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Supporting Information

5 **Development of a nanobody tagged with streptavidin-binding peptide and its**

6 **application in a Luminex fluoroimmunoassay for alpha fetal protein in serum**

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37 **Table S1** The 1st round PCR system

Reagent	Volum (μL)
5 \times Prime STAR Buffer	5.0
dNTP Mixture (2.5 mM each)	2.0
Prime AlpVh-LD	1.0
Prime CH2-R	1.0
Prime STAR (2.5 U/ μL)	0.25
cDNA	1.0
H ₂ O	14.75

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39 **Table S2** The 2nd round PCR system

Reagent	Volum (μL)
5 \times Prime STAR Buffer	5.0
dNTP Mixture (2.5 mM each)	2.0
Prime Alp-Vh-F1	1.0
Prime AlpVhh-R1/AlpVhh-R2	1.0
Prime STAR (2.5 U/ μL)	0.25
cDNA	1.0
H ₂ O	14.75

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41 **Table S3** The biopanning conditions for the selection of AFP-specific VHH phages

Rounds	AFP concentration ($\mu\text{g/mL}$)	Blocking solution	Input phage (cfu)	incubation time	PBST concentration	PBST washing times	PBS washing times
1	10	3% BSA	5.0×10^{11}	1.5 h	0.1%	8	8
2	5	3% OVA	5.0×10^{11}	1 h	0.25%	12	12
3	2.5	3% BSA	5.0×10^{11}	1 h	0.5%	15	15

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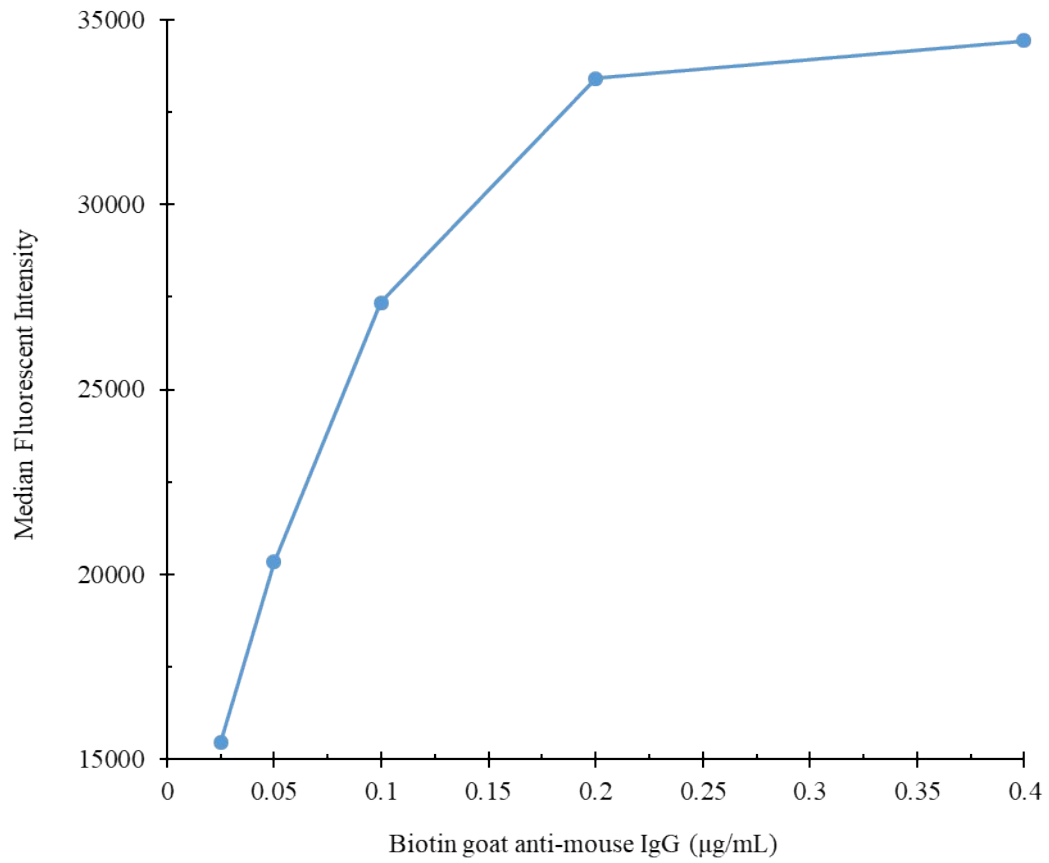
44 **Table S4** The enrichment of phage in three rounds biopanning

Rounds	AFP concentration ($\mu\text{g/mL}$)	Input phage (cfu)	Output phage (cfu)	Recovery rate	Enrichment
1	10	5.0×10^{11}	1.8×10^6	0.0036‰	-
2	5	5.0×10^{11}	3.1×10^8	0.62‰	172.22
3	2.5	5.0×10^{11}	1.6×10^9	3.2‰	5.16

Recovery rate=Output phage particles/Input phage particles; Enrichment=The later round of recovery rate/The former round of recovery rate

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48 **figure S1** The validation of monoclonal antibody-conjugated magnetic microspheres.

49 **Table S5** The screening of paired antibodies based on Luminex-200 system

Samples	14 Monoclonal Antibodies of AFP													
	M3008-1	100015	LDZL0001	H036001	M3008-2	4A3	5H7	100014	H036002	5153-01M	XA2	6483-01M	XFPA	JFZL0002
Control	23	25	12.5	20	25	17.5	23.5	26	19	29	22.5	18	20	18
Sample 1 (4.88 ng/mL)	26	32	14	23	23.5	16.5	45	24	20	24	21.5	21.5	19.5	17.5
Sample 2 (62.7 ng/mL)	402	644.5	77	254	38	31	951	73	39	37.5	38	171.5	40	22.5

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Table S6 The orthogonal test for parameter optimization of the NS-LFIA

Treatment Name	Factors			Results
	mAb-5H7 ($\mu\text{g/mL}$)	A1-SBP ($\mu\text{g/mL}$)	SA-PE ($\mu\text{g/mL}$)	
1	2.5	1	1.5	447
2	2.5	2	2.5	514.5
3	2.5	3	3.5	640.5
4	5	1	2.5	569.5
5	5	2	3.5	694.5
6	5	3	1.5	457
7	10	1	3.5	581
8	10	2	1.5	476
9	10	3	2.5	507.5

53 **Table S7** The selectivity of the NS-LFIA

Cancer Biomarkers	Spiked Concentration	Median Fluorescent Intensity	Recovered concentration (ng/ml)
AFP	50 ng/mL	1708	49.7
CA12-5	1 µg/mL	25	0.14
CA15-3	1 µg/mL	25	0.14
CA19-9	1 µg/mL	26	0.18
CEA	1 µg/mL	24	0.09
CY21-1	1 µg/mL	24	0.09
HE4	1 µg/mL	25.5	0.16
PSA	1 µg/mL	27	0.22
SCCA	1 µg/mL	24.5	0.12
Blank control	0	24	0.09

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56 **Table S8** The LOD of the NS-LFIA.

AFP Concentration (ng/mL)	Median Fluorescence Intensity (MFI)	Calculated Concentration (ng/mL)	Mean \pm SD (ng/mL)	LOD (ng/mL)
0	25	0.136		
	23.5	0.074		
	22	0.011		
	20.5	-0.051		
	21	-0.030		
	21	-0.030		
	21	-0.030		
	20	-0.072		
	26	0.178		
	22	0.011	-0.00020875	0.237
	23	0.053	± 0.079	
	20	-0.072		
	23.5	0.074		
	20	-0.072		
	19	-0.114		
	19.5	-0.093		
	21	-0.030		
	20.5	-0.051		
	24	0.095		
	22	0.011		

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