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TITLE

Biosensor-based High Throughput Biopanning and Bioinformatics Analysis Strategy for the Global Validation of Drug-protein Interactions

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KEYWORDS

biosensor, QCM, phage, drug, small molecule, peptide, protein, segment, interaction, docking, dynamics, RELIC

SUMMARY

This study aimed to present a strategy for identifying drug-peptide interactions. The strategy involves the biopanning of drug-recognizing short peptides based on a quartz-crystal microbalance (QCM) biosensor, followed by bioinformatics analysis for quantitatively assessing the information obtained for the drug recognition and annotation of the drug-binding sites on proteins.

ABSTRACT

Receptors and enzyme proteins are important biomolecules that act as binding targets for bioactive small molecules. Thus, the rapid and global validation of the drug-protein interactions is highly desirable for not only understanding the molecular mechanisms underlying therapeutic efficacy but also for assessing drug characteristics, such as adsorption, distribution, metabolism, excretion, and toxicity (ADMET) for clinical use. Here, we present a biosensor-based high throughput strategy for the biopanning of T7 phage-displayed short peptides that can be easily displayed on the phage capsid. Subsequent analysis of the amino acid sequences of peptides containing short segments, as “broken relics”, of the drug-binding sites using bioinformatics programs in receptor ligand contact (RELIC) suite, is also shown. By applying this method to two clinically approved drugs, an anti-tumor irinotecan, and an anti-flu oseltamivir, the detailed process for collecting the drug-recognizing peptide sequences and highlighting the drug-binding sites of the target proteins are explained in this paper. The strategy described herein can be applied for any small molecules of interest.

INTRODUCTION

Identification of drug-binding targets is an essential for the development of drugs as well as for understanding the molecular mechanisms of diseases. In particular, receptor and enzyme proteins are the most important molecular targets of bioactive small molecules[1]. Although affinity capture is a well-established technique for identifying the drug-binding proteins[2],

technical limitations, such as low solubility of proteins, often hamper the validation of drug targets[2]. Most importantly, the immobilized small molecules lose the degree of freedom necessary for docking and may be inaccessible to the internally located binding sites on larger target proteins. Furthermore, protein misfolding, inability to analyze co-crystallization conditions, and limitations due to molecular size often hamper the use of X-ray crystallography, nuclear magnetic resonance (NMR), and other such experimental analyses for studying drug-protein interactions.

The use of the T7 phage display biopanning is an efficient way for determining the binding site on proteins for small molecule baits[3]. In particular, a T7 phage-displayed random peptide library, which can be constructed by inserting synthetic DNA into a multi-cloning site, is effective. Compared to the T7 phage library displaying proteins, the short peptides can be easily engineered to be displayed on the T7 phage capsid without physical restrictions, which can sterically contact with any small molecule drug fixed on a solid support[2]. Furthermore, the introduction of a quartz-crystal microbalance (QCM) biosensor into the T7 phage display biopanning platform allows the identification of such weak but specific interactions of short peptides with drugs by monitoring the reduction in QCM frequency[4,5]. The bound T7 phage is then directly recovered by infecting the host *Escherichia coli* (BLT5615), and the DNA sequence of the region that encodes the affinity-selected peptide harboring drug-recognizing amino acids is determined. Subsequent analysis of the amino acid sequence of the peptide population provides information regarding drug recognition. In silico pairwise alignment of the rescued amino acid sequences can be used to obtain information regarding the biological target of the drug within a selected proteome. This high throughput identification of protein fragments with affinity towards a drug can be used to heuristically reconstruct the drug-binding site in a manner similar to that of reconstructing an ancient artefact from pottery shards[6]. In particular, this unique approach can be useful when conventional proteomics approaches fail.

Here, we present a biosensor-based strategy for the biopanning of T7 phage-displayed peptides and bioinformatics analysis for the target validation of the small molecules. Beyond technical limitations on conventional methods, this strategy enables the identification of drug-binding sites on target proteins for any small molecule of interest under the identical protocol.

PROTOCOL

NOTE: The following are the steps for screening drug-recognizing T7 phages using a QCM biosensor and recovering the screened phages via *E. coli* (BLT5615) infection. The protocols for the synthesis of a derivative of a small molecule that forms a self-assembled monolayer (SAM) and for the construction of the T7 phage-displayed 15-mer random peptide library can be found elsewhere[6,7].

1. Preparation of the QCM sensor chip

1.1. Attach a ceramic sensor chip on the oscillator of a 27-MHz QCM apparatus, and record the intrinsic frequency (Hz) in the air phase before small molecule immobilization.

1.2. Detach the chip and drop 20 μL of a solution (1 mM in 70% ethanol) of a small molecule derivative that forms SAM onto the gold electrode of the sensor chip using a pipette.

CAUTION: The sensor chip crystal where the gold electrode (Au, 0.1 mm thick, 2.5 mm i.d., 4.9 mm^2) is located is extremely thin and may crack easily (SiO_2, 0.06 mm thick, 9 mm i.d.). Hence, pipette carefully.

1.3. Leave for 1 h at room temperature (around 20 °C) in a Petri dish with moistened tissue and shielded from room lights.

1.4. Wash the electrode surface gently with ultrapure water; then, remove the water drops by blowing air with a syringe or air duster.

1.5. Set up the sensor chip for the QCM apparatus and record the reduction in frequency in the air phase to measure the amount of the small molecule that has been immobilized.

NOTE: At least, 100 Hz of intrinsic frequency is necessary for successful small molecule immobilization (1 Hz immobilizes 30 pg).

2. Biopanning of the T7 phage library using a QCM biosensor (Figure 1)

2.1. Set a cuvette with a dedicated magnetic stirrer on the QCM biosensor and pour 8 mL of the reaction buffer (10 mM Tris-HCl, 200 mM NaCl, pH 7-8) into the cuvette.

2.2. Attach the QCM sensor chip to the oscillator and pull down the arm of oscillator to immerse the chip into the buffer being stirred at 1000 rpm.

2.3. Start monitoring the QCM frequency on the personal computer (PC) and wait until the sensorgram equilibrates to around 3 Hz/min of the frequency drift.

2.4. Inject 8 μL of a T7 phage library ($1–2 \times 10^{10}$ pfu/mL) into the cuvette (final concentration: $1–2 \times 10^7$ pfu/mL) and mark the injection point on the sensor.

2.5. Monitor the frequency reduction caused by the binding of T7 phages to the small molecule immobilized on the gold electrode surface for 10 min.

2.6. Stop the QCM frequency monitor, dislodge the sensor chip from the oscillator, and remove the buffer by blowing air and/or wicking away with wipes.

2.7. Put the sensor chip into a humid Petri dish and drop the 20 μL of the suspension of *E. coli* (BLT5615) (OD_{600} = 0.5–1.0 after shaking at 37 °C by adding IPTG to 1 mM) host cells in the log phase onto the gold electrode.

2.8. Close the lid of the dish and cover it with aluminum foil to block out light.

2.9. Incubate the dish at 37 °C for 30 min on a 96-well microplate mixer (~500 rpm) for enhancing the recovery of the bound T7 phages.

2.10. Recover the 20 μL of the solution and suspend it into 200 μL of LB medium.

NOTE: The samples obtained in this step can be preserved at 4 °C by one week.

2.11. Prepare a dilution series of the phage solution for plaque isolation and DNA sequencing according to the general procedure described in the manufacturer's instructions[8,9].

2.12. Wipe the gold electrode surface with a cotton swab soaked with 1% sodium dodecyl sulfate solution.

2.13. Wash the gold surface with ultrapure water from the washing bottle and then remove water drops by blowing air with a syringe or air duster.

2.14. Drop 5 μL of piranha solution (Conc. H_2SO_4: 30% H_2O_2 = 3:1) on the gold surface and leave for 5 min.

2.15. Wash the gold surface again with water and then dry by blowing air and/or wicking away with wipes.

2.16. Repeat steps 2.14 and 2.15.

CAUTION: Prepare the piranha solution immediately prior to use. Use this liquid carefully, as it is a very strong acid. Treatment for longer than 5 min erodes the sensor chip.

3. Bioinformatics analysis using Receptor Ligand Contact (RELIC) program suite (Figure 2)[10,11]

3.1. Unzip the stand-alone RELIC program on a PC with an MS Windows operating system.

3.2. Align the amino acid sequences of the 15-mer peptides affinity-selected using the drug or randomly selected from unscreened parent library in each text format file (name.txt).

3.3. Type the amino acid sequence of single or multiple protein(s) in each text file with FASTA format, or download the database text files in FASTA format from any protein database (e.g. UniProt (http://www.uniprot.org/) or DrugBank (https://www.drugbank.ca/).

3.4. Place the text files (and PDB files for HETEROalign) in the folder necessary for running each RELIC program.

3.5. Click the executable file (program.exe) for AADIV, INFO, MOTIF, MATCH, HETEROalign, FASTAcon, and FASTAskan in the independent folder to open the Personal Version of FTN95.

3.6. Type the appropriate filename, along with the extension (name.txt), in the command message to execute each program and obtain the required text format file.

3.7. Export the resulting text file to a spreadsheet software (e.g., Excel) to generate a plot of information content (INFO) or cumulative similarity scores calculated using a BLOSUM62 (MATCH, HETEROalign).

NOTE: The original RELIC server (http://relic.bio.anl.gov) is no longer available and some stand-alone type RELIC programs that work on PCs with a Windows platform can be obtained from the corresponding author (tkksg@rs.noda.tus.ac.jp).

REPRESENTATIVE RESULTS

The representative results for two clinically approved drugs are shown in **Figure 3**. Irinotecan (**Figure 3A**), a water-soluble prodrug of natural camptothecin used for treating advanced colorectal cancer and non-small cell lung cancer, is converted to SN-38 in the liver, which inhibits topoisomerase I in cancer cells[12]. Furthermore, this compound directly inhibits acetylcholinesterase (AChE)[13,14]. Through the strategy, 29 peptides that recognized Iri immobilized as an SAM was identified by QCM biosensor-based one-cycle biopanning subsets. Subsequent pairwise alignment of the 29 peptides and AChE yielded maximal scores for Y121, Q225, F290, E327, H440, and Y442 and highlighted the portion in the three-dimensional structure. These amino acid residues were consistent with those making up the Iri-binding site of AChE. The same subset of peptides successfully identified E99, L100, L252, L305, I387, and V474 in the vicinity of the catalytic triad (S221, E353, and H467) in carboxylesterase (CE), indicating that these amino acids form a scaffold for Iri recognition during de-esterification of Iri[15]. Such amino acid residues in the catalytic site cannot be identified directly using conventional X-ray crystallography or NMR analysis, as the enzyme reaction proceeds smoothly and does not form the static complex stably under general experimental conditions. Thus, that combinatorial detection of drug-binding sites of multiple proteins, including those in the intermediate complexes possibly formed with enzymes during metabolic reactions for a particular drug, is possible using the affinity-selected peptides determined for one drug.

Figure 3B shows the other results obtained for oseltamivir (Osel), an anti-flu drug that is activated to oseltamivir carboxylate, which in turn inhibits the neuraminidase (NA) of the influenza virus[16]. The 27 of peptides that recognized Osel covering the QCM sensor chip gold electrode surface successfully detected the the Osel-binding site in NA[16]. This binding site consists of unstructured peptide loops that potentially undergo dynamic movement while docking with Osel. The Osel-recognizing peptides on the T7 phage capsid might mimic this dynamic docking when binding to the Osel fixed on the gold electrode surface of the QCM sensor chip. Neuropsychiatric adverse events (NPAEs) have been identified in young patients with influenza, which effect on patients are possibly related to the disease itself rather than the drug. Studies have demonstrated that Osel is actively exported from the central nervous system (CNS) of rodents via multidrug

resistance (MDR) protein in the blood-brain barrier (BBB)[17]. Indeed, one of the proteins of the class of this MDR showed a high score (top 5% out of 4,396 in the DrugBank 1.0 protein database[18]), in addition to other transporters, neurotransmitter-related enzymes, and receptors, in our study. The pharmacological significance of these proteins with regard to the appearance of adverse effects of Osel is being investigated.

So far, single and multiple small molecule-binding sites on the target proteins have been successfully identified for six small molecule drugs been using our strategy (**Figure 4**). For Brz2001 and roxithromycin (RXM), identical drug-binding sites on a target protein were identified using different pools of peptides, the numbers and amino acid sequences for which varied completely[7,19]. Furthermore, single peptide pools obtained for Iri, RXM and Osel led to the identification of multiple binding sites on different proteins for each drug, such as AChE and CE for Iri (Figure 3A)[20], angiomotin and CYP3A4 for RXM[19,21], and NA and MDR-associated protein for Osel. An unknown molecular target was identified for the anti-tumor compound doxorubicin (FANCF)[22], and the anti-angiogenic macrolide antibiotic RXM (angiomotin)[19].

Figure 1: Schematic representation of the QCM biosensor-based biopanning of the T7 phage-displayed peptide library. A T7 phage library that displays random peptides is injected into the cuvette containing the buffer (under stirring) where the QCM biosensor chip is immersed and the frequency is stabilized. After monitoring the frequency reduction due to the binding of T7 phages to small molecules immobilized on the gold electrode surface of the sensor chip, the sensor chip is detached from the oscillator. DNA from the bound T7 phage is then directly recovered after host *E. coli* (BLT5615) infection. The resulting T7 phages are isolated via plaque formation and, finally, the amino acid sequence of the drug affinity-selected peptide displayed on the T7 phage capsid is determined according to the general phage display method.

Figure 2: Schematic representation of the quantitative assessment of the sequence comparison between drug-selected peptides and single or multiple proteins. Drug-selected peptide sequences are respectively aligned with the primary amino acid sequences of single and multiple proteins, and the similarity in each 3–5 amino acid sets is cumulatively scored via pairwise alignment according to a modified BLOSUM62 matrix. The resulting plot or diagram indicates the residues or portions that constitute a potential drug-binding site on the protein. Further analysis using an appropriate RELIC program highlights the binding site on the three-dimensional structure (if PDB file is available) or ranks entire proteins that are possibly the binding target (HETEROalign program is currently unavailable).

Figure 3: Representative results of peptide collection and subsequent bioinformatics analysis. (**A**) Irinotecan (anti-tumor prodrug, topoisomerase I inhibitor). T7 phage interaction was monitored for 10 min as a reduction in QCM frequency. The DNA of the bound T7 phage was recovered and sequenced to determine the corresponding amino acid sequence. The amino acid sequences of 29 15-mer peptides, collected using a subset of one-cycle biopanning, highlighted the amino acids that make up the Iri-binding site of AChE [PDB ID 1U65]. Further assessment using the same 29 peptides highlighted the neighboring amino acid residues (scaffold residues for de-esterification) of the catalytic triad in CE, a liver enzyme that converts Iri to SN-38 (active

form) [PDB ID: 1K4Y]. Similarity scores of 103 randomly selected peptides from the unscreened parent library[7,19] have been subtracted from these scores to remove library bias. These figures are reproduced from Ref. 20, with permission from Elsevier. **(B)** Oseltamivir (anti-flu drug). The 27 peptides containing Osel-recognizing amino acids highlighted disordered portions of the Osel-binding site for neuraminidase (NA) (virus enzyme) [PDB ID: 2HT7]. Global validation of the sequence similarity between 27 peptides and 4,396 proteins in DrugBank 1.0[18] revealed NA to be within the top 5% range, in addition to the host human proteins associated with the functions of the central nervous system.

Figure 4: Summary of the small molecules, the binding targets of which were identified using this strategy.

DISCUSSION

Here, a strategy for the QCM biosensor-based biopanning of drug-recognizing peptides, followed by bioinformatics analysis for validating drug-protein interactions using the identified peptides, has been presented. Designing of the small molecule derivatives for immobilization on the gold electrode of the biosensor is an important step, as the introduced linker may hinder the binding and collection of the peptide that recognizes the drug. To avoid this, derivatives with different positions of the introduced linker are prepared[23]. Alternatively, for immobilizing hydrophobic small molecules, the sensor chip is immersed in bulk water in a 10 cm Petri dish, and a 5 μL solution of the small molecule (10 mM solution in dimethyl sulfoxide) is dropped onto the gold electrode of the biosensor, to cover its surface, and incubated for 5 min. This allows retention of a sub-hundred hertz intrinsic frequency of small molecules, which is held for at least 10 min during the biopanning. Indeed, using such immobilization, the Osel affinity-selected peptides clearly highlighted the Osel-binding site in NA (**Figure 3**).

The T7 phage used for preparing the peptide library here is genetically engineered using the NNK_{15} cassette that encodes 32 codons for all 20 of standard amino acids and represses the emergence of 2 stop codons (UAA, UGA) and emerges only UAG (**Figure 1**)[6,7]. This is important for displaying 15-mer full-length peptides and increasing the diversity of the library. The T7 phage display system has a technical display limit of 10^7–10^9 T7 phages. However, the diversity of the 15-mer peptide library is theoretically 20^{15} (3.27×10^{19}); thus, it cannot be used for complete library construction. Nevertheless, similarity search or mining of conserved motifs allows the detection of the amino acids comprising the drug-binding sites of proteins even with this limited diversity of peptides in the library. In addition, 3–5 amino acid stretches within the library peptide (the appearance rate is between $1/20^3$ and $1/20^5$, which can be realized using the T7 phage display system) are involved in the recognition of small molecule drugs; therefore, a 100% match of the peptide sequences with 15-mer amino acid sequences constituting the drug binding site of the target protein is not required. Indeed, approximately 30 affinity-selected peptides successfully highlighted the binding site of the target protein for each drug tested (**Figure 4**). Thus, the diversity of the parent T7 phage library used (1.7×10^7 pfu/mL) can be used to heuristically reconstruct the drug-binding site.

Typically, 3–5 copies of T7 phages that displayed the same sequences as those of the 15-mer

amino acid sequences harboring drug-recognizing amino acid stretches emerged within the 16 plaques arbitrarily isolated, indicating the success of the affinity selection under our protocol. This indicates that 18–30 different drug-recognizing peptide sequences are collected within the 96 plaques isolated (the number is associated with the microplate format), which are identified subsequently using sequencing of the DNA and obtaining the corresponding amino acid sequence. In the present strategy, injection of 8 μL of the T7 phage library into the cuvette containing 8 mL buffer (1000-fold dilution of the library) is suitable for reducing the non-specific binding of T7 phages. To increase the diversity of the affinity-selected peptides, repeating one-cycle selection several times and using 16 or 32 plaque isolations per screening proved to be more effective than isolation from a single solution at a time. For example, to effectively collect approximately 30 differently sequenced affinity-selected peptides, 3–6 sets of one-cycle selection were conducted, 16 or 32 plaques were isolated in each experiment. Appearance of identical sequences in all 16 or 32 T7 phage plaques are indicated accidental detection of background or might contamination as a carryover. In contrast, the absence of T7 phages with the same sequence or appearance of many T7 phages with shorter peptides than the 15-mer length indicates that T7 phages in the population non-specifically emerged with high probability. As QCM frequency reduction occurs to the same extent even in such cases, the success of the selection should be comprehensively evaluated by sequencing the DNA of the isolated T7 phage, followed by bioinformatics analysis of the amino acid sequences of the peptides. Furthermore, unlike the conventional T7 phage display protocol, repeating rounds of selection is less effective, as the variation and number of the T7 phages are small and are not concentrated even after repeating the amplification and selection steps[23].

Importantly, this method is applicable for the mining of small molecule-binding sites in the proteomes of humans, pathogenic viruses, and even plants. Interestingly, the possibly unstructured short display length of peptides on the T7 phage capsid can mimic the molecular dynamics of peptides of proteins during docking with a small molecule; this can reflect dynamic binding[24]. Beyond the technical limitations of conventional methods, this strategy, applicable to identical protocols for small molecules, may expand the druggable proteome as well as provide more granularity regarding drug-protein interaction analysis.

Certain technical limitations of this approach should be considered. Organic synthesis is necessary for small molecule immobilization on the gold electrode surface of the biosensor chip. For the non-experts in organic chemistry, some immobilization reagents are commercially available to mechanically fix the small molecule by coupling. Furthermore, certain nonsense portions of the peptides might result in the detection of a portion of the protein not relevant for drug docking as false positives. This corresponds empirically to beta-sheet or leucine-rich domains rich in leucine or valine residues, which are encoded by more codons than other standard amino acids, when copies of the T7 phage are produced. Controlling the library peptide length might control the occurrence of false positives. In contrast, there may be cases where amino acid residues in the drug-binding site that are involved in docking, as demonstrated using X-ray crystallography or NMR analysis, are not detected. This may be solved by collecting a larger number of the drug-recognizing peptides or changing the direction of fixing of the small molecules on the gold electrode.

Many drug-protein interactions that are related to the main and secondary effects of drug use may yet be unidentified in the proteome; in addition, enzymes and transporters responsible for drug absorption, distribution, metabolism, excretion, and toxicity, might also be still unidentified. Protein binding is not always responsible for the bioactivity of a drug. Thus, a combination of other information from biological assays will improve the identification of essential drug targets responsible for the main and adverse effects of drugs. Further adaptations of this concise technique will increase the practicality and throughput for mining of the protein-binding sites of a wide range of small molecule drugs. The method presented herein will largely contribute to not only conduct basic researches in the related fields but also clarify the molecular mechanisms underlying therapeutic efficacy or other biological effects of drugs in clinical use.

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DISCLOSURES

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The stand-alone RELIC programs and the sequence data of affinity-selected peptides for drugs as well as the protein sequences from proteome database used in this paper are available from this author upon request (tkksg@rs.noda.tus.ac.jp).

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Figure 1

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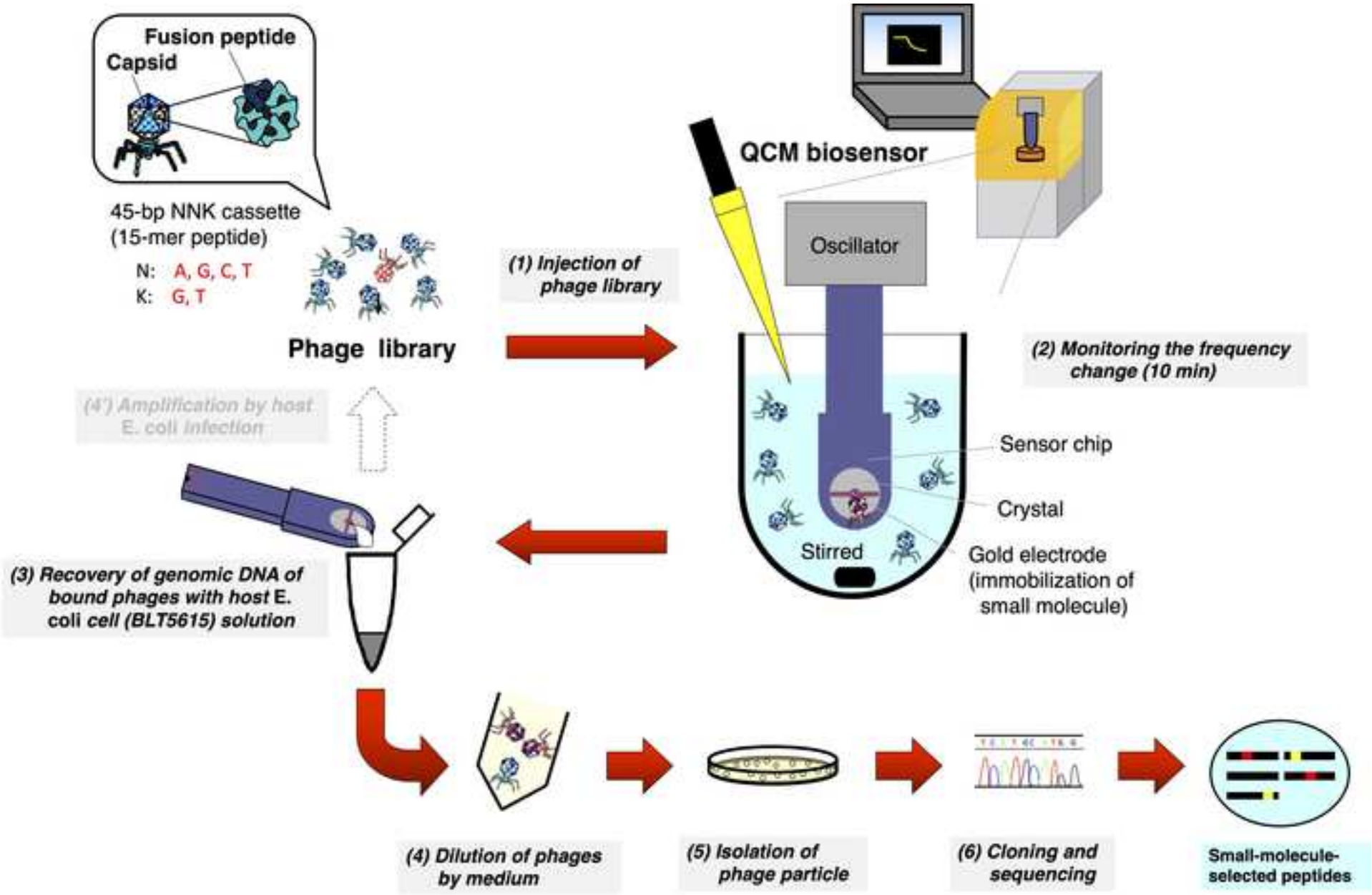

Figure 2

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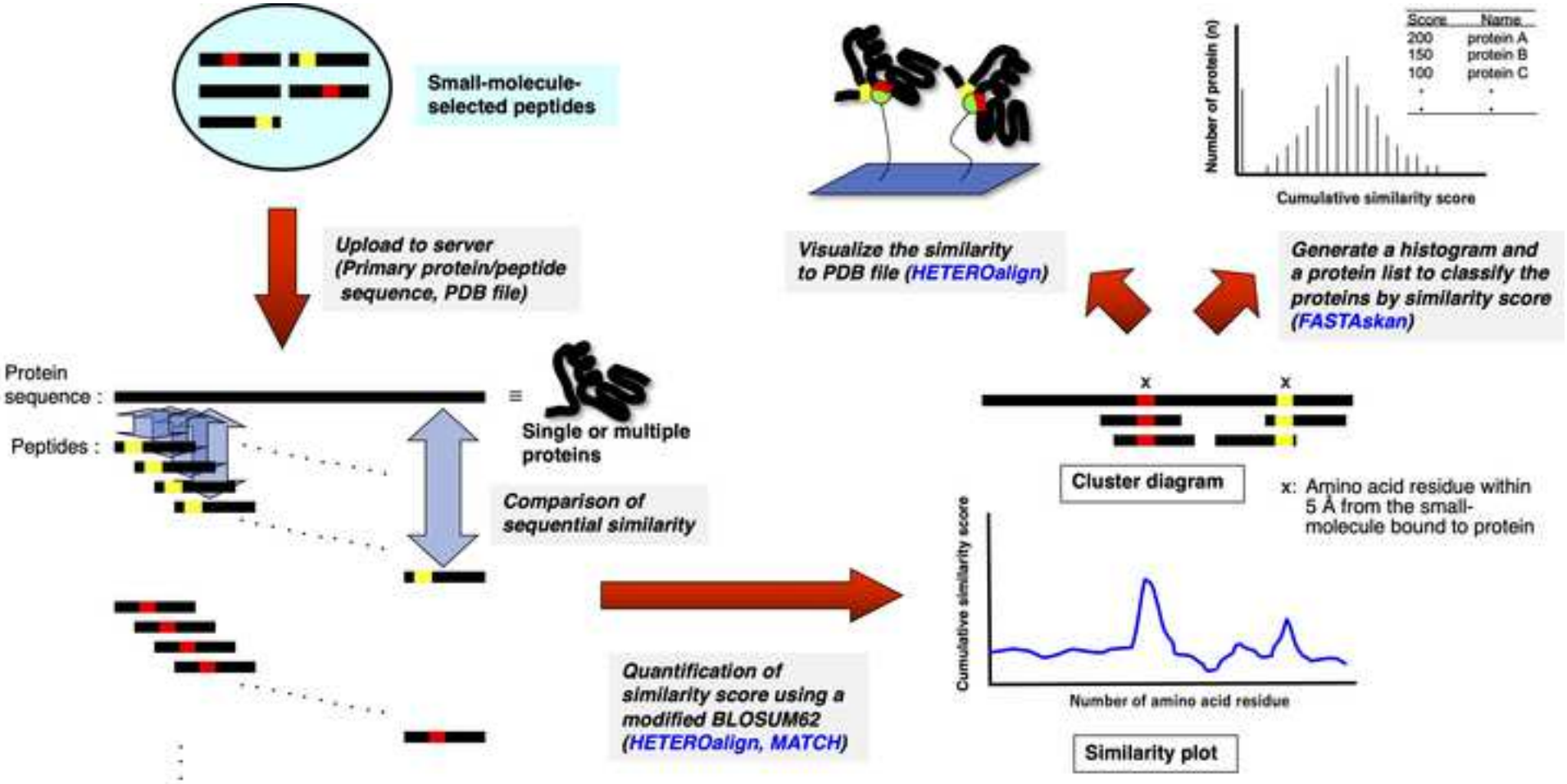


Figure 3

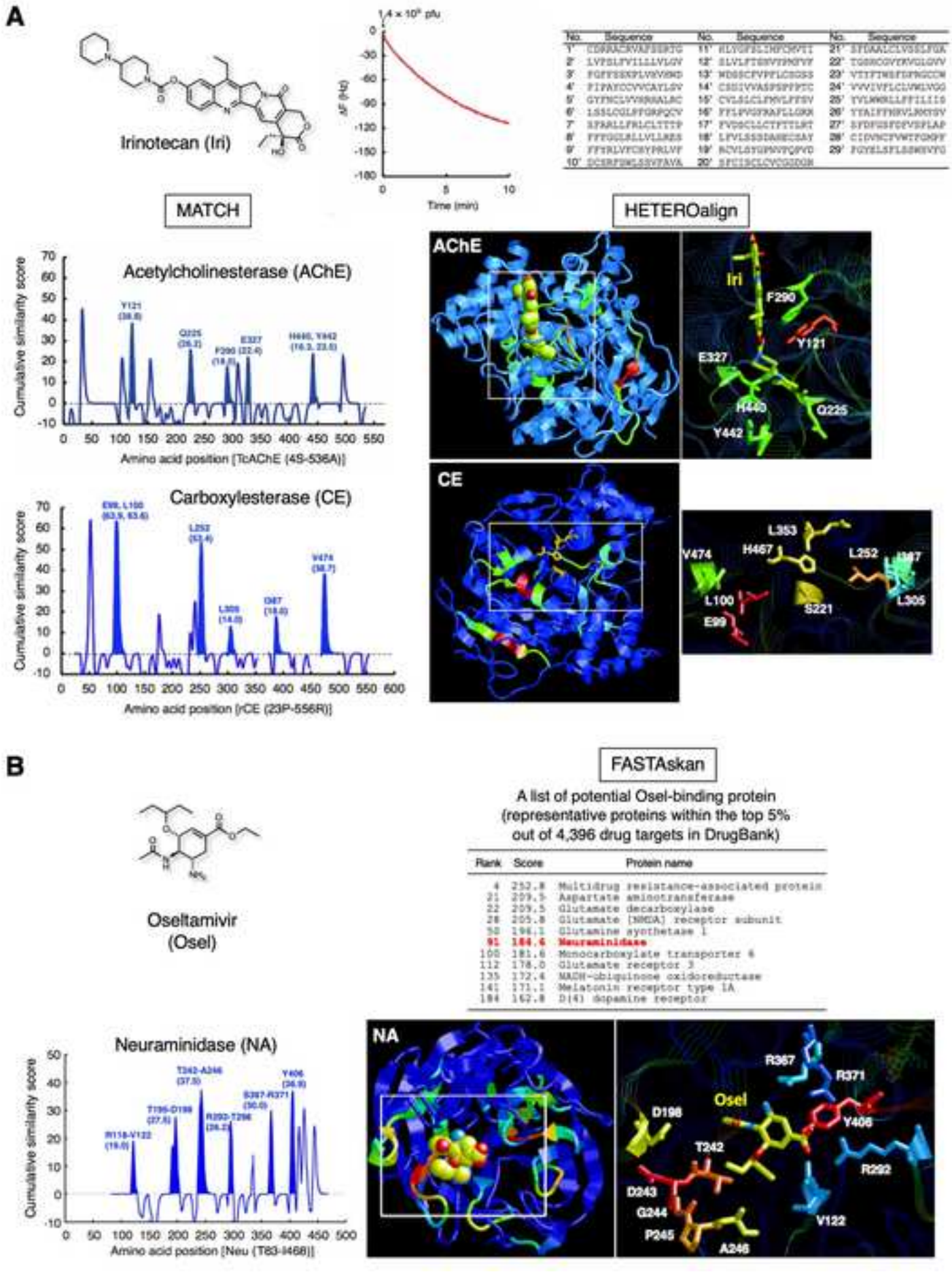
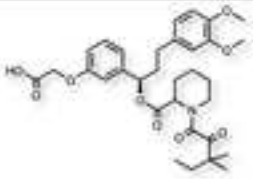
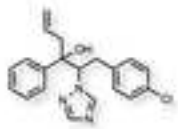
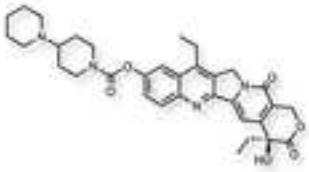
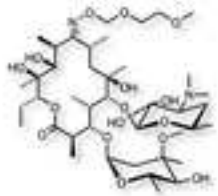
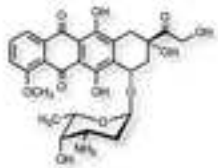
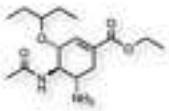


Figure 4

Small molecule	Molecular structure	Activity	Number of 15-mer peptides collected by the QCM-PD	Target	Reference
Synthetic ligand for FKBP (SLF)		· Immunosuppressant	35	FKBP12	20
Brz2001		· Inhibitor of plant hormone biosynthesis	34, 26	DWARF4	6, 7
Irinotecan		· Inhibitor of AChE · Substrate for CE	29	AChE CE	20
Roxithromycin		· Anti-angiogenic · Substrate for CYP3A4	92, 25	Angiomotin CYP3A4	19, 21
Doxorubicin		· Anti-tumor	27	FANCF	22
Oseltamivir		· Anti-influenza virus	27	NA MDR-associated protein	

Name of Material/Equipment	Company	Catalog Number	Comments/Description
AFFINIXQ_N	ULVAC, Inc. (Tokyo, Japan)	QCM2008-STKIT	Contains Glass cuvette, stir magnet, operation and analysis software with a Windows PC
AADIV	Northeastern University (Lee Makowski)	AADIV.exe	Calculates the frequency of occurrence of each of the 20 amino acids at each recombinant insert position, as well as the overall position-independent frequency of each amino acid within that set of peptide sequences. Also roughly estimates the sequence diversity of a display library by statistical sampling method based upon sequences obtained from a limited number of randomly sampled members of the library.
Ceramic Sensor Chip	ULVAC, Inc. (Tokyo, Japan)	QCMST27C	4 sensor chips/package
Dimethyl sulfoxide	Sigma-Aldrich (St. Louis, MO, USA)	D8418	
Ethanol	Merck (Kenilworth, NJ, USA)	09-0850	
FASTAcon	Northeastern University (Lee Makowski)	FASTAcon.exe	Identifies proteins from a population with short consensus sequences.

FASTAskan	Northeastern University (Lee Makowski)	FASTAskan.exe	Lists proteins with high similarity to a peptide population.
Immiblization kit for AFFINIX	ULVAC, Inc. (Tokyo, Japan)	QCMIMKT	SAM reagent and amine coupling reagent
INFO	Northeastern University (Lee Makowski)	INFO.exe	Provides mathematical measure of the probability of observing a particular peptide sequence by random chance (i.e., nonspecific binding) as opposed to by selection for a specific property (affinity to small molecule).
Liguid LB medium	Sigma-Aldrich (St. Louis, MO, USA)	L3522	Autoclave for 20 min
MATCH	Northeastern University (Lee Makowski)	MATCH.exe	Identifies any stretches of amino acid residues within a particular protein that exhibit significant similarity to a group of affinity-selected peptides. Outputs as cluster dia-gram and cumulative similarity plot calculated from a modified BLOSUM62 matrix with a short window (5–6 amino acids in length).

MOTIF1	Northeastern University (Lee Makowski)	MOTIF1.exe	Searches for three continuous amino acid sequence motifs within a peptide population.
MOTIF2	Northeastern University (Lee Makowski)	MOTIF2.exe	Searches for patterns of three amino acids and does not allow conservative amino acid substitutions, but does allow identical gap lengths.
NaCl	Merck (Kenilworth, NJ, USA)	S3014	
Recptor ligand contacts (RELIC)	Argonne National Laboratory (Lemont, IL, USA)	https://www.relic.anl.gov	Currently unavailable (Stand-alone program can be used from correspondence author upon request)
Tris	Merck (Kenilworth, NJ, USA)	252859	

Institute of Quantum Life Science,
National Institutes for Quantum and
Radiological Science and Technology (QST)
4-9-1 Anagawa, Inage, Chiba 263–8555, Japan

[October 21st]

[Vineeta Bajaj]
[Review Editor]
Journal of Visualized Experiments

Dear Editor:

We wish to re-submit the attached manuscript title "**Biosensor-based High Throughput Biopanning and Bioinformatics Analysis Strategy for the Global Validation of Drug-protein Interactions"** as an Original Article. The manuscript ID is JoVE61873.

The manuscript has been rechecked and appropriate changes have been made in accordance with the reviewers' and editors' suggestions. The responses to their comments have been prepared and attached herewith/given below.

We thank you and the reviewers for your thoughtful suggestions and insights, which have enriched the manuscript and produced a better and more balanced account of the research. We hope that the revised manuscript is now suitable for publication in your journal.

Please note that we have added a version of the revised manuscript, showing changes made by us as blue.

Thank you in advance for your cooperation in this severe COVID-19 situation.
Any delay is no problem for us.

Again, thank you for your consideration, and I look forward to hearing from you.

Sincerely,
Yoichi Takakusagi

Institute of Quantum Life Science,
National Institutes for Quantum and Radiological Science and Technology
[81-43-206-4067]
[81-43-206-3276]
Email address: takakusagi.yoichi@qst.go.jp, tkksg@rs.noda.tus.ac.jp

Editorial Office Comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
3. Please ensure that the Summary is between 10-50 word limit.
4. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.
5. Please define all abbreviations during the first-time use. .e.g., ADMET?
6. Please revise the Introduction to include all of the following:

a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.
8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
9. Please ensure you answer the “how” question, i.e., how is the step performed?
10. The Protocol should contain only action items that direct the reader to do something.

11. 1.2: Which small molecule is used in your case? When and how do you deposit the gold electrode?
12. 2.4: What kind of Phage library is used? How do you decide on the injector point?
13. 2.9: please include citations for plaque isolation and sequencing?
14. 3: Please expand on how the bioinformatic analysis is performed. Please include button clicks in the software, knob turns, command lines etc. If using large scripts please include as a supplementary file.
15. Is RELIC hosted by any server somewhere or not available at all?
16. Is RELIC Commercial? If yes, please remove and use generic term.
17. Only one note can follow one step. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that providesdetails about how to perform a particular step should either be included in the step itself or added as a sub-step. Other details should be moved to the discussion.
18. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.
19. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.
20. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
21. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as

a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

22. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

>Response to the comment

Thank you for the advice. The revised manuscript has been proofread by a professional editor of English language.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

>Response to the comment

The manuscript has been formatted in the appropriate style.

3. Please ensure that the Summary is between 10-50 word limit.

>Response to the comment

The Summary is now within the 10–50-word limit.

4. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

>Response to the comment

The Abstract is now within the 150–300-word limit.

5. Please define all abbreviations during the first-time use. .e.g., ADMET?

>Response to the comment

ADMET indicates adsorption, distribution, metabolism, excretion and toxicity. All abbreviations have been defined where they appear for the first time in the text and have been highlighted in blue.

6. Please revise the Introduction to include all of the following:
    a) A clear statement of the overall goal of this method
    b) The rationale behind the development and/or use of this technique
    c) The advantages over alternative techniques with applicable references to previous studies
    d) A description of the context of the technique in the wider body of literature
    e) Information to help readers to determine whether the method is appropriate for their application

>Response to the comment

The Abstract and Introduction have been structured considering the requirements mentioned above (from a) to e)). Additional references have been cited in the Introduction.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

>Response to the comment

Not applicable

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

>Response to the comment

The text in the protocol section has been written in the imperative tense and phrases have been avoided.

9. Please ensure you answer the "how" question, i.e., how is the step performed?

>Response to the comment

The sentences in the protocol section have been written to indicate how each step was performed.

10. The Protocol should contain only action items that direct the reader to do something.

>Response to the comment

This requirement has been met.

11. 1.2: Which small molecule is used in your case? When and how do you deposit the gold electrode?

>Response to the comment

Irinotecan (Iri), an anti-tumor compound, was used. In this case, an Iri derivative that forms a self-assembled monolayer on the gold electrode of the sensor chip was used (Takakusagi Y. *et al. Bioorg Med Chem* 16(22), 9837-9846, 2008).

12. 2.4: What kind of Phage library is used? How do you decide on the injector point?

>Response to the comment

The 15-mer of T7 phage-displayed library, which was originally engineered by inserting the synthetic NNK_{15} cassette into the cloning site of the T7 phage DNA and packaged using the T7 phage packaging kit (Novagen), was used. After immersing the oscillator-attached sensor chip into the buffer-filled cuvette and adjusting the QCM frequency until the frequency drift

of the sensor was within 3 Hz/min, 8 μL of the phage library was injected into the cuvette and marked as injector pointer. The drift, which is an index of the injector point, is now described in section 2 of Protocol.

13. 2.9: please include citations for plaque isolation and sequencing?

>Response to the comment

The original protocol from Millipore (Novagen) is now cited as references 8 and 9.

14. 3: Please expand on how the bioinformatic analysis is performed. Please include button clicks in the software, knob turns, command lines etc. If using large scripts please include as a supplementary file.

>Response to the comment

The Protocol section regarding the bioinformatics analysis has been written in detail in section 3 of the revised manuscript.

15. Is RELIC hosted by any server somewhere or not available at all?

>Response to the comment

The RELIC server originally functioned on Linux in the Argonne National Institute (Ref. 10). However, since 2009, this server is no longer available. Instead, the developers generated a stand-alone type RELIC program that works on PCs with a Windows OS. These executable programs are available upon request.

16. Is RELIC Commercial? If yes, please remove and use generic term.

>Response to the comment

No, RELIC is not a commercial item.

17. Only one note can follow one step. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Other details should be moved to the discussion.

>Response to the comment

In light of the above comments, the Protocol section has been largely amended in the revised manuscript.

18. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

>Response to the comment

The embedded Figures have been removed from the revised manuscript and have been uploaded separately.

19. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

>Response to the comment

The embedded Table has been removed from the revised manuscript and has been uploaded separately.

20. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that

identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

>Response to the comment

The revised manuscript has been arranged appropriately.

21. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

>Response to the comment

The copyright permission has been already obtained from Elsevier (Ref. 20). The PDF has been uploaded during resubmission.

22. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

>Response to the comment

The Discussion has been largely rewritten and contains six paragraphs with citations, per the criteria described above. Critical steps as well as tips for modifications and troubleshooting have also been mentioned. Technical limitations regarding library variation have been mentioned.

Response to the Reviewers' comments and revisions that have been made

I thank all the Reviewers for the constructive comments on the manuscript. I have revised the manuscript per the Reviewers' comments. Please check the following point-by-point responses to the Reviewers' comments as well as the attached the revised file ("Instructions_for_Authors_(Revised_MS).docx"). I have also attached a copy of the revised file showing changes in colored text ("Instructions_for_Authors_correction_highlighted). docx") for the benefit of the Reviewers. I hope that my responses will resolve all the Reviewers' concerns.

Original comments from Reviewer #1

Reviewer #1:

Manuscript Summary:

The manuscript presents an interesting technique to map small molecule binding sites onto proteins, which has possible ADMET applications. It provides procedural details for forming SAM layers on silicon biosensors, and using these sensor chips to inform on real-time binding events to perform successful T7 bio-panning experiments. It then describes how to perform an analysis to map these peptides to proteins of interest. The manuscript provides a good level of detail that should allow the practical aspects of this methodology to be reproduced.

Major Concerns:

1) The written manuscript contains numerous typos and grammatical errors.
2) It is not readily apparent how you would use the phage display results to interrogate a database of proteins to identify unexpected binding sites. I think the bioinformatic analysis needs to be explained in more detail how it is performed. At the moment all the details seem to be in only one table and figure 3.
3) There seems to be no statement on how many peptides you need to sequence to perform a successful experiment from a phage selection. Do these experiment always return 30-50 peptides. Or do they return just a few .... Also how does this influence a successful experiment.
4) With the biosensor realtime detection system - does it still show phage binding in unsuccessful experiments. Does it inform on poorly immobilized ligand? They only show the result for a successful experiment.
5) The authors show that they identify high similarity hit proteins to predict possible unknown interaction sites - are there any possible methodology for inferring the chance that these hits may be false positives?

Point-by-point response to the comments from Reviewer #1

I thank the Reviewer for the constructive suggestions. I have provided answers to all the comments below.

1) The written manuscript contains numerous typos and grammatical errors.

>Response to the comment

Thank you for the advice. The revised manuscript has been proofread by a professional editor of English language.

2) It is not readily apparent how you would use the phage display results to interrogate a database of proteins to identify unexpected binding sites. I think the bioinformatic analysis needs to be explained in more detail how it is performed. At the moment all the details seem to be in only one table and figure 3.

>Response to the comment

Thank you for the comments. The section on bioinformatics analysis (section 3 of Protocol) has been expanded in the revised manuscript. The details regarding each program have been substantiated with citations.

3) There seems to be no statement on how many peptides you need to sequence to perform a successful experiment from a phage selection. Do these experiment always return 30-50 peptides. Or do they return just a few .... Also how does this influence a successful experiment.

>Response to the comment

Approximately 30 affinity-selected peptides are sufficient for highlighting the drug-binding sites on target proteins, as shown in our previous study. Examples of successful experiments have been summarized in Figure 4. In general, drug-recognizing amino acid stretches are considered to consist of 3–5 residues. Although a 15-mer peptide is technically sufficient to

reveal the drug-recognizing motif within a peptide (e.g., a five-amino acid motif appears more than thrice as frequently in a 15-mer peptide library as in a 5-mer peptide library), increasing the number of selections concurrently increases the number of false positives, owing to matching of the non-sense intervening amino acid sequence with the database query sequence by chance. Thus, collection of approximately 30 peptides is suitable for a subsequent bioinformatics analysis, as shown for the six compounds in Figure 4. Details regarding the number of selections required for collecting 30 peptides and other technical details have been provided in the Discussion of the revised manuscript.

4) With the biosensor realtime detection system - does it still show phage binding in unsuccessful experiments. Does it inform on poorly immobilized ligand? They only show the result for a successful experiment.

>Response to the comment

As pointed out by the Reviewer, the QCM frequency decreases to the same extent in cases where selection is unsuccessful and those that are successful. The success of the selection should be comprehensively evaluated after DNA sequencing of the isolated T7 phage and subsequent bioinformatics analysis of the amino acid sequences of the displayed peptides. This has been discussed at the end of the third paragraph of the Discussion in the revised manuscript.

5) The authors show that they identify high similarity hit proteins to predict possible unknown interaction sites - are there any possible methodology for inferring the chance that these hits may be false positives?

>Response to the comment

Following binding assays, such as bead pull down or Biacore assay, assays are required to identify the putative binding site of a protein. Even if the binding is confirmed in vitro, the chemically-defined in vitro binding strength does not always result in identification of the proteins responsible for the bioactivity of the drug in vivo. Thus, versatile validation

involving other biological assays is required to demonstrate whether the binding proteins or sites identified in this method are also the “biological targets” responsible for the bioactivity of the drug.

Original comments from Reviewer #2

Reviewer #2:

Manuscript Summary:

Let me state up front that I don't believe this method has any value is really finding the unknown protein targets of drugs. If the target is known, I am sure it is possible to stitch together a story that some random collection of peptides "looks" like a part of the known target. However, I would be happy to be proved wrong.

One deal breaker for this paper is that a required software component (RELIC) is no longer available. Unless the authors can state exactly where the readers can obtain this software (and stating "obtainable from the developer" is not good enough), the methodology describes cannot be done so is not suitable for Jove.

If this can be fixed then other items that need to be addressed are:

Major and Minor Concerns:

Line 18

"drug-peptide", not "drug-protein". The phage display only a 15-mer

Line 33-34

This statement, while true is not practical. There are 20^15 possible 15-mer peptides. That is 3.27 x 10^19. So 1 mL of phage lysate can maximally contain 10^12 phage, that is just 0.00001% of the compete library. The amounts used in this Jove report is 8 uL of 10^10 solution (line 118-119) so this is less than 0.000000001% of all possible 15-mers. Another way of looking at this is you wanted just one copy of every possible 15-mer you would have to make the library in 100,000,000 L of 0.6 OD log phase E. coli.

Even then, many would be missing due to sequence bias. So, clearly impossible. The best that can be hopped for is a very sparse matrix with no guarantee that anything like the binding site of the drug is in there. This point needs to be made near lines 72-73.

There is a general lack of references in the introduction and gratuitous self-citations. References are required after the comma on line 51, after the period on line 52, after the period on line 64.

Self citations 2,3 (line 60 citations should be replaced by references to other labs that use phage display for target ID. References 2,3 can be moved to after the period on line 66.

The sentence that starts on line 52 and ends on line 55 makes no sense and could be deleted or rephrased if it is important.

Line 57

Analyses

lines 68-74 is very difficult to understand and too long:

"The subsequent algorithmic analysis of the amino acid sequence of the population of the peptide reveals information content for drug recognition, which allows mining of all binding sites for the small molecule drug by the pairwise alignment of the amino acid sequence of the drug-recognizing peptides and proteins within proteome in theory. Thus, a concise, high precision and throughput collection of the "broken relics" and subsequent archaeology-like heuristic restore of the drug-binding site is realized for any small molecule of interest under the identical protocol."

Suggested:

The computational analysis, through pairwise alignment of the rescued amino acid sequences, can be used to uncover information about the drugs biological target within a selected proteome. This high throughput collection of protein shards can be used to

heuristically restore the drug-binding site in a similar manner to reconstructing an ancient artifact from pottery shards.

Note: there is no evidence that this is "high precision" as the method failed to find many known targets (Figure 4)

Line 98. Please explain how water drops are "removed"

Line 135. Provide the "general procedure" or a reference to the literature. There is also no details on the sequencing, PCR or electrophoresis(?) methods used.

Line 137.

"after recovering the DNA.." - how?

Figure 1

This figure is chaotic and needs to be improved before publication. I suggest turning it into a cycle with the library going into the cycle and the sublibrary going out and leading to sequencing.

Lines 162.

If RELIC is no longer available, where is the reader supposed to get this? If it is no longer available then this whole paper should be rejected as nonviable.

Line 203

Change "are" to "is"

Line 218.

Delete "was"

Line 230

Makes no sense.

Lines 263-265

As the power of phage display is the iterative nature of selections, I fail to see the advantage of using a QCM for just one round. Would not any surface that the drug could stick to be just as good? Please explain. I get that the authors want to avoid amplification bias but they are doing one round of amplification anyway.

Lines 265-268

Rewrite - see comments on lines 68-74.

Line 281, "…domain, which is rich in.."?

Line 281

Replace "many" with "more"

Point-by-point response to the comments from Reviewer #2

I thank the Reviewer for the constructive suggestions. I have provided answers to all the comments below.

Let me state up front that I don't believe this method has any value is really finding the unknown protein targets of drugs. If the target is known, I am sure it is possible to stitch together a story that some random collection of peptides "looks" like a part of the known target. However, I would be happy to be proved wrong.

>Response to the comment

Thank you for the comments. Even using the conventional affinity-guided techniques, identification of unknown protein targets is still a time-consuming and expensive process requiring extensive evaluation. Information from other biological assays is also indispensable for determining unknown protein targets, as affinity-guided methods rely on the chemically-defined binding strength in vitro, which does not always result in identification of the proteins responsible for bioactivity of the drug in vivo. Furthermore, the drug-binding to proteins is not always directly involved in the bioactivity of a compound (e.g., serum albumin).

Hence, I do not believe that researchers can comprehensively understand the ADMET of drugs using only conventional methods. The affinity-based methods chemically evaluate the interaction between a drug and potential binding proteins but do not necessarily reflect the bioactivity or bioavailability of the drug. Thus, I strongly believe that, owing to the technical limitations of conventional methods, the unique approach described in the present manuscript is worthy of publication as a technical paper. In particular, rapid and comprehensive validation based on heuristic analysis will widely target the proteome and annotate the potential drug-binding sites. Combined with the biological information obtained from biological assays, the data obtained using this method will validate the drug-protein interactome (possibly, including unknown interactions) and accelerate research and development in the field of drug discovery, which cannot be achieved using conventional proteomics-based approaches alone.

One deal breaker for this paper is that a required software component (RELIC) is no longer available. Unless the authors can state exactly where the readers can obtain this software (and stating "obtainable from the developer" is not good enough), the methodology describes cannot be done so is not suitable for Jove.

>Response to the comment

Thank you for the important comments. The RELIC program was obtained from its developers and can be obtained from me upon request. This has been described in NOTE 2 and DATA AVAILABILITY STATEMENT of the revised manuscript.

Major and Minor Concerns:

Line 18

"drug-peptide", not "drug-protein". The phage display only a 15-mer

>Response to the comment

This has been corrected in the revised manuscript.

Line 33-34

This statement, while true is not practical. There are 20^15 possible 15-mer peptides. That is 3.27 x 10^19. So 1 mL of phage lysate can maximally contain 10^12 phage, that is just 0.00001% of the compete library. The amounts used in this Jove report is 8 uL of 10^10 solution (line 118-119) so this is less than 0.000000001% of all possible 15-mers. Another way of looking at this is you wanted just one copy of every possible 15-mer you would have to make the library in 100,000,000 L of 0.6 OD log phase E. coli.

Even then, many would be missing due to sequence bias. So, clearly impossible. The best that can be hopped for is a very sparse matrix with no guarantee that anything like the binding site of the drug is in there. This point needs to be made near lines 72-73.

>Response to the comment

Thank you for the important comments. I agree with the calculation regarding the complete size of the 15-mer peptide library. However, because of technical limitations of the T7 phage display system, the diversity of T7 phages range from 10^7 to 10^9. Nevertheless, this is sufficient to cover the drug-recognizing amino acid stretches (consisting of 3–5 residues; between $1/20^3$ and $1/20^5$). In addition, a similarity search or mining of conserved motifs allows the detection of amino acids making up the drug-binding site on proteins despite the limited diversity of the peptide. In such cases, collection of 15-mer peptides corresponding to these amino acids (100% match) are not necessarily required.

These details have been mentioned in the third paragraph of the revised manuscript.

There is a general lack of references in the introduction and gratuitous self-citations. References are required after the comma on line 51, after the period on line 52, after the period on line 64.

>Response to the comment

A reference [Ziegler S. *et al. Angewandte Chemie.* 52 (10), 2744-2792, (2020)] has been cited for the above-mentioned points.

Self citations 2,3 (line 60 citations should be replaced by references to other labs that use phage display for target ID. References 2,3 can be moved to after the period on line 66.

The sentence that starts on line 52 and ends on line 55 makes no sense and could be deleted or rephrased if it is important.

>Response to the comment

Thank you for the comments. Our reviews (Ref. 2 and 3 in the original manuscript) have been cited in line 66. The sentence from line 52 to 55 has been rephrased in the revised manuscript.

Line 57

Analyses

>Response to the comment

This has been corrected in the revised manuscript.

lines 68-74 is very difficult to understand and too long:

"The subsequent algorithmic analysis of the amino acid sequence of the population of the peptide reveals information content for drug recognition, which allows mining of all binding sites for the small molecule drug by the pairwise alignment of the amino acid sequence of the drug-recognizing peptides and proteins within proteome in theory. Thus, a concise, high precision and throughput collection of the "broken relics" and subsequent archaeology-like heuristic restore of the drug-binding site is realized for any small molecule of interest under the identical protocol."

Suggested:

The computational analysis, through pairwise alignment of the rescued amino acid sequences, can be used to uncover information about the drugs biological target within a selected proteome. This high throughput collection of protein shards can be used to

heuristically restore the drug-binding site in a similar manner to reconstructing an ancient artifact from pottery shards.

>Response to the comment

Thank you for your comment. As suggested, the corresponding texts have been amended in the revised manuscript.

Note: there is no evidence that this is "high precision" as the method failed to find many known targets (Figure 4)

>Response to the comment

This has been deleted in the revised manuscript.

Line 98. Please explain how water drops are "removed"

>Response to the comment

The following sentence has been added in the revised manuscript:

4. Wash the electrode surface gently with ultrapure water; and then, remove the water drops by blowing air with a syringe or air duster.

Line 135. Provide the "general procedure" or a reference to the literature. There is also no details on the sequencing, PCR or electrophoresis(?) methods used.

>Response to the comment

The original protocols from Novagen have been cited as Ref. 8 and 9 in the revised manuscript.

Line 137.

"after recovering the DNA.." - how?

>Response to the comment

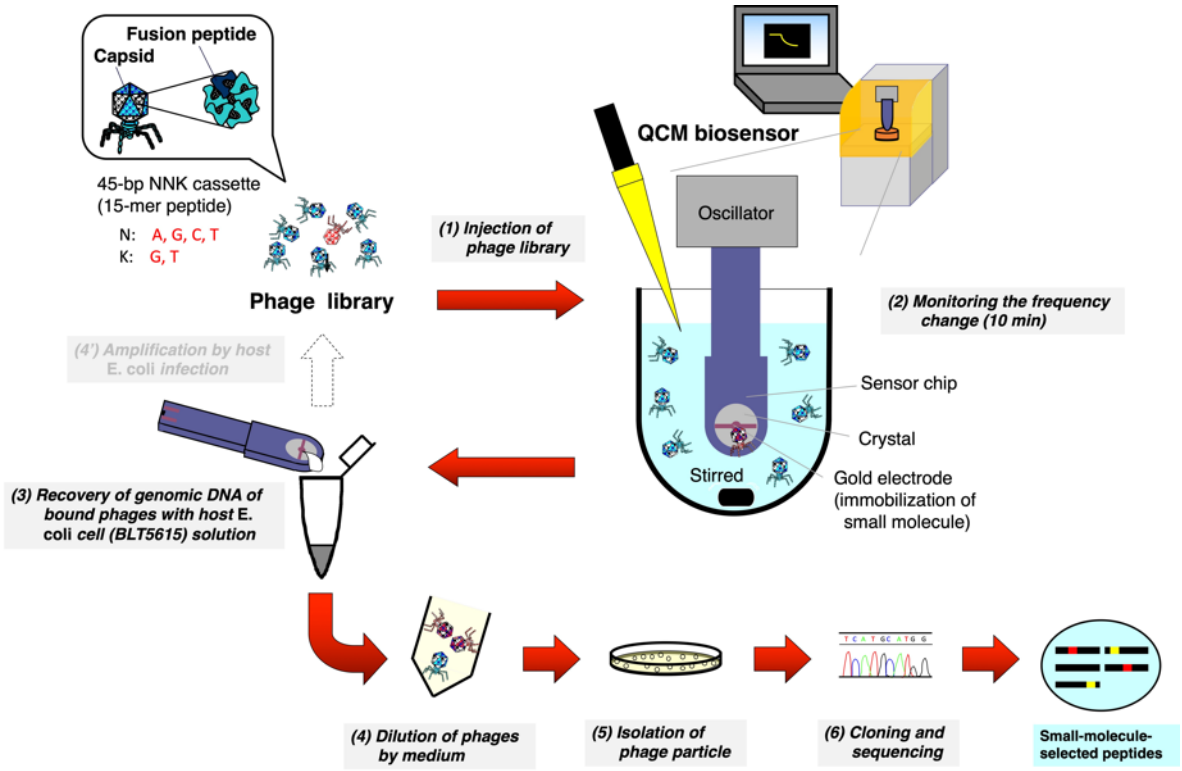
The T7 phage DNA was recovered by using host *E. coli* (BLT5615) culture. This text has been deleted in the revised manuscript. Instead, the manufacturer's instructions describing the detail have been cited as mentioned above.

Figure 1

This figure is chaotic and needs to be improved before publication. I suggest turning it into a cycle with the library going into the cycle and the sublibrary going out and leading to sequencing.

>Response to the comment

Figure 1 has been improved, as suggested by the Reviewer.



Lines 162.

If RELIC is no longer available, where is the reader supposed to get this? If it is no longer available then this whole paper should be rejected as nonviable.

>Response to the comment

Thank you for the important comments. The RELIC program was supplied by its developers and can be obtained from me upon request. This has been described in NOTE 2 and DATA AVAILABILITY STATEMENT in the revised manuscript.

Line 203

Change "are" to "is"

>Response to the comment

This has been corrected.

Line 218.

Delete "was"

>Response to the comment

This has been deleted.

Line 230

Makes no sense.

>Response to the comment

This sentence has been amended in the revised manuscript.

Lines 263-265

As the power of phage display is the iterative nature of selections, I fail to see the advantage of using a QCM for just one round. Would not any surface that the drug could stick to be just as good? Please explain. I get that the authors want to avoid amplification bias but they are doing one round of amplification anyway.

>Response to the comment

Thank you for your interesting comment. As reported previously [Takakusagi Y *et al. Bioorg Med Chem*. 15, 7590-7598, (2007)], repeated rounds of selection using this QCM platform makes little sense. This is because a few, but specific, T7 phages are collected under the selection protocol, and the contents do not change even after repeating the amplification and selection steps. Multiple sets of one-cycle selections can effectively increase the number of affinity-selected peptides using this method. These have been mentioned at the end of the third paragraph in the Discussion with appropriate citation in the revised manuscript.

Lines 265-268

Rewrite - see comments on lines 68-74.

>Response to the comment

This sentence has been deleted in the revised manuscript.

Line 281, "…domain, which is rich in.."?

>Response to the comment

This has been corrected in the revised manuscript.

Line 281

Replace "many" with "more"

>Response to the comment

Per the suggestion, “many” has been replaced with “more”.

∗ All other collections have been conducted and highlighted in blue.

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