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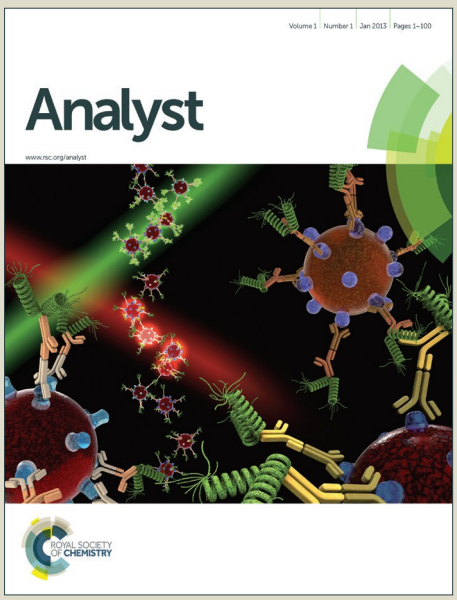

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Sensitive measurement of total protein phosphorylation level in complex protein samples

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ABSTRACT

Measurement of protein phosphorylation plays an essential role in delineating cell signaling pathways. Although the detection of a specific phosphoprotein has been largely accomplished by immunological methods, a specific and sensitive assay to measure total protein phosphorylation level in complex samples such as whole cell extracts has yet to be established. Here, we present a sensitive phosphorylation assay on a microwell plate to determine total protein phosphorylation level calibrated to a phosphoprotein standard. The core of the assay is a reagent termed pIMAGO that is multi-functionalized with titanium ions for its superior selectivity towards phosphorylated proteins and with fluorophores for quantification. The specificity, sensitivity, and quantitative nature of the assay were demonstrated with standard proteins and whole cell lysates. The method was then employed to measure the overall protein phosphorylation level of human cells under different treatments. At last, we investigated the practicability of the assay to serve as a sensitive tool to estimate the amount of phosphorylated samples prior to a mass spectrometry-based phosphoproteomic analysis.

INTRODUCTION

Protein phosphorylation is one of the most important post-translational modifications and plays a crucial role in the regulation of nearly every aspect of cellular life.[1] Thus, detection of protein phosphorylation, especially quantitative measurement of changes in protein phosphorylation, is essential for a better understanding of how signaling pathways function in cells under both normal and diseased states.[2] While many researchers focus on specific proteins and phosphorylation events, global analysis of protein phosphorylation as those in whole cell lysates or protein complexes could be as beneficial.

Many biological experiments frequently require the estimation of total protein amount. However, the estimation of total protein phosphorylation level in biological samples, while equivalently important, has seldom been practiced. This is largely due to the fact that current method for phosphorylation measurement is neither convenient nor sensitive. Existing methods for global phosphorylation analyses include the use of ^{32}P radioactive labeling, molybdate-based colorimetric determination, phospho-specific antibodies, phospho-staining reagents, and high performance mass spectrometry. One of the major limitations of the classic radioactive labeling with ^{32}P-orthophosphate, in addition to its safety issues, is that long-time incubations of whole cells with large doses of radioisotopes are very toxic in nature, leading to DNA damage, cell cycle arrest, and eventually apoptosis.[3, 4] Thus, it has nowadays more commonly been used to detect the phosphorylation of protein substrates in *in vitro* kinase assays. Molybdate colorimetric method is based on the alkaline hydrolysis of phosphate from seryl and threonyl residues and quantification of the released phosphate with malachite green and ammonium molybdate.[5] It has been applied to the estimation of the phosphate content of purified

samples or the staining of proteins separated by polyacrylamide gel electrophoresis (SDS-PAGE).[5, 6] However, the detection is indirect, relatively insensitive, and most of all, limited to proteins that are phosphorylated at serine or threonine residues.[6] Antibodies such as anti-phosphotyrosine antibodies have facilitated the detection of total tyrosine phosphorylation in protein complexes, but these antibodies are still sequence-dependent.[7-9] Moreover, at present there is still no reliable antibody available that can recognize serine- and threonine-phosphorylated proteins, independent of sequences. To overcome the limitations of antibodies, there have been attempts recently in applying novel chemical reagents to staining phosphoproteins in gels or on membrane, including Phos-tag, Pro-Q Diamond, Stains-All and other similar reagents or methods.[10-15] These staining techniques are most useful for qualitative detection of protein phosphorylation by mobility shift or relative measurement of phosphorylation changes in individual protein bands after separation in one- or two-dimensional gels.[16-18] Besides, there are methods specifically developed to measure the activities of kinases or phosphatases, such as ADP-Glo kinase assay, Omnia kinase assay, and IMAP FP kinase and phosphatase assays.[19-22] However, all these methods are devised to measure phosphorylation or dephosphorylation on specific proteins or peptides.

In the past decade, mass spectrometry has evolved into a powerful tool for large-scale analysis of proteins and post-translational modifications, including the identification of novel phosphorylated proteins and phosphorylation sites.[23-25] Challenges still remain due to the relatively low stoichiometric nature and ionization efficiency of phosphopeptides, so additional steps for phosphopeptide enrichment are usually required.[25, 26] Furthermore, samples for mass spectrometric analyses often vary significantly in terms of the protein

phosphorylation level. Samples extracted from yeast and plant cells, for instance, have typically much lower levels of phosphorylation than those from human cells with the same total amount of proteins. Thus, a larger starting amount is desirable for phosphoproteomic studies with yeast or plant samples. The exact sample amount to be used, however, is mostly judged by individual laboratories based on either previous reports or trial-and-error experiments. Therefore, it would be extremely useful to develop a simple and sensitive assay to measure total protein phosphorylation level in individual samples, in analogy to the bicinchoninic acid (BCA) protein assay.

We present here a novel strategy to quantitatively measure total protein phosphorylation level on a microwell plate, which features a multi-functionalized chemical reagent termed pIMAGO. It is based on a polyamidoamine dendrimer that is derivatized with titanium ions for its known superior selectivity towards phosphorylated residues and with fluorophores for quantification.[27-29] We have demonstrated the initial applications of pIMAGO to analyze individual phosphoproteins.[30-32] The measurement of total phosphorylation level in biological samples presents distinctive technical challenges due to sample complexity. Here, the specificity, sensitivity and quantitative nature of the pIMAGO reagent for the measurement of the total phosphoprotein level were examined with standard proteins and *E. coli* whole cell lysates. The pIMAGO phosphorylation assay was then employed to measure the total protein phosphorylation levels of multiple cell lysates and samples from human cell lines before and after treatment. Finally, we showed that the technique can serve as a simple and useful tool to estimate the sample amount to be used for a typical phosphoproteomic analysis using mass spectrometry.

EXPERIMENTAL

Experimental details in materials, synthesis of the pIMAGO reagent, preparations of cell lysates and plant samples, LC-MS/MS analysis, ICP-MS analysis, and Supplementary Figures are included in the Supplementary Information.

Measurement of Protein Phosphorylation by pIMAGO

Standard proteins (e.g. α-casein) and prepared cell lysates of interest were immobilized onto a polystyrene 96-well microplate (Corning® 96-well EIA/RIA clear polystyrene high binding microplate) at the designated amounts in each well in 100 μL of carbonate buffer, pH 9.6, for either 2 h at room temperature or overnight at 4 °C. The solution was removed then and the wells were washed three times with 200 μL of the blocking buffer, 1% BSA (bovine serum albumin) in TBST (0.1% Tween-20, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5), and blocked with the same buffer for 1 h. Then, each well was incubated with 0.5 μL of the pIMAGO reagent in 100 μL of 200 mM glycolic acid/1% trifluoroacetic acid solution for 1 h. After washing with 200 μL of 200 mM glycolic acid/1% trifluoroacetic acid solution for three times and then with 200 μL of TBST for three times, the wells were rinsed once with methanol and emptied. The plate was scanned with an infrared imaging system (Odyssey® LI-COR, Lincoln, NE) and signals were recorded. The protein phosphorylation levels of cell lysates of interest can then be determined by interpolating from the α-casein standard curve established.

Phosphoprotein Dephosphorylation and *In Vitro* Kinase Assay

For phosphoprotein dephosphorylation, *E. coli* cell lysate was incubated with CIAP (calf intestine alkaline phosphatase) in 1× CIAP buffer (50 mM Tris-Cl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol) for 12 h at 37°C.

For *in vitro* kinase assay, 500 ng of purified Band 3 per well was immobilized onto a polystyrene microplate and blocked as described above. The wells were then incubated with 500 μM of ATP and 2 mM of $MnCl_2$ with or without the presence of the Syk kinase (50 ng per well). The kinase reaction was stopped with the addition of 200 mM glycolic acid/1% trifluoroacetic acid solution at the specified time points, and the remaining reagents were washed away. Then, each well was incubated with pIMAGO, and then washed, and detected as described above.

RESULTS AND DISCUSSION

Design of pIMAGO strategy for measuring total protein phosphorylation level

The development of an efficient assay for measuring total protein phosphorylation level in complex samples would be extremely useful for the field of signal transduction. A reliable phosphorylation assay should be specific, sensitive, quantitative, and can be readily calibrated using an affordable and common phosphoprotein standard. The strategy we use to establish the assay features a recently developed reagent, termed pIMAGO, which is based on a polyamidoamine dendrimer functionalized to chelate multiple titanium ions on its surface for selective binding to the phosphate groups on phosphorylated proteins and also with infrared fluorescent dyes for sensitive detection and quantification (Figure 1A).[30] The workflow of the pIMAGO phosphorylation assay is similar to that of the classic enzyme-linked immunosorbent assay (ELISA). As shown in Figure 1B, a complex protein sample such as cell lysates is first immobilized onto a polystyrene 96-well microplate along with a phosphoprotein standard (e.g. α-casein) with known amounts ranging from 3 ng to 50 ng per well. The wells are then blocked with BSA and incubated with the pIMAGO reagent, which binds to all phosphorylated proteins regardless of their sequences. After

washing away unbound pIMAGO, the microplate can be scanned using an infrared imaging system (e.g. LI-COR Odyssey) and the fluorescent signal is recorded. A common phosphoprotein, α-casein, at different known concentrations is used to construct a calibration curve before each measurement.

Specificity and Sensitivity of pIMAGO-based Phosphoprotein Detection

The specificity and sensitivity of pIMAGO for phosphoprotein detection was first examined using a number of standard phosphorylated and non-phosphorylated proteins. As shown in Figure 2A, the pIMAGO fluorescent signals derived from 50 ng of the phosphoproteins α-casein and β-casein were approximately 80- and 40-fold stronger, respectively, than those from the non-phosphoproteins BSA and α-lactalbumin of the same amount, with high reproducibility (shown as Mean + SD; CV% < 10%, n = 6). The specificity was further demonstrated with *E. coli* cell lysates treated with or without CIAP (calf intestine alkaline phosphatase). The phosphorylation signals from *E. coli* cell lysates (containing 500 ng of proteins based on the bicinchoninic acid assay) decreased significantly after the CIAP treatment to slightly above the background of the non-phosphoproteins, indicating that the pIMAGO signals were indeed due to protein phosphorylation (Figure 2A).

Next, we investigated whether there were significant interferences from other phosphate-containing molecules such as nucleic acids and phospholipids that may still remain in cell lysates, despite of the fact that the polystyrene microplates used in the assay are intended to bind only large biomolecules as proteins and antibodies through mainly hydrophobic interactions. Cell lysates of human cell line DG-75 were carefully prepared per standard protocols and subjected to either nuclease treatment to degrade all forms of DNA and RNA

or protein precipitation using acetone to remove both phospholipids and nucleic acids. DG-75 cell lysates with or without treatments were then immobilized onto the plate and their phosphorylation levels quantified by pIMAGO. As shown in Supporting Information Figure S1, neither nuclease treatment nor protein precipitation resulted in any significant difference in total phosphorylation levels of DG-75 cell lysates (with 500 ng of proteins per well). This indicates that the potential interference from nucleic acids and phospholipids remaining in cell lysates are trivial, probably due to the fact that pIMAGO preferably binds to phosphoproteins rather than nucleic acids or phospholipids. In addition, polystyrene microplates likely prefer to bind to proteins as well.

Quantitative Capabilities of pIMAGO-based Phosphoprotein Detection

Next, the quantitative capabilities of pIMAGO-based phosphoprotein detection were evaluated. In order to relatively quantify the protein phosphorylation levels of samples of interest, a calibration curve of a standard phosphoprotein needs to be established first. We chose α-casein with its attractive features as a calibration standard: commercially available with relatively low cost and stable phosphate content over more than 6-month storage period. We also measured the absolute amount of phosphorus in α-casein using inductively coupled plasma mass spectrometric (ICP-MS) analysis (Supplementary Information). The result showed that 1 ng of α-casein contained 1.06 pg of phosphorus (i.e. 0.106% phosphorus by weight). Therefore, the assay can determine absolute phosphorus amount in samples of interest indirectly after the calibration using known amount of α-casein. Quantitative detection of α-casein with two-fold serial dilutions ranging from 3 ng to 50 ng per well is shown in Figure 2B and Figure S2. Similarly, DG-75 cell lysates with different protein amounts ranging from 30 ng to 500 ng were added to different wells and

detected with pIMAGO (Figure 2C). Both data resulted in excellent linearity ($r^2 > 0.97$). The outstanding quantitative capabilities of pIMAGO were further demonstrated when it was employed to monitor an *in vitro* kinase assay. Band 3 is a known protein substrate for spleen tyrosine kinase (Syk).[33] Purified Band 3 (500 ng per well) was immobilized onto the microplate and then after blocking, the wells were incubated with Syk kinase (50 ng per well) for different lengths of time. As illustrated in Figure 2D, pIMAGO was able to efficiently capture the increase in phosphorylation of Band 3 over time. Altogether, these results demonstrated that pIMAGO can be used for sensitive and quantitative measurement of phosphorylation change of individual proteins or complex mixtures such as cell lysates in a straightforward format similar to ELISA.

Detection of Total Phosphorylation Level Changes in Cell Lysates

Since pIMAGO is capable of quantitatively detecting protein phosphorylation levels in cell lysates, it would be particularly useful to measure potential changes in total protein phosphorylation levels upon treatment of cells. To evaluate whether the perturbation of an upstream kinase would dramatically change the overall phosphorylation levels of cells, we selected three different human cell lines (Jurkat, MCF7, and HeLa cells), treated with PMA (phorbol-12-myristate-13-acetate), a potent activator of PKC (protein kinase C). PKC is involved in the regulation of a variety of cellular functions including migration and polarity, proliferation, differentiation and apoptosis, by directly phosphorylating substrates or activating downstream signaling pathways.[34, 35] The established pIMAGO assay was employed to measure the phosphorylation levels of cell lysates from Jurkat, MCF7, and HeLa cells, upon PMA treatment (Figure 3). Interestingly, when compared to control lysates, stimulation by PMA failed to result in significant increases in the total

phosphorylation levels of Jurkat, MCF7, or HeLa cell lysates using unpaired *t*-tests (Figure 3B and 3C). As a comparison, Western blotting was performed using an anti-phospho-threonine antibody, which also showed similar phosphorylation levels between control and PMA-treated cell lysates, although only of threonine sites (Figure S3). These results indicated that a large portion of phosphoproteins in cells remained unchanged upon stimulation. While the activation of an upstream kinase such as PKC would increase the phosphorylation of a subset of downstream substrates and pathways, these effects may not be strong enough to disturb the balanced steady state of equilibrium in overall cellular phosphorylation level. We expect that with the emergence of the pIMAGO assay capable of measuring total protein phosphorylation, more interesting studies will follow up on the dynamics of overall phosphorylation level and its effects on cellular status.

Use of pIMAGO Phosphorylation Assay for the Estimation of Starting Sample Amount for Mass Spectrometry-based Phosphoproteomic Studies

Finally, we investigated whether the pIMAGO phosphorylation assay can serve as a sensitive tool to help determine the sample amount required for a mass spectrometry-based phosphoproteomic analysis. Before performing a phosphoproteomic experiment, it is important to estimate how much phosphorylation is in a test sample so that a proper sample preparation protocol can be applied, including potential fractionations and phosphopeptide enrichment.[25, 36, 37] Samples like yeast and plant cell extracts are typically lower in phosphorylation than human samples and thus increasing the starting amount of yeast or plant samples for analysis is desirable for a global phosphoproteomic profiling. However, there is no simple solution to estimate the starting sample amount except performing a series of preliminary experiments, which can be both costly and inefficient. Thus, we

attempt to use the pIMAGO assay to measure total protein phosphorylation level just as the bicinchoninic acid (BCA) assay measures protein concentration.

To validate this idea, we have collected different types of samples from several model organisms that are commonly used in mass spectrometry-based phosphoproteomic analysis: human cell line DG-75, yeast, mature leaves and seedlings of Arabidopsis (*Arabidopsis thaliana*). Cell lysates were carefully obtained as described in Supplementary Information. The protein concentration of each cell lysate was first measured by the BCA assay and then the pIMAGO phosphorylation assay was performed to quantify the total protein phosphorylation level of each cell lysate (500 ng of proteins) by interpolating from the α-casein standard curve established in the same experiment. As expected, DG-75 human cell lysate displayed much higher phosphorylation level than yeast or plant samples, of which seedlings also showed stronger phosphorylation than mature leaves (Figure 4). For example, 500 ng of DG-75 cell lysate has a total phosphorylation level equivalent to 38 ng of α-casein, while 500 ng of yeast and seedlings are equivalent to 10 and 9 ng of α-casein, respectively, about one fourth of that of DG-75. This also indicated that significantly more protein amount of yeast or seedling cell lysate may be needed to achieve similar level of protein phosphorylation of DG-75 cell lysate.

A complex protein sample may be dominated by a few high abundant proteins while the concentration of low abundant proteins may be too low to be easily detected. Therefore, total protein concentration does not correlate with the number of proteins in the sample. Similarly, the total phosphorylation level does not necessarily reflect the number of phosphorylated proteins in the sample. However, for similar systems, the total protein phosphorylation level may be practically used to estimate total phosphorylation numbers.[25,

[26] We applied DG-75 cell lysates with different protein amounts (10, 50, 100, and 300 μg) to a series of phosphoproteomic analyses under the same instrument condition. The total number of unique phosphopeptides identified was plotted against the starting protein amount. As shown in Figure 5A, the identification number increased along with the protein amount where 100 μg of DG-75 cell lysate resulted in an average of around 2500 unique phosphopeptides identified in a single LC-MS/MS run with a total 90-min LC gradient. According to the pIMAGO phosphorylation results, the total phosphorylation level of 400 μg of yeast or Arabidopsis seedling lysate may be equivalent to that of 100 μg of DG-75 lysate. Therefore, 100 and 400 μg of yeast and Arabidopsis seedling protein extracts were also applied to phosphoproteomic analyses. As a result, an average of 1996 and 1821 unique phosphopeptides were identified from 400 μg of yeast and seedling lysates, respectively, doubling the number of identifications when 100 μg of these samples were used under the same LC-MS/MS condition.

CONCLUSION

In summary, we are reporting a novel phosphorylation assay on microwell plates to measure total protein phosphorylation level in complex samples such as cell lysates. The pIMAGO-based phosphorylation assay is sensitive, relatively simple and efficient for specific and quantitative measurement of overall protein phosphorylation in an ELISA-like format. The technique can be employed to measure either total protein phosphorylation levels of cell lysates calibrated to a phosphoprotein standard with known concentration or global changes in phosphorylation levels of cell lysates upon treatment. The assay can also serve as a useful tool to estimate the sample amount required for a mass spectrometry-based phosphoproteomic analysis to ensure enough phosphoproteins-containing samples

are present. The new strategy has the potential to become a routine analytical tool for general signal transduction and phosphoproteomic studies.

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FIGURE CAPTIONS

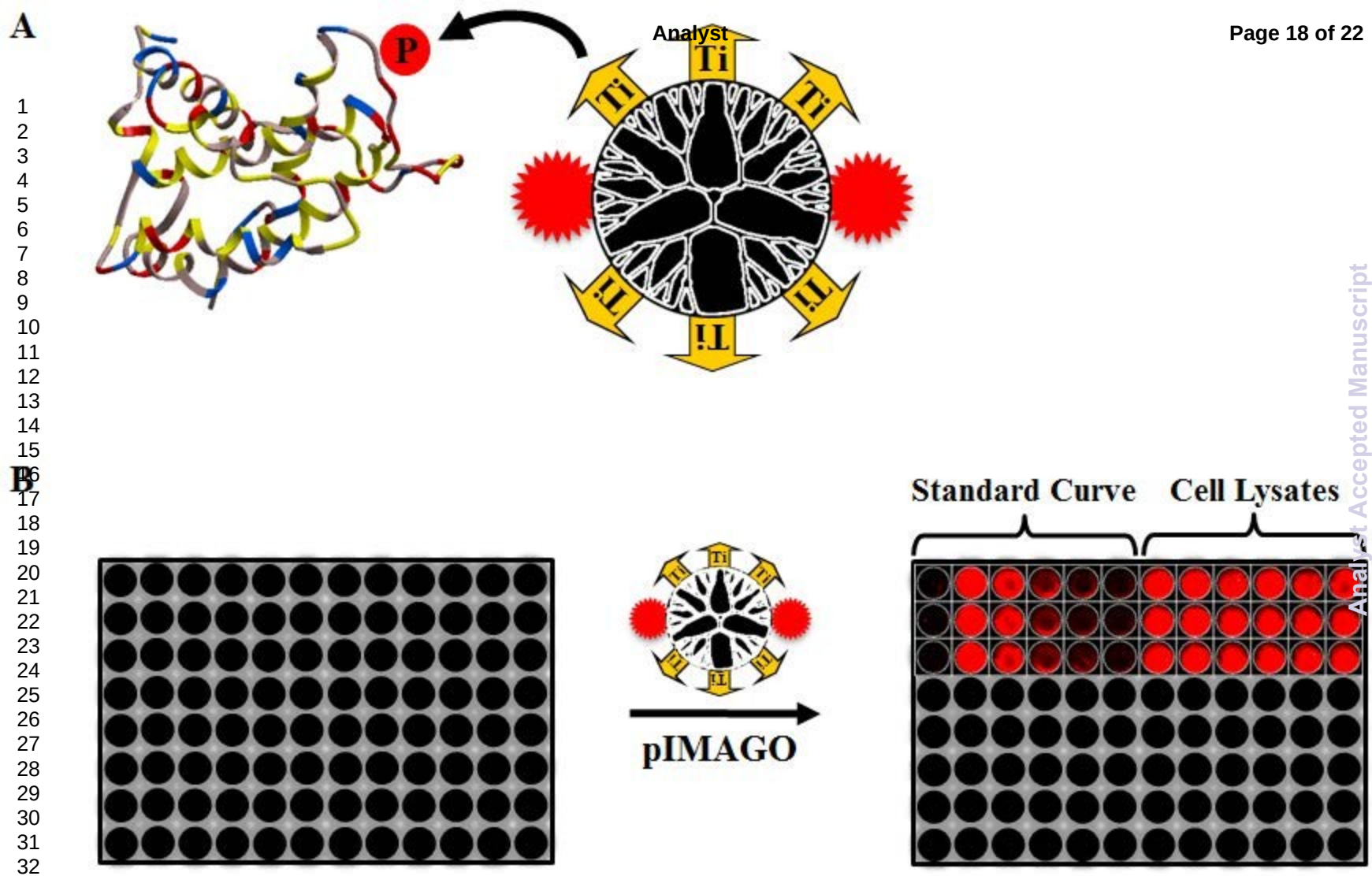
Figure 1. (A) Schematic representation of the pIMAGO reagent. (B) Experimental workflow for pIMAGO-based quantitation of total protein phosphorylation level in a complex protein sample.

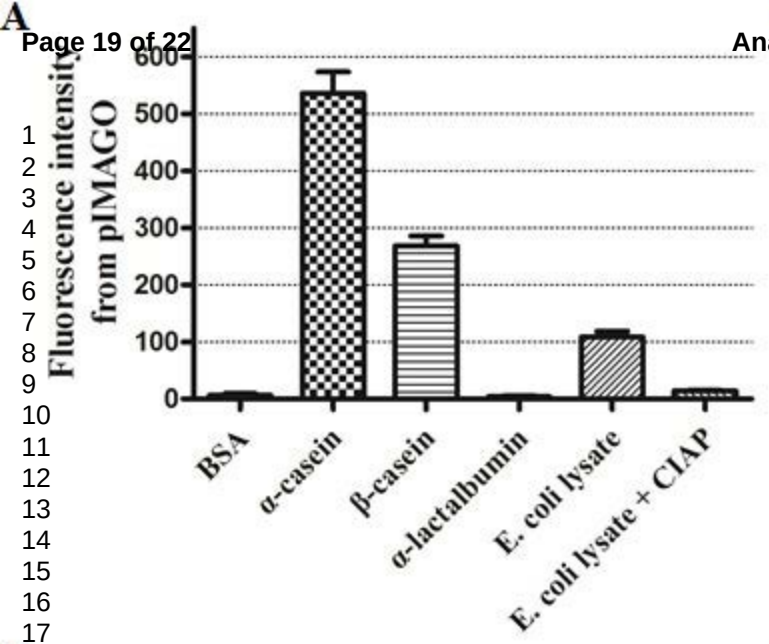
Figure 2. Specificity and quantitative nature of pIMAGO-based phosphoprotein assay. (A) Specific detection of protein phosphorylation from standard proteins and *E. coli* cell lysates by pIMAGO. (B) Quantitation of fluorescent signals from pIMAGO-based detection of the phosphoprotein standard α-casein (shown as Mean ± SD). (C) Quantitation of fluorescent signals from pIMAGO-based detection of DG-75 cell lysates (shown as Mean ± SD). (D) Quantitative measurement of fluorescent signals from pIMAGO-based detection of phosphorylation of Band 3 by *in vitro* Syk kinase assay.

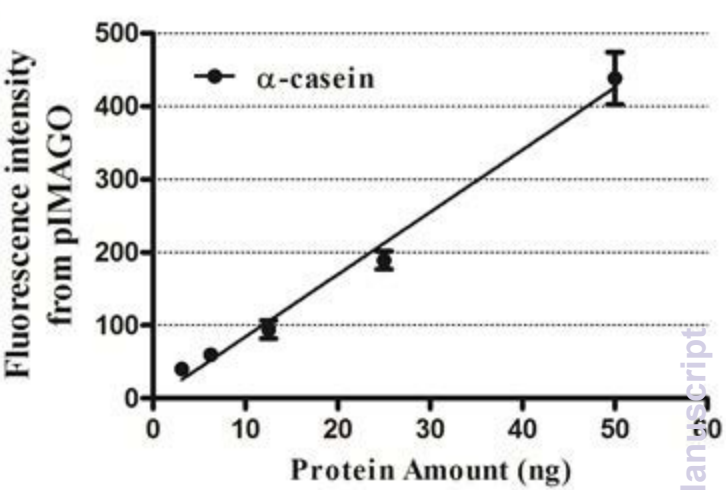
Figure 3. Detection of total phosphorylation level changes in human cell lysates by pIMAGO phosphorylation assay. A) Quantitation of fluorescent signals from pIMAGO-based detection of the phosphoprotein standard α-casein (shown as Mean ± SD). B) Measurement of fluorescent signals from pIMAGO-based detection of human cell lysates with and without PMA treatment (shown as Mean + SD). C) The corresponding fluorescence image scanned at 700 nm.

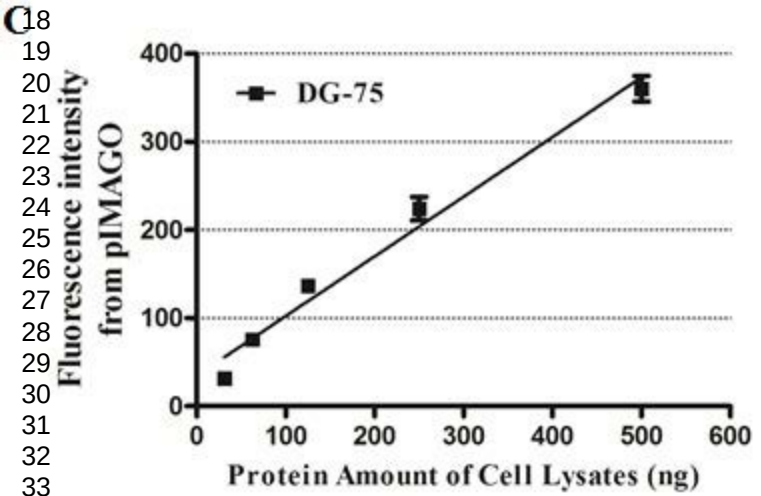
Figure 4. Quantitation of total phosphorylation levels of four cell lysates from three model organisms commonly used in mass spectrometry-based phosphoproteomic analysis by pIMAGO phosphorylation assay. 500 ng of proteins of each cell lysate was immobilized, measured, and interpolated to the α-casein standard curve established.

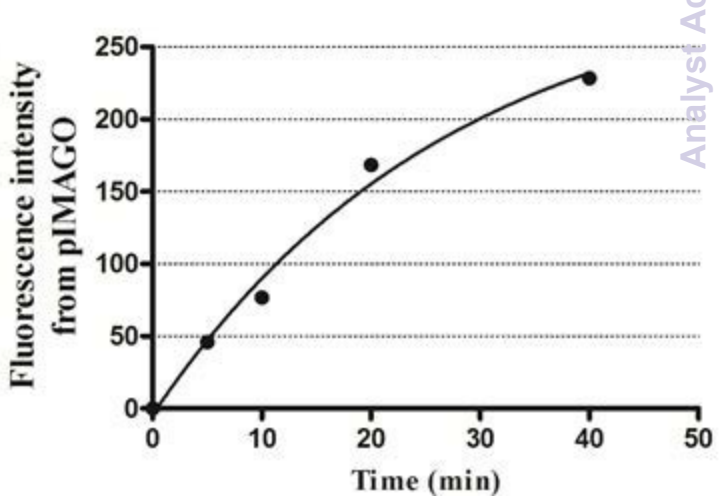
Figure 5. Use of pIMAGO phosphorylation assay to estimate starting sample amount for mass spectrometry-based phosphoproteomic analysis. (A) Total number of unique phosphopeptides identified when different protein amounts of DG-75 cell lysates were employed. (B) Total number of unique phosphopeptides identified when different protein amounts of Arabidopsis seedling and yeast cell lysates were employed (shown as Mean + SD).

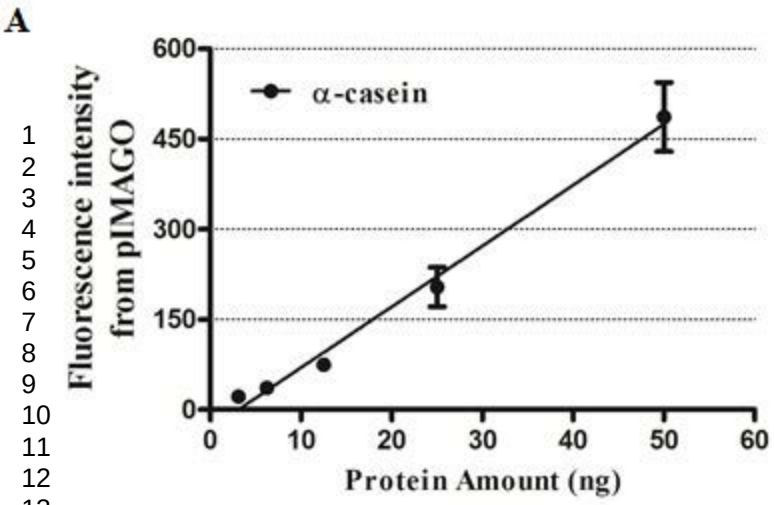


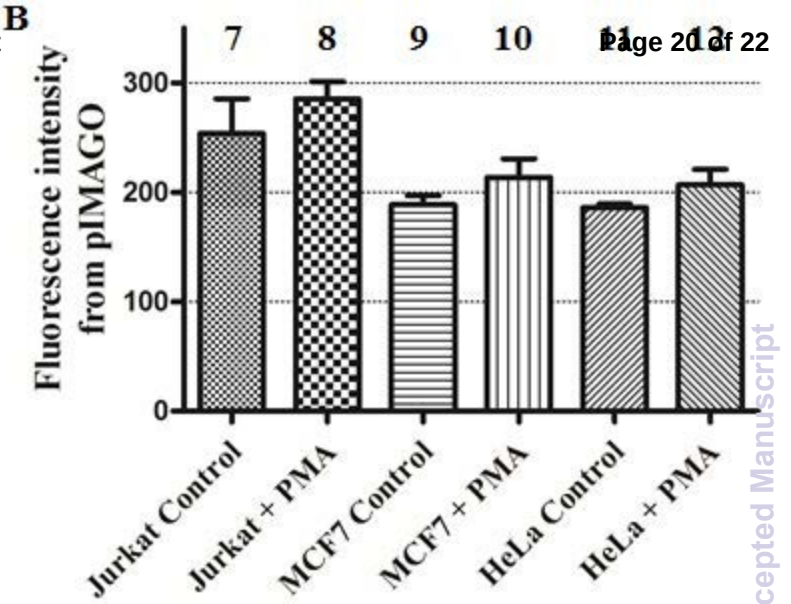


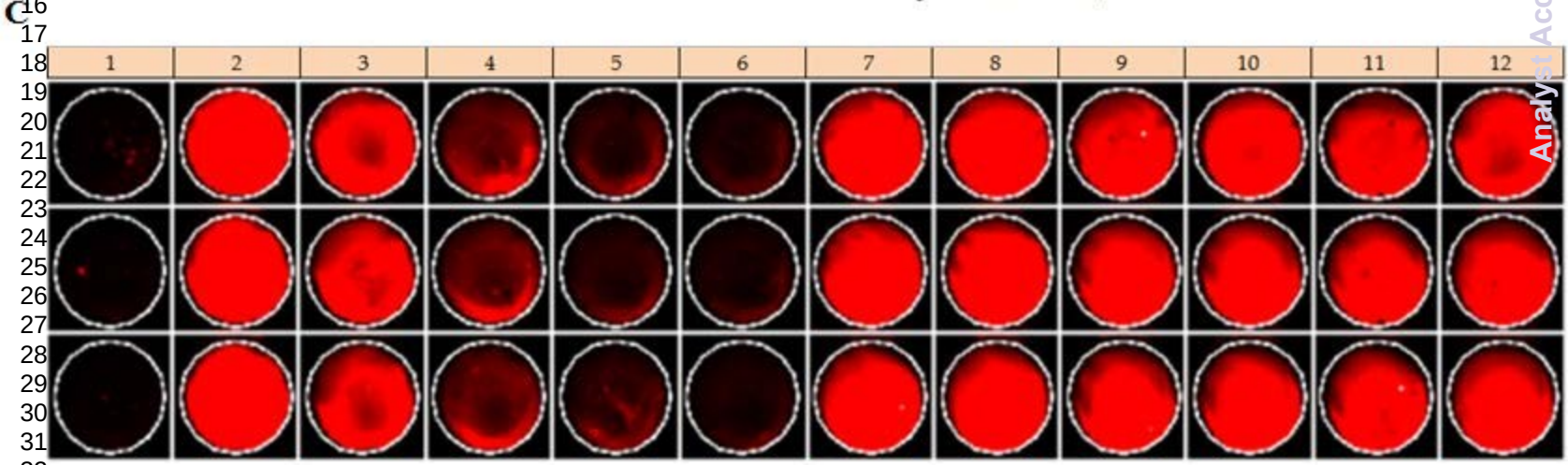


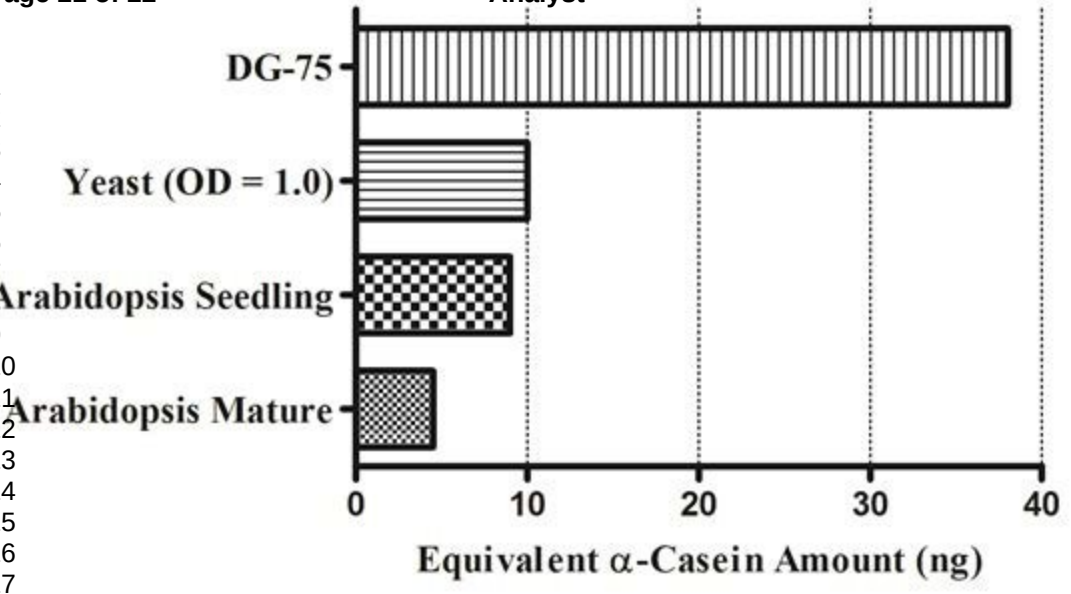




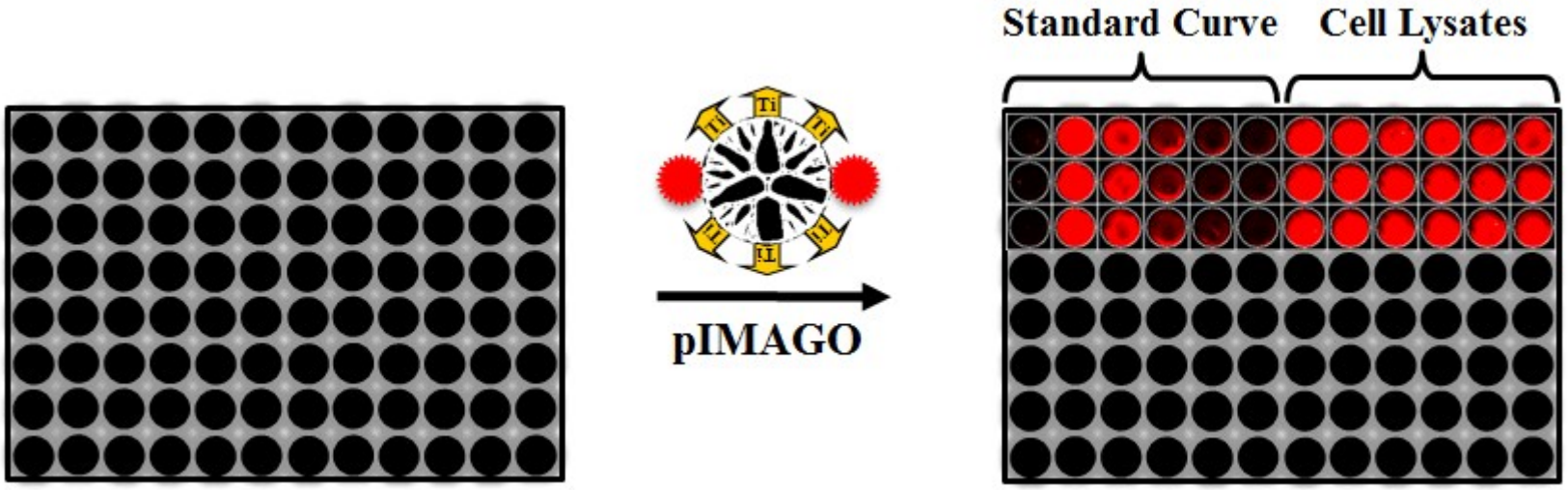








TOC Entry



Need to know the protein phosphorylation level in your samples? Multi-functionalized nanopolymer provides a simple solution.