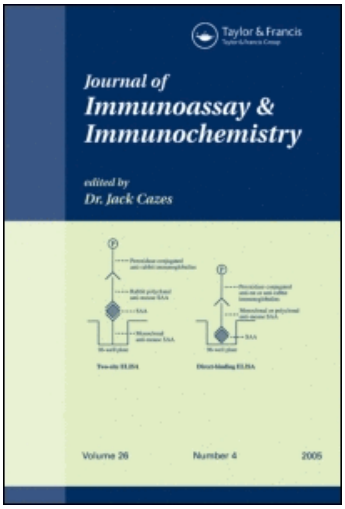

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A Sensitive and Rapid Chemiluminescence ELISA for Filamentous Bacteriophages

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Abstract: Filamentous bacteriophage (Ff) displayed random peptide and antibody libraries are widely used to identify specific, high affinity, binding ligands. A critical element in the identification of target-specific phages is to determine phage titers, not only at every round of selection, but also for normalizing phage titers of a set of individual clones for their comparative binding analysis. The conventional ELISA-based Ff titration methods require a minimum of 4–5 hr assay time and their lowest reported detection limit is ~50,000 particles/well.

In this report, we present a sandwich ELISA that allows detection of ~1000 Ff particles/well in less than 2.5 hr. The values of correlation of coefficient (r^2) for the curves at low phage concentrations (up to 10^6 TU/well) were about 0.999 in our ELISA. Experiments conducted at different temperatures suggest using 40°C incubations when titering low phage concentration samples. Experiments were also conducted with conventional ELISA for comparison. Our ELISA method derives an advantage from using a chemiluminescence substrate that gives much larger signals and wide linear range of measurement, thus allowing discrimination between background and low Ff phage concentrations. In conclusion, the Ff titration method presented here is highly sensitive, rapid, and amenable to high throughput analysis.

Keywords: Filamentous phage, Titration, ELISA, Phage-display selection

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INTRODUCTION

Filamentous bacteriophage (Ff) displayed random peptide and antibody libraries that represent rich sources of high-diversity ligands are widely used to probe protein-protein interactions, identify interacting partners and binding domains, and even home small molecules against these sites.[1,2] Specific, high affinity binding ligands are identified by several rounds of selection on target of interest to enrich the binding phage. A critical element in the identification of target-specific phages is to determine phage titers before and after every round of selection and for normalizing phage titers of a set of clones for their comparative binding study. Traditional biological methods of Ff titration, which rely on infection of an *E. coli* test strain, are satisfactorily sensitive, but time-consuming and, in spite of some developments,[3] they are far from automation for high-throughput systems. Recently developed procedures using real-time PCR appear to be promising, but are still to be tested widely.[4] Most commonly used physical methods for phage titration, independent of biological functions, include spectrophotometry and enzyme immunoassays. Spectrophotometric procedures require sample purification and are not very accurate.[5] The conventional ELISA-based Ff titration methods are accurate; however, they require a minimum of 4–5 hr assay time and their lowest reported detection limit ranges from 50,000 to 100,000 particles/well.[6–9]

In this report, we present a sandwich ELISA that allows detection of ~1000 Ff particles/well in less than 2.5 hr. The sensitivities of the published ELISA methods are expressed in terms of number of particles/well that generally imply the biological titers (TU, colony- or plaque-forming units, i.e., cfu or pfu), which differ based on the vectors/hosts and the conditions of the assays. Therefore, we conducted parallel experiments with conventional ELISA using the same Ff stock with a defined titer for a head-to-head comparison with our assay method.

EXPERIMENTAL

The phage-displayed random peptide library (RPL) used for the present study was constructed in our laboratory using fd-tet-derived fUSE5 vector that we received from George Smith.[10] The insert peptide sequences ($X_4CX_{10}CX_4$, where X = any amino acid) in the library were expressed as N-terminus fusion to minor coat pIII protein. The library phage titer was determined as tetracycline transducing units (TU) from three separate sets of experiments using K91/Kan *E. coli*.[5]

We used white polystyrene microplate wells (F96 Maxisorb™ Immuno plate; Nulge Nunc International, Roskilde, Denmark) that were coated overnight, in cold, with 100 μL (500 ng)/well of rabbit anti-fd bacteriophage (Sigma Chemical Co., St. Louis, MO) diluted in carbonate (15 mM

carbonate-35 mM bicarbonate) buffer, pH 9.6. The next day, wells were washed 3x with TBST (TBS, 25 mM Tris-HCl, 150 mM NaCl, pH 7.4, containing 0.05% Tween-20) and blocked for 15 min with 2% Hammerstein grade casein (HSGC; ICN Biochemical Inc., Aurora, OH) in TBS. We conducted blocking experiments with 2% bovine serum albumin (Sigma), 5% non-fat dry milk (Bio-Rad labs, Hercules, CA), or Pierce blocker casein (Pierce, Rockford, IL) also; however, 2% HSGC produced the best signal-noise ratio. Wells were washed 3x with TBST and once with TBS. 100 μL of diluted phage preparations were added to wells and incubated for 1 hr. Wells were washed 4x with TBST. 100 μL of HRP/anti-M13 monoclonal conjugate (Amersham Pharmacia Biotech, Little Chalfont, UK), diluted 3000x in TBST containing 0.2% HSGC, were added and incubated for 30 min. This antibody, which reacts specifically with the major phage coat protein pVIII, was purified from mouse ascites fluid. Wells were washed (3 min each) 5x with TBST and once with TBS. Chemiluminescence was developed with 100 μL of equally mixed (v/v) luminol/enhancer and stable peroxide buffer (SuperSignal femto substrate; Pierce, Rockford, IL) and read immediately using a microplate luminometer (Turner Biosystems, Sunnyvale, CA).

It is difficult to formulate one protocol that represents all reported phage ELISA methods because these procedures employ different phage immobilization strategies, blocking agents, antibodies, reporter enzymes, and their substrates.[6–9] The method representing conventional ELISA in our report uses longer time of incubations and colorimetric detection method, which are two common conditions found in all published phage ELISA procedures. For conventional ELISA, similar microplates, but neutral in color (F96 Maxisorb™ Immuno plate), were coated with rabbit anti-fd bacteriophage exactly the way described above. Wells were washed 3x with TBST and blocked for 1 hr with 2% HSGC. Following 3 washes with TBST and once with TBS, 100 μL of phage dilutions were added and incubated for 2 hr. Wells were washed 4x with TBST and incubated for 1 hr with 100 μL of HRP/anti-M13 monoclonal conjugate diluted as above. Following 5 washes (3 min each) with TBST and once with TBS, 100 μL ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma) was added and, after 30 min, HRP was assayed at A_{405nm}. In both assays, the incubation steps with blocker, phage and mAb-HRP were conducted at room temperature; however, in some experiments, they were done at higher temperatures (30–45°C) as well.

RESULTS AND DISCUSSION

The data presented in Figure 1 show that measurable signals appeared at 1000 phage TU in the chemiluminescence assay (A) and at 100,000 TU in conventional ELISA (B), indicating about a 100-fold higher detection sensitivity with

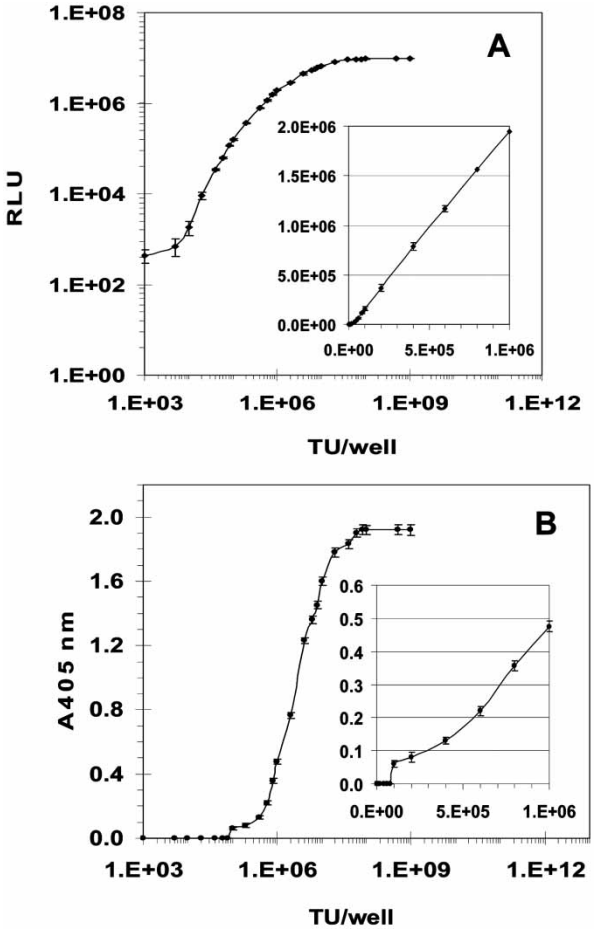

Figure 1. Titrations of filamentous bacteriophage conducted by the present chemiluminescence ELISA (A) and conventional ELISA (B). TU on the x-axis represents phage titers as determined by biological assay. Relative light units (RLU) or A_{405} nm on the y-axis represent chemiluminescence or absorbance, respectively. Each data point with a bar represents mean $\pm$ standard deviation of triplicate samples ($n = 3$). The in-set figures highlight the assay performances at the low concentration range.

our procedure. A comparison of the two graphs demonstrates better proportional values at lower phage concentrations in our assay, in comparison to the conventional procedure. Furthermore, a wider linear range is also evident in chemiluminescence ELISA. The data in Figures 2A and 2B were analyzed and drawn, for linear regression, at low TU range (up to 10^6) by using Prism 4.0 software (GraphPad, San Diego, CA). The values for correlation coefficient (r^2) were about 0.999 in our ELISA. The data from

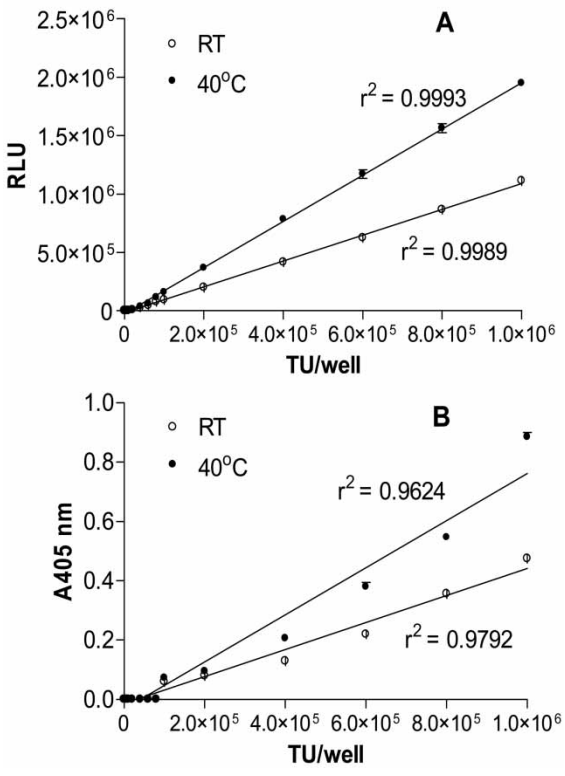

Figure 2. Linear graphs of filamentous bacteriophage titrations done at different temperatures using present chemiluminescence ELISA (A) and conventional ELISA (B). TU on the x-axis represents phage titers as determined by biological assay. Relative light units (RLU) and A_{405} nm on the y-axis represent chemiluminescence and absorbance, respectively. Each data point with a bar represents mean $\pm$ standard deviation of triplicate samples ($n = 3$). Incubation steps were conducted at room temperature (RT) or at 40°C. The values of correlation of coefficient (r^2) of each assay are presented next to the respective lines.

incubations at higher temperatures demonstrated maximum signals at 40°C for both the assay procedures. This suggests using 40°C incubations when titering low phage concentration samples. Figure 2 shows the linear graphs at 40°C for both the ELISAs.

These results show a higher sensitivity of our chemiluminescence ELISA method for phage quantification at low concentrations. This ELISA requires less than 2.5 hr, which is about half the time taken by a conventional ELISA. Our ELISA method derives an advantage from using chemiluminescence substrate which gives much larger signals, thus allowing discrimination

between background and low phage concentrations in these experiments. Chemiluminescence detection also provides a wide linear range of measurement. In addition, the use of anti-pVIII, which recognizes a multivalent target (major coat protein, ~2800 copies), helped to achieve a higher sensitivity compared to the methods based on minor coat protein pIII (5 copies) detection.[8] Literature searches revealed no phage ELISA that could match the rapidity of our method and only one report[12] that demonstrated equal or better sensitivity than the present ELISA. However, the plotted mean absorbance values (without error bars) in this report[12] show low and almost equal ODs ($\leq$0.1) for 100 and 1000 fd-phage particles. This method uses three enzymes and their substrates to conduct sequential reactions for signal amplification, and requires 4.5 hr exclusively for different incubations.

In the course of our experiments, repeated chemiluminescence ELISA of one distinct stock of phage under consistent conditions as described earlier gave titers which were highly reproducible. Multiple assays of the same sample in an experiment produced 1% to 7% (intra-assay) variation. In these experiments, only the linear part of the standard curves was used to derive values of unknown samples. The coefficient of variation (inter-assay) in this part of the curves ranged from 3 to 19%, as calculated from 6 different sets of experiments. Spiking experiments performed to address the issue of sample matrix effects demonstrated that phage samples in LB media could be used directly (without any purification step) in our ELISA titration.

The improved ELISA described here can be used with excellent reproducibility and ease for following an enrichment of target-binding clones at successive rounds of panning using filamentous phage displayed libraries as a source of high diversity ligands. Since the biological titers of phage preparations from different vectors/hosts cannot be compared for their actual phage concentrations, this ELISA would find use in equalizing (in terms of RLU) Ff concentrations in preparations from different hosts, vectors, and libraries before comparing their binding affinities to a target. Our ELISA method can be directly adopted for the binding assessment of antibody/peptide-clones to a target, which is not possible with methods that require phage disaggregation before detection.[8] Furthermore, this procedure can also be used for the titration of non-infective phages in selectively infective phage (SIP) technology.[11] In conclusion, this study describes a chemiluminescence ELISA that allows rapid quantification of small numbers of Ff particles in microplate setting that can be adapted in high-throughput experiments using phage display technology.

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