Electronic Supplementary Material (ESI) for Lab on a Chip.

Supporting Information

Highly paralleled emulsion droplets for efficient isolation, amplification, and screening of cancer biomarker binding phages

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Supporting Material Contents

Experimental Procedures

Materials and cell lines The phage display library Ph.D.-12 (diversity 2.7×10^9) was purchased from New England Biolabs (Ipswich, MA, USA). This kit includes the -28 gIII sequencing primer (5'-GTA TGG GAT TTT GCT AAA CAA C-3'), -96 gIII sequencing primer (5'-CCC TCA TAG TTA GCG TAA CG-3'), the host bacteria *E. coli* ER2738 (tetracycline-resistant), streptavidin (SA) and biotin. Transferrin receptor (CD71) and glypican-1 (GPC1) were purchased from R&D Systems (Minneapolis, MN, USA). HRP-labeled M13 antibody was purchased from the Sino Biological Inc (Beijing, China). Streptavidin (R-PE), sulfo-NHS-biotin, HRP-conjugated streptavidin (SA-HRP), TFRC siRNA (AM16708), CD71 antibody (MEM-75) and nickel-coated plates (8-well stripe) were purchased from Thermo Fisher Scientific Inc (Shanghai, China). Streptavidin (SA) sepharose beads and nickel sepharose beads were purchased from GE Healthcare (Chicago, IL, USA). The control peptide was unrelated to the target (NYYQTHLHLTEL), and all peptides were synthesized with a GGGSKKK-biotin linker at the C-terminus after the 12 amino acids by Synpeptide Co., Ltd. (Shanghai, China). The SU-8-negative tone photo-epoxy GM 1070 was purchased from MicroChem (Gersteltec Sarl, Switzerland). Polydimethylsiloxane (PDMS, Sylgard 184) and curing agent were obtained from Dow Corning (Shanghai, China). The film photomask was produced by Qingyi Precision Maskmaking Co. Ltd. (Shenzhen, China). 1H, 1H, 2H, 2H-perfluorooctyldimethylchlorosilane was obtained from Alfa Aesar (Tianjin, China). Fluorinated oil 7500 was obtained from Lianqun Co. Ltd. (Shenzhen, China). All reagents used in this experiment were analytical pure, the water was 18.2 MΩ.cm ultrapure water, and part of the reagents were sterilized at high temperature and high pressure or filtered through a 0.22 μm Millipore filter membrane.All cell lines (U251, Jurkat, K-562, HEK-293T, NIH-3T3) were purchased from the Cell Bank of the

Chinese Academy of Science (Shanghai, China). They were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin at 37 °C in a humid atmosphere with 5% CO_2.

Fabrication of microfluidic chip The chip structure was designed using AutoCAD. The design drawing was sent to Shenzhen Qingyi precision photoelectric company (Shenzhen, China) for preparation for a chrome mask film. The SU-8 pattern was fabricated by a conventional photolithographic method. In brief, GM 1070 photoresist was coated on a silica wafer at a spin rate of 800 rpm to produce a film with a thickness of 150 μm. The photomask pattern was transferred onto SU-8 photoresist via UV exposure. PDMS and curing agent were mixed well in a 10:1 ratio by weight, and after removing the air bubbles by vacuum pump, poured onto the GM 1070 patterned silicon wafer and spun at 500 rpm for 20 s to obtain a patterned PDMS layer with a thickness of about 200 μm. The patterned PDMS layer was peeled from the SU-8 patterned silica wafer post by heating at 75 °C for 20 min to form the microfluidic structures, and a droplet capture well was excised using a scalpel along the well outline. The patterned layer was then bonded to a flat PDMS covering layer, which was peeled from another polished glass slide. The module was punched with two inlets for injecting mixed target coated beads and *E. coli* sample and pre-enriched phage library separately, one inlet for oil and one outlet for collecting the droplets before bonding onto a flat bottom PDMS layer to seal the channels and well. Finally, the entire PDMS module was bonded to a glass slide to provide high rigidity and low air permeability. Bonding of PDMS to PDMS and PDMS to glass was carried out immediately after oxygen plasma treatment. After bonding, 2% 1H, 1H, 2H, 2H-perfluorooctyldimethyl-chlorosilane/GH-135 (v/v) solution was injected into the channels and incubated at 65 °C for 1 day to produce fluorinated surfaces.

The growth curve of *E. coli* ER2738 and amplification of phages in droplet The *E. coli,* nucleic acid dye and LB medium were injected into microfluidic chips, packaged into the droplets, and cultured at 37 °C in an incubator. Fluorescence images were taken by a fluorescence microscope every hour under the same experimental conditions. The DNA of *E. coli* was stained with nucleic acid dye and emitted fluorescence when excited; thus the fluorescence intensity responded to the concentration of the *E. coli*, and the fluorescence images were analyzed using ImageJ software to obtain the average gray value. Finally, the growth curve of *E. coli* was determined by normalization of the average gray value. To verify the monodisperse of phage in droplets, phages were spread into 1% agarose droplets at a final concentration of 0.3 phage per droplet, corresponding to 4.5×10^5 pfu/mL phages enclosed (for droplets with diameter 75 μm, volume 2.2×10^{-7} mL). The agarose droplets were cultured for 4 hr. Half of the droplets were pipetted into warm TBS to dilute to the appropriate concentration for titer. The other droplets were gelatinized and the emulsion was broken. Twenty gelatinized droplets were extracted individually by a capillary, amplified and sequenced. Methods for sequencing are described below.

Simulation of dm-Display in tube A mixture of target-coated beads, *E. coli* and a small number of initial libraries was diluted in bacterial cultural medium and cultured at 37 °C in an incubator for 4.5 hr. After culture, the mixture was washed by 0.5% Tween-TBS and beads were stained with M13 phage antibody (Sino Biological, 11973-MM05T-H) followed by a secondary antibody anti-rabbit-AF488 (Alexa Fluor 488, R&D). After the final incubation, the beads were washed 3 times in 0.5% Tween-TBS and imaged under fluorescence microscopy.

Monoclonal phage amplification and purification To avoid bead loss during transfer, amplification was achieved in two batches. Phages were first amplified in situ. Forty μL *E. coli*

and culture medium were added to the 8-strip tubes and incubated on a table concentrator at 37 °C for 4 hr. Second, they were transferred to 15 mL culture medium for bulk amplification. The amplified phages were purified twice using a universal PEG/NaCl method as described in the literature5. Finally, phages were resuspended in 1 mL TBS and quantified by titer.

Enzyme-linked immunosorbent assay (ELISA) of phages ELISA was used to determine the binding affinity between phages and the target protein. First, the target protein (0.2 μg/well for CD71 and 0.1 μg/well for GPC1, diluted in 0.1 M $NaHCO_3$, pH= 8.6) was added to a 96-well plate and placed in a sealed wet box at 4 °C for overnight adsorption. Then the excess solution was removed and the well was blocked with 300 μL 5 mg/mL BSA-TBS at room temperature for 1 hr. Afterward, the well was washed 6 times with 300 μL 0.5%Tween-TBS (washing buffer). Then, 2×10^6 pfu corresponding phages were added and incubated at room temperature for 1 hr. After washing 6 times, the HRP-labeled anti-M13 antibody (diluted in blocking buffer at 1: 5000) was added and incubated for 1 hr at room temperature. After washing 6 times, 100 μL of commercially available TMB substrate was added to each well for 10 min color-development, and then 20 μL 2 M H_2SO_4 was added to stop the reaction. Finally, the OD450 was recorded using a microplate reader.

Determination of phage affinity to cells U251 cells were cultured in a 24-well plate. The cells were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature before incubation with phages. Phages (1×10^8 pfu, quantified by titer) were mixed with 400 μL TBS containing 0.5% (w/v) skim milk powder by the vortex. The mixture was incubated with fixed cells for 1 hr with gently shaking in a 37 °C incubator. Cells were washed twice with 1 mL TBS to remove non-specifically adsorbed phages. Bound phages were individually eluted by elution

buffer and neutralization buffer (described in library pre-enrichment). Finally, the eluted phages were titrated to determine the number of phages bound with U251 cells.

TFRC siRNA silence on U251 U251 cells were cultured on a 6-well plate to be 70% confluent at the time of transfection. TFRC siRNA was transferred into U251 at a final concentration of 100 nM with Lipofectamine® 3000 Reagent. The RNA-lipid mixture was cultured with cells for 48 h. After culture, cells in 6-well plates were dispersed with a cell scraper. The cells were then stained with anti-CD71-biotin or biotin-peptide as described in binding behavior of anti-CD71 and GWW peptide.

Peptides K_ds against CD71 First, the CD71 protein (0.1 μg/well, diluted in 0.1 M $NaHCO_3$ pH 8.6) was added to a 96-well plate and placed in a sealed wet box at 4 °C for overnight absorption. The next day, the excess solution was removed and the well was blocked with 300 μL 5 mg/ml BSA-TBS at room temperature for 1 hr. The well was washed 4 times with 300 μL 0.2% Tween-TBS (washing buffer). Peptides with increasing concentrations diluted in 0.05 mg/mL BSA 0.2% Tween-TBS were added into each well and incubated at room temperature for 1 hr. After washing 4 times, the diluted SA-HRP (diluted in 0.05 mg/mL BSA 0.2% Tween-TBS at 1: 5000) was added and incubated for 40 min at room temperature. After 3 washes, 100 μL of commercially available TMB substrate was added to each well for 10 min color-development, and 20 μL 2 M H_2SO_4 was added to stop the reaction. Finally, the OD450 was recorded using a microplate reader. The results were fitted by GraphPad Prism software by the single point adsorption equation $Y = Bmax*X/(K_d + X)$.

Binding behavior of anti-CD71 and GWW peptide The adherent cells (U251, 293T and 3T3) were cultured on a 24-well plate and incubated with 30 μM biotin-peptide or 0.4 μg/test anti-CD71 with slow shaking for 30 min in a total volume of 400 μL. Then streptavidin-

phycoerythrin (SA-PE) or anti-IgG-PE was added. After each incubation, cells were washed twice with 500 μL TBS. Finally, cells were dislodged with a cell scraper and resuspended in 200 μL TBS. For suspension cells (Jurkat), biotin-peptides and antibodies were treated similarly but were washed by centrifugation for 3 min at 1200 rpm. The fluorescence intensities of all cells were measured by flow cytometry. The median value of fluorescence was calculated and the cell autofluorescence background was subtracted to obtain the binding fluorescence of each cell. The histogram was plotted as the background-corrected fluorescence intensity.

K_d Determination of monoclonal phages The K_d determination protocol was similar to that for the affinity ELISA. First, the target protein (0.1 μg/well, diluted in 0.1 M $NaHCO_3$ pH 8.6) was added to a 96-well plate and placed in a sealed wet box at 4 °C for overnight absorption. The next day, the excess solution was removed and the well was blocked with 300 μL 5 mg/ml BSA-TBS at room temperature for 1 hr. The well was washed 6 times with 300 μL 0.5%Tween-TBS (washing buffer). Phages with increasing concentrations were added to each well (minimum 1.0×10^8 pfu/well, maximum 7.5×10^{10} pfu/well) and incubated at room temperature for 1 hr. After washing 6 times, the HRP-labeled anti-M13 antibody (diluted in blocking buffer at 1: 5000) was added and incubated for 1 hr at room temperature. After 6 washes, 100 μL of commercially available TMB substrate was added to each well for 10 min color-development, and 20 μL 2 M H_2SO_4 was added to stop the reaction. Finally, the OD450 was recorded using a microplate reader. The results were fitted by Sigma Plot software.

Phage sequencing Phages were amplified individually for 4.5 hr as mentioned above. Amplification products were centrifuged at 10,000 rpm for 10 min, and 0.5 mL supernatant was pipetted into another tube. The supernatant was mixed with 0.2 mL 20% (w/v) PEG/NaCl and allowed to stand for 10 min at room temperature. A 10,000-rpm centrifugation was performed

for 10 min to remove supernatant and precipitate the phages. The sediment was resuspended in 100 μL lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 4 M NaI) to lyse phages. The lysis product was mixed gently with 250 μL ethanol and allowed to sit for 10 min at room temperature to separate DNA. The DNA was washed twice with 70% ethanol by 10,000-rpm centrifugation. Finally, the DNA sediment was resuspended in 20 μL H_2O and sent to Sangon Biotech (ShangHai, China) for sequencing (using universal primer -96Ⅲ for automatic sequencing).

Characterization of peptides In order to verify peptide selectivity towards different cells, it was incubated with five kinds of cells and affinities were determined individually. The adherent cells (U251, 293T and 3T3) were cultured on a 24-well plate and incubated with 10 μM biotin-peptide with slow shaking for 30 min in a total volume of 400 μL. Then streptavidin-phycoerythrin (SA-PE) was added to make the peptide fluorescent. After each incubation, cells were washed twice with 500 μL TBS. Finally, cells were dislodged with a cell scraper and resuspended in 200 μL TBS for flow cytometry analysis. For half-adherent cells (K562) and suspension cells (Jurkat), 10 μM biotin-peptides were treated in a similar manner but washed by centrifugation for 3 min at 1200 rpm. In order to measure the equilibrium dissociation constant (K_d) of the peptide, a series of concentrations of the peptide was incubated with cells, followed by SA-PE staining, as described above. The mean fluorescence intensity was obtained through the flow cytometry data. The K_ds of the peptides were determined by the single point adsorption equation $Y = Bmax*X/(K_d + X)$, fitted by Sigma Plot 10.0 software.

Peptide characterization by laser scanning confocal microscopy K-562 and HEK-293T cells were cultured in a glass culture dish. Ten μM peptides or 1 μL CD71-antibody was

incubated with cells for 30 min in 200 μL TBS. Then cells were fixed in 4% PFA for 30 min. After fixing, cells were incubated with SA-PE for 10 min and stained with nuclear dye DAPI for 10 min. Afterward, cells were washed twice with 1 mL TBS. Finally, the culture dish was imaged under confocal microscope Leica SP8.

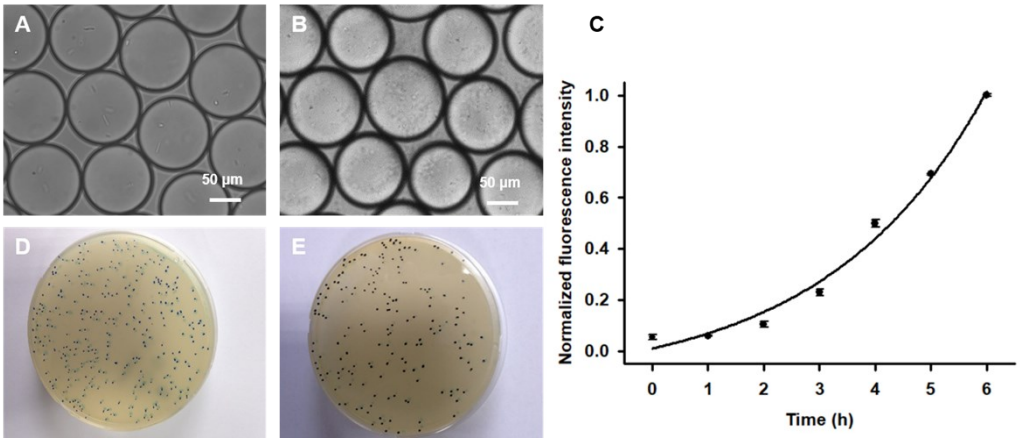


Fig. S1. *E. coli* and phage propagation in droplets.

Images of *E. coli* (A) before and (B) after amplification in droplets. (C) Growth curve of *E. coli* in droplets, indicated by the fluorescence intensity of the enclosed nucleic acid dye GeneFinder. (D and E) The growth concentration of phage after culturing in droplets for 4 hr.

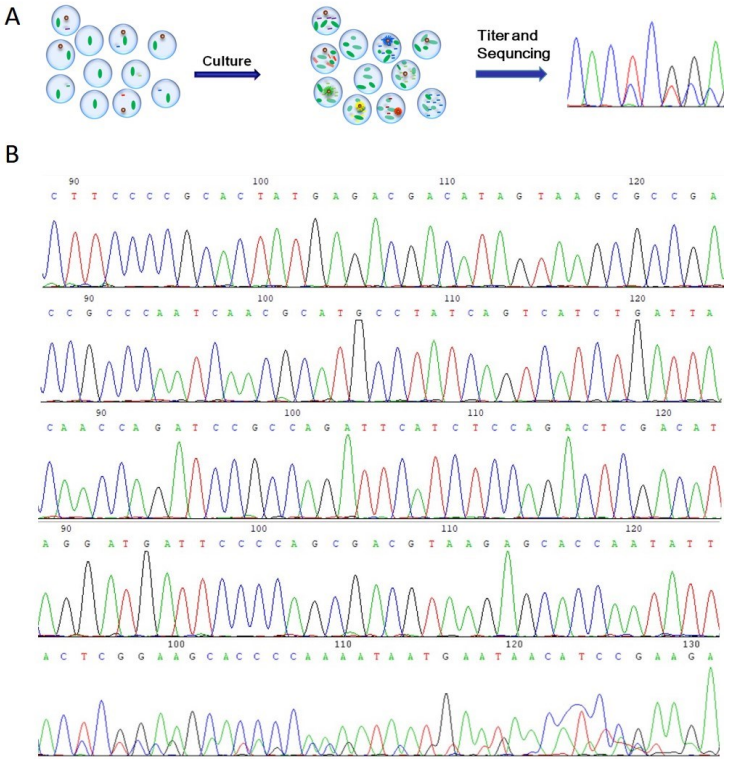


Fig. S2. Phage distribution in droplets consistent with Poisson statistics.

(A) Scheme for demonstration for phage distribution in droplets. Titer was used to verify whether there were phages enclosed in droplets and whether they met the Poisson distribution (results shown in Table S3). Sequencing was performed to further prove the monodispersity of the enclosed phages. (B) Sequencing of 5 phage-containing droplets from 20 selected droplets. Five sequences represent the phages contained in five droplets. The upper four were deemed to be monoclones, while the last was a polyclone, in agreement with the Poisson distribution.

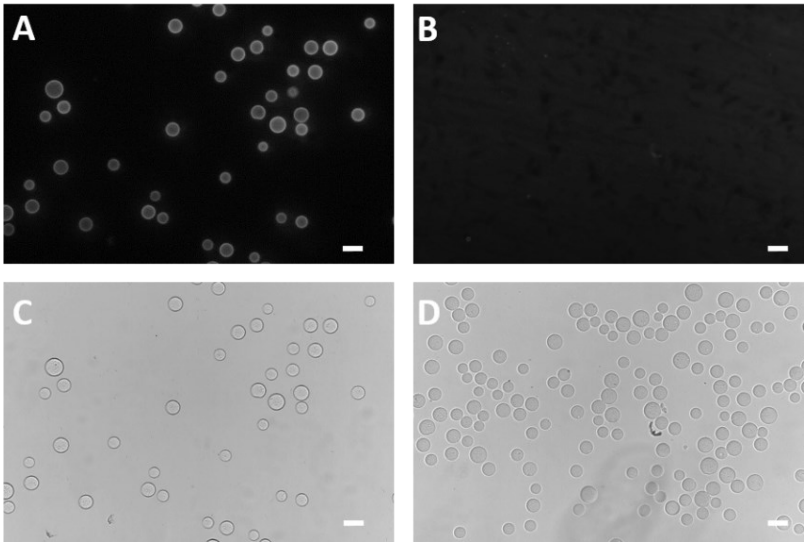


Fig. S3 Simulation of dm-Display in tube.

SA sepharose beads were coupled with CD71 protein and incubated with a small amount of phage library and *E. coli* for 4 hr in-tube culture, which was stained with fluorescent M13 antibody after culture. (A) (C) Fluorescence image and bright field image with a small amount of phage library added. (B) (D) Fluorescence image and bright field image without phage but also stained with the fluorescent M13 phage antibody to verify the specificity of the antibody.

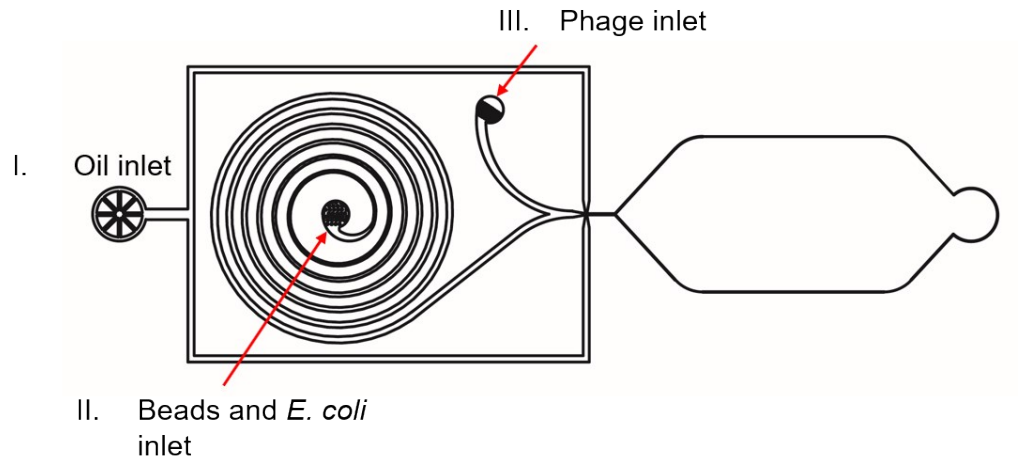


Fig. S4 The design of droplet generation chip.

Each phase was injected as diagram showing. The helical structure for beads and *E. coli* injection was designed to prevent beads sediment before droplets generation.

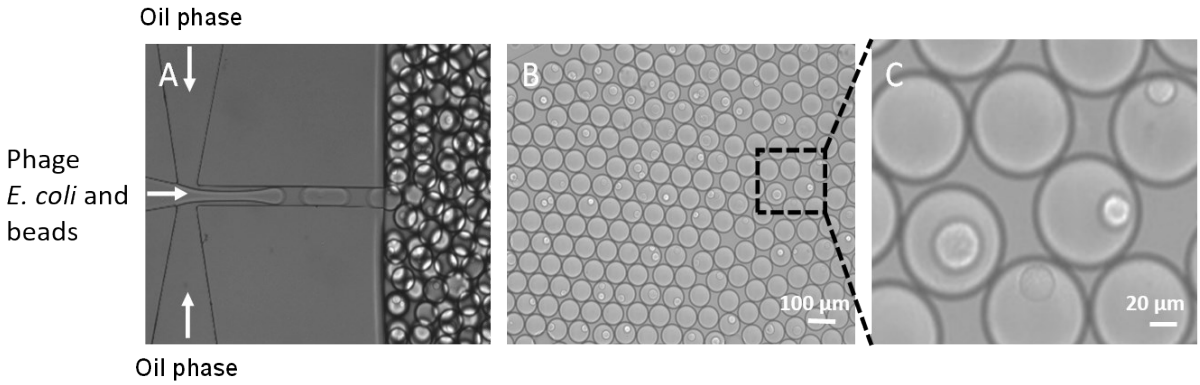


Fig. S5. Uniform droplet generated by dm-Display.

Phages, beads and *E. coli* are mixed and injected in the middle channel, and oil enters from two side channels. The water-in-oil droplets are formed at the cross-section and then enter the storage pool. (B) Bead encapsulation in droplets, counted as 27% single-bead occupation. The scale bar is 100 µm. (C) High magnification image of the local area of droplets in B.

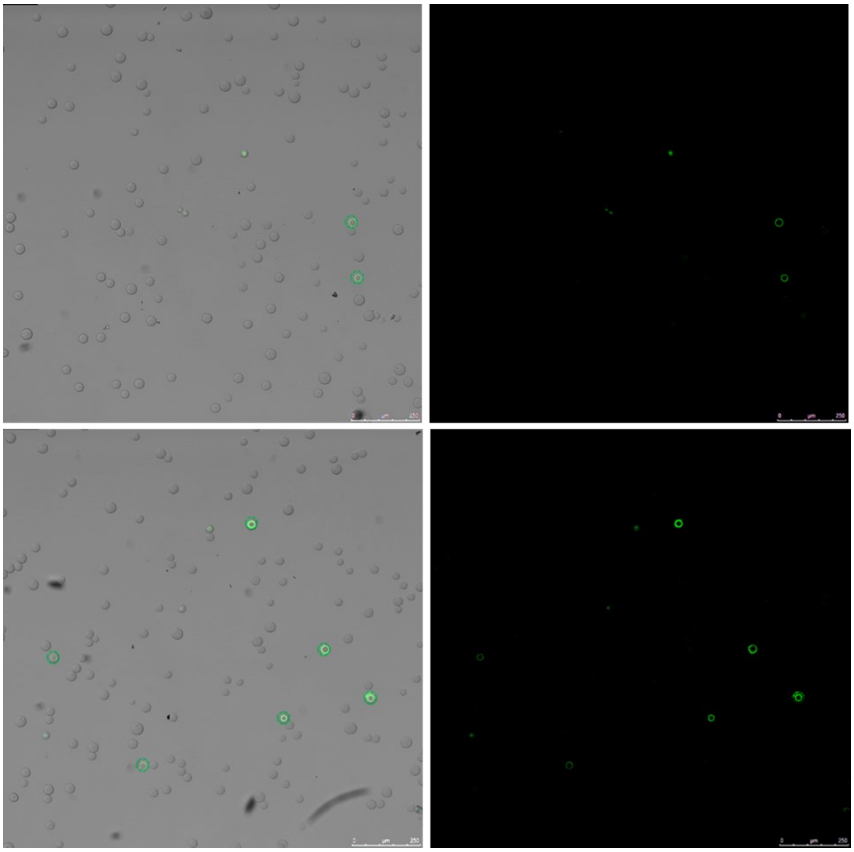


Fig. S6 Large-field images for CD71-coated beads after staining with fluorescent phage antibody.

The beads circled in green are fluorescent. (A) (C) Overlay images of the bright and fluorescent fields; (B) (D) Fluorescent field images. The scale bar was 250 μm.

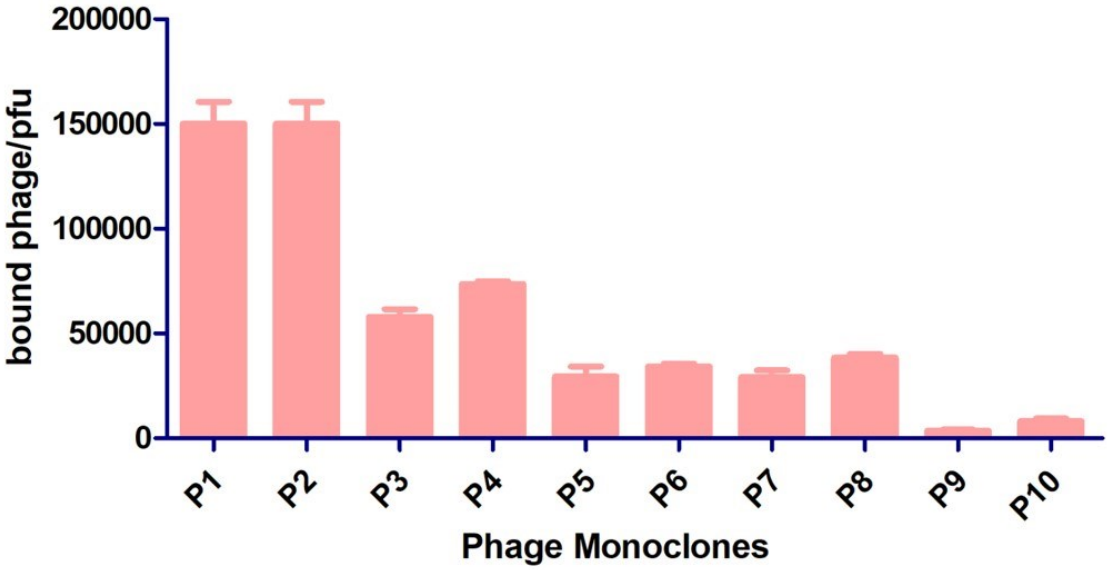


Fig. S7 Phage affinity against CD71-posive cell line U251

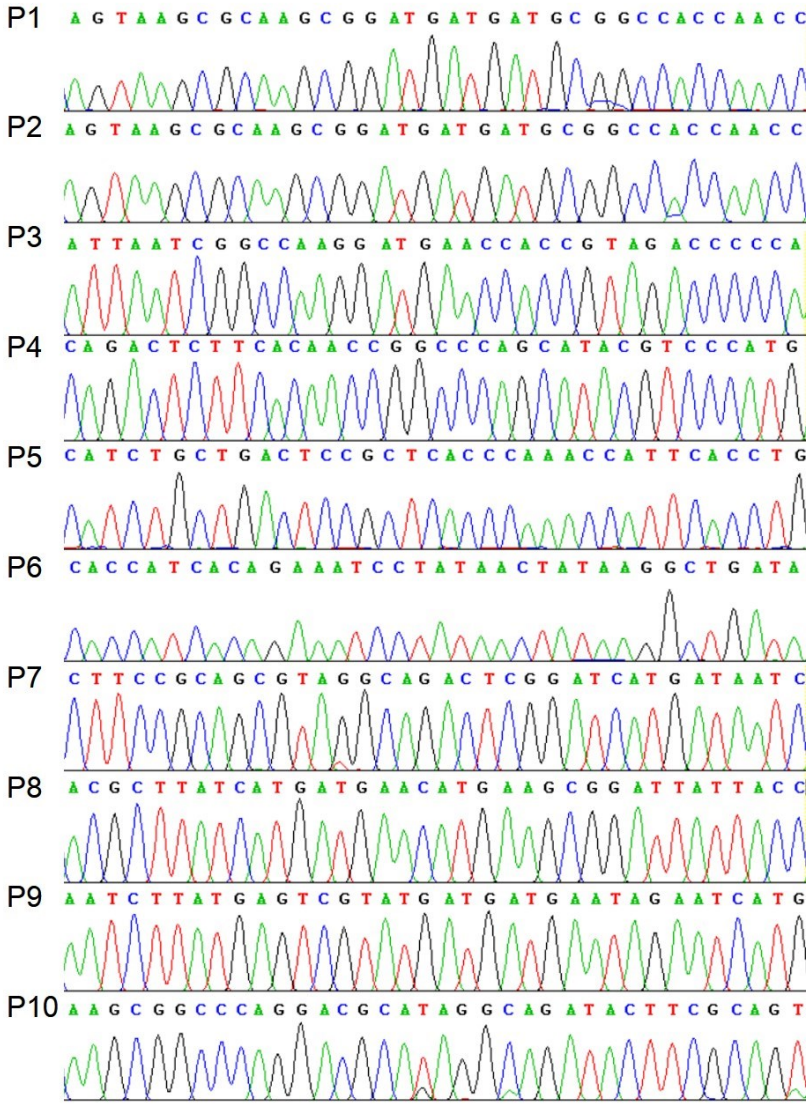


Fig. S8 DNA sequencing of 10 positive beads in dm-Display against CD71.

Each row is a bead, and each position in a row is a reading base with four colors representing four kinds of bases.

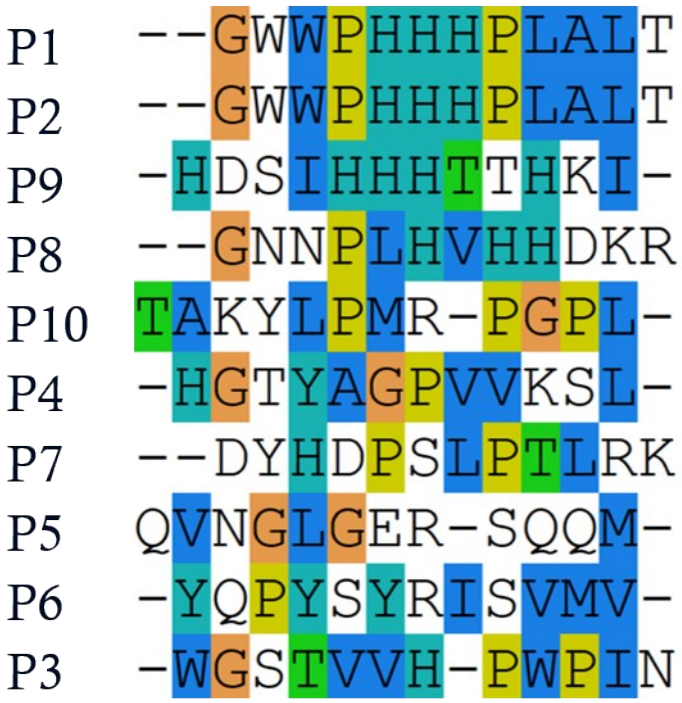
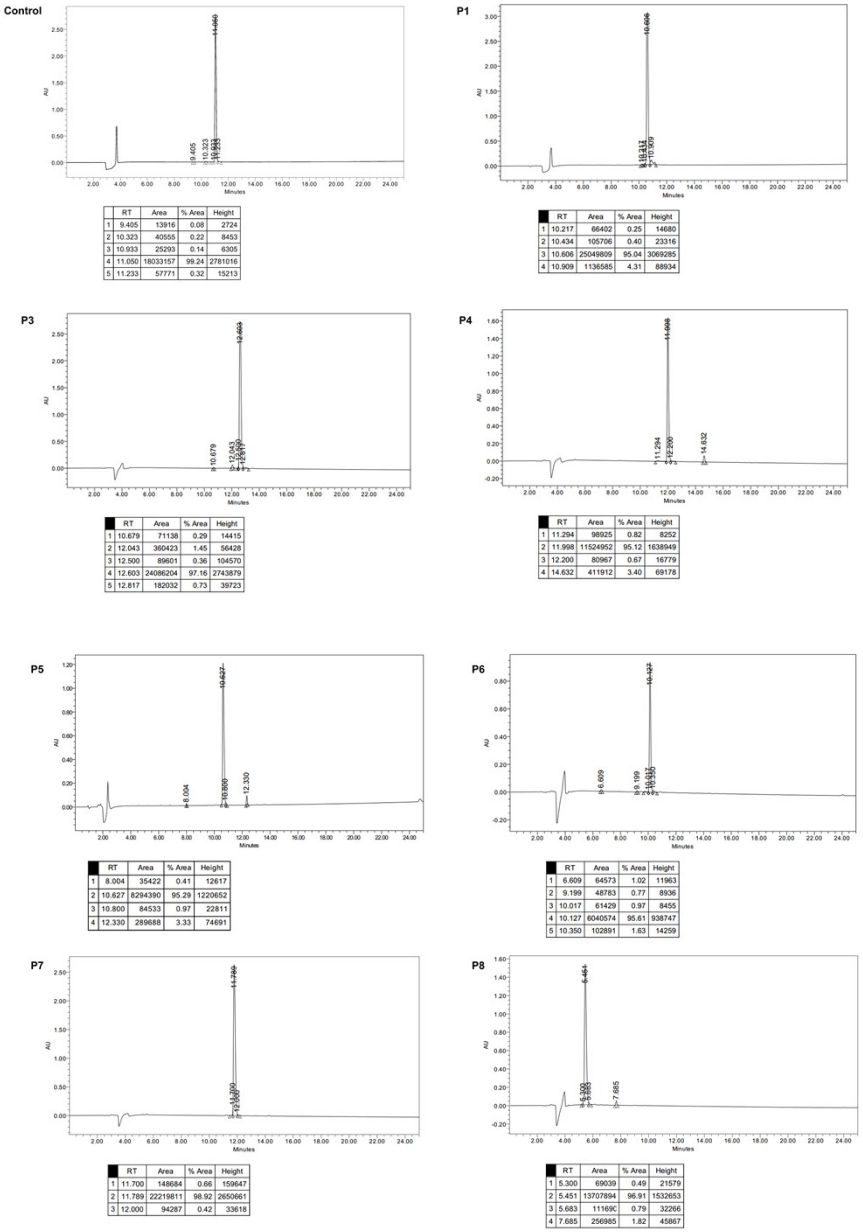


Fig. S9 Peptide sequence alignment of 10 positive beads in dm-Display against CD71.

Alignment was finished by Clustal X software. The order of sequences was arranged by sequence similarity and the same or similar amino acids are colored by the software if the amino acid at the position was conversed.



	RT	Area	% Area	Height
1	9.405	13916	0.08	2724
2	10.323	40555	0.22	8453
3	10.933	25293	0.14	6305
4	11.050	18033157	99.24	2781016
5	11.233	57771	0.32	15213

	RT	Area	% Area	Height
1	10.217	66402	0.25	14680
2	10.434	105706	0.40	23316
3	10.606	25049809	95.04	3069285
4	10.909	1136585	4.31	88934

	RT	Area	% Area	Height
1	10.679	71138	0.29	14415
2	12.043	360423	1.45	56428
3	12.500	89601	0.36	104570
4	12.603	24086204	97.16	2743879
5	12.817	182032	0.73	39723

	RT	Area	% Area	Height
1	11.294	98925	0.82	8252
2	11.998	11524952	95.12	1638949
3	12.200	80967	0.67	16779
4	14.632	411912	3.40	69178

	RT	Area	% Area	Height
1	8.004	35422	0.41	12617
2	10.627	8294390	95.29	1220652
3	10.800	84533	0.97	22811
4	12.330	289688	3.33	74691

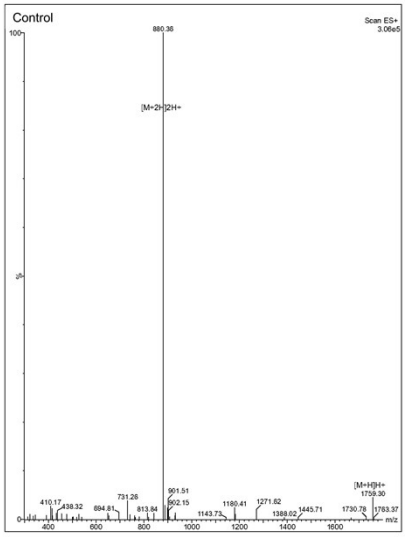
	RT	Area	% Area	Height
1	6.609	64573	1.02	11963
2	9.199	48783	0.77	8936
3	10.017	61429	0.97	8455
4	10.127	6040574	95.61	938747
5	10.350	102891	1.63	14259

	RT	Area	% Area	Height
1	11.700	148684	0.66	159647
2	11.789	22219811	98.92	2650661
3	12.000	94287	0.42	33618

	RT	Area	% Area	Height
1	5.300	69039	0.49	21579
2	5.451	13707894	96.91	1532653
3	5.683	11169C	0.79	32266
4	7.685	256985	1.82	45867

Fig. S10. Characterization of the purified peptides by High Performance Liquid Chromatography (HPLC).

All the peptides accounted for over 95% according to the area statistics.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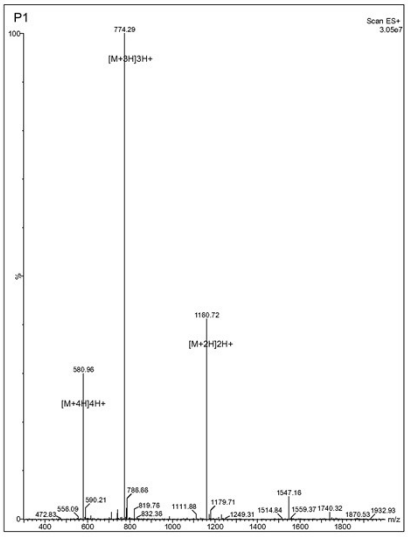
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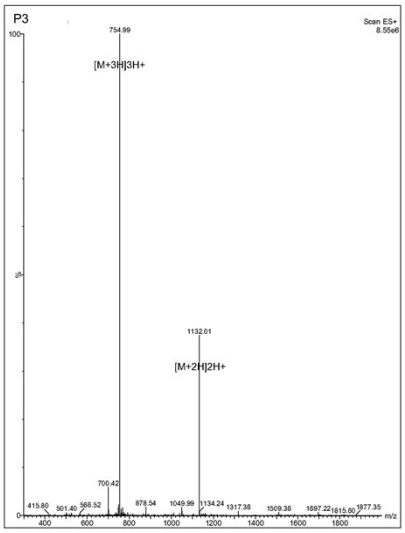
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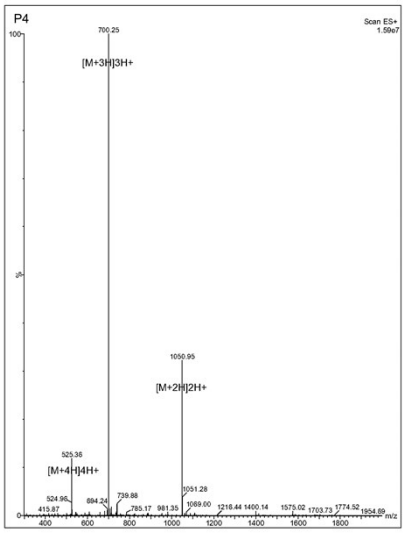
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739.88
785.17
981.35
1051.28
1069.60
1216.44
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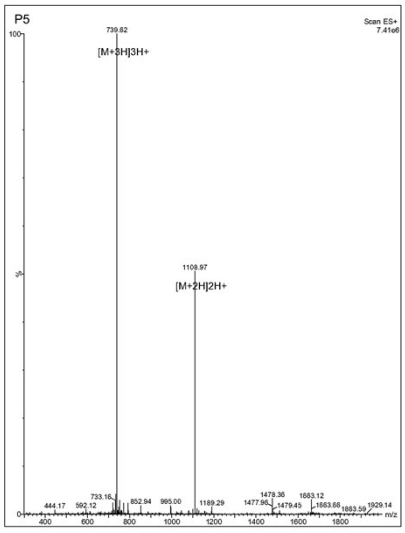
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1929.14
400
600
800
1000
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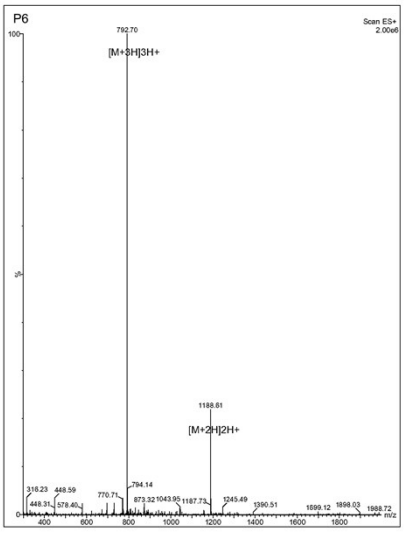
1800
m/z


P6
Scan ES+
2.00e6
100
%
0
792.70
[M+3H]3H+
1188.81
[M+2H]2H+
316.23
448.59
448.31
578.40
770.71
784.14
873.32
1043.95
1187.73
1245.49
1390.51
1699.12
1898.03
1988.72
400
600
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1800
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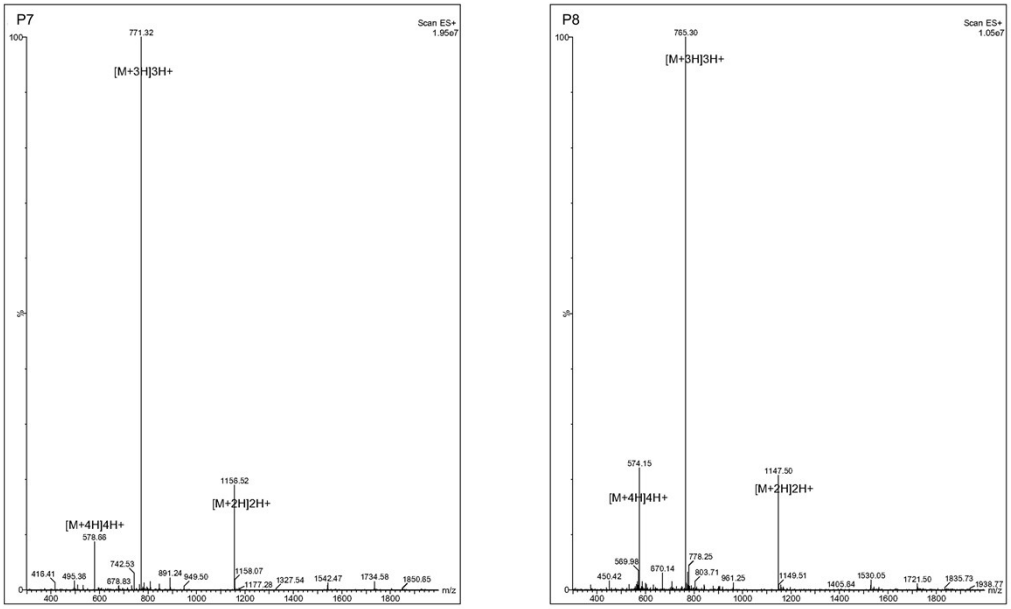

Fig. S11. Characterization of the purified peptides by Mass Spectrophotometer (MS).

The ion peaks at different charges were marked on the graph, which were corresponding to the molecular weight of selected peptides.

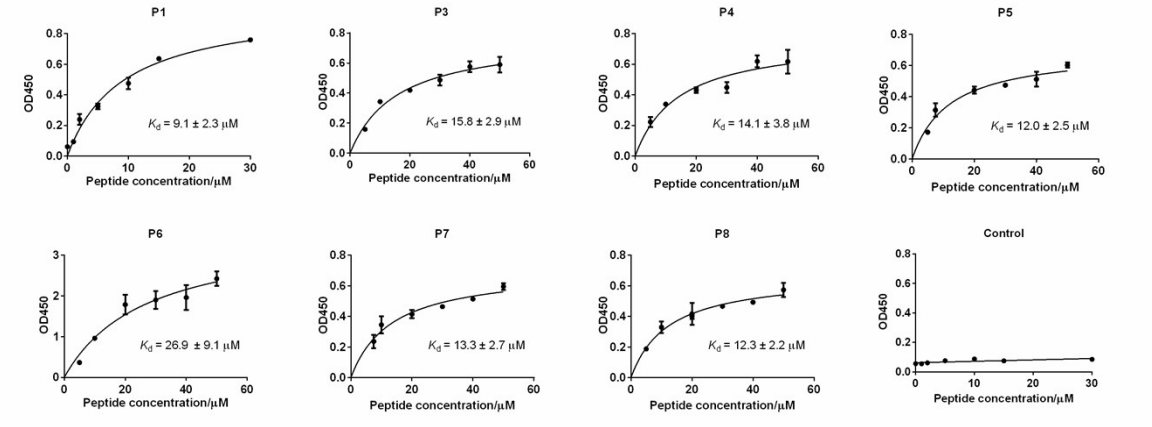

Fig. S12. K_d determination of the selected peptides against CD71 by ELISA.

P1 peptide was also named as GWW peptide. CD71 protein was pre-immobilized on 96-well plates before incubation with biotin-labeled peptides and SA-HRP. OD450 was read after the TMB catalytic chromogenic reaction.

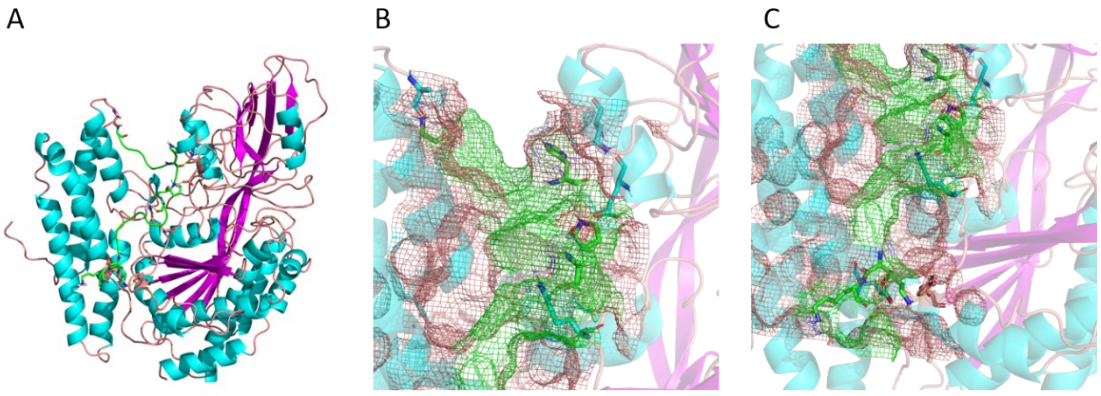

Fig. S13. Molecular Docking detains of GWW-CD71 complex.

(A) Docking poses of CD71-GWW (Ribbons). The structure of GWW peptide, α helix, β sheet and loop of CD71 were shown in green, cyan, purple and brown, respectively. (B)(C) Zoom in of Figure 5C III.

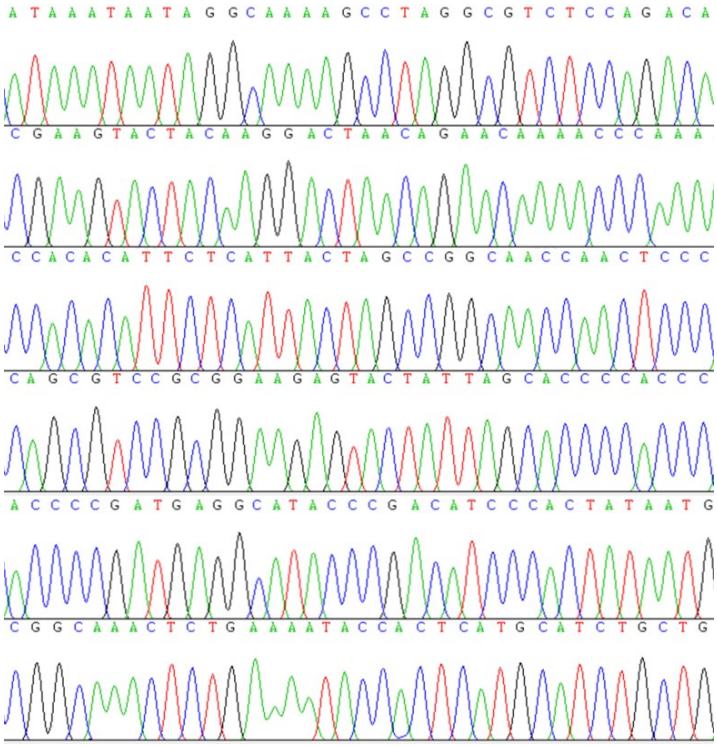

Fig. S14. DNA sequencing of 6 positive beads in dm-Display against GPC1.

Each row is a bead, and each position in a row is a reading base with four colors representing four kinds of bases.

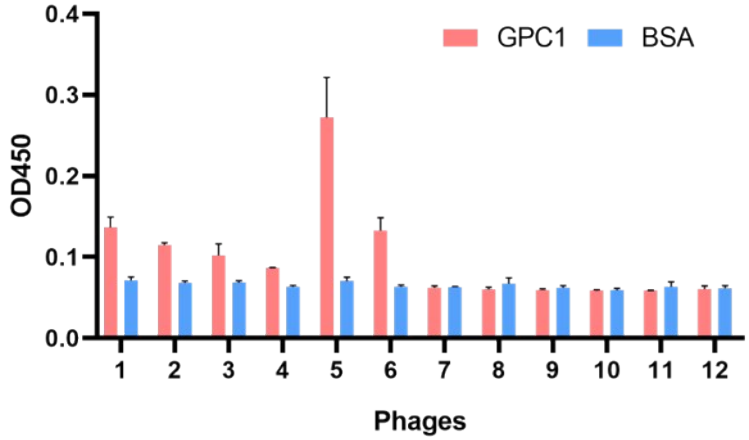

Fig. S15. Target bound ELISA of selected phages in dm-Display against GPC1.

Phage 1-6 and 7-12 were bright and dim beads, respectively. The value of OD450 reflected the number of phages bound to GPC1 protein, with more bound phages indicating greater affinity. Bovine Serum Albumin (BSA) was chosen as control protein to observe non-specific absorbance.

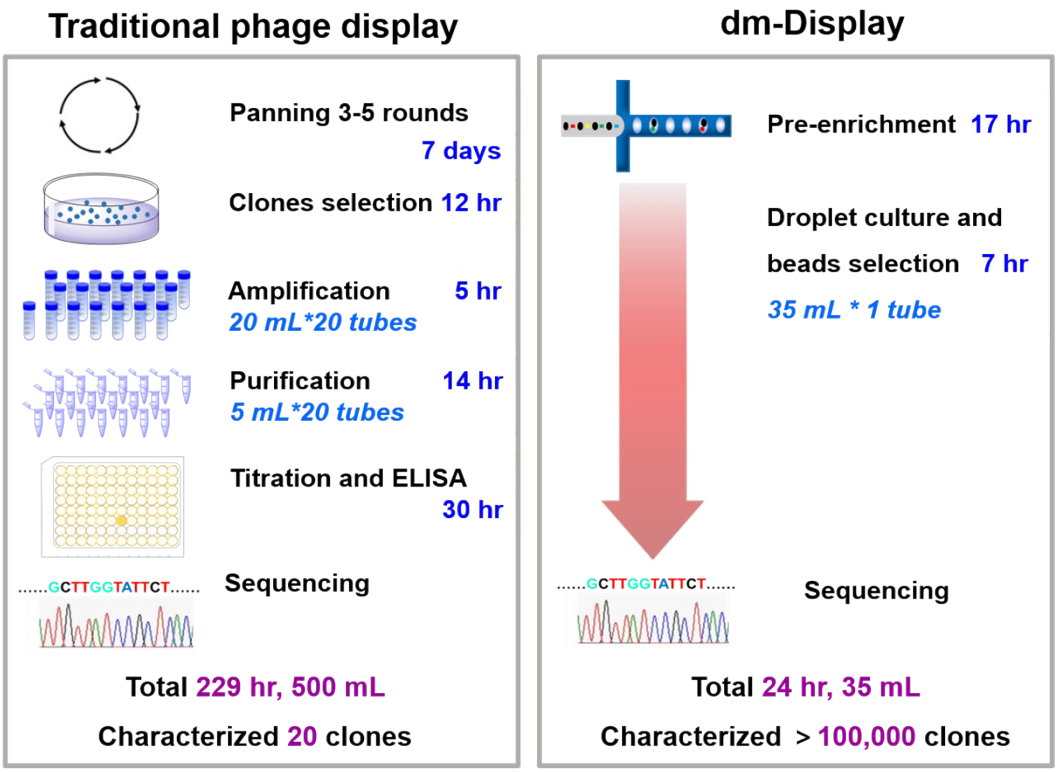

Fig. S16. Comparison of traditional phage display and dm-Display in terms of time requirement, reagent consumption and characterization of clones.

The traditional approach was evaluated according to the instruction manual for the Ph.D.12 phage display library.

Table S1 Distribution pf phages in droplets corresponded to Poisson distribution

	Empty droplets	**Phage droplets**
Predicted values	14.9	5.1
Experimental values	15	5

20 droplets were extracted after droplet culture and the encapsulation of phages was expected to follow Poisson statistics and experimentally quantified by titer.

Table S2 Analysis of the peptide sequences against CD71

Numbers	Peptide Sequences	Predicted Structures
P1、P2	**GWWPHHHPLALT**GGGS	CCCCTCCCEEEESCCC
P3	**WGSTVVHPWPIN**GGGS	CCCEEECCCCCCTCCC
P4	**HGTYAGPVVKSL**GGGS	CCCECSCEEEETTCCC
P5	**QVNGLGERSQQM**GGGS	CCCTCCHHHHHTTCCC
P6	**YQPYSYRISVMV**GGGS	CCCEEEEEEEEEECCC
P7	**DYHDPSLPTLRK**GGGS	CCCCTCCCCHCTTCCC
P8	**GNNPLHVHHDKR**GGGS	CCCCEEEEECTTTCCC
P9	**HDSIHHHTTHKI**GGGS	CCCEEECCEEEECCCC
P10	**TAKYLPMRPGPL**GGGS	CCCECCCCCCCTCCCC

Structures were predicted by SSpro 8, H: alpha-helix, E: extended strand, T: turn, S: bend, C: the rest (random coil).

GGGS is the universal linker between displayed peptide and PⅢ protein. The bold 12 amino acids are the displayed peptide.

Table S3 Binding energy of the simulated GWW-CD71 complex

Van der waal (KJ/mol)	Electrostatic (KJ/mol)	Polar salvation (KJ/mol)	Non-polar salvation (KJ/mol)	Binding energy (KJ/mol)
-453.17±1.63	-26.83±2.59	19.49±3.47	-15.91±2.83	-475.42±6.31

Table S4 Interaction residues between GWW and CD71

CD71 Protein	GWW peptide
725ALA	1GLY
230ASN	6HIS
266GLU	8PRO
534LYS	10ALA
503TRY	17LYS
613GLY	18LYS

Table S5 Analysis of the peptide sequences against GPC1

Numbers	Peptide Sequences	Predicted Structures
1	**CLETPRLLPIIY**GGGS	CCCCCTCCCEEEECCC
2	**FGFCSVSPCSTS**GGGS	CCEEEECCCCCCSCCC
3	**GSWLPASNENVW**GGGS	CCECCCCCCEEECCCC
4	**GWGANSTLPRTL**GGGS	CCCCCCCCCCCCTCCC
5	**HYSGMSGMPHRG**GGGS	CCCCCCSCCCCCCCCC
6	**QQMHEWYFQSLP**GGGS	CCCEEEEEEECTTCCC

Structures were predicted by SSpro 8, E: extended strand, T: turn, S: bend, C: the rest (random coil).

GGGS is the universal linker between displayed peptide and PⅢ protein. The bold 12 amino acids are the displayed peptide.

Table S6. Comparison of traditional panning and dm-Display.

Traditional Panning [a]			**dm-Display**		
Process	**Duration**	**Reagents**	**Process**	**Duration**	**Reagents**
Panning for 3-5 rounds	At least 7 days		**Library pre-enrichment**	17 hr	
Monoclonal phage picking	12 hr [b]		**Droplet generation and culture**	5 hr	1 mL× 1 tube
Phage amplification	5 hr	20 mL× 20 tubes [c]	**Bead staining and selection**	2 hr	34 mL× 1 tube [d]
Phage purification	14 hr [e]	5 mL× 20 tubes			
Titration	12 hr				
Phage ELISA	18 hr [f]	100 μL ×96 wells [g]			
Total	229 hr	＞500 mL	**Total**	24 hr	35 mL
Number of clones characterized	20-200		＞100,000		

[a]. According to the instruction manual of Ph.D.™ phage display libraries by New England Biolabs (NEB). In the literature, the number characterized varied from 20 to 200. Consumption of reagents and time were all calculated for 20 clones. [b]. Titration-based monoclone selection takes 12 hr (overnight). [c]. 20 tubes for 20 monoclones; more tubes are required for more monoclones. [d]. The 34 mL includes 30 mL dilution buffer to avoid cross-

contamination and 4 mL buffer for washing. [e]. The 14 hr purification includes two 10-min centrifugations for removing *E. coli*, one 12-hr phage sediment, two 10-min centrifugations for phage concentration, one 1-hr phage sediment and two 10-min centrifugations for phage concentration. In fact, most centrifuges can handle only 6 tubes at a time; thus, treatment of 20 tubes usually takes more than 24 hr. [f]. The 18 hr ELISA includes 12 hr for adsorption of target, 2 hr for blocking, 2 hr for phage incubation, 1 hr for antibody incubation and 1 hr for chromogenic reaction. [g]. The number of 96-well plates depends on the number of phage dilutions and number of monoclones.