

# Phospho-specific recognition by 14-3-3 proteins and antibodies monitored by a high throughput label-free optical biosensor

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**Abstract** Label-free detection of molecular interactions has considerable potential in facilitating assay development. When combined with high throughput capability, it may be applied to small molecule screens for drug candidates. Phosphorylation is a key posttranslational process that confers diverse regulation in biological systems involving specific protein–protein interactions recognizing the phosphorylated motifs. Using a resonant waveguide grating biosensor, the Epic™ System, we have developed a generic assay to quantitatively measure phospho-specific interactions between a trafficking signal-phosphorylated SWTY peptide and 14-3-3 proteins or anti-phosphopeptide antibodies. Compared with a solution-based fluorescence anisotropy assay, our results support that the high throughput resonant waveguide grating biosensor system has favorable technical profiles in detecting protein–protein interactions that recognize phosphorylated motifs. Hence it provides a new generic HTS platform for phospho-detection.

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**Keywords:** Optical biosensor; High throughput; Resonant waveguide grating; Label-free; 14-3-3 and phosphopeptide

## 1. Introduction

Phosphorylation is a major posttranslational process which often results in reorganization of protein–protein interactions

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**Abbreviations:** Ahx, 6-aminohexyl-; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DMSO, dimethylsulfoxide; DTT, dithiothreitol; ELISA, enzyme linked immunosorbant assay; GST, glutathione S-transferase; LOD, limit of detection; LR, linear range; NH<sub>2</sub>-SWpTY, NH<sub>2</sub>Ahx-AhxFRGRSWpTY-COOH; NH<sub>2</sub>-SWTY, NH<sub>2</sub>Ahx-Ahx-FRGRSWTY-COOH; HEPES, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid; NP40, octylphenolpoly(ethyleneglycoether); PEG-amine, O,O'-Bis(2-aminopropyl) polyethylene glycol 1900; R18, NH<sub>2</sub>-PHCVPR-DLSWL-DLEANMCLP-COOH; RWG, resonant waveguide grating; SWpTY, RGRSWpTY-COOH; SWTY, RGRSWTY-COOH; SWpTD, RGRSWpTD-COOH; SWpTP, RGRSWpTP-COOH; SWpTG, RGRSWpTG-COOH; SPR, surface plasmon resonance

and therefore converts an enzymatic reaction into a cellular event [1]. Changes in phosphorylation patterns and protein interactions are hallmarks of human diseases, especially prominent in cancers where kinases and phosphatases are critical players [2]. Ubiquitous in all eukaryotic cells, dimeric 14-3-3 proteins are one of the first phospho-residue recognition protein modules to be identified. 14-3-3 proteins interact with numerous binding proteins and are involved in a wide variety of physiological functions including signal transduction, apoptosis, protein trafficking and localization, metabolism, cell motility, and malignant transformation (see reviews [3–5]). Furthermore, 14-3-3 proteins have been used as a diagnostic marker for prion-related diseases such as Creutzfeldt-Jakob disease or mad cow disease [6]. Based on studies using synthetic peptides [4], three modes of 14-3-3 mediated interaction were reported [7–9] and a variety of kinases have been implicated in target phosphorylation. Because of the critical role in signaling pathways, 14-3-3 proteins also represent a potential target for therapeutic intervention in cancer [10]. Therefore, rapid and reliable detection of phosphorylation-induced interactions, such as those mediated by 14-3-3 proteins, is of considerable interest both for detection and for identifying small molecule modulators.

In addition to 14-3-3 proteins which recognize phosphorylated sequences, antibodies have been raised in order to identify proteins only after they have been phosphorylated. The discriminatory ability of antibodies in recognizing the same amino acid sequences with or without phosphate is therefore the key in judging the utilities of anti-phosphopeptide antibodies.

Label-free detection technologies have long been recognized for their ability to probe biomolecular interactions [11] without the need of labeling the target molecules. Different techniques, such as surface plasmon resonance (SPR), optical ellipsometry, quartz crystal microbalance, and calorimetry, have been developed. Among them, SPR has been widely applied for diverse research. However, its compatibility with high throughput screening remains a challenge, although the development of SPR imaging has shown the potential for increasing the throughput. On the other hand, resonant waveguide grating (RWG) sensors have recently been developed into a high throughput, microplate-based biosensor system, the Corning® Epic™ System, combining the features of label-free detection with high throughput capability [12]. Label-free optical biosensors offer the possibility of developing a generic assay for evaluating protein–protein interactions specific to phosphorylated

target sequences. Future developments may allow for detection of protein–protein interaction using crude cell lysates.

Using a genetic screen of random peptides for trafficking signals, we have identified a series of membrane transport signals [13]. Further characterization has demonstrated that SWTY motif upon phosphorylation recruits 14-3-3. As results, it has enhanced forward transport activities to increase steady state surface expression of membrane proteins [9,13]. Solution assays have been developed for quantifying the SWTY-14-3-3 interaction [14]. In addition, we have developed specific anti-phosphopeptide antibodies for SWTY motifs. Hence this interaction complex offers an ideal system to evaluate the optical sensor for its ability to detect and evaluate the specificity of phospho-interactions.

## 2. Materials and methods

### 2.1. Reagents

All inorganic salts were of analytical purity and were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. PEG-amine (O,O'-Bis(2-aminopropyl)polyethylene glycol 1900), ethanolamine, boric acid, dimethylsulfoxide (DMSO), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), octylphenolpoly(ethylene-glycolether) (NP40), Triton X-100, and dithiothreitol (DTT) were all purchased from Sigma–Aldrich (St. Louis, MO). All solutions were prepared in a 10 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7.3) unless indicated. Anti-SWpTY antibody produced from rabbit was from Sigma Genosys (St. Louis, MO). Peroxidase labeled anti-Rabbit IgG (H+G, made in goat) was from Vector laboratories, Inc. (Burlingame, CA), anti-14-3-3 antibody (made in rabbit) from Santa Cruz Biotechnology (Santa Cruz, CA), and Supersignal enzyme linked immunosorbant assay (ELISA) Femto Maximum Sensitivity substrate kit from Pierce (Rockford, IL).

### 2.2. Preparation and purification of recombinant 14-3-3 proteins

14-3-3 $\zeta$  protein was expressed as a glutathione *S*-transferase (GST)-tagged fusion and purified from *E. coli* strain BL21-SI (Invitrogen, Carlsbad, CA) as previously described [14]. Recombinant GST-tagged 14-3-3 proteins were purified using Glutathione–Sepharose 4B beads (Invitrogen, Carlsbad, CA). Fluorescence anisotropy measurements were used to determine the binding affinity of 14-3-3 $\zeta$  for SWpTY [14].

### 2.3. Synthesis of peptides

NH2-SWpTY (NH2AhxAhx-FRGRSWpTY-COOH, Ahx: 6-amino-hexyl-) peptide and non-phosphorylated NH2-SWTY (NH2-AhxAhx-FRGRSWTY-COOH) peptide were synthesized by Biomer (San Diego, CA). The peptides SWpTY, RGRSWpTY-COOH; SWTY, RGRSWTY-COOH; SWpTD, RGRSWpTD-COOH; SWpTP, RGRSWpTP-COOH; and SWpTP, RGRSWpTP-COOH were from New England Peptides (Boston, MA). The peptides were prepared as stock solutions by dissolving in water, and if necessary with the addition of a minimal amount of acetonitrile.

### 2.4. Resonant waveguide grating biosensor detection

A beta version of the Corning<sup>®</sup> Epic<sup>™</sup> System with an on-board liquid handling system was used for all experiments. The details of the instrument have been described elsewhere [12,15]. Briefly, each well in the 384-well Epic<sup>™</sup> microplate contains an RWG sensor, which consists of an optical grating and a high index of refraction waveguide coating. When illuminated with broadband light at a fixed angle of incidence, these sensors reflect only a narrow band of wavelengths that is a sensitive function of the effective index of refraction of the waveguide. The sensors are coated with a surface chemistry layer that enables covalent attachment (via peptide bond formation) of peptides/proteins or other biomolecules. Binding of molecular recognition partners to the immobilized target induces a change in the effective index of refraction of the waveguide, and this is manifest as a shift in the wavelength of light that is reflected from the sensor. The magnitude of this wavelength shift is proportional to the amount of analyte that binds to

the immobilized target. Unlike most commercial SPR biosensors, which generally use a continuous flow system for the determination of the kinetics of binding, the Epic<sup>™</sup> System does not utilize flow channels. RWG sensors are evanescent in nature which means that the magnitude of the electric field in the medium adjacent to the sensor surface decays exponentially from the sensor surface. The distance from the sensor surface at which the electric field strength has decreased to 1/e of its initial value is the penetration depth. For the Epic<sup>™</sup> System, the penetration depth is ~150 nm. Thus, the system is selective and sensitive to binding events that take place within this penetration. For most of the experiments described here, a well adjacent to the sample well was used as a reference. The most recent version of the Epic<sup>™</sup> microplate utilizes a self-referencing scheme in which each well of the plate has its own reference region. This is enabled by a plate design that provides protein binding chemistry on only half of the sensor surface so that when a solution of peptide/protein target is added to the well, it only binds to half of the sensor surface, leaving the other half as an in-well or self-reference.

### 2.5. 14-3-3 detection protocol on the Epic<sup>™</sup> System

As a common protocol for 14-3-3 detection (Fig. 1), the first step is to immobilize NH2-SWpTY peptide (50  $\mu$ g/ml, pH 7.5), as a 14-3-3 binding motif, on the Epic<sup>™</sup> plate. This was followed by the addition of ethanolamine to quench the remaining active sites on the surface. Reference wells were made by the addition of PEG-amine (50  $\mu$ g/ml, pH 9.2) or the non-phosphorylated peptide NH2-SWTY (50  $\mu$ g/ml, pH 7.5). The second step is the addition of 14-3-3 protein solutions into both the sample wells and the reference wells. As seen in Fig. 1C, the wavelength shift after the subtraction of the signals from the reference wells is termed the referenced signals for the 14-3-3 binding event. The signal without subtraction of the reference is termed the unreferenced signal. Usually six sample wells and two reference wells were used for each binding reaction unless otherwise described.

To further validate the 14-3-3 binding event on the Epic<sup>™</sup> plates, the same Epic<sup>™</sup> plates above were then subjected to an ELISA. After washing 5 times with PBS buffer, 50  $\mu$ l of the anti-14-3-3 antibody (1:200 dilution) was added and incubated at 4 °C for 0.5 h. After washing with PBS buffer with 0.1% Tween, 50  $\mu$ l of peroxidase labeled anti-rabbit IgG (1:1000 dilution) was added and incubated at 4 °C for 0.5 h. After washing with PBS buffer with 0.1% Tween again, 50  $\mu$ l of luminescence substrate from Supersignal ELISA Femto Maximum Sensitivity substrate kit was added to each well and subjected to luminescence detection on a multifunctional plate reader Safire<sup>2</sup> (Tecan US, Research Triangle Park, NC).

### 2.6. Affinity ranking by competitive replacement

The rank order of affinity of the peptide motifs was determined by competition assays. After immobilization of NH2-SWpTY on the Epic<sup>™</sup> plate, a pre-mixed solution of 14-3-3 and varying concentrations of the competitive peptides were added into the Epic<sup>™</sup> wells. The referenced signals were applied in Eq. (1) to determine the relative affinities of the corresponding competitive peptides

$$y = B * \left\{ \left( \frac{K_{D_{\text{NH-SWpTY}}}}{K_{D_{\text{competitor}}}} x + K_{D_{\text{NH-SWpTY}}} + D_0 + P_0 \right) - \sqrt{\left( \frac{K_{D_{\text{NH-SWpTY}}}}{K_{D_{\text{competitor}}}} x + K_{D_{\text{NH-SWpTY}}} + D_0 + P_0 \right)^2 - 4 * D_0 * P_0} \right\}. \quad (1)$$

The fitting was done with Eq. (1) using Origin 7.0 (OriginLab, Northampton, MA), with  $y$  as the referenced binding signal;  $B$  as a constant;  $D_0$  as the concentration of NH2-SWpTY that conferred 90% of saturated GST-14-3-3 $\zeta$  response;  $P_0$  as the concentration of GST-14-3-3;  $K_{D_{\text{NH-SWpTY}}}$  as the  $K_D$  (=2.1  $\mu$ M) for NH2-SWpTY binding to GST-14-3-3 $\zeta$  as determined by the concentration response of GST-14-3-3 $\zeta$  with 50  $\mu$ g/ml of immobilized NH2-SWpTY;  $K_{D_{\text{competitor}}}$  as  $K_D$  for competitive peptides, and  $x$  as the concentration of competitor peptide.

### 2.7. Characterization for high throughput screening

To characterize the capability of the Epic<sup>™</sup> System for high throughput screening of 14-3-3 phospho-specific interactions, 50  $\mu$ g/ml of NH2-SWpTY at pH 7.5 was immobilized in one set of rows and PEG-amine was immobilized in a second set of rows on the Epic<sup>™</sup>

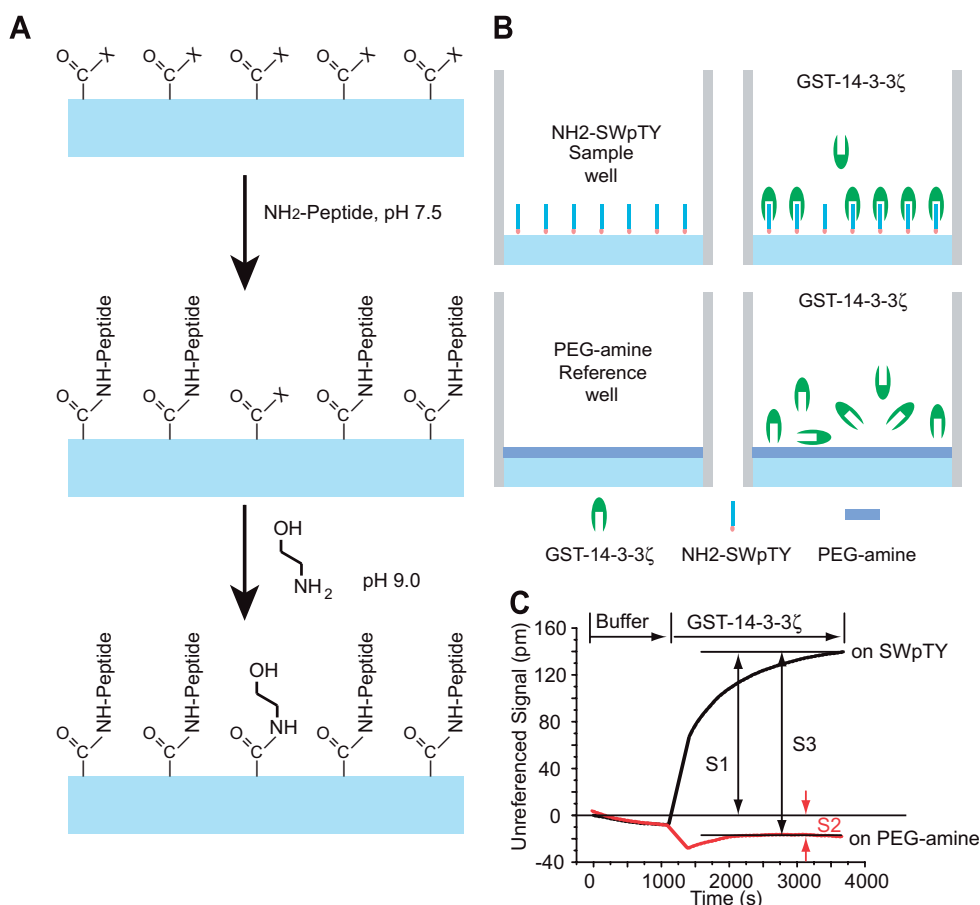


Fig. 1. Scheme of Epic™ detection. (A) Surface reaction scheme for immobilization of N-terminal amino group modified peptides. X, reactive group; NH<sub>2</sub>-Peptide, N-terminal amino group modified peptides. (B) Binding scheme for 14-3-3. Left two panels show the immobilized substrate (NH<sub>2</sub>-SWpTY) and reference (PEG-amine). Right two panels show GST-14-3-3ζ binding. In the sample well, where GST-14-3-3ζ is added, GST-14-3-3ζ binds to the immobilized NH<sub>2</sub>-SWpTY peptide. In the reference well containing immobilized PEG-amine, there is no binding of GST-14-3-3ζ. (C) The time-course charts of the respective responses from sample wells and reference wells, with S1 as an unreferenced signal from GST-14-3-3ζ on NH<sub>2</sub>-SWpTY (50 μg/ml at pH 5.5, GST14-3-3 at 1 μM), S2 as an unreferenced signal from GST-14-3-3ζ on PEG-amine (50 μg/ml, pH 9.0 GST14-3-3 at 1 μM), and S3 as a referenced signal obtained by subtraction of S1 with S2.

plate with PEG-amine immobilized wells used as a reference. GST-14-3-3ζ at 1 μM with varying DMSO amounts was added to test the compatibility of adding small molecule library in DMSO solution. GST-14-3-3ζ at 1 μM with or without pre-incubated 20 μM SWpTY (as a positive inhibition control) was added into the wells of one Epic™ plate with intrawell self-referencing for the statistic data. The Z factor and S/N ratio [16] were calculated from Eqs. (2) and (3), respectively, where  $A_v$  is the average and S.D. is the standard deviation of the referenced signals

$$Z = 1 - 3 * (S.D._{\text{With 14-3-3}} + S.D._{\text{Without 14-3-3}}) / (A_{v_{\text{With 14-3-3}}} - A_{v_{\text{Without 14-3-3}}}) \quad (2)$$

$$S/N = (A_{v_{\text{With 14-3-3}}} - A_{v_{\text{With 14-3-3 and SWpTY}}}) / \sqrt{(S.D._{\text{With 14-3-3}})^2 + (S.D._{\text{With 14-3-3 and SWpTY}})^2} \quad (3)$$

### 3. Results

#### 3.1. Interaction between 14-3-3 and NH<sub>2</sub>-SWpTY peptide

The label-free detection of 14-3-3 interactions with its phosphorylated partners was performed using a RWG system known as the Epic™ System (see Section 2). The detection

scheme is based on the direct covalent immobilization of NH<sub>2</sub>-SWpTY (NH<sub>2</sub>AhxAhx-FRGRSWpTY-COOH) peptide on the surface of the Epic™ plates (Fig. 1A). SWpTY is a member of the mode III consensus motif family that interacts with 14-3-3 (see review, [17]). In solution, synthetic SWpTY peptide binds to recombinant 14-3-3 with a dissociation constant ( $K_D$ ) of 0.17 μM, comparable to the affinity of canonical modes I and II binding [14]. The Epic™ System exploits the evanescent wave that is generated by the resonant coupling of light into a waveguide via a diffraction grating [18]. Hence, the interactions within ~150 nm vertical range may be detected, with no requirement for removal of the interaction solution. Furthermore, the micro-titer plate format of the Epic™ System allows for the open access for parallel and sequential additions of analytes, a feature that is more difficult to implement in a flow cell-based system.

Fig. 1B shows, upon GST-14-3-3ζ addition, the sample well with bound GST-14-3-3ζ on the NH<sub>2</sub>-SWpTY-immobilized surface gives a binding signal while the unbound 14-3-3 out of the ~150 nm penetration depth is not detected. Conversely the reference well with PEG-amine immobilized surface gives very low signal upon GST-14-3-3ζ addition.

Fig. 1C shows the evolution of the binding signal as a function of time. Unlike SPR experiments performed with flow channels in which true kinetic measurements can be made, the binding in the Epic™ plates is diffusion limited and, therefore, kinetic measurements are also diffusion limited. Nonetheless, equilibrium binding measurements and estimation of affinity can be accurately determined.

To determine the feasibility of detection with the above mentioned scheme, different concentrations of NH<sub>2</sub>-SWpTY were immobilized and tested for the interaction with 0.4 μM of recombinant 14-3-3ζ. The signal increased in a dose-dependent manner (Fig. 2, open squares). To validate that this increase of signal indeed reflects increased binding of 14-3-3 proteins, the same Epic™ plates were then subjected to an ELISA using an anti-14-3-3 antibody (see Section 2). The ELISA signals proportionally matched those of the Epic™ System (Fig. 2, filled circles). In contrast, reference wells with PEG-amine did not produce any ELISA signal (Fig. 2, open circles). Hence, immobilization of NH<sub>2</sub>-SWpTY peptide at 50 μg/ml allows for approximately 80% of the maximal signal and therefore was used for the subsequent experiments.

To evaluate the specificity of the interaction, 14-3-3 was added into wells immobilized with either NH<sub>2</sub>-SWpTY or non-phosphorylated NH<sub>2</sub>-SWTY (Fig. 3A). Only wells immobilized with the phosphorylated peptide NH<sub>2</sub>-SWpTY gave detectable signals. Furthermore, upon addition of competitive 14-3-3 binding peptides, SWpTY or a non-homologous 14-3-3 binding peptide NH<sub>2</sub>-PHCVPRDLSWLDLEANMCLP-COOH (R18), the binding signals were significantly reduced (Fig. 3B). In contrast, the non-phosphorylated SWTY peptide did not affect the binding of 14-3-3. To be more definitive, the SWpTY peptide binding was tested with wild type 14-3-3 and 14-3-3 K49E, which has a mutation at the binding site [19]. Consistently, the binding signal was obtained only in wild type 14-3-3 proteins (Fig. 3C). Therefore, these experimental results provide the evidence for the specific detection of 14-3-3 interactions with substrate peptides.

### 3.2. Optimization of biosensor detection

To optimize the detection, we examined conditions for both substrate immobilization and binding reactions. First, the immobilization efficiency of NH<sub>2</sub>-SWpTY on the Epic™ plate surfaces was compared under different pH and immobilization time. Fig. 4A shows that the immobilization of NH<sub>2</sub>-SWpTY peptide is more effective at a pH between 7.0 and 10.0. With respect to immobilization time, we found little effect on the overall 14-3-3 signal (Fig. 4B). Hence, the standard experiments were performed at pH 7.5 for 20 min. Because the mode III binding requires free carboxyl terminus and arginine [9,13], the results suggest the attached primary amino-terminal amine group is suitable for immobilization and the side chains of arginine residues in RGRSWpTY were not the primary immobilization sites. Thus, these immobilization conditions should be generally applicable to peptides with a similar conjugation group.

To compare and contrast potential differences in monitoring 14-3-3 binding in solution by fluorescence anisotropy versus that by an optical biosensor, we further examined the following binding conditions: ionic strength, bulk refractive index matching reagents (such as bovine serum albumin, BSA), pH and detergents.

Fig. 5A shows that NaCl concentration affects the 14-3-3 signals and gives a higher signal at higher concentrations. In contrast, previous results with fluorescence anisotropy have shown little effect of NaCl concentration in a homogeneous binding assay [14]. Further analysis suggested that the increased referenced signal with NaCl is mainly due to the decrease of un-referenced signals in reference (PEG-amine) wells (data not shown). Higher ionic strength presumably reduces the non-specific association of proteins with the PEG-amine-treated reference wells.

Common to SPR and RWG, a feature of the Epic™ System is that the bulk refractive index can have a large impact on the signals. This effect can be effectively eliminated by proper use of referencing. Fig. 5B shows that the signals after correction with the reference wells are similar compared with different un-referenced signals (data not shown) at different concentrations of

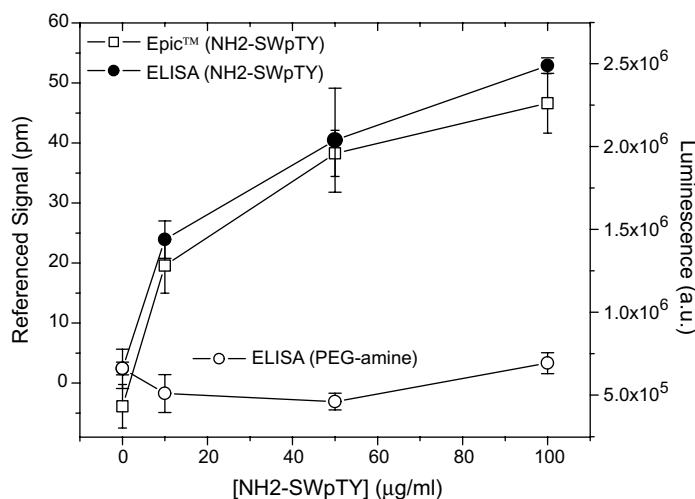


Fig. 2. Binding detection of 14-3-3 to immobilized NH<sub>2</sub>-SWpTY peptide. NH<sub>2</sub>-SWpTY in different concentrations (pH 5.5) was immobilized on the Epic™ plate. After addition of GST-14-3-3ζ (0.4 μM), the referenced signals were plotted on the left axis against the concentrations of NH<sub>2</sub>-SWpTY. The same plate was used for ELISA detection as described in Section 2 and the luminescence signals were plotted on the right axis.

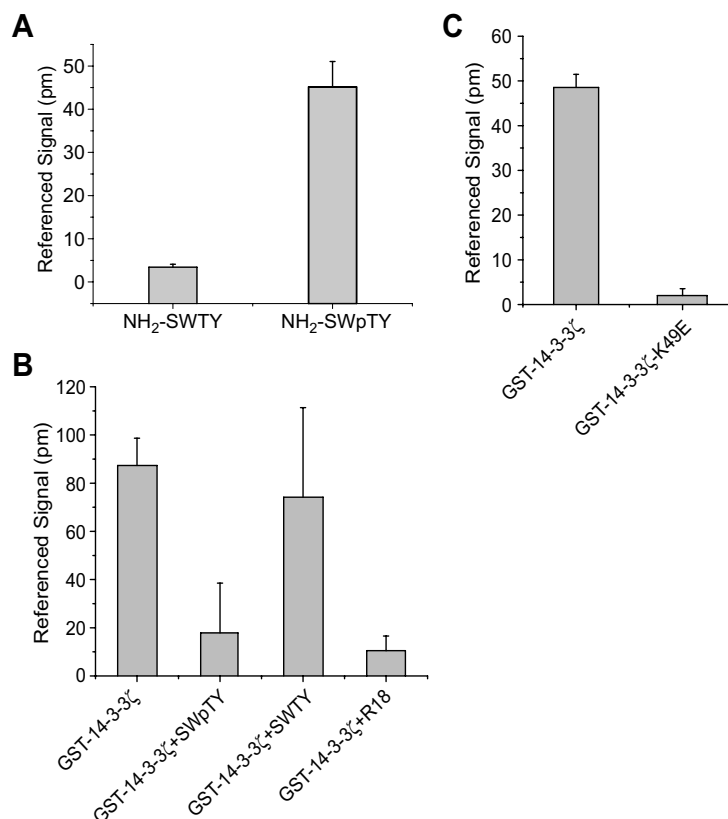


Fig. 3. Specificity of detection. (A) The referenced signals for the binding of 14-3-3 to immobilized SWpTY, and SWTY. SWpTY, and SWTY were immobilized at 50  $\mu\text{g/ml}$ , pH 5.5, and GST-14-3-3 $\zeta$  was added at 0.4  $\mu\text{M}$ . (B) Competition assay with competitors and control. 1  $\mu\text{M}$  of competitors (SWpTY and R18) and control peptide (SWTY) were pre-incubated with 0.4  $\mu\text{M}$  GST-14-3-3 $\zeta$  and applied on the immobilized NH2-SWpTY (50  $\mu\text{g/ml}$ ). (C) Comparison of the referenced signals from the GST-14-3-3 $\zeta$  and its validated binding mutant GST-14-3-3 $\zeta$  (K49E). GST-14-3-3 was used at 0.4  $\mu\text{M}$ .

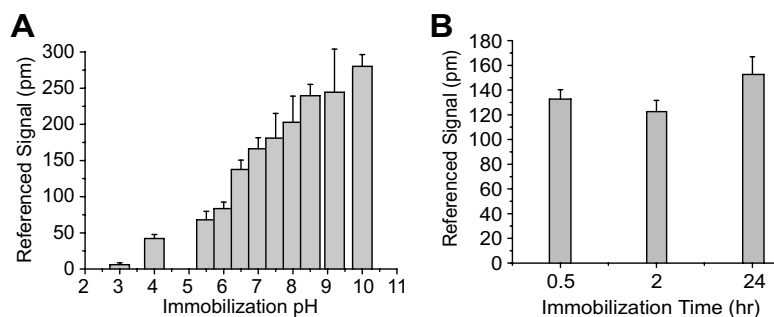


Fig. 4. Optimization of peptide immobilization. (A) pH effect on the immobilization of NH2-SWpTY on Epic™ plates. The peptides were immobilized in HEPES buffers of different pHs. GST-14-3-3 $\zeta$  was used at 0.4  $\mu\text{M}$ . (B) Immobilization time effect on 14-3-3 binding. NH2-SWpTY was immobilized at 50  $\mu\text{g/ml}$ , pH 5.5 and GST-14-3-3 $\zeta$  was used at 0.4  $\mu\text{M}$ .

BSA. The addition of 0.1% BSA caused a  $\sim 57$  pm shift in the unreferenced signal, with no change for the referenced signals, and the referenced signal levels were independent on the amount of BSA present (Fig. 5B). These results also suggest that there is little to no non-specific binding of BSA to the surface.

Fig. 5C shows the effect of pH on the binding signals. At pH 8.5, significantly reduced binding was observed. This is likely due to the effect on binding itself and was observed by fluorescence anisotropy as well [14], although it is possible that higher pH may also affect conformation of 14-3-3 and its binding partners.

The inclusion of detergents often results in the improvement of the stringency of binding. In some cases, it can reduce the aggregation of proteins in vitro. Binding buffers supplemented with 1% of CHAPS, NP40, or Triton X-100 did not have significant effects on the 14-3-3 signals (Fig. 5D).

### 3.3. Specification of biosensor detection

Using the above optimized conditions, the assay sensitivity was studied. The whole range of GST-14-3-3 $\zeta$  binding signals is shown in Fig. 6. The limit of detection (LOD) for GST-14-3-3 $\zeta$  is shown in inset of Fig. 6. For GST-14-3-3 $\zeta$ , the

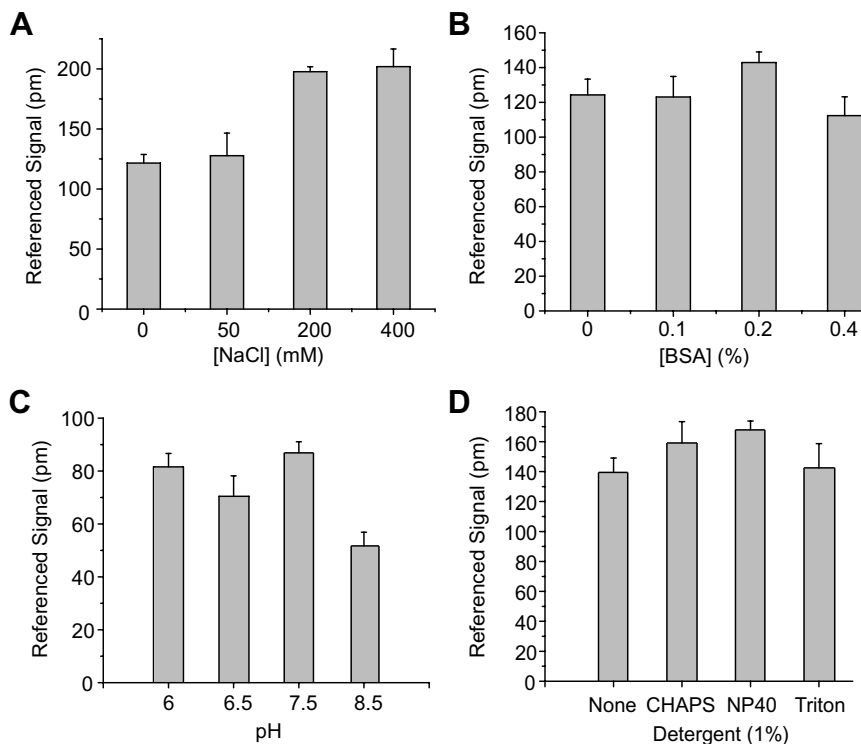


Fig. 5. Optimization of Epic™ binding conditions. (A) Ionic strength effect on Epic™ signals. GST-14-3-3 $\zeta$  (0.4  $\mu$ M) was applied to immobilized NH2-SWpTY (50  $\mu$ g/ml) in the presence of different concentrations of NaCl. (B) Bulk refractive index effect on Epic™ signals. Bulk refractive index was modulated with BSA. GST-14-3-3 $\zeta$  (0.4  $\mu$ M) was applied to immobilized NH2-SWpTY (50  $\mu$ g/ml) in the presence of different concentrations of BSA. (C) pH effect on Epic™ signals. GST-14-3-3 $\zeta$  (0.4  $\mu$ M) in a HEPES buffer of varying pH was applied to immobilized NH2-SWpTY (50  $\mu$ g/ml). (D) Effect of detergents on the Epic™ signals. GST-14-3-3 $\zeta$  (0.4  $\mu$ M) was applied to immobilized NH2-SWpTY (50  $\mu$ g/ml, pH 7.5) in the presence of different detergents at 1%.

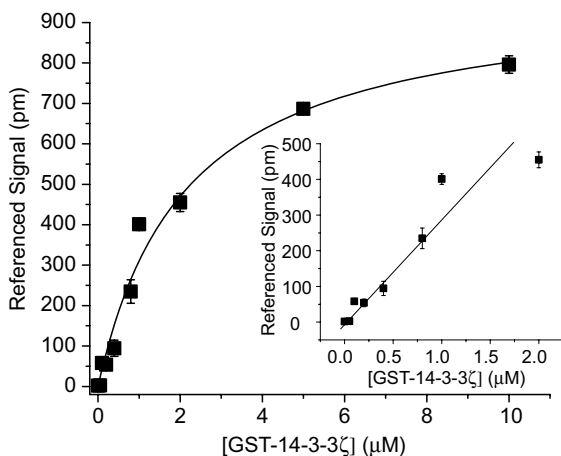


Fig. 6.  $K_D$  estimation and LOD and LR for 14-3-3 binding. The referenced signals were plotted against GST-14-3-3 $\zeta$  concentrations. NH2-SWpTY was immobilized at 50  $\mu$ g/ml at pH 7.5.  $K_D$  was estimated by single site binding model. Inset: LOD and LR for GST-14-3-3 $\zeta$  binding when 50  $\mu$ g/ml of NH2-SWpTY was immobilized at pH 7.5.

LOD is 38 nM with a linear range (LR) of 0.038–2  $\mu$ M ( $R^2 = 0.96$ ).

The density of immobilized NH2-SWpTY influences the detection range of GST-14-3-3 binding. At lower densities of NH2-SWpTY (achieved by performing the immobilization

reaction at pH 5.5), the upper limit of the LR was reduced to 800 nM. The LOD, however, was not significantly affected by the amount of immobilized peptide.

Solid phase-based interactions can be used to estimate the affinity of interaction between the interacting pairs. The direct fitting of the one binding site with the data shown in Fig. 6 can offer a direct estimation of the equilibrium affinity of GST-14-3-3 $\zeta$  with immobilized NH2-SWpTY, i.e., 2.0  $\mu$ M, which is similar to the fluorescence anisotropy detection in solution. Change in the density of the immobilized phosphopeptide (with different pH) has not caused significant alteration in binding curve. The lower density only results in lower signals and a narrower detection range (Fig. 6).

To determine the rank order of the affinity of various peptides for 14-3-3, a competition assay was conducted. In contrast to the direct detection using the immobilized binding partner, the competition assay uses the binding competitors to measure binding affinity in solution. Affinity detection through competitive replacement has been a routine method [20,21]. The synthetic peptides, SWpTY, SWTY, SWpTD, and SWpTP have  $K_D$  values of  $0.17 \pm 0.04$   $\mu$ M,  $>100$   $\mu$ M,  $2.2 \pm 0.3$   $\mu$ M, and  $45 \pm 11$   $\mu$ M, respectively for His-14-3-3 $\zeta$ , as determined by a homogeneous fluorescence anisotropy assay [14]. Fig. 7A shows the distinct signals by competitor peptides with considerably different affinities using the Epic™ System. The fitting results for  $K_D$  values of the competitors SWpTY, SWTY, SWpTD, and SWpTP are  $0.12 \pm 0.05$   $\mu$ M,  $>50$   $\mu$ M,  $1.75 \pm 0.14$   $\mu$ M, and  $6 \pm 1.67$   $\mu$ M, respectively. Direct comparison of this method with the previous detection

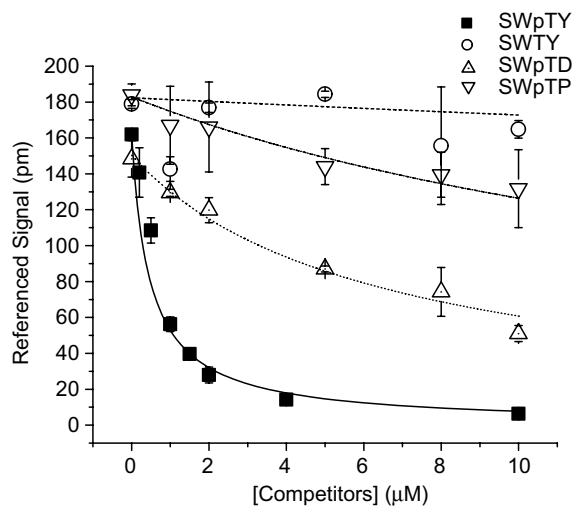


Fig. 7. Affinity ranking measurement using different competitive peptides. The competition assay was carried out with pre-incubation of the competitors with 1.5  $\mu\text{M}$  of GST-14-3-3 $\zeta$  before adding into the Epic<sup>TM</sup> sample wells. 50  $\mu\text{g}/\text{ml}$  of NH<sub>2</sub>-SWpTY was immobilized at pH 4.0. (■) SWpTY, (○) SWTY, (△) SWpTD, (▽) SWpTP.

of SWTY-14-3-3 interaction with fluorescence anisotropy suggests a similar rank order of the affinity.

### 3.4. Comparison of two different phosphopeptide-detection systems

To further explore the applicability of the Epic<sup>TM</sup> System to different phospho-recognizing modules, an anti-SWpTY antibody has been applied using the same detection format. The LOD for anti-SWpTY antibody is 2.9 nM, which is approximately 10-fold higher in sensitivity than that of 14-3-3, with a LR from 2.9 to 100 nM (Fig. 8A). This is also in accordance with the LOD and  $K_D$  correlation [22].

The affinity difference between anti-SWpTY and GST-14-3-3 $\zeta$  can be best visualized in Fig. 8B. The normalized Epic<sup>TM</sup> signal indicates that the anti-SWpTY antibody has a much higher affinity for the immobilized NH<sub>2</sub>-SWpTY relative to 14-3-3. The detection with anti-SWpTY antibody binding to its antigen offers a possibility of using the Epic<sup>TM</sup> System for the characterization and profiling of phospho-specific interactions by antibodies.

### 3.5. Compatibility of the Epic<sup>TM</sup> System for high throughput screening

The detection of 14-3-3 binding by the Epic<sup>TM</sup> System was further examined for the capability of high throughput screening. Since most of the small molecule libraries are dissolved in DMSO, consequently the effect of DMSO, or the tolerance of DMSO should be checked for high throughput screen compatibility. As expected, the presence of DMSO did result in a change of bulk refractive index and hence a change in the *unreferenced* signals of both test wells and reference wells (data not shown). However, as shown in Fig. 9A, proper use of referencing eliminates this effect and thus there is no significant difference for the referenced signals between 0% DMSO and 10% DMSO.

The responses of GST-14-3-3 $\zeta$  with or without pre-incubated SWpTY were also studied using SWpTY as a positive inhibition control for the robustness and statistic data of the

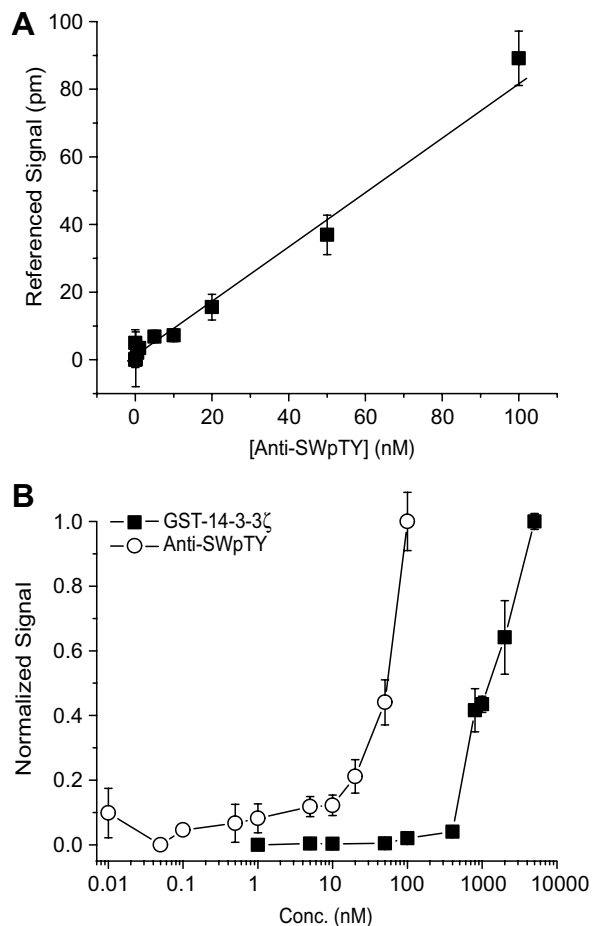


Fig. 8. Comparison of two different phospho-detection systems. (A) LOD and LR of anti-SWpTY binding, when 50  $\mu\text{g}/\text{ml}$  of NH<sub>2</sub>-SWpTY was immobilized at pH 7.5. (B) Comparison of two different phospho-detection system of 14-3-3 and anti-SWpTY antibody. The referenced signals were normalized between 0 and 1. (○) anti-SWpTY; (■) GST-14-3-3 $\zeta$ .

assay. The signals upon addition of 14-3-3 addition with or without competitor SWpTY peptide are shown in Fig. 9B. The assay showed a good signal to noise ratio of  $\sim 15$ . The addition of 20  $\mu\text{M}$  competitor SWpTY peptide in the solution resulted in a 94% decrease of the referenced signals.

As a common indicator for the feasibility for high throughput screening, Z factors for the 14-3-3 detection on the Epic<sup>TM</sup> System were calculated (Fig. 9C). The addition of 4% DMSO and up to 10 mM DTT does not affect significantly the Z factor, indicating the robustness and compatibility of the assay for high throughput screening.

## 4. Discussion

A label-free assay for the phospho-specific interactions of 14-3-3 proteins has been developed using the RWG Epic<sup>TM</sup> System. When the NH<sub>2</sub>-SWpTY (NH<sub>2</sub>AhxAhx-FRGRSWpTY-COOH) peptide is covalently immobilized to the surface, binding of 14-3-3 proteins can be detected at as low as 38 nM, with adjustable LRs depending on the density of immobilized NH<sub>2</sub>-SWpTY. The specificity of detection is validated using the competition experiments with a

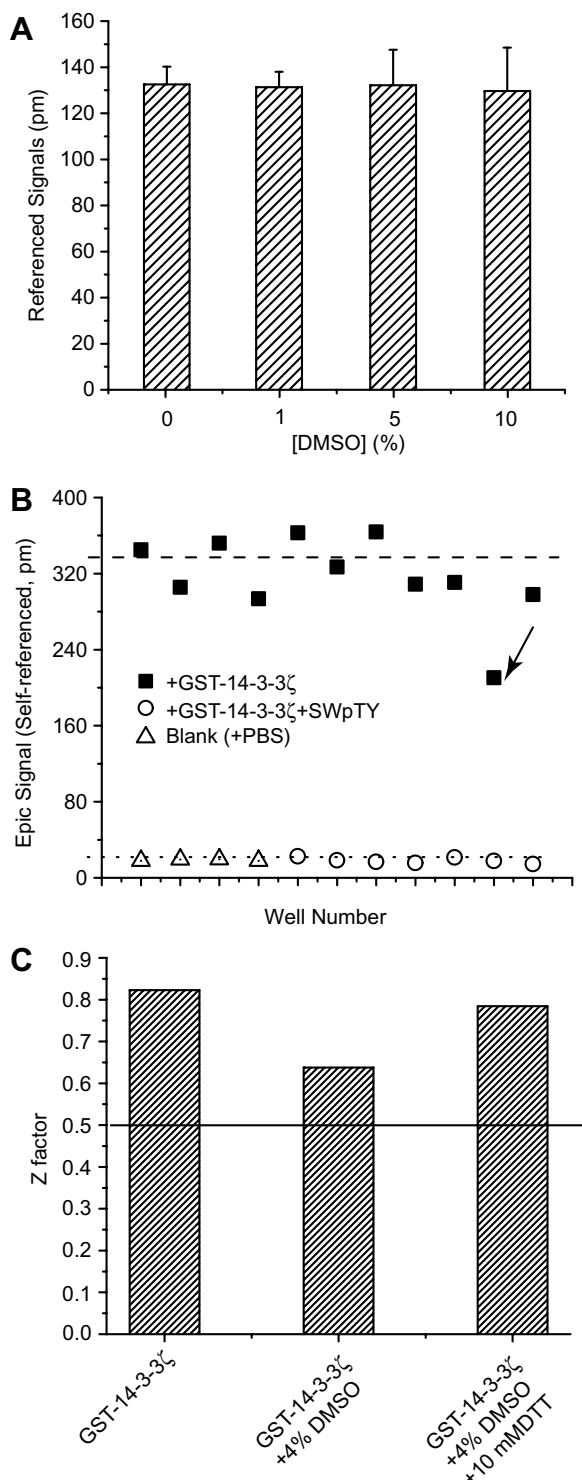


Fig. 9. Compatibility of the Epic™ System for 14-3-3 high throughput screening. (A) DMSO tolerance of the detection, referenced signals from sample wells. GST-14-3-3 $\zeta$  (0.4  $\mu$ M) was added with different concentrations of DMSO into the wells immobilized with NH<sub>2</sub>-SWpTY (50  $\mu$ g/ml, pH 7.5). (B) Data set of binding signal with 1.0  $\mu$ M of GST-14-3-3 $\zeta$  (■), 1.0  $\mu$ M of GST-14-3-3 $\zeta$  pre-incubated with 20  $\mu$ M SWpTY (○), and PBS alone (△) when NH<sub>2</sub>-SWpTY was immobilized at 50  $\mu$ g/ml at pH 7.5. Arrow indicates the outlier. Here an Epic™ plate with intrawell self-referencing was used and referenced signals were denoted as Epic Signal. (C) Z factor for the 14-3-3 detection with 1.0  $\mu$ M of GST-14-3-3 $\zeta$ , 1.0  $\mu$ M of GST-14-3-3 $\zeta$  with 4% DMSO, and 1.0  $\mu$ M of GST-14-3-3 $\zeta$  with 4% DMSO and 10 mM DTT.

non-phosphorylated SWTY peptide and a binding mutant of 14-3-3 protein. Furthermore, competition assays were performed to determine the rank order of binding affinities of the different peptide motifs. The Epic™ System was also applied for the detection with a phosphorylation-specific antibody. In addition, the assay is compatible with high throughput screening with a Z factor larger than 0.5 and up to 10% DMSO tolerance. Therefore, the reported assay offers a label-free screening system for phospho-specific interactions applicable at the quantitative level. With minor modifications, the reported assay can be applied for the screening of modulators for 14-3-3 protein–protein interactions. Since the kinase/phosphatase reactions can introduce the phosphorylation and dephosphorylation of the SWTY/SWpTY sequences immobilized on the surface of the sensor plate, the current assay can be further developed into a high throughput label-free protocol for modulators of kinases and phosphatases.

The reported assay conditions are tuned for 14-3-3 interactions with the SWpTY peptide. Many aspects of the experimental conditions may be transferable to other interaction systems, especially protein–peptide interactions that are exemplified by phospho-specific antibody to the SWTY peptide. Comparison with solution-based assays such as fluorescence polarization suggests that at least for the interaction between SWpTY and 14-3-3, the Epic™ System gave comparable characteristics in terms of sensitivity and dynamic range. Because Epic™ allows for quick estimation of binding affinity, it provides an attractive means for quantitative assessment of interactions between different ligands and one protein or between one ligand and different interacting proteins. This could be applicable to a number of assays such as antibody evaluation and detection of interacting components in cell lysates.

Optical biosensors including both SPR and RWG employ evanescent waves and have wide applications [23]. The reported assay system for the 14-3-3 interaction demonstrates its feasibility for quantitative evaluation of protein–protein interactions in a high throughput manner. The 384-well open detection format offers future possibilities of performing multiplexed assays and monitoring progressive reactions.

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