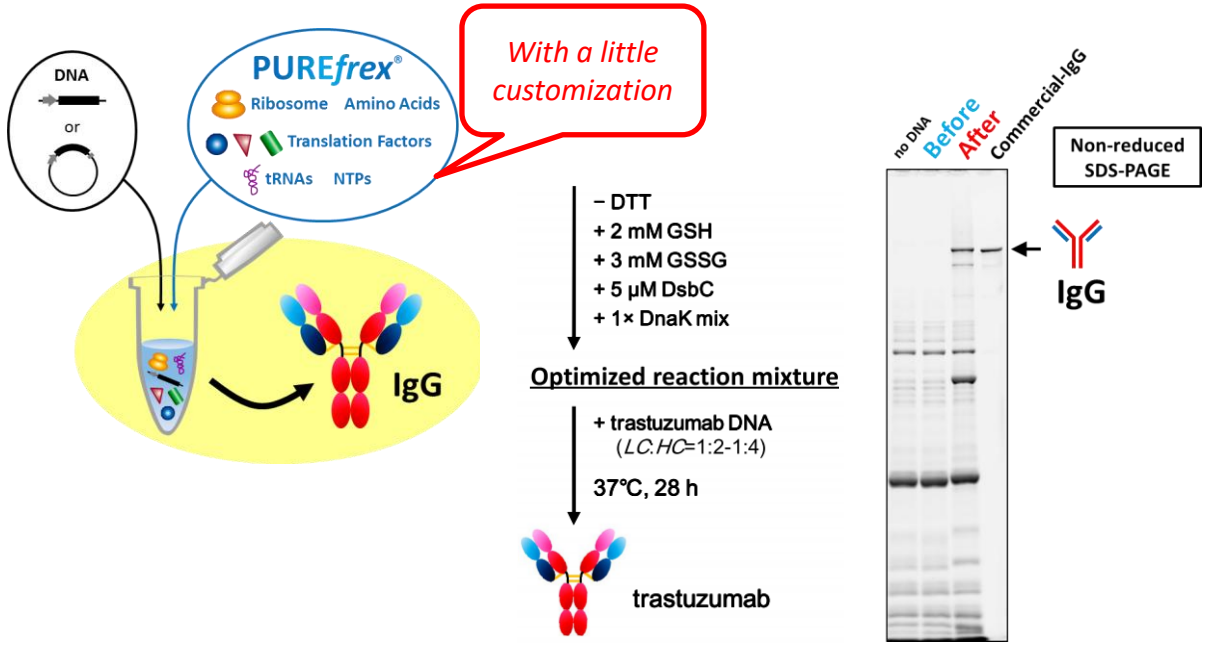
anti-CD3-scFv/CD19-scFv





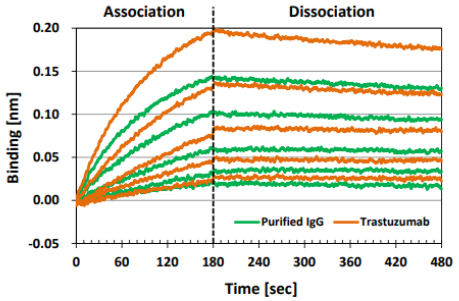
✓ 0.6mg/mL active BiTE was produced under optimized condition.

-Application for IgG-



Only 2 days for IgG!!

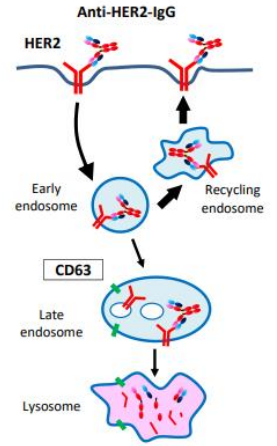
Binding kinetics analysis



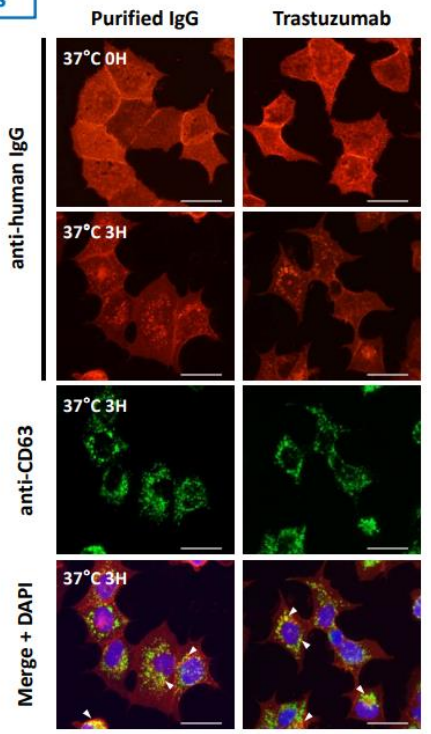
	K_D [M]	k_{on} [1/Ms]	k_{off} [1/s]
Purified IgG	4.24E-10	6.38E+05	2.70E-04
Trastuzumab	4.03E-10	6.29E+05	2.53E-04

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.**

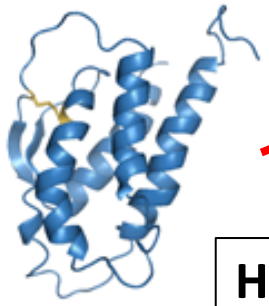
-Application for Cell based assay-

PURE*frex*® +/- DsbC Set

As disulfide bridge enhancer

+ IL-2 DNA

Synthesis of IL-2 protein



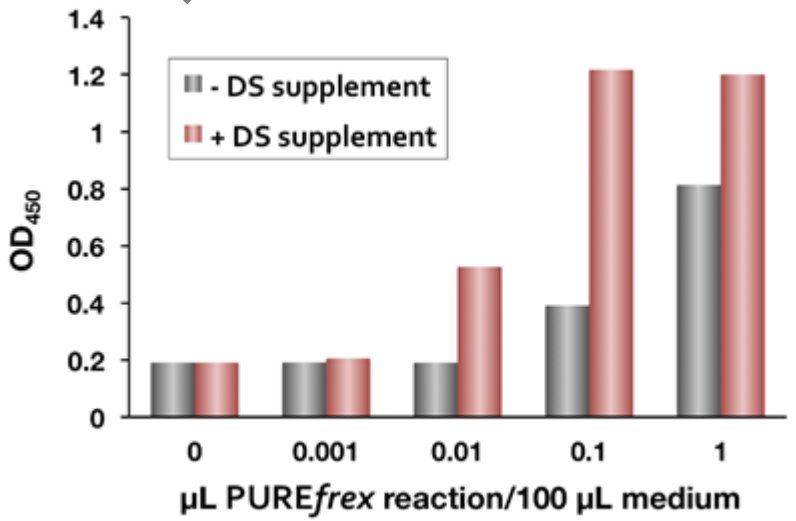
Human IL-2
MW: 15.5 kDa
1 S-S bond

KHYG-1 cells

+ synthesized IL-2

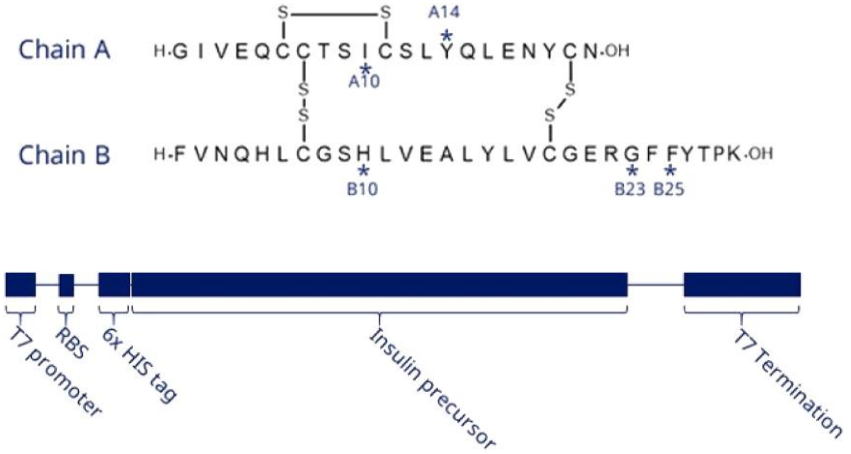
Cell cultivation with IL-2

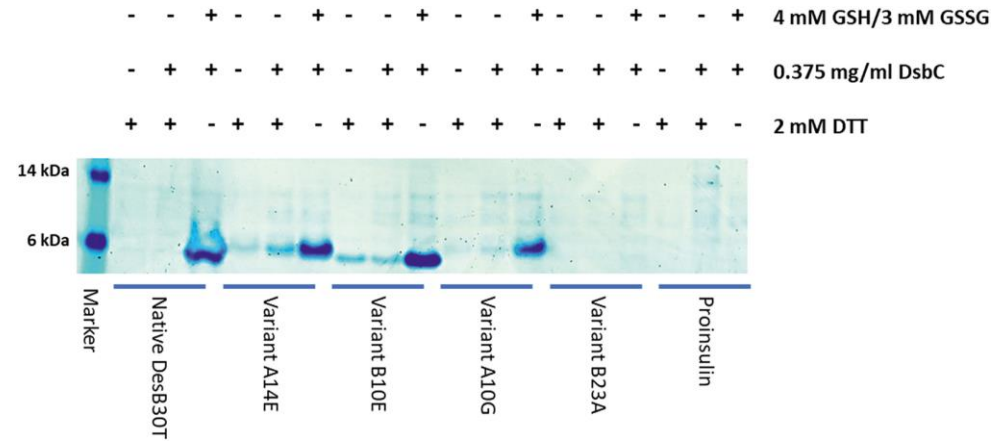
Cell viability assay (WST assay)

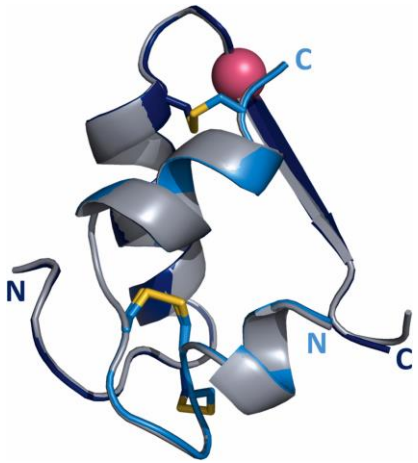


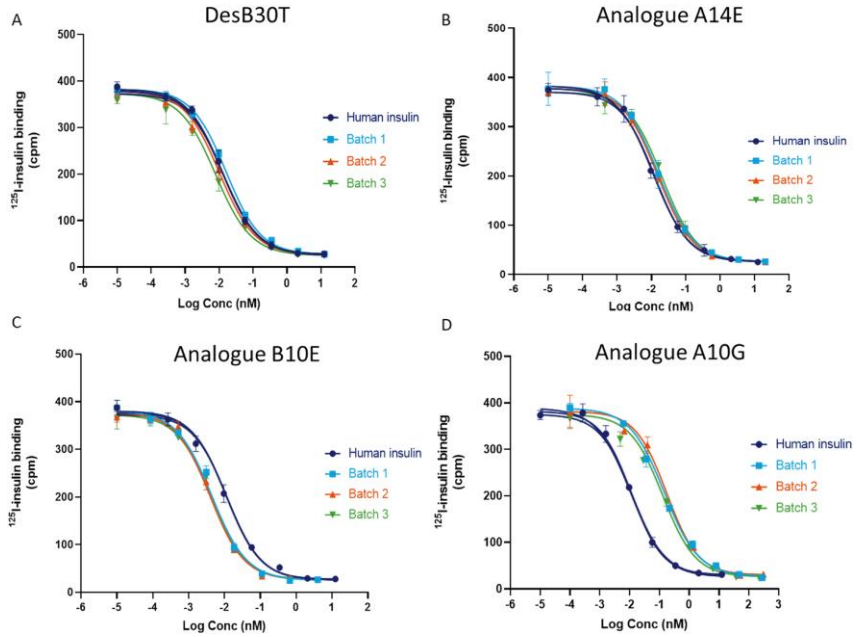
✓ **Directly applicable to cell-based assay.**

-Application for complex molecule-









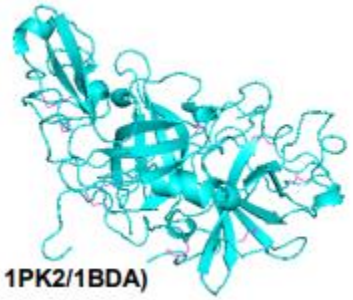
Jensen et al. (2021) Protein Expr. Purif., 186, 105910.

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

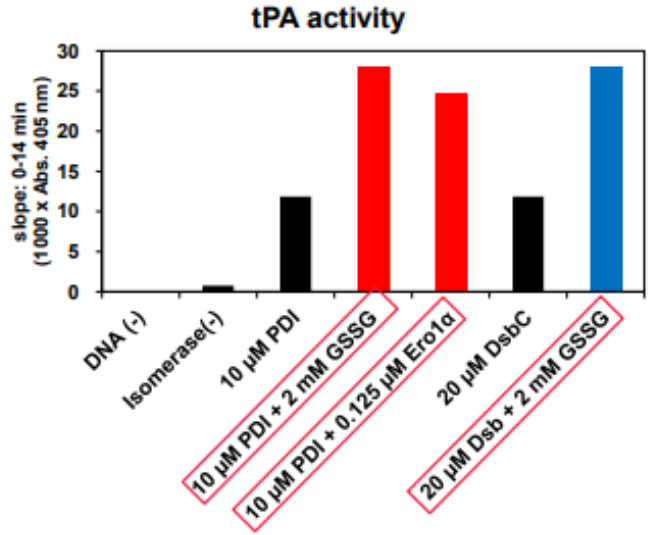
-Application for complex molecule-

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

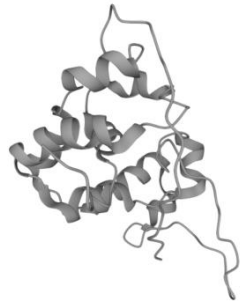


(PDB ID: 1PK2/1BDA)

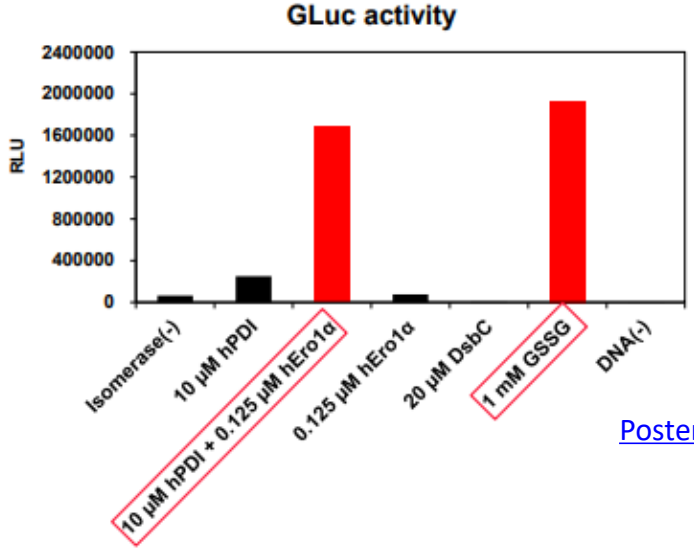


Gaussia Luciferase (GLuc)

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

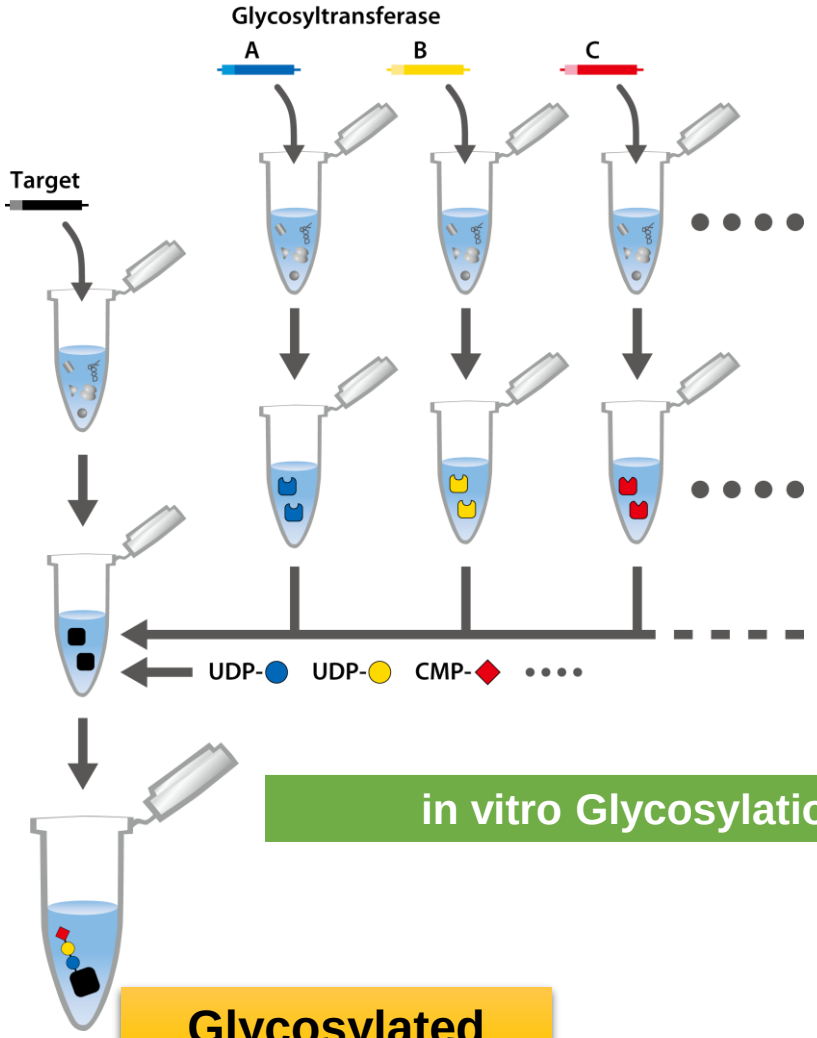
Reconstituted cell-free protein synthesis kit

PURE*frex*®

For more information

GeneFrontier

-Application for glycosylation; CFPS-IVG-

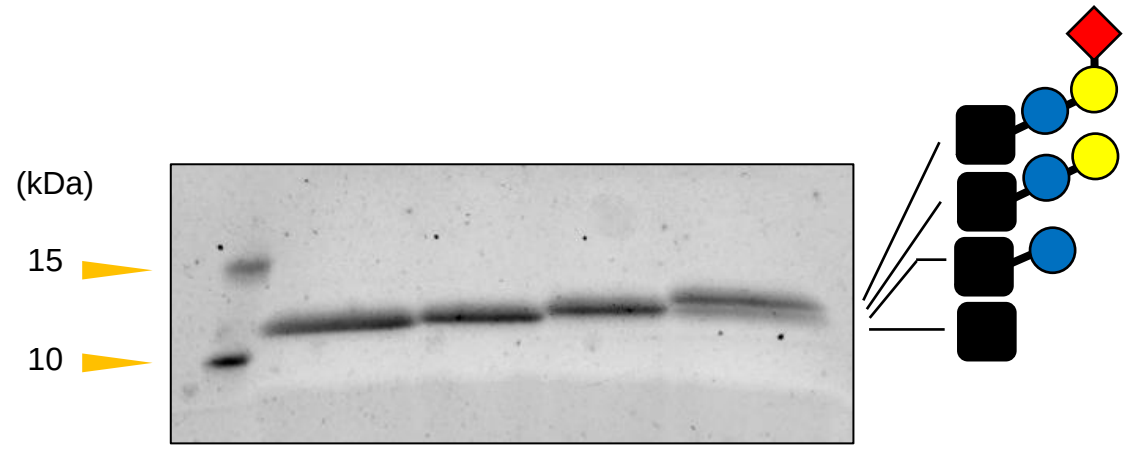


Cell-free Protein Synthesis (CFPS)

PURE*frex*®

in vitro Glycosylation (IVG)

Glycosylated Protein

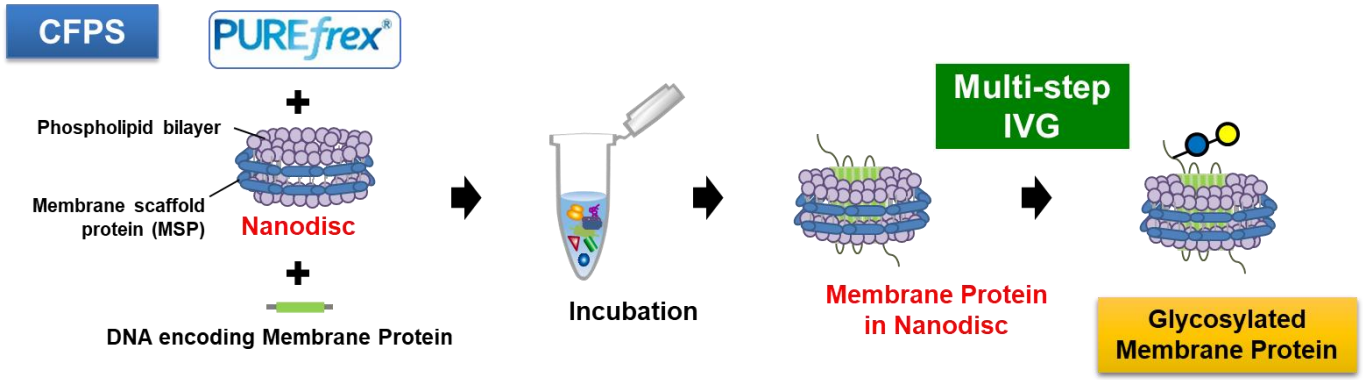


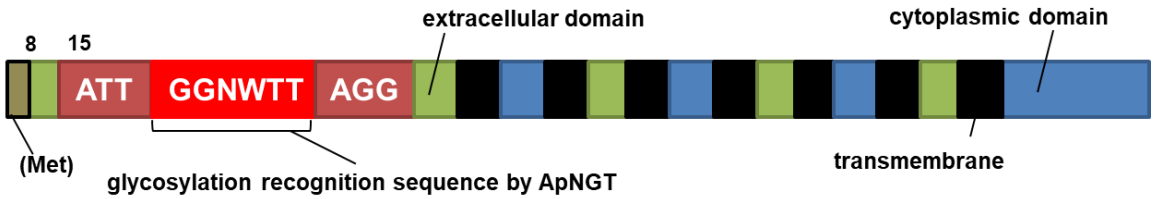
UDP-●/ Enzyme A	-	+	+	+
UDP-●/ Enzyme B	-	-	+	+
CMP-◆/ Enzyme C	-	-	-	+

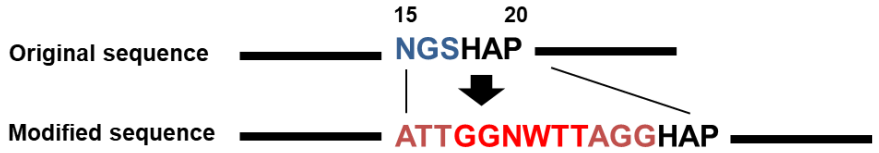
Applicable to glycosylation of target proteins.
Various reaction system can be designed.

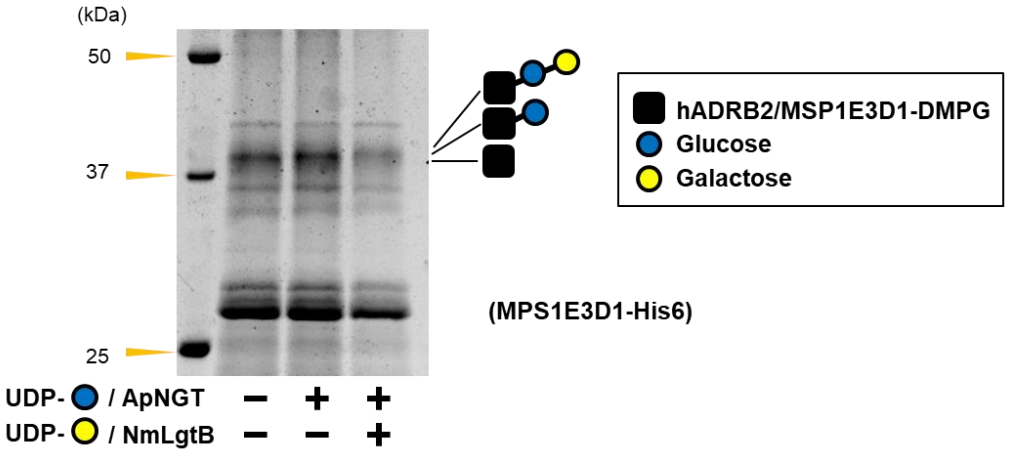
Poster_MBSJ 2021

-Application for glycosylation; CFPS-IVG-







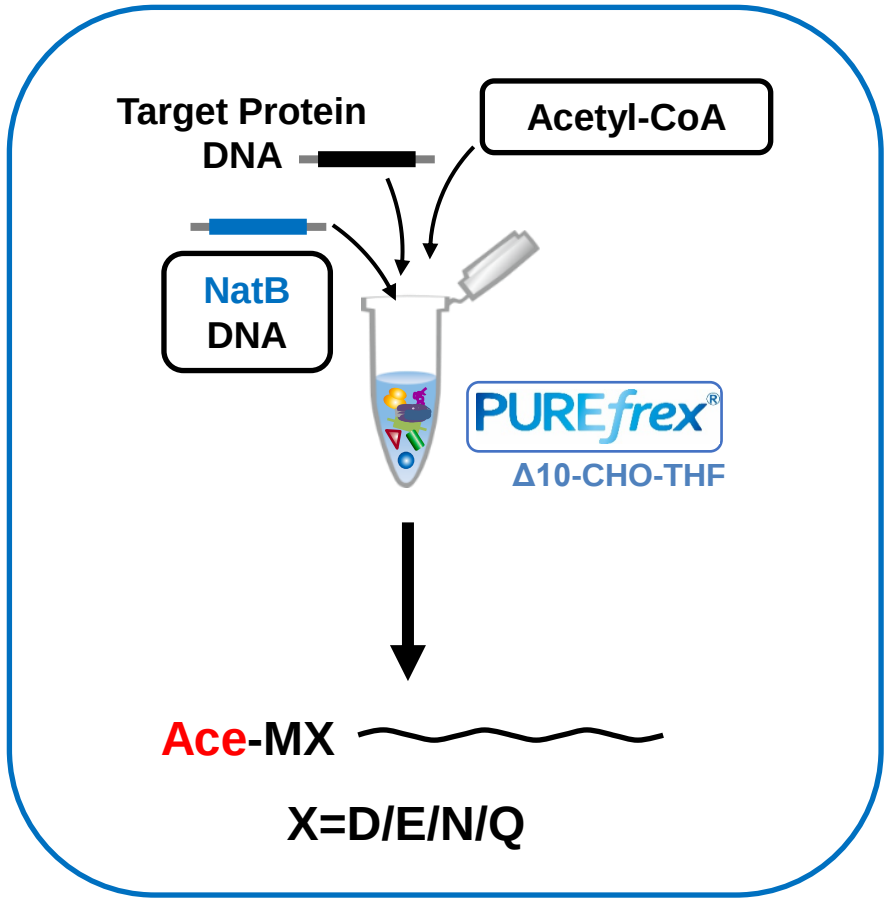


Solubilized membrane protein, hADRB2, are also glycosylated by multi-step IVG

Poster_MBSJ 2021

-Application for acetylation-

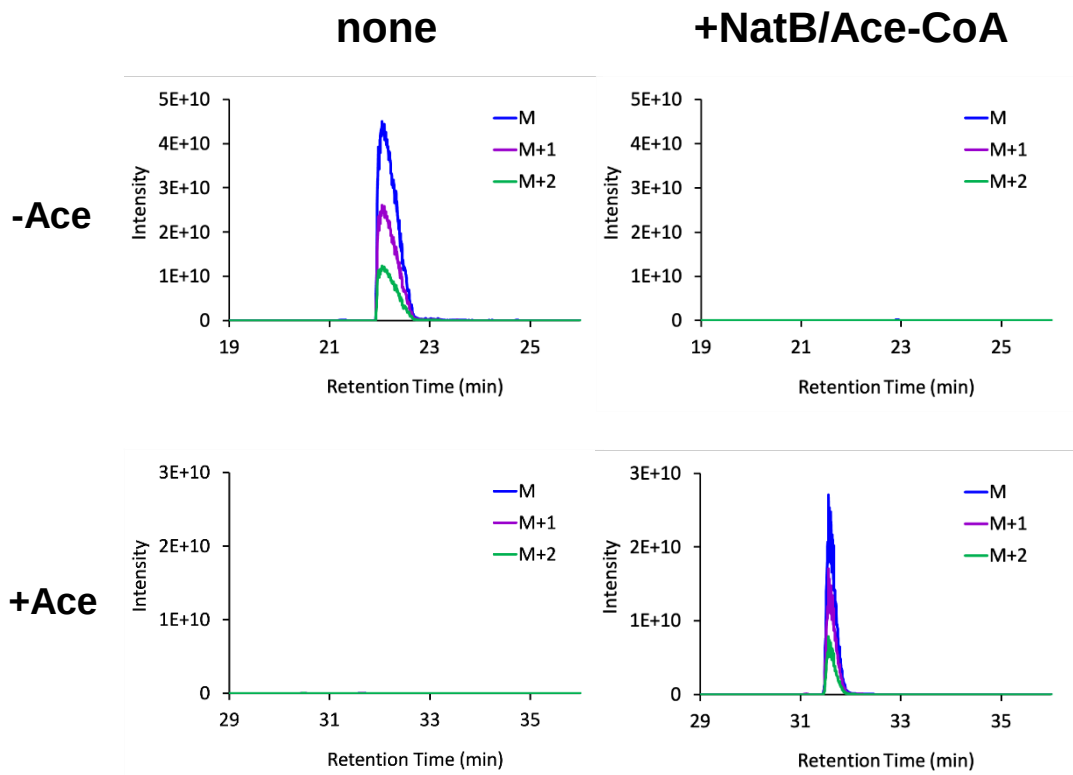
Acetylation (by NatB)



target: α-synuclein (K6A)

MDVFMAGLSK･･･ Hisx6

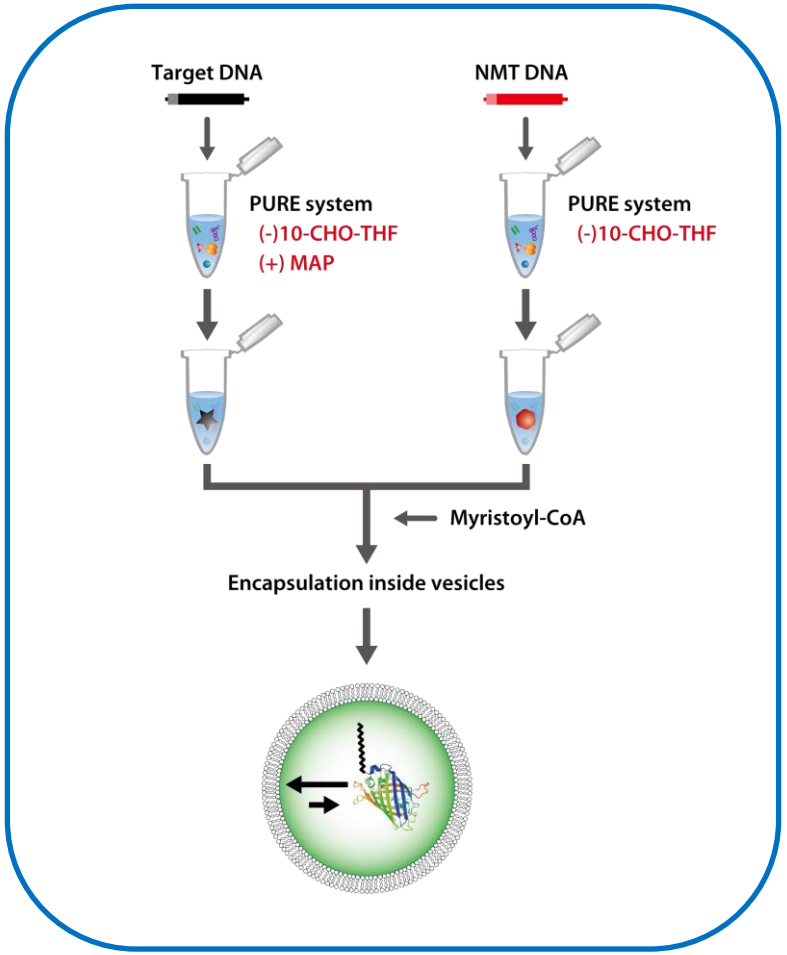
Chromatogram of MS1 intensity

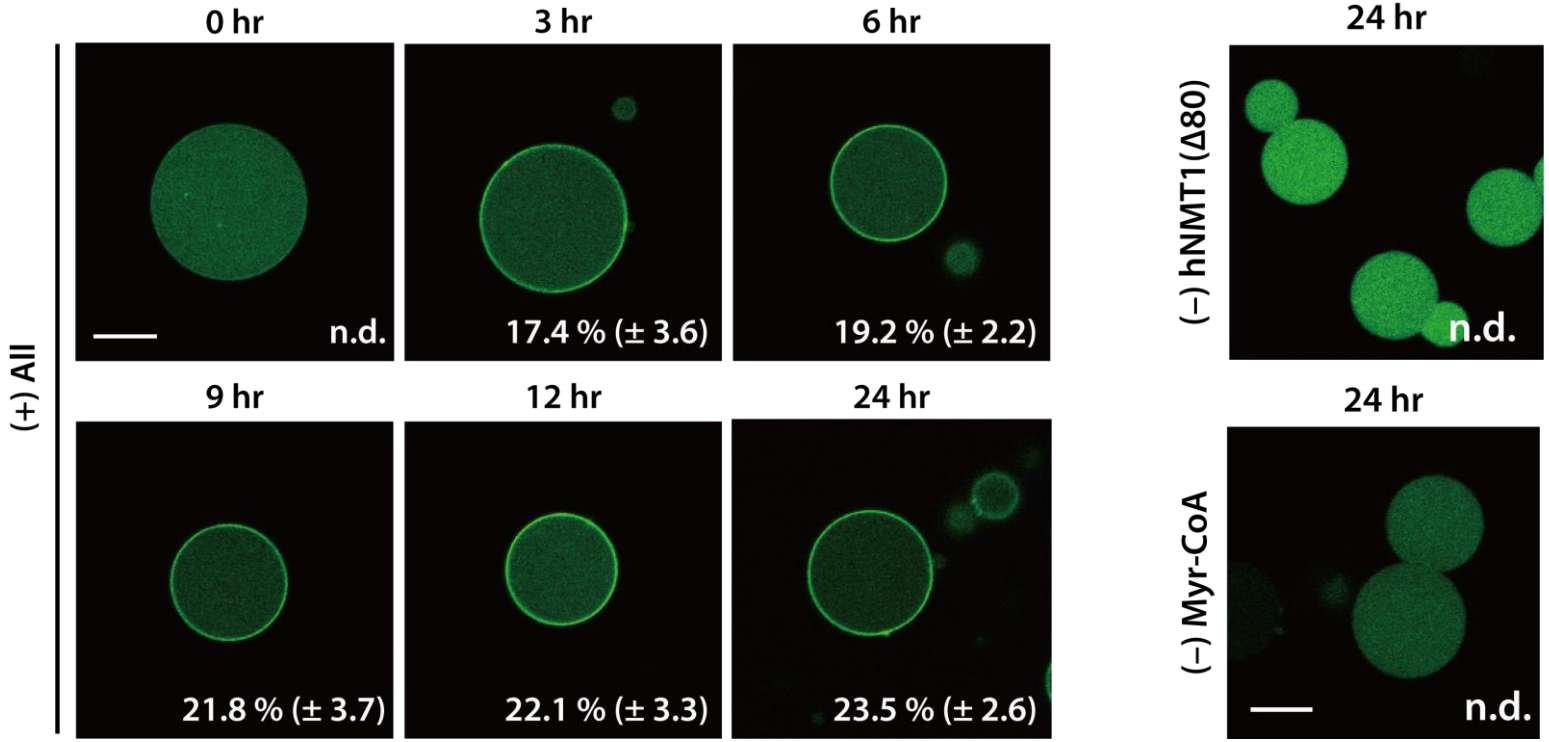


Perfect control of N-terminal acetylation of α-synuclein

-Application for myristoylation-

Myristoylation (by NMT)





Functional N-terminal myristoylation confirmed by localization

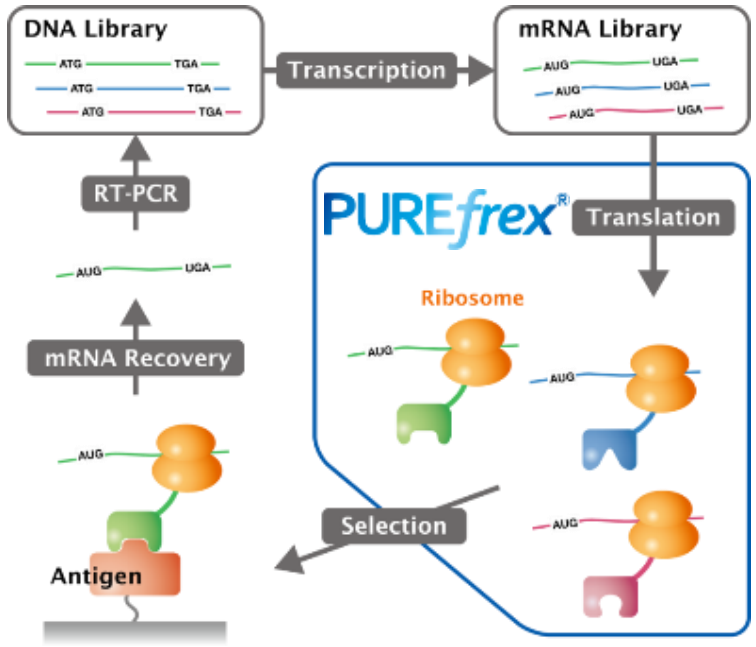
*Ribosome display system using PURE*frex

◆ Advanced screening system for Biologics

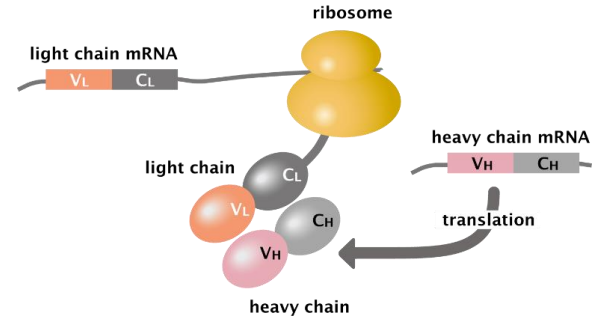
- mAb (scFv / Fab)
- VHH
- Cyclic peptide

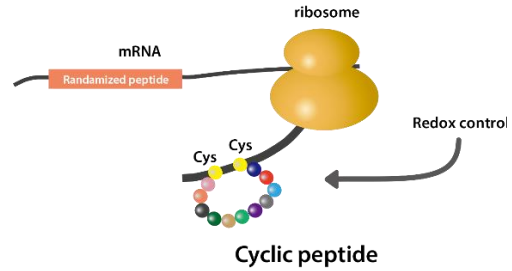
◆ High Selection Efficiency

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



Licensed technology under JP4931135 etc.

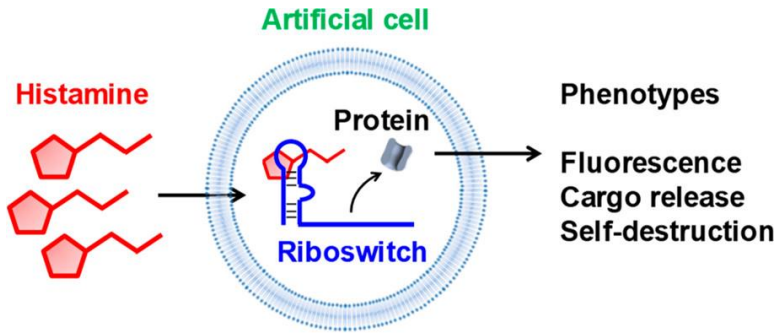




Disulfide Rich peptide

Bicyclic peptide

-Broad applications, yet to come!-



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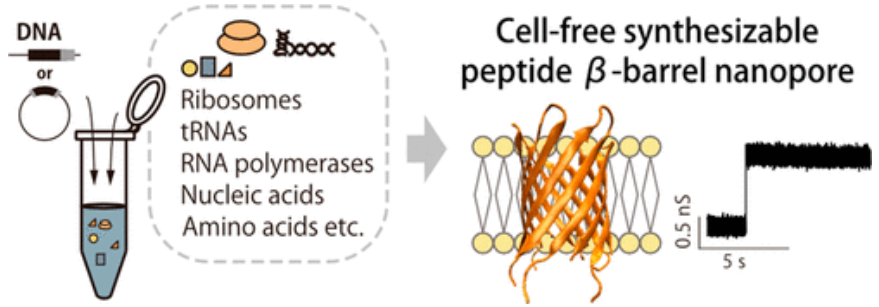

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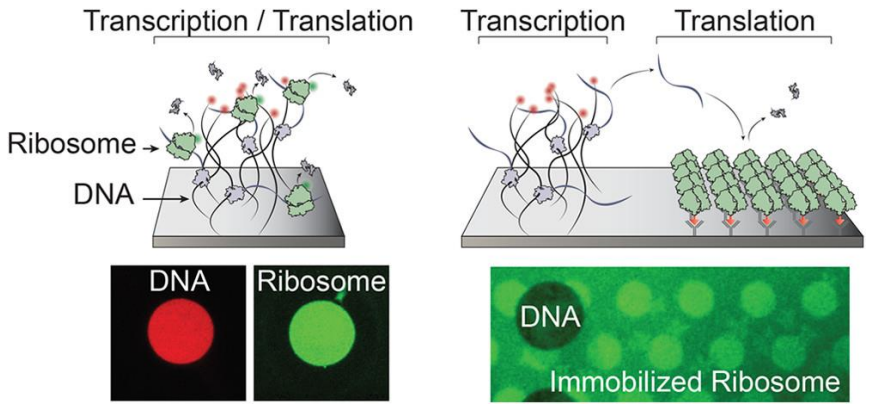

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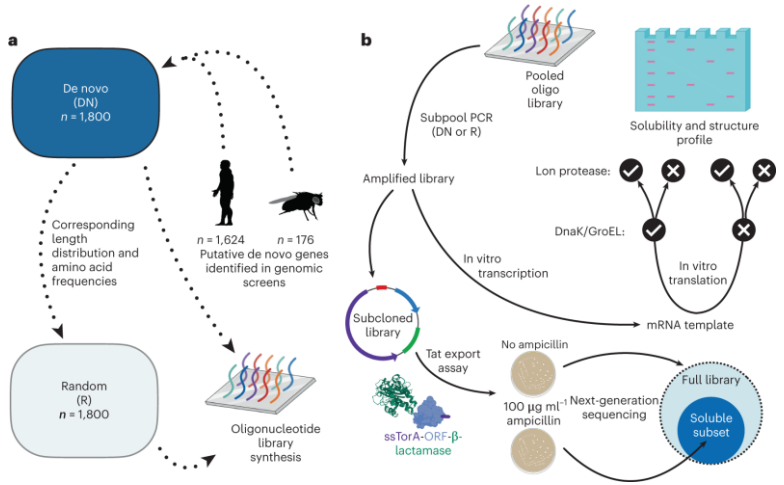

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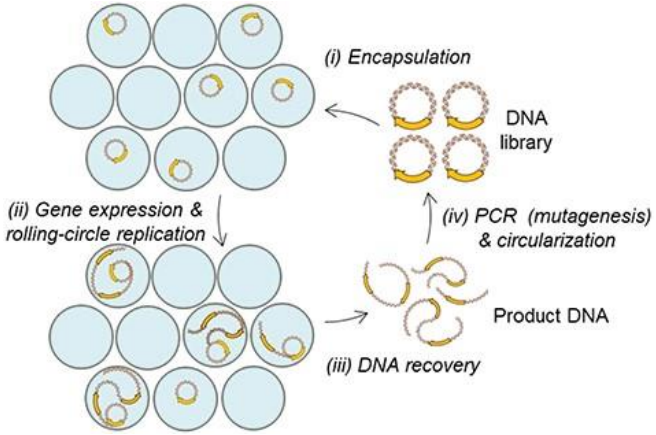
-Broad applications, yet to come!-



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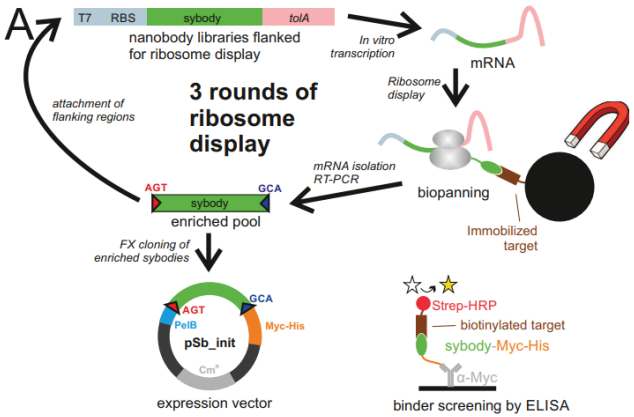

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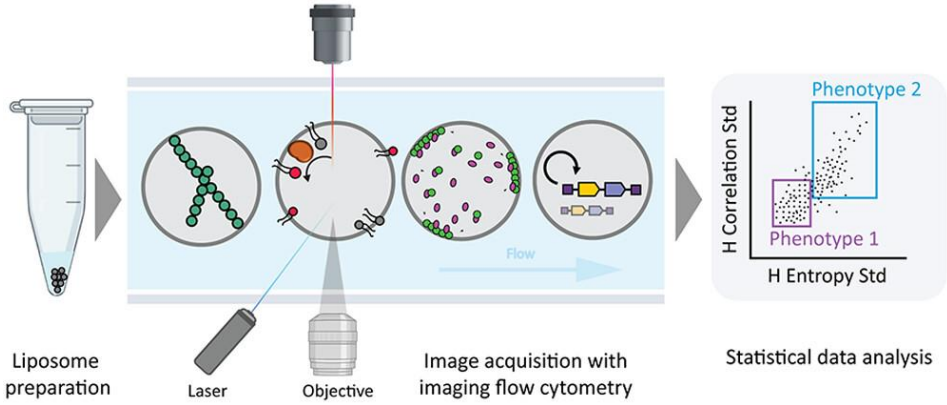

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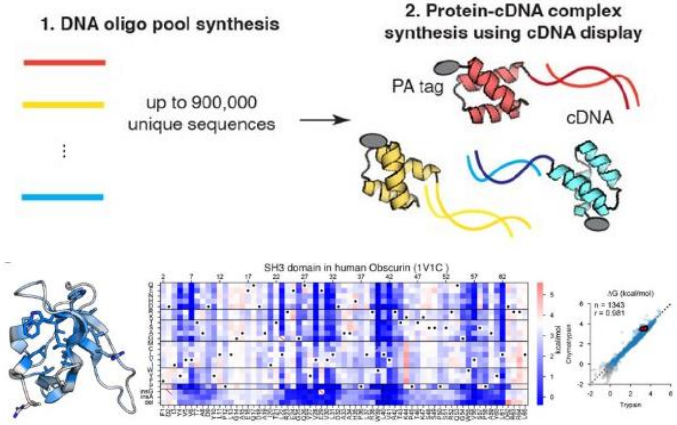

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