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Robust, Quantitative Analysis of Proteins using Peptide Immunoreagents, in Vitro Translation, and an Ultrasensitive Acoustic Resonant Sensor

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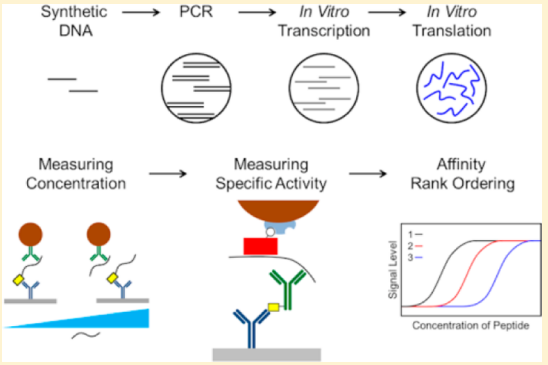
Supporting Information

ABSTRACT: A major benefit of proteomic and genomic data is the potential for developing thousands of novel diagnostic and analytical tests of cells, tissues, and clinical samples. Monoclonal antibody technologies, phage display and mRNA display, are methods that could be used to generate affinity ligands against each member of the proteome. Increasingly, the challenge is not ligand generation, rather the analysis and affinity rank-ordering of the many ligands generated by these methods. Here, we developed a quantitative method to analyze protein interactions using in vitro translated ligands. In this assay, in vitro translated ligands generate a signal by simultaneously binding to a target immobilized on a magnetic bead and to a sensor surface in a commercial acoustic sensing device. We then normalize the binding of each ligand with its relative translation efficiency in order to rank-order the different ligands. We demonstrate the method with peptides directed against the cancer marker Bcl-x_L. Our method has 4- to 10-fold higher sensitivity, using 100-fold less protein and 5-fold less antibody per sample, as compared directly with ELISA. Additionally, all analysis can be conducted in complex mixtures at physiological ionic strength. Lastly, we demonstrate the ability to use peptides as ultrahigh affinity reagents that function in complex matrices, as would be needed in diagnostic applications.



Advances in mass spectrometry[1] and microarrays[2] have provided a better perspective of biological systems, but a long-term goal in proteomics is the development of affinity reagents against all members of the proteome. Monoclonal antibody methods,[3] phage display,[4] ribosome display,[5] and mRNA display[6] can all generate tens to hundreds of potential polypeptide ligands against targets of interest. Recent advances combining in vitro selection with high-throughput sequencing has greatly accelerated the process of generating a large list of potential ligands.[7] Many of these techniques do not use antibodies as affinity reagents but rather use either small protein scaffolds or peptides for protein recognition, offering the potential of antibody-free diagnostics. Peptides are especially attractive as antibody replacements because they can be chemically synthesized (avoiding issues with expression and purification), are renewable, and are generally stable without refrigeration.[8] However, in order to generate a set of proteome-wide affinity reagents, high-throughput methods will not only be required for initial ligand discovery but also will be needed for screening and characterization to determine the best ligand for an application. Current methods (e.g., ELISA) are slow, laborious, and do not correct for differences in ligand expression levels.[9] While new technologies such as optical resonators[10] and nanowire sensors[11] provide the advantage of direct ligand−target affinity measurements, these methods are challenging to implement in complex media, at physiological salt, and in a high-throughput fashion. There is thus a pressing need for high-throughput, robust methods that are sensitive, utilize little reagent, and function in complex media.

In vitro translation provides an appealing route to screen ligands in a high-throughput fashion, since no cloning is required, allowing ligands to be generated quickly. Typically, very little material is synthesized and translation levels are highly variable, requiring both a highly sensitive assay and the ability to normalize the signal for expression. Additionally, detection of proteins in crude translation reactions (a complex media) is essential because purification steps after translation can be costly, inconsistent, and significantly hinder the throughput of the method.

Here, we developed a method to accurately assess the relative affinity of multiple ligands for a specific protein. To do this, we quantify the specific activity of a clone (i.e., binding to the protein of interest) and normalize the binding signal for protein expression. This approach was implemented in a general way on a commercial acoustic resonant biosensor platform, the ViBE BioAnalyzer (BioScale, Lexington, MA.) The ViBE BioAnalyzer uses acoustic membrane micro-particle technology (AMMP) to detect the presence of the analyte using a sandwich format assay. The analyte is linked to the surface of the sensor using an antihapten antibody at one end and to a

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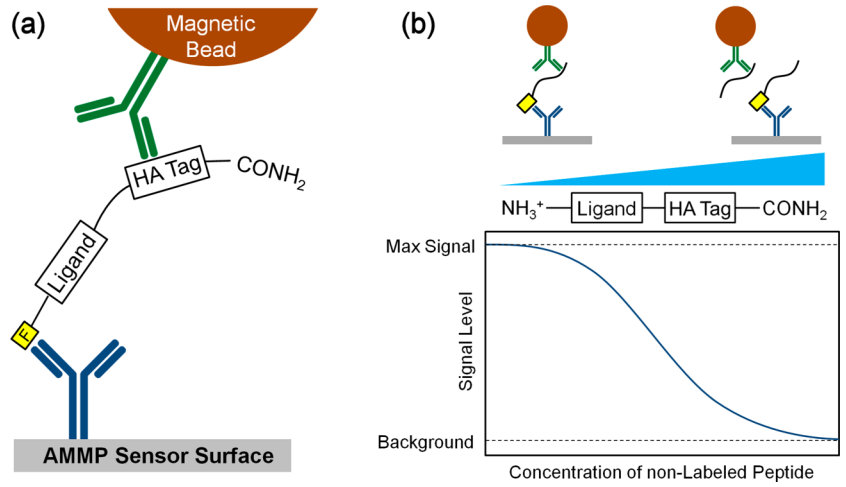

Figure 1. Schematic of HA tag competition assay. (a) Fluoresceinated HA-tagged peptides bind to anti-HA antibodies on magnetic beads and antifluorescein antibodies on sensor surface to generate signal. (b) HA-tagged peptides or proteins without fluorescein cannot interact with sensor surface and reduce signal levels by competing with the fluorescein-labeled peptide for anti-HA antibody.

magnetic bead on the other end.[12] The sensor uses piezoelectric properties of a vibrating membrane, where the membrane's in-liquid resonance frequency shifts when an external mass influences the sensor acoustic loading.[13] The beads act to amplify the loading, resulting in much higher levels of sensitivity.

An advantage of our approach, using the acoustic resonant devices, is that the assays are sensitive enough to be done using in vitro synthesized proteins, enabling rapid analysis in less than 1 day starting with synthetic DNA.

MATERIALS AND METHODS

AMMP Assays. In vitro translated peptides and proteins were diluted 1:10, initially using an assay buffer [1× PBS + 1% (w/v) BSA + 0.1% (v/v) Tween-20], and the subsequent dilutions were performed in a 10% translation solution in assay buffer. A sample of synthetic Pep1 at 30 nM in 10% translation solution was prepared and diluted serially to generate the standard curve. All samples and standards were run in duplicate. Samples and standards were incubated with magnetic beads and fluorescein-labeled analyte for 4 h. Because each AMMP assay run time is 10 min, constant incubation time for all samples was achieved by separating the start of incubation for each column on the 96 well plates by 10 min. Run buffer was 1× PBS + 1% (v/v) Tween-20 + 1% (v/v) heat-treated FBS (Invitrogen; FBS was heat treated for 15 min at 65 °C and filtered.) BioScale Universal Detection Cartridges were used in performing all assays. The device was used per the manufacturer's instructions.[12]

For the AMMP HA tag competition assay, 60 μL of sample was incubated with 30 μL of anti-HA antibody immobilized on epoxy magnetic beads (6 μg of beads/mL) and 30 μL of 240 pM fluorescein-labeled synthetic Pep1 for 4 h and analyzed using the ViBE BioAnalyzer. Both anti-HA beads and Pep1 were diluted using assay buffer.

For the AMMP target-binding assay using in vitro translated peptides, 60 μL of sample was incubated with 30 μL of biotinylated Bcl-x_L immobilized on streptavidin magnetic beads (8.3 μg of beads/mL) and 30 μL of 10 nM fluorescein-labeled anti-HA antibody for 4 h. For the AMMP target-binding assay using mRNA-peptide fusions, 60 μL of sample (diluted in 1× assay buffer) was added to 30 μL of biotinylated Bcl-x_L immobilized on streptavidin magnetic beads and 30 μL of assay buffer and incubated for 4 h.

The concentration of the HA tag competition assay samples were determined using synthetic Pep1 standards and fit to a simple 4-parameter logistics curve (Figure S-1a of the Supporting Information).

ELISA Target-Binding Assay. ELISA plates were incubated overnight at 4 °C with 1.5 nmol of anti-HA antibody per well. Plates were washed with wash buffer [1× PBS + 0.1% (v/v) Tween-20] and blocked with 1× PBS + 5% (w/v) BSA for 2 h. In each well, 100 μL of sample and 100 μL of 33 nM biotinylated Bcl-x_L were added and incubated for 4 h. Plates were washed, incubated with streptavidin horseradish peroxidase conjugate (Strep-HRP, Thermo Scientific) for 1 h, washed, and incubated with TMB substrate (Thermo Scientific). Reactions were stopped after approximately 10 min with 2 M sulfuric acid, and the absorbance at 450 nm was measured via a plate reader (Molecular Devices).

RESULTS AND DISCUSSION

Quantitation of in Vitro Translated Proteins. Overall, our goal was to develop a general quantitative method to rank-order protein-binding ligands using in vitro translated polypeptides. This simple aim is complicated by several factors: (1) the small amount of protein produced by in vitro translation systems, (2) detection of binding in complex media, and (3) the fact that most existing methods (e.g., sandwich ELISA) require two orthogonal affinity reagents to function.

Our solution was to develop a competition assay that required only a single affinity tag added to each sequence (described below). Unfortunately, only modest levels of polypeptides are synthesized via in vitro translation, and the crude sample matrix (the solution containing the sample, which includes proteins, surfactants, nucleic acids, salts, etc.) can interfere with the assay, further reducing sensitivity.[14] The problem with existing competition assays using immobilized affinity reagents is that they are insensitive. There, the lack of sensitivity arises from two features of these assays. First, for the competition to give the maximum signal, competitor concentrations must be scanned from below the analyte concentration to significantly above it (>10 fold). Second, in

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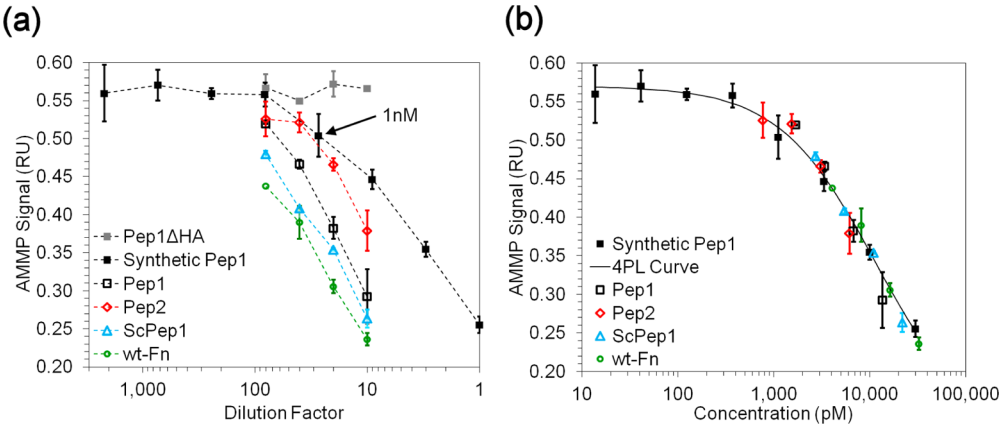

Figure 2. Competition assay using HA-tagged peptide. (a) Reduction of AMMP signal via competition with translated peptide is dependent on the absence (Pep1ΔHA) or presence (all other samples) of the HA tag. (b) Sample concentrations were calculated based on a simple 4-parameter logistics curve, and the results show dilutional linearity. Pep1ΔHA concentration cannot be estimated using this method.

order to deplete the analyte signal, the competitor must be present at concentrations significantly in excess of the K_d for the interaction between the affinity reagent and the analyte.[15] Assays with high levels of signal for the analyte using low concentrations of the affinity reagent are able to achieve high degrees of sensitivity. ELISA assays require high amounts of affinity reagents immobilized on the plate in order to achieve high sensitivity for the analyte. This means a high level of competitor is necessary to deplete the available binding sites on the immobilized affinity reagent, leading to a low level of sensitivity. In line with these views, we were unsuccessful in our attempts to create a competition ELISA assay sensitive enough to detect translated peptides and proteins (data not shown). We decided to use AMMP technology, which was ideal for our analysis due to its high sensitivity, low reagent consumption, ability to detect analytes in complex matrices, and the potential for automation.[12]

The AMMP competition assay we developed is shown in Figure 1. In this assay, a synthetic peptide is labeled with fluorescein, allowing it to bind to an antifluorescein antibody on the sensor surface. The synthetic peptide also contains an HA tag (NH_2–YPYDVPDYA–COOH) that binds to an anti-HA antibody on a magnetic bead, thus linking the bead to the sensor surface. This enables the detection of the fluorescein-HA peptide (Figure 1a). As synthetic or translated HA tagged (nonfluoresceinated) peptide is added, it competes with the fluorescein-HA peptide for binding to the anti-HA antibody. This decreases the fraction of antibody available to bind the fluorescein-HA peptide, thus reducing the signal in a dose-dependent fashion (Figure 1b).

We chose the HA tag in the competition assay because it is small, widely used in biotechnology, and several inexpensive anti-HA antibodies are commercially available. Since each in vitro translated peptide or protein in our assay could be designed to include a single HA tag, each peptide or protein should interact with the anti-HA antibody with equal affinity. Thus, appending an HA tag to our peptides or proteins would enable us to quantify in vitro translated polypeptides independent of the sequence N-terminal to the HA tag using this competition assay.

In order to test and validate our HA tag competition assay, we needed to show that the assay could be used to quantify proteins or peptides with different sequences N-terminal to the HA tag. We also needed sequences with different affinities for their target so that we could test our ability to rank-order ligands by binding affinity. We chose two peptides from an mRNA display selection against the B-Cell lymphoma-extra large protein (Bcl-x_L) (T.T. Takahashi, R.W. Roberts, manuscript in preparation). The first peptide, peptide 1 (Pep1), binds to Bcl-x_L with high affinity (~250 pM), while the second peptide, peptide 2 (Pep2), has a lower affinity for Bcl-x_L (~65 nM). We also used a scrambled version of peptide 1 (ScPep1) as a negative control in the specific binding assays to demonstrate the binding selectivity to Bcl-x_L. We synthesized the DNA that coded for these peptides followed by an HA tag on the C-terminus of each peptide. Through the use of a simple radiolabeled binding experiment, we were able to show that translated peptides with the C-terminal HA tag interact with equal affinity to the HA antibody immobilized on beads (Figure S-1 of the Supporting Information).

HA Tag Competition Assay. We first generated the signals using the synthetic fluoresceinated HA peptide. Using the synthetic unlabeled peptide as a competitor, we observed significant reduction of signal for concentrations higher than 1 nM (Figure 2a). We then in vitro translated Pep1, Pep2, and ScPep1 samples, as well as Pep1 lacking the HA tag (Pep1ΔHA) as a negative control. We also translated the 10th fibronectin type III domain of human fibronectin[16] with a C-terminal HA tag (wt-Fn) to determine if we could quantify in vitro translated proteins using our HA tag competition assay as well. All samples were run in duplicate, starting with a 1:10 dilution followed by four subsequent 1:2 dilutions. We normalized matrix interference effects by diluting all samples and standards into the same solution, which contained 10% (v/v) translation solution. Pep1, Pep2, and ScPep1 all showed a reduction of signal up to a dilution factor of 40. As expected, the Pep1ΔHA control, which lacks the HA tag, did not reduce the signal at any tested dilution. Lastly, wt-Fn containing a C-terminal HA tag also showed a significant reduction in signal, demonstrating we could also quantify in vitro translated proteins in complex matrices.

Using the unlabeled synthetic HA peptide as a competitor, we generated a calibration curve to calculate the concentration of our translated samples. To do this, we fit the synthetic HA peptide data to a simple four-parameter logistic curve (Figure 2b and Figure S-2a of the Supporting Information). We then determined the concentration of the translated samples by interpolation of all four dilutions and took the mean. The

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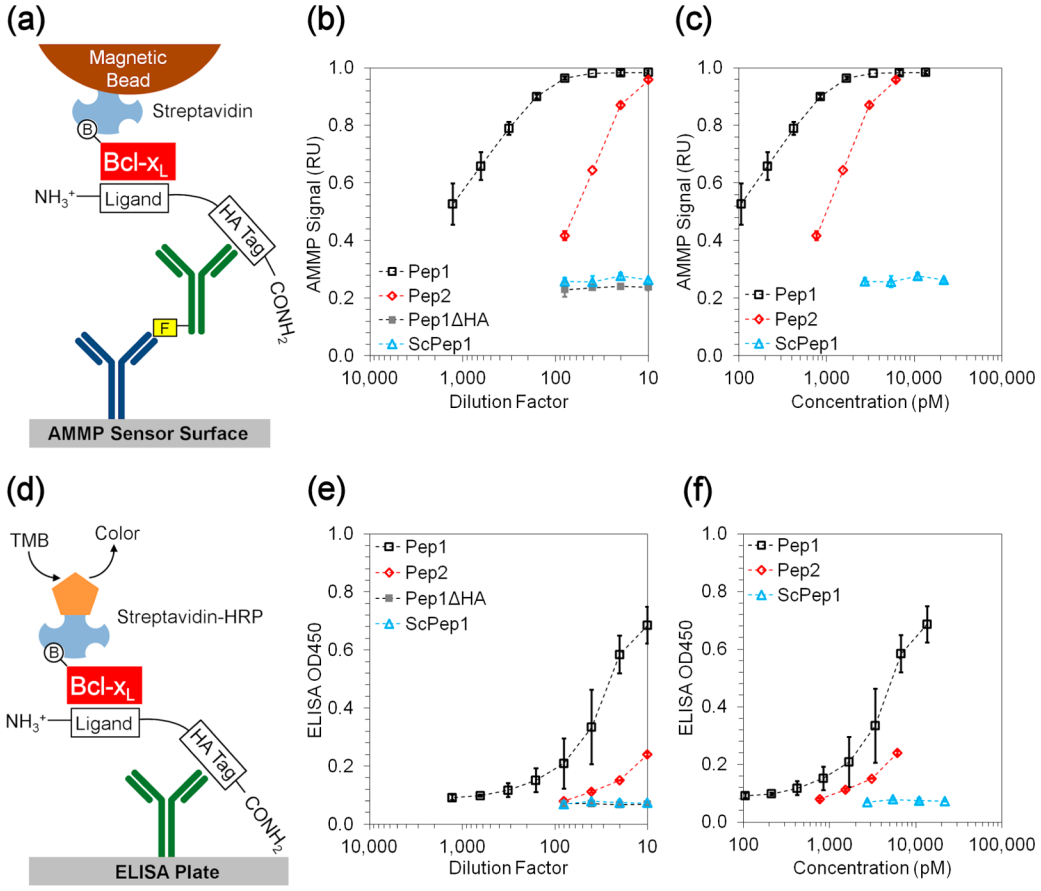

Figure 3. AMMP and ELISA target-binding assays. (a) Schematic of the AMMP target-binding assay. An HA-tagged ligand binds to target (Bcl-x_L) immobilized on streptavidin magnetic beads. Anti-HA antibody, labeled with fluorescein, binds to the HA tag and to antifluorescein antibody on the sensor surface. (b) AMMP signal in the target-binding assay is a function of dilution factor and ligand affinity. At the same dilution, Pep1 generates higher signal levels than Pep2, whereas ScPep1 and Pep1ΔHA show background levels of signal. (c) AMMP target-binding assay adjusted for peptide concentration as measured using the HA tag competition assay, demonstrating true relative affinities. (d) Schematic of the ELISA target-binding assay. An HA-tagged ligand binds to biotinylated target (Bcl-x_L) and anti-HA antibody on the ELISA plate. Streptavidin-HRP binds to biotin, resulting in an ELISA signal. (e) Target-binding ELISA assay is much less sensitive than the AMMP assay [described in (b)], and the respective samples behave equivalently: Pep1 generates a higher signal level than Pep2 and ScPep1 and Pep1ΔHA generate no signal over the background. (f) ELISA target-binding assay adjusted for peptide concentration as measured using the HA tag competition assay.

concentration (in nanomolar) and coefficient of variation (CV) for the different in vitro translated samples are shown in Figure S-2b of the Supporting Information. Wt-Fn had the highest translation efficiency, with its concentration measured at 327 nM, followed by ScPep1 (219 nM), Pep1 (136 nM), and Pep2 (62 nM).

Our data demonstrate that the amount of peptide or protein synthesized using in vitro translation is highly sequence-dependent. The difference between the lowest (Pep2) and the highest (wt-Fn) concentrations of in vitro translated polypeptides was a factor of 5. This variability could drastically skew rank-ordering of potential ligands. Moreover, the difference in expression level between Pep1 and ScPep1 is approximately 2-fold. This result is somewhat surprising as the sequences use ~70% identical codons (Table S-1 of the Supporting Information). In summary, we demonstrate that different sequences translate with significantly different efficiencies. Thus, only determining a clone's specific binding is not enough for rank-ordering ligands. Instead, it is necessary to normalize the signal by each clone's expression level in order to determine the highest affinity clones.

To confirm that the signal generated in the assay was not due to nonspecific adhering of antibody modified magnetic beads or the unlabeled peptide to the sensor surface, we first eliminated the peptide analyte from the complex and observed background levels of signal (~10%, Figure S-3a of the Supporting Information). We then added an excess (30 nM) of nonfluoresceinated peptide to the antibody modified magnetic beads and still observed background levels of signal.

Design of a Target-Binding Assay. Once we developed a method to quantify the amount of sample in an in vitro translation reaction, we then designed an assay to measure each sample's relative binding affinity. We used a simple sandwich assay, where we could test different peptides for binding to their target (here, Bcl-x_L). In the AMMP target-binding assay (Figure 3a), immobilized Bcl-x_L on magnetic beads binds to HA-tagged synthetic peptide. The fluoresceinated anti-HA antibody binds to the HA-tagged peptide as well as the antifluorescein antibody on the sensor surface, thus linking the magnetic bead to the sensor surface.

To demonstrate that our peptides bound to Bcl-x_L with different affinities, we used a radiolabeled binding assay, where we immobilized Bcl-x_L on magnetic beads and tested the binding of ^{35}S-labeled, HA-tagged Pep1, Pep2, and ScPep1 (Figure S-4 of the Supporting Information). Pep1 shows the highest level of binding to immobilized Bcl-x_L (~80%), while

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Pep2 shows modest binding (~1%). Scrambled peptide 1 (ScPep1) shows negligible binding to immobilized Bcl-x_L (<0.1%). No peptide showed any appreciable binding to magnetic beads without Bcl-x_L. These results agree with experiments that show Pep1 has higher affinity for Bcl-x_L than Pep2 (T.T. Takahashi, R.W. Roberts, in preparation). Our data here also show that the addition of the HA tag (with a six-amino acid spacer) does not interfere with the binding of the peptides to Bcl-x_L.

Measuring Relative Binding Affinity Using the AMMP Target-Binding Assay. We translated the Bcl-x_L binding peptide (Pep1 and Pep2) as well as the negative control peptides (ScPep1 and Pep1ΔHA) and analyzed the dilutions of each sample on the AMMP target-binding assay (Figure 3, panels b and c). Both Pep1 (the high-affinity binder) and Pep2 (the moderate-affinity binder) show saturated signal levels for the 1:10 dilution samples. Dilutions of 1:80 for Pep2 and over 1:1000 for Pep1 give a robust signal over the background. In vitro translated ScPep1 and Pep1ΔHA (two negative controls) showed background levels of signal at every dilution. Omitting various components of the sandwich (synthetic Pep1 or fluoresceinated anti-HA antibody) results in no signal over background levels, confirming the specificity of the assay (Figure S-3b of the Supporting Information).

In order to accurately rank the relative affinities of Pep1 and Pep2, we used the concentrations calculated for each from the HA tag competition assay (Figure 3c). Pep2 provides robust signal over background at 1 nM, whereas Pep1 gives a robust signal even at 100 pM. ScPep1 has undetectable levels of binding for Bcl-x_L at the tested concentrations. These results are in agreement with the radiolabeled binding assay and previous observations. The performance characteristics of Pep1 are comparable to antibody-based ligands, previously analyzed using this device, in terms of assay range and sensitivity.[12]

Comparison of AMMP Technology and ELISA. In order to directly compare AMMP technology to widely used ELISA methods, we used the same reagents to analyze the same standards and samples from the AMMP assay on a similarly formatted ELISA assay (Figure 3d). The results show that in vitro translated Pep1 is functional in ELISA and gives a dilution profile similar to what would be expected of an antibody. In vitro translated Pep2 also gives significant ELISA signal over background for the lowest two dilutions (Figure 3e). The highest dilutions for Pep1 and Pep2 are at background levels of signal for ELISA, while they are significantly above the background level of signal on the AMMP assay. In this experiment, the difference in sensitivity between the ELISA and the AMMP assays for the in vitro translated Pep1 is ~20-fold. Pep1ΔHA and ScPep1 showed no appreciable binding to Bcl-x_L at any dilution.

We also performed several independent experiments with known concentrations of synthetic Pep1 in order to confirm the sensitivity difference we observe between AMMP assays and ELISA (Table 1). In buffer, the AMMP assay is on average an order of magnitude more sensitive than ELISA. In 10% translation solution, the AMMP assay remains more sensitive than ELISA by a factor of 4. Perhaps more notable, with regard to high-throughput ligand analysis, is that the AMMP assay offers this higher sensitivity with 5-fold less antibody and over 100-fold less target per sample.

One of the advantages of higher sensitivity assays is lower sample consumption and less sample matrix interference, since higher dilutions in a simple buffer reduces the amount of the interfering matrix. The decreased amount of antibody required in AMMP assays has the additional benefit of enabling highly sensitive competition assays. The AMMP assay, by using significantly lower amounts of ligand than the ELISA, can attain a level of sensitivity in competition assays that is difficult to reach, or unreachable, using ELISA.

Table 1. Comparison between ELISA and AMMP Target-Binding Assays[a]

	ELISA	AMMP
mean LOD in assay buffer (pM)	51	5
mean LOD in 10% translation solution (pM)	288	67
range (logs of concentration)	2	2
sample volume (uL)	100	60
Anti-HA antibody/sample (fmols)	1500	300
Biotin-labeled Bcl-x_L/sample (fmols)	3300	30

[a]On average, AMMP assays are ~10-fold more sensitive than ELISA in assay buffer and four-fold more sensitive in a 10% translation solution. Both assays show a similar range of quantitation, although the AMMP assay uses five-fold less anti-HA antibody and 110-fold less biotinylated Bcl-x_L.

Comparison of Different Assays to Determine Translated Peptide Concentration. We were interested in using the three assay formats described above (competition assay, target-binding assay, and ELISA-based assays) to determine the concentration of a translated sample for comparison. Ideally, each assay would give the same result for a single sample, but previous comparisons of methods[17] have shown biases using different assay formats. To compare these assays, we calculated the concentration of in vitro translated Pep1 using synthetic Pep1 standards and a four-parameter logistic calibration curve for each assay. The concentration of translated Pep1 was measured at 136 nM using the HA tag competition assay, 300 nM using AMMP target-binding assay, and 1100 nM using the ELISA target-binding assay (Figure S-5a of the Supporting Information). A value between 100 and 300 nM is more consistent with previous analyses on this in vitro translation system.[18] The bias in concentration measurement was consistent (R^2 = 0.92) over three translated samples tested in three independent trials when comparing the target-binding assays using AMMP or ELISA (Figure S-5b of the Supporting Information). Though the reason for the bias is unknown, consistent biases of different methods of concentration measurement are common.[17] Lastly, as long as the relative concentrations that are determined for a set of samples using a single assay format are consistent, the bias from different assays will not affect our ability to rank order different ligands. Since we are determining the relative activity of a set of ligands, normalizing each ligand's activity by its relative concentration should not change the overall rank ordering.

Sample Matrix Effects. For the AMMP and ELISA assays, we diluted all samples and standards into an assay buffer containing 10% translation solution to negate matrix effects on the generated signal. In the competition assay, translation solution present in samples is reported as artifactual higher concentration while in the target-binding assay, translation solution is reported as artifactual lower concentration (Figure S-6 of the Supporting Information). Thus, assays that do not adjust for matrix effects can over- or underestimate the level of analyte present in solution, depending on the assay used.

Binding of Peptide-mRNA Fusion Molecules. One application of the AMMP sandwich assay we developed is for

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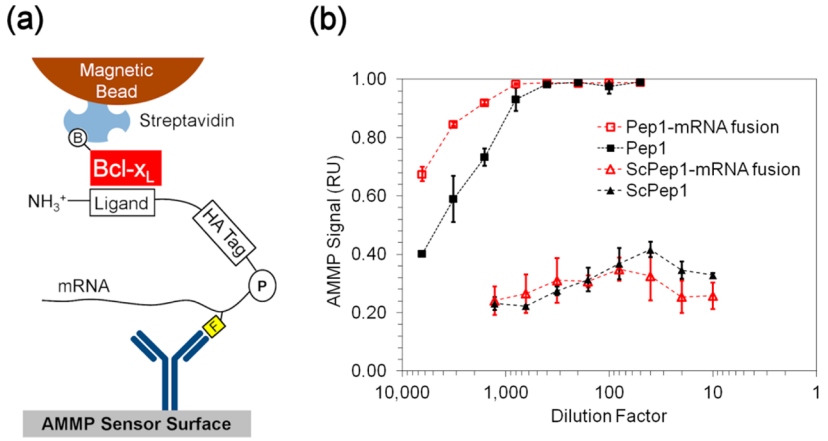

Figure 4. Peptide-mRNA fusions are easily adapted to a target-binding assay. (a) Fluorescein on the fusion molecules bind to antifluorescein antibodies on the sensor surface and fused peptide binds to target (Bcl-x_L) immobilized on a magnetic bead, generating an AMMP signal. (b) Comparison of fused peptide (Pep1 or ScPep1-mRNA fusion) and peptide alone (Pep1 or ScPep1) shows mRNA fusion to peptide does not perturb ligand activity in the target-binding assay.

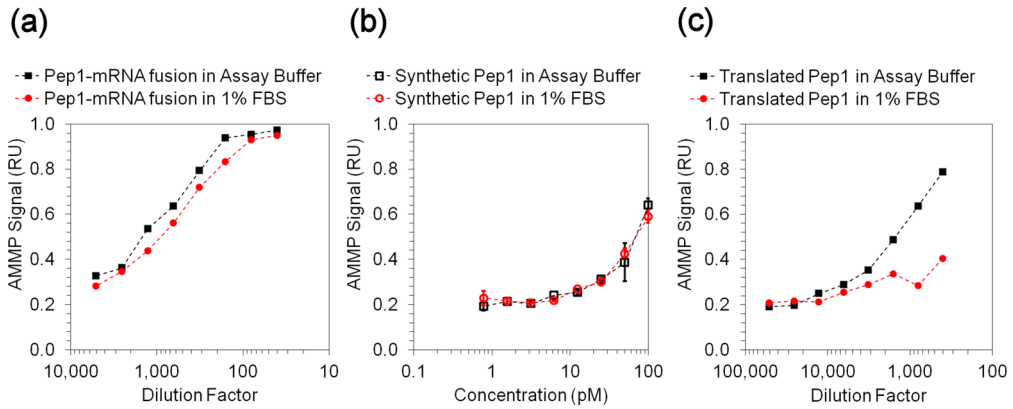

Figure 5. Peptides have modification-dependent stability in serum. (a) Pep1-mRNA fusions containing C-terminal puromycin show a slightly lower signal in 1% FBS buffer versus assay buffer. (b) Pep1 modified with a C-terminal amide shows activity independent of buffer. (c) Pep1 with an unmodified C-terminus shows much lower activity in 1% FBS, indicating degradation.

analysis of mRNA-peptide fusions used in mRNA display.[6] In mRNA display, an mRNA template is covalently linked to the polypeptide that it encodes using a puromycin linker. This step is essential in mRNA display, which is widely used for the generation of high-affinity ligands by in vitro selection of high-diversity libraries.[19]

Currently, radioactive binding assays are used to evaluate the function of mRNA-peptide fusions.[18] We were interested in testing if our AMMP assays were sensitive enough to evaluate mRNA–peptide function, which would increase throughput and avoid the use of radiation. To do this, we designed a target–binding assay for the mRNA–peptide fusion molecule (Figure 4a). We ligated the mRNA of our peptides to the puromycin-containing DNA linker, where the DNA linker possessed a fluorescein label. By using the fluorescein-labeled linker, we could avoid the use of a C-terminal HA tag/antibody to form the sandwich. In this assay, the fluorescein tag on the mRNA-puromycin molecule binds to the antifluorescein antibody on the sensor surface. The peptide of the fusion molecules binds to the biotinylated Bcl-xL immobilized on streptavidin magnetic beads, thus connecting the magnetic bead to the sensor surface (Figure 4a).

To test this assay, we in vitro translated mRNA or mRNA-puromycin-linked molecules.[20] Samples of in vitro translated peptide were incubated with fluorescently labeled anti-HA antibody and Bcl-x_L immobilized on the magnetic beads, while samples of in vitro translated mRNA-peptide fusions were incubated simply with Bcl-x_L magnetic beads. The scrambled ScPep1 negative control showed low levels of signal as either peptide or fusion (Figure 4b). Interestingly, we observed that the Pep1-mRNA fusion gives higher signal levels than Pep1 peptide. This is surprising given that generally less than half of the translated peptides are fused to their encoding mRNA.[18] This result, which increases the sensitivity of the experiment by 5-fold, is likely due to eliminating the HA tag/anti-HA antibody interaction necessary in the peptide format. In the fusion format, every peptide is covalently joined through puromycin to the fluorescein that immobilizes the construct on the sensor chip. In addition to the advantage of not needing a protein tag, this assay can be used in cases where higher sensitivities are needed either due to poor target-ligand interaction or poor translation efficiency, to distinguish between ligands' relative affinities. However, because we do not use the HA tag/anti-HA antibody in the sandwich, we cannot calculate the concentration of fusions by competition with a known standard.

Peptides as Diagnostic Reagents. Peptides as affinity reagents for immunoassays offer several advantages over antibodies in terms of stability, storage, cost of production,

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and purification.[8] They have largely been ignored as immunoreagents due to their instability in the presence of protease and peptidases, as well as their generally lower affinities for their targets. Recent advances in display technologies have enabled generation of high-affinity peptides with the potential of being attractive immunoreagents.[21] Previous studies have shown that protecting the C-terminus of the peptides can greatly increase resistance to proteolysis.[22]

We sought to demonstrate that our assays could be performed in serum, a matrix which would be encountered frequently in diagnostic settings. To do this, we tested Pep1 in a solution containing 1% nonheat-treated FBS in assay buffer for 4 h. FBS is known to contain active proteases that could degrade linear peptides.[23]

Both the mRNA-peptide fusion (C-terminus covalently bound puromycin) and synthetic peptide (C-terminal amide) show very similar signal levels in FBS and in the assay buffer (Figure 5, panels a and b). This observation is consistent with the molecules being stable in FBS over the 4 h incubation time. On the other hand, in vitro translated Pep1 (natural carboxy C-terminus and therefore sensitive to carboxypeptidase degradation)[24] shows a distinctly lower signal in FBS versus assay buffer, consistent with peptide degradation. Overall, our results show that C-terminally blocked peptides (fusions and amidated peptides) show excellent performance in 1% FBS and argue that these reagents can be used in complex media-containing proteases and peptidases.

CONCLUSIONS

We have demonstrated a robust, sensitive, specific, and scalable method to assess the relative affinity of ligands for the protein of interest in a complex matrix, while normalizing for expression levels. We measured the difference in expression levels of two similar sequences to be a factor of 2, while different sequences showed a 5-fold difference in expression levels, which can introduce significant error in relative affinity assessments. Our method is approximately an order of magnitude more sensitive than ELISA in a similarly formatted target-binding assay, while using 100-fold less target and 5-fold less antibody per sample. Lastly, we used AMMP technology to test mRNA–peptide fusion function without the need for the HA tag/anti-HA antibody and showed a more general implementation of the method.

The rank-ordering method described above is not limited to in vitro translated proteins and peptides. We have shown that detection can occur in complex matrices such as reticulocyte lysate or fetal bovine serum; therefore, our methods can likely be modified to work in various cell lysates. Our methods can also likely be adapted to screen antibodies, for which the HA tag and the anti-HA antibody can be switched with the Fc region of antibodies and immunoreagents against the Fc region.

As diagnostic reagent candidates, peptides offer several advantages over antibodies: they are smaller, have a longer shelf life, can be stored at room temperature while lyophilized, and are much easier to synthesize and purify. The main disadvantages over antibodies in diagnostics are peptide instability in complex matrices containing peptidase and proteases as well as lower affinities. We have demonstrated that with a simple modification to the C-terminus, our peptides were able to detect target in a serum solution containing active proteases. We have also shown that peptides are capable of detecting the target with very high affinities in complex media and are suitable as high-affinity diagnostic reagents.

ASSOCIATED CONTENT

Supporting Information

Detailed protocols on peptide synthesis, hapten labeling, Bcl-x_L expression, in vitro translation, radiolabeled binding assays, DNA sequences, and further details on HA tag competition and target-binding assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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