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Gene cloning and utility phophorylation assay of a protein-fused substrate for a highly sensitive detection of cdc2 protein kinase using a radioisotope detection technique for the development of a protein biochip

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The prototype of the cdc2 protein kinase in mammalian cells regulates its entry into mitosis by phosphorylating a group of key proteins in the major cell cycle transitions. In this study, using the mep45 gene encoding the 45 kDa major envelope protein (Mep45) of *Selenomonas ruminantium*, a rumen bacteria, a Mep45-fused substrate (PKTPKKAKKL-Mep45, MFS-cdc2) was cloned to detect the activity of cdc2 protein kinase. We report here on a strategy for the detection of a phosphorylation of a substrate catalyzed by cdc2 protein kinase by using a radioisotope detection technique. It is possible to constantly obtain a reasonable quantity of MFS-cdc2 for the cdc2 protein kinase assay and its cost can be as low as a synthesized peptide. Results of the study indicate that the Mep45-fused protein can be used effectively as a substrate for detecting the activity of cdc2 protein kinase and it can be used in developing a protein biochip for a high-throughput screening and also for studying protein–protein interactions.

Keywords: radioisotope detection; phosphorylation detection; protein-fused substrate; cdc2 protein kinase; biochip

Introduction

Protein microarrays function as a powerful emerging technology for proteomics and clinical applications. In recent years, they have been used to screen thousands of binding events in a parallel and high-throughput manner, and are of major importance not only in the detection of a disease but also aid in the discovery and development of a drug.[1] A protein microarray is a piece of glass slide on which various proteins are impregnated, and it is used to identify protein–protein interactions. Protein chips have the potential to function in many other applications including the study of protein–protein interactions, protein–drug interactions, DNA–protein interactions, a protein localization, antigen–antibody interactions, enzyme–substrate interactions, and receptor–ligand interactions all of which may be amendable to an array-type high-throughput screening (HTS).[2,3] For example, they are used to identify the substrates of various enzymes or the targets of biologically active small molecules. Their signal detection can be assayed by autoradiography and quantified by a densitometer.[4,5] There are generally two kinds of detection strategies for protein microarrays such as label-free methods and labeled probe methods. Labeled probe methods are those that employ the use of fluorescence, chemiluminescence, electrochemiluminescence, and radioisotope (RI) for detection.[1,6]

Phosphorylation is an ubiquitous cellular regulatory mechanism found in all cells of the body. It is a reversible and covalent modification of a lipid or protein that serves to modify the activity of a phosphorylated molecule by inducing conformational changes within a molecule. It occurs either through the addition of a phosphate group via the transfer of a terminal phosphate from an adenosine triphosphate (ATP) molecule catalyzed by kinases or phosphatases to an amino acid residue and/or by their removal.[7–9] Kinases and phosphatases are of great interest to researchers involved in the detection of diseases and drug discovery because of their wide relevance to diseases and human health. Cancer and other metabolic disorders, neurological diseases, proliferative diseases, and inflammatory diseases are among those in which the protein phosphorylation plays an important role.

Cell cycle-dependent protein kinases (CDKs) are a family of serine/threonine protein kinases that include cyclin-dependent kinase 1 (cdk1) [formerly called cdc2 (or $p34^{cdc2}$)], cdk2–cdk7, and the RbKs. In eukaryotes, it regulates the cell entry into mitosis, the transition from the G2 to the M phase, by phosphorylating a group of key proteins including RNA

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polymerase II and histone H1.[10,11] The sequence of a substrate for the cdc2 protein kinase, PKTPKKAKKL, is derived from the p34^{cdc2} *in vitro* phosphorylation sites of histone H1.[12]

Protein fusion tags have become the mainstay of recombinant protein technology, mainly because of their convenient purification and monitoring of expressed recombinant proteins. Various tags with a high affinity to their binding partner are effective tools to immobilize peptides or proteins on the surface of a chip. The advantages of using fusion proteins to facilitate in the purification and detection of recombinant proteins are well recognized.[13]

The use of RI is traditionally considered to be one of the most sensitive detection methods. Radioactive labeling is mainly performed using different RIs such as ^{32}P or ^{33}P incorporated into ATP for phosphorylation. Today this technique has been developed to detect active proteins and to allow for a precise quantification of the amount of protein assayed. We have already reported on a feasibility study using [γ-^{32}P]ATP for determining the activity of a cAMP-dependent protein kinase assay on a glass chip.[14] The results were found to be promising and can be applied when using an RI detection technique for the biochip to determine an enzyme activity.

In the present investigation, we propose a suitable strategy for the detection of phosphorylation of a substrate catalyzed by cdc2 protein kinase by using an RI detection technique, which can be used in developing a protein biochip.

Results and discussion

An RI assay for a protein–peptide-fused protein interaction with an enzyme was developed by Park *et al.*[14] The strategy was progressed by a substrate–enzyme interaction by using an RI detection on a protein microarray. In this study, we investigated the feasibility of applying an RI detection technique to the cdc2 protein kinase assay for the development of a protein biochip.

A substrate (PKTPKKAKKL) of the cdc2 protein kinase was fused to the N-terminus of major envelope protein (Mep45) of *Selenomonas ruminantium*. The *mfs-cdc2* gene encoding the Mep45-fused substrate was amplified by polymerase chain reaction (PCR). As a result, the 1.4-kb products were amplified and the DNA fragments were cloned into the pGEM-T vector and they were sequenced using T7 promoter and T7 terminator primers. The *mfs-cdc2* was cloned to the pET-28a vector and the recombinant MFS-cdc2 protein was expressed as C-terminal His6-tag fusion proteins in *Escherichia coli* BL21 (DE3). Therefore, the Mep45-fused protein for a substrate was expressed as a recombinant protein from a plasmid pMFS-cdc2 in *E. coli* and it was purified. Protein samples obtained from each purification step were evaluated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The final step yielded a protein with a molecular weight of 44 kDa, which was identical to the estimated molecular weight (Figure 1).

The resultant MFS-cdc2 protein was used as a substrate for the cdc2 protein kinase assay. For a protein microarray construction, a protein immobilization on the surface of a glass slide is intrinsic. Substrate immobilization was done by spotting the MFS-cdc2 protein onto aldehyde-coated glass slides. The amine groups of the protein reacted immediately with the aldehyde groups on the slide layer to form a Schiff base linkage.

Phosphorylation of the cdc2 protein kinase and *S. ruminantium* Mep45-substrate fusion protein using [γ-^{32}P]ATP is illustrated in Figure 2. It occurs through the addition of a phosphate group to

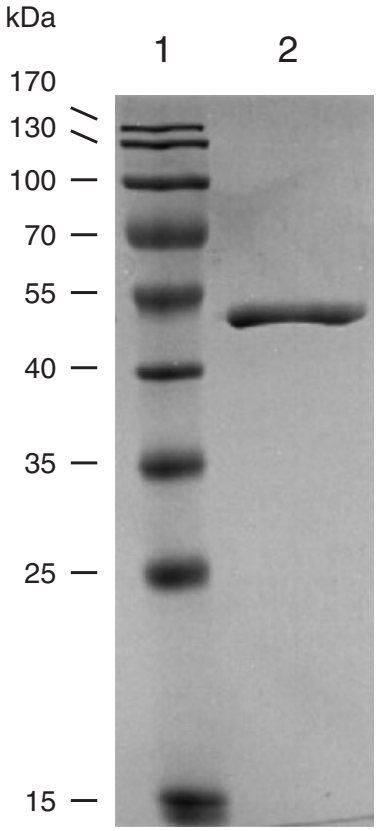

Figure 1. SDS-PAGE analysis of the purified protein-fused substrate for cdc2 protein kinase. 1, molecular mass standards; 2, purified protein-fused substrate.

the threonine of the substrate (PKTPKKAKKL) via a transfer of phosphate from [γ-^{32}P]ATP molecule catalyzed by cdc2 protein kinase. Purified *S. ruminantium* Mep45-substrate fusion protein was spotted and immobilized on aldehyde-terminated glass slides as explained before and the glass slides were incubated in a reaction mixture that contained a kinase buffer with ATP mixture (50 μM ATP, 0.5 mCi/mL [γ-^{32}P]ATP) and 10 μg/mL of cdc2 protein kinase. The glass slides were then washed and detected by using an X-ray film or phosphoimager analysis. Specific signals for the reaction of the cdc2 protein kinase were only detected for the MFS-cdc2, but not for the bovine serum albumin (BSA), which was used as a negative control (Figure 3). It was found that the kinase activities were detected for the specific protein-fused substrate spots phosphorylated by RI.

We then examined the signal of the kinase assay system with varying concentrations of the cdc2 protein kinase. The reaction mixture consisted of a kinase buffer supplemented with an ATP mixture (50 μM ATP, 0.5 mCi/mL [γ-^{32}P]ATP) and various concentrations of the cdc2 protein kinase (1×10^{-13}–1 μg/mL). It was observed that the signal of the phosphorylation was detected even at the lowest concentration tested (1×10^{-13} μg/mL) although the intensity was weak. The signal intensity of the cdc2 protein kinase was found to increase gradually up to 1 μg/mL of the enzyme concentration (Figure 4). The signal intensity can be increased by spotting a high concentration of the substrate onto the glass slide.

Enzyme-linked immunosorbent assay (ELISA) has generally been used in immunology to detect the presence of an antibody or an antigen in a sample. It has been used as a diagnostic tool in medicine and plant pathology, as well as a quality control check in

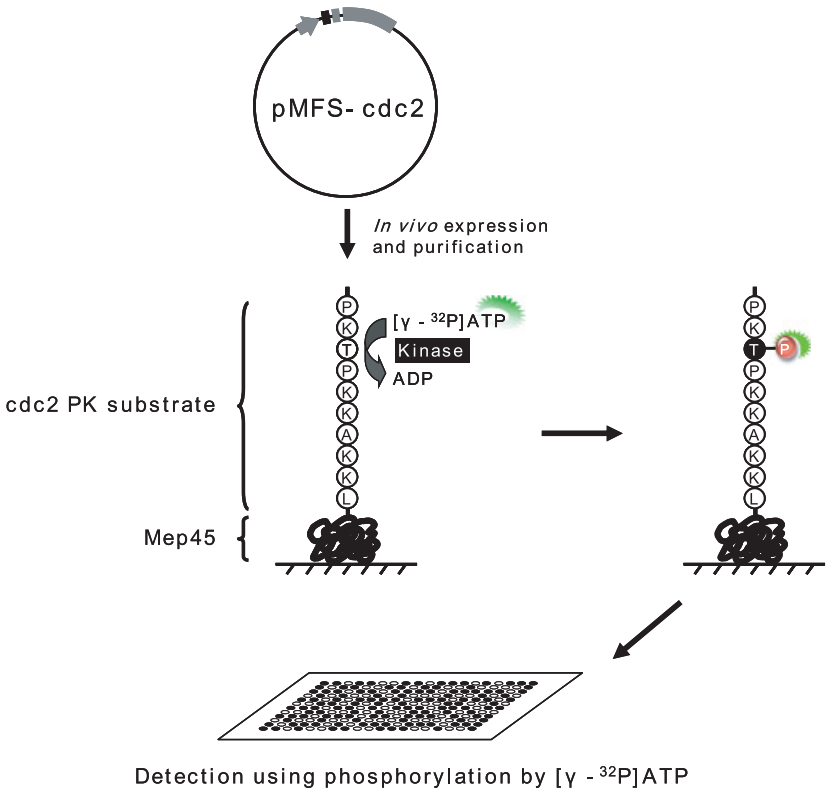

Figure 2. Scheme of the recombinant Mep45-fused substrate for cdc2 protein kinase assay on radioactive isotope. The gene, protein, peptide, and transferred [^{32}P]phosphate are marked as follows: pMFS-cdc2, plasmid encoding Mep45-fused protein for substrate; cdc2 PK substrate, PKTPKKAKKL; Mep45, *Selenomonas ruminantium* major envelope protein; circled P, phosphorylated [^{32}P]phosphate.

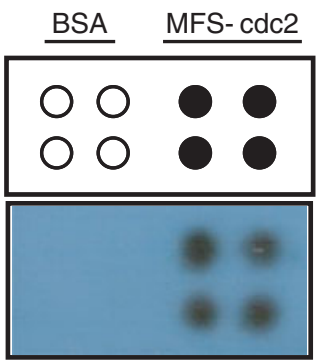

Figure 3. Detecting the substrate of cdc2 protein kinase with [γ-^{32}P]ATP on a glass slide. BSA, bovine serum albumin; MFS-cdc2, Mep45-fused substrate.

various industries. BSA, which is used during a blocking step, binds to any remaining protein binding site after the primary antigen or antibody is bound to a solid-phase surface. As a diluent for antibodies, antigens, and other proteins, BSA ensures a minimal non-specific interaction of these reactants with a solid-phase surface on a glass slide. In the conventional ELISA, interaction between a kinase and a peptide substrate is hindered because of the interference caused by a small peptide (substrate) that gets buried in a bigger BSA.[15] Therefore, it is difficult to detect the phosphorylated peptide with fluorescence-labeled antibodies by ELISA. A minimal non-specific binding of biomolecules on a biochip is crucial for high-quality microarray experiments.

We have reported in our previous investigation that avoiding the use of BSA in a blocking step provides certain advantages. The method is very simple and more sensitive than other conventional methods as it makes use of an RI detection technique. Moreover, it considerably reduces the experimental time as the use of a blocking agent can be avoided.[14] Further, it also facilitates in the immobilization and identification of small molecules on a biochip, which is not possible with other methods.

In this study, we performed a feasibility study using [γ-^{32}P]ATP to determine the activity of the cdc2 protein kinase assay on a glass chip. The detection of phosphorylation of the Mep45 protein-fused substrate catalyzed by the cdc2 protein kinase was determined by using an RI detection technique. Moreover, we found that it is possible to obtain a reasonable quantity of the Mep45 protein-fused substrate for the kinase at a constant rate and its cost can be as low as a synthesized peptide. The results of this study are found to be promising, and can be applied when developing biochips to determine an enzyme activity during a disease diagnosis and a new drug development.

Experimental

Materials

The cdc2 protein kinase was purchased from Abcam (Cambridge, UK). The aldehyde-terminated slides were from Nuricell Inc. (Seoul, Korea). [γ-^{32}P]ATP was purchased from IZOTOP (Budapest, Hungary). Unless specified, all other reagents were obtained from Sigma Chemical Co. (St Louis, MO).

Bacterial strains, plasmids, and culture conditions

The strains used in this study included *S. ruminantium* subsp. *lactilytica* and *E. coli* BL21 (DE3) (Novagen), which were used as host strains. Culture media included a trypticase peptone

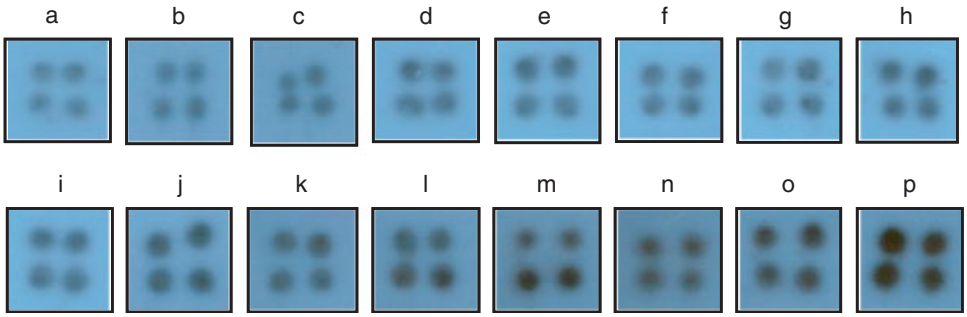

Figure 4. Effect of cdc2 protein kinase concentration on kinase assay. a, 1×10^{-13} μg/mL; b, 1×10^{-11} μg/mL; c, 5×10^{-9} μg/mL; d, 1×10^{-8} μg/mL; e, 5×10^{-7} μg/mL; f, 1×10^{-6} μg/mL; g, 5×10^{-6} μg/mL; h, 1×10^{-5} μg/mL; I, 5×10^{-5} μg/mL; j, 1×10^{-4} μg/mL; k, 5×10^{-4} μg/mL; l, 1×10^{-3} μg/mL; m, 5×10^{-3} μg/mL; n, 1×10^{-1} μg/mL; o, 5×10^{-1} μg/mL; p, 1 μg/mL of cdc2 protein kinase.

Table 1. The nucleotide sequences of the primers designed for the protein-fused substrate of cdc2 protein kinase

Primers	Sequences
cdc2-Fw-Nde	5′-CAT **CAT ATG** CCT AAA ACT CCT AAA AAA GCT AAA AAA CTT GCT AGC AAC CCG TTC TCC GAT G-3′
Rv-Bam	5′-GAC **GGA TCC** TTA GAA GAA GAA CTG AAC GCG ACC GAA G-3′

Boldface, restriction enzyme sites; underline, amino acid sequences of substrate for cdc2 protein kinase; box, stop codon.

(0.2%)–bacto-yeast extract (0.2%)–sodium lactate (1.25%) medium (TYL medium) for *S. ruminantium* and an Luria-Bertani (LB) medium for *E. coli* strains. *S. ruminantium* was grown at 37°C under anaerobic conditions as described previously.[16]

Preparation of the oligonucleotide primers for the cloning of a protein-fused substrate for the cdc2 protein kinase

The 1296 bp of the *mep45* gene encoding the 45 kDa major envelope protein (Mep45) of 432 amino acids in *S. ruminantium* was registered by Ko *et al.* (GenBank Accession No. AB252707). The chromosomal DNA of *S. ruminantium* was isolated and purified by a standard method. The peptide (Pro[1]-Lys-Thr-Pro-Lys-Lys-Ala-Lys-Lys-Leu[10]) is a highly selective substrate of the cdc2 protein kinase, and the oligonucleotide primers cdc2-Fw-Nde (5′-CAT CAT ATG CCT AAA ACT CCT AAA AAA GCT AAA AAA CTT GCT AGC AAC CCG TTC TCC GAT G-3′) and Rv-Bam (5′-GAC GGA TCC TTA GAA GAA GAA CTG AAC GCG ACC GAA G-3′) were designed for a Mep45-fused substrate (MFS-cdc2) for the cdc2 protein kinase. In combination with each primer, a 1.4-kb fragment was approximately amplified from the chromosomal DNA of *S. ruminantium* by PCR using a *Taq* polymerase. Hence, the 1.4-kb PCR fragment was used for cloning of MFS-cdc2 for a phosphorylation assay (Table 1).

Cloning of a protein-fused substrate for the cdc2 protein kinase

PCR amplification was performed in a PCR thermal cycler (Corbett 9600) using the chromosomal DNA of *S. ruminantium* as a template. The reaction mixture contained chromosomal DNA reaction mixture as described above, 1 pmol each of sense and antisense strand primers, 1 mM deoxyribonucleotide triphosphates, 2.0 units of *Taq* DNA polymerase (Takara Bio Inc.), and a *Taq* DNA polymerase reaction buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, pH 9.0). The cycling conditions were as follows: one cycle of 94°C for 5 min for the denaturation of the DNA; 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; and a final extension of 5 min at 72°C. PCR products were visualized on 0.8% agarose gels stained with GelGreen (Biotium, Inc.) and purified by a gel extraction spin column (BIO-RAD). The fragment for *MFS-cdc2* was ligated with pGEM-T vector (Promega), which is designated as the pGEM-T-MFS-cdc2, with *E. coli* JM109 as a host. Isolated plasmid pGEM-T-MFS-cdc2 was digested with *Nde* I and *Bam*H I, and insert fragment of *MFS-cdc2* was ligated with pET-28a vector (Novagen, Madison, WI). The resultant plasmid pMFS-cdc2 was introduced to *E. coli* BL21 (DE3) for the production of the Mep45-fused substrate for the cdc2 protein kinase.

Expression and purification of the recombinant fusion proteins

For the preparation of the Mep45-fused substrate from the cells, the organism was grown in an LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) containing the final concentration of 50 μg/mL kanamycine at 30°C, and it was expressed in 1 mM isopropyl β-D-1-thiogalactopyranoside. The cells were harvested by centrifugation and disrupted by sonication in an ice-cold phosphate-buffered saline (PBS) buffer (200 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, 1 mM Na_2HPO_4, pH 7.5) containing protease inhibitor cocktail, 1% (v/v) Triton X-100, and 10 mM EDTA. After a centrifugation for 5 min at $1000 \times g$, the supernatant was collected, and then centrifuged for 20 min at $16\,000 \times g$ to pellet the MFS-cdc2. The inclusion body of the MFS-cdc2 was repeatedly washed in the 20 mM Tris–HCl buffer (pH 7.5) containing 1% (v/v) Triton X-100 to extract the membrane-associated materials. It was dissolved in 20 mM sodium phosphate containing 6 M guanidine and 500 mM NaCl at pH 7.8. The crude recombinant fusion protein with His-tag at its C-terminal was purified by an Ni-chelating resin (GE Healthcare, Uppsala, Sweden) using a gradient from pH 7.8 to 4.0 in 20 mM

sodium phosphate containing 8 M urea and 500 mM NaCl. The eluted recombinant fusion protein was dialyzed with PBS buffer and the precipitated protein was dissolved in 20 mM sodium phosphate containing 6 M guanidine and 500 mM NaCl at pH 7.8. This recombinant fusion protein was spotted directly and immobilized on a glass slide without any further purification process. The protein compositions of the MFP-cdc2 were determined by SDS-PAGE. Protein concentration was measured by Bradford's method (Bio-Rad protein assay, BIO-RAD) using BSA as a standard.[17]

Determination of cdc2 protein kinase activity

Three hundred and fifty nanograms of the MFP-cdc2 diluted with a PBS buffer containing 1% glycerol was spotted onto the aldehyde-terminated slides. For the immobilization of the substrate, the slides were prepared by incubation in a humid chamber for 1 h at 30°C. The slides were then washed with the PBS buffer and kinase buffer in sequence. The slides were incubated with reaction mixture for 1 h at 37°C. The reaction mixtures were composed of kinase buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 40 mM β-glycerophosphate, 20 mM ρ-nitrophenylphosphate, 0.1 mM sodium vanadate, 0.01% Brij 35, pH 7.5) supplemented with an ATP mixture (50 μM ATP, 0.5 mCi/mL [γ-^{32}P]ATP, 1% BSA) and 10 μg/mL of the cdc2 protein kinase. After reaction, the slides were washed three times for 30 s. After the slides were exposed to an X-ray film (Fuji), they were then developed and analyzed.

Conclusion

Protein microarrays offer an ideal system for rapid and parallel identification of the substrates for a protein kinase. However, several hurdles remain before this protein microarray technology can be deployed on a commercial scale. Thus, it is currently used only as a research tool, while it awaits the development of various biomolecules, a highly sensitive detection method, and an inexpensive price for its production. The use of RIs in a protein microarray is widely preferred to other detection methods because it is highly sensitive, thus facilitating in the detection of even minor quantities of a protein.

In this study, we have synthesized a Mep45-fused substrate for cdc2 protein kinase and studied the phosphorylation of the substrate by varying the concentrations of the enzyme to check on the possibility of using a fused protein substrate in developing a protein biochip. Results of our study showed that the Mep45-fused substrate was highly effective and the phosphorylation of the substrate was detected even with the lowest concentration (1×10^{-13} μg/mL) of cdc2 protein kinase.

The major problem associated with the use of BSA when used as a blocking agent during immunostaining to prevent the non-specific binding of proteins to tissue sections or cytological samples is that it causes the immobilized peptide to become hardly accessible to macromolecules such as enzymes and antibodies.[6,18] Our previous study has already confirmed that there is no need for a blocking step in a protein microarray.[14] Since it does not make use of blocking agents that offer minimum non-specific binding, it even facilitates in the identification of smaller molecules, which is practically not feasible with the other conventional methods. Further, it is possible to obtain a reasonable quantity of a protein-fused substrate at a constant rate and its cost can be as low as a synthesized peptide. We have also reported that the use of an RI detection technique reduces the overall detection time, and it is also highly sensitive thus facilitating in an easy detection.

Hence we conclude that the use of Mep45-fused substrate in developing a protein biochip for the detection of the activity of cdc2 protein kinase by using an RI detection technique is highly economical and can be used for both HTS and studying protein–protein interactions.

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