July 15th, 2004.

Creation of virus-binding proteins (VBPs) for removal of pathogenic viruses in water

[forseeing new technology for virus removal from water]

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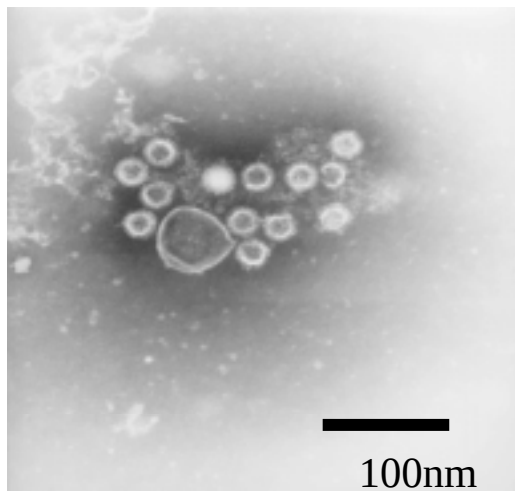
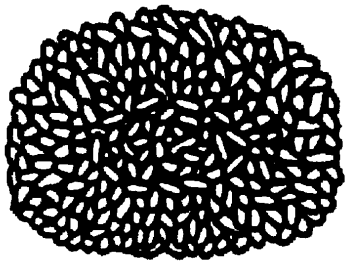


Photograph of *Norovirus* by Immuno-electron microscopy (IEM)

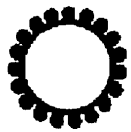
Configuration of viruses



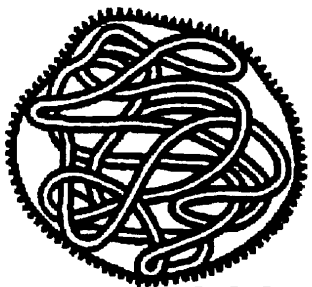
Poxviridae

Rhabdoviridae

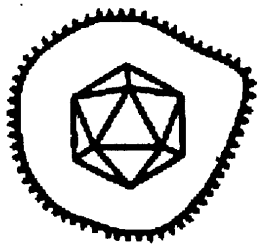
Orthomyxoviridae



Coronaviridae



Paramyxoviridae



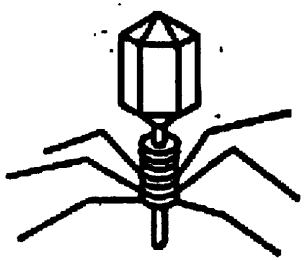
Herpesviridae

Adenoviridae

Papovaviridae

Reoviridae

Picornaviridae



Bacteriophage T2

Tabaco mosaic virus

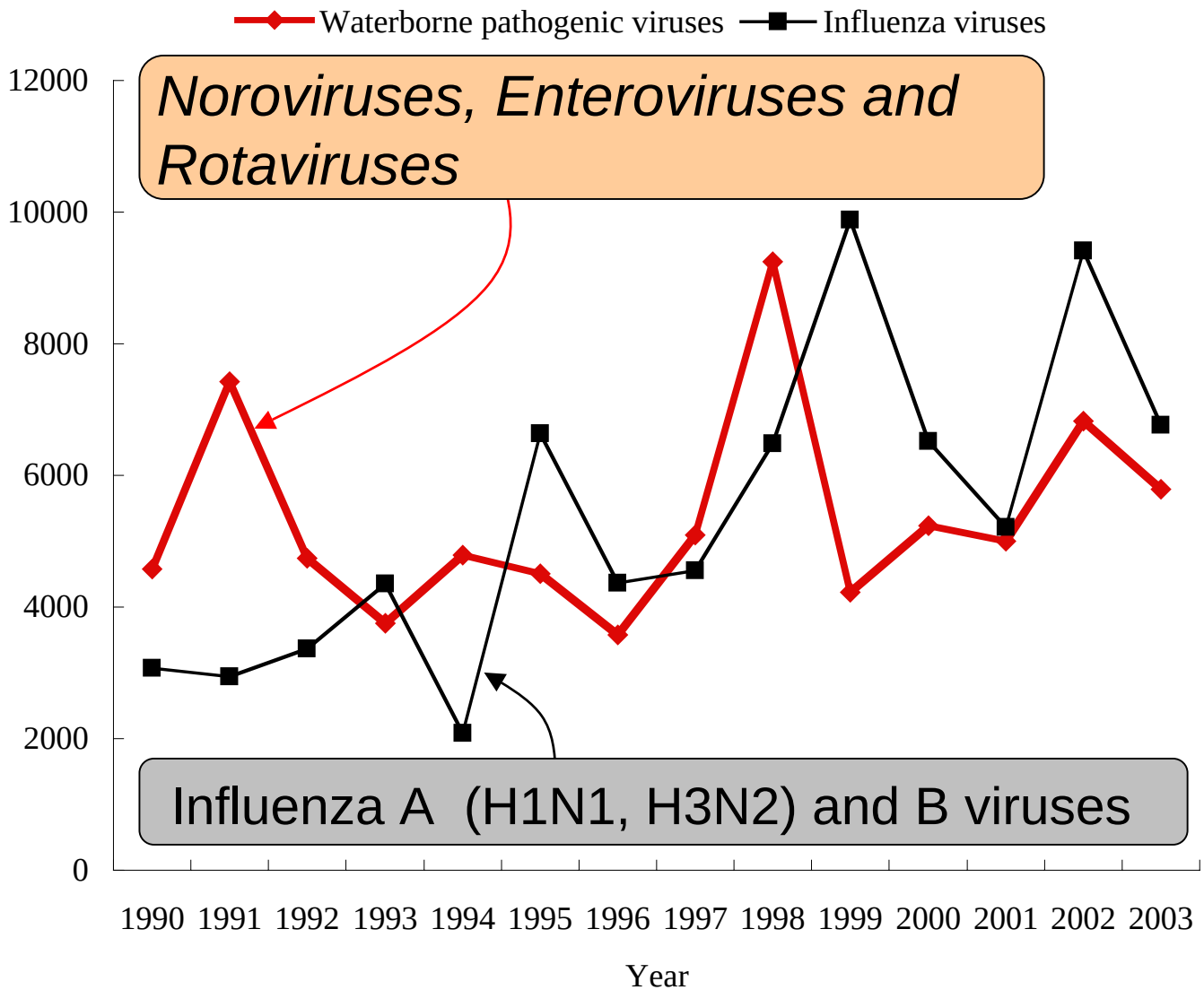
Togaviridae

100nm

Waterborne pathogenic viruses

Family	Genus	Species	Main symptoms
Picornaviridae	*Enteroviruses*	Polioviruses	Infantile paralysis
		Coxsackie A Viruses	Respiratory disease
		Coxsackie B Viruses	Epidemic myalgia
		ECHO viruses	Vomiting, Diarrhea
		Enteroviruses	Vomiting, Diarrhea
	Hepatovirus	Hepatitis A virus	Hepatitis
Reoviridae	*Reovirus*	Reoviruses	Diarrhea, Fever
	Rotavirus	Rotaviruses	Gastroenteritis
Astroviridae	*Astrovirus*	Human astroviruses	Gastroenteritis
Caliciviridae	*Calicivirus*	Human caliciviruses	Gastroenteritis
	Noroviruses	Norwalk virus	Gastroenteritis
	Sapoviruses	Sapporo virus	Gastroenteritis
Adenoviridae	Mastadenovirus	Human adenovirus	Gastroenteritis, Conjunctivitis
Unclassfied		Hepatitis E virus	Hepatitis

Number of viral isolates from Human in Japan (1990-2003)



Infectious Agents Surveillance Report

Backgrounds

Pollution of water environment with viruses

- Viruses are detected from various water samples

Increasing in viral infectious diseases

- Population explosion, urban congestion, water shortage, and so on might be main factors.

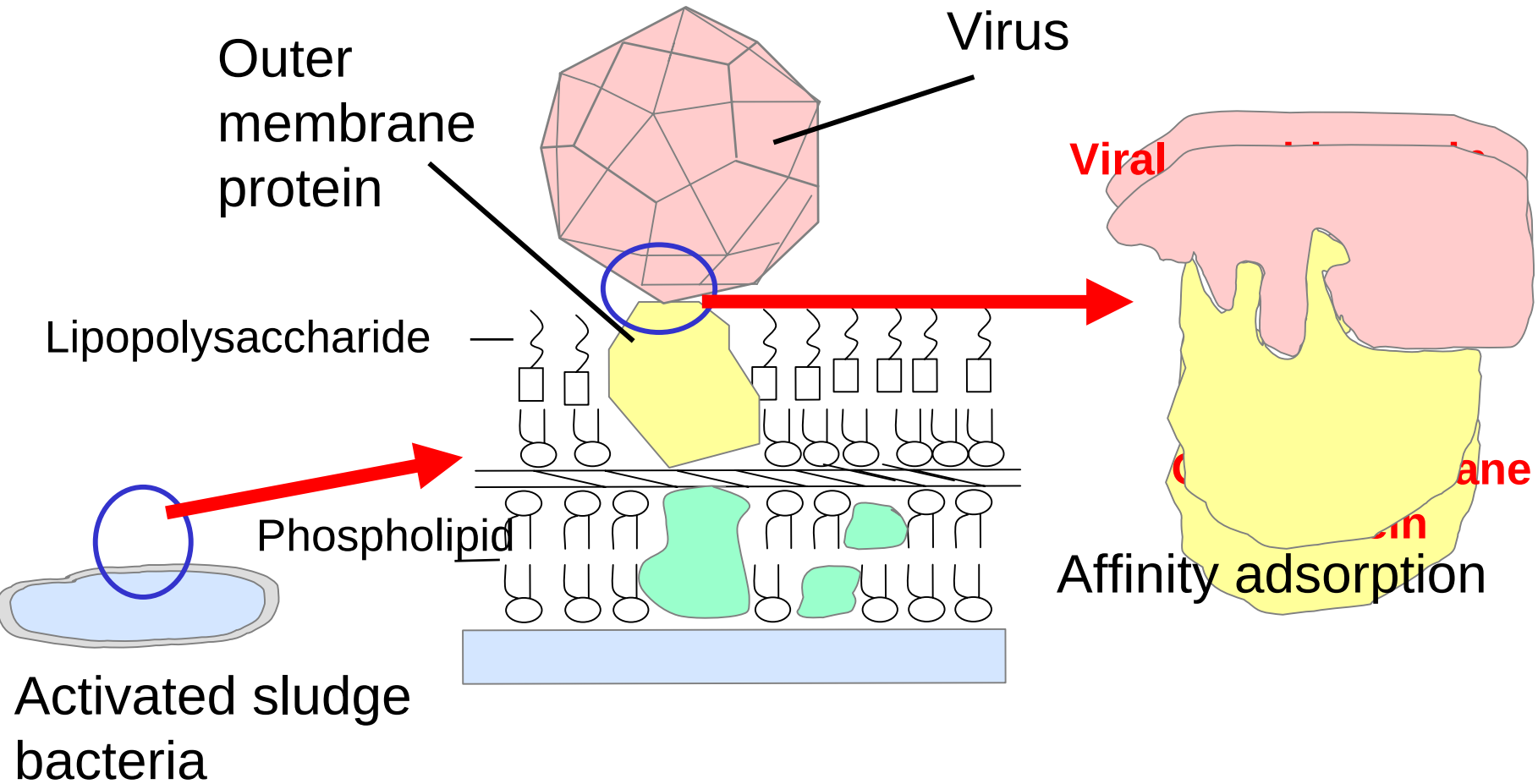
Difficulty in removing and inactivating viruses

- too small to be removed in filtration process
- high tolerance to chlorine

New approach for virus removal is need to be developed

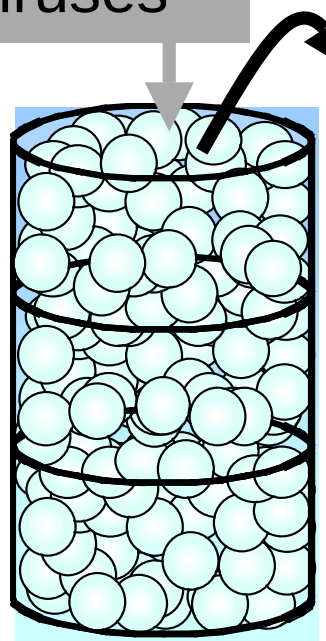
New technology for virus removal

- Viruses are well captured by activated sludge flocs
- Proteins of activated sludge bacteria play an important role in the adsorption



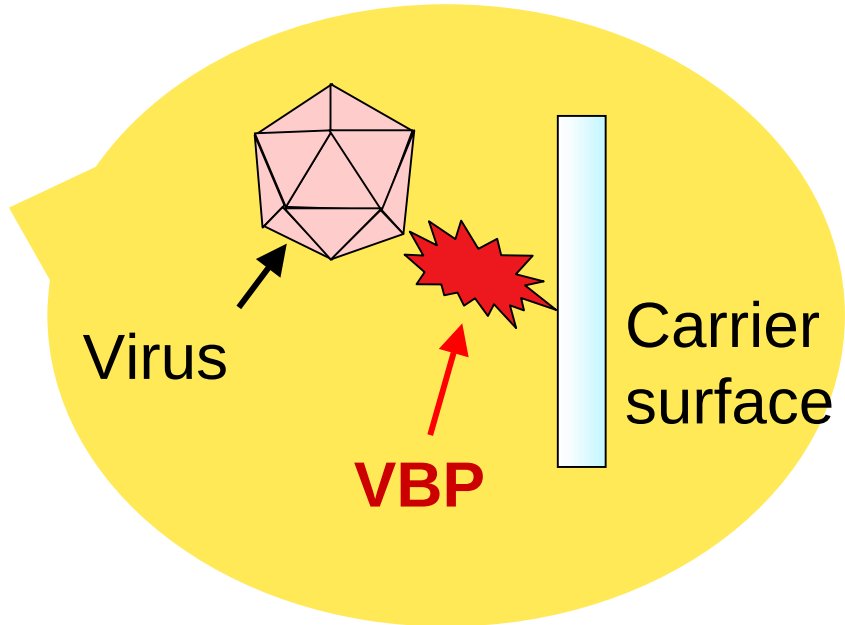
New technology for virus removal

Polluted water with pathogenic viruses



Virus removing column

Virus-free water



VBP immobilization

- Covalent binding
- Oriented immobilization

Identification of VBPs

Identification of VBPs

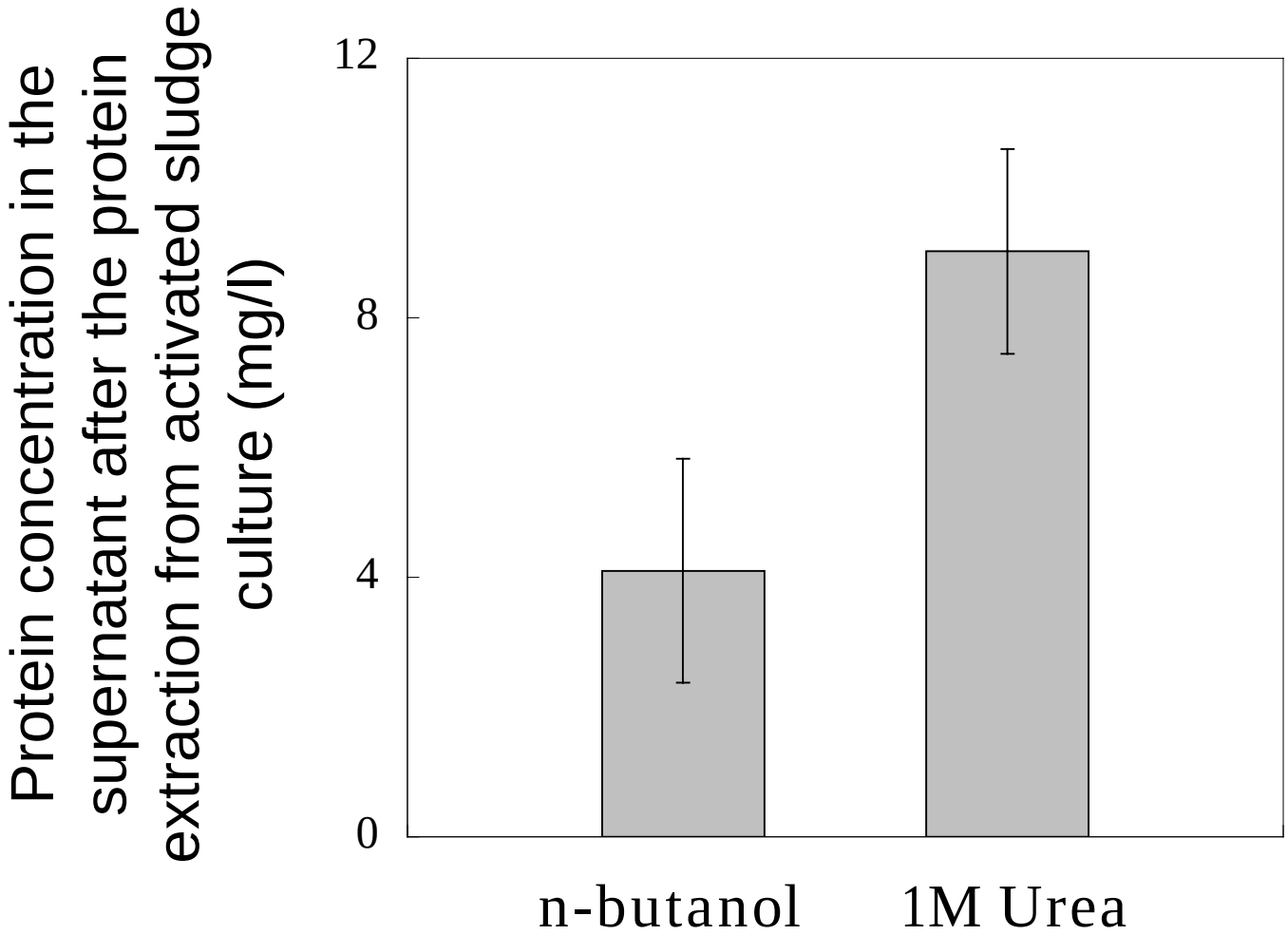
Isolation and characterization of VBPs

- Cultivation of activated sludge bacteria
- Extraction of bacterial proteins with urea
- VBP isolation with affinity chromatography
- Evaluation of virus binding ability of VBPs with ELISA
- Estimation of molecular weight of VBPs with SDS-PAGE
- Evaluation of net surface charge of VBPs with ion exchange chromatography

Identification of VBPs

- Separation of VBPs with 2-dimensional electrophoresis
- Determination of N terminal amino acid sequences
- Homology search for amino acid sequences

Extraction of bacterial proteins

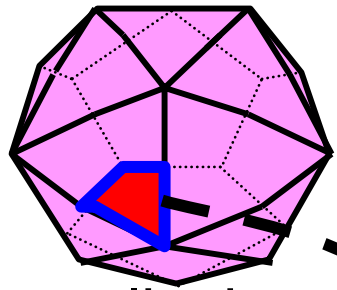


VBP isolation with affinity chromatography

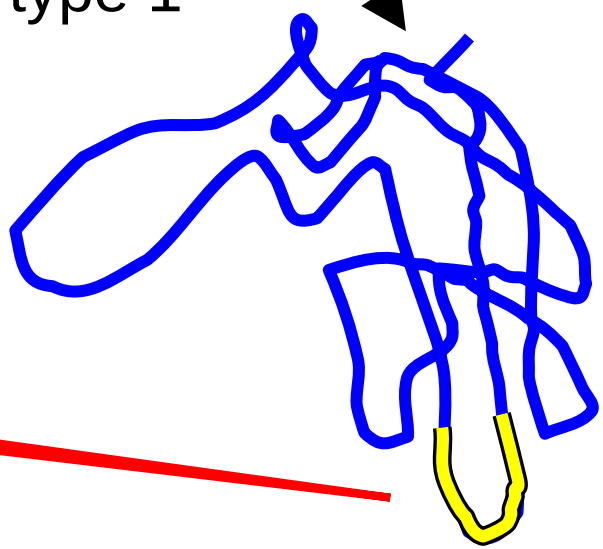
Affinity ligand

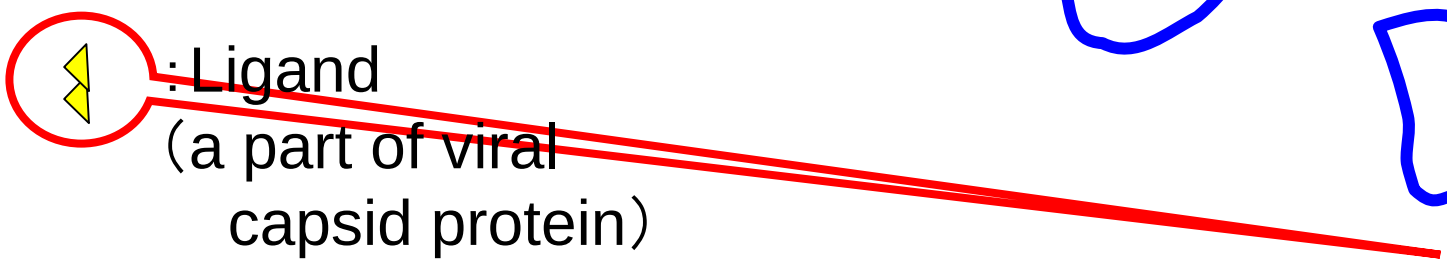
NH_2-DNPASTTNKDKL-COOH

(Hogle, J. et al., 1985)



Poliovirus type 1





：Ligand
（a part of viral capsid protein）

Protruding part responsible for antigen-antibody interaction

VBP isolation with affinity chromatography

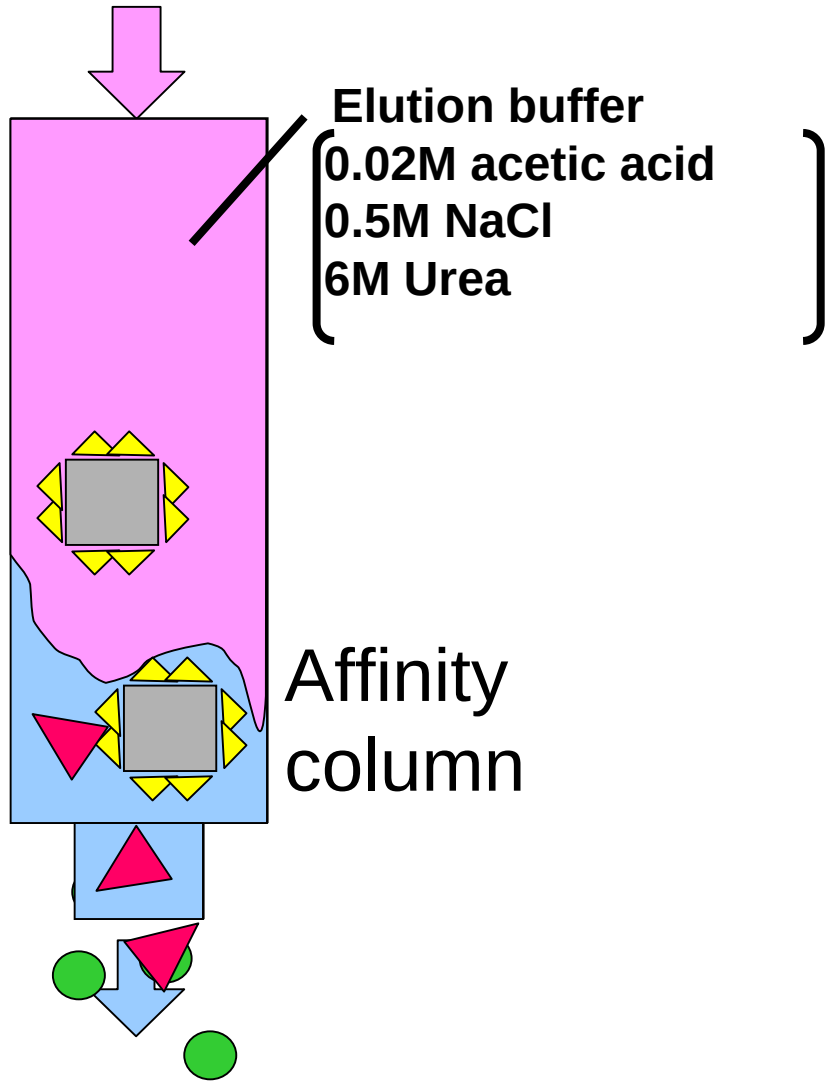
：VBP

：Impurities

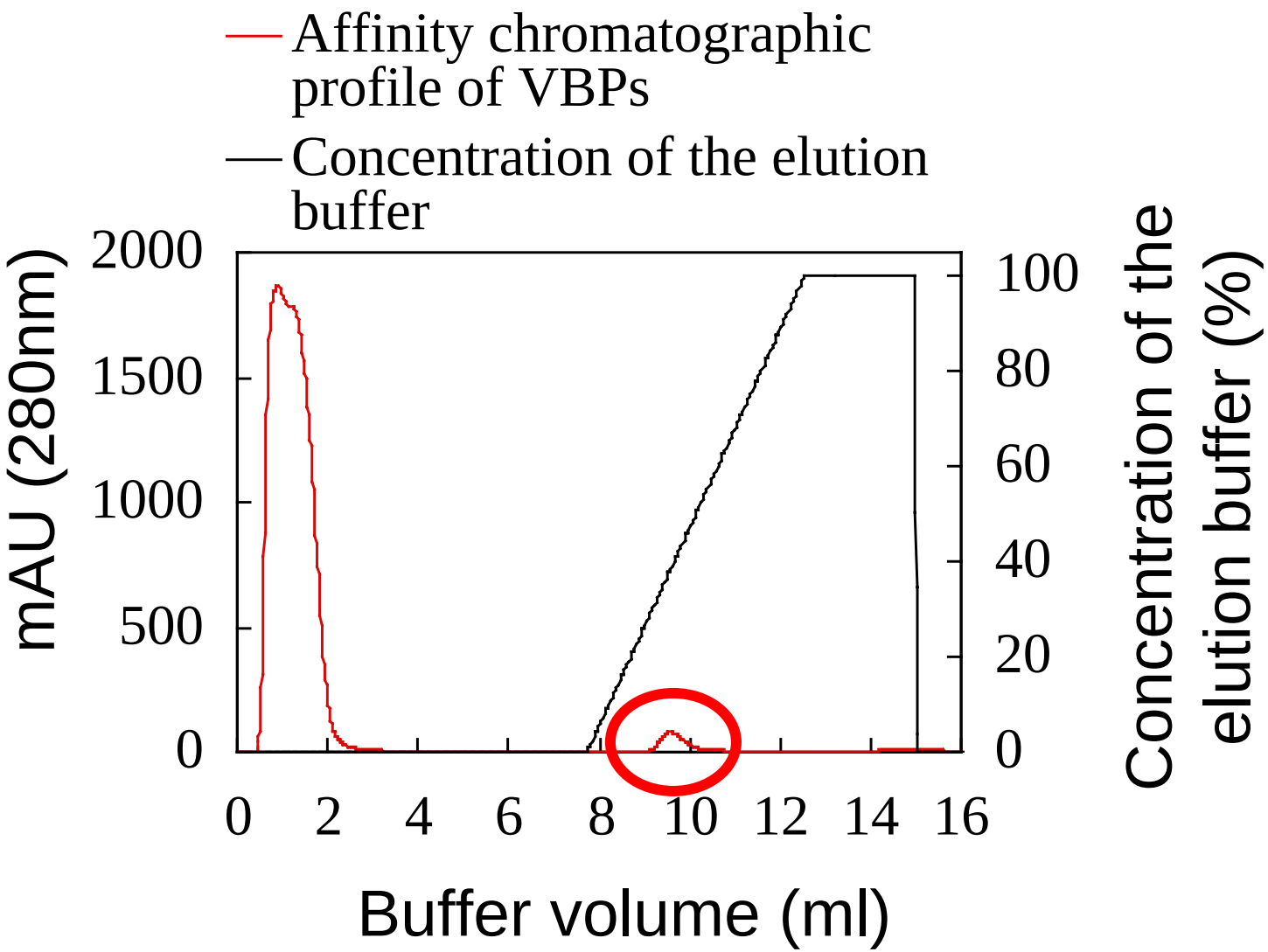
：carrier
(NHS activated)

：Ligand
（a part of viral
capsid protein

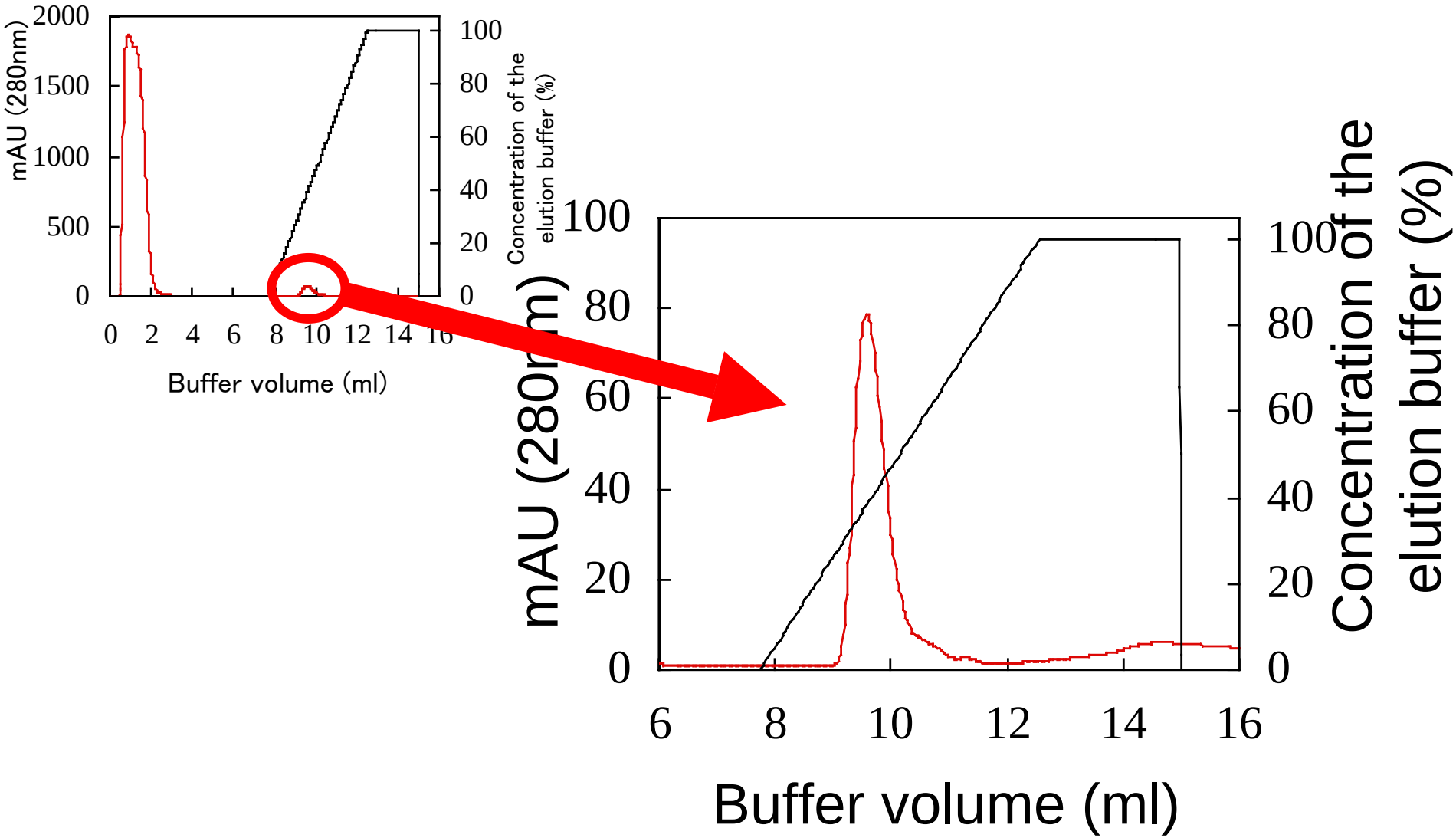
- Start buffer: **Tris-HCl**
（**2mM**, **pH8.0**）



VBP isolation with affinity chromatography



VBP isolation with affinity chromatography



Evaluation of virus binding ability of VBP with ELISA

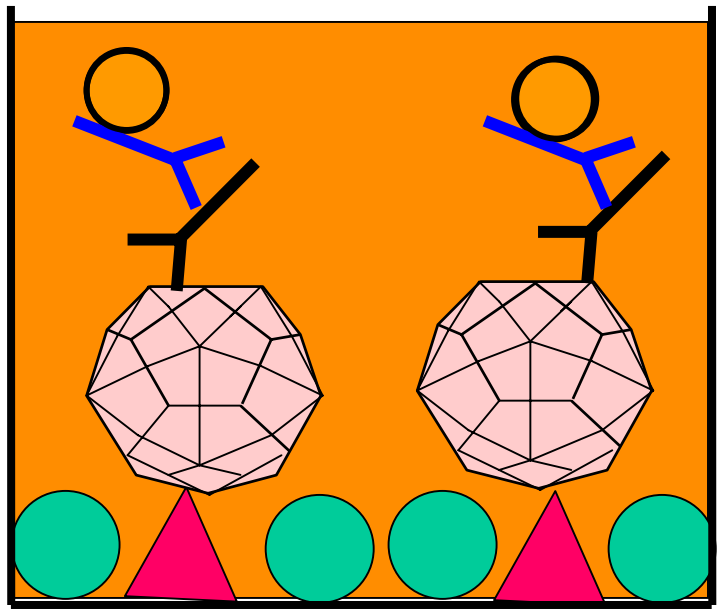
: VBP

: Bovine serum albumin

: Poliovirus type 1 (PV1)

: Primary antibody
（Mouse anti-PV1 antibody）

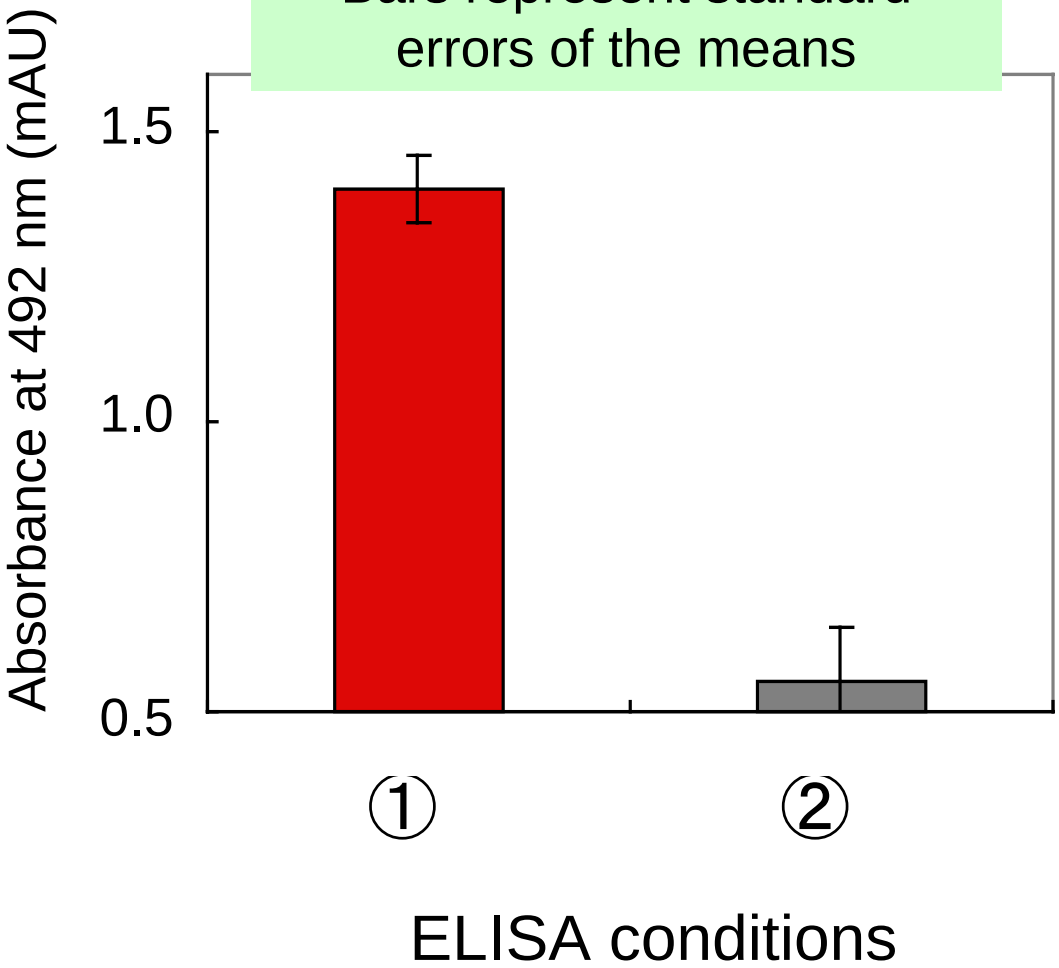
: Secondary antibody modified with HRP
（rabbit anti-mouse antibody）



ELISA plate

Evaluation of virus binding ability of VBP with ELISA

Bars represent standard errors of the means

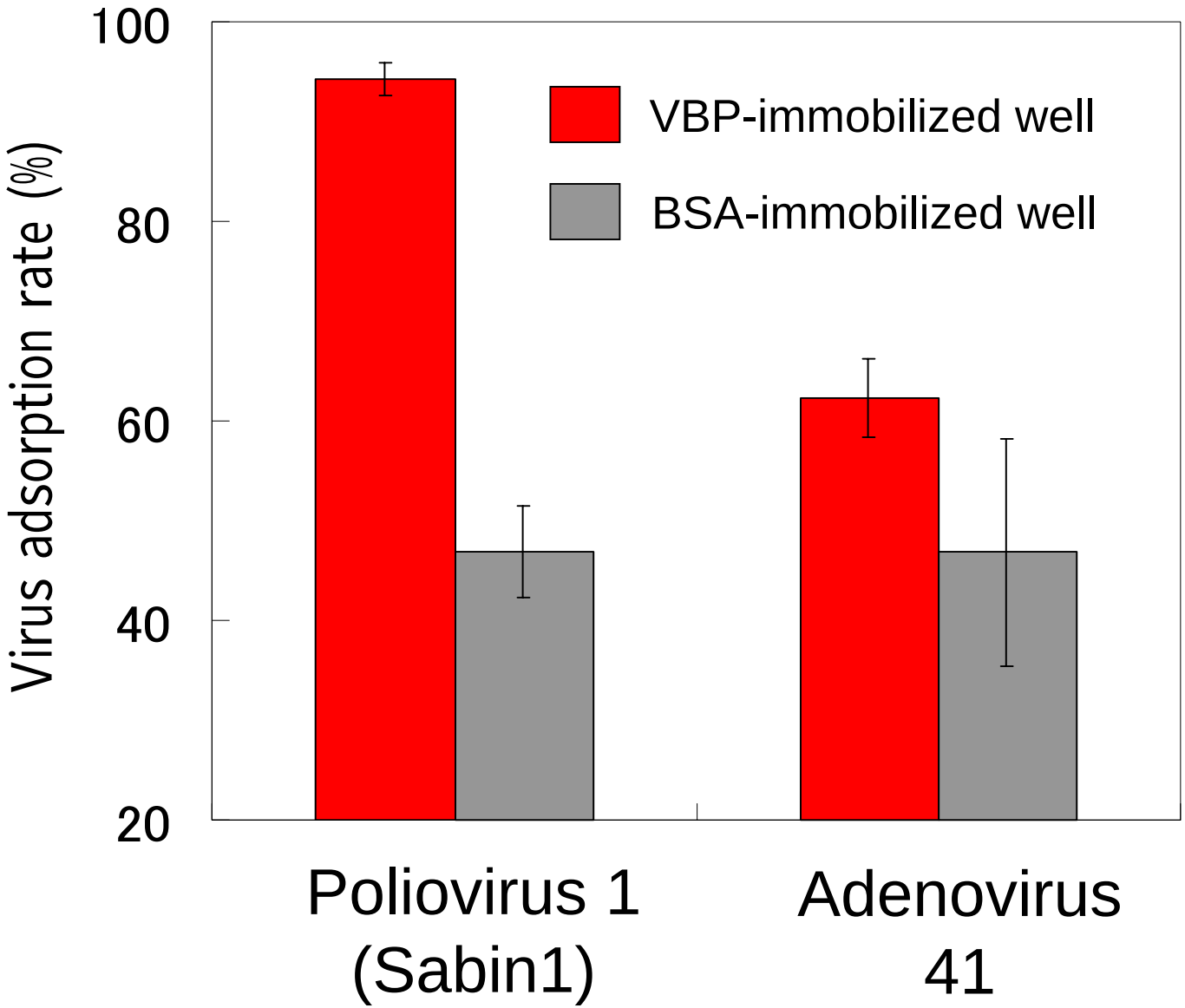


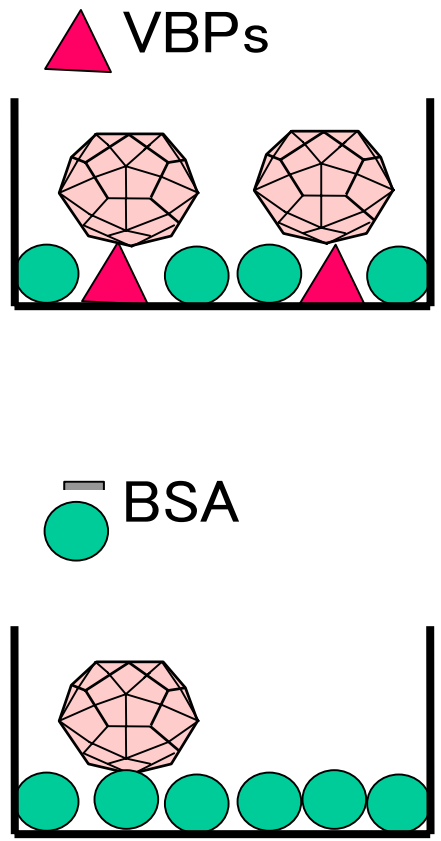
①　②

①：PV1 was added to VBP-immobilized well.

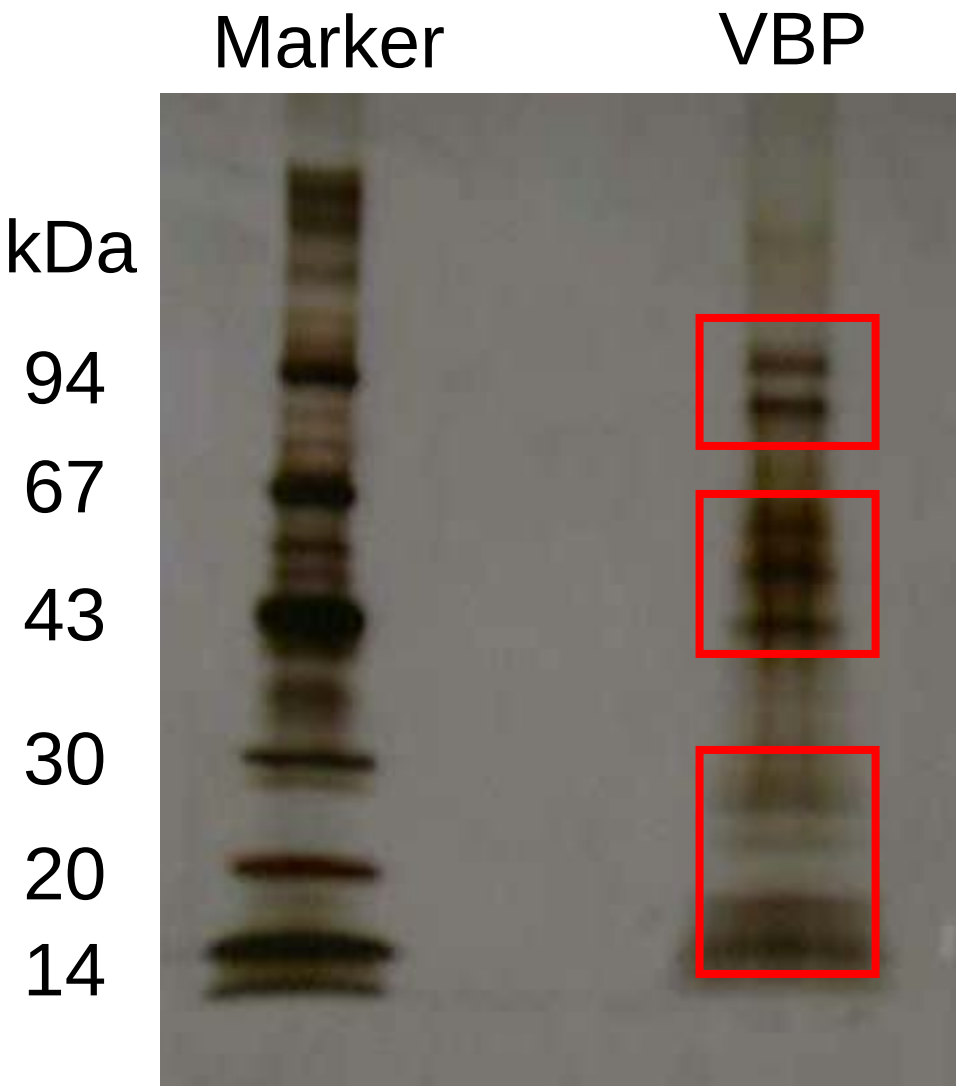
②：PV1 was not added to VBP-immobilized well.

Evaluation of virus binding ability of VBP





Estimation of molecular weight of VBP with SDS-PAGE

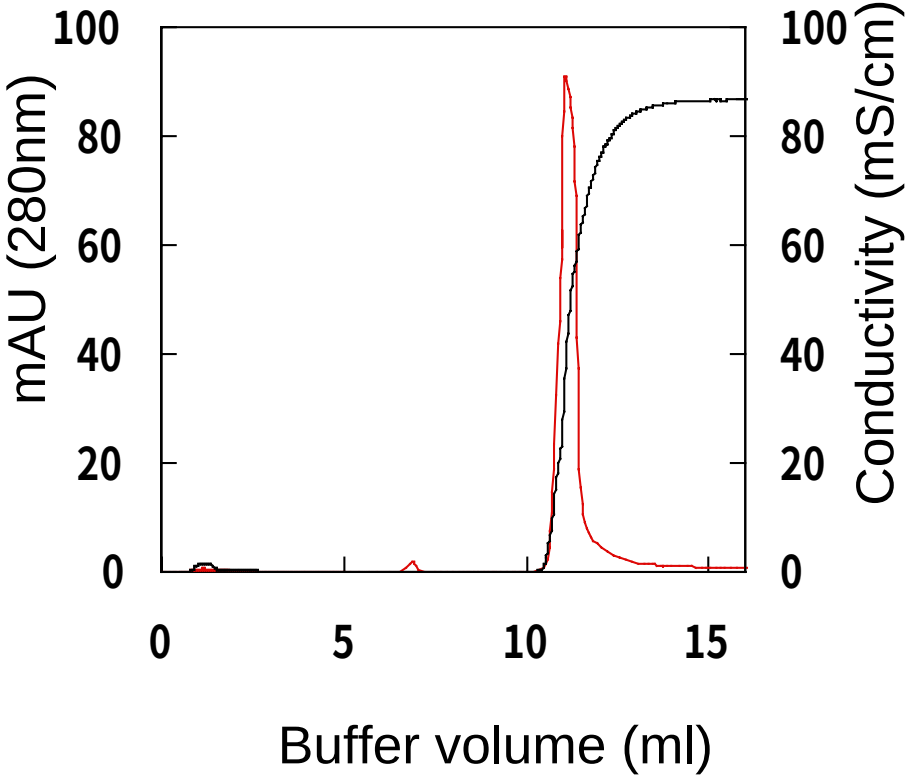


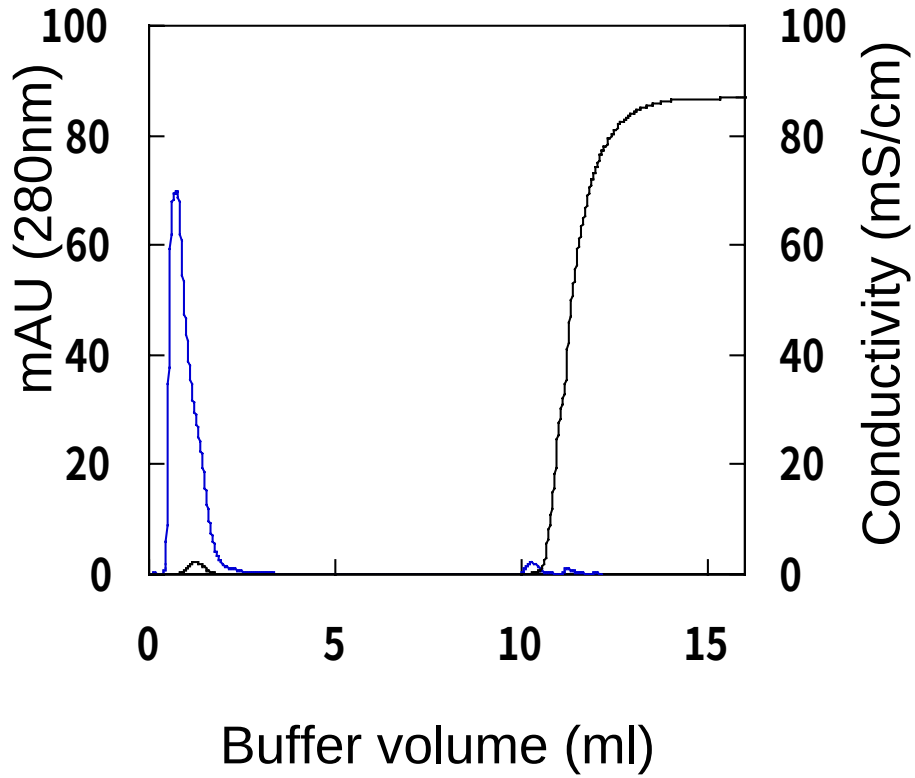
Evaluation of net surface charge of VBP with ion exchange chromatography

: Anion exchange chromatographic profile of VBPs

: Cation exchange chromatographic profile of VBPs

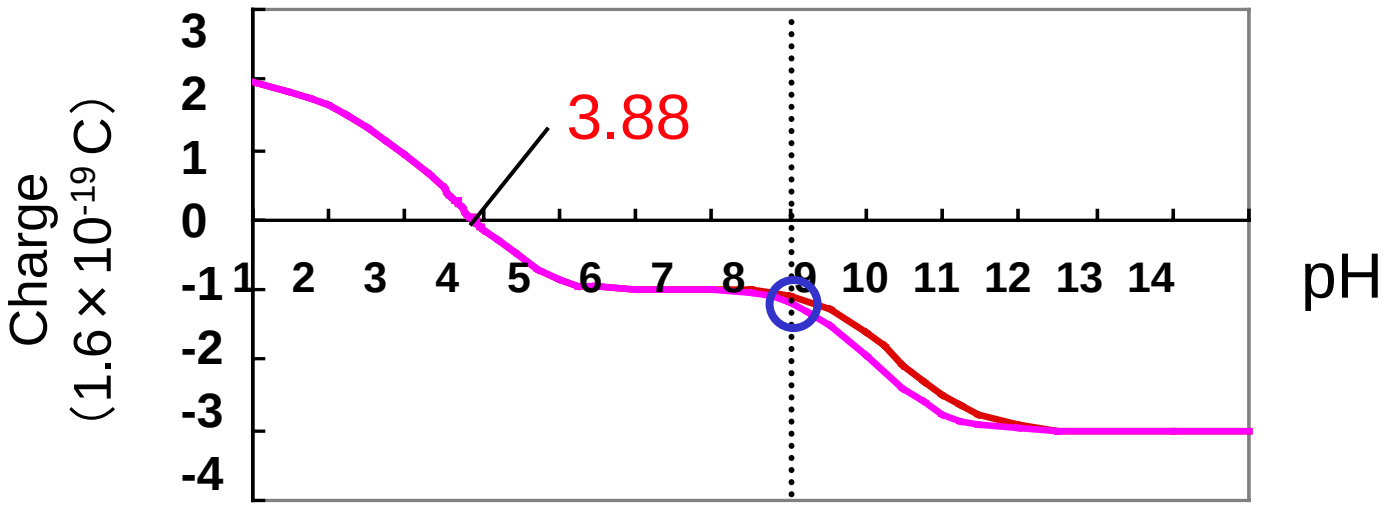
: Conductivity





Evaluation of net charge of viral capsid peptide in affinity column

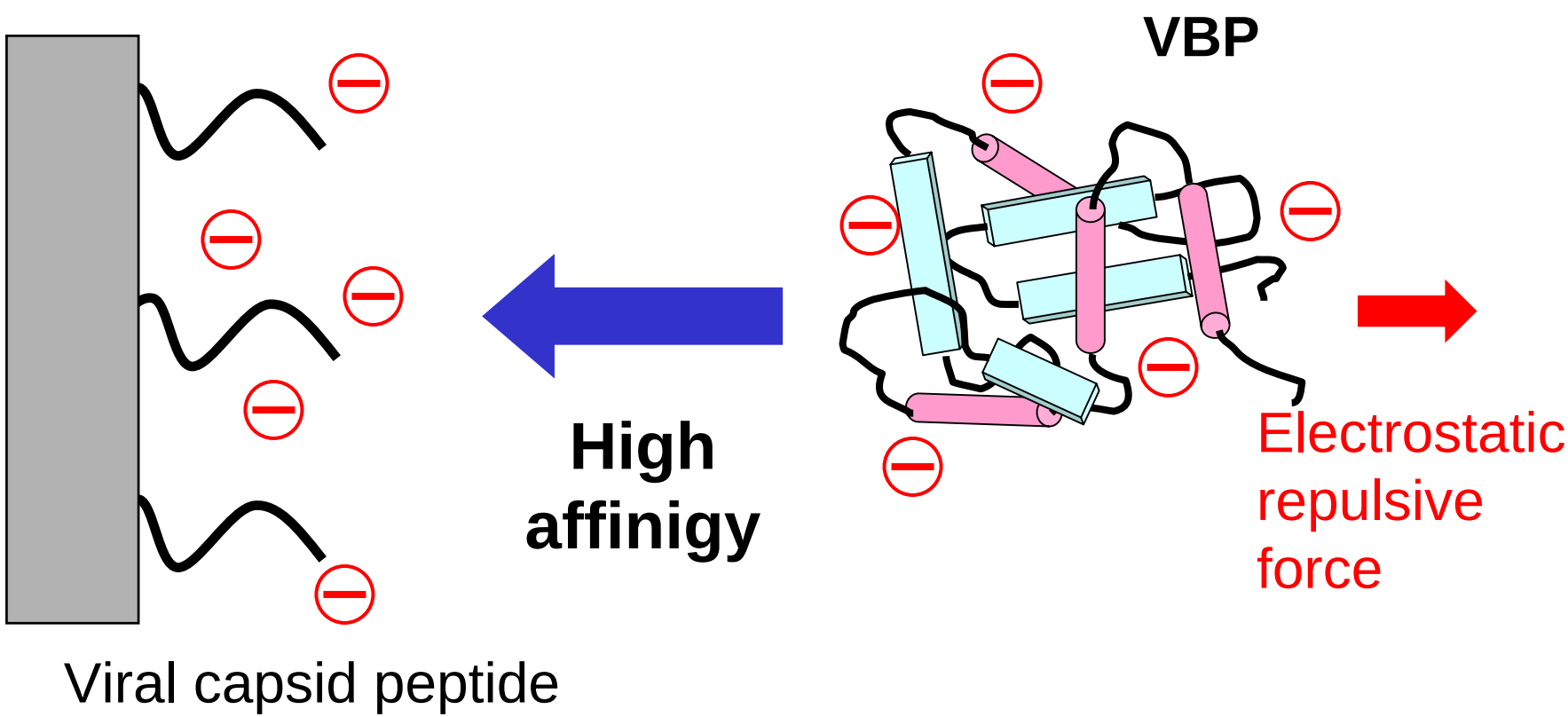
- Poliovirus capside peptide is negatively charged
  (NH_2-DNPASTTNKDKL-COOH)



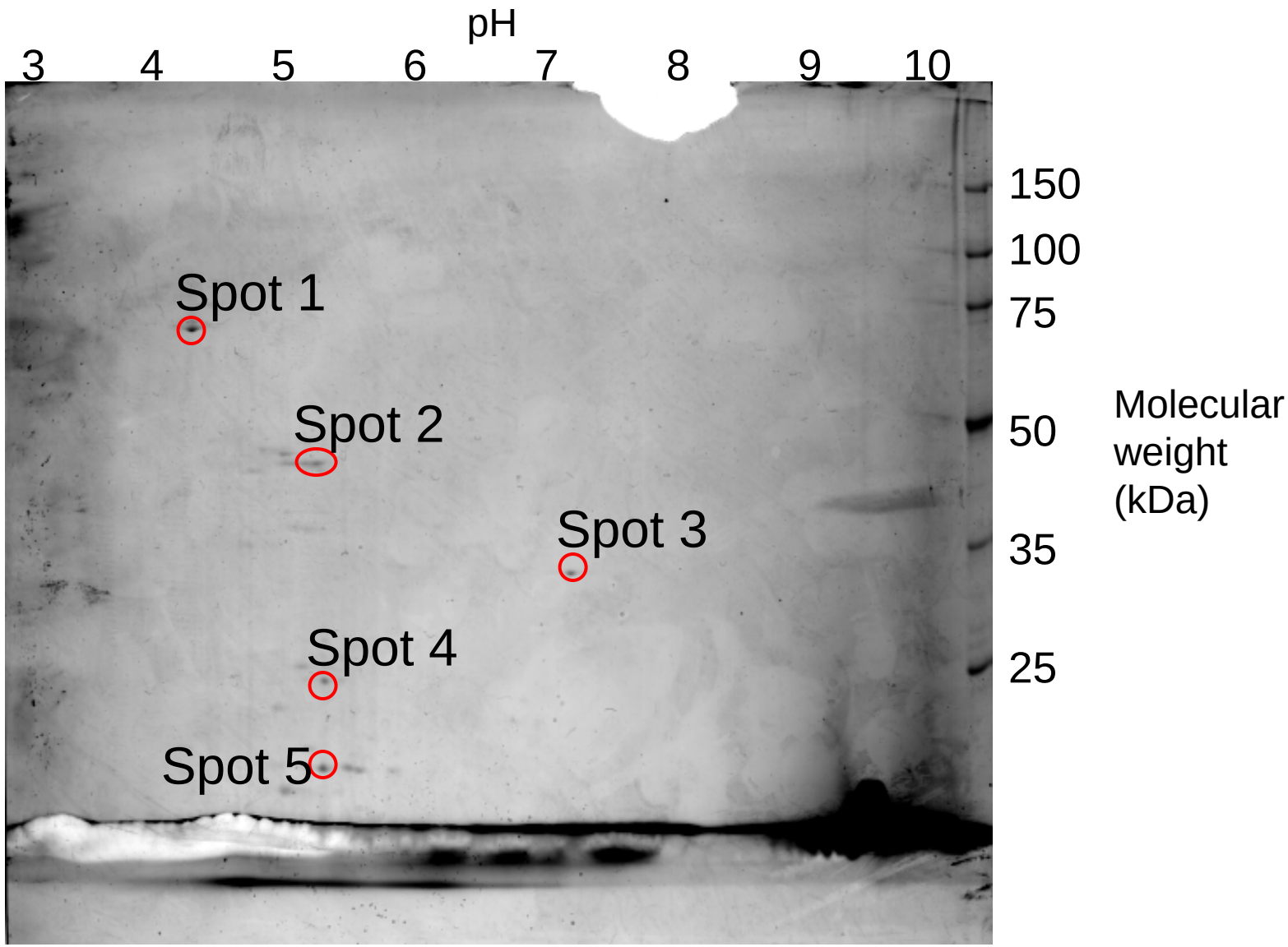
$$RNH_3^+ \rightleftharpoons RNH_2 + H^+$$

$$\begin{cases} K = \dfrac{[RNH_2][H^+]}{[RNH_3^+]} \\ [RNH_2] + [RNH_3^+] = X \end{cases} \Rightarrow [RNH_3^+] = \frac{X[H^+]}{K + [H^+]}$$

Interaction between VBPs and viral capsid peptide



Separation of VBP with 2-dimensional electrophoresis



Determination of amino acid sequences of VBPs

Spot Number	N-terminus → direction of analys																								
	1				5					10					15					20						25
1	A	V	V	Y	D	K	D	G	T	S	F	D	I	Y	G	R	V	Q	A	N	Y	Y			
2	V	D	F	H	G	Y	F	R	P	Q	V	G													
3	M	D	X	Q	E	D	X	A																	
4	A	D	Y	S	G	D	I	H	K	N	D	Y	K	W	F	Q	F	N	L	M	G	T	X	D	X	L
5	G	D	A	F	S	Y	A	K	G	S	X	T	G	A	H	T	K	S	D	Y						
	1				5					10					15					20						25

A: alanine, V: valine, Y: tyrosine, D: aspartic acid, K: lysine, G: glycin, T: threoni

F: phenylalanine, I: isoleucine, R: arginine, Q: glutamine, N: asparagine, H: histidir

M: methionine, E: glutamic acid, W: tryptophan, L: leucine, X: nonidentifiable.

Homology search of VBP sequences

Homology search of the determined amino acid sequences of VBPs was conducted against all amino acid sequences (more than 1.6 million sequences) in protein data bases (DAD, PRF, PIR, Swiss-Prot) with the Blast program provieded by DNA Data Bank of Japan on the web.

DAD: DNA Data Bank of Japan, Amino Acid Sequence Database

PRF: Protein Research Fundation (Japan)

PIR: Protein Identification Resource, (Japan, USA and Europe)

SWISS-PROT (Switzerland)

PDB: Protein Data Bank (USA)

Homology search of amino acid sequences of VBPs

Spot Number	Number of analyzed residue	Molecular weight of VBPs (kDa)	Number of proteins that have more than 80 % homology	Proteins that provoke the highest homology			
				Name of protein	number of residue	Homology	The site with high homology
1	22	75	5	Aeromonas hydrophila outer membrane protein	355	90	21-42
4	26	25	2	Vibrio cholerae outer membrane protein OmpK protein	296	81	51-77

VBP cloning and evaluation of virus binding protein of VBP clone

VBP cloning

Isolation of VBP gene

- Construction of DNA probe
-Construction of DNA library for activated sludge bacteria
- Colony hybridization

VBP cloning

- Construction of a vector for VBP cloning
- VBP expression and purification

Evaluation of virus binding ability of VBP clone

- ELISA

Construction of DNA probe

DNA probes for VBPs in Spot 1 and Spot 4 were constructed.

Amino acid sequence　　DNA sequence

Spot 1

A	V	V	Y	D	K	D	G	T	S	F	D	I	Y	G	R	V	Q	A	N	Y	Y
gct	gtt	gtt	tat	gat	aaa	gat	ggt	act	tct	ttt	gat	att	tat	ggt	cgt	gtt	caa	gct	aat	tat	tat
gcc	gtc	gtc	tac	gac	aag	gac	ggc	acc	tcc	ttc	gac	atc	tac	ggc	cgc	gtc	cag	gcc	aac	tac	tac
gca	gta	gta					gga	aca	tca			ata		gga	cga	gta		gca			
gcg	gtg	gtg					ggg	acg	tcg					ggg	cgg	gtg		gcg			
									agt	36864 sets					aga						
									agc						agg						

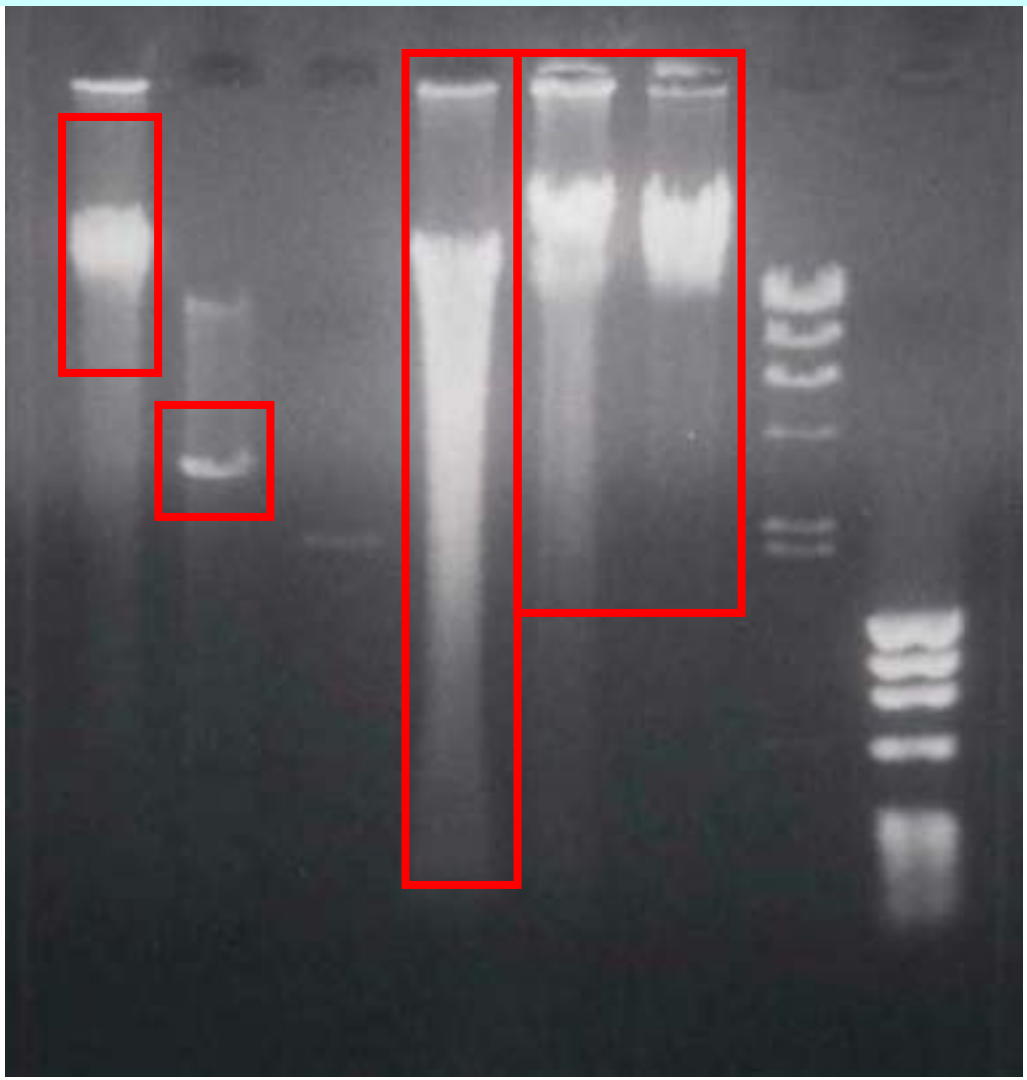
Spot 4

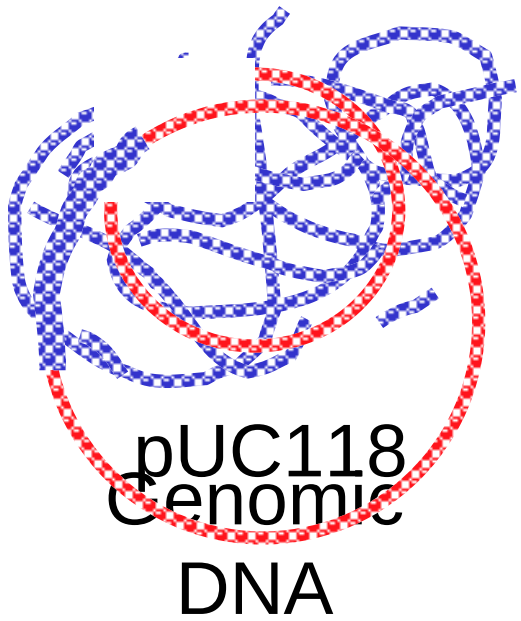
A	D	Y	S	G	D	I	H	K	N	D	Y	K	W	F	Q	F	N	L	M	G	T
gct	gat	tat	tct	ggt	gat	att	cat	aaa	aat	gat	tat	aaa	tgg	ttt	caa	ttt	aat	tta	atg	ggt	act
gcc	gac	tac	tcc	ggc	gac	atc	cac	aag	aac	gac	tac	aag		ttc	cag	ttc	aac	ttg		ggc	acc
gca			tca	gga		ata												ctt		gga	aca
gcg			tcg	ggg														ctc		ggg	acg
			agt											6144 sets				cta			
			agc															ctg			

Letters in red are probe regions

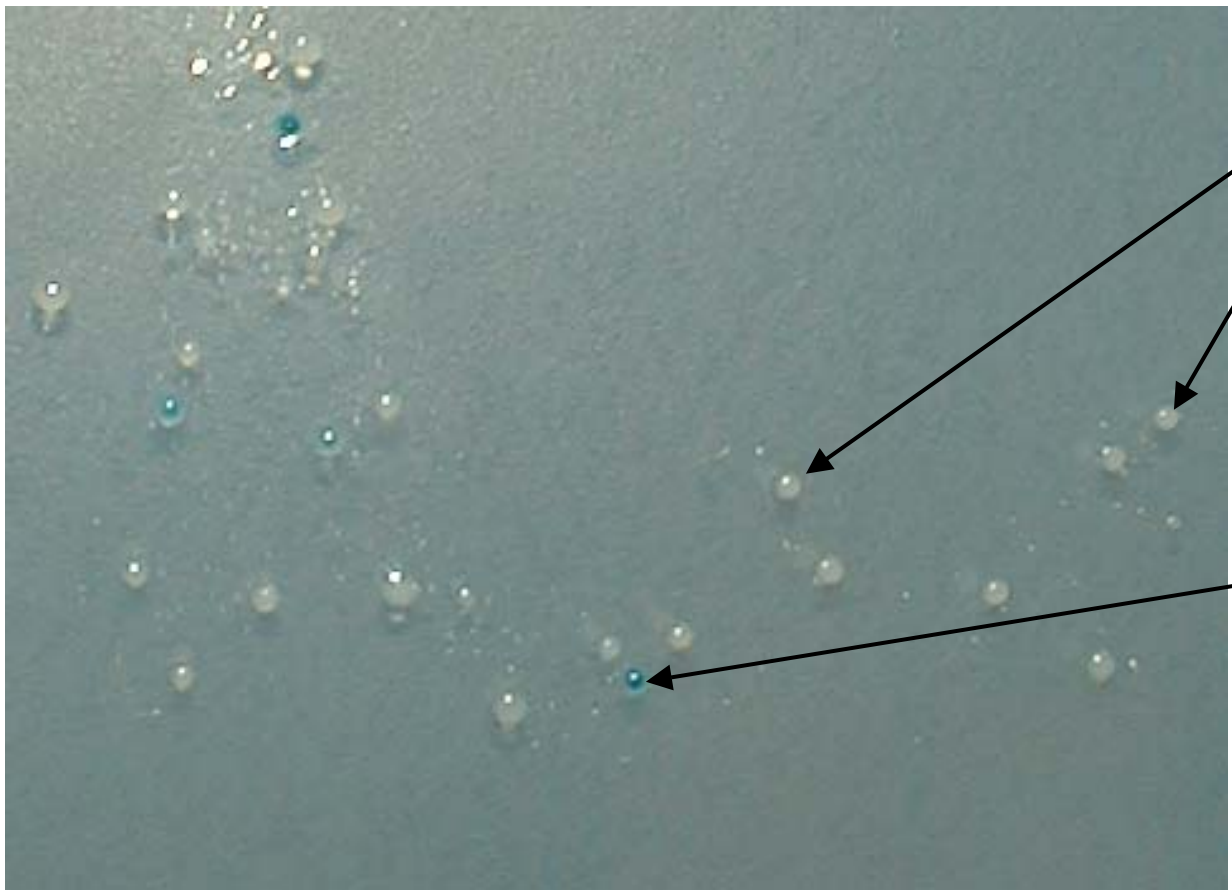
Construction of DNA library for activated sludge bacteria

pUC118 with extracted genomic DNA





Construction of DNA library for activated sludge bacteria



White colony has pUC118 with insert DNA.

Blue colony has pUC118 without insert DNA.

DNA library for activated sludge bacteria was used for isolating VBP genes with colony hybridization.

Construction of DNA library for activated sludge bacteria

Library code	No. of white colony	No. of blue colony	The ratio of No. of white colony to No. of blue colony
DH5α-pUC118 A	1825	175	10.4
DH5α-pUC118 B	3750	650	5.77
DH5α-pUC118 C	2150	500	4.30
DH5α-pUC118 D	2550	275	9.27

Two thousand white colonies can cover whole length of genomic DNA, because the insert DNA is 2 kbp in length and genomic DNA of bacteria is generally 4 million bp in length.

Isolation of VBP gene with colony hybridization

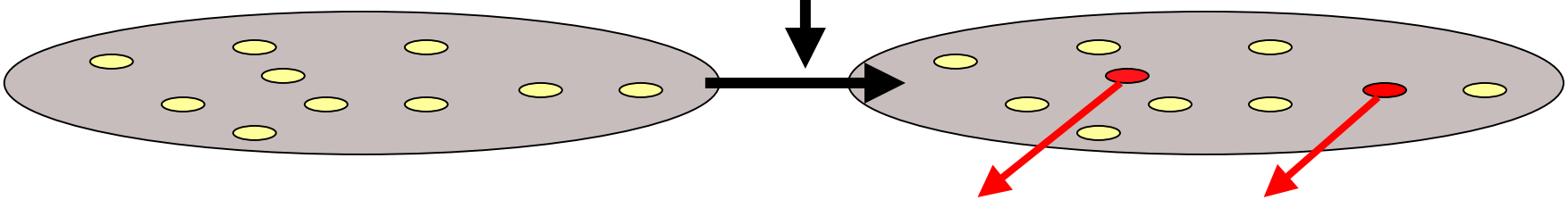
DNA probe

Example: DNA probe for VBP in Spot 4

DIG-5'- g a y a t h c a y a a r ～～～c a r t t y a a y -3'

DNA library for activated sludge bacteria

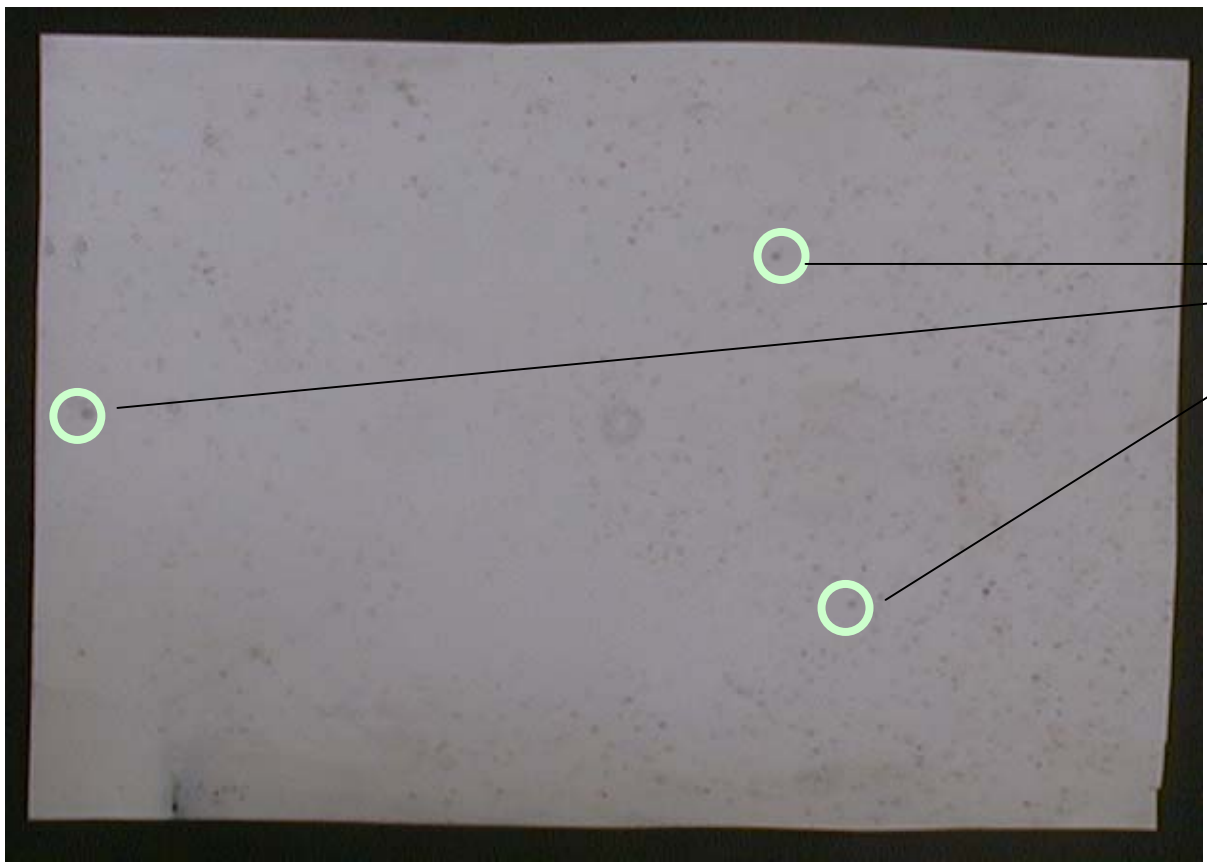
Colony hybridization



Colored colonies were picked up

Isolation of VBP gene with colony hybridization

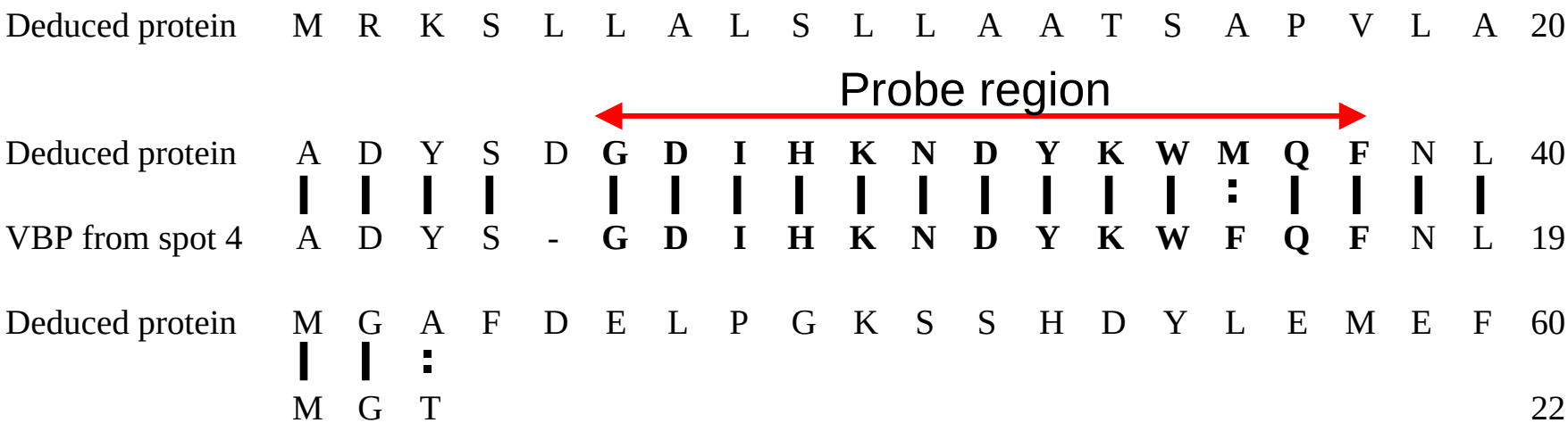
Colored filter



Colored spots were picked up.

Isolation of VBP gene with colony hybridization

One sequence that have almost the same sequence with VBP in spot 4 was isolated.

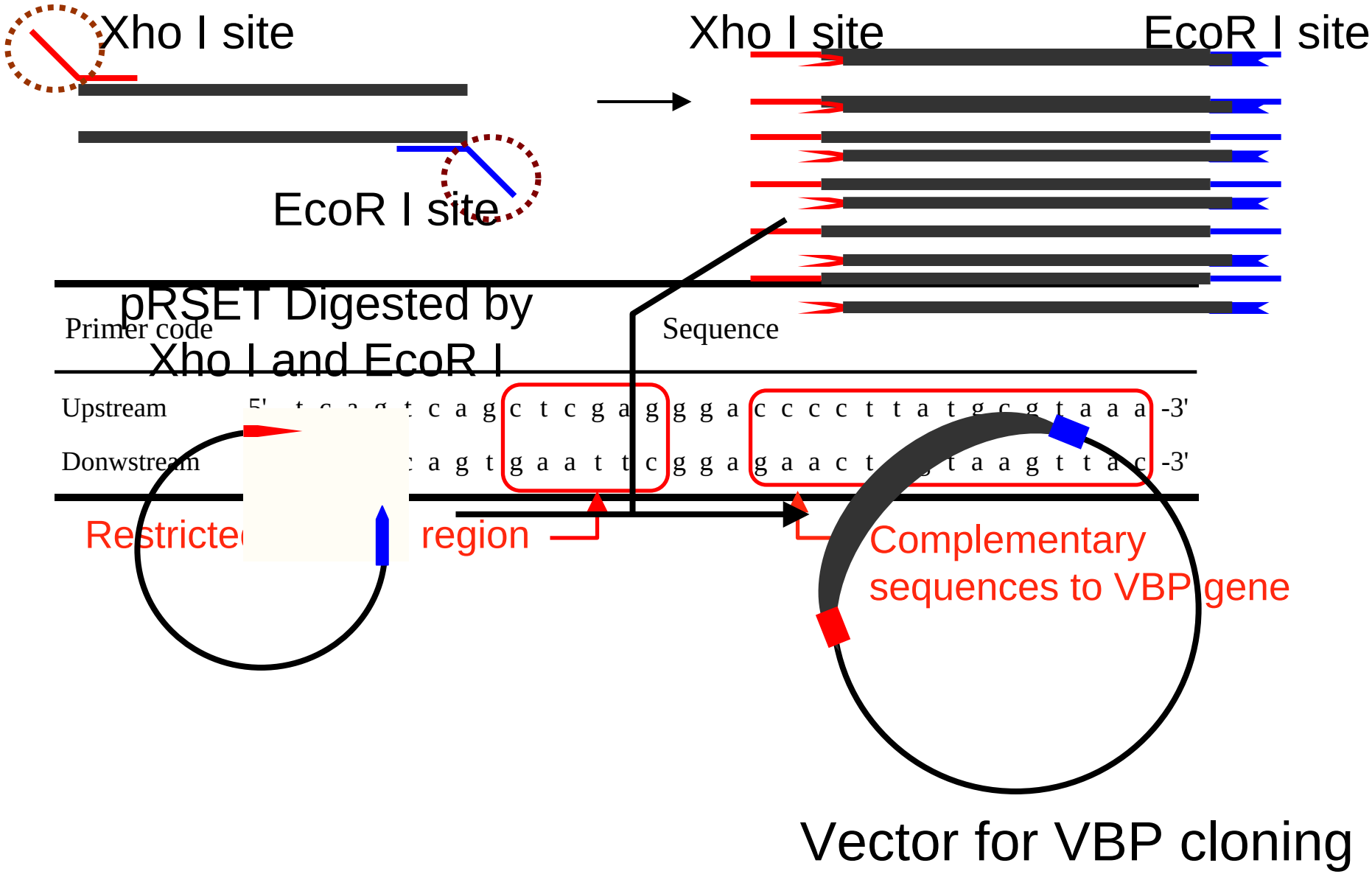


ORF	1	atg	cgt	aaa	tca	ctt	ctt	gct	tta	agc	cta	ttg	gca	gca	act	tca	gca	cct	gta	ctg	gcc
Deduced protein	1	M	R	K	S	L	L	A	L	S	L	L	A	A	T	S	A	P	V	L	A
ORF	61	gct	gac	tac	tca	gat	ggc	gat	atc	cac	aaa	aac	gat	tac	aag	tgg	atg	caa	ttt	aac	ctg
Deduced protein	21	A	D	Y	S	D	G	D	I	H	K	N	D	Y	K	W	M	Q	F	N	L
ORF	121	atg	ggt	gca	ttc	gac	gaa	ctt	cca	ggc	aaa	tca	tct	cat	gat	tat	ctg	gaa	atg	gaa	ttt
Deduced protein	41	M	G	A	F	D	E	L	P	G	K	S	S	H	D	Y	L	E	M	E	F
ORF	181	ggc	ggt	cgt	tct	ggc	atc	ttt	gac	ctg	tac	ggt	tac	gtt	gat	gtg	ttc	aac	ctg	acc	agt
Deduced protein	61	G	G	R	S	G	I	F	D	L	Y	G	Y	V	D	V	F	N	L	T	S
ORF	241	gac	aaa	ggc	agc	gac	aaa	aac	ggc	aaa	gaa	aaa	atc	ttc	atg	aag	ttt	gct	cca	cgt	gtg
Deduced protein	81	D	K	G	S	D	K	N	G	K	E	K	I	F	M	K	F	A	P	R	V
ORF	301	tca	ctg	gat	gca	ttg	act	ggc	gcg	gat	atg	tca	ttt	ggc	cca	gta	caa	gaa	atg	tac	ttg
Deduced protein	101	S	L	D	A	L	T	G	A	D	M	S	F	G	P	V	Q	E	M	Y	L
ORF	361	gca	act	ctg	atc	gaa	tgg	ggc	ggt	aac	tca	gat	gtt	aac	tct	caa	aaa	atc	ggt	ctg	ggt
Deduced protein	121	A	T	L	I	E	W	G	G	N	S	D	V	N	S	Q	K	I	G	L	G
ORF	421	tca	gac	gtc	atg	gtt	cca	tgg	ttt	ggc	aaa	gtt	ggt	cta	aac	cta	tac	ggt	act	tac	gac
Deduced protein	141	S	D	V	M	V	P	W	F	G	K	V	G	L	N	L	Y	G	T	Y	D
ORF	481	tca	aat	gaa	aaa	gac	tgg	aac	ggc	ttc	acc	atc	tca	act	aac	tgg	ttt	aaa	cct	ttc	tac
Deduced protein	161	S	N	E	K	D	W	N	G	F	Q	I	S	T	N	W	F	K	P	F	Y
ORF	541	ttc	ctt	gag	aat	ggt	tca	ttc	atc	tcc	tac	caa	ggc	tat	atc	gat	tac	caa	ttt	ggt	atg
Deduced protein	181	F	L	E	N	G	S	F	I	S	Y	Q	G	Y	I	D	Y	Q	F	G	M
ORF	601	gat	aac	gat	aac	aaa	gca	tta	aac	acc	tct	aac	ggt	ggt	gca	atg	ttc	aat	ggt	att	tac
Deduced protein	201	D	N	D	N	K	A	L	N	T	S	N	G	G	A	M	F	N	G	I	Y
ORF	661	tgg	cac	tca	gat	cgc	ttc	gct	gta	ggc	tat	ggc	tta	aaa	gcc	tac	aaa	gat	gtt	tat	ggt
Deduced protein	221	W	H	S	D	R	F	A	V	G	Y	G	L	K	A	Y	K	D	V	Y	G
ORF	721	ttg	aaa	gat	gat	ggt	ctt	gct	ggt	aaa	aca	agt	gga	ttt	ggt	cac	tac	gta	gca	gta	act
Deduced protein	241	L	K	D	D	G	L	A	G	K	T	S	G	F	G	H	Y	V	A	V	T
ORF	781	tac	aag	ttc	tcc	gaa	ttc	gaa	gct	tga											
Deduced protein	261	Y	K	F	S	E	F	E	A	*											

probe like sequence

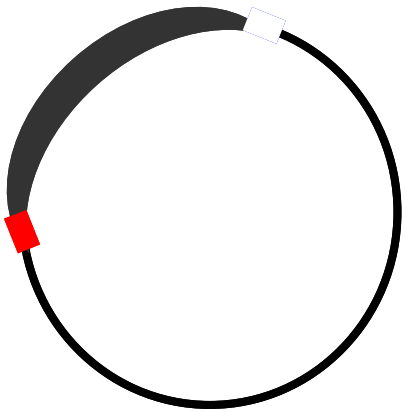
The isolated gene consisted of 807 bp and code a protein of 268 residues in length.

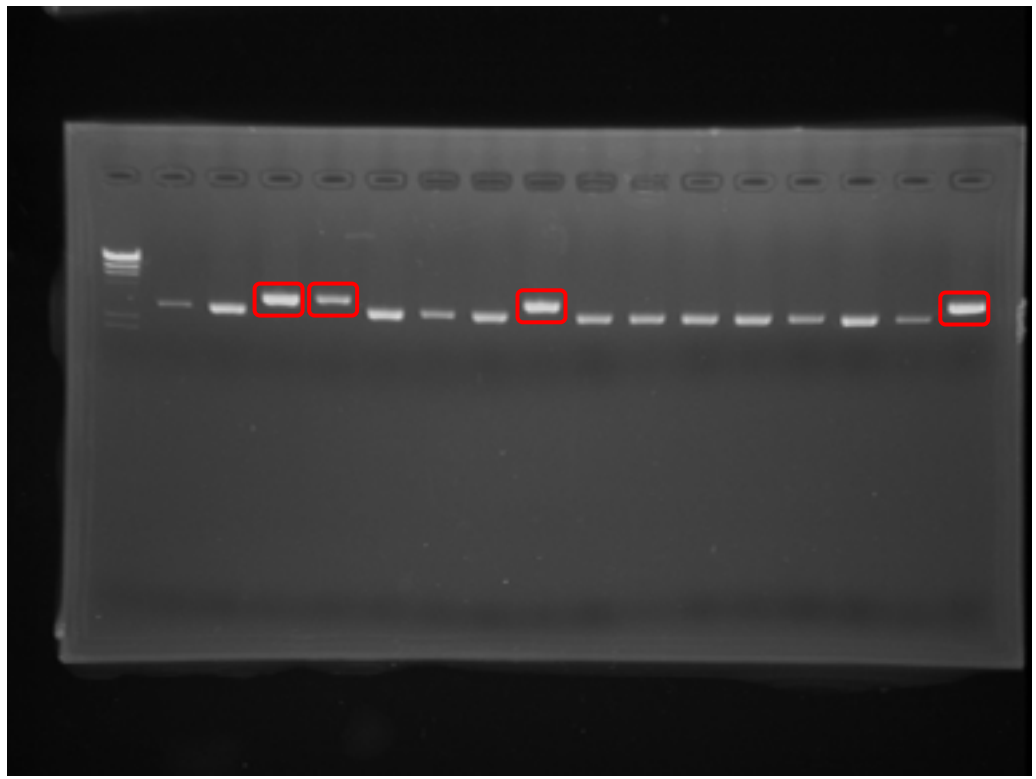
Construction of vector for VBP cloning



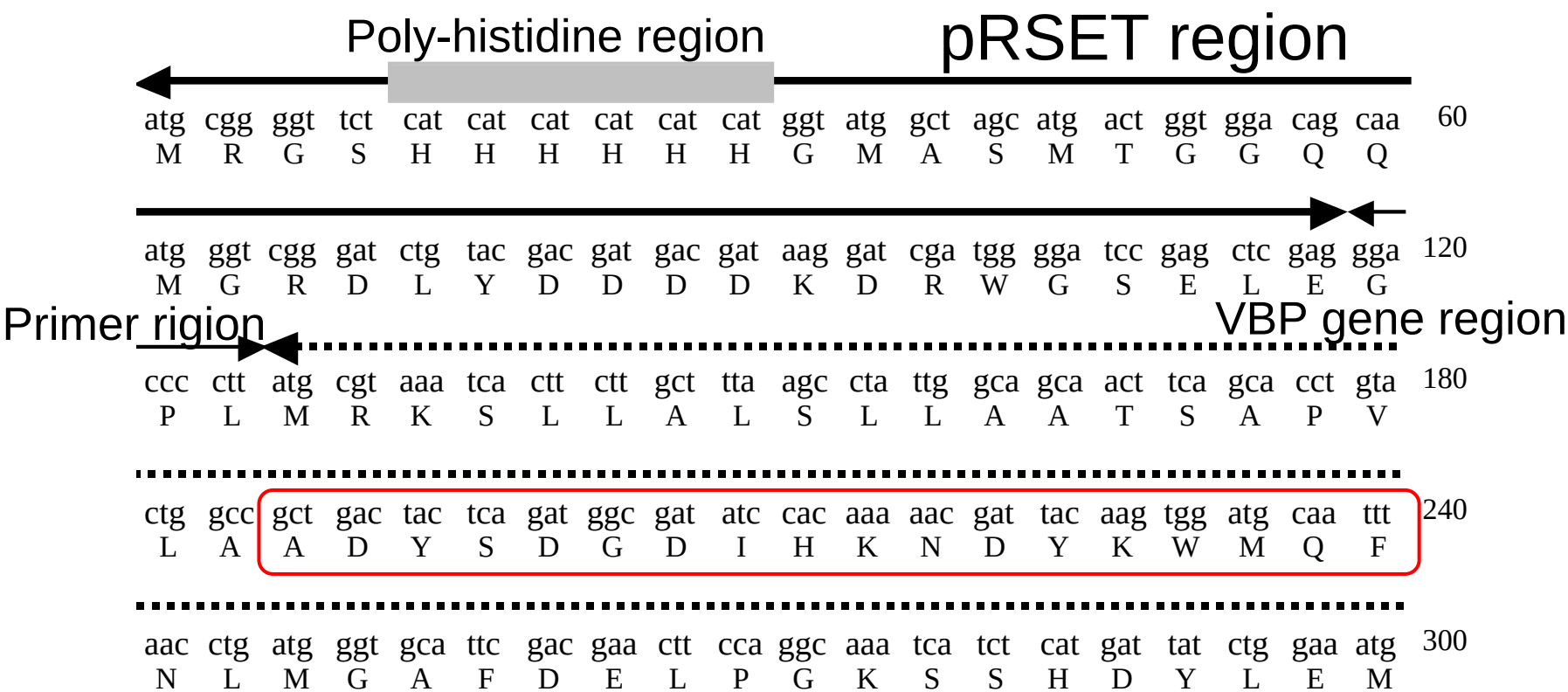
Construction of vector for VBP cloning

Vector was digested by EcoRI after ligation





Construction of vector for VBP cloning



It is expected that one protein of about 33 kDa was produced.

VBP expression and purification

⑴Transformation of *E. coli* and expression of VBP

E. coli BL21 was transformed with the constructed vector, and the expression of VBP was induced by IPTG.

IPTG: Isopropyl-beta-D-thiogalactopyranoside

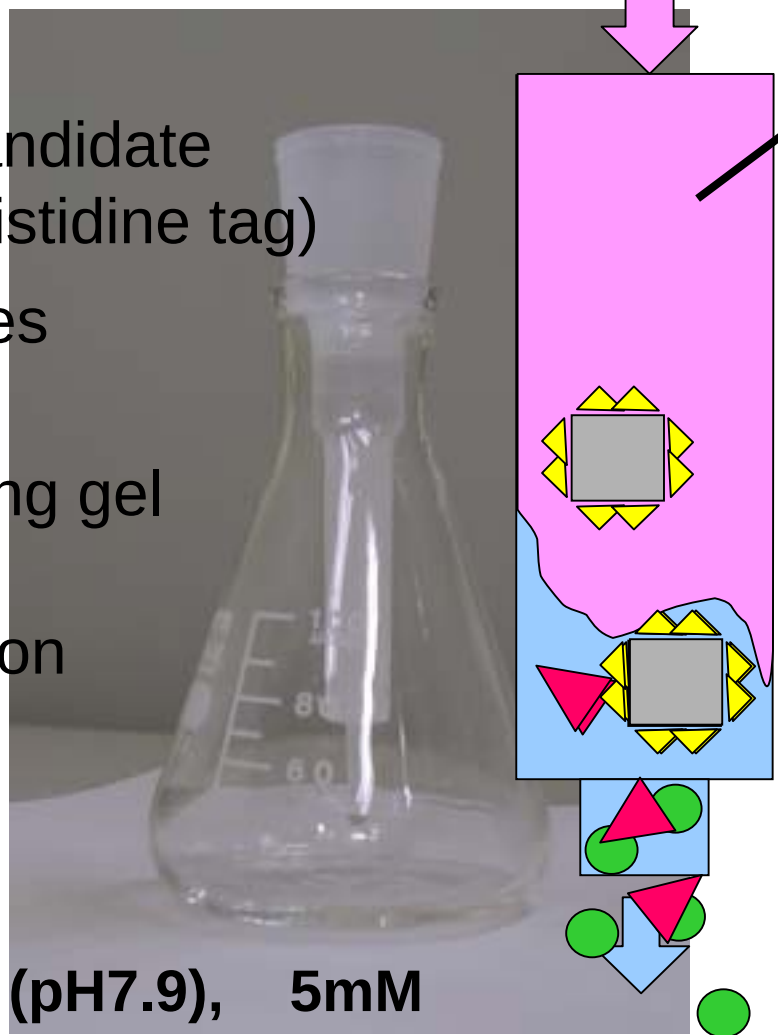
⑵Confirmation of VBP expression

VBP expression was confirmed with SDS-PAGE.

VBP expression and purification

IPTG 1mM

VBP expression and purification



: VBP candidate (with Histidine tag)

: Inpurities

: Chelating gel

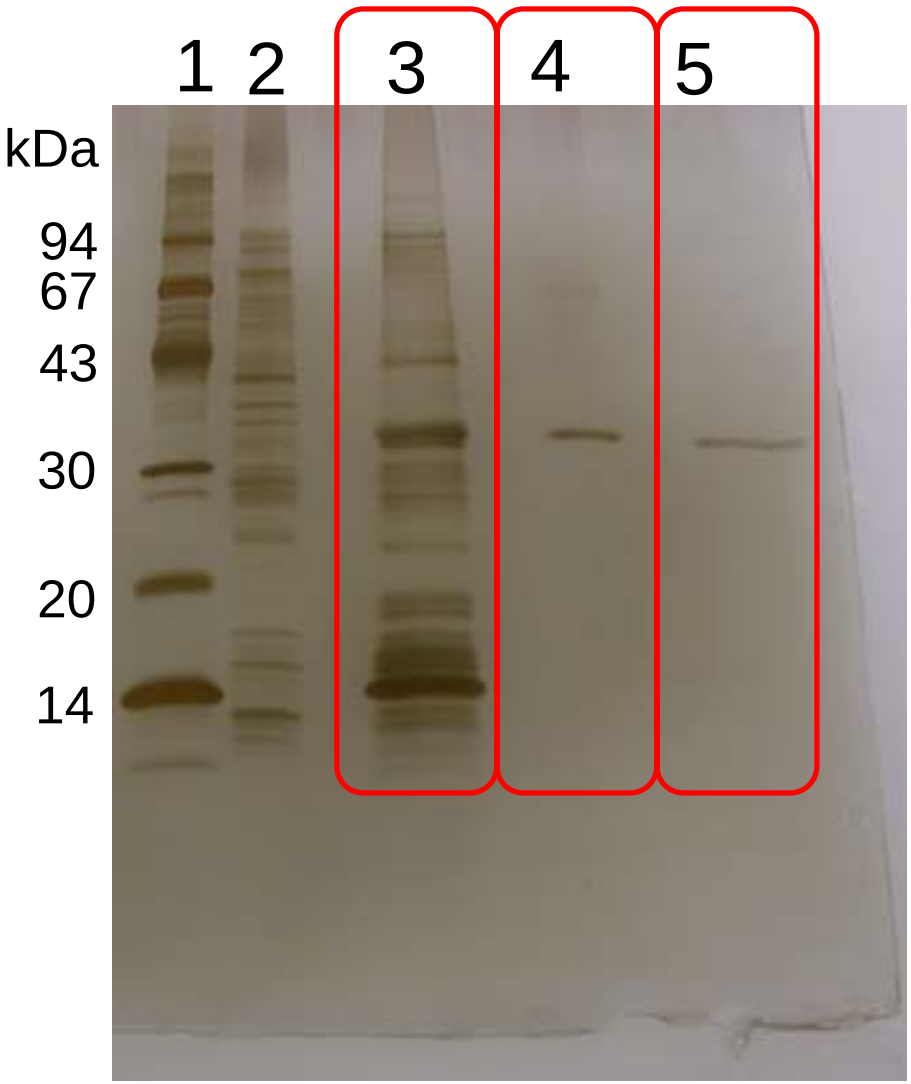
: Nickel ion

Elution buffer
20mM Tris
1M imidazole
0.5M NaCl

Chelate column

・Start buffer
20mM Tris-HCl (pH7.9), 5mM imidazole, 0.5M NaCl

VBP expression and purification



Lanes

1: Marker

2: Water-dissolved proteins

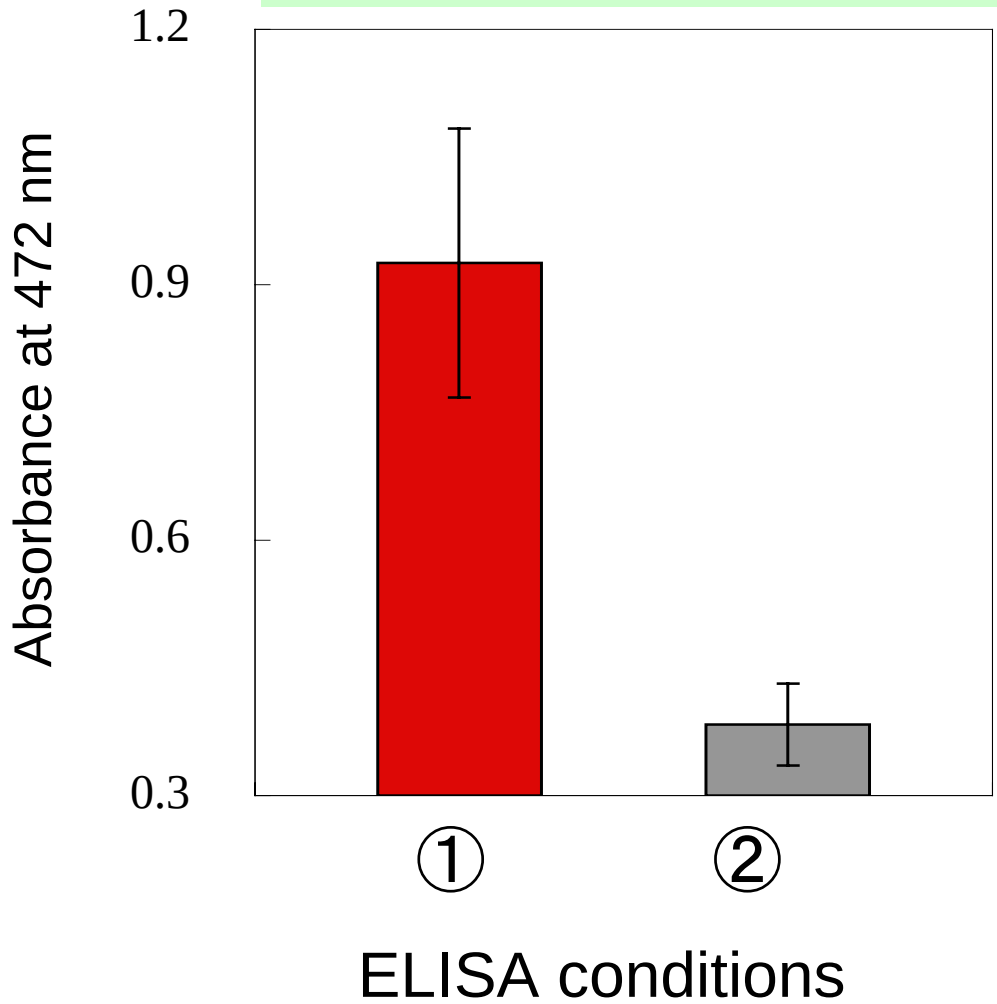
3: Urea-dissolved proteins

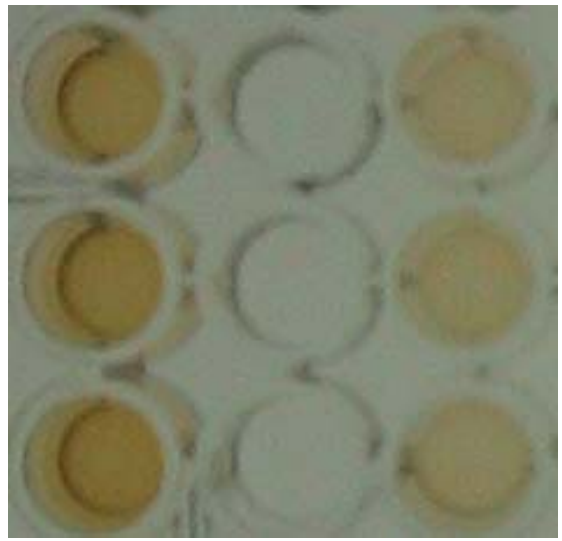
4: Purified VBP

5: Dialyzed VBP

Evaluation of virus binding ability of VBP clone with ELISA

Error bar represents standard error for triplicates





①：PV1 was inoculated to VBP-immobilized well.

②：PV1 was not inoculated to VBP-immobilized well.

VBP immobilization

VBP immobilization on carrier

VBP immobilization on glass particle

Silane-coupling reagent and glutaraldecyde were used for the VBP immobilization

Production of poly-lysine tag VBP (Lys-Tag VBP)

Introduction of poly-lysine tag on C-terminus of VBP.

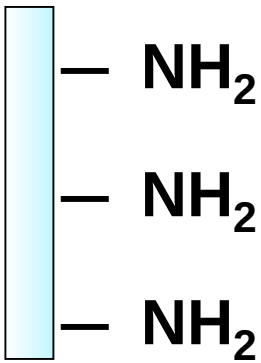
Practical experiment

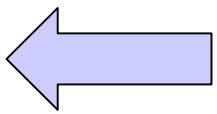
Construction of virus removing column using VBP.

Isolation of new VBPs

Isolation of Adenovirus-Binding Protein: (ADVBP)

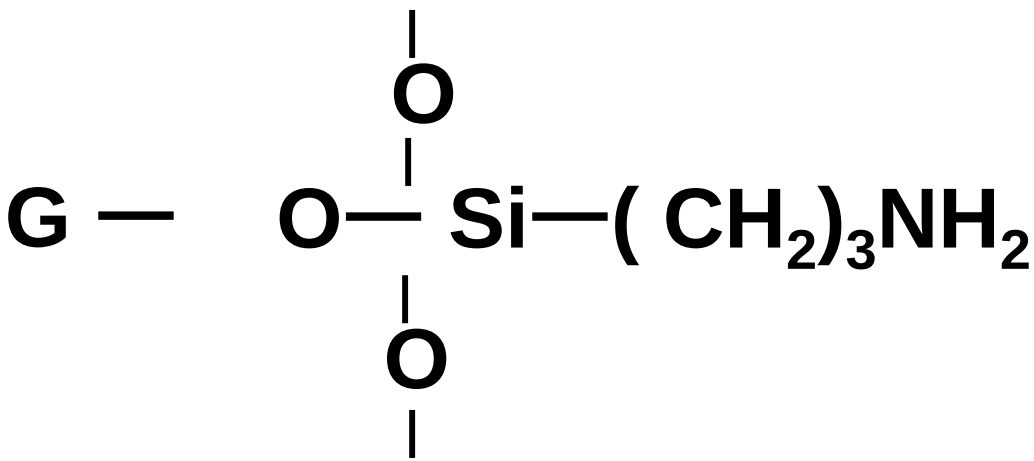
VBP immobilization with cross-linkage reagent





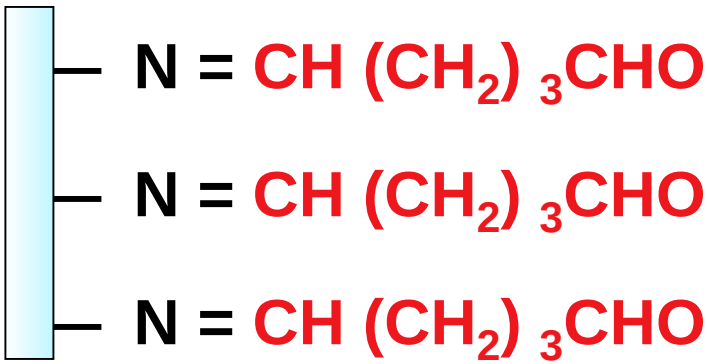
OHC $(CH_2)_3$CHO

glutaraldehyde

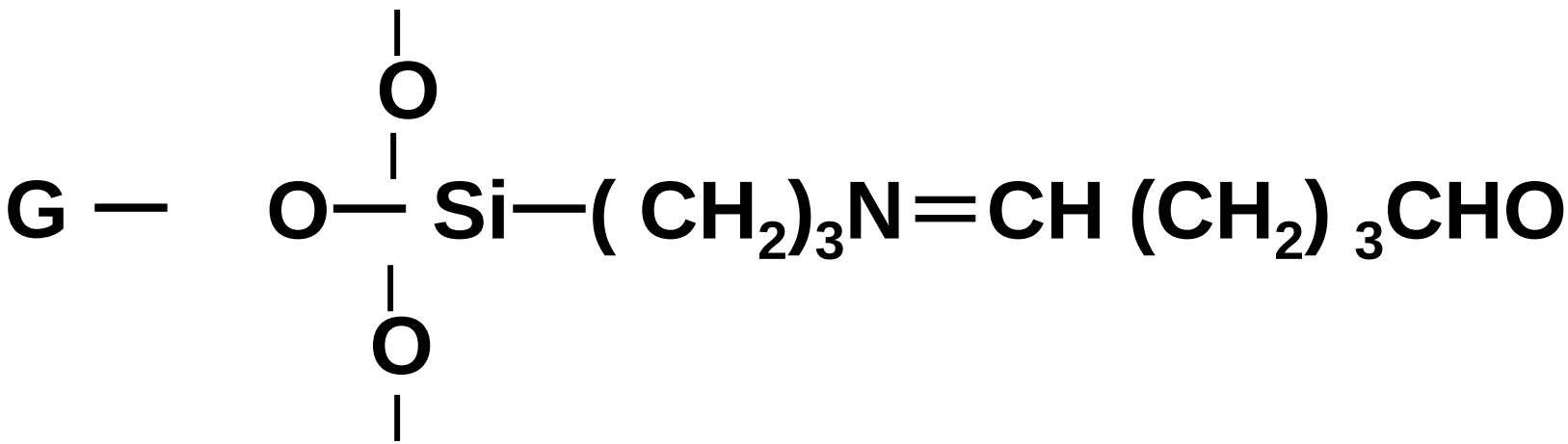


3-aminopropyltryethoxysilane

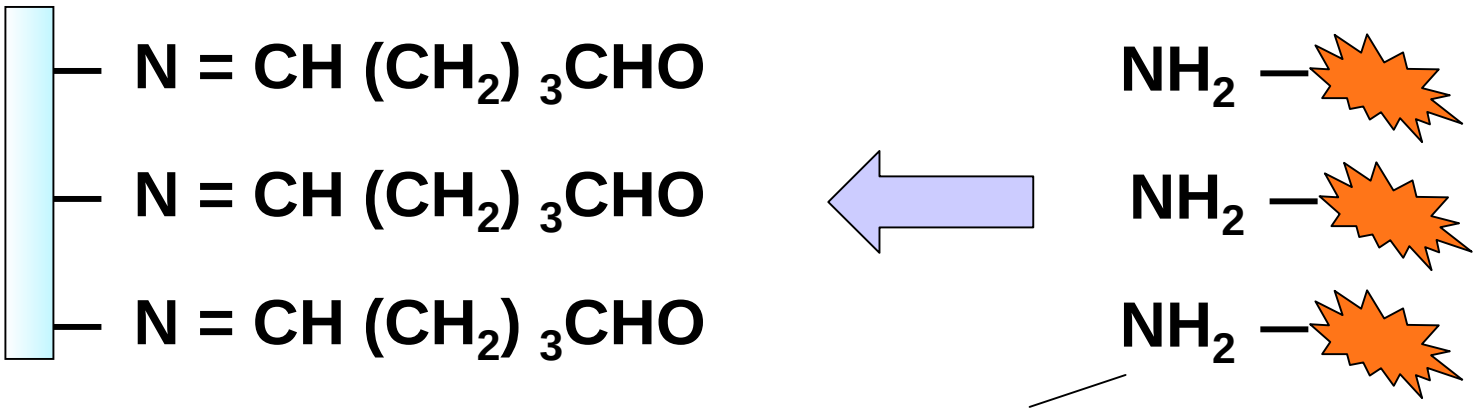
VBP immobilization with cross-linkage reagent

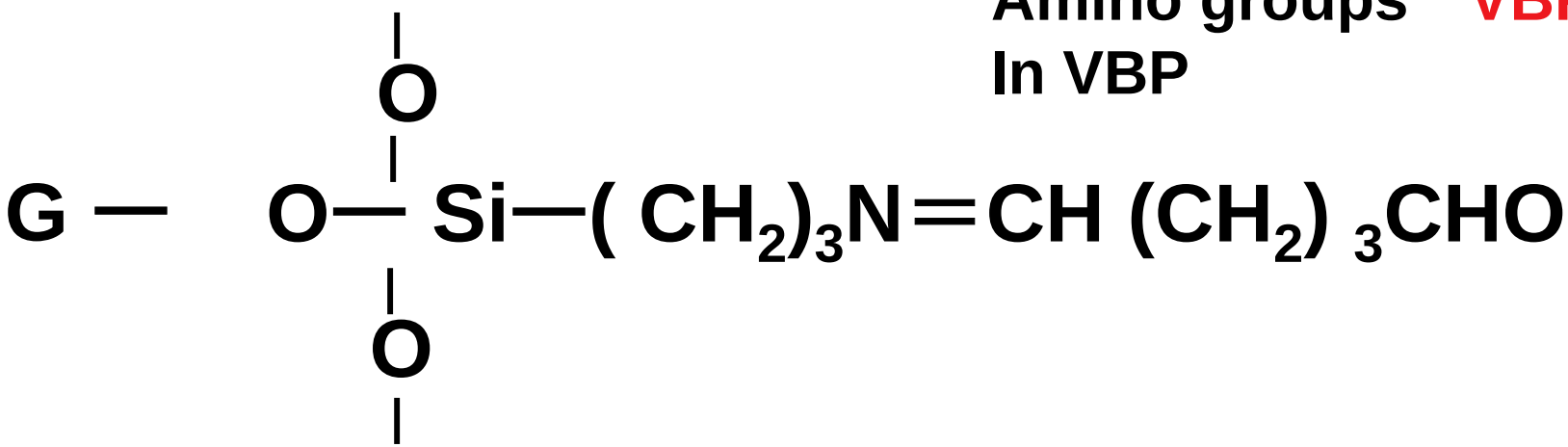


glutaraldehyde

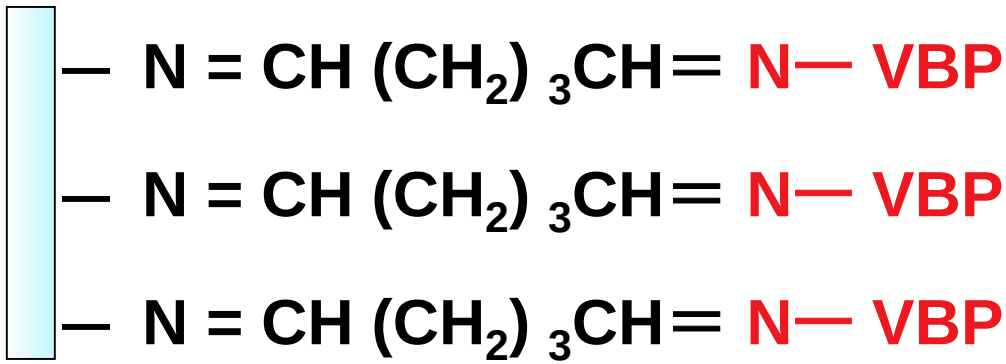


VBP immobilization with cross-linkage reagent

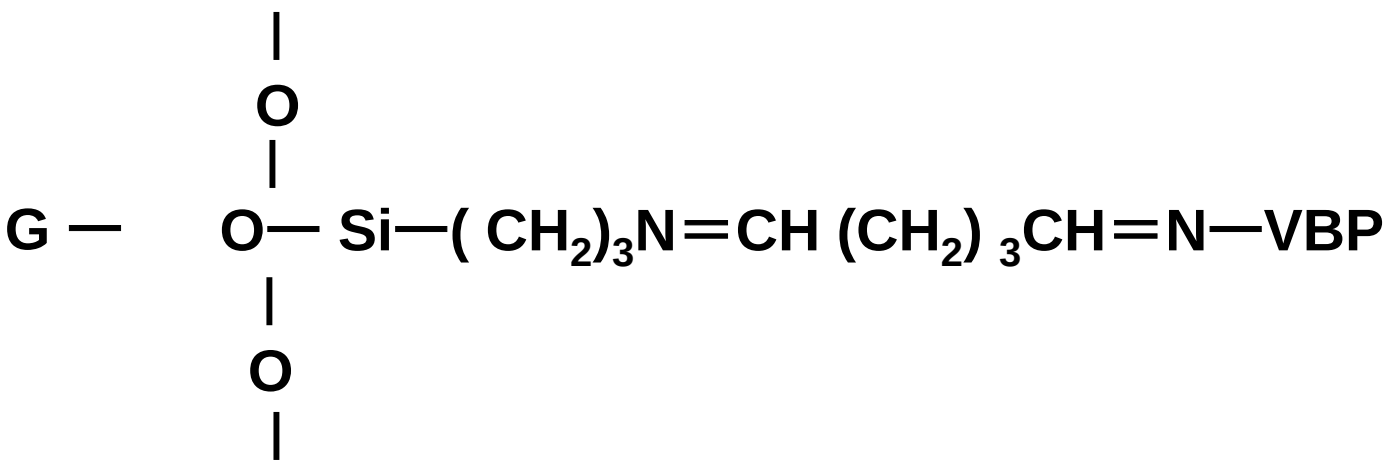




VBP immobilization with cross-linkage reagent



VBP immobilization



Strategy for VBP immobilization (2) (Oriented immobilization of VBP)

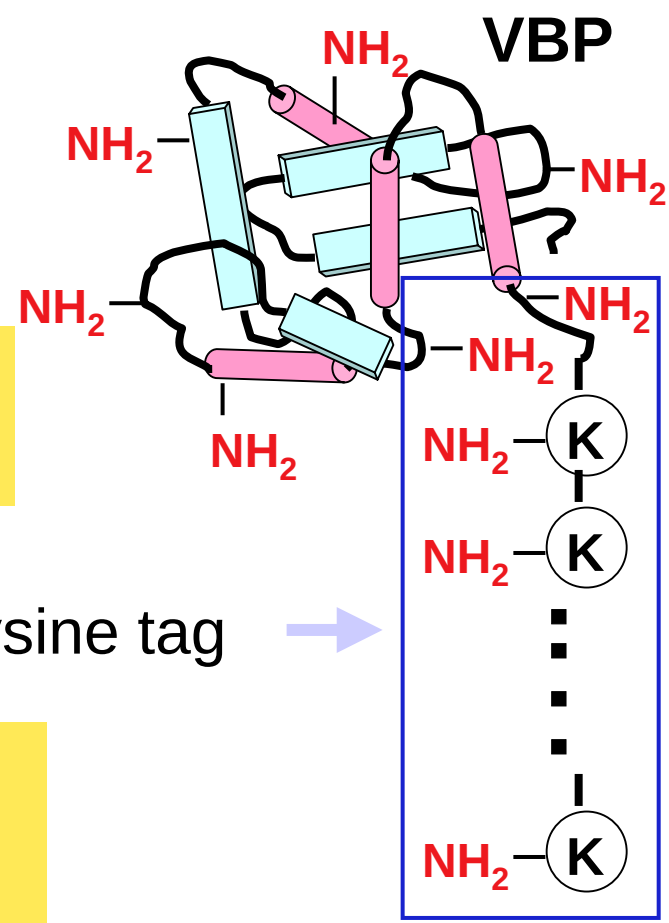
Covalent binding between VBP and carrier surface

VBP has a number of amino groups

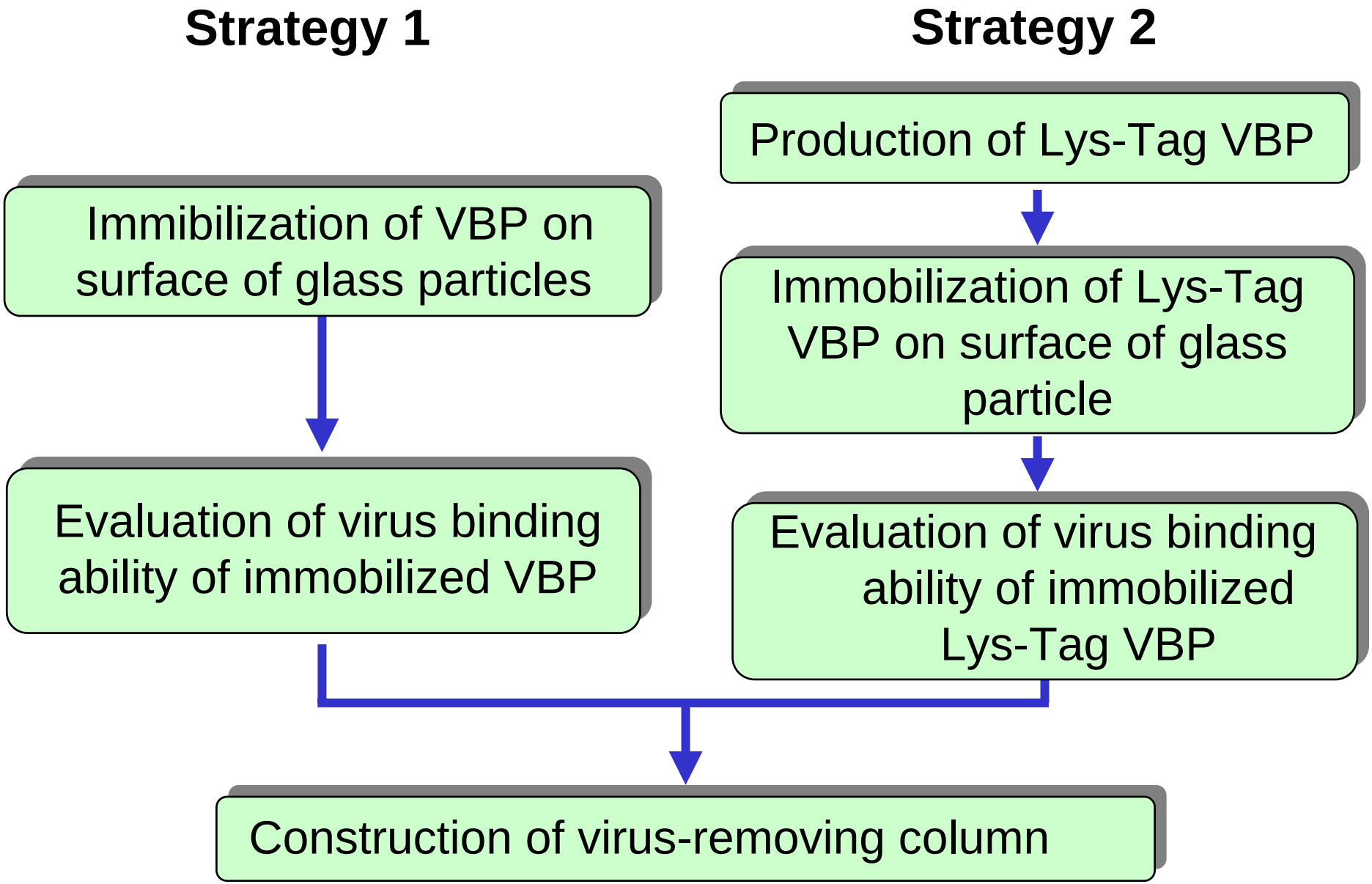
Impossible to orientationally immobilize VBP

Poly-lysine tag was added to VBP

It is possible to Orientationally immobilize VBP with binding between glutaraldehyde and poly-lysine tag.

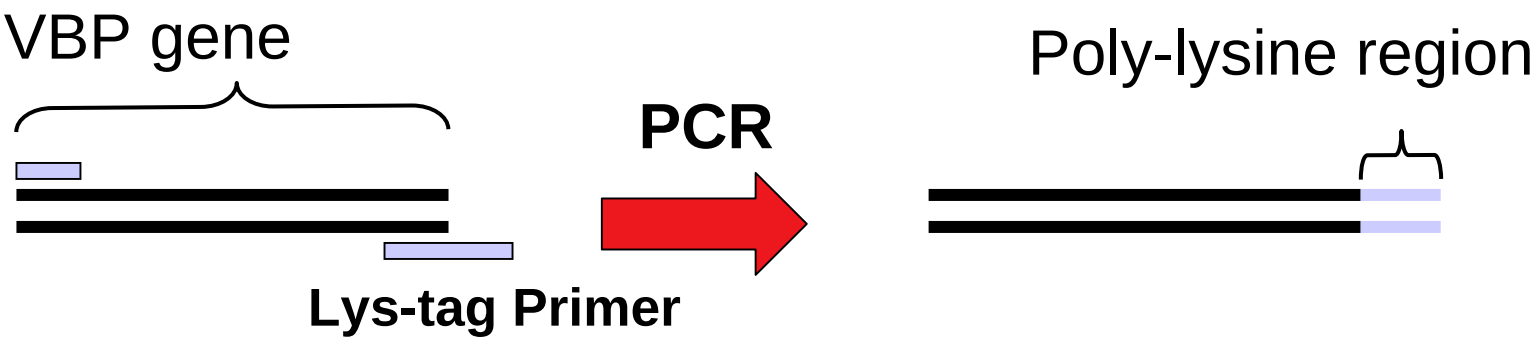


Experimental flow

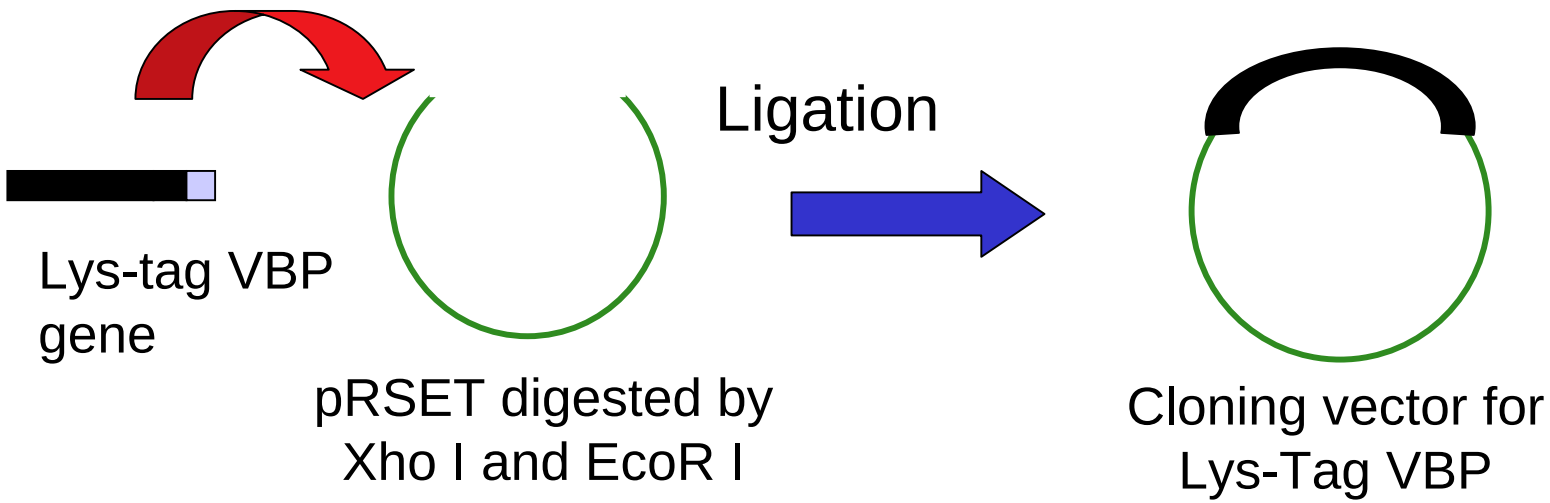


Production of Lys-Tag VBP

① Creation of gene for Lys-Tag VBP

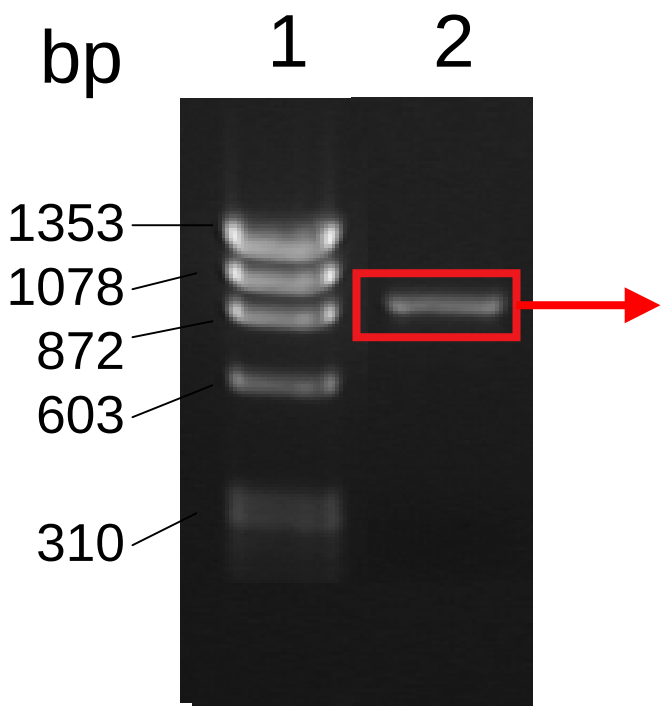


② Construction of cloning vector for Lys-TagVBP



Production of Lys-Tag VBP

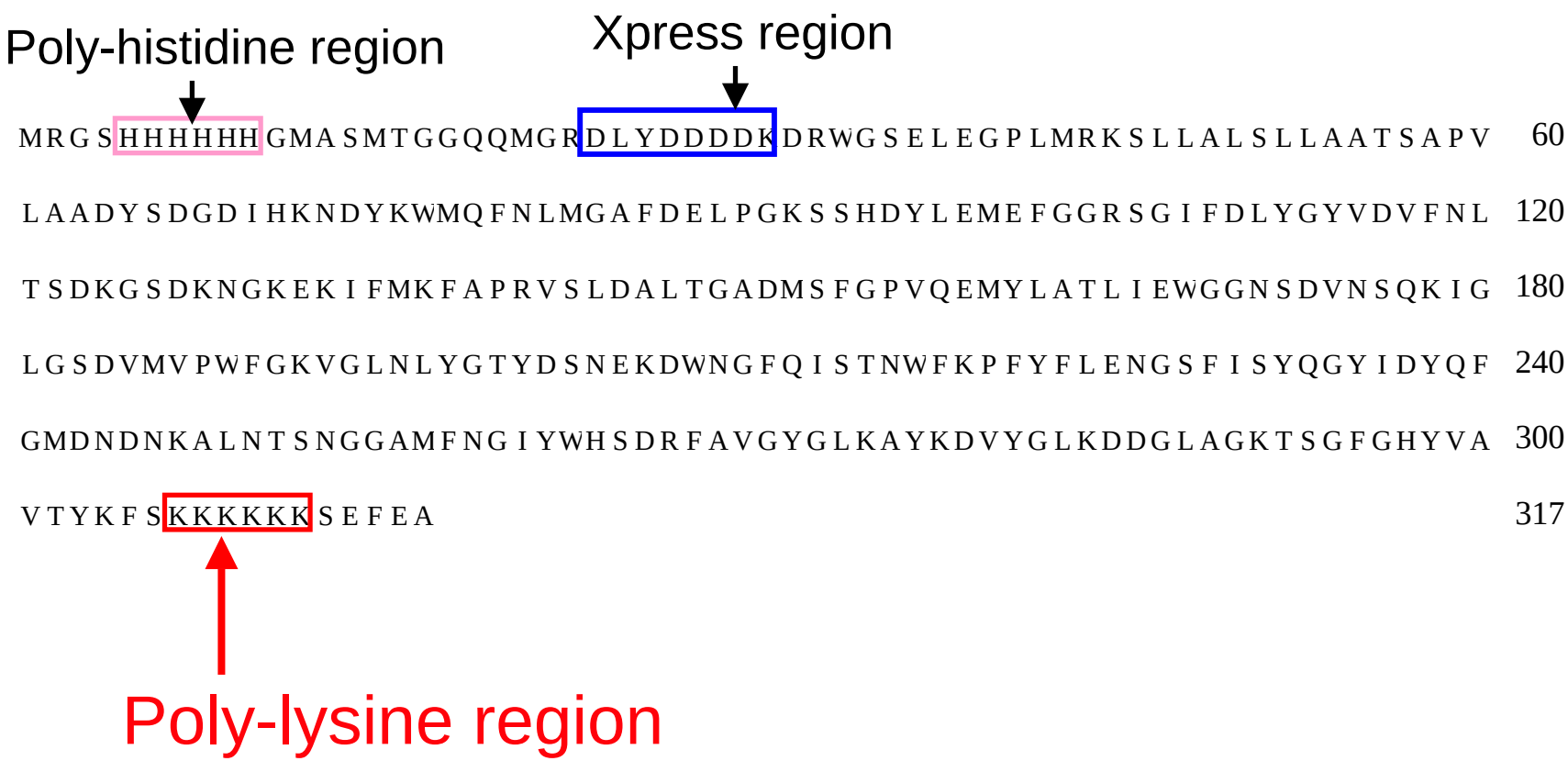
Result of agarose gel electrophoresis



Lys-tag VBP gene

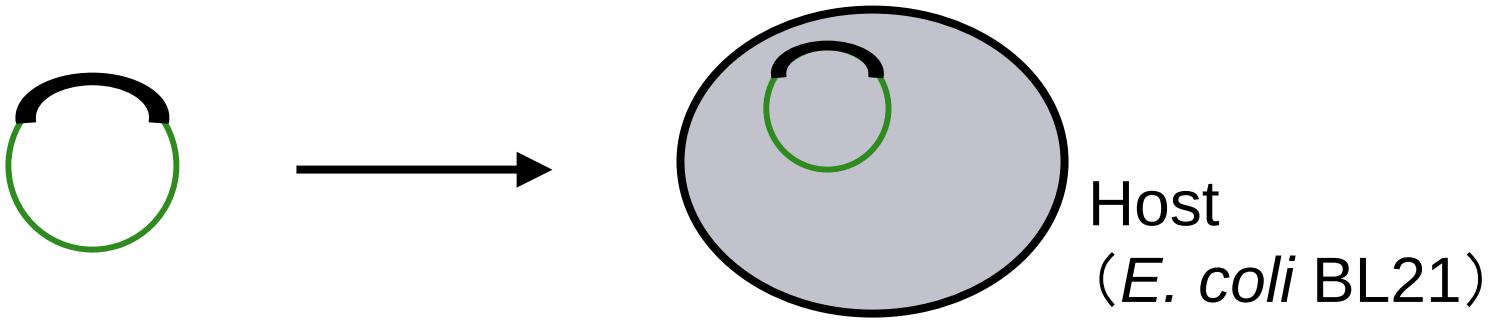
Lane 1: Marker
Lane 2: PCR product

Production of Lys-Tag VBP



Production of Lys-Tag VBP

① Transformation of *E. coli* cells with cloning vector

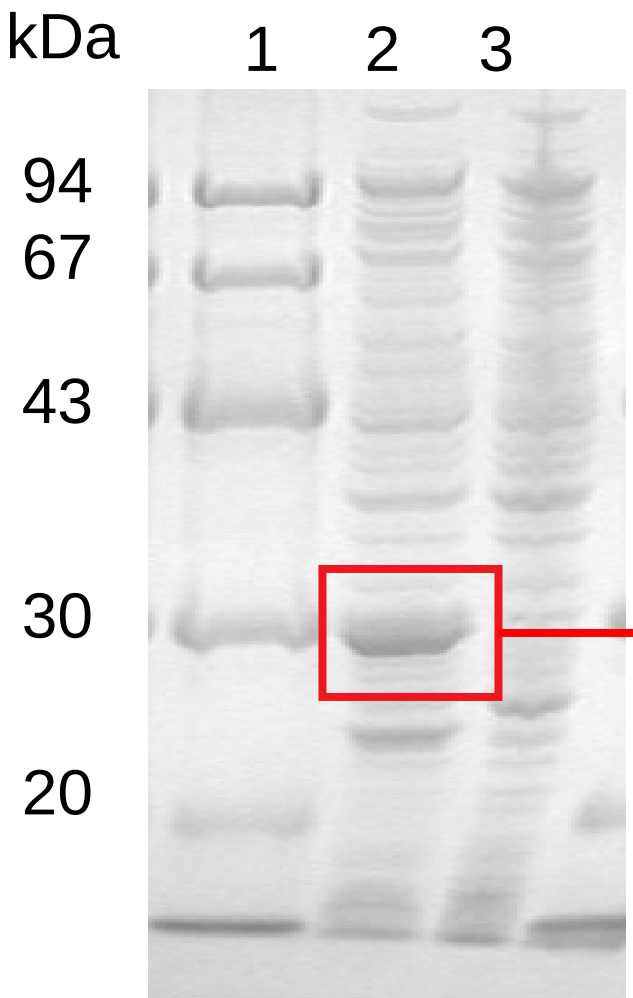


Exression of Lys-Tag VBP
was induced by IPTG

② Confirmation of the expression of Lys-Tag VBP

The expresson of the Lys-Tag VBP was
confirmed with SDS-PAGE

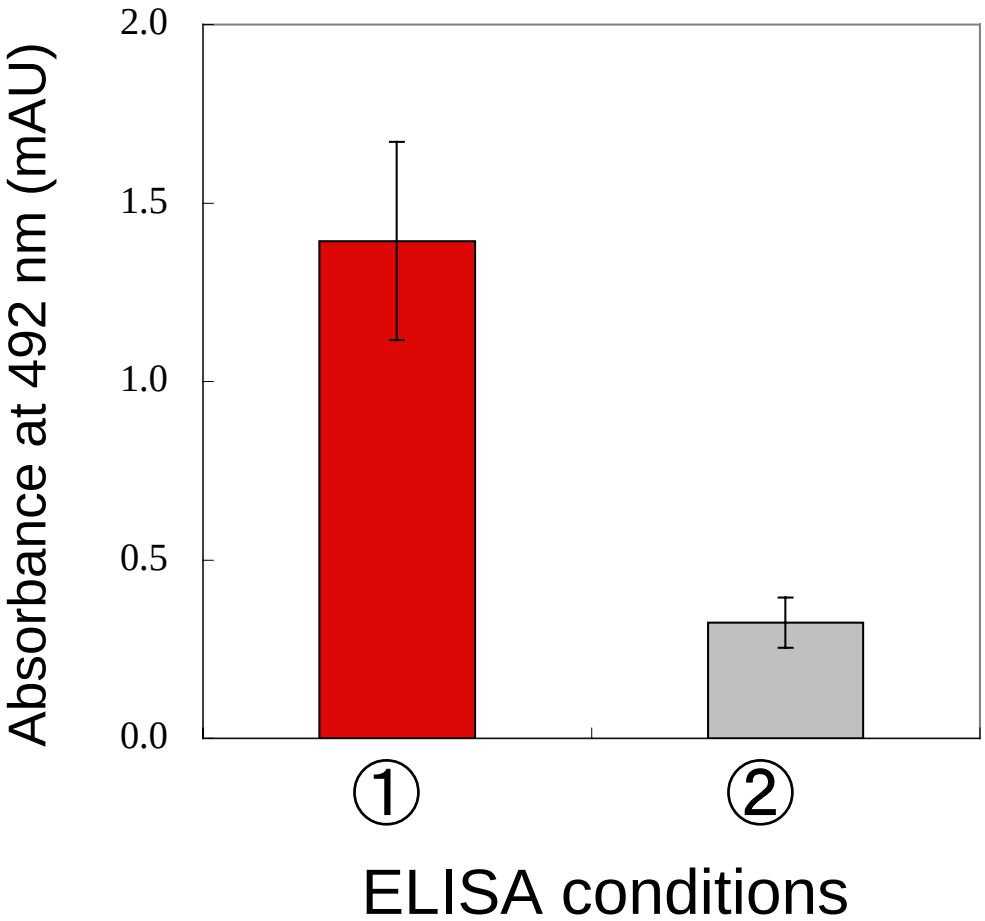
Expression of Lys-Tag VBP



Lane 1: Marker
Lane 2: Proteins from transformants induced by IPTG
Lane 3: Proteins from transformants

Successful expression of The Lys-Tag VBP was confirmed

Evaluation of virus binding ability of Lys-Tag VBP



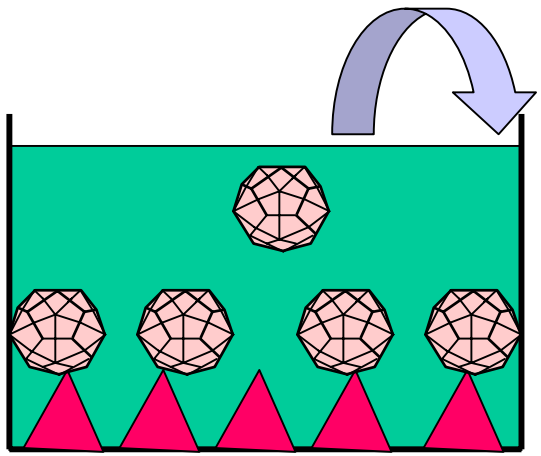
① ②

①: PV1 was added to Lys-Tag VBP-immobilized well

②: PV1 was not added to Lys-Tag VBP-immobilized well

Evaluation of virus-binding ability of Lys-Tag VBP （Binding constant）

Sampling



: Lys-Tag VBP

: PV1

Adsorption of PV1 to Lys-Tag VBP

↓ **Sampling of supernatant**

Enumeration of PV1 in supernatant

↓

$$K = \frac{[VBP\text{-}PV1]}{[VBP]\,[PV1]}$$

Calculation of binding constant

Evaluation of virus-binding ability of Lys-Tag VBP （Binding constant）

	K ($M^{-1} \times 10^6$)
Lys-tag VBP ― PV1	1.10
Protein A ― Prosep-rA	0.34
Protein A ― Sepharose 4 FF	0.14

＊

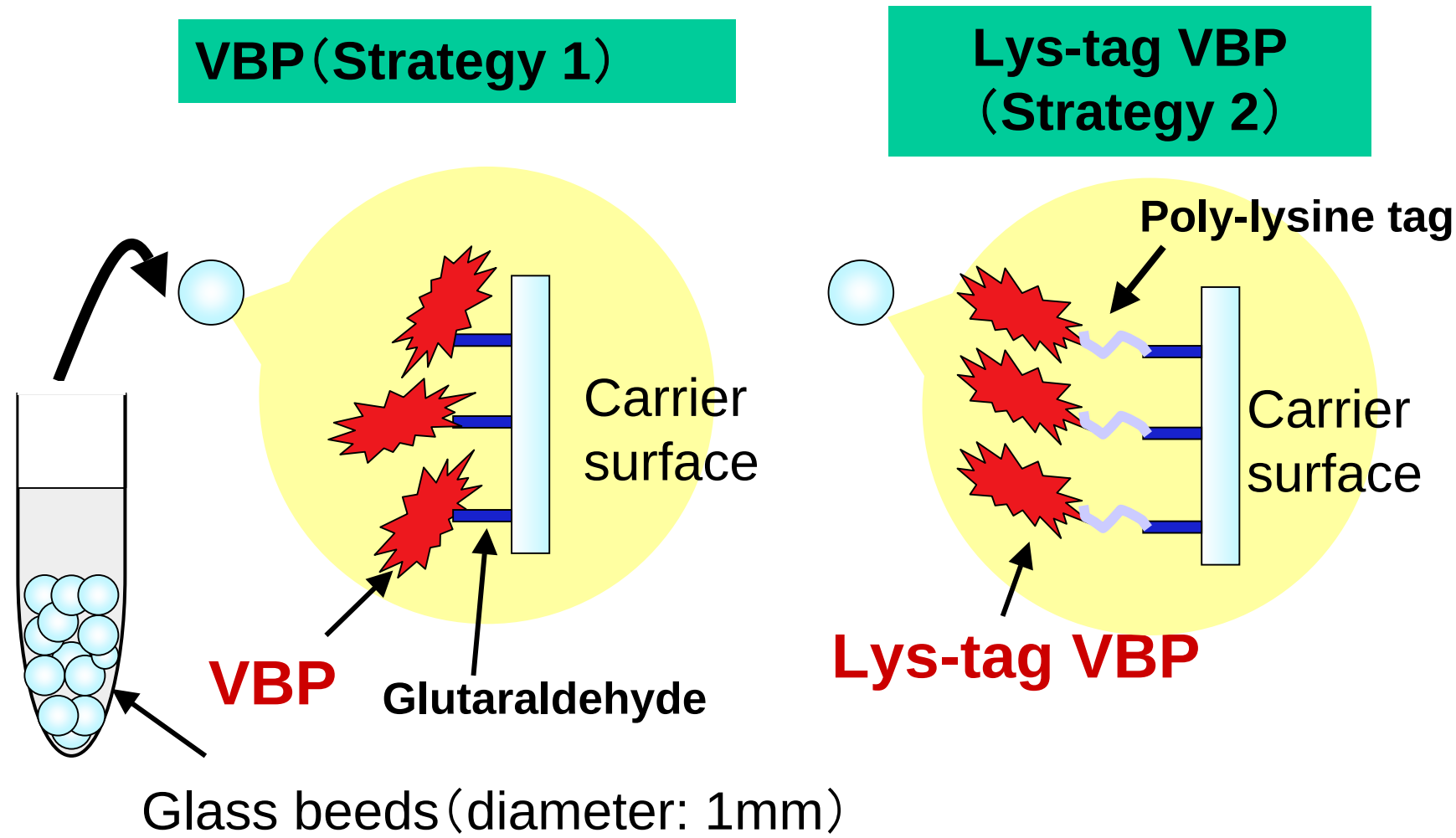
＊R Hahn et al.（2003）Comparison of protein A affinity sorbents

The larger K value is, the stronger the binding ability is.

It was confirmed that there is strong affinity between PV1 and Lys-Tag VBP.

Binding between PV1 and immobilized VBP

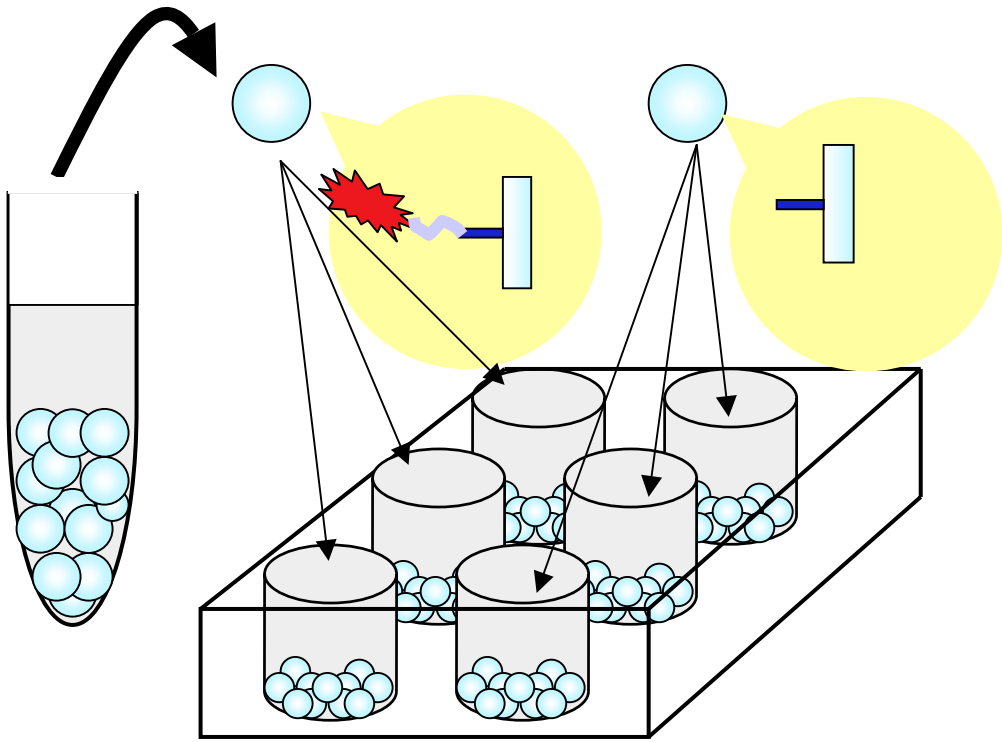
① VBP immobilization on surface of glass particle



Binding between PV1 and immobilized VBP

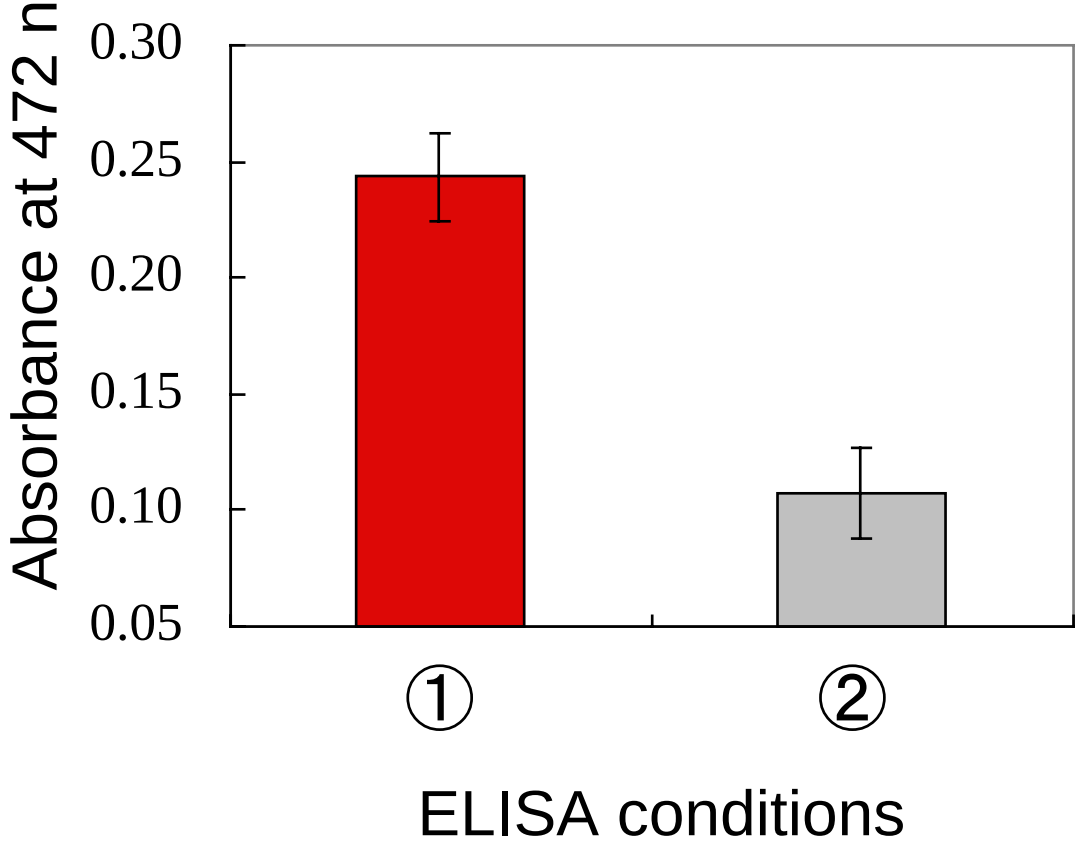
② Confirmation of VBP immibolization

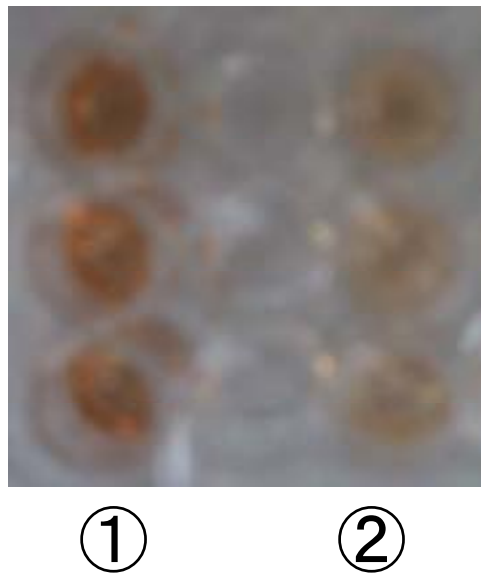
VBP immobilization was confirmed with ELISA, which can detect Xpress region of VBP



ELISA plate

Confirmation of VBP immobilization





①: Lys-tagVBP-immobilized glass beeds

②: Glass beeds

Binding between PV1 and immobilized VBP

③ Binding constant

	K (10^6 M^{-1})
VBP (Strategy 1)	2.10（±0.80）
Lys-tag VBP (Strategy 2)	4.38（±3.05）

The larger K value is, the stronger the binding is.

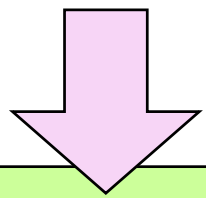
Construction of virus-removing column

・VBP immobilization on surface of glass particle

・Evaluation of virus removal efficiency

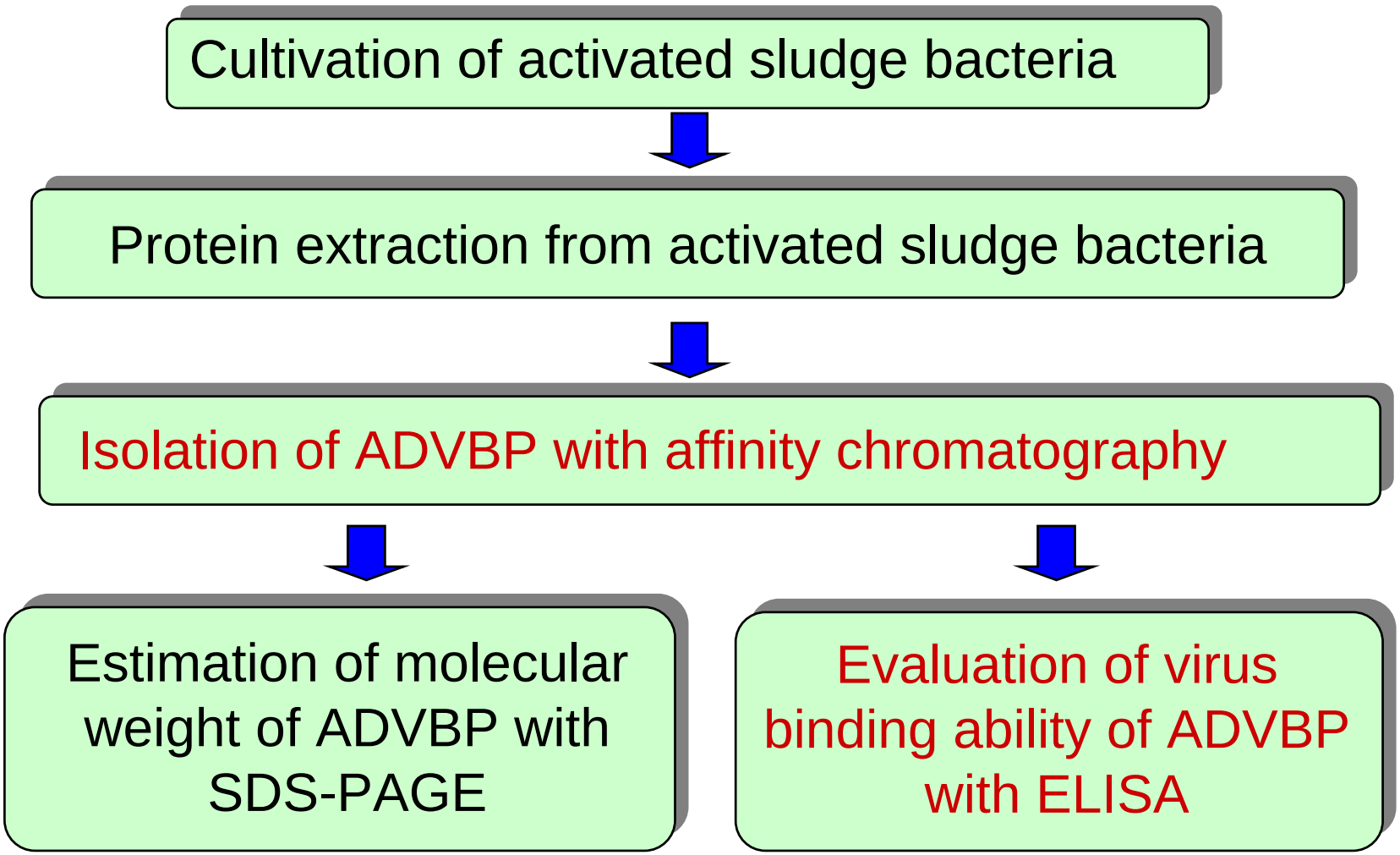
Isolation of new VBPs

- Isolation of Adenovirus-Binding Protein：(ADVBP) from activated sludge bacteria
- Evaluation of virus-binding ability of ADVBP



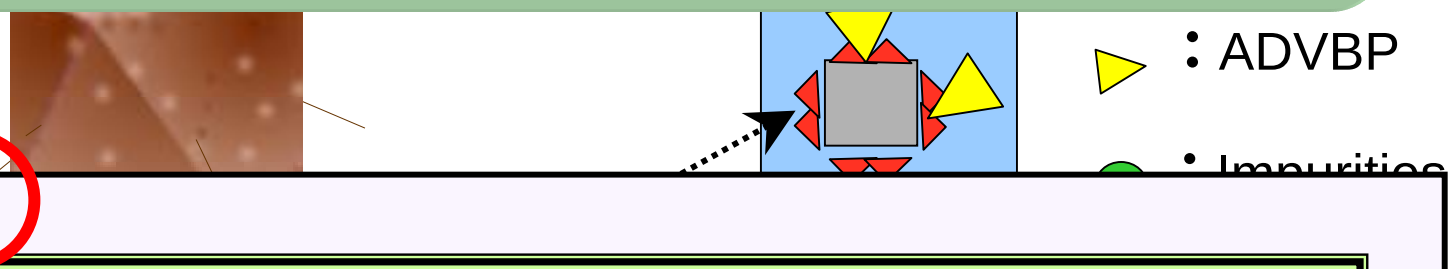
ADVBPs can be used as viral adsorbents ?

Experimemtal flow



Isolation of ADVBP with affinity chromatography

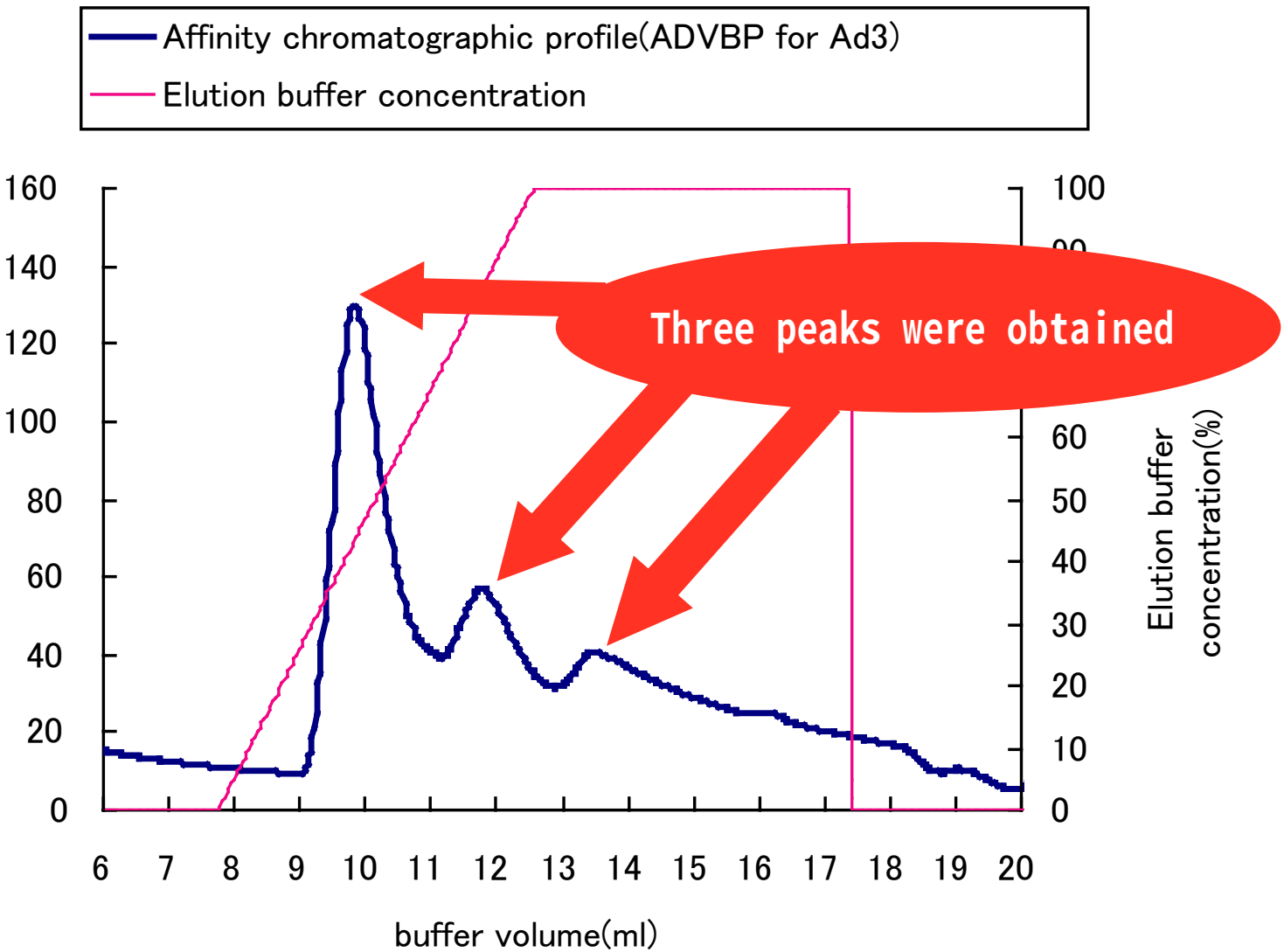
Capsid peptides of AD3 and AD40/41were used as ligands



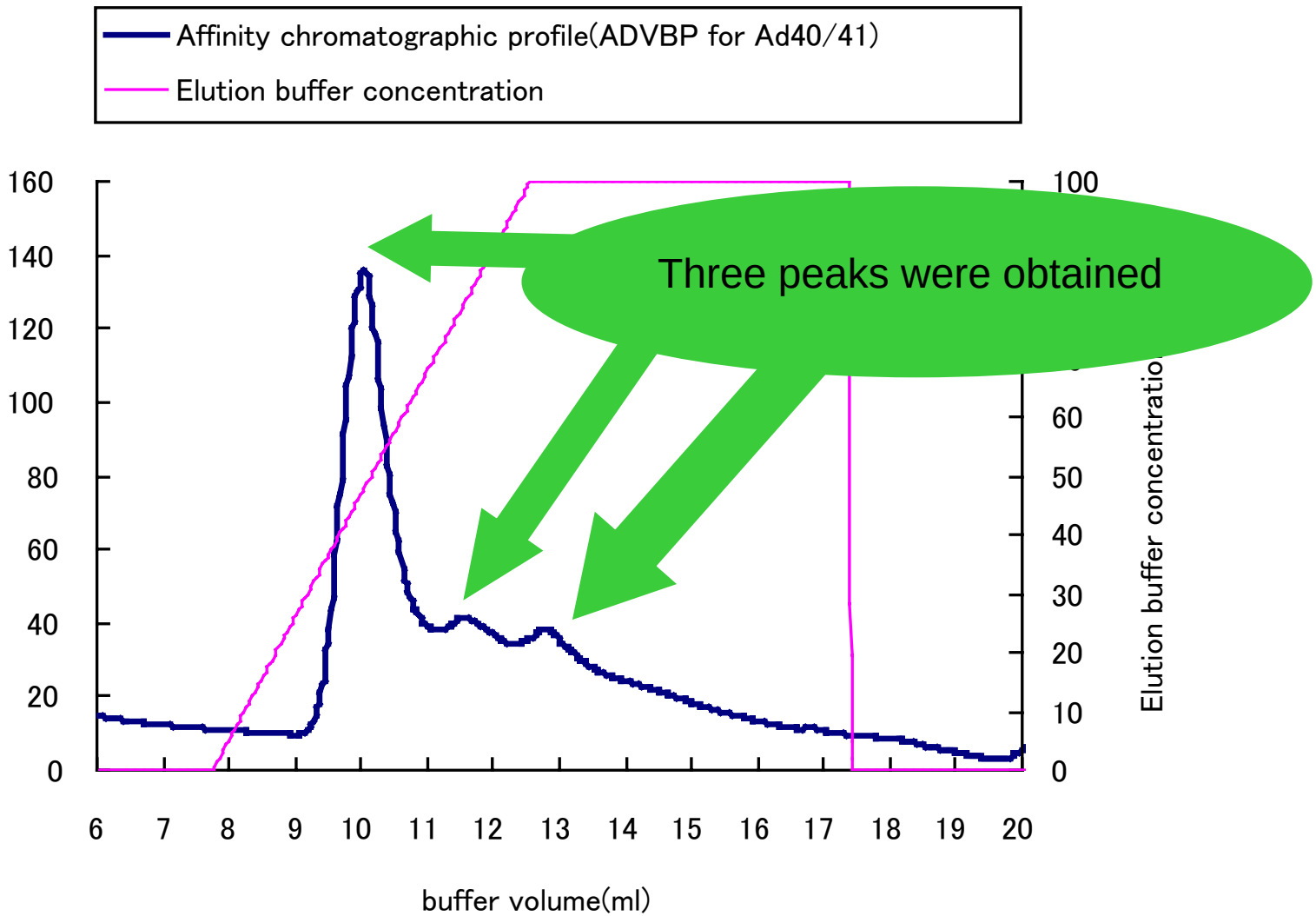
Amino acid sequences of ligand

AD3	NCYYK ASDGA LF
AD40/41	MALTY TFLQG DP

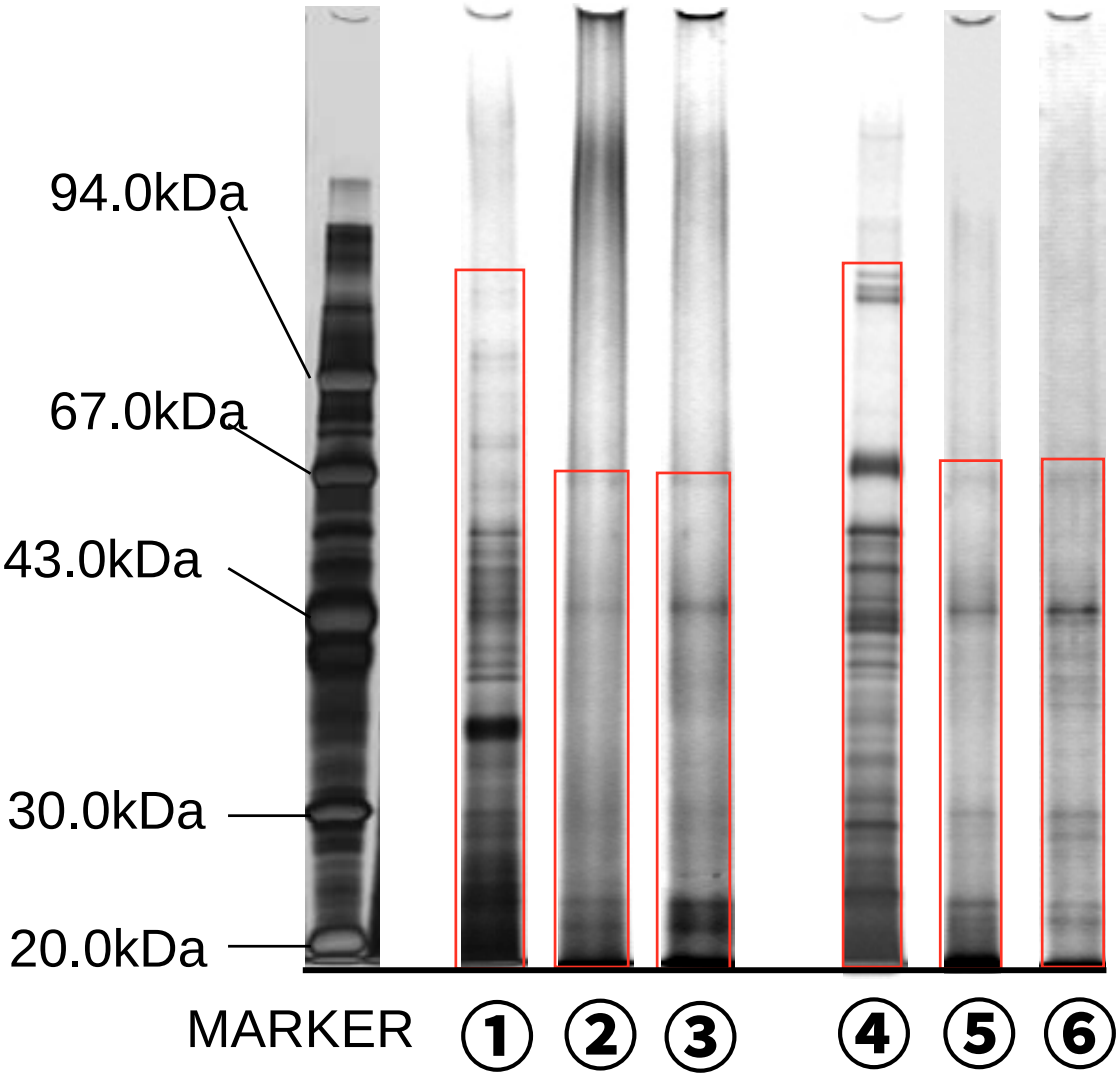
Isolation of ADVBP for AD3

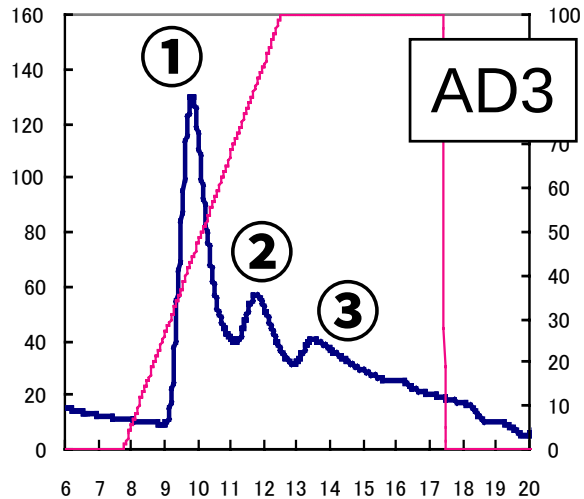


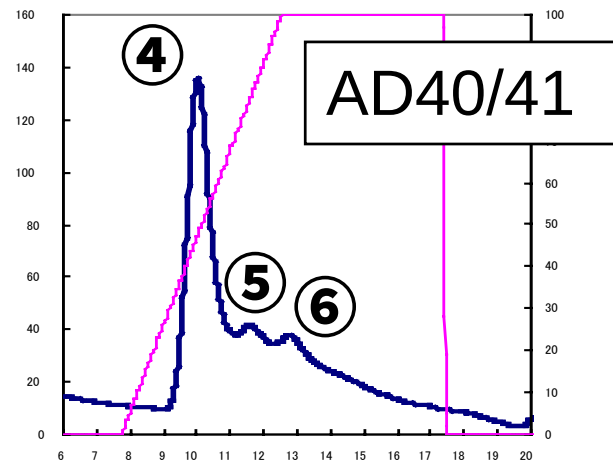
Isolation of ADVBP for AD40/41



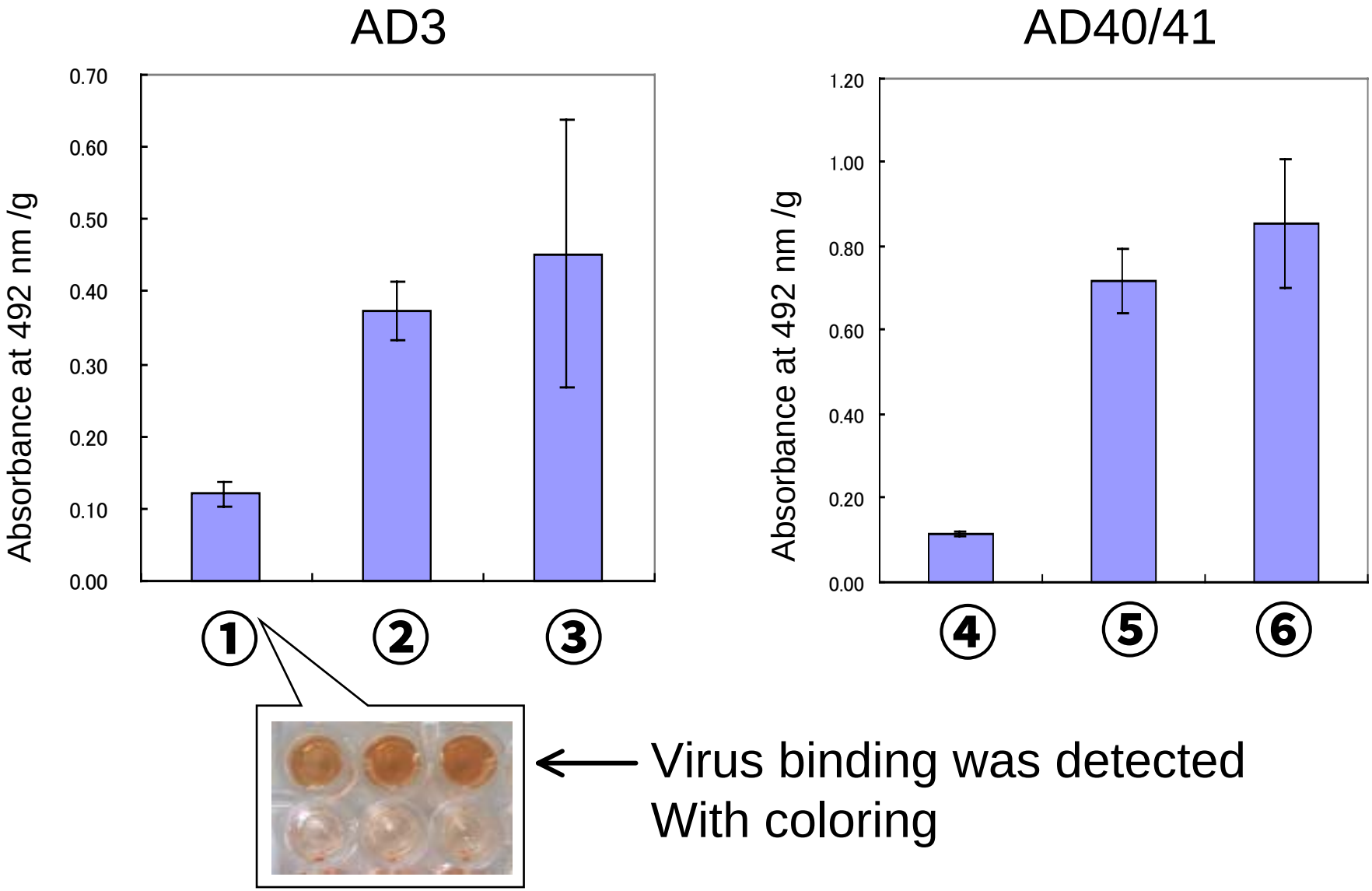
Estimation of molecular weight of ADVBP with SDS-PAGE







Evaluation of virus binding ability of ADVBPs with ELISA



Summaries (1)

Poliovirus-binding protein was successfully isolated with affinity chromatography using polioviral capsid peptide as a ligand.

VBP gene (807bp) can be obtained from genomic DNA library of activated sludge bacteria with colony hybridization.

PVBP clones produced by *E. coli* cells exhibited virus binding ability.

Summaries(2)

Lys-Tag VBP that has poly-lysine tag at its C-terminus was successfully produced.

VBP was successfully immobilized on surface of glass particles with silane coupling reagent and glutaraldehyde.

Adenovirus-binding protein (ADVBP) was successfully isolated with affinity chromatography.

Future works

Isolation of VBPs for other pathogenic viruses such as Noroviruses and Rotaviruses.

Isolation of each VBP gene and construction of cloning system.

Development of virus detection technique using VBP as specific adsorbent.