HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2016 January 01.

Published in final edited form as:

Methods Mol Biol. 2015 ; 1248: 189–199. doi:10.1007/978-1-4939-2020-4_13.

Identifying reactive peptides from phage-displayed libraries

Glenn M. Eldridge and **Gregory A. Weiss**

Department of Chemistry, University of California, Irvine, Irvine, CA 92697-2025 USA

Summary

Phage display enables the synthesis, selection and screening of large, polypeptide libraries (>1 × 10^{10}). Selections from such libraries can identify binding partners to essentially any desired target (1, 2). Peptide with affinity or reactivity to small molecule probes are attractive for numerous uses including the targeted, site-specific labeling of proteins. Here, we describe selection and screening protocols for the identification of short peptides that can selectively bind to and/or react with small molecules.

Keywords

phage display; molecular evolution; site-specific protein labeling; bioorthogonal chemistry

1. Introduction

Site-specific protein labeling (SSPL) has emerged as a very effective method to study individual proteins within cells, tissues, and living organisms (3). Recent advances in microscopy have driven the visualization of the spatial and temporal properties of proteins within living cells (4). Such studies can illuminate both robust cellular processes and temporary functions of cellular proteins (5). However, all proteins are composed of the same 20 amino acids, and only a few distinctive proteins (e.g., hemoglobin or green fluorescent protein) possess the unique spectral qualities necessary for visualization within the cellular milieu. In the last twenty years, multiple generic site-specific protein labeling techniques have been developed to visualize targeted proteins (6). However, each of these techniques has inherent limitations, such as the large size of the conjugate protein or the toxicity of the probe. Such limitations must be considered before the labeling technique is implemented, and the consequent compromises inspire improvements to SSPL.

A large toolbox of bioorthogonal labels could allow tailoring of the labeling technique to address the required experimental conditions. This approach contrasts with the current paradigm in which experiments and their interpretation are necessarily filtered through the limitations of the available tools. Additionally, collections of bioorthogonal labels could enable the simultaneous visualization of multiple proteins present within a living cell. For example, multiple proteins could be visualized during either normal or diseased states to provide spatiotemporal insights into protein function and dysfunction. Thus, the discovery of additional bioorthogonal labels is a high priority challenge in chemical biology.

Reactive peptides present an attractive potential source of bioorthogonal labels, provided several conditions can be met. First, such peptides must be not present in the proteome of the experimental system (e.g., human or *Drosophila*) to prevent background labeling. Second, short peptide lengths are preferred to minimize disruption of the fusion protein. Ideally, such peptide sequences can strongly bind to or react with specific bioorthogonal functional groups to allow the visualization of many different proteins simultaneously.

Phage display has been used to identify short peptides that react with functional groups (7). For example, we have recently selected from a phage-displayed peptide library a set of peptides that react with the hydrazide functional group under biological conditions (8). These selection methods could uncover other short peptide-bioorthogonal reagent pairs to diversify the SSPL toolbox.

In our initial experiments, the hydrazide-reactive peptides reacted covalently, yet inefficiently, with two different hydrazide-based probes. The library design and selection parameters, while successful in selecting hydrazide reactive peptides, likely contributed to the selection of inefficiently reactive peptides. For example, displaying short peptides on the phage major coat protein, P8, can result in many copies of short peptides attached to each phage, as thousands of copies of this coat protein compose the outer surface of the phage. Furthermore, each copy of the P8-displayed peptide can access a large number of low energy conformations. A phage can be selected and amplified on the basis of a relatively minor contribution to the peptide's distribution of conformational states.

Furthermore, peptides with weak binding and/or reactivity can populate the selected sequences unless the selection conditions correct for the multivalency of P8-displayed peptides. We have designed additional selection criteria that address the multivalency of peptides fused to the P8 coat protein of M13 phage to select for efficiently reactive peptides. This selection is termed a negative selection to distinguish it from the more conventional anti-selections, which are described below.

Negative selections remove selectants with fast off-rates, through exchange between ligands in solution and ligands attached to a solid support. In the first step, the phage are incubated with the target in solution. After sufficient time for the reaction to take place, the phage are next transferred to a vessel with the target attached to a solid support. The phage binding strongly to the target will remain in solution, and ignore the solid support-bound target. In this negative selection, only phage that display peptides capable of reacting quickly and irreversibly with the target in solution are propagated for the next round of selection. Phage-displayed peptides capable of reversible binding, which results in the exchange the initial target for the solid support-bound target, are removed. As an added bonus, the selection condition also removes phage-displayed peptides binding non-specifically to the solid support (e.g., to the polystyrene).

In positive selections, the phage encounter the target functionality attached to a solid support miscible in water (e.g., Tentagel). In addition, we apply extremely stringent washing conditions, such as large volumes of wash solution supplemented with the target, again to remove readily reversible peptide sequences. Such conditions can select for the sequences

with the lowest off-rates. As in the negative selection, weakly or reversibly binding peptide sequences are removed from the population of selectants during the positive selection.

Anti-selections remove peptides binding non-specifically to the solid support. The solid support used for the positive selections can be used, if the target functionality is removed or hidden by a protecting group. In anti-selections, the phage remaining in solution or unbound to the solid support are used immediately for a positive selection. In general, a positive selection must follow an anti-selection before propagation of the phage to avoid amplification of phage lacking a displayed peptide on their surface.

To be useful, selectants must retain activity when removed from the surface of the phage, a caveat for all molecular display formats. The small sizes of both reactive peptides and their small molecule ligands adds to this consideration; the composite surface of phage and peptide could provide an artificial binding pocket, which fails to materialize upon removal of the peptide from the phage surface. Dimerization or higher order oligomerization on the phage surface can further complicate this crucial step of recapitulating selectant activity in a non-phage format. An avidity effect could increase the apparent off-rate for the phage-displayed selectant, but not the resynthesized peptide (9). Thus, for multiple reasons, peptides displayed with multiple copies per phage could appear to bind the target strongly during phage-based screening, but then bind the target only weakly when removed from the phage surface.

A standard protocol to elute phage displaying peptides or proteins is treatment with an acidic elution buffer to disrupt the interaction between the displayed peptide and the target. However, this approach could have little effect on peptides reacting covalently or charge-independently with the target. Alternatively, the target can be used to specifically elute phage-displaying peptides; however, such elution conditions can preferentially release the most reversible binding peptides, and miss dislodging peptides with the lowest off-rate. Another method to specifically elute the target-bound phage is proteolysis, but incubation times of six hours or more are typical for many proteases (10). Preferably, phage should be cleaved from the solid support prior to re-infection and amplification. We have observed successful selections using phage bound to solid phase resin to directly infect *E. coli*, and re-amplify the bound phage.

In our optimized selections for selecting peptides that bind to or react with a small molecule, anti- and positive selections with increasing stringency are used in the 1st, 2nd and 4th rounds of selection. Negative selections are used in the 3rd and the 5th rounds (see Figure 1).

After three to five rounds of selection, the selected peptides should be removed from the phage surface before individual assay to examine their activities. This strategy of removing the peptide from the phage surface addresses the transferability caveat described above. The new format can also identify sequences with binding properties influenced by the phage surface to guide the design of the next generation library.

Ideally, conditions mimicking the intended use of the peptide are used for screening. To assay prospective hydrazide binding peptides, the selected peptides and their variants were synthesized on filter paper using a technique known as spot synthesis (11, 12). The arrays of

peptides were then screened for binding and reactivity with a small fluorescent molecule, rhodamine hydrazide. Since rhodamine hydrazide was not employed during the selections, only those peptides that react with the hydrazide functionality should result in a positive signal in this screen. As illustrated here, a fluorescent spot-synthesis assay produces a strong signal for visualization in a single step assay.

2. Materials

Prepare all solutions using ultrapure water (18 MΩ at 25 °C).

1. An M13 bacteriophage-displayed peptide library. The preparation and re-amplification of phage-displayed peptide libraries with diversities up to 1×10^{11} has been described previously (13).
2. A solid phase resin derivatized with a water soluble polymer (e.g., PEG) and the desired functionality (e.g., hydrazide). (*see* Note 1)
3. A sintered glass funnel. (*see* Note 2)
4. Phosphate-buffered saline (PBS; 1×)
5. PBS containing 0.05% Tween-20 (PT)
6. XL-1 (Agilent) or other *E. coli* cell line harboring the F′ episome necessary for infection with M13 bacteriophage.
7. Helper phage, 1×10^{13} cfu/mL (KO7 [from NEB] or VCS [from Stratagene])
8. 20% Polyethyleneglycol 8000/2.5 M NaCl “PEG/NaCl”
9. 2YT media (10 g bacto-yeast extract, 5 g NaCl, 17 g bacto-tryptone in 1 liter of water. The pH of the solution should be adjusted to neutral, and then the media sterilized by 20–30 min treatment in an autoclave.
10. Oliogonucleotide primers that hybridize at least 100 bp before the start of the library peptides, for sequencing and determination of the effective binding peptides.

3. Methods

Prior to each round of selection, an anti-selection removes non-target binding peptides. The anti-selection consists of all the components used during the selection *except* the target functionality. Solid-phase resins that swell in water, such as Tentagel (*see* Note 3), provide a large number of binding opportunities for a random peptide library. Resin binding peptides could dominate the pool of selectants if anti-selections are not integrated into the selection

[1]Solid phase resins preferably include a cleavable linker to release the target with its bound phage. However, we found hydrazide Tentagel, which lacks a cleavable linker, to provide an excellent target to select hydrazide binding peptides. No issues were encountered in propagating phage eluted from hydrazide-derivatized Tentagel.

[2]The pore size of a sintered glass funnel should be tested to verify that phage can easily flow through the funnel (i.e., by titering both the phage passing through and retained by the sintered glass). Small pore sizes of negatively charged silica could retain unbound phage, and poison the selection conditions with phage selected for the wrong reason.

[3]Tentagel, a water-miscible solid support, enables stringent wash steps to remove non-specific binding peptides during positive selections. For example, up to half a liter of different wash buffers were applied to 20 mg of resin during each round of selection.

strategy. A second aliquot of the solid phase resin derivatized with the target functionality is then used in positive selections to isolate phage-displayed peptides with affinity for the target.

3.1 Choosing a functional group

The first step of any molecular evolution experiment is selecting the target molecule. Only a narrow range of functionalities are appropriate for site-specific protein labeling within living cells due to the rich set of functional groups found *in cellulo*. For example, the potential small molecule targets cannot react indiscriminately with any of the natural amino acid side chain functionalities. This criteria excludes aldehydes, maleimides, and many activated esters. Other less obvious, electrophilic functionalities are excluded such as β-lactams; highly hydrophobic functionalities, such as pyrene, also cannot be used due to strong non-specific binding. Conversely, high stability of the target functionality can be an obstacle to the selection of reactive peptides, which can offer only the naturally occurring amino acid sidechain functionalities for the reaction. For example, in our hands, both azides and alkynes appear indifferent to reactions with random peptide libraries.

In general, electrophilic functionalities are particularly well-suited to sitespecific protein labeling. Proteins generally have abundant nucleophiles available for reaction (6). For example, we selected peptides with a nucleophile capable of attacking the carbonyl of the hydrazide. Other electrophilic carbonyls with different adjacent leaving groups might be better suited to reactivity with specific peptide sequences.

1. In the very first step, perform an appropriate binding assay (e.g., a phage-based ELISA) between the target molecule and wild-type phage. This initial experiment should identify and remove from consideration functional groups that are too reactive with random protein functionalities for experiments with phage-displayed peptide libraries. High inherent reactivity, defined as a signal three-times the signal of the background, will result in ineffective selections. Attempting to use functionalities with such high background binding will allow every member of the phage library to react indiscriminately with the target, preventing selection of individual peptide sequences.

3.2 Anti-selections of phage-displayed peptide libraries

1. Equilibrate the solid phase resin in buffer (*see* Note 4 when using Tentagel).
2. Dilute 1 mL (or an appropriate volume to include 100-copies of each member of the library) of the phage library in PBS. Then add the phage solution to ~20 mg of the target functionality-modified solid support for 1 h with gentle mixing, either by bubbling N_2 gas or a stirring with a small stir bar.
3. Remove the unbound phage library from the anti-selection, and transfer the library to the sintered glass funnel with the equilibrated target-modified solid support for the positive selection.

[4]Before use, swell the Tentagel resin in 50 mL of dichloromethane for 2 h. Then wash the resin three times with 100 mL PBS.

3.3 Positive selections with the phage-displayed peptide library

1. Prepare a second aliquot (*see* Notes 5 and [6]) of the solid-phase resin with the target. Wash three times with 100 mL PBS to neutralize the pH.
2. Add the phage library from the anti-selection, and incubate the resin and library for 1 h. (*see* Note 7)
3. Wash the resin 5 × with 50 mL of PT for 6 min each wash to remove unbound phage. (*see* Note 8)
4. Resuspend the resin, bound with phage, in 1.5 mL of PBS
5. Optional: While the phage are bound to the target in a positive selection, wash with 100 mL of ~1 mM target for 2 h to remove reversibly bound phage. Then, equilibrate with PBS before re-propagation. (*see* Note 9)

3.4 Repropagation of the phage-displayed peptide library. (*see* Note 10)

1. Add 0.75 mL of the phage-bound resin to a log-phase culture of Xl-1 *E. coli* (10 mL). Incubate 20 min at 37 °C with shaking.
2. Titer the infected culture onto carbenicillin-supplemented LB plates to determine the number of phage selectants, and incubate the titer plates overnight at 37 °C. (*see* Note 11)
3. Add 10 μL of helper phage (1×10^{13} cfu/mL) to the infected bacterial culture, and continue the incubation for an additional 20 min.
4. Transfer 10 mL of the infected culture to 200 mL of 2YT containing 50 μg/mL carbenicillin and 20 μg/mL kanamycin. Incubate this culture overnight (16–18 h) at 37 °C with 250 rpm shaking.

3.5 Isolation of the repropagated selectants

1. Centrifuge the overnight culture at 10 krpm for 20 min at 4 °C.

---

[5] Deprotect a second aliquot of the Boc-hydrazide Tentagel using 50 mL 1:1 solution of TFA:DCM for 2 h. Then, wash three times with 100 mL of PBS to neutralize the Tentagel.

[6] The quantity or concentration of the positive selection can be decreased in subsequent rounds of selection to increase the stringency and select for phage-displayed peptides that bind with faster on-rates.

[7] The time of the positive selection can be decreased in subsequent rounds (i.e. from 30 to 10 to 3 min) to select for phage-displayed peptides that bind with faster on-rates.

[8] The number and type of washes (different pH values, detergents, cellular lysate, etc.) can be altered in subsequent selection rounds to isolate stable peptide-target interactions under specific conditions.

[9] In the second round of selection, a competition selection is used to remove weakly bound phage (Figure 1). A large excess of target is added to the solid phase-bound phage. Weakly bound and irreversibly bound peptides could form interactions with the excess target in solution, while only irreversibly bound phage should remain tethered to the solid phase.

[10] Contamination is a common problem with phage selections. One troublesome contaminant is a lytic phage that amplifies after a few rounds of selection; the resultant lysis of the culture decreases the number of M13 phage particles produced, and derails the selection. This lytic phage can be removed by heat treatment, since M13 is a thermostable phage. After a round of selection, M13 phage can be heat-treated at 95 °C for 10 min to destroy possible contaminating phage particles, before re-amplification. Non-phage contaminants, such as fungi, can be removed by sterile filtration after rounds of selections; repeating the filtration again after re-propagation further reduces the chances of contamination.

[11] A typical indicator for successful selections is increasing phage titers from one round to the next. This criterion holds true if the selection conditions remain constant during each round of selection. However, if the selection conditions are varied, especially in favor of more stringent selections, as described here, then the titer results are less reliably an indicator for the success of the selection. Individual selectants should be sequenced, and screened regardless of titer.

2. Transfer the phage-containing supernatant to a new centrifuge tube, mix with 40 mL of PEG/NaCl and incubate on ice for 20 min at 4 °C.
3. Re-centrifuge at 10 krpm for 20 min at 4°C.
4. Re-suspend the fragile small white phage pellets (occasionally observed as streaks) in 5 mL of PBS, and sterile filter the solution.
5. Continue with additional rounds of anti-selections, negative selections, or positive selections. Up to five rounds of repropagation can be used, though the risk of selecting truncated sequences based upon faster growth in *E. coli* increases in later rounds.

3.6 Negative selection to select for phage-displaying peptides that react irreversibly, quickly, and completely with small molecule target. (*see* Note 12)

1. Incubate the repropagated phage library with ~10 mM target small molecule at RT for 3 h. (*see* Note 13).
2. Add the small molecule-phage mixture to the target-modified solid support, and incubate for 5 h at RT with stirring.
3. Elute the unbound phage and repropagate from this pool of phage (i.e., not the phage that is bound to the solid support).
4. Add 10 μL of the phage-hydrazide mixture to a 10 mL culture of log-phase XL-1 *E. coli* and continue with the phage re-propagation and isolation (steps **3.3** and **3.4**).

3.7 Screening of selected peptides

1. After DNA sequencing of individual selectants, synthesize the selected peptides on cellulose as outlined in Hilpert et. al.(12)
2. Screen with a different target than was used for either positive or anti-selections. Rhodamine hydrazide was used during screening of spot-synthesized peptides derived from hydrazide-modified Tentagel selections. Start with low concentrations and screen with higher and higher concentrations until an appropriate signal is visualized.

Acknowledgments

We gratefully acknowledge the support of the NIGMS of the NIH (1 R01 GM100700-01 and 1 R01 GM078528-01).

[12]Different small molecules with the target functionality can be used during negative selections to eliminate cross-reactivity with various functionalities. For example, in our selections for peptide reactivity with hydrazide, acetahydrazide was used during Round 3, and AMCA hydrazide was used in Round 5.

[13]Only phage that display peptides that completely and irreversibly react with the target in solution should emerge from the negative selection. Those peptides that release the target in solution and bind to the target on the solid support will be removed during the negative selection. A true negative selection (in which phage do not bind to a solid phase) should not be employed before the 3rd round of selection to allow removal of truncated and other non-binding phage in earlier rounds.

References

1. Sarikaya M, Tamerler C, Schwartz DT, Baneyx F. Materials Assembly and Formation Using Engineered Polypeptides. Annu Rev Mater Res. 2004; 34:373–408.
2. Deutscher SL. Phage display in molecular imaging and diagnosis of cancer. Chem Rev. 2010; 110:3196–211. [PubMed: 20170129]
3. Giepmans BNG, Adams SR, Ellisman MH, Tsien RY. The fluorescent toolbox for assessing protein location and function. Science. 2006; 312:217–24. [PubMed: 16614209]
4. Joo C, Balci H, Ishitsuka Y, Buranachai C, Ha T. Advances in single-molecule fluorescence methods for molecular biology. Ann Rev Biochem. 2008; 77:51–76. [PubMed: 18412538]
5. Nooren IM, Thornton JM. Structural Characterisation and Functional Significance of Transient Protein–Protein Interactions. J Mol Biol. 2003; 325:991–1018. [PubMed: 12527304]
6. Sletten EM, Bertozzi CR. Bioorthogonal Chemistry: Fishing for Selectivity in a Sea of Functionality. Angew Chem, Int Ed. 2009; 48:6974–6998.
7. Tanaka F, Fuller R, Asawapornmongkol L, Warsinke A, Gobuty S, Barbas CF III. Development of a Small Peptide Tag for Covalent Labeling of Proteins. Bioconjugate Chem. 2007; 18:1318–1324.
8. Eldridge GM, Weiss GA. Hydrazide reactive peptide tags for site-specific protein labeling. Bioconjugate Chem. 2011; 22:2143–53.
9. Cwirla SE, Peters Ea, Barrett RW, Dower WJ. Peptides on phage: a vast library of peptides for identifying ligands. Proc Natl Acad Sci U S A. 1990; 87:6378–82. [PubMed: 2201029]
10. Goodyear, CS.; Silverman, GJ. Protein-Protein Interactions. Golemis, EA.; Adams, PD., editors. Vol. Chapter 9. Cold Spring Harbor Laboratory; New York: 2008.
11. Frank R. Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. Tetrahedron. 1992; 48:9217–9232.
12. Hilpert K, Winkler DFH, Hancock REW. Peptide arrays on cellulose support: SPOT synthesis, a time and cost efficient method for synthesis of large numbers of peptides in a parallel and addressable fashion. Nat Prot. 2007; 2:1333–1349.
13. Sidhu SS, Weiss GA. No Title. Phage Display: A practical approach. 2004:27–41.

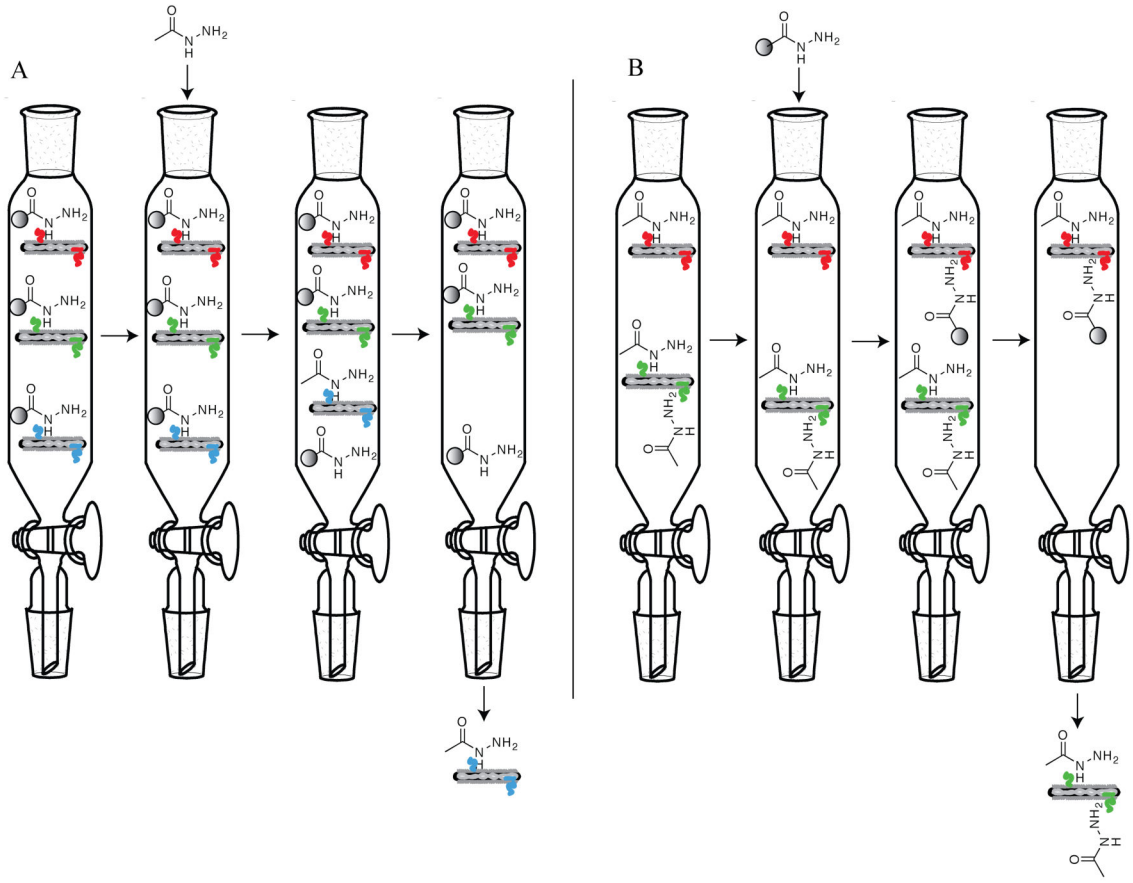
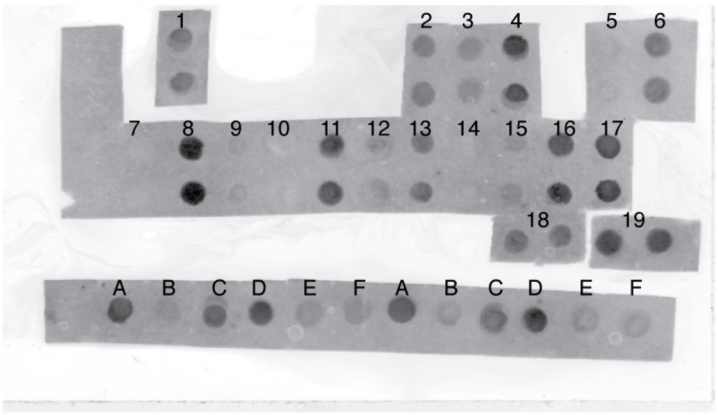


Figure 1.

(**A**) In the optional wash step, excess acetyl hydrazide selectively elutes weakly bound peptides from the hydrazide Tentagel resin to select for tightly binding peptides still adhered to the resin. (**B**) In the negative selection described here, pre-incubating the phage library with a dilute solution of acetyl hydrazide, and then applying the reaction mixture to hydrazide Tentagel isolates only those phage in which each copy of the peptide quickly and irreversibly reacts with hydrazide.



SPOT	SEQUENCE	SPOT	SEQUENCE
1	LSCGCVQWLDAKNCNV	15	GRNNHACEDCGLPGCHKNKL
2	EEPKHTCKTNKTHHCDKSNS	16	NKGHKGCTEGGGKTCHTTRT
3	ENNSNRCHDQNHQTCNKHRP	17	HQNIESCKKDTKTACNPRVS
4	KKTAPQHCDSTECNTQPTSR		
5	AHASNTCHHTNNTGCATHKP	18	HKSNHSSKNRE
6	NKASTHCHPPNSRQCKAERT	19	HKSNHSSKNR
7	HKTNHSCPRGTPPRCHHATT		
8	VPQTPDNCRQDHCHPRPRAA	A	EHHKEHCKPR
9	ASRHENCSHRPQTTCQTQNN	B	PRQCTTATPQ
10	STDAPHCQTHTPRSCLDGQI	C	QECTHSKTRT
11	DPHHRECTRQTTHNCNHKRH	D	ECQKKPHTR
12	KQHHQVCKHKQETRCPNNTK	E	KTKSSSQK
13	KEHTERCNNNHKERCYARNP	F	NKTKSSSQK
14	IPEHHTCDNEQKTTCRRHND		

Figure 2.

Rhodamine hydrazide screening of spot-synthesized peptides from optimized selections. The first 17 sequences were identified in the later rounds of the selection conditions described here to isolate highly efficient hydrazide-reacting peptides. Peptides 18, 19, and A–F were identified in other spot synthesis screens as potential hydrazide interacting peptides.