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

Auto-Panning: a highly integrated and automated biopanning platform for peptide screening

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Materials and methods

Materials

Horseradish peroxidase (HRP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Silicone oil PMX-200 (10 mPa·s) was obtained from Aladdin (Shanghai, China). TMB substrate was purchased from tiangen (Beijing, China). Streptavidin-R-phycoerythrin conjugation SA-PE (SA100-41), Dynabeads M-280 Tosyl-activated (14204) were purchased from Thermo Fisher Scientific Inc (Shanghai, China). Kimwipers were purchased from Kimberly-Clark (Kimberly-Clark, Irving, TX, USA).The control proteins including human serum albumin (HSA), human immunoglobulin G (IgG), bovine serum albumin (BSA), and the bacterial culture reagents including LB Lennox culture medium, LB Top Agar, LB Lennox agar, isopropyl β-D-thiogalactopyranoside (IPTG), 5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal), and tetracycline hydrochloride (Tet) were all purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Transferrin receptor CD71 (2474-TR-050) and human EphA2 Alexa Fluor 488-conjugated Antibody (FAB3035G) were purchased from R&D Systems (Minneapolis, MN, USA). Teflon AF1600 was purchased from Dupont (Shanghai, China). SU-8 (GM 1040) was purchased from Gersteltec Sarl (Pully, Switzerland).Tetronic 90R4.

Two kinds of washing buffer were used to increase selection stringency with increasing rounds. Washing buffer A used in the first round of selection contained 1 mM $CaCl_2$, 0.1% (w/v) Tween 20 in TBS (25 mM Tris, 0.15M NaCl, pH 7.2~7.5); washing buffer B used in the second and third rounds of selection contained 1 mM $CaCl_2$, 0.5 % (w/v) Tween 20 in TBS. The LB Lennox medium contained 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl. The culture medium in Auto-panning was 50 times diluted late logarithmic phase *E.coli* in 1.5 × LB Lennox medium. The acid elution buffer contained 0.2M glycine-HCl, 1 mg/mL BSA, pH 2.2. The neutralization buffer contained 0.1M Tris—HCl, pH 9.0. The buffers were prepared with 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 the buffers to be used on chip contained 0.1% (w/v) Tetronic 90R4 (a nonionic low-foaming surfactant) to ensure the correct contact angle change.

Preparation of protein-coated magnetic beads

The M280 tosylated Dynabeads were coupled with target protein EphA2 or counter selection protein HSA, based on covalent bonds via primary amino or sulfhydryl groups. To adjust selection stringency, magnet beads with different protein concentration were coupled as follows: Two kinds of EphA2-beads were involved to decrease the target protein concentration in selection in order to increase selection stringency with increasing rounds. For positive selection EphA2-beads A, 20 μL 30mg/mL M280 tosylactived Dynabeads were gently washed with 0.1 M borate buffer (pH 9.5), and the beads were resuspended in 5 μL 1μg/μL EphA2, and 25μL 0.1 M borate buffer (pH 9.5) was added to give a total volume of 30 μL. Twelve μL of 3 M ammonium sulfate in 0.1 M borate buffer (pH 9.5) was added, and the mixture was incubated on a roller at 37°C for 12–18 hours. After that, the beads were resuspended in 200 μL 0.5% (w/v) BSA-PBS (pH 7.4), and incubated at 37°C for an hour to block the unreacted tosyl sites. The beads were washed twice with 200 μL 0.1% (w/v) BSA-PBS (pH 7.4), and finally resuspended to reach a final concentration of 30 mg beads/mL and 0.25 mg EphA2/mL. EphA2-beads B were coupled as described above, except that the protein content was reduced to 1.2 μL 1μg/μL EphA2, reaching a final concentration of 30 mg beads/mL and 0.06 mg EphA2/mL. For counter selection HSA-beads, the amount of beads was increased to 80 μL, and all the volumes of the other liquids were accordingly scaled up as EphA2-beads B, reaching a final concentration of 30 mg beads/mL and 0.06 mg HSA/mL. All the coupled beads were finally resuspended at a concentration of 30 mg beads/mL in washing buffer A (1 mM $CaCl_2$, 0.1% Tween 20 in TBS), and a final concentration of 0.1% Tetronic 90R4 was added before loading into reservoirs.

DMF working station

The DMF Auto-Panning working station includes a DMF driving system (function generator) with a laptop, microfluidic software, DMF chip, chip holder, webcam, a ball-point pen magnet, and a heater. The basic configuration and operation of DMF system were described in our previous paper[1]. A ball-point pen magnet was positioned on the chip for bidirectional magnetic separation. A heater was placed under the chip to maintain a suitable temperature.

Chip design

The chip of DMF Auto-Panning consists of two parallel glass plates. As shown in Figure 1, the bottom plate was formed with 27 actuation electrodes (4 mm × 4 mm), 6 medium electrodes A (3 mm × 2 mm), 9 medium electrodes B (2 mm × 2 mm) and 6 reservoir electrodes (6 mm × 8 mm) with 40 μm gaps between each. The width of lines connecting electrodes and pins was 60 μm.

Chip fabrication

The bottom plate of DMF chip was bottom-up assembled on glass with 300 nm-height chromium as an electrode layer, 11.7 μm-height SU-8 1040 photoresist as a dielectric layer, and 200 nm-height Teflon AF 1600 as a hydrophobic layer, and the top plate was made of indium tin oxide (ITO)-coated glass as a ground electrode. Four layers (480 μm) of double-sided tape were used as spacers between the top and bottom plates.

Pre-treatment and optimization before device operation

The chip was sterilized by soaking in 75% alcohol for more than 10 min, and then exposed under UV light for over 30 min in a clean bench before using. The chip could be reused after alcohol and UV light treatment.

The droplet size was optimized to be 2/8 μL for droplet generation, 16~32 μL for incubation, 8~16 μL for magnetic separation. The voltage of DMF system was set at 90-110 Vrms, 10 kHz. The beads concentration was 30 mg/mL in reservoir, and dispersed at 2 μL/unit.

Before all the droplet manipulations, silicone oil PMX-200 was pipetted and filled the gap between the top and bottom plates to construct a lubricated environment for droplet movement. All the reagents were loaded into corresponding reservoirs. The library, washing buffer, elution buffer, and culture medium were dispersed at 8 μL/unit. The EphA2-beads, HSA-beads, and neutralization buffer were dispersed at 2 μL/unit.

To ensure selection stringency, 10 μL initial library was diluted to reach a final volume of 100 μL before screening and was divided into two 50 μL aliquots, each of which was incubated with 2 μL positive selection beads for 1 h on chip.

Magnetic separation on DMF chip

The bidirectional separation method we described in our previous work was utilized[1]. As shown in Figure S2, a ball-point pen magnet was employed above the chip, pointing to the edge of the droplet. The bead droplet was driven across the magnet back and forth to make beads focus on the ball-point of the magnet. Then electrodes were switched on sequentially (Figure S2A-B). Afterwards, another electrode was switched on to stretch the droplet in contact with three electrodes, immediately followed by switching the middle electrode off. A combined effort of tension and magnet force made the droplet unequally split into two droplets (Figure S2C), a small droplet containing magnetic bead pellet and a large droplet of supernatant. The ball-point magnet was only put above droplet when magnetic separation was needed, otherwise it was removed.

Characterization of droplet generation

Droplets of all kinds of reagents were generated and photographed 10 times on chip. The corresponding volumes were calculated by the average area in photographs processed by image J software, normalized with that of the pipette-generated droplets.

Characterization of bead retention on chip

Chromogenic reaction of HRP-TMB was used for characterization of bead retention after magnetic separation, as described in our previous paper[1]. For magnetic separations on chip, 2 μL of HRP-beads (30 mg/mL) were introduced into the chip and washed 8 times with 8 μL washing buffer. After washing, the beads were resuspended in 8 μL washing buffer, extracted and resuspended in 200 μL PBS in a tube, 20 μL of the which was transferred to a 96-well plate with 250 μL of TMB substrate added inside each well. After incubation at room temperature for 15 minutes, 50 μL of 2 M H_2SO_4 was added to stop the reaction. Absorbance at 450 nm was read by a microplate reader (EnSpire® Multimode Plate Reader, PerkinElmer, Waltham, MA, USA). For magnet separations in tube, 2 μL of HRP-beads (30 mg/mL) were suspended in 100 μL PBS and washed 8 times with washing buffer, and finally resuspended in 208 μL PBS. After that, the bead mixtures were processed in the same way as washed beads on chip. Bead retention (r) was calculated by $r = A_w/A_0$, where A_w represents the absorbance at 450 nm of washed beads and A_0 represents the absorbance at 450 nm of unwashed beads, which were obtained by suspending 2 μL of unwashed HRP-beads (30 mg/mL) in 208 μL PBS.

Characterization of washing efficiency on chip

The chromogenic reaction of HRP-TMB was used for washing efficiency characterization on chip as presented in our previous paper[1]. Two μL BSA-beads (30 mg/mL) were mixed with 8 uL HRP solution on chip for 10 minutes, followed by magnetic separation and 7 cycles of washing with 8 μL washing buffer on chip. After each magnetic separation and washing, the supernatant was collected and added to PBS in a tube. Afterwards, the diluted supernatant was added to a 96-well plate and mixed with 200 μL TMB substrate. After incubation at room temperature for 15 minutes, 50 μL of 2 M H_2SO_4 was added to quench the reaction. Absorbance at 450 nm was recorded on a microplate reader. For washing in tube, 8 μL HRP solution was diluted and mixed with 2 μL BSA-beads (30 mg/mL) in a total volume of 100 μL for 30 min,

followed by magnetic separation and 7 cycles of washing with 100 μL washing buffer. The supernatant was collected and processed in the same way as the supernatant obtained from washing process on chip. HRP% in supernatant of the i^{th} washing step (E_i) was defined as $E_i = A_i/A_t$, where A_i represents the absorbance at 450 nm of the supernatant sample from the i^{th} washing step (0^{th} represents the magnetic separation after original incubation), and A_t represents the absorbance at 450 nm of 8 μL HRP solution.

Phage growth curve

E.coli ER2738 was cultured overnight to reach OD~1.0, and was then diluted 1:100 with LB Lennox as the culture medium. A phage concentration of 1000 pfu/μL was chosen as the initial concentration in the curve. For incubator culture, 5 mL of culture medium was pipetted into a clean 12 mL culture tube, which was rotated at 250 rpm at 37°C for 5 h and then transferred to ice at a certain time point for titration. For chip culture, a 16 μL culture droplet (8 μL/unit) underwent a programmed active 5-h incubation at 37°C by continuously cycling on four electrodes. The bottom of the chip was heated with a heating sheet wrapped with foil, and a handheld infrared thermometer was used to measure and control the droplet temperature. At each time point,1 μL of mixture was pipetted into a clean tube placed on ice for titration. The growth curve was constructed by plotting the time point when culture was stopped on the abscissa and the phage concentration at that time on the ordinate.

Feasibility of phage enrichment

A positive phage specially bound to CD71 named GWW phage and a control phage unbound to CD71 named DYH phage were involved. A mimetic phage library was prepared by mixing GWW and DYH phages at different ratios (1:10, 1:100, 1:1000) to a final concentration of 5×10^8 pfu/μL. Binding buffer was 0.2 mg/mL BSA in washing buffer. Washing buffer was 1 mM $CaCl_2$, 0.5% (w/v) Tween 20 in TBS (25 mM Tris, 0.15M NaCl, pH 7.2~7.5). For tube and DMF enrichment, 32 μL phage library was incubated with 2 μL EphA2-beads (30 mg beads/mL, 0.25 mg EphA2/mL) for 30 min at room temperature. After incubation, the beads were washed 6 times with 32 μL washing buffer, which was then resuspended in 10 μL elution buffer for 10-min incubation, and rapidly neutralized with 2 μL neutralization buffer. For 96-well plate enrichment, a high-adsorption polystyrene well was incubated with 1.5 μg EphA2 diluted in 100 μL 0.1 M $NaHCO_3$, pH 8.6, for overnight adsorption. The well was then blocked with 10 mg/mL BSA at room temperature for 2 h. One hundred μL mimetic phage library was added to incubate with coated EphA2 for 1 h. After that, the well was washed 6 times with 200 μL washing buffer. Then, the bound phages were eluted with 100 μL elution buffer for 10-min incubation and recovered with 20 μL neutralization buffer. DYH phage is a special phage that forms transparent plaques on IPTG/X-gal culture plates, while GWW turns blue. The proportion of GWW phage was easy to calculate according to the titer.

The enrichment was calculated according to the magnification of the proportion of the positive phage (GWW). The enrichment E was defined as $E = A_o/A_i$ with 3 sets of parallel acquisitions; A_i represents the input GWW phage proportion before enrichment, and A_o represents the output GWW phage proportion after enrichment.

Peptide selectivity

In order to validate its binding selectivity towards various cells, the peptide was incubated with four different kinds of cells, and the binding affinities were characterized individually. The adherent cells (U251, HEK-293T and U87-Mg) were cultured overnight in a 24-well plate, incubated with 10 μM 200 μL biotin-peptide diluted in 1mM $CaCl_2$-TBS buffer with slow shaking for 30 min. Then the peptide binding to cells was fluorescently by the addition of 1:400 diluted SA-PE. After incubation, cells were washed 2 times with 1 mL 1mM $CaCl_2$-TBS. Finally, cells were dislodged with a cell scraper and resuspended in 200 μL 1mM $CaCl_2$-TBS for flow cytometry analysis. As for half-adherent cells (K-562), 10 μM biotin-peptides were treated in the same way but washed by centrifugation at 1200 rpm for 3 min.

Confocal imaging

U87-MG cells were cultured overnight in a glass-bottom petri dish. The adherent cells were cultured with 200 μL 10 μM biotin-labeled-peptides in 1mM $CaCl_2$-TBS for 30 min at room temperature. Afterwards, cells were cultured with 1:400 diluted SA-PE for 10 min, fixed, and stained with nuclear dye Hoechst 33342 for 10 min at room temperature. After each incubation, cells were washed twice with 1 mL 1mM $CaCl_2$-TBS. K-562 cells were similarly treated, but washed by centrifugation at 1200 rpm for 3 min. Finally, the confocal microscope Leica SP8 was used to observe the cells and capture the cell images.

For peptide internalization analysis, U87-MG cells were cultured overnight in a glass culture dish. The cells and the peptides were placed at 4°C/room temperature/37 °C for 10 min to adapt to the incubation temperature. After 10-min temperature adaption, 200 μL 10 μM biotin-labeled-peptides in 1mM $CaCl_2$-TBS were added to the dishes, and the dishes were incubated at 4 °C for 10 min, room temperature for 10 min, and 37 °C for 20 min. Then, cells were fixed in 4% PFA for 30 min. After fixing, cells were incubated with

1:400 diluted SA-PE for 10 min, and stained with nuclear dye Hoechst 33342 for 10 min at room temperature. After each incubation, cells were washed twice with 1 mL 1mM $CaCl_2$-TBS. Finally, the cells were observed, and the images were captured under confocal microscope Leica SP8.

For EphA2 relocation analysis, U251 cells were cultured overnight in a glass-bottom petri dish. The adherent cells were treated with 500 μL 10 μM biotin-labeled-peptides in non-FBS DMEM culture medium for 30 min at 37 °C. After treatment, cells were fixed with 4% PFA for 15 min, permeabilized with 0.5% Triton X 100 for 15 min, and blocked with 5 mg/mL BSA-PBS for 30 min. Cells were then stained with anti-EphA2-AF488 for 30 min and Hoechst 33342(1:5000) for 10 min. Finally, the confocal microscope Leica SP8 was used to observe the cells and capture the cell images.

Peptide internalization confirmation

For trypsin digest tests, U87-MG cells were cultured overnight on three 24-well plates. The plates and peptides were placed at 4°C/room temperature/37 °C for 10 min to adapt to the following incubation temperature. After 10-min temperature adaption, 200 μL 10 μM biotin-labeled-peptides in 1mM $CaCl_2$-TBS were added to wells, and incubated at 4 °C for 10 min, room temperature for 10 min, and 37 °C for 20 min. After that, cells were incubated with 1:400 diluted SA-PE for 10 min. The trypsin digest groups were then treated with 200 μL 0.25% EDTA-trypsin at 37 °C for 3 min, and the digest was stopped by adding 1 mL 10% FBS-DMEM. After that, the cells were dislodged with a cell scraper and resuspended in 200 μL 1mM $CaCl_2$-TBS for flow cytometry analysis.

Wound healing assay

U251 cells were cultured overnight in the 6-well petri dish to reach a nearly 100% density of cells. The adherent cells were scarified by a 200 μL pipette, and treated with 1200 μL 10 μM biotin-labeled-peptides in non-FBS DMEM culture medium for 5 h at 37 °C, 5% CO_2. The wounds were photographed before/after treatment to analyze the healing effects.

Table S1. Selection details in Auto-Panning against EphA2

Round	Volume /μL	Counter selection details	Positive selection details	Washing details
1st	100	N/A	100 μL diluted library +4 μL EphA2-beads A, 60 min incubation	Washing buffer A, 6 times
2nd	30	30 μL amplified product +2 μL HSA-beads, incubation 20 min	30 μL counter selection product +4 μL EphA2-beads B, 45 min incubation	Washing buffer B, 6 times
3rd	30	30 μL amplified product +4 μL HSA-beads, incubation 30 min	30 μL counter selection product +2 μL EphA2-beads B, 30 min incubation	Washing buffer B, 8 times

Note:

EphA2-beads A were coupled at 30mg beads/mL, 0.25mg protein/mL. EphA2-beads B were coupled at 30mg beads/mL, 0.06mg protein/mL. HSA-beads were coupled at 30mg beads/mL, 0.06mg protein/mL.

Table S2. Variations of droplet volume used in Auto-Panning

Reagents	Library	Washing buffer	Elution buffer	Culture medium	Positive selection beads	Counter selection beads	Neutralization buffer
CV/%	3.9	2.1	2.4	4.7	5.1	4.3	3.9
Volumes/μL	7.85±0.31	8.11±0.17	9.03±0.22	9.05±0.42	1.78±0.09	1.98±0.10	1.96±0.08

Note:

The volume of droplet was approximately estimated by the average area of droplet in photographs processed by image J software, normalized with that of the pipette-generated droplets.

Table S3. Comparison of reagents and time consumption for 3 rounds of phage display

round	Processes	Conventional approach		Auto-Panning	
		Duration/h	Reagents/mL	Time /h	Reagents /mL
1st	Counter selection	N/A	0.8	N/A	0.5 mL
	Positive selection	1		2	
	Washing (6 times)	0.5		0.5	
	Elution	0.17		0.17	
	Amplification	4.5	20	5	
	Purification	＞15	5	/	
	Titration	＞12	/		
2nd	Counter selection	0.33	1.1	0.33	0.5 mL
	Positive selection	0.75		0.75	
	Washing (9 times)	0.5		0.5	
	Elution	0.17		0.17	
	Amplification	4.5	20	5	
	Purification	＞15	5	/	
	Titration	＞12	/		
3rd	Counter selection	0.5	1.4	0.5	0.5 mL
	Positive selection	0.5		0.5	
	Washing (12 times)	0.5		0.5	
	Elution	0.17	/	0.17	
	Summarization	~70	53	~16	1.5

Note:

Counter selection was removed in the first round of selection.

Purification of amplified phages was by the double precipitation method by PEG/NaCl.

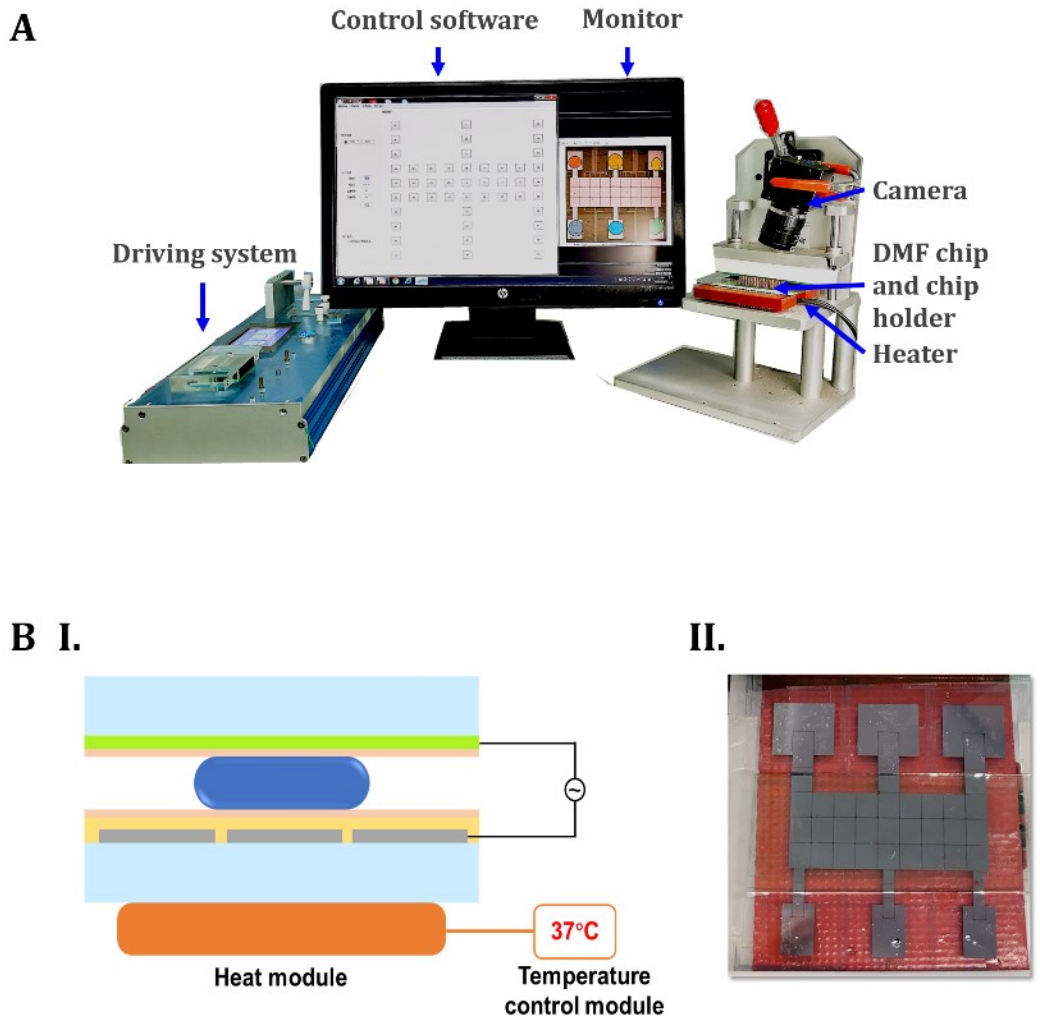


Figure S1. Photograph of Auto-Panning system

(A)Photograph of the entire Auto-Panning system. (B) Schematic illustration (I) and photograph (II) of the heater placed under the chip. The brown-red sheet is the heater.

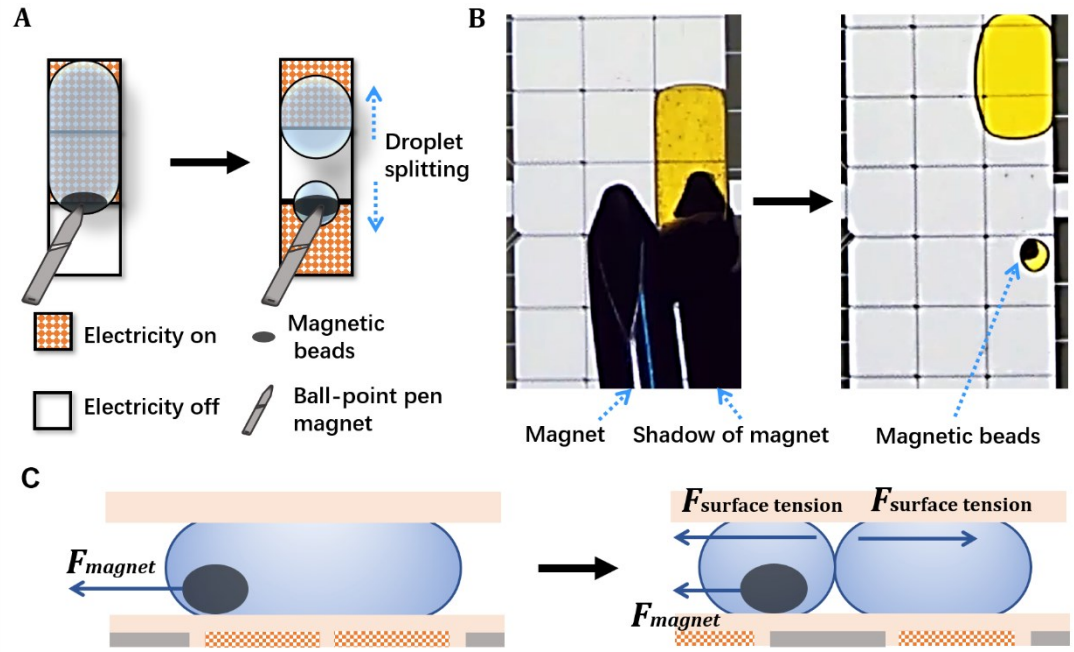


Figure S2. Magnetic separation on Auto-Panning.

Schematic illustration of (A) electrode control, (B) image of separation, and (C) force bearing of droplets splitting.

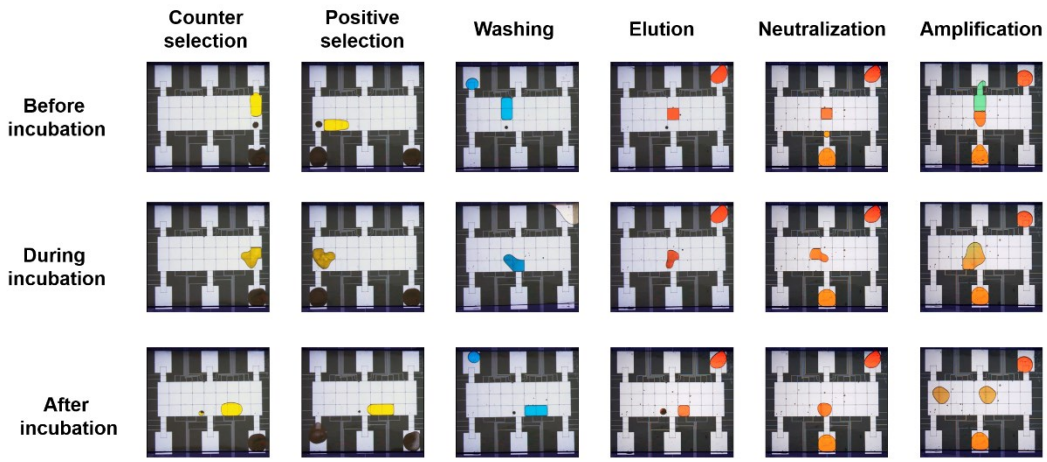


Figure S3. Screenshots of Auto-Panning procedures.

The colorful liquids were used for observation, not the real reagents in Auto-Panning.

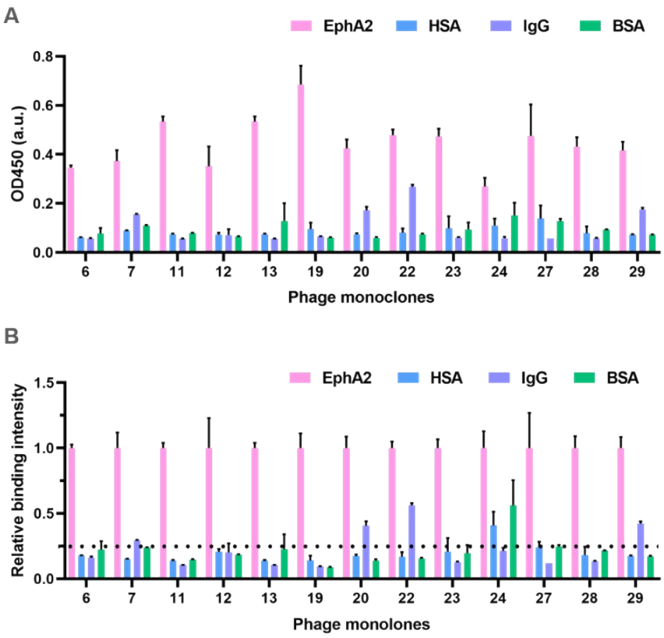


Figure S4. Protein selectivity of high-affinity phages

(A) Selectivity of 13 phage clones against different proteins measured with an ELISA method. (B) Normalized cross-reactive intensity of clones with their binding intensity against EphA2. The black dotted threshold line was set at 0.25.

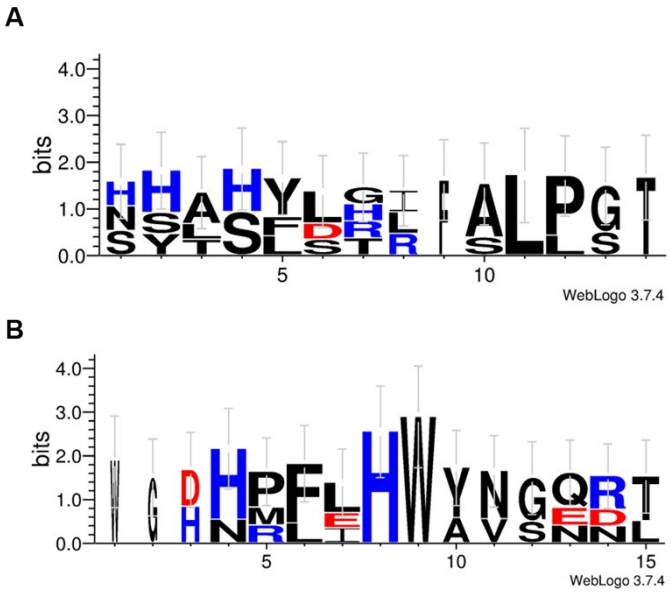
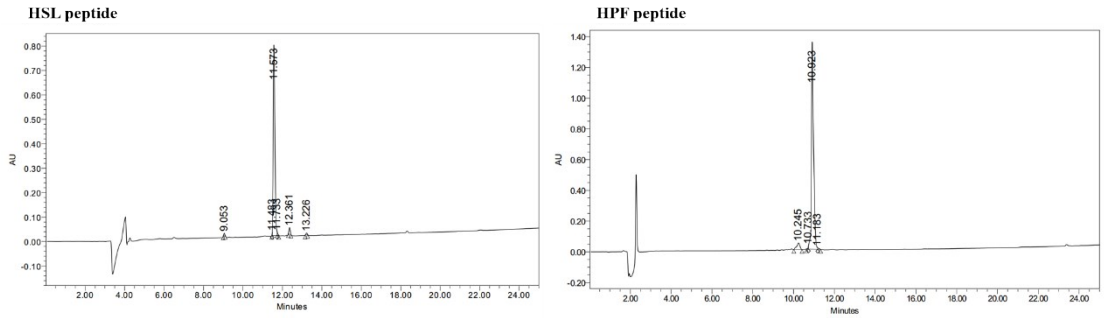


Figure S5. Sequence alignment of the 8 clones.

(A) ALP motif. (B) FLHW motif.The sequences were first aligned by Clustal X, and the obtained aln file was used to draw the weblogo; the height of each symbol indicates the relative frequency of each amino acid at that position, and the width of each symbol is proportional to the fraction of valid symbols at that position. The error bars indicate an approximate Bayesian 95% confidence interval. For coloring, the positively charged amino acids (K, R, H) are colored blue, while the negatively charged amino acids (D, E) are colored red.



	RT	Area	% Area	Height
1	9.053	39286	0.80	10920
2	11.483	6727	0.14	13453
3	11.573	4674010	95.27	781203
4	11.733	31619	0.64	14657
5	12.361	121354	2.47	27244
6	13.226	32910	0.67	7340

	RT	Area	% Area	Height
1	10.245	436691	4.25	40965
2	10.733	26853	0.26	20697
3	10.923	9776784	95.08	1348441
4	11.183	42709	0.42	9670

Figure S6. Characterization of the purified peptides after peptide synthesis by High Performance Liquid Chromatography (HPLC).

All the peptides accounted for over 95% according to the area statistics.

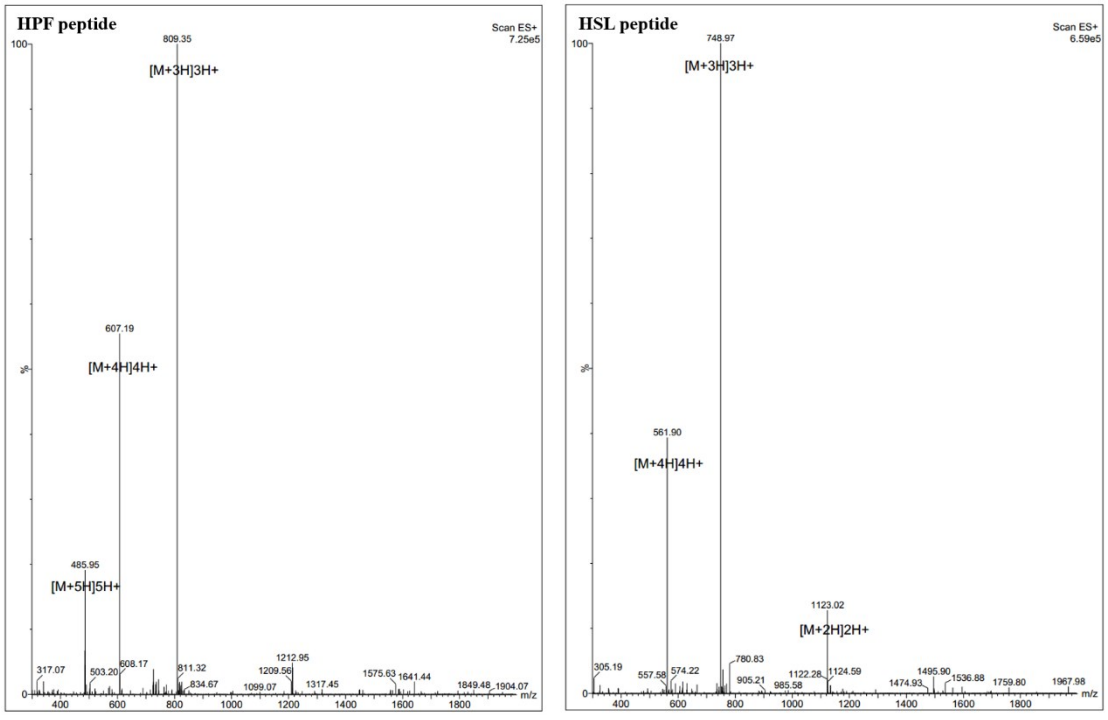


Figure S7. Characterization of the purified peptides after peptide synthesis by Mass Spectrometer (MS).

The ion peaks at different m/z ratios marked on the graph correspond to the designated peptide.

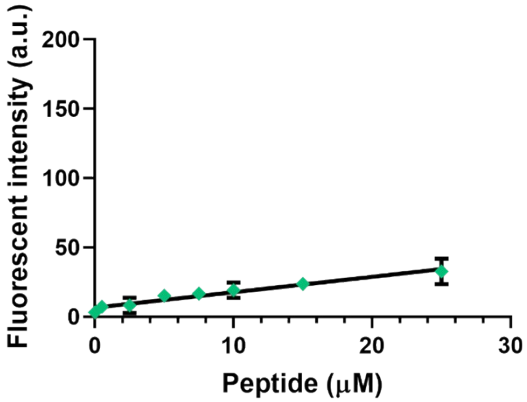


Figure S8. Non-specific adsorption of the control peptide (NYYQTHLHLTEL) on EphA2 with increasing concentration.

The fluorescent intensity of beads-peptide complex was blanked with that of beads.

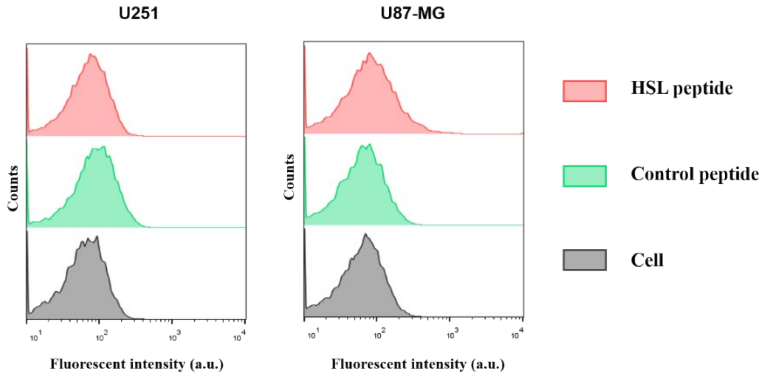


Figure S9. Non-binding of HSL peptide and EphA2-positive cells.

The cells were incubated with peptide and SA-PE, sequentially, and measured by flow cytometry. The HSL peptide was 10 μM.

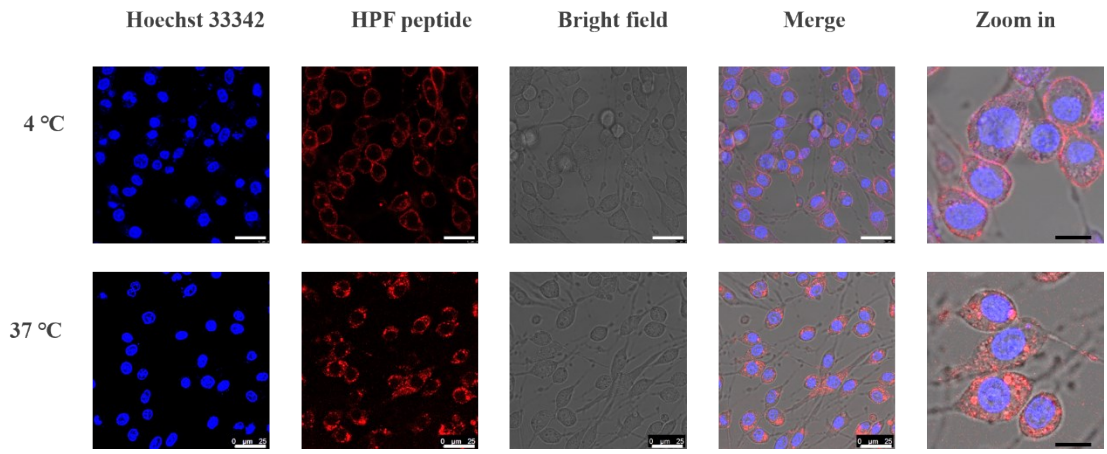


Figure S10. Initialization study of HPF peptide.

Peptide internalization into U87-MG cells was observed under the 80ᔕ objective of the Leica SP8 confocal microscope. The white scale bar corresponds to 25 μm, and the black scale bar in zoom-in corresponds to 10 μm. The peptide concentrations were 10 μM.

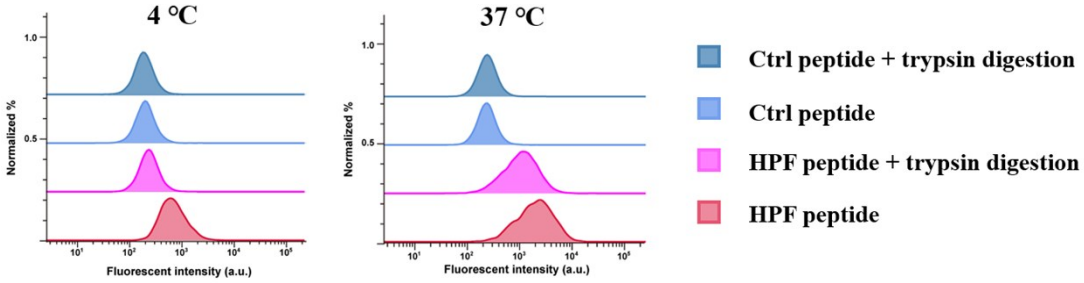


Figure S11. Trypsin digest of surface binding peptides on U87-MG cells.

The peptide concentrations were 10 μM.

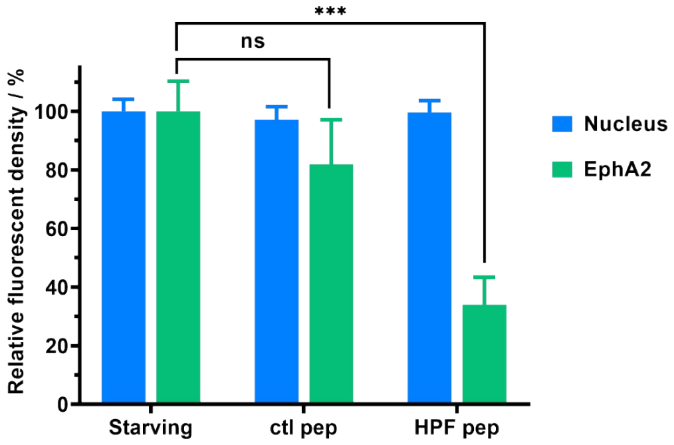


Figure S12. Relative EphA2 expression after peptide treatment.

The relative fluorescence intensity was analyzed by gray analysis by ImageJ software. Batch effects between different pictures were corrected for the fluorescence of Hoechst, and normalized with the EphA2 expression at the starting state.

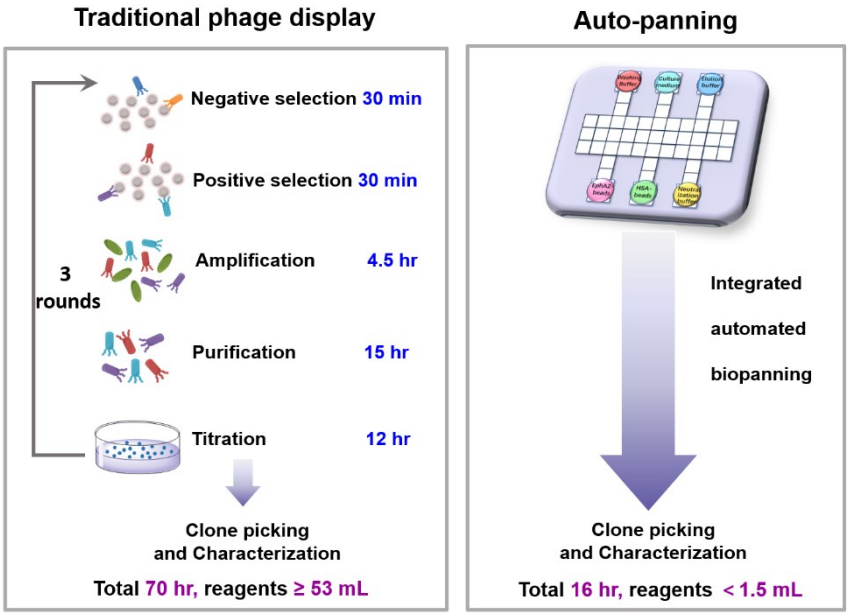


Figure S13. Method comparisons of the traditional approach and Auto-Panning.

The details of time and reagents consumption were listed in Table S3.

References

1. J. Guo, L. Lin, K. Zhao, Y. Song, M. Huang, Z. Zhu, L. Zhou and C. Yang, *Lab Chip*, 2020, **20**, 1577-1585.