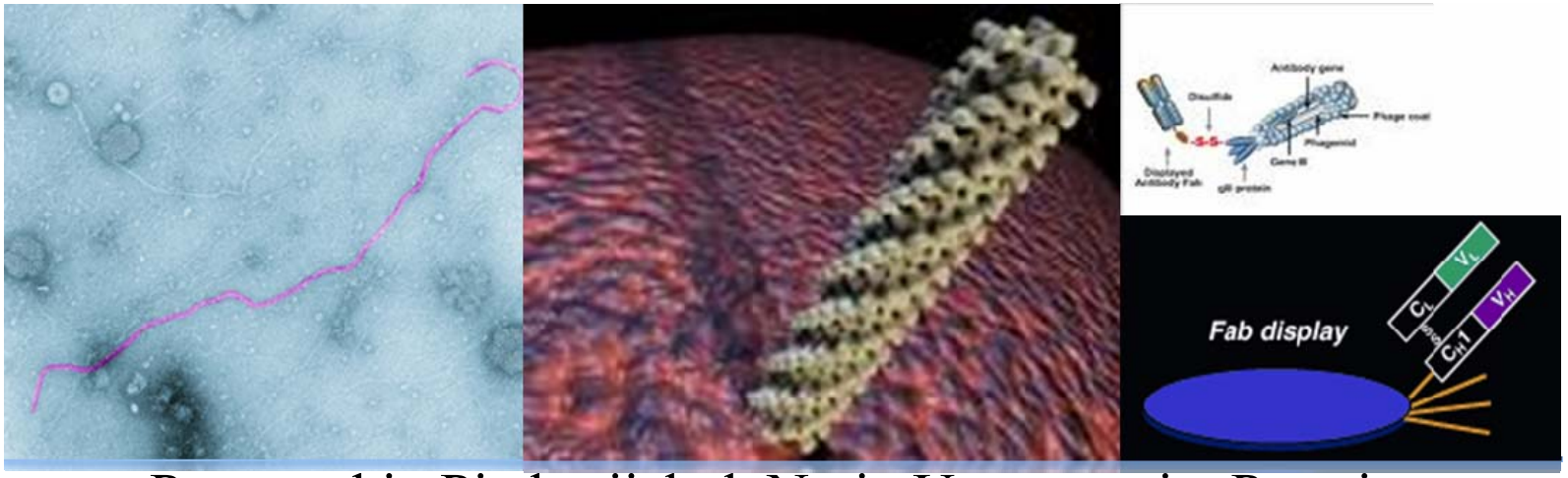
RECOMBINANT FAB ANTIBODY THAT SPECIFIC TO HAMAGGLUTININ PROTEIN of H5N1 VIRUS DEVELOPED FOR THERAPEUTIC AND DIAGNOSTIC APPLICATION



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Topics

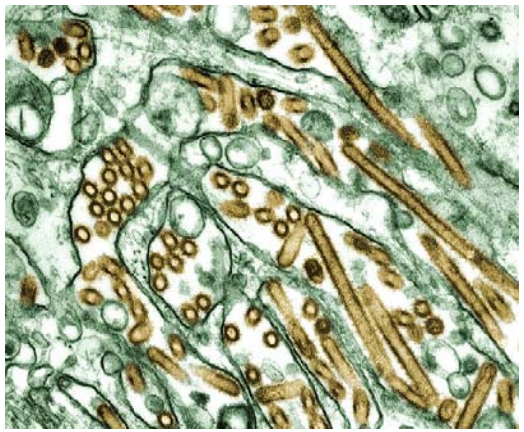
- Background & Problem
- Materials & Methods
- Results
- Discussions
- Conclusions

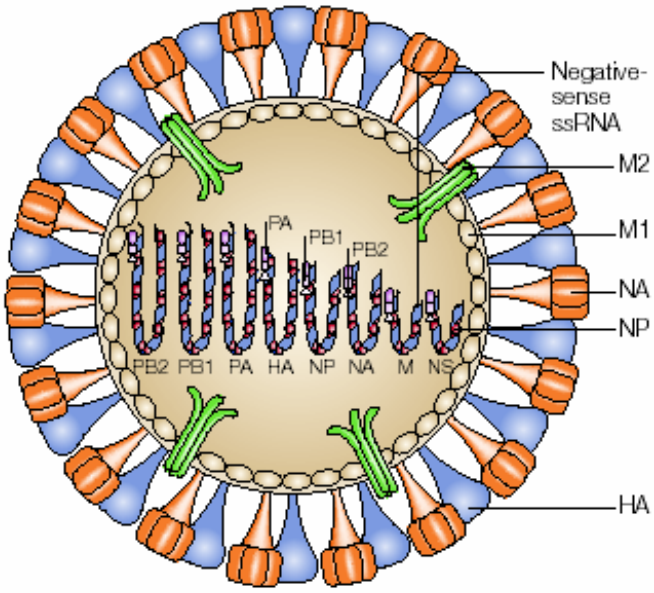
Avian Influenza virus

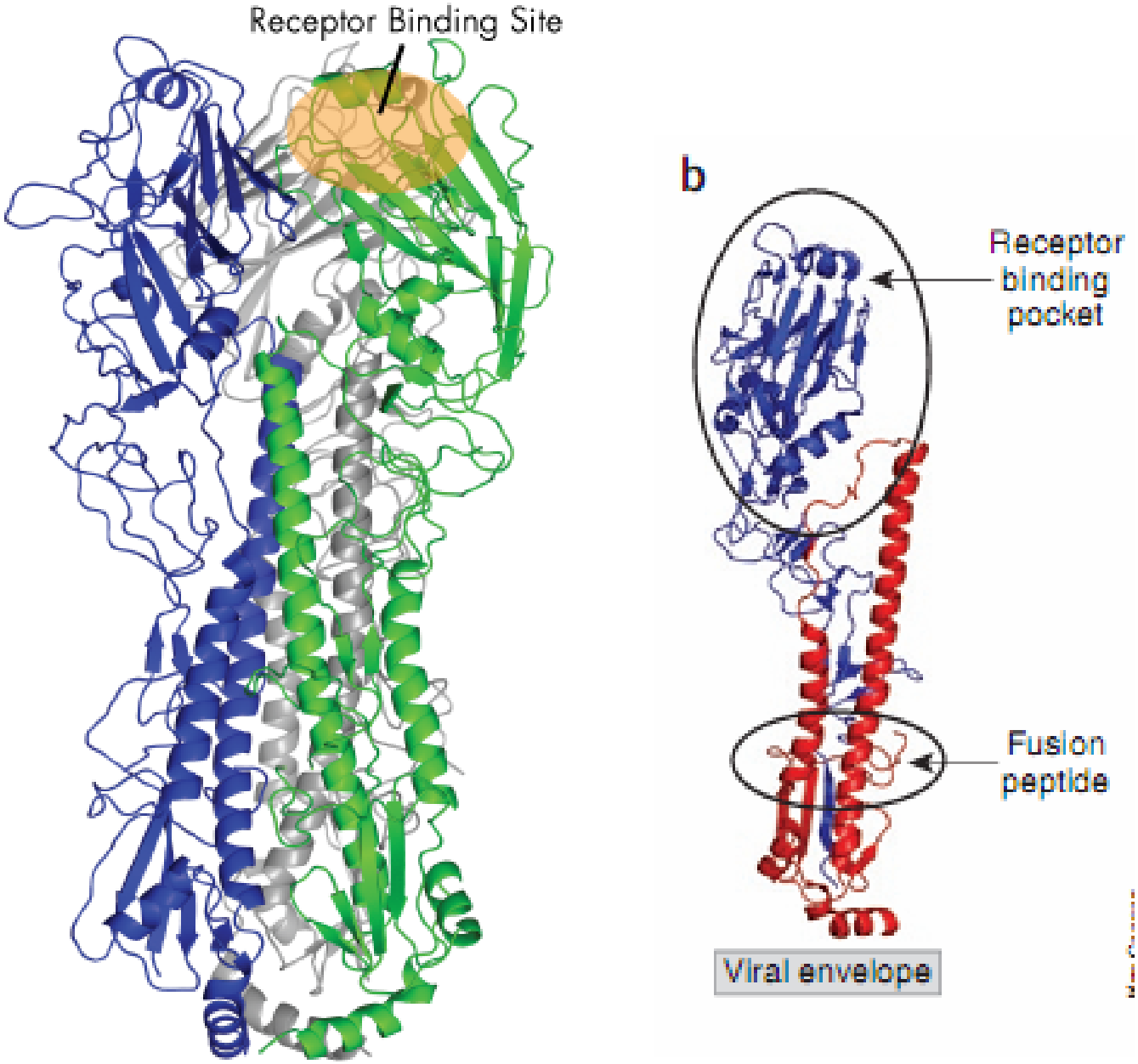
- **Avian Influenza virus is the influenza A virus that infect to avian species**
- **Direct transmission from this avian influenza virus to human was first reported in 1997**
- **Then, since 2003, this strains was re-emerged, and continued to be a cause of disease in both human and poultry.**

Avian Influenza Virus – Structure and Biology

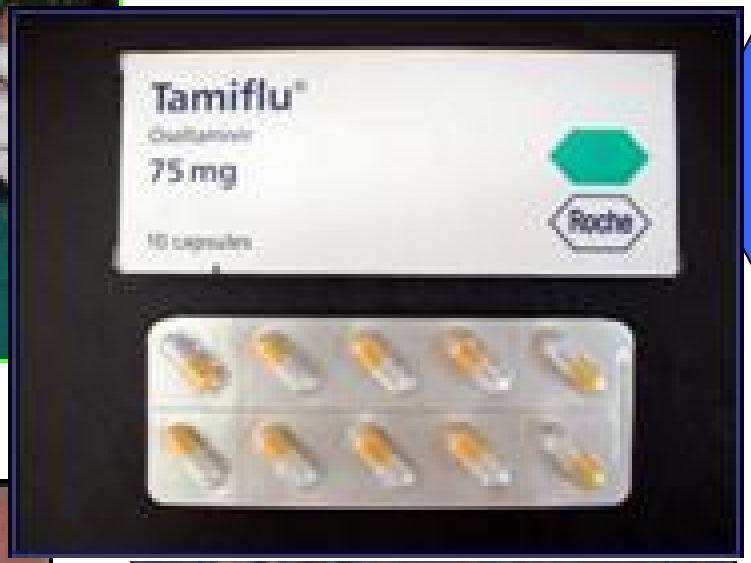
- Spherical or longitudinal shaped enveloped
- Negative sense single strand RNA
- Avian Influenza are classified as:
  - Family orthomyxoviridae
  - Genus of InfluenzavirusA
  - 9 internal proteins
    - **PA, PB1, PB1-F2, PB2, M1, M2, NP, NS1, NS2**
  - 2 external glycoproteins
    - **Hemagglutinin (HA)**
    - **Neuraminidase (NA)**



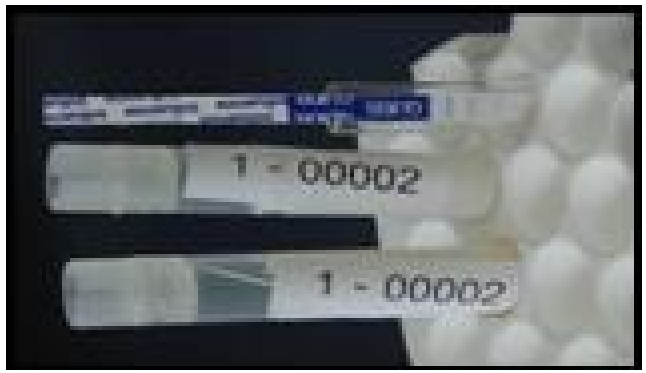



Receptor Binding Site
b
Receptor
binding
pocket
Fusion
peptide
Viral envelope

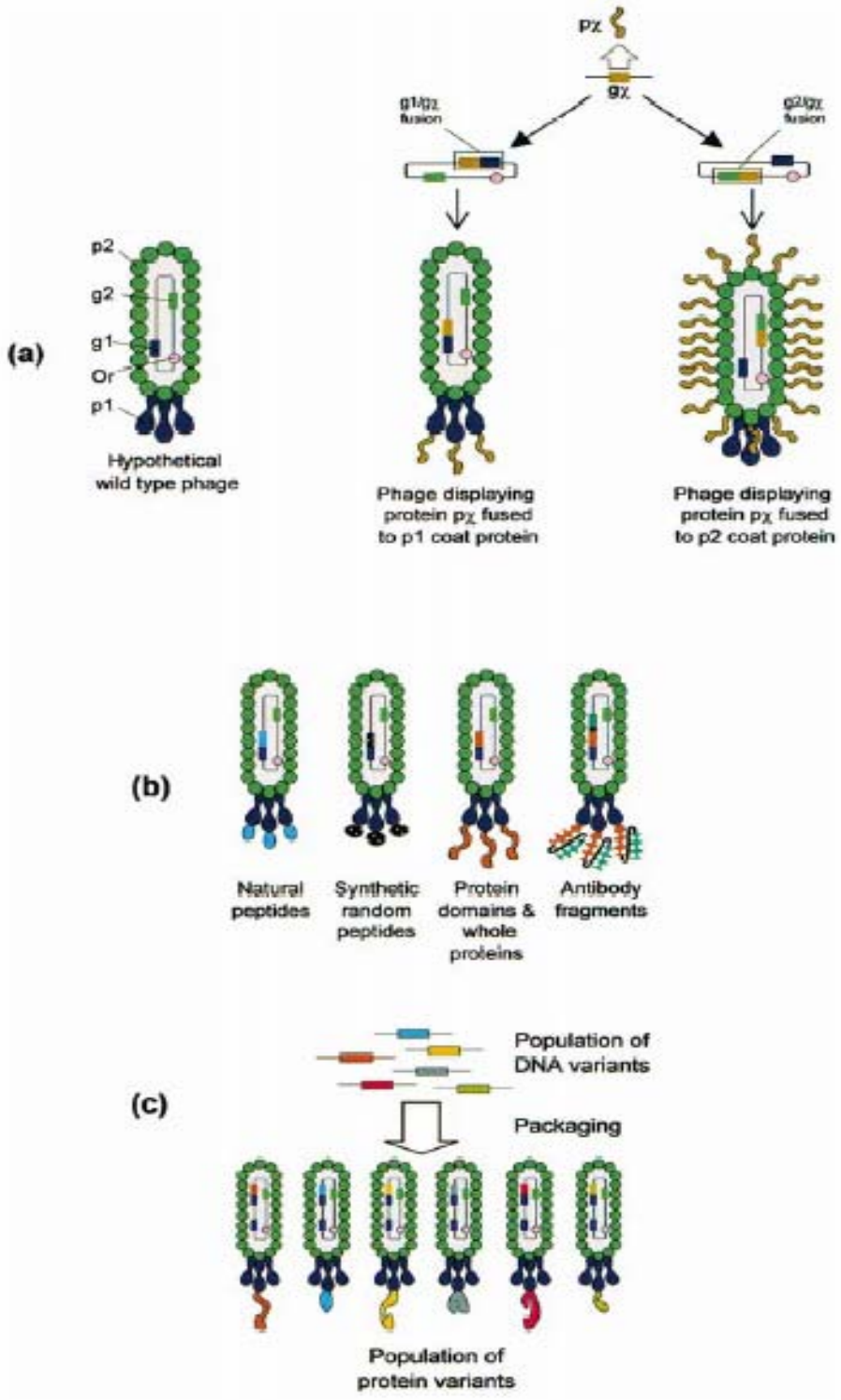
Prevention & Stockpiling



- Vaccine
- Antiviral agents
- Diagnostic test kits
- Respirators



Phage Display Technology

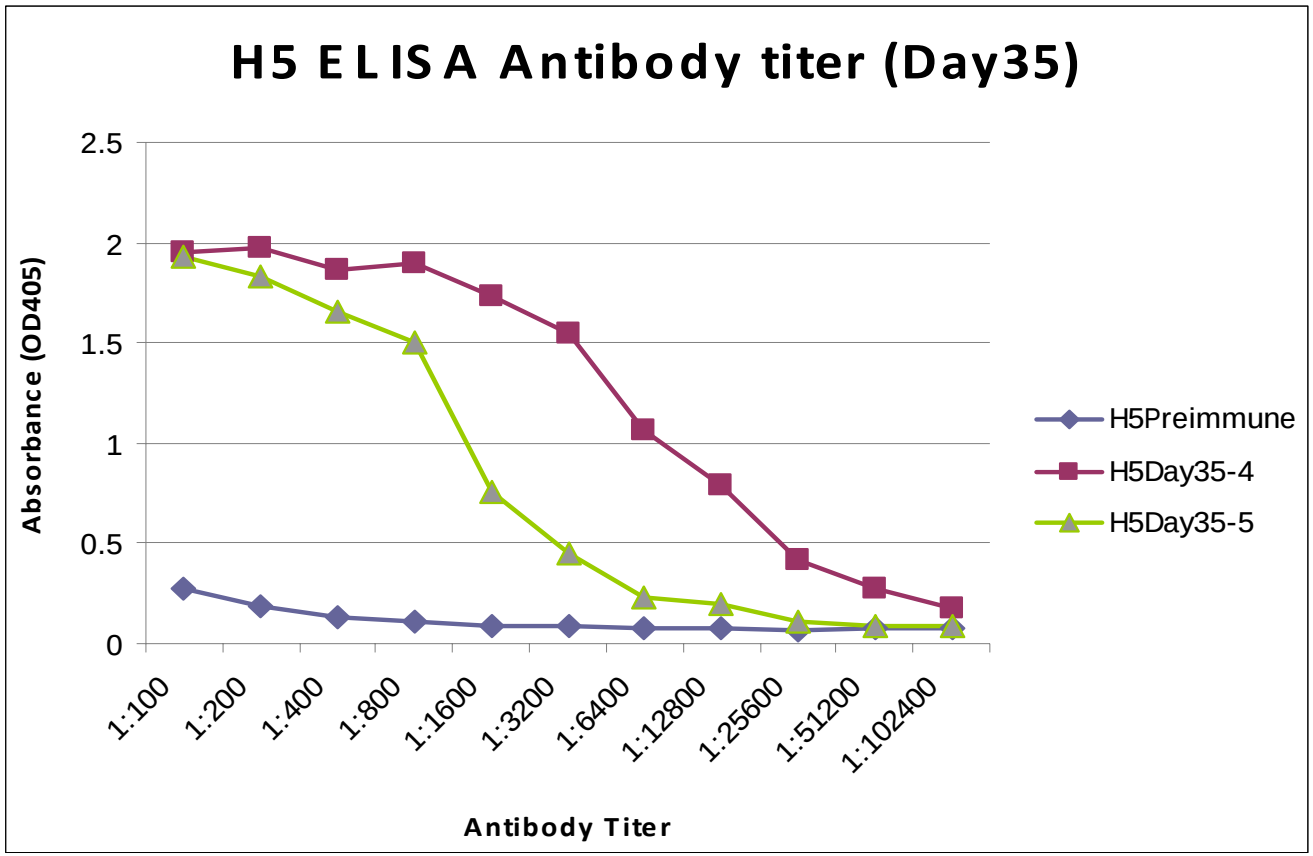


OBJECTIVES

- **Development of Fab MAb specific to hemagglutinin of H5N1 influenza virus using Phage Display Technology**
- **Characterization of the specific Fab MAbs**

Materials & Methods
&
Results

Immunization (1st Selection in vivo)



High antibody responses against H5 protein (1:10,000)

Fab genes amplification

RNA

cDNA

VH

V λ

pComb3XTT

Cκ

CH1

V λ

Sense Extension Primer

Cκ

pelB Sequence

Lead-B
Reverse Primer

VH

Lead VH

CH1

dpseq
Reverse Primer

Sense Extension Primer

Chimeric Light Chain

Fd

**dpEx Reverse
Extension Primer**

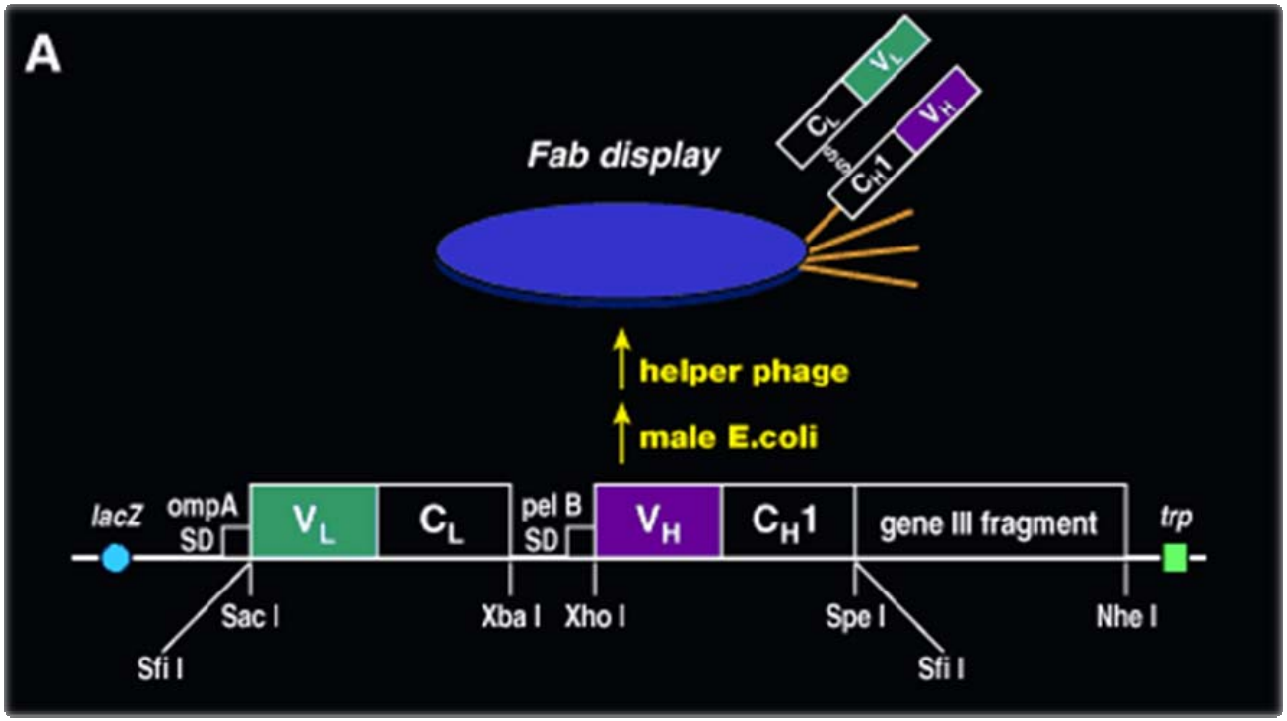
Fab fragment

Fab and pComb3Xss *Sfi*I digestion and Ligation

⬇

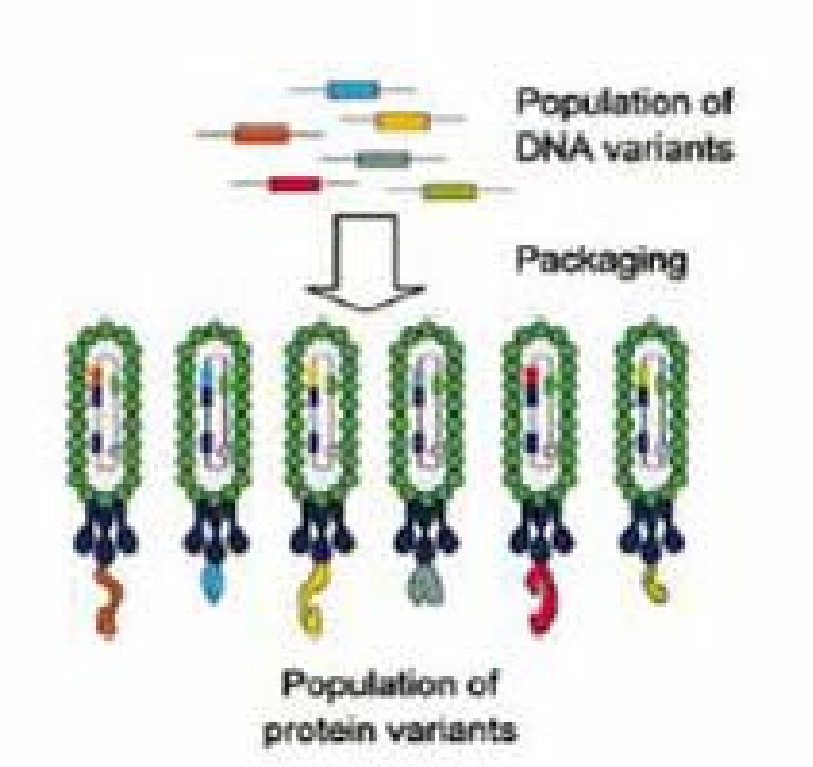
Transform to ER2738 *E. coli* cell

⬇



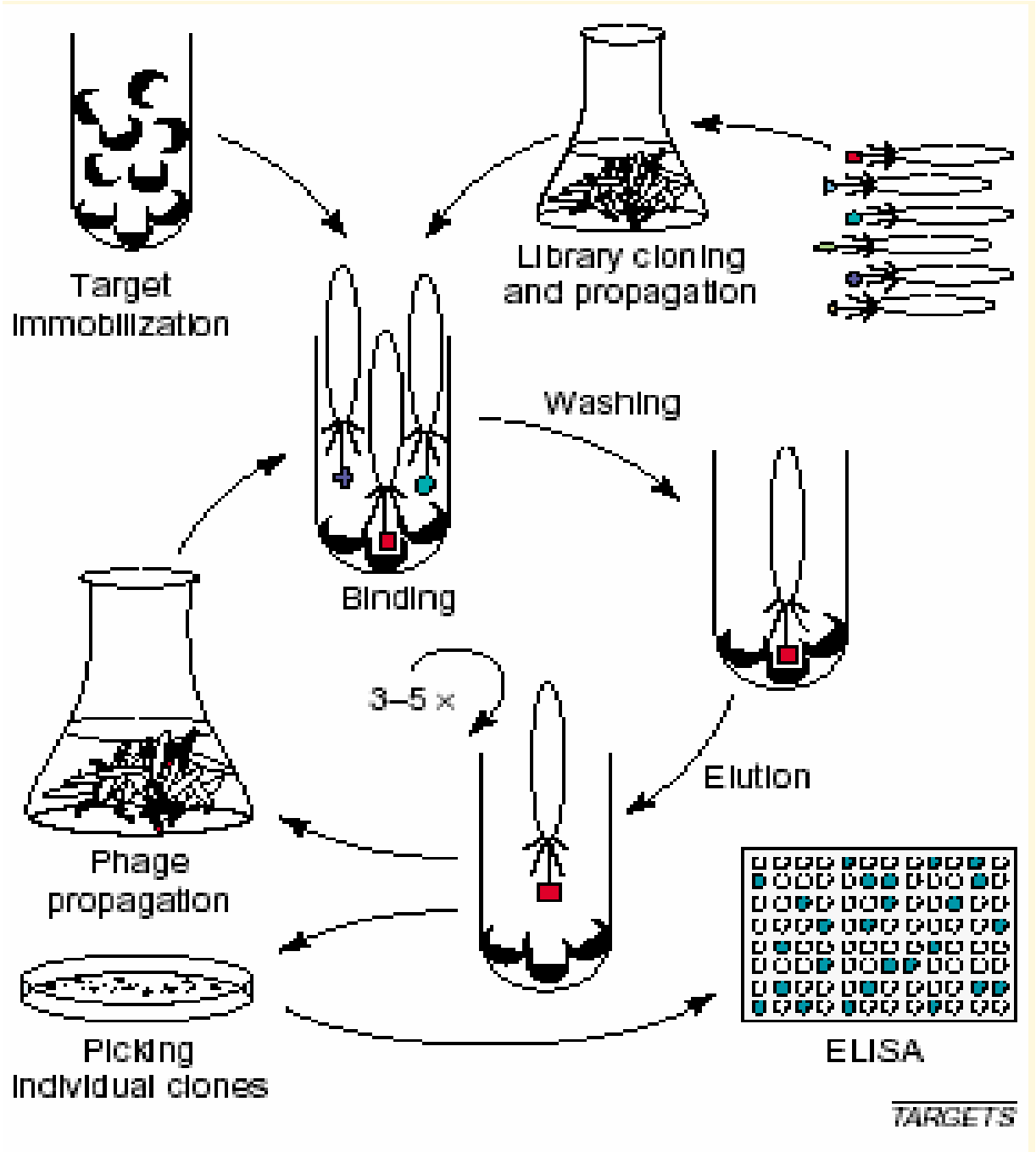
A large library with high diversity at 10^8 were produced

Antibody Library Construction



10^8 size of immunized antibody library were successfully constructed

Bio-panning (2^{nd} selection in vitro)



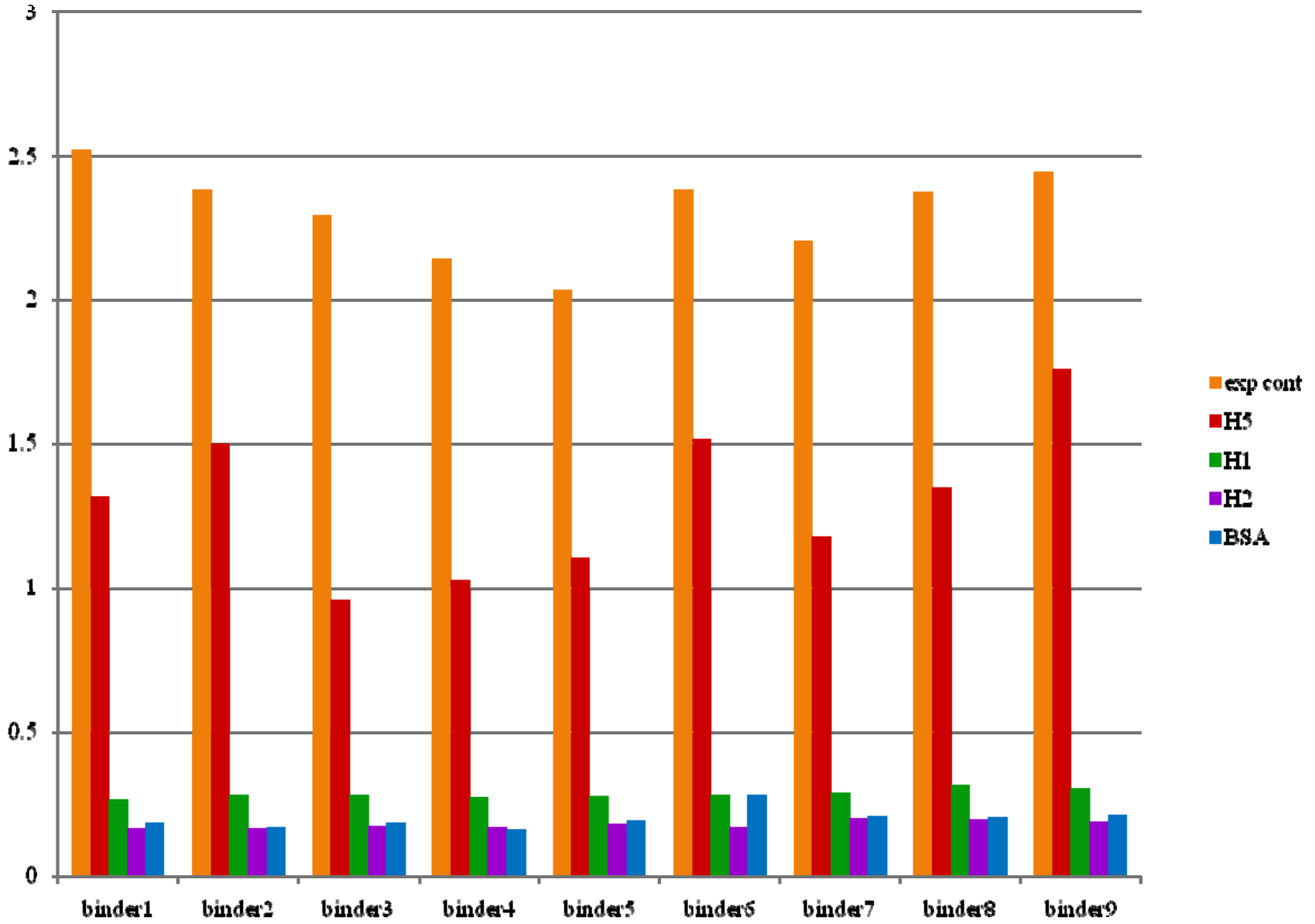
Sequences analysis

55/144 binders were ELISA positive

pComb plasmid isolation and sequencing

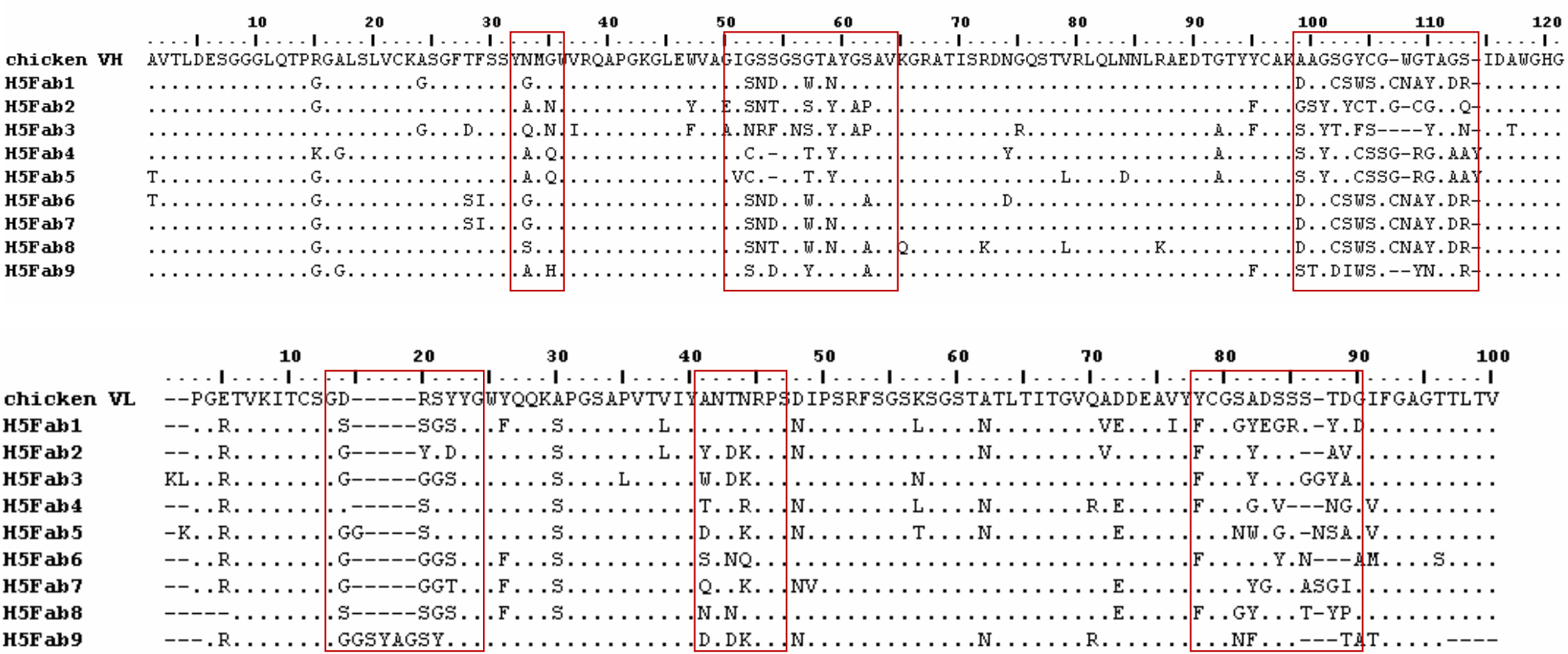
Compare amino acid sequence of both heavy chain and light chain by using clustalW software

9 different clones



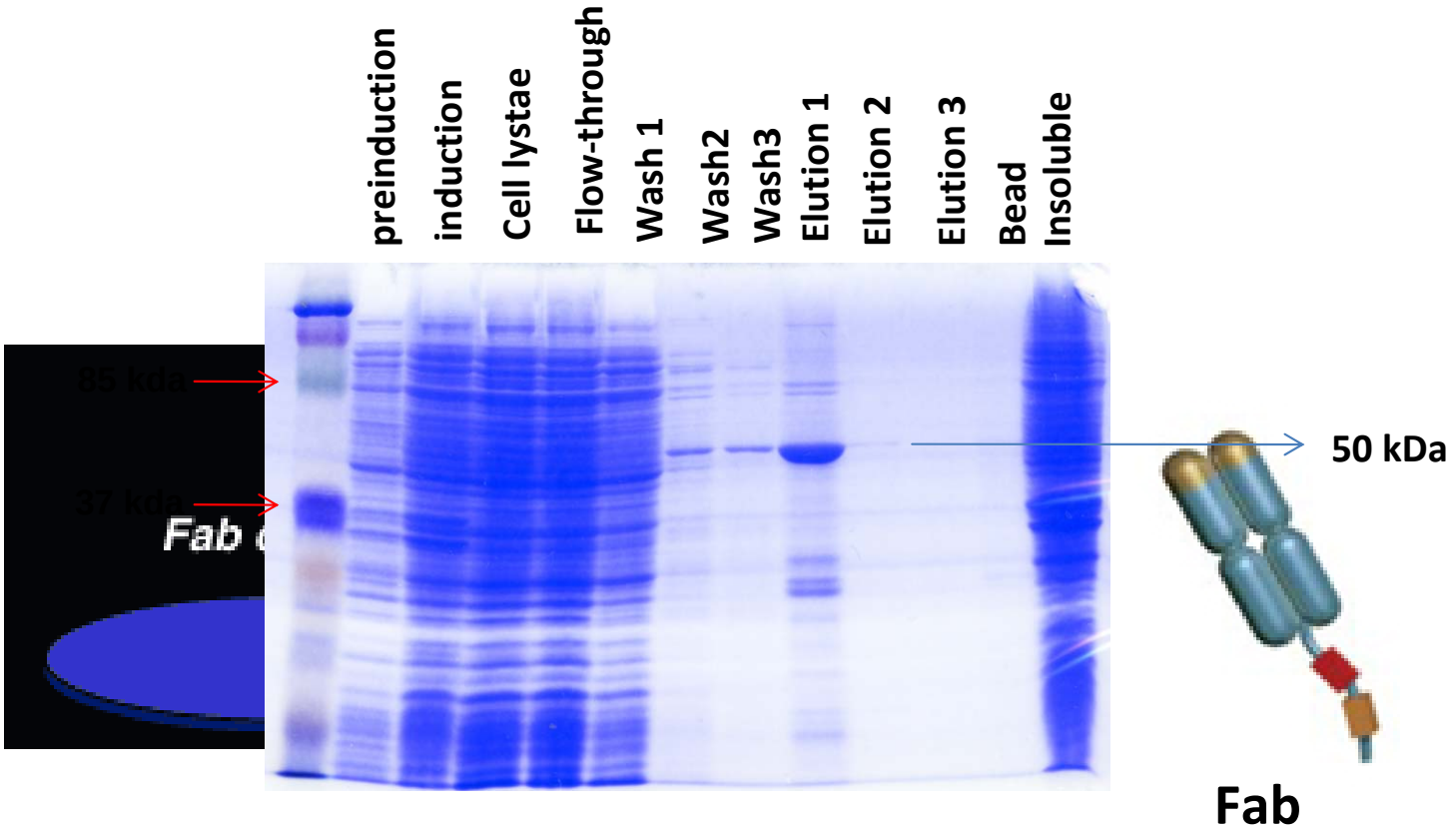
9 distinct Fab Mabs were obtained

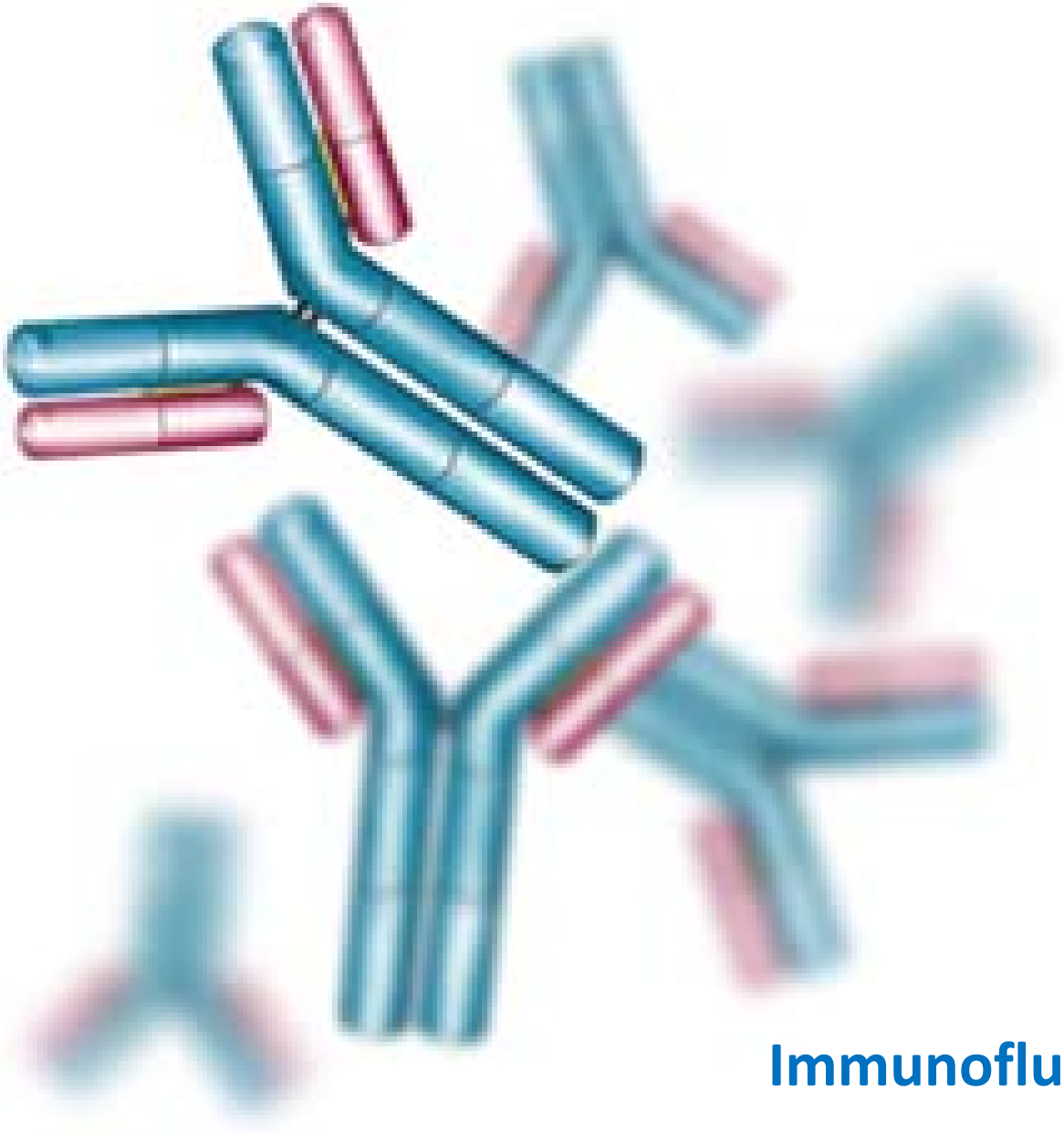
Sequences analysis



Expression & Purification of Fab MAbs

- **A large amount of soluble Fab fragments can be produced and purified in *E. coli* system**

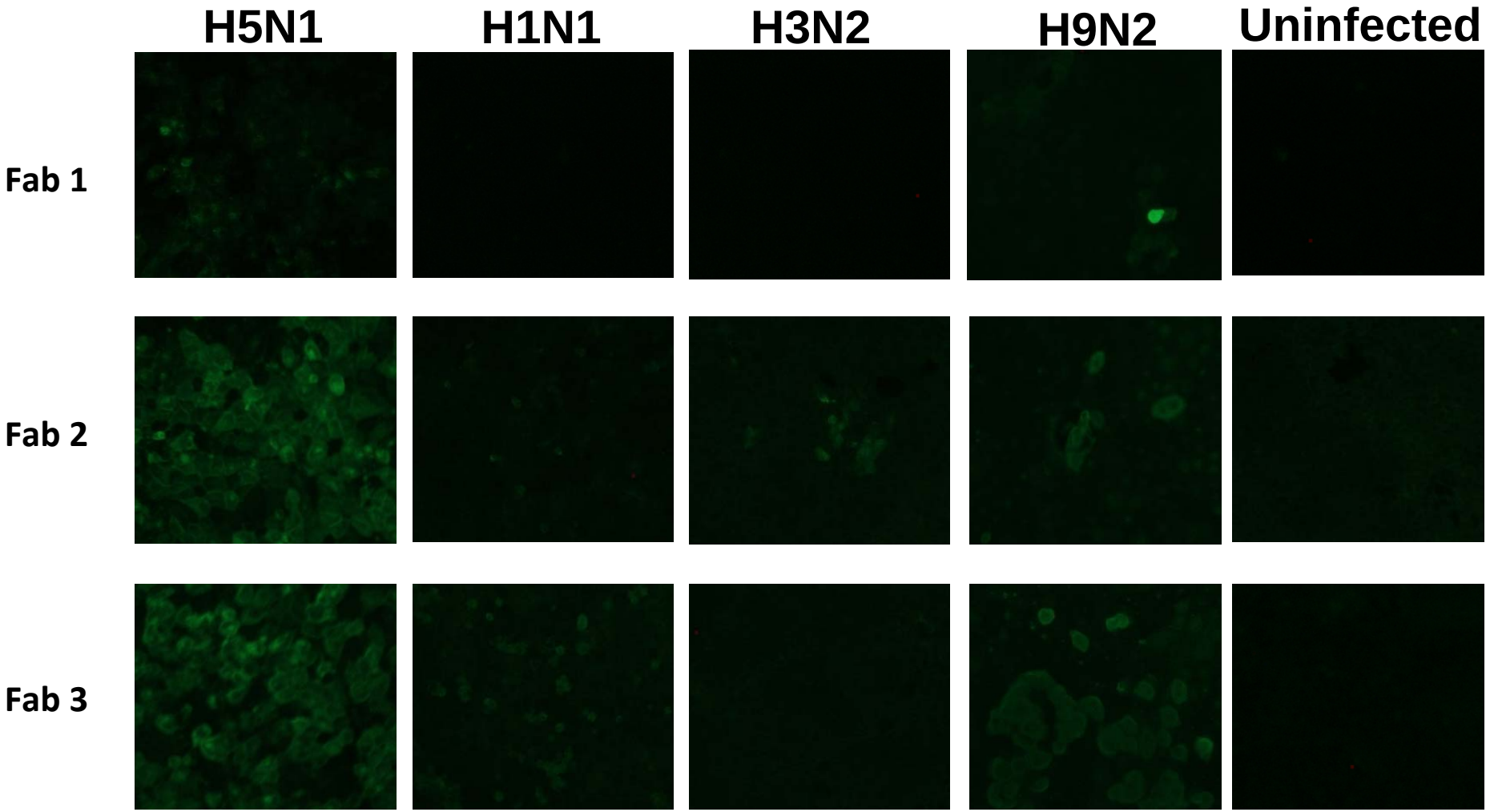




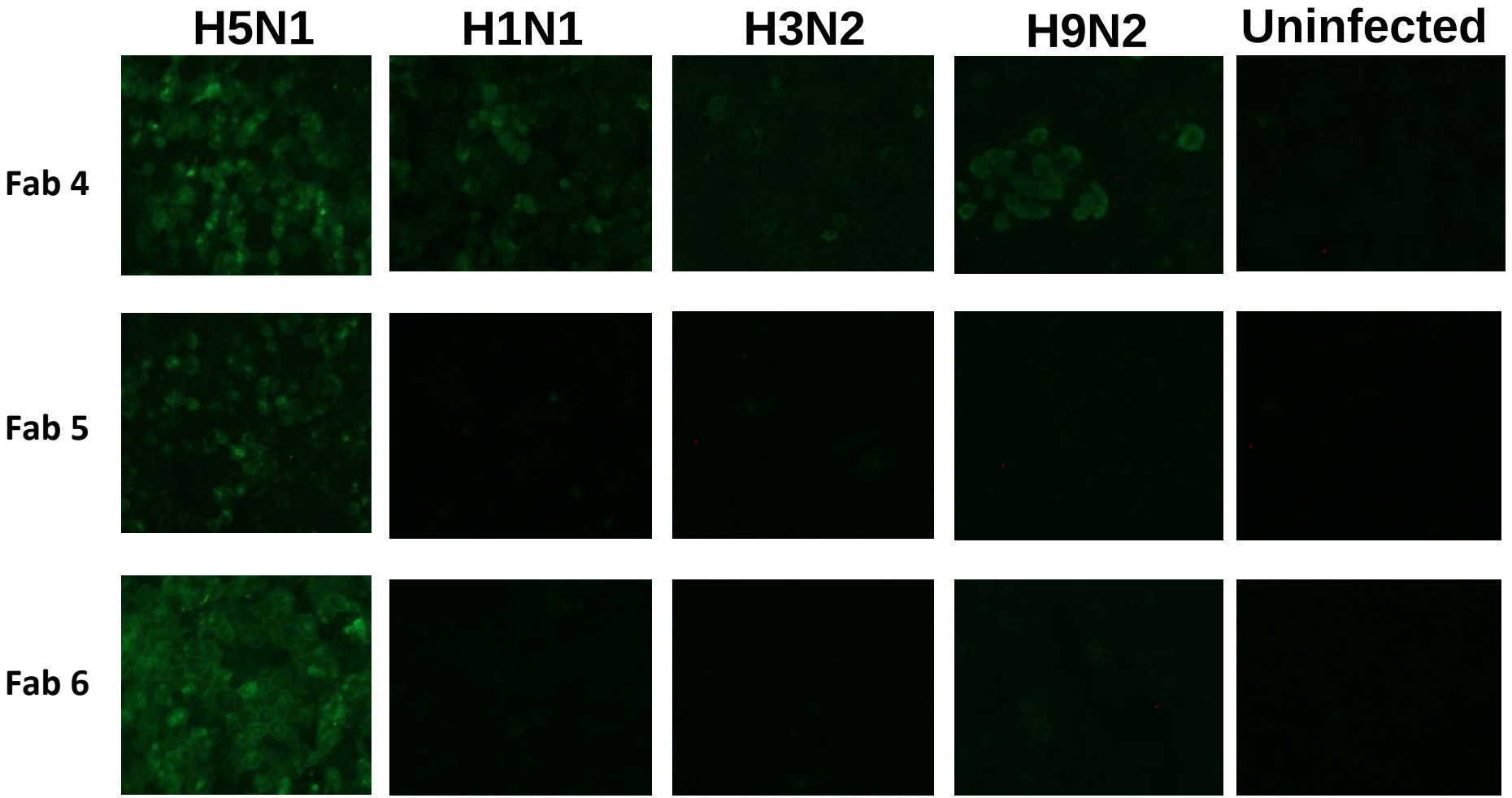
Immunofluorescense Assay

SPECIFICITY TEST

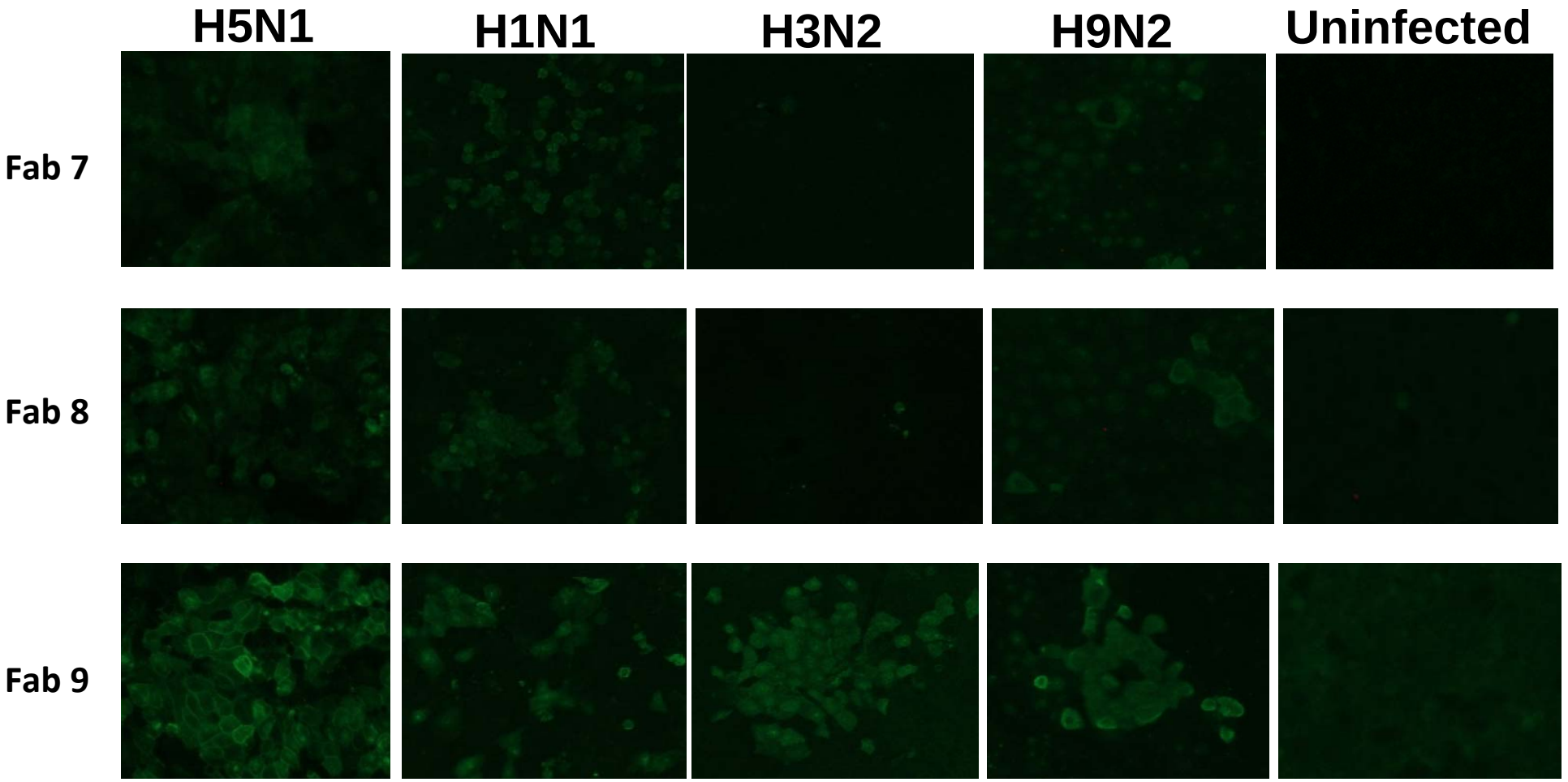
Immunofluorescense of 9 Fab MAbs



Immunofluorescense of 9 Fab MAbs



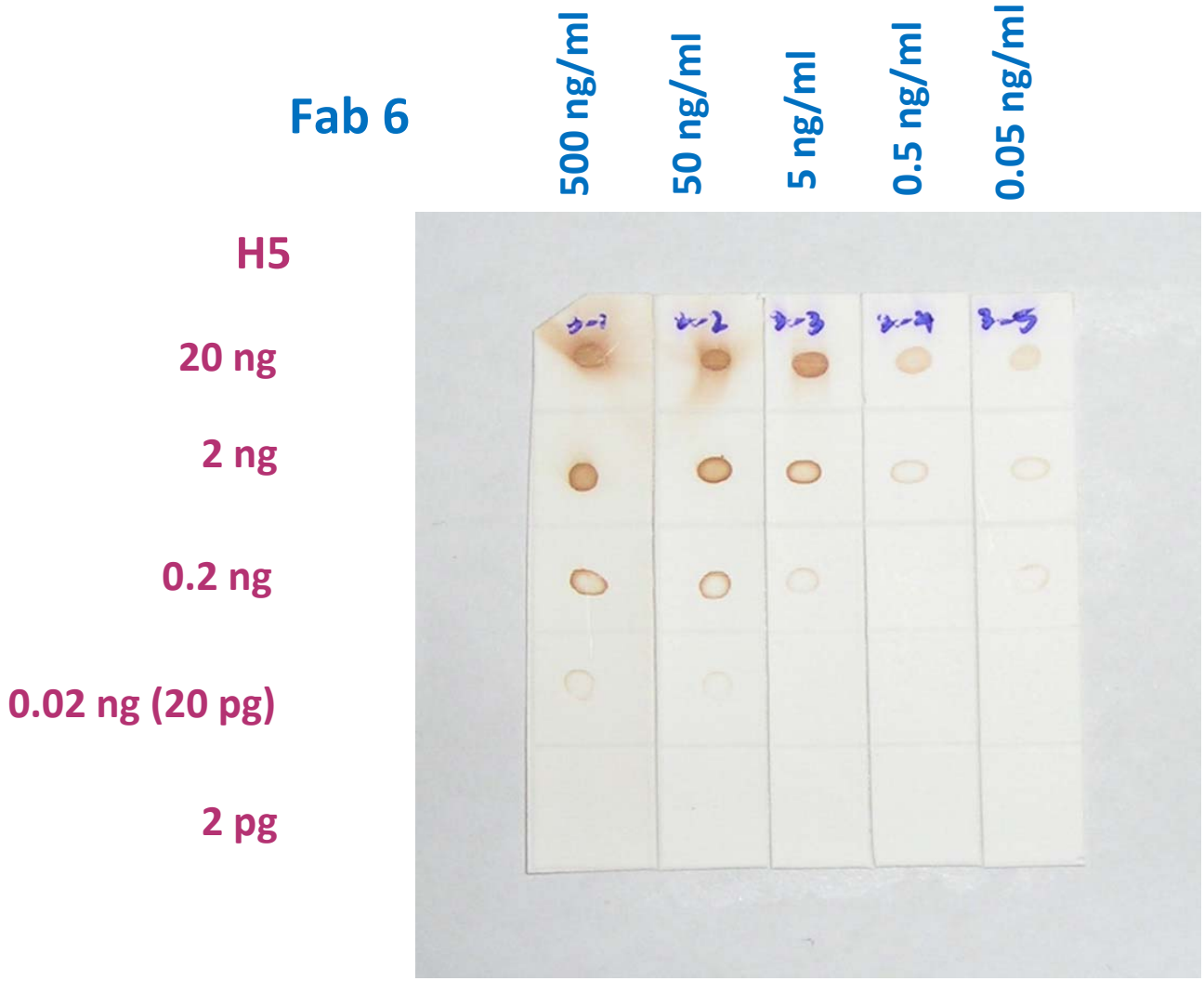
Immunofluorescense of 9 Fab MAbs



Binding reactivity of the 9 Fab MAbs to different subtypes of influenza A virus

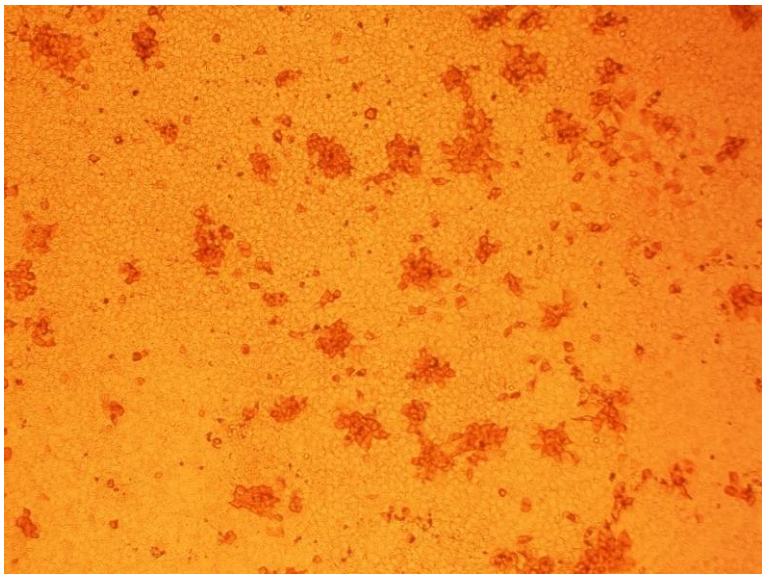
Fab	Influenza A virus			
	A/crow/Kyoto/53/2004 (H5N1)	A/PuertoRico/8/1934 (H1N1)	A/Sydney/5/1997 (H3N2)	A/Turkey/Wisconsin/1/66 (H9N2)
H5Fab1	+	-	-	-
H5Fab2	+	-	+	+
H5Fab3	+	+	-	+
H5Fab4	+	+	+	+
H5Fab5	+	-	-	-
H5Fab6	+	-	-	-
H5Fab7	+	+	-	+
H5Fab8	+	+	-	+
H5Fab9	+	+	+	+

SENSITIVITY TEST

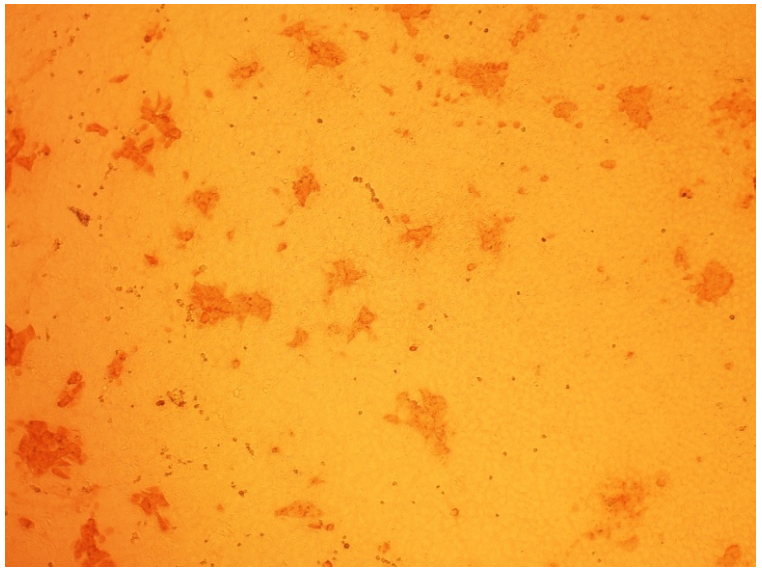


NEUTRALIZING ACTIVITY

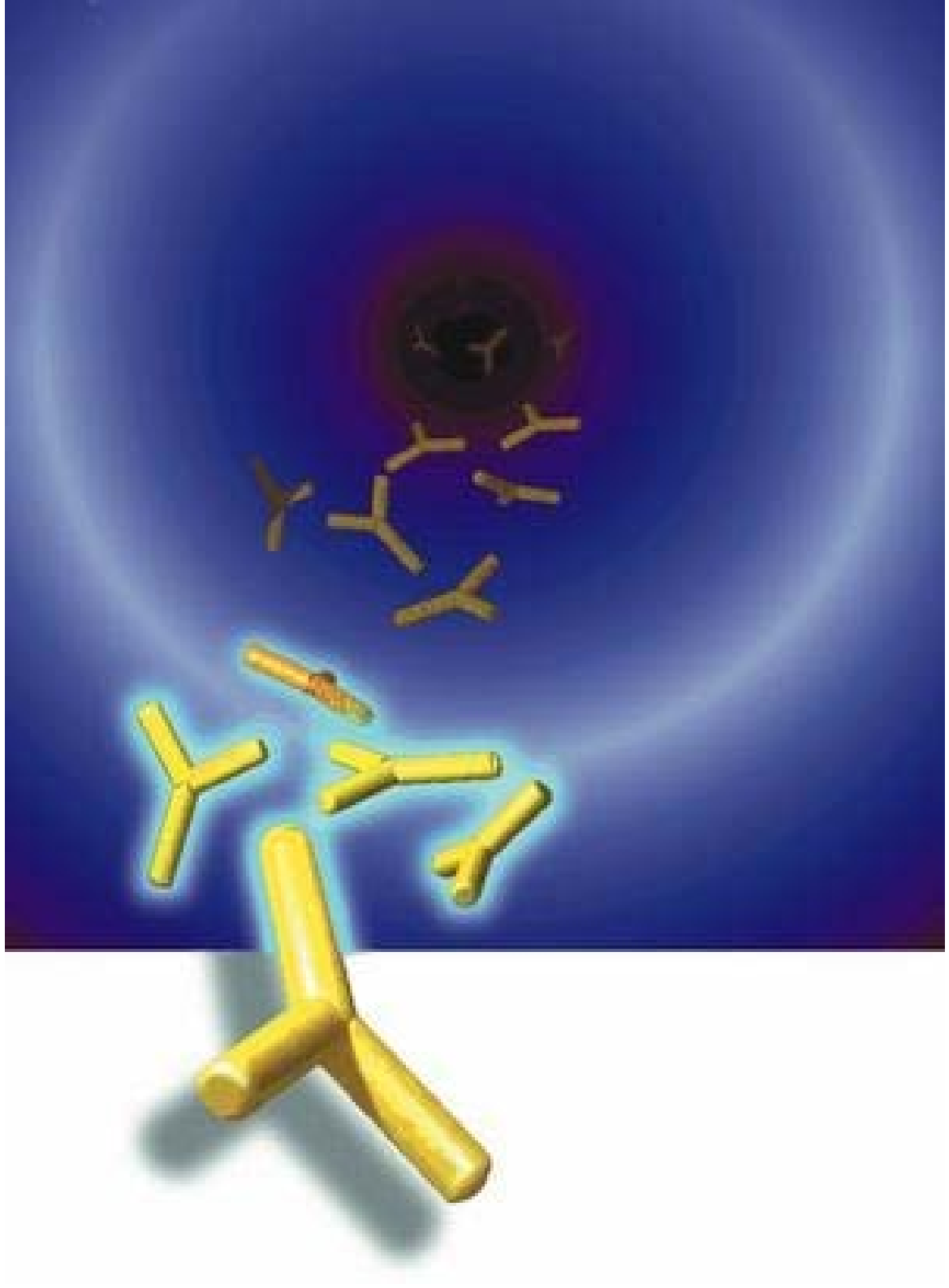
One clone (Fab2) can inhibit virus infection to MDCK cell



No antibody control



Fab2 at 25 μg/ml



DISCUSSIONS & CONCLUSION

DISCUSSION & CONCLUSION

MAb production

Hybridoma Technology

Whole IgG is the final product
Required cell culture facility

Phage Display Technology

Functional region of IgG were constructed
Using recombinant DNA technology

Phage Display Technology

Naïve library

Only one library can be used for diverse antigen
Antibody against non-immunogenic antigen
can be produced
Very large library is required for high affinity antibody
(10^9 ~ 10^{10})
Longer time is required
Sometime impossible for normal lab scale

Immunized library

Library needed to be constructed for each Ag
High affinity Ab can be obtained smaller size
of library 10^8

DISCUSSION & CONCLUSION

- **The immunized chimeric chicken/human Fab antibody library were constructed (10^8 size)**

- **For H5 detection test development, the Hemagglutinin protein (H5) were used as the target antigen**

DISCUSSION & CONCLUSION

- After sequence analysis, 9 unique Fab were obtained.
- For further characterization, soluble Fab were produced.
  - Unassembled heavy and light were observed
- Reactivity of each clones to influenza A virus by IFA
  - 3 Fab (H5Fab1, 5, 6) were highly specific to H5N1 virus, that can be used for rapid detection test development
  - 1 Fab (H5Fab9) is cross-reacted to all subtypes of influenza A virus tested
  - 1 Fab (H5Fab2) show neutralization activity
    - Humanization needed to perform
    - Conversion of Fab to whole IgG may enhance the neutralization activity (Fleury et al., 1999)

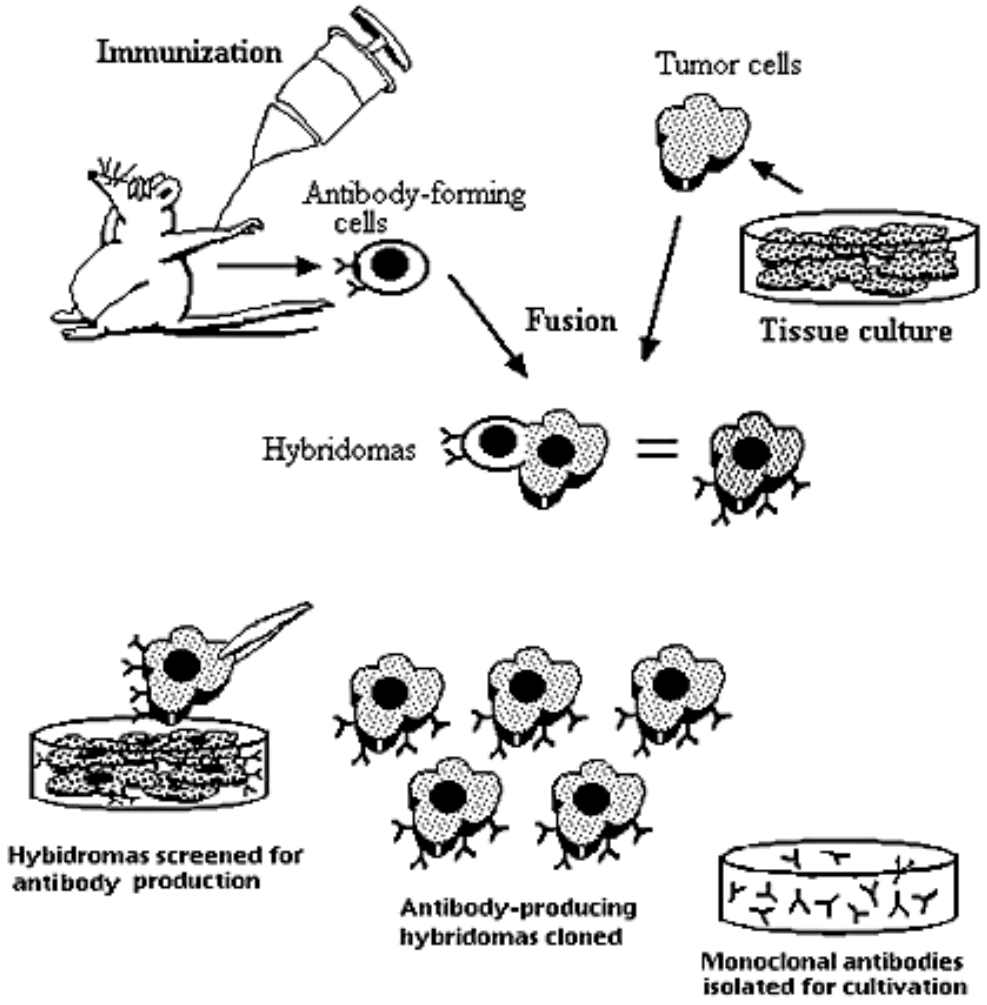
Acknowledgement

- TRF Research Scholar (The Thailand Research Fund).
- Commission on Higher Education, Thailand.
- The Scripps Research Institute, USA.
- Osaka University

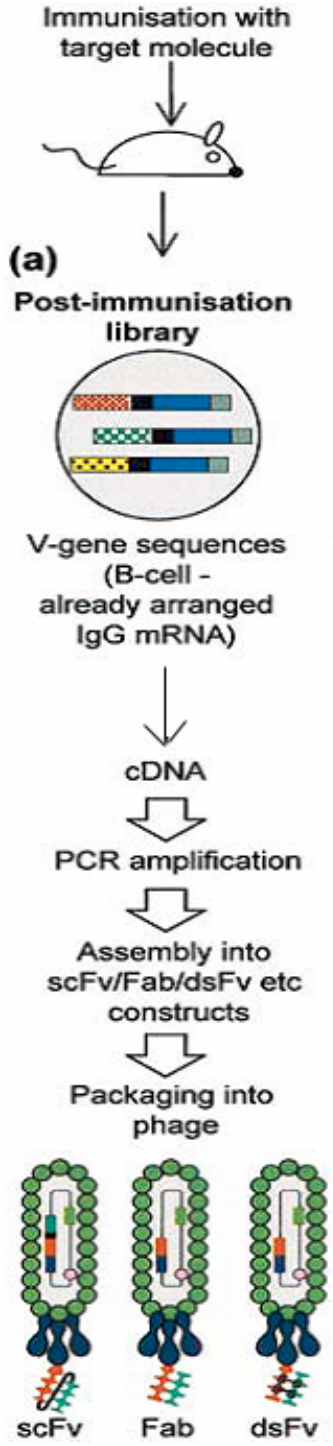
Technology for MAb production

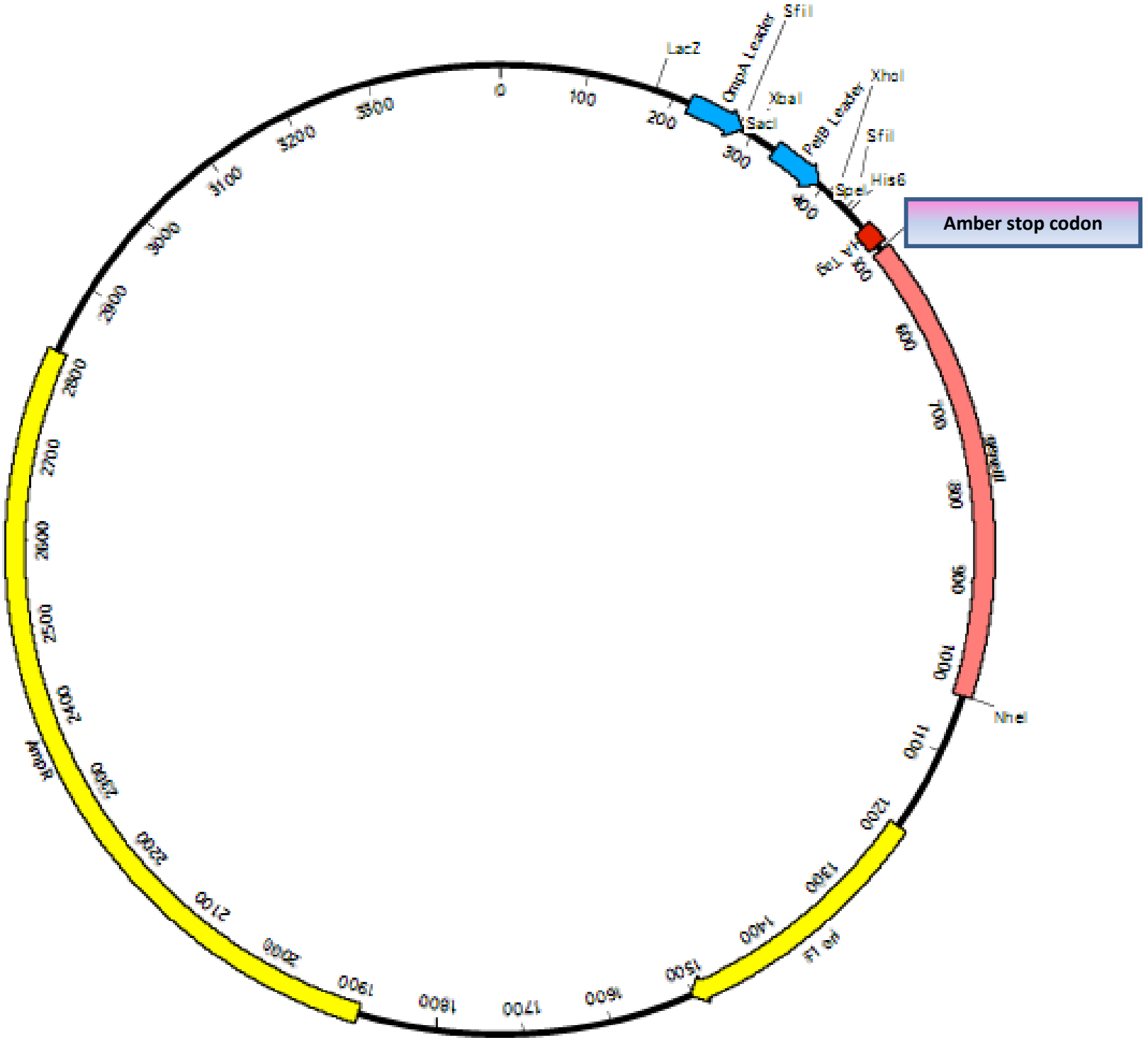
Pros & Cons	**Hybridoma technology 1970** (Tissue culture based)	**Phage display technology 1990** (Genetic engineering)
Sensitivity, & Specificity Stability	high moderate	high high
Labor intensive	high	moderate
Reagent Cost	high	Low
Equipment Cost	high	Low

Monoclonal antibody Production

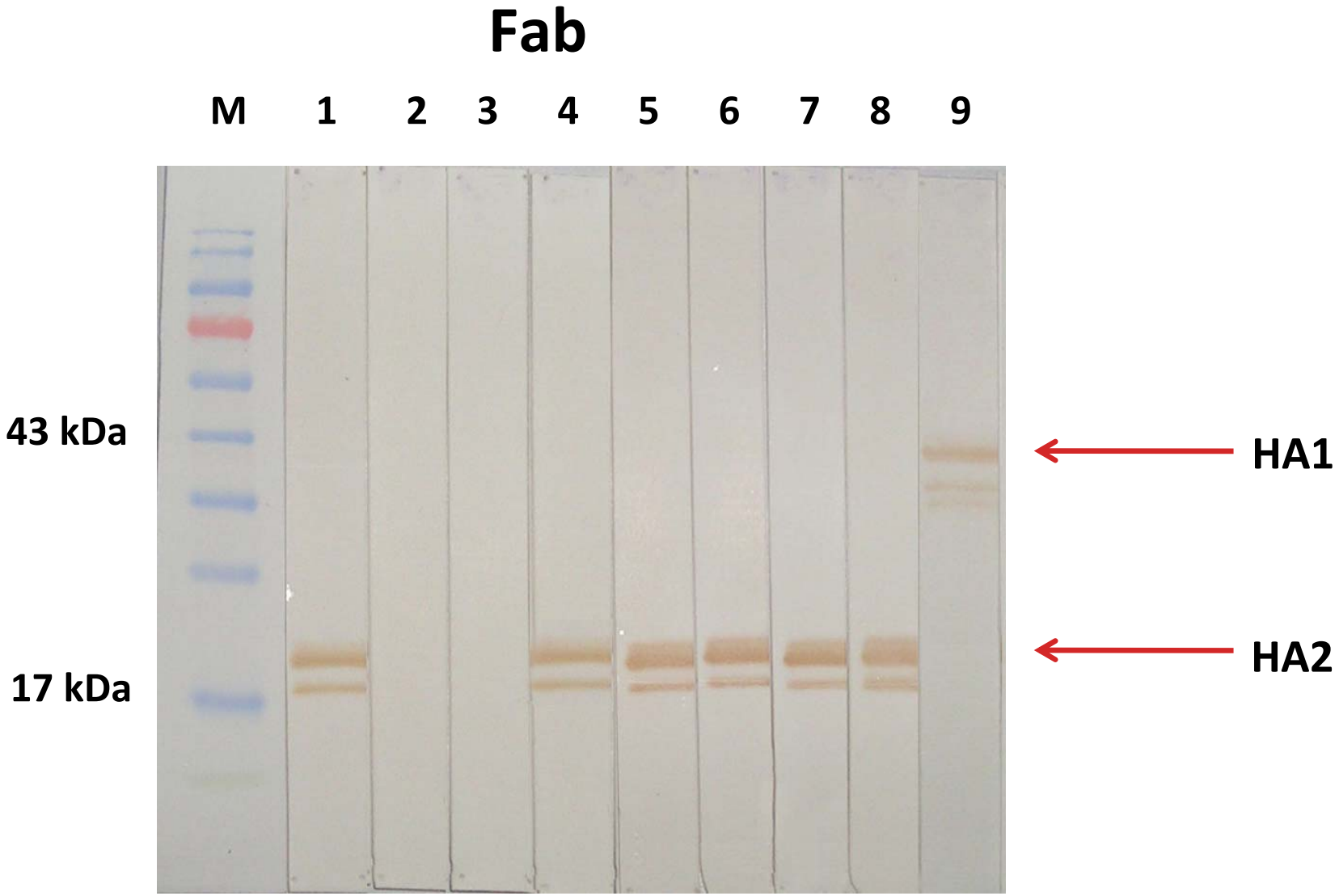


Monoclonal Antibody Production

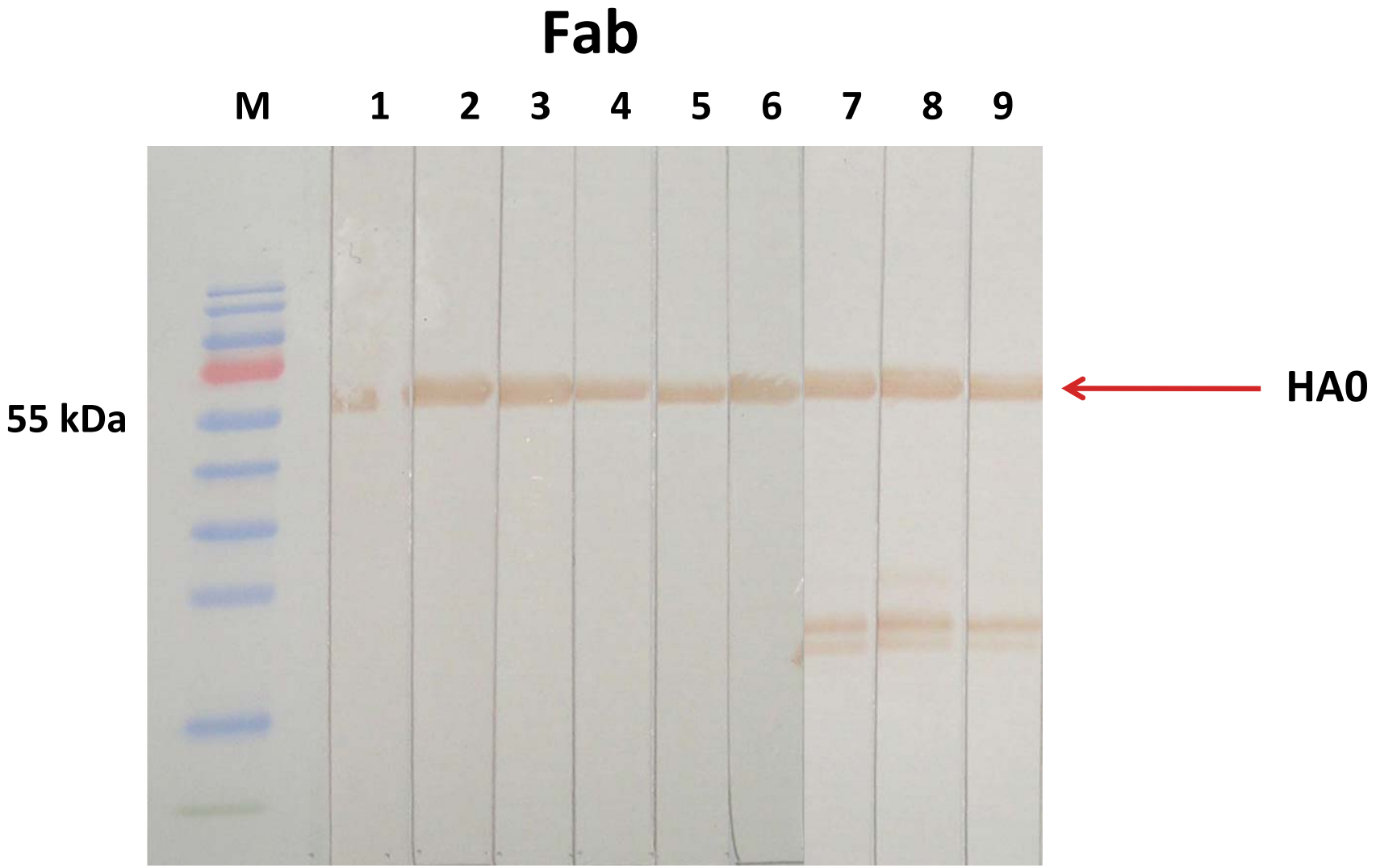



LacZ
OmpA Leader
SfiI
XbaI
SacI
PelB Leader
XhoI
SfiI
SpeI
His6
Amber stop codon
NheI
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000
2100
2200
2300
2400
2500
2600
2700
2800
2900
3000
3100
3200
3300

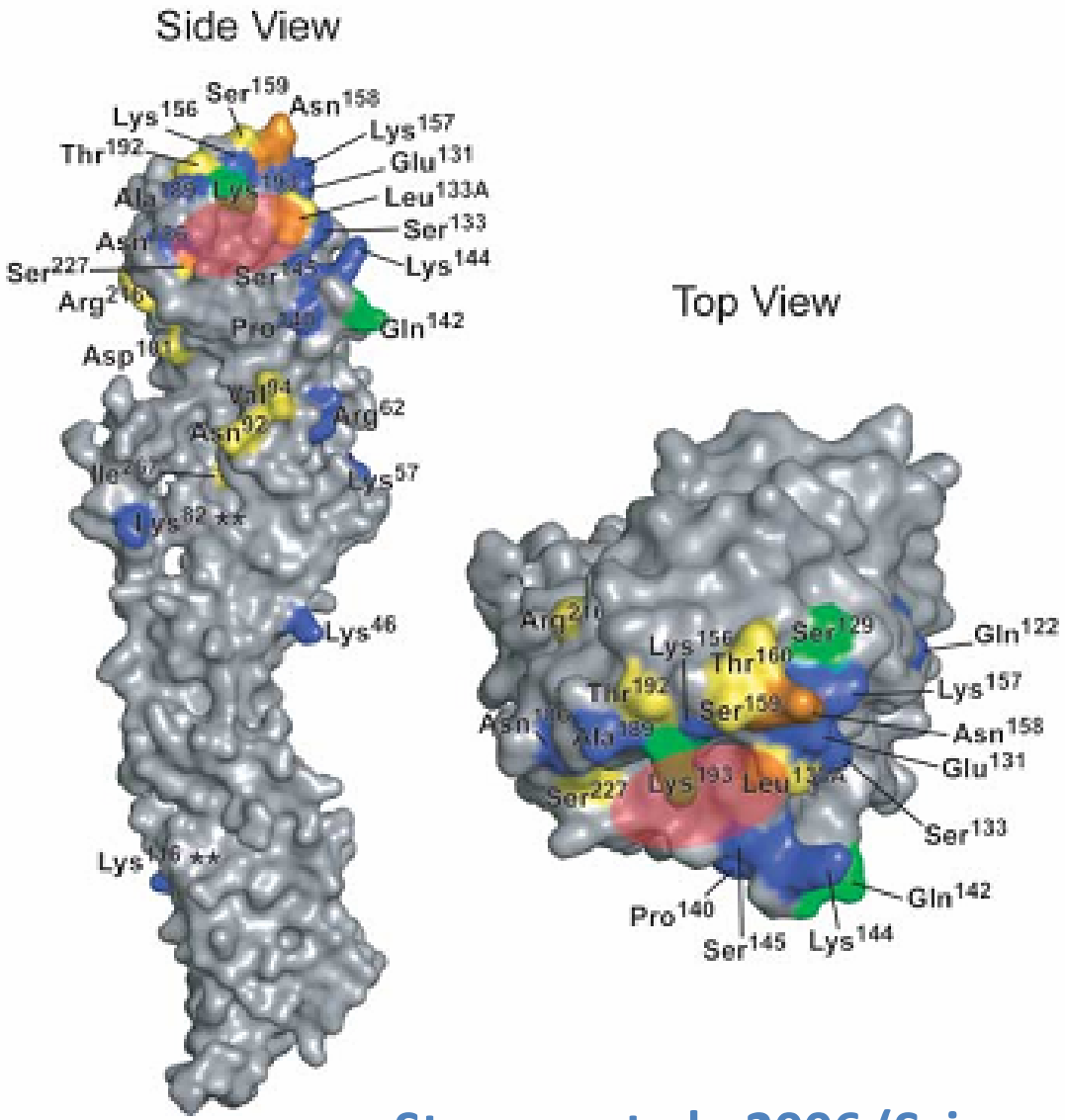
Western Blot analysis (reducing)



Western Blot analysis (non-reducing)



DISCUSSION & CONCLUSION



Stevens et al., 2006 (Science)

- Natural mutations are colored yellow
- Escape mutants are colored blue
- Overlap positions are colored green
- New glycosylation position is colored orange
- Receptor binding site is highlight in red oval