Anti-HCV IgM Detections Using Reporter Molecule Generated by Directed Molecular Evolution

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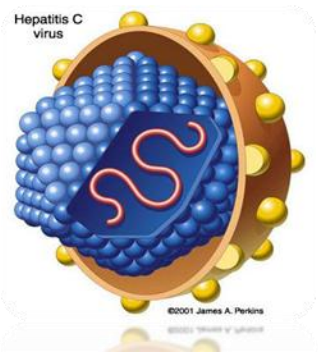
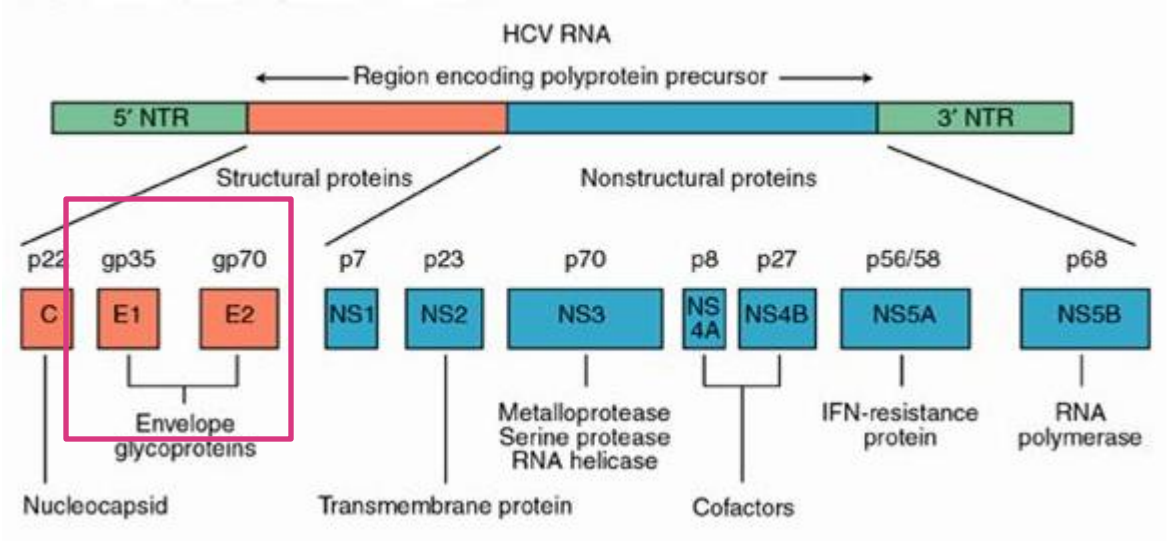
Background

Hepatitis C virus (HCV)

RNA-enveloped virus belonging to the *Flaviviridae.*





Hepatitis C Virus (HCV)

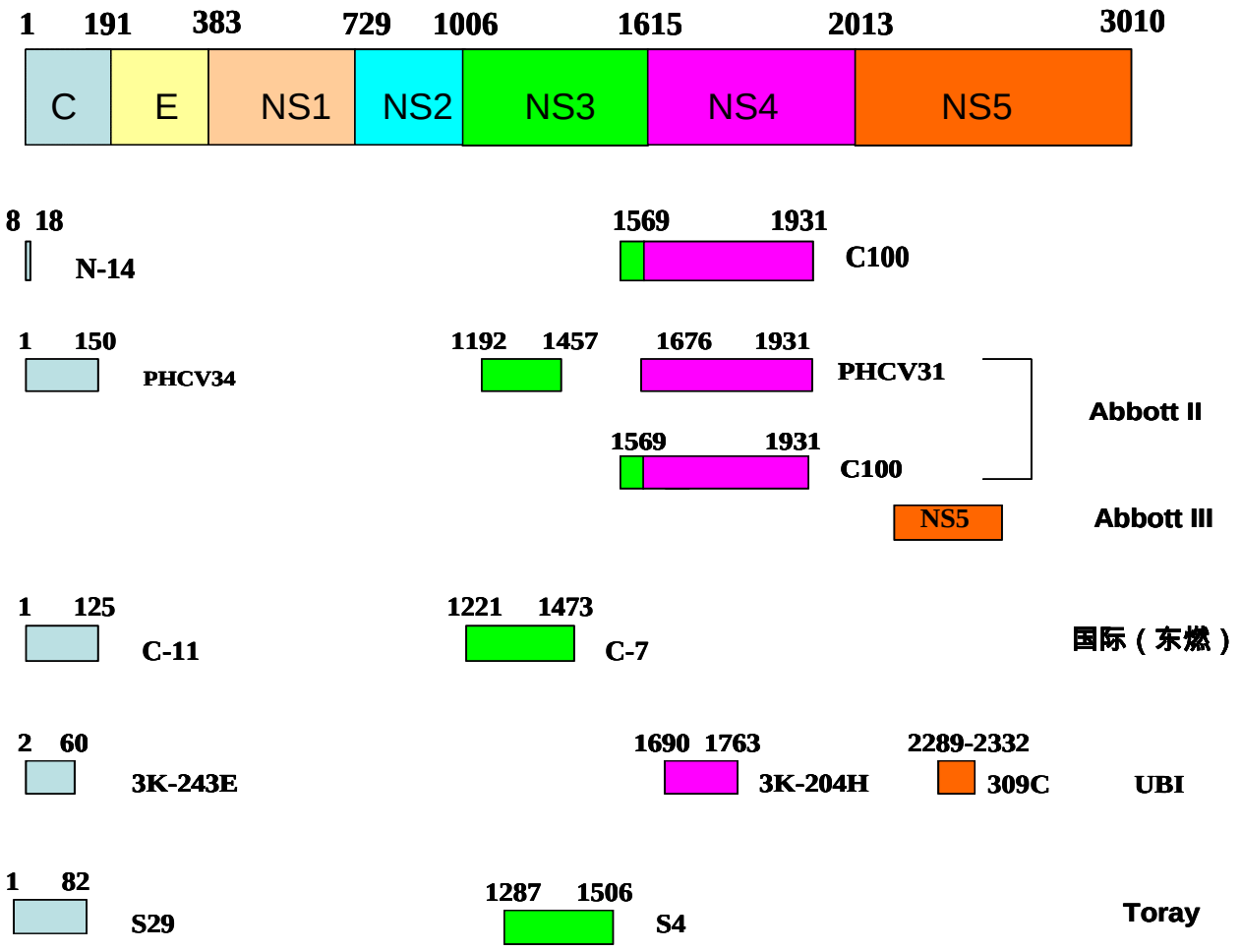
An estimated 170 million people are infected with hepatitis C virus (HCV) worldwide and at least 3–4 million people are infected each year .

World Health Organization,1999,Crabb, 2001

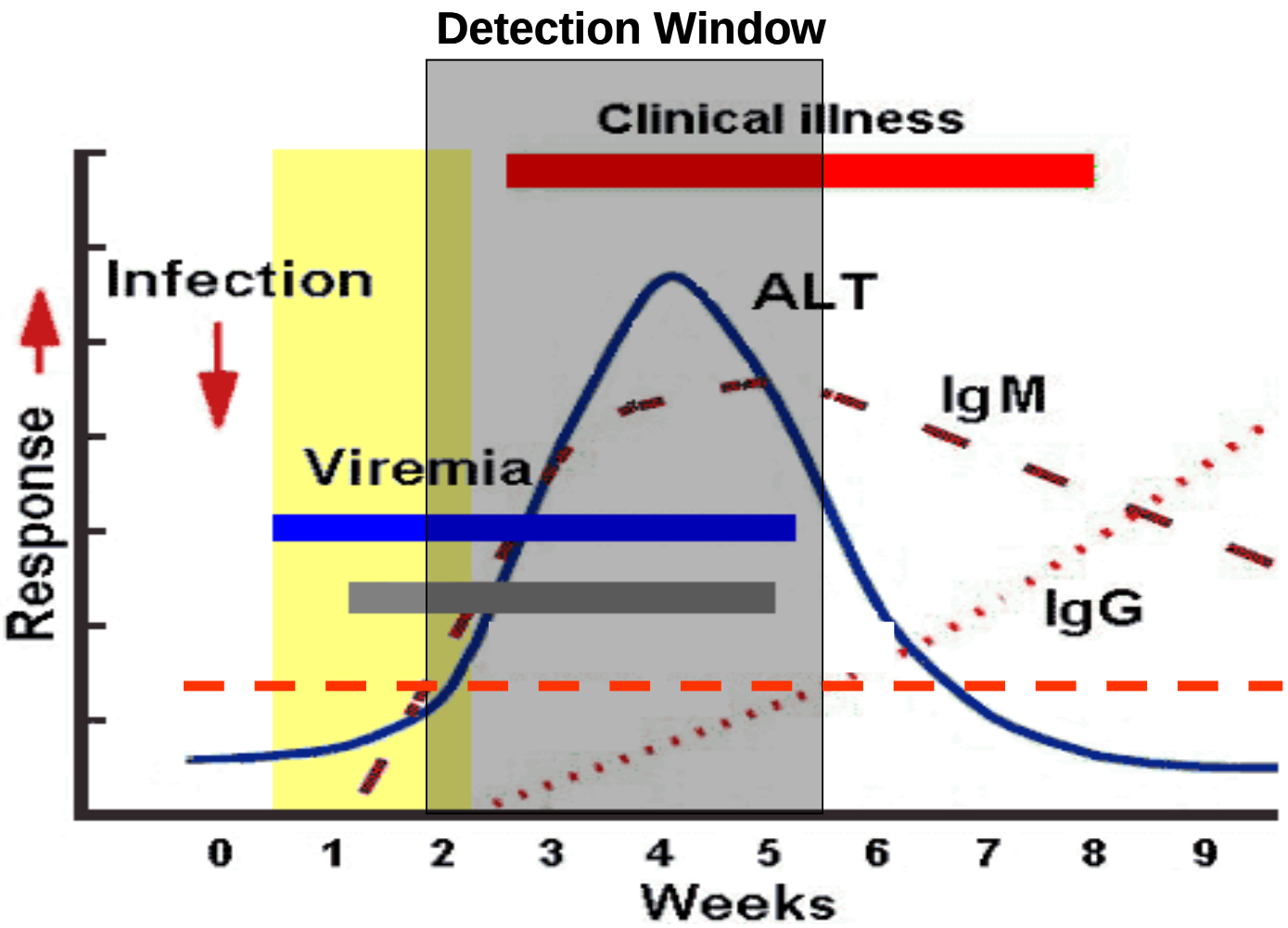
Over 70 % of acute infections eventually become persistent, of which a significant proportion develop liver cirrhosis and ultimately hepatocellular carcinoma

Crabb, 2001; Lavanchy & McMahon, 2000

Anti-HCV EIA Improved by Adding New Antigens



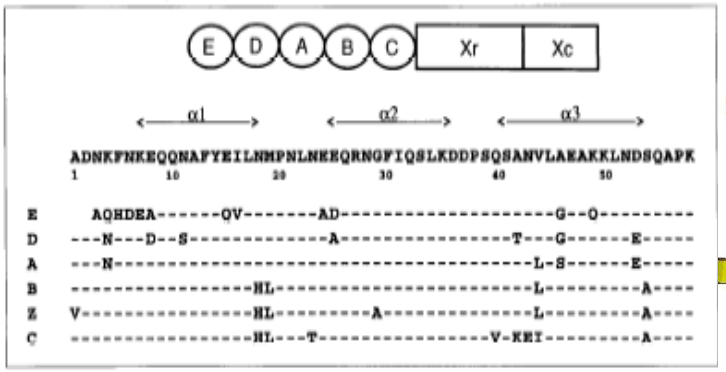
Detection Window: A Biggest Problem of Anti-HCV Assay

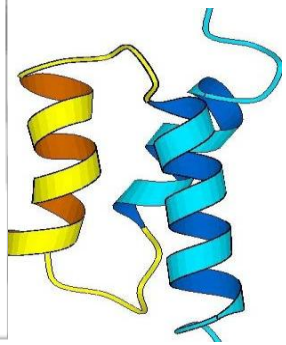


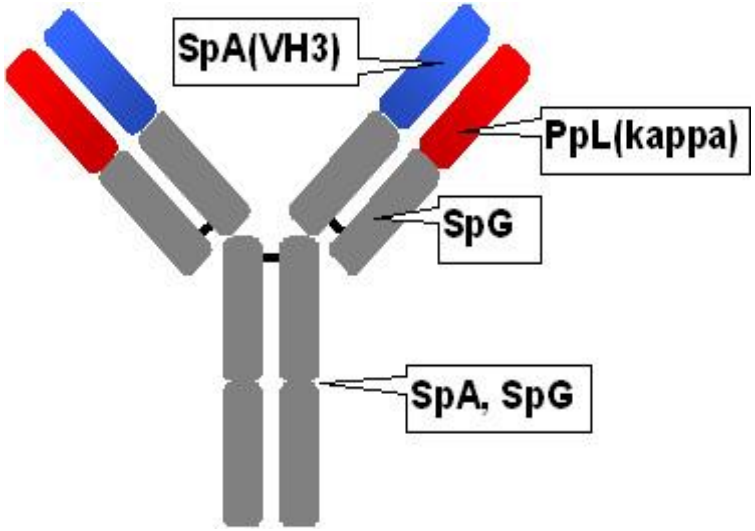
Bacterial Immunoglobulin Binding Proteins (IBPs)

Staphylococcal protein A (SpA)

IgG, IgM, IgA, IgD, IgE

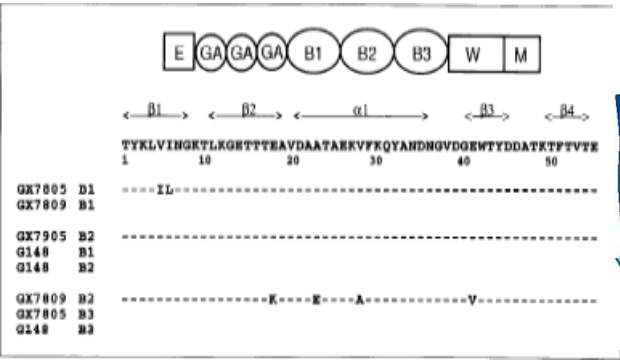


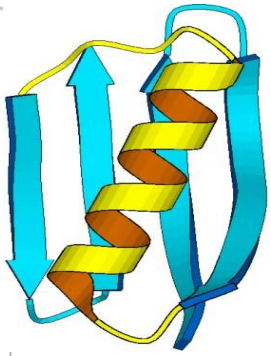




Streptococcal protein G (SpG)

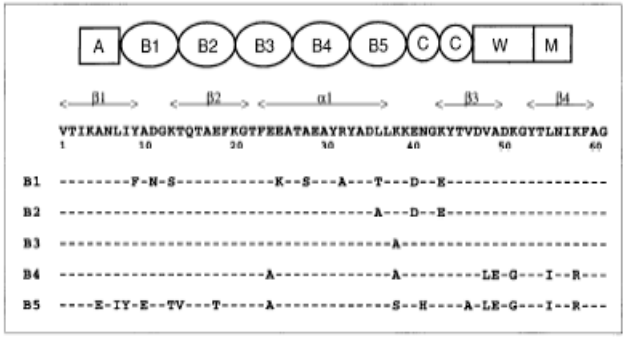
IgG

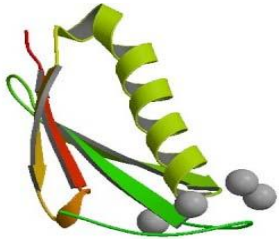




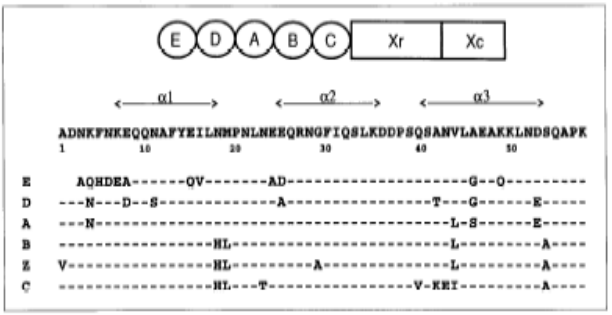
Peptostreptococcus magnus protein L (PpL)

IgG, IgM, IgA, IgD, IgE

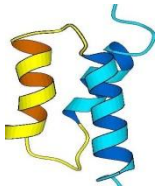




Phage Library Displaying Randomly Linked Binding Domains of SpA and PpL

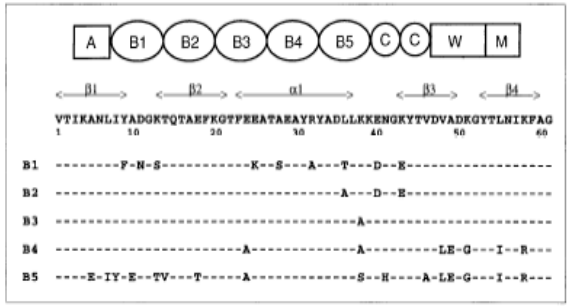


Protein A



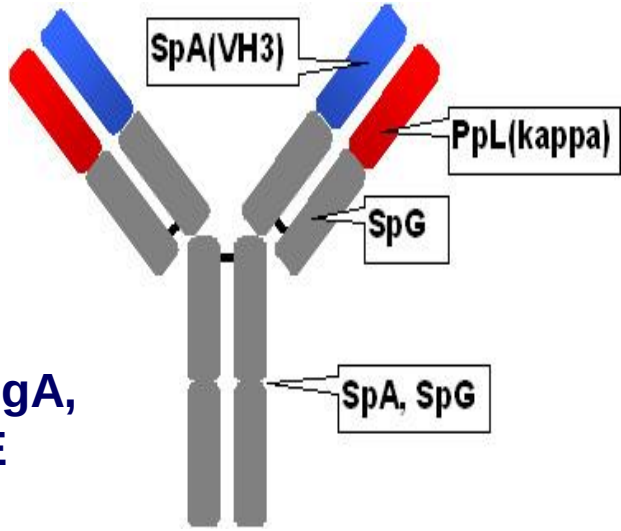
IgG,

IgM, IgA, IgD, IgE



Protein L

IgG, IgM, IgA, IgD, IgE



B A C D

B1 B2 B3

B B3 A

d B2

a D B2 C

B1 C B3

D A

B1 C B2 a b3

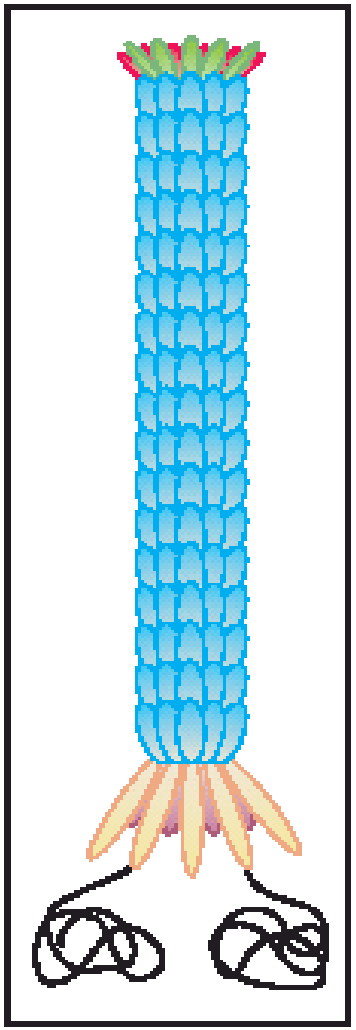
. . . . . .

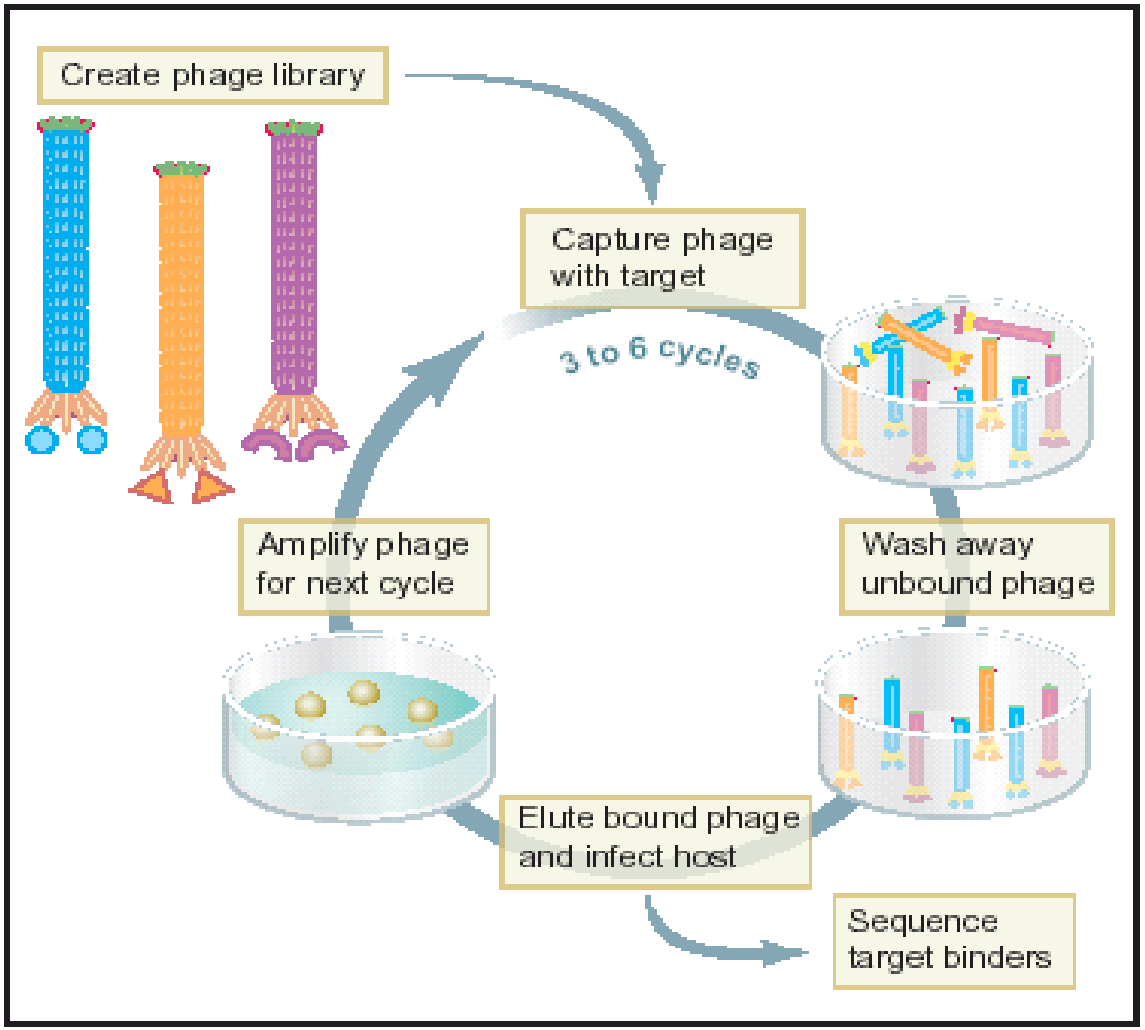
Note:

The Capital letter refers to forward sequence

The Lower case letter refers to reverse sequence

Molecular Evolution of the Phage Library Directed By Human Immunoglobulin





Novel Evolved Immunoglobulin Binding Molecules (NEIBMs) Characterized by Alternate Arrangement of PpL B3 Domain and SpA D Domain

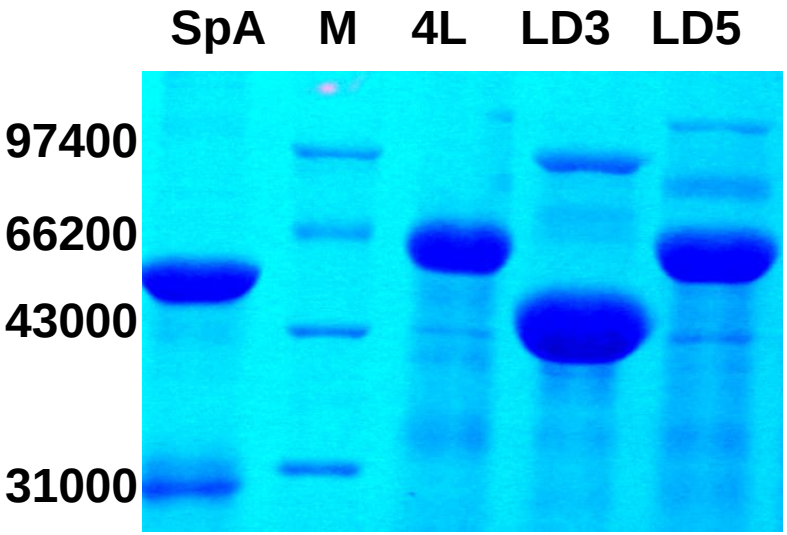
Table 4 Sequence analysis of phage displayed fragments of Ig affinity selection of combinatorial molecular library

The original library		The third round selection			The fourth round selection		
1domain	2 domain	2 domain	3 domain	>3 domain	2 domain	3 domain	>3 domain
3# A	7# A-Lr	4# A-D	6# B-A-L	3# L-D-L-A	1# L-L	3# L-D-L	4# L-D-L-D-L
8# L	9# L-Dr	7# L-A	26# L-D-L	9# L-A-L-D-L	8# L-A	6# L-D-L	5# L-C-L-D-L
10# Br	11# Lr-L	8# B-L	31# L-C-L	27# L-D-L-D-L	10# B-L	9# A-D-L	13# L-D-L-C
14# Lr	18# Br-L	19# D-L	34# A-B-L	35# A-D-L-B-L	21# L-C	19# L-C-L	26# L-A-L-B
40# D	23# A-D	33# L-C	38# L-D-L	36# L-A-L-B	25# L-L	29# L-B-D	33# L-D-L-D-L
未测出	33# Lr-C	42# L-L	40# A-D-L	44# L-D-L-D-L	37# A-L	37# A-B-L	41# A-D-L-D-L

A,B,C,D represent the A,B,C,D domain of protein A respectively. L represents B3 domain of protein L. "#" is the number of different clone of original library and third and fourth round Ig affinity selection library. "r" represents arrange in reverse. Underlined represents clones containing $(MDPL\text{-}MDPA)_n$ structure.

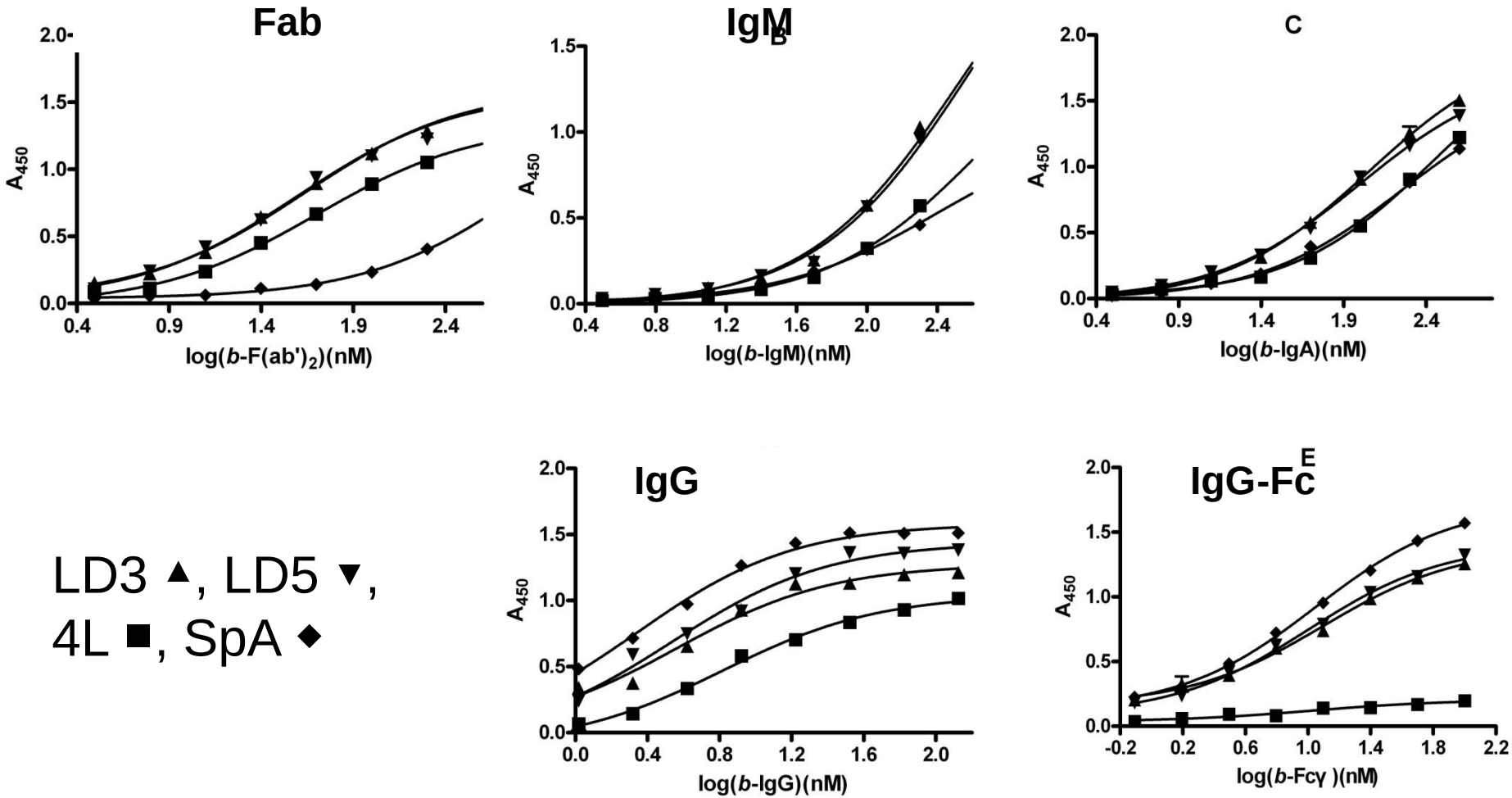
Production of The Representative Molecules of NEIBMs:

L-D-L (LD3) and L-D-L-D-L (LD5)



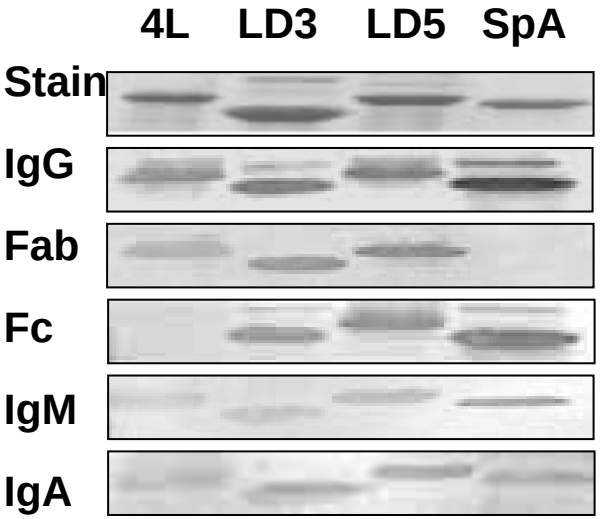
SDS-PAGE analysis of purified 4L、LD3、LD5 and SpA

LD3/LD5 Showed Increased Binding Activity With IgG-Fab、IgM、IgA than SpA and PpL By ELISA



LD3 ▲, LD5 ▼,
4L ■, SpA ◆

LD3/LD5 Showed Increased Binding Activity With IgG-Fab、IgM、IgA than SpA and PpL By Western Blot and Dot Blot



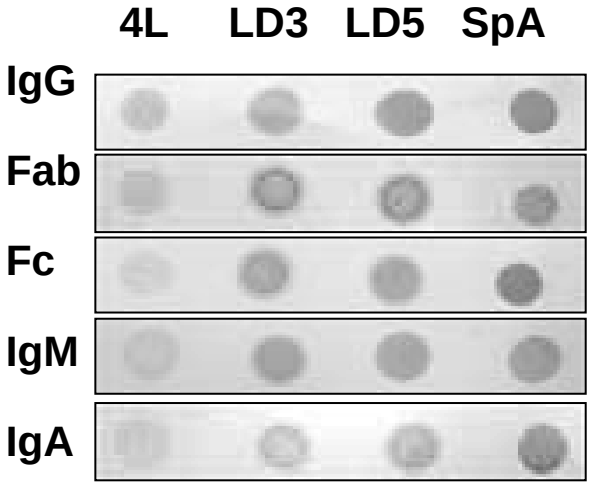


Figure 4. Western blotting and dot blotting analysis.

(A) 4L, LD3, LD5, SPA were separated by 12% SDS-PAGE under denaturing condition and eletrotransferred to PVDF membranes, then probed with biotinylated IgG, F(ab')2, Fcγ, IgM and IgA. (B) 3 μg of 4L, LD3 , LD5 and SpA was applied to five identical nitrocellulose membranes, and incubated with biotinylated IgG, F(ab')2, Fcγ, IgM and IgA. The data presented was representative of 3 replicated experiments.

LD3/LD5 Showed Stronger Binding Capability with IgG-Fab、IgM、IgA than SpA and PpL By Competitive ELISA

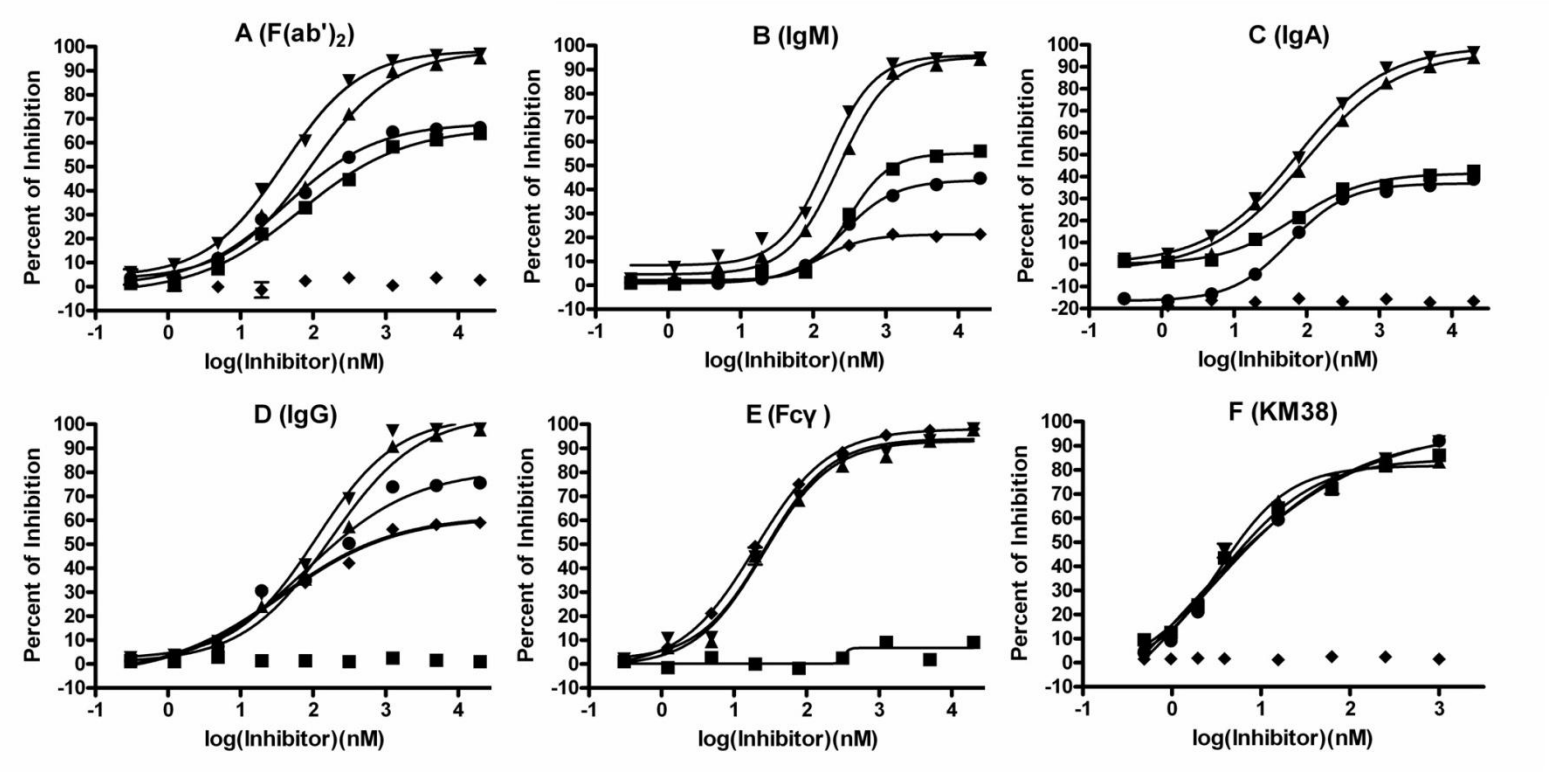


Figure 6. Inhibition of b-LD5 binding to immobilized Igs. Wells were coated with IgG, F(ab')2, Fcγ, IgM, IgA and scFv KM38 respectively, and serial dilutions of LD3 (▲), LD5 (▼), 4L (■), SpA (◆) and 4L+SPA(●) with a fixed concentration of b-LD5 were added and incubated. Then washed, incubated with HRP-streptoavidin conjugate and followed by TMB substrate. The immobilized Igs were labeled on top of each graph. The inhibition percentage of LD3/5 in (A), (B) and (C) were determined to be significantly different from that of 4L+SpA or 4L using the ANOVA and student's t test ($p<0.001$).

LD3/LD5 Showed Stronger Binding Affinity to IgM、IgA than SpA and PpL By SPR

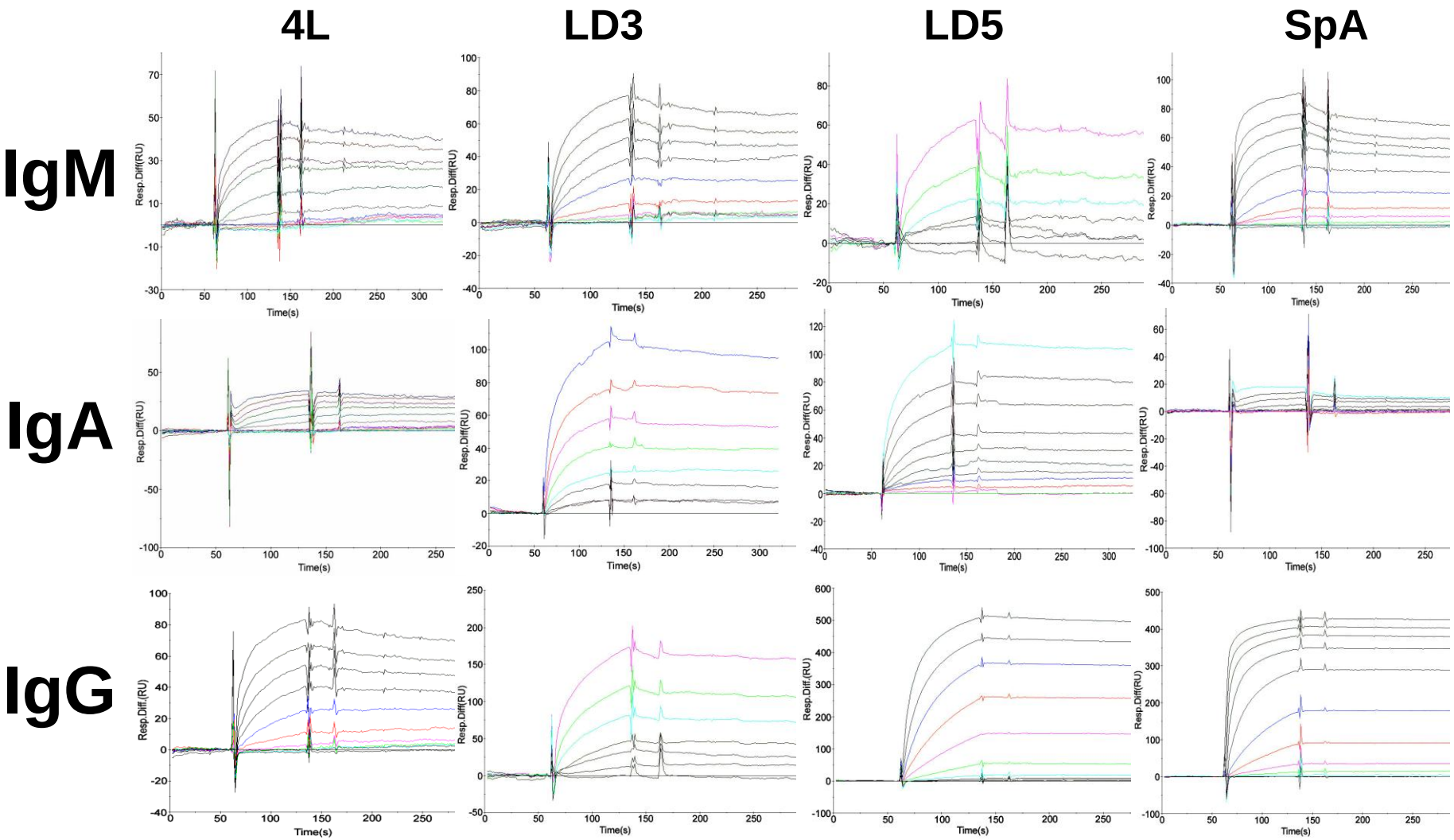


TABLE 1： Surface plasmon resonance analysis of the interactions between 4L, LD3, LD5, or SpA and Igs.

Analyte	Constant	Ligand		
		IgG	IgM	IgA
4L	*ka*($M^{-1}s^{-1}$)	9.46×10^{4}	1.3×10^{5}	8.91×10^{4}
	kd(s^{-1})	5.3×10^{-4}	3.24×10^{-4}	3.99×10^{-4}
	K_D(nM)	5.6	2.49	4.47
LD3	*ka*($M^{-1}s^{-1}$)	6.15×10^{4}	6.19×10^{4}	1.66×10^{5}
	kd(s^{-1})	5.58×10^{-5}	6.94×10^{-5}	4.96×10^{-4}
	K_D(nM)	0.907	1.12	2.99
LD5	*ka*($M^{-1}s^{-1}$)	1.23×10^{5}	7.24×10^{4}	3.21×10^{4}
	kd(s^{-1})	1.09×10^{-4}	2.24×10^{-5}	9.42×10^{-5}
	K_D(nM)	0.882	0.309	2.93
SpA	*ka*($M^{-1}s^{-1}$)	1.53×10^{5}	1.14×10^{5}	4.12×10^{5}
	kd(s^{-1})	4.45×10^{-5}	8.14×10^{-4}	2.93×10^{-3}
	K_D(nM)	0.29	7.16	7.11

LD5 didn't Show Stronger Binding Capability with scFV VH3-VκFab By Inhibitory ELISA

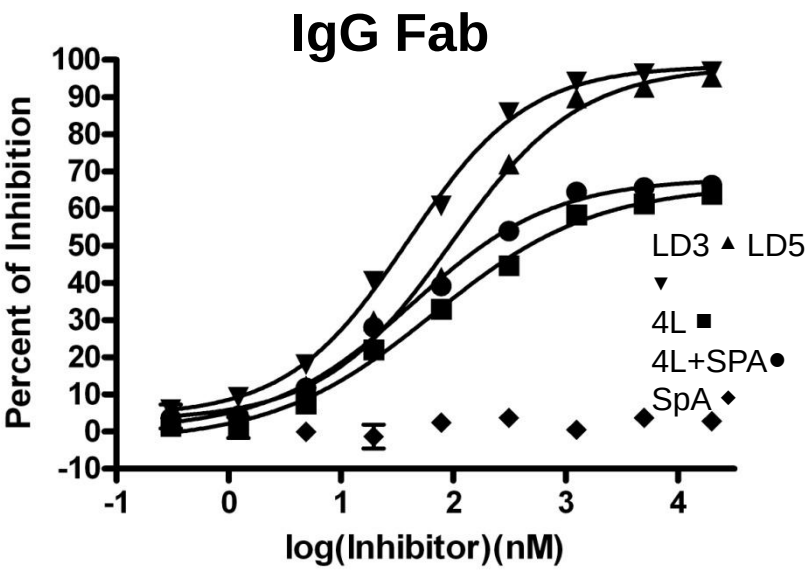


FIGURE 3：Inhibition of the interaction of IgG Fab with LD5 by LD3,LD5, SpA and 4L.

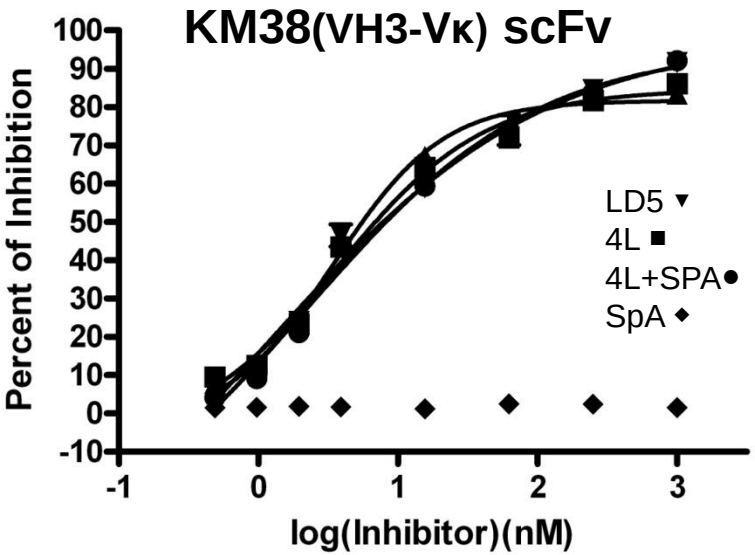


FIGURE 4：Inhibition of the interaction of KM38(VH3-Vκ) scFv antibodies with LD5 by LD3,LD5, SpA and 4L.

Simultaneously Double-Site Binding to VH3-VκFab Is Conformation Restricted

Simultaneously Double-Site Binding to VH3-Vκ Fab

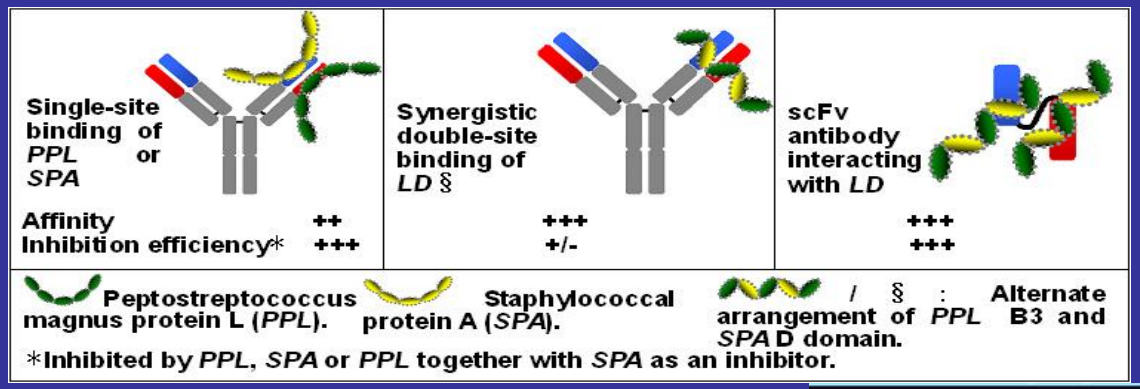
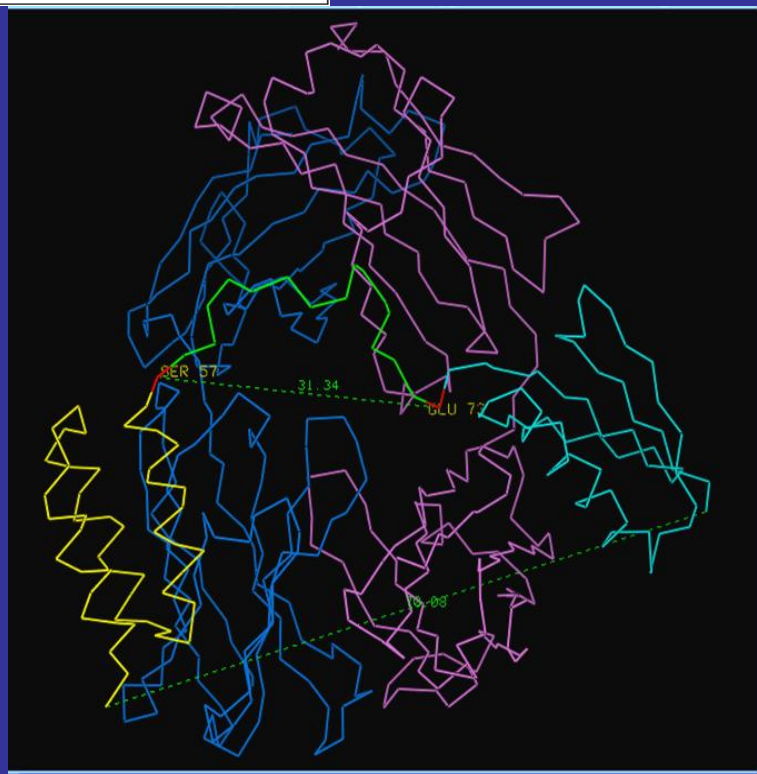
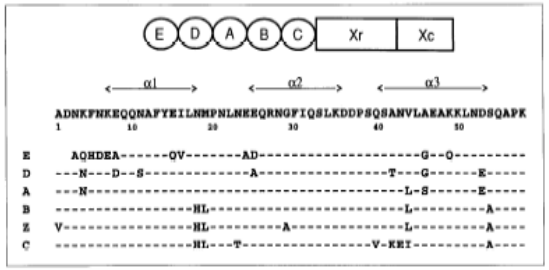


Fig7 The computer simulation of complex of Fab, pro_A and pro_L domain

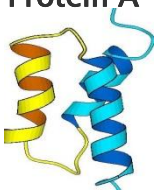
The VH3 and Vκof were depicted in dark blue and purple respectively. The D domain of SPA and B3 domain of pPL were colored in yellow and light blue respectively. While the linker between pro_a and pro_L was in green. In LD3/LD5 construct, there are two kinds of possible arrangement forms ,LD or DL, which may be responsible for the double sites binding. In this modeled complex, the distance between helix Ⅲ in the C-terminal of pro_A D domain and β Ⅰ in the N-terminal of pro_L B3 domain was about 31.34Å, while DL arrangement form could produce a 17 aa linking peptide, which was shown long enough and flexible for the linking. However, for LD arrangement , the β4 in C-terminal of pro_L B3 domain and helix I in N-terminal of pro_A D domain was about 70Å The LD arrangement form could only produce a 13 aa linking peptide,which too short to complete this linkage which needed at least 22 residues for the linking.



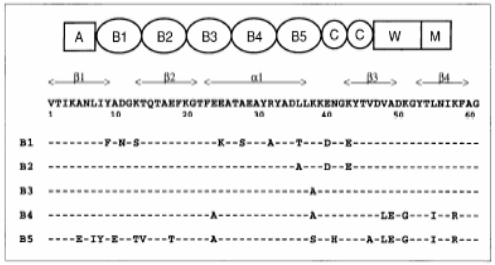
Phage Library Displaying Randomly Linked Binding Domains of SpA, SpG and PpL



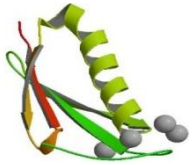
Protein A



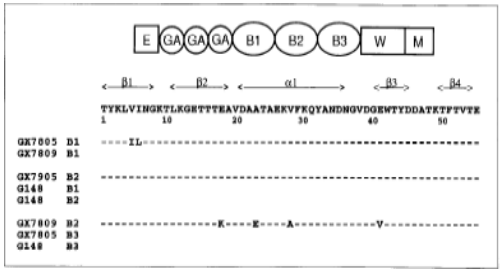
A D



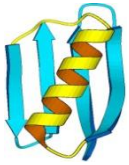
Protein L



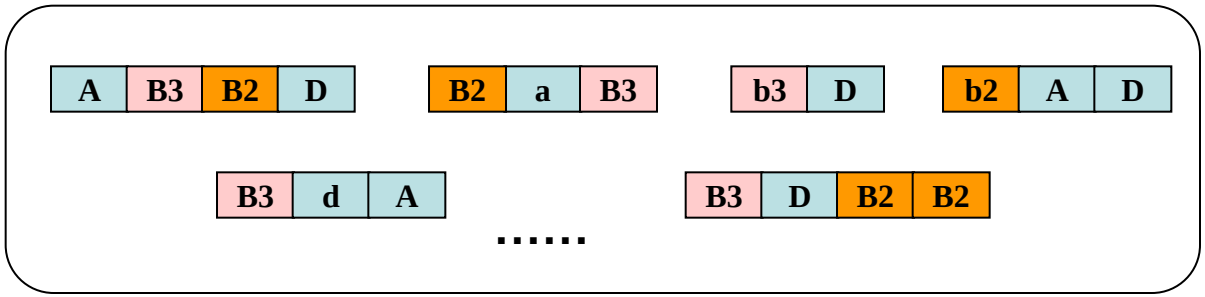
B3



sPG



B2



Note:

The Capital letters refer to forward sequence

The Lower case letters refer to reverse sequence

Novel Evolved Immunoglobulin Binding Molecules (NEIBM) Characterized by Alternate Arrangement of SpA A Domain and PpL B2 Domain

Table 1: Sequence analyses of inserted fragments on phage clones in the original library and the third or fourth post-selection libraries with four Ig molecules

Phage Libraries	*Composition of single domains of inserted fragment*
The original phage library (5*)	PG-PL_{9N}; PA(A)-PG-PL_{6N}; PG_{3N}^{R}-PG^{R}-$PA(D)_{6N}$; PL_{3N}-PA(D); PG_{6N}-$PA(A)_{3N}$-PL^{R}
The 4th round of selection with hIgG (10)	PA(A)-PG_{9N}(5**); PA(A)-PG(2); PA(A)-PG_{3N}(2); $PA(A)_{6N}$-PG_{3N}
The 4th round of selection with hIgG1-Fc (10)	PA(A)-PG(7); $PA(A)_{6N}$-PG(2); $PA(A)_{3N}$-PG-PA(A)
The third round of selection with hIgM (11)	PA(A)-PL-PL_{9N}(4); $PA(A)_{6N}$-PL_{9N}(2); PA(A)-PL_{3N}(3); $PA(A)_{9N}$-PL_{3N}; $PA(A)_{9N}$-PL
The 4th round of selection with hIgA (10)	PA(A)-PL-PL_{9N}(5); $PA(A)_{6N}$-PL_{9N}(2); PA(A)-PL_{3N}(2); $PA(A)_{9N}$-PL_{3N}

*: Number of sequenced phage clones; **: number of phage clones with the same inserted fragment; PA(A) and PA(D): A and D domains of protein A; PG: B2 domain of protein G; PL: B3 domain of protein L; 3N, 6N and 9N: the sequence of random linking peptides composed of three, six and nine nucleotides; R: reverse sequence of original sequence.

The HRP-LD5 Exhibits Higher Binding Activity for IgM and IgA Than PcAb

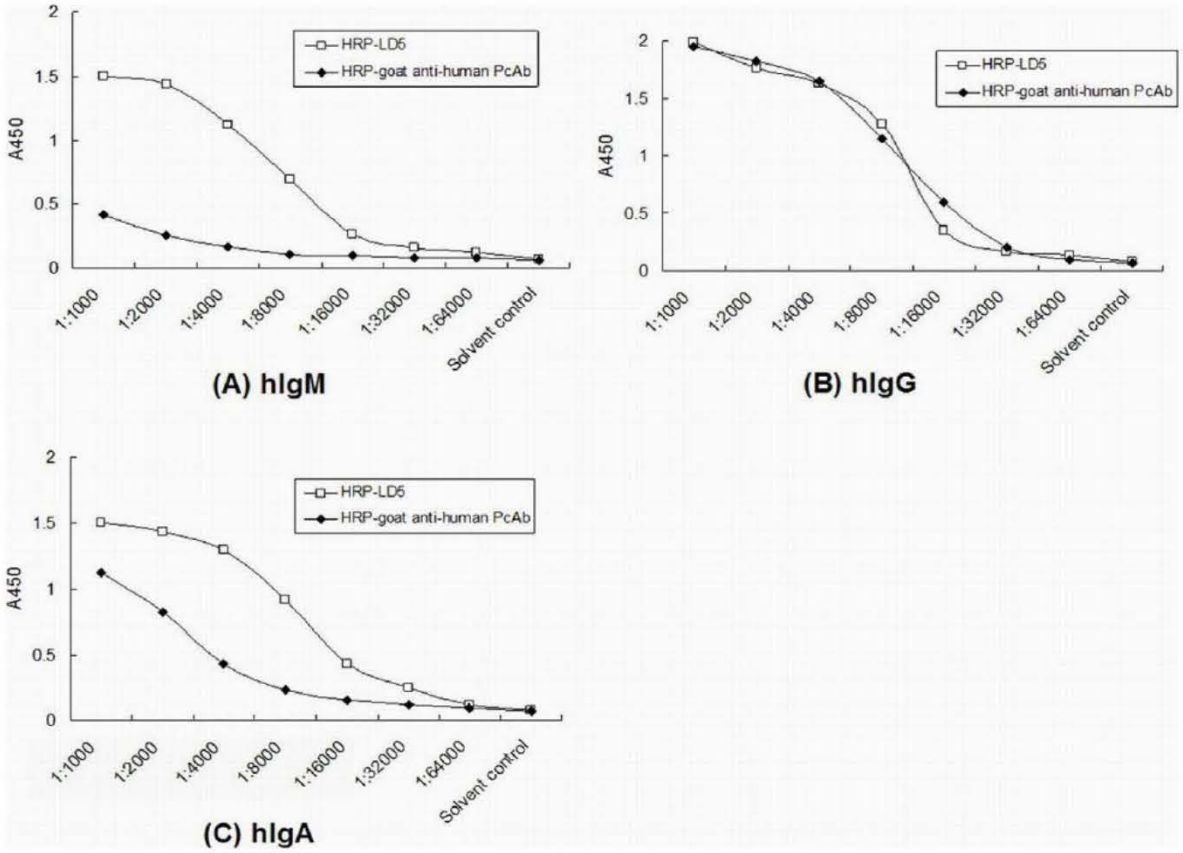
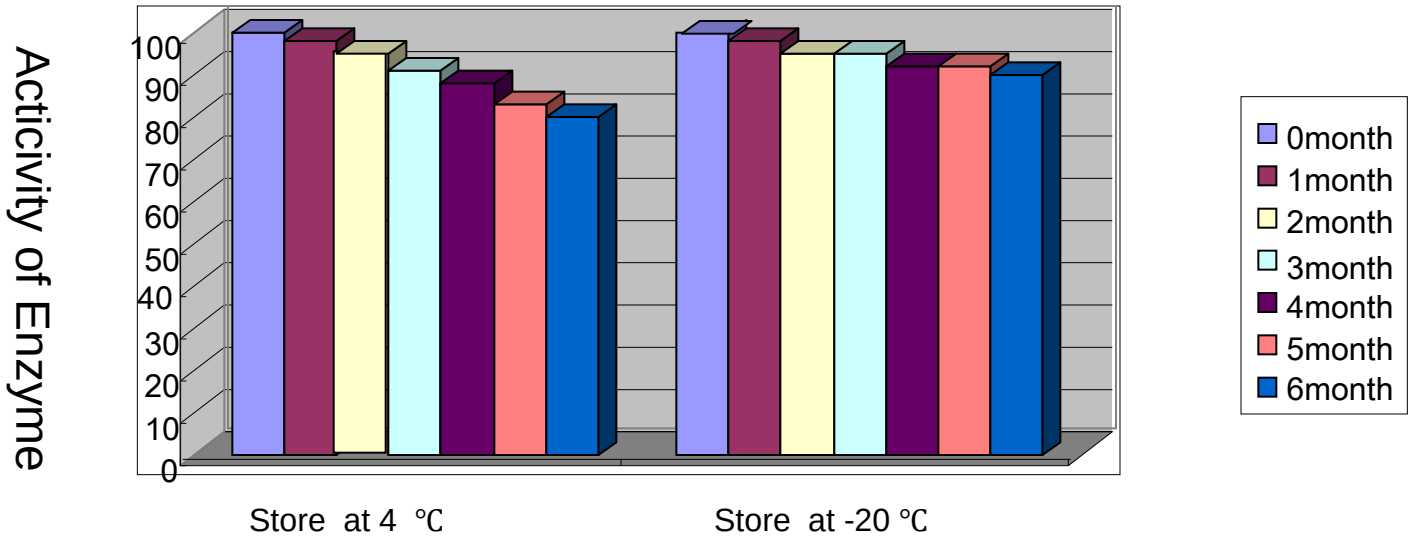


Figure 1. The enzyme-labeled LD5 exhibits enhanced binding activities for IgM and IgA. The binding activities of horseradish peroxidase (HRP)-labeled LD5 (HRP-LD5) or HRP-conjugated goat anti-human polyclonal antibodies (HRP-goat anti-human PcAb) to coated hIgM (A), hIgG (B) or hIgA (C) were examined by ELISA. The coating buffer was used as solvent control.
doi:10.1371/journal.pone.0018477.g001

The Enzyme Activity of HRP-LD5 Molecule Stored for Various Periods at 4℃ And -20℃



The binding activity of the enzyme complex is hardly lost when stored at -20℃ for 6 months ; when stored at 4℃ for 6 months , more than 80%of its binding activity is remained.

HRP-LD5 Based Anti-HCV Assay Meet the Requirement of Chinese National Standard for Batch Production Quality Control of Anti-HCV EIA Kits

Table 2. Comparison of anti-HCV detection against the national reference panel of HRP-LD5 assay with three commercial diagnostic kits.

HRP-conjugated molecules	True positive(a)	False negative(b)	True negative(c)	False positive (d)	Sensitivity(%) a/(a+b) (95% CI)[1]	Specificity(%) c/(c+d) (95% CI)[1]	Relative accuracy (%) (a+c)/(a+b+c+d)
Abbott 3.0 kit	30	0	29	1	100 (0.88–1.00)	96.66 (0.83–1.00)	98.33
Ke Hua kit	29	1	30	0	96.66 (0.83–1.00)	100 (0.88–1.00)	98.33
Chang Zheng kit	29	1	30	0	96.66 (0.83–1.00)	100 (0.88–1.00)	98.33
HRP-LD5 assay	30	0	29	1	100 (0.88–1.00)	96.66 (0.83–1.00)	98.33

[1]95% CI, 95% confidence interval.

doi:10.1371/journal.pone.0018477.t002

Detection Performance of Various Assays in 195 Serum Specimens From Hemodialysis Patients

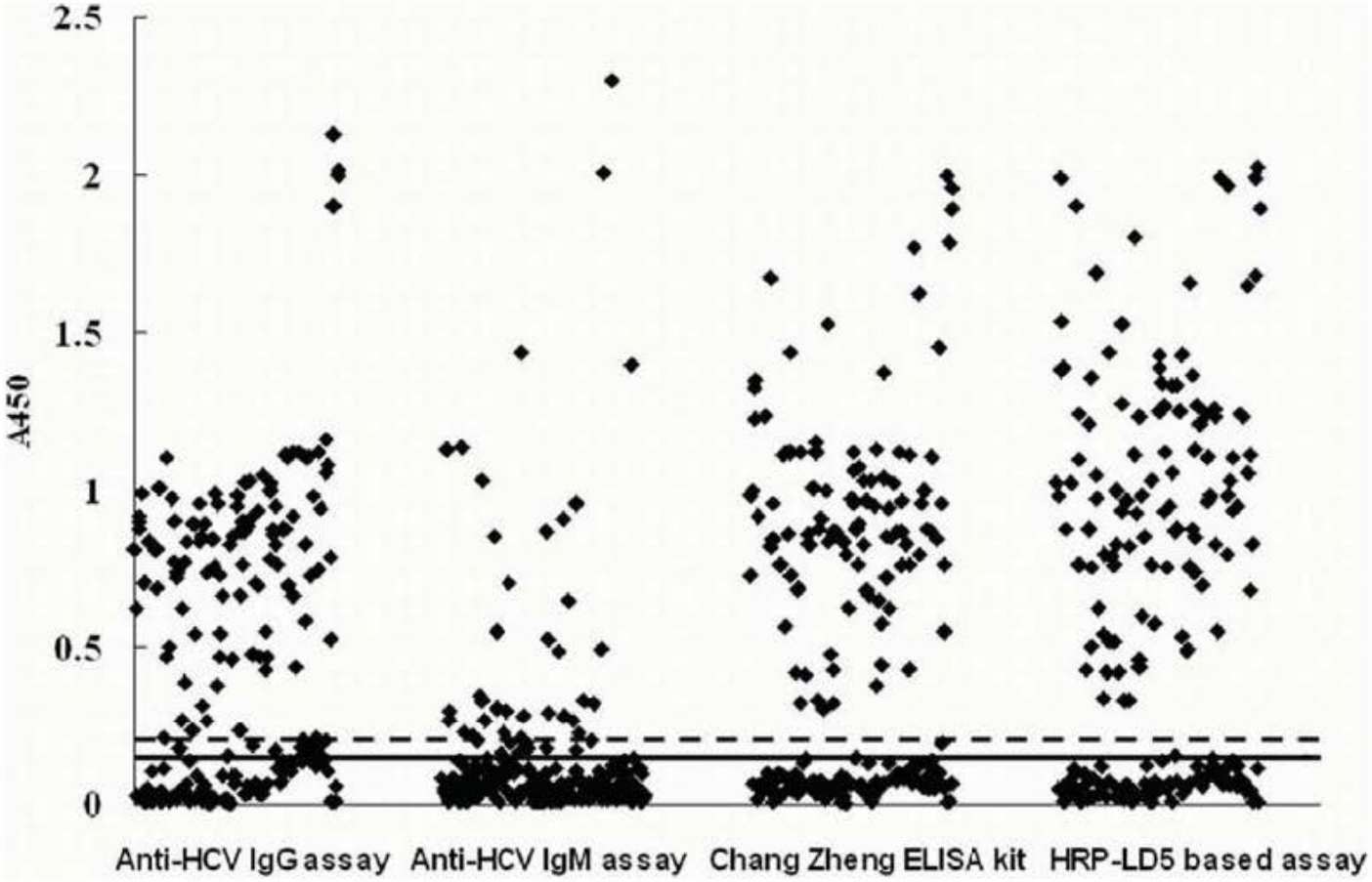


Figure 2. Anti-HCV detection in 195 serum specimens from hemodialysis patients by different ELISA assays. ◆, A_{450} values, with ELISA cut-off values of either 0.150 indicated in solid line (established anti-HCV IgG assay and Chang Zheng anti-HCV diagnostic ELISA kit) or 0.200 indicated in dotted line (established anti-HCV IgM assay and HRP-LD5 based assay).

doi:10.1371/journal.pone.0018477.g002

Five Negative Specimens by Chang Zheng kit Were Detected by HRP-LD5 Based Assay

Table 3. Comparison of anti-HCV antibody detection in serum samples from 195 hemodialysis patients of HRP-LD5 based assay with Chang Zheng anti-HCV kit.

The established anti-HCV IgG assay or IgM assay		Chang Zheng kit		HRP-LD5 assay	
		+	−	+	−
IgG(+) or gM(+)[1]	108(55.4%)	104(53.3%)	4 (2.1%)	108(55.4%)	0
95% CI[2]	0.484–0.624	463–0.603	0.001–0.041	0.484–0.524	
IgG(+), IgM(+)[1]	33(16.9%)	33(16.9%)	0	33(16.9%)	0
95% CI[2]	0.116–0.222	0.116–0.222		0.116–0.222	
IgG(+), IgM(−)[1]	71(36.4%)	71(36.4%)	0	71(36.4%)	0
95% CI[2]	0.296–0.432	0.296–0.432		0.296–0.432	
IgG(−), IgM(+)[1]	4(2.1%)	0	0	4(2.1%)	0
95% CI[2]	0.001–0.041			0.001–0.041	
IgG( ), IgM( )[1]	87(44.6%)	0	87(44.6%)	1(0.5%)	86(44.1%)
95% CI[2]	0.376–0.516		0.376–0.516	−0.005–0.015	0.371–0.511
Total samples	195	104	91	109	86

[1]Data shown are the number of cases with each test result. Data shown in parentheses are the percentages of detected cases in the 195 total serum samples
[2]95% CI, 95% confidence interval.

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The IgM Nature of the Five Specimens Was Demonstrated by IgM Comfirmation Test

Table 4. IgM confirmation detection of five cases of HRP-LD5 assay positive alone serum samples*.

No. of individual serum	First collected samples						Second collected samples (after three weeks)					
	Chang Zheng kit		HRP-LD5 based assay		anti-HCV IgM assay		Chang Zheng kit		HRP-LD5 based assay		anti-HCV IgM assay	
	BME		BME		BME		BME		BME		BME	
	−	+	−	+	−	+	−	+	−	+	−	+
1	0.110	0.078	0.433	0.054	0.216	0.048	0.503	0.258	1.354	0.372	1.121	0.053
2	0.178	0.060	0.358	0.047	0.187	0.064	0.280	0.263	0.957	0.675	0.565	0.042
3	0.021	0.045	0.465	0.049	0.312	0.071	0.287	0.117	1.255	0.136	1.010	0.039
4	0.142	0.076	0.876	0.068	0.550	0.055	0.803	0.426	1.876	0.463	1.233	0.064
5	0.090	0.053	0.332	0.032	0.228	0.051	0.652	0.289	1.479	0.244	0.980	0.057

*Serum samples treated without (−) or with (+) β-mercaptoethanol (BME) were used to test anti-HCV IgM antibodies. Data shown are the A_{450} values, and the cut-off values of the ELISA were either 0.200 (HRP-LD5 based assay and established anti-HCV IgM assay) or 0.150 (Chang Zheng kit).

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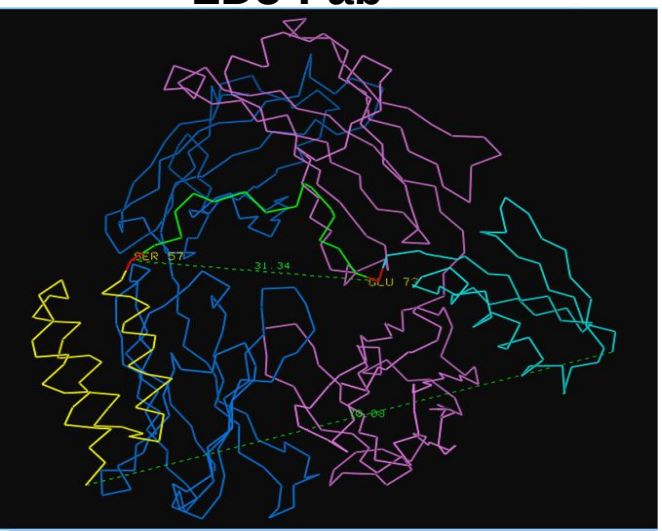
Distinct Binding Characteristics of HRP-LD5

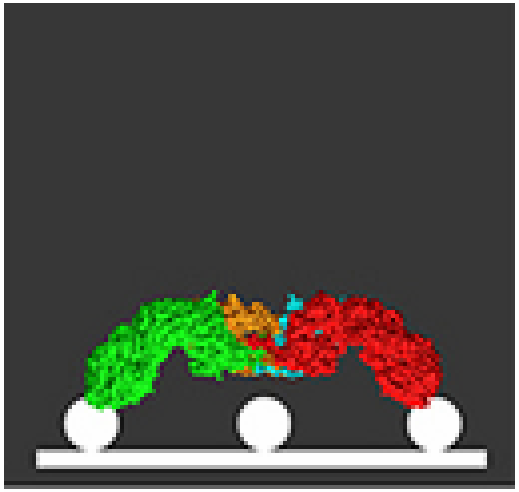
Antibody-Antigen

- **High affinity**
- **Good accessible**
- **Defined binding sites**
- **Conformation restricted binding**

LD5-Fab



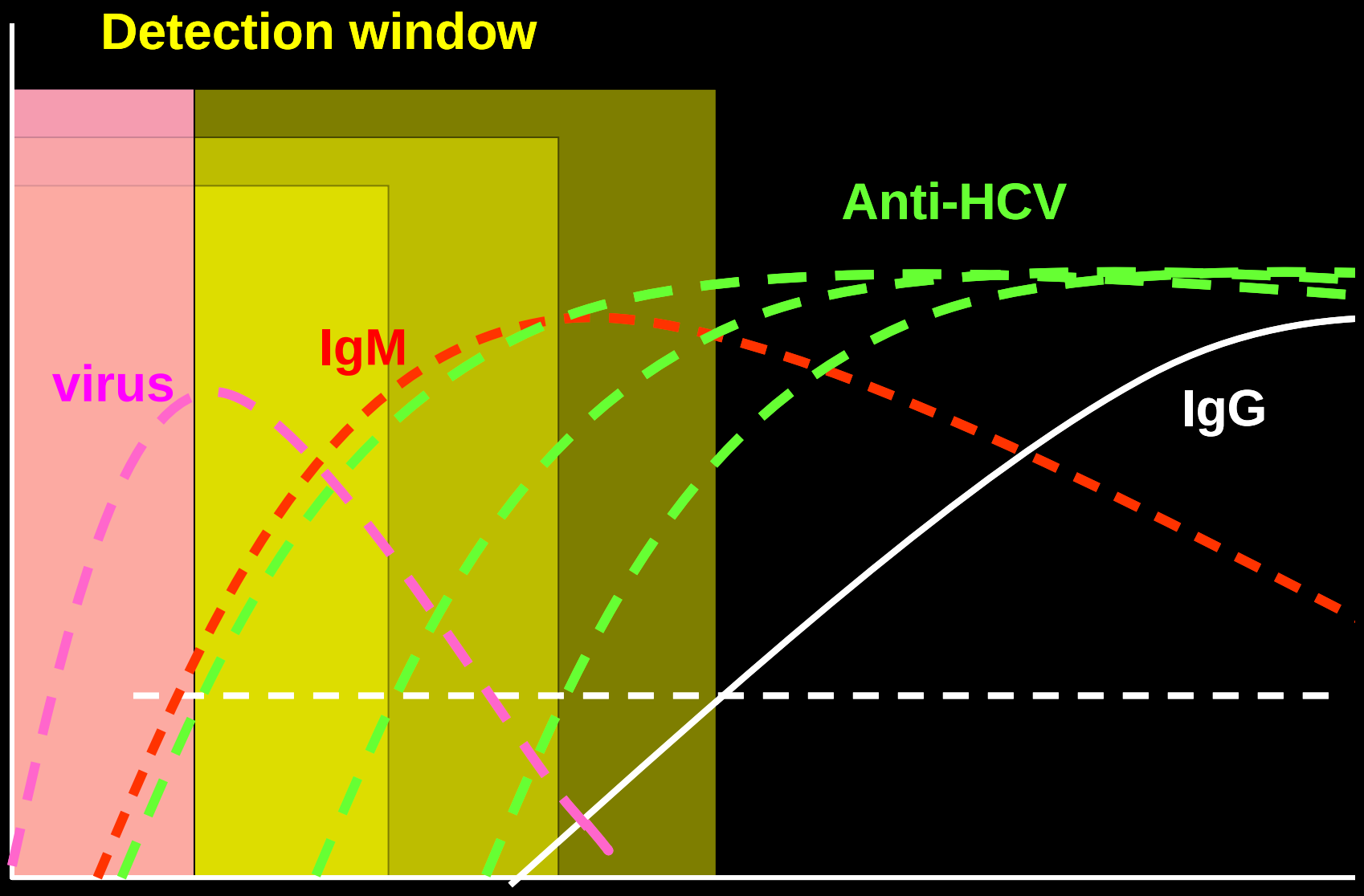
IgM -Antigen



Conclusion

- **HRP-LD5 a novel reporting molecule.**
- **Novel antibody binding characteristic.**
- **Good performance for anti-HCV IgM detection.**
- **Detection window of anti-HCV assay could be shorten by improved IgM detection.**

Anti-HCV Detection Window Shorten by Improved IgM Detection



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THANK YOU !