Nonradioactive Assay of FLAG[®]-Tagged MAPK Using ANTI-FLAG[®] Antibody-Coated Multiwell Plates

L. Zhang, S. Uder, T. Juehne, B. Brizzard, and K. Song
Sigma-Aldrich, St. Louis, MO, USA

BioTechniques 32:442-447 (February 2002)

ABSTRACT

We have developed a rapid, sensitive, and quantitative 96-well microplate-based nonradioactive immunoprecipitation/kinase assay to evaluate mitogen-activated protein kinase (MAPK) activity. Three quantitative nonradioactive immunoprecipitation/kinase assays of MAPK were demonstrated on a 96-well microplate coated with ANTI-FLAG[®] M2 antibody (ANTI-FLAG M2 plate): (i) the capture of phosphorylated FLAG[®]-tagged MAPK fusion protein (FLAG-MAPK) from phorbol esters-stimulated, FLAG-MAPK-transfected COS-7 cells, coupled with a very sensitive ELISA procedure to quantify the level of phosphorylation of FLAG-MAPK; (ii) the in vitro kinase reaction of FLAG-MAPK activity with a substrate and ATP in the same well used to capture the phosphorylated FLAG-MAPK; and (iii) the in vitro kinase reaction of captured non-activated FLAG-MAPK by its upstream kinase from phorbol 12-myristate 13-acetate (PMA)-stimulated COS-7 cells. These results demonstrate that the ANTI-FLAG M2 plate allows for the rapid and quantitative determination of phosphorylation of FLAG-MAPK directly from stimulated, transfected cell lysate. Captured, phosphorylated FLAG-MAPK retains catalytic activity as demonstrated by the phosphorylation of Elk-1 in the same well. Furthermore, phosphorylation of captured FLAG-MAPK by the upstream kinases can be observed directly on the plate. These assays are sensitive, specific, and suitable for handling multiple samples. Thus, the ANTI-FLAG M2 plate forms the basis of a high-throughput screening platform in kinase analysis.

INTRODUCTION

Epitope tags provide a convenient way to isolate interacting proteins without the need for specific antibodies to each new protein. To date, there are numerous tags that have been reported for the expression of recombinant proteins in mammalian expression systems, with the FLAG[®] epitope tag being

one of the most common. The system relies on the small FLAG octapeptide (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) that can be detected by the ANTI-FLAG[®] M2 antibody (9). The FLAG epitope system has been used in expression systems for the detection and purification of heterologous proteins in many biological systems including *E. coli* (2,9), *Saccharomyces cerevisiae* (1,10), and mammalian systems (12,15).

A common immunoprecipitation/kinase assay is an in vitro procedure utilizing antibody-coated resin to study the function of protein kinases. Basically, the procedure involves immunoprecipitation of the protein kinase of interest with an antibody-coated affinity resin, followed by an in vitro kinase reaction catalyzed by the kinase bound to the resin with the addition of [γ -³²P]ATP or nonradiolabeled ATP and substrates. Gel electrophoresis followed by autoradiography and Western analysis using specific phospho-antibodies is then employed to examine the ³²P-labeled substrates or phosphorylated substrates, respectively. Although it is widely used, this method has its limitations, such as numerous centrifugation and time-consuming washing steps, and is thus not readily amenable to use with a large number of samples. Also, special safety measures need to be addressed if [γ -³²P]ATP is used for the kinase reaction. Moreover, the aspirations required for removing the washing buffer often result in a loss of the affinity resin and bound kinase, which makes reproducibility between experiments a major concern. We report here a nonradioactive plate-based system for studying kinase activity using an ANTI-FLAG M2 antibody coated 96-well microplate (ANTI-FLAG M2 plate) that allows efficient capture of FLAG-tagged kinase from a transfected cell lysate and, subsequently, functionally characterizes the captured kinase on the plate.

The ANTI-FLAG M2 plate is prepared by covalently attaching the ANTI-FLAG M2 monoclonal antibody (mouse IgG1 isotype) to the surface of a 96-well clear microplate via

the Fc portion of the antibody. This attachment provides a favorable orientation for the antibody on the surface for maximum capture of FLAG-fusion protein. The ANTI-FLAG M2 monoclonal antibody binds to the FLAG epitope regardless of its location in the fusion protein (2).

To demonstrate ANTI-FLAG M2 plate-based analysis of protein kinase activity, we selected one of the well-studied kinase pathways, the mitogen-activated protein kinase (MAPK) pathway. The MAPK signaling pathway has been shown to play an important role in cell proliferation and differentiation (17). The MAPK pathway is activated by a variety of extracellular signals, including growth factors, mitogens, and cellular stress (4,5,16). The activation of receptor tyrosine kinase and G-protein-coupled receptors can lead to the activation of the ERK1/2 (p44/p42 MAPK) proteins. Activation occurs because of the phosphorylation of the Thr183 and Tyr185 by a dual specificity kinase, mitogen or extracellular signal-regulated protein kinase (MEK) (3). Downstream targets of MAPK include the Elk-1 and Stats transcription factors (8,11). In this report, p42 MAPK was expressed as a FLAG-MAPK fusion protein, and three different types of FLAG-MAPK activity analysis were performed to study FLAG-MAPK within the ANTI-FLAG M2 plate format. The plate-based immunoprecipitation/kinase assay overcomes the disadvantage of the traditional resin-based technique because there are no centrifugation steps involved and analyzing multiple samples can be performed with relative ease. Moreover, because the use of radioisotope is eliminated in these assays, safety and disposal issues involved with the use of radioisotope are avoided. In addition, because the ANTI-FLAG M2 antibody is covalently coupled to the plate and uniformly distributed, high-throughput quantitative measurement of the kinase becomes feasible.

MATERIALS AND METHODS

Unless otherwise indicated, all of the materials used in this research are from Sigma (St. Louis, MO, USA).

Binding Capacity of the ANTI-FLAG M2 Plate

The maximum binding capacity per well of the ANTI-FLAG M2 plate was determined by using purified FLAG-tagged bacterial alkaline phosphatase (FLAG-BAP) protein and estimated to be about 400 ng or 1 pmol per well. In addition, the detection sensitivity (based on a standard BAP enzyme activity assay) (6) is about 1 ng or 20 fmol. The standard deviation of binding capacity from plate to plate and well to well is less than 10%.

Constructs

The pFLAG-CMVTM-2-MAPK vector expresses the N-terminal tagged FLAG fusion protein containing full-length human p42 MAPK. For the preparation of pFLAG-CMV-2-MAPK, DNA encoding the full-length open reading frame between base pairs 328 and 1410 of human p42/MAPK was generated by RT-PCR from Jurkat cells with a pair of primers pMAPK forward (5'-CAGTAAGCTTATGGCGGCGGCGGC-GGCGGCGGGCGCG-3') and pMAPK reverse (5'-CTAGTCTAGATTAAGATCTGTATCCTGGCTGGAATCT-3') using

Taq ReadyTM Mix. The conditions for PCR were 30 s of denaturation at 94°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C for 25 cycles. The resulting PCR fragment contains *Hind*III sites on the 5' and *Xba*I on the 3' sites, respectively. The PCR products were then digested with *Hind*III and *Xba*I and ligated into the *Hind*III/*Xba*I-digested pFLAG-CMV-2 vector. The sequence of the resulting plasmid, pFLAG-CMV-2-MAPK, was confirmed using the BigDyeTM terminator cycle sequencing kit and an ABI PRISM[®] 373 DNA sequencer (both from Applied Biosystems, Foster City, CA, USA).

Cell Culture and Transfection

The COS-7 mammalian cell line was obtained from ATCC (Manassas, VA, USA). Cells were maintained at 37°C in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 500 mg/L L-glutamine. For transfection, the cells were plated in 6-well microplates at 4 × 10⁵/well, 18 h before the transfection with the SuperFectTM transfection reagent according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). Cells were transfected with 2 µg pFLAG-CMV-2-MAPK using 10 µL SuperFect.

Stimulation of the Cells with PMA

Unless otherwise indicated, cells were incubated for 15 min with 100 ng/mL phorbol 12-myristate 13-acetate (PMA), 24 h after transfection. When specified, cells were pretreated with 30 µM synthetic MEK inhibitor, PD098059, for 30 min before PMA stimulation.

Preparation of Cell Lysate

The cells were harvested in 0.5 mL ice-cold cell lysis buffer supplemented with mammalian protease inhibitors (10 µL/1 mL cell lysis buffer). The cell lysis buffer contains 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% Triton[®] X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, and 1 mM Na₃V₄O₄. The cells were homogenized with a Kinematica AG Polytron[®] PT 1200 (Brinkmann Instruments, Westbury, NY, USA), followed by incubation on ice for 30 min and microcentrifugation at 16 000× g for 10 min at 4°C. Total protein of the supernatant was determined using a Bicinchoninic Acid Kit.

Capture of FLAG-MAPK and Phospho-FLAG-MAPK with the ANTI-FLAG M2 Plate

The ANTI-FLAG M2 plate was incubated with various amounts of cell lysate, as indicated in the figure legends, on a rotary shaker with gentle rocking for 1 h at 4°C. The final volume of each well was brought to 150 µL with cell lysis buffer. At the end of the incubation, the plate was washed four times with 200 µL 1× kinase buffer containing 25 mM Tris, pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na₃V₄O₄, and 10 mM MgCl₂.

Detection of the Phospho-FLAG-MAPK with Anti-MAPK, Activated (Diphosphorylated ERK-1&2) Alkaline Phosphatase Conjugate on the ANTI-FLAG M2 Plate

Anti-MAPK, Activated Alkaline Phosphatase Conjugate (200 µL) diluted 1:250 in the blocking buffer was added to

each well. The plate was incubated for 1 h at room temperature on a rotary shaker with gentle agitation. The blocking buffer contains 3% nonfat milk in TBST (Tris-buffered saline with Tween[®] 20, pH 8.0). At the end of incubation, liquid was removed from the well and the plate was washed four times with 200 μ L TBST. The plate was then incubated with 200 μ L pNPP substrate. The plate was allowed to incubate at room temperature for 15–30 min or until a yellow color developed. The reaction was stopped with 50 μ L stopping solution and 3 M NaOH. The absorbance reading was taken at 405 nm (A_{405}).

“In-Well” Kinase Assay of Phospho-FLAG-MAPK Captured on ANTI-FLAG M2 Plate

Fifteen micrograms of each cell lysate, as described in Figure 2, were incubated with ANTI-FLAG M2 plate as described above. At the end of the incubation, the plate was washed four times with 200 μ L 1 $\times$ kinase buffer. To initiate the kinase reaction, the following components were added in a final volume of 40 μ L: 38.4 μ L 1 $\times$ kinase buffer, 0.8 μ g substrate GST-Elk-1 fusion protein (Cell Signaling Technology, Beverly, MA, USA), and 0.8 μ L 10 mM ATP. The phosphorylation reaction was initiated by incubating the plate at 30°C for 30 min.

Examination of Phosphorylated GST-Elk-1

Phosphorylation of substrate GST-Elk-1 was analyzed by two methods: ELISA and Western analysis. For ELISA, the

kinase reaction was terminated by adding 160 μ L 1 $\times$ kinase buffer to the wells, and the entire reaction (200 μ L from the ANTI-FLAG M2 plate was transferred to a Reacti-Bond[™] Glutathione Coated Plate (GSH plate) (Pierce Chemical, Rockford, IL, USA). The plate was allowed to incubate for 1 h at room temperature on a rotary shaker with gentle agitation. At the end of incubation, the GSH plate was washed five times with 200 μ L 1 $\times$ kinase buffer; anti-phospho-Elk-1 diluted in the blocking buffer was added at 200 μ L/well and incubated at room temperature for 1 h at 4°C. Anti-rabbit IgG peroxidase conjugate (200 μ L) diluted 1:10 000 in the blocking buffer was added as the secondary antibody to each well and incubated for 1 h at room temperature. At the end of incubation, the plates were washed four times with TBST, followed by incubation with peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB) at 200 μ L/well for 20 min. Reactions were stopped with 100 μ L H₂SO₄. The absorbance reading was taken at 450 nm (A_{450}).

For Western analysis, the kinase reaction in the ANTI-FLAG M2 plate was terminated by adding 20 μ L 4 $\times$ SDS sample buffer and 10% 2-mercaptoethanol (β ME) to the wells and the entire reaction (60 μ L) was boiled for 5 min and subsequently analyzed by Western blotting.

Western Analysis

Western analysis was carried out following standard procedures. Briefly, proteins were resolved on a 4%–20% gradi-

ent SDS-PAGE gel and electrotransferred to a PVDF membrane. The blot was blocked in blocking buffer and probed with affinity-purified polyclonal anti-phospho-Elk-1 antibody diluted 1:1000 in blocking buffer. Goat anti-rabbit IgG peroxidase conjugate was used as the secondary antibody diluted 1:10000 in the blocking buffer. Reactive bands were detected with ECL plus™ according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA).

“In-Well” Phosphorylation of FLAG-MAPK Captured on ANTI-FLAG M2 Plate

Various amounts of FLAG-MAPK cell lysate were incubated with the ANTI-FLAG M2 plate as described above. At the end of the incubation, wells were washed with 1× kinase buffer, followed by the phosphorylation reaction. The “In-Well” phosphorylation reaction was assembled by adding (in a final volume of 150 μ L) 75 μ L of 1× kinase buffer, 72 μ L cell lysate (40 μ g) prepared from PMA-stimulated, non-transfected COS-7 cells, and 3 μ L 10 mM ATP. The plate was then incubated for 30 min at 30°C, and the reactions were terminated by discarding the solution, followed by washing the wells four times with 1× kinase buffer.

RESULTS AND DISCUSSION

COS-7 cells were transfected with pFLAG-CMV-2-MAPK and, 24 h after transfection, stimulated with PMA for various times as indicated in Figure 1. Detection of the plate-bound phosphorylated FLAG-MAPK was measured by Anti-MAPK, Activated (Diphosphorylated ERK-1&2) Alkaline

Figure 1. Capture of the phospho-FLAG-MAPK from PMA-stimulated pFLAG-CMV-2-MAPK-transfected COS-7 cells on the ANTI-FLAG M2 plate. Capture of the phospho-FLAG-MAPK was performed by incubating the ANTI-FLAG M2 plate with 5 μ g cell lysates prepared from the transfected cells harvested at different time points after stimulation as indicated. Upper panel, ELISA of the captured phospho-MAPK. Values represent the means $\pm$ SD from three independent experiments. Lower panel, Western analysis of the whole cell lysates used in the upper panel (7 μ g/lane). Total FLAG-MAPK was detected with ANTI-FLAG M2 antibody, followed by anti-mouse IgG peroxidase conjugate as the secondary antibody. C, cell lysate prepared from COS-7 cell transfected with empty vector pFLAG-CMV-2.

Phosphatase Conjugate. Figure 1 (upper panel) shows that the ANTI-FLAG M2 plate successfully captured phospho-FLAG-MAPK from PMA-treated cells. Treatment of FLAG-MAPK-expressing cells with PMA markedly enhanced the phosphorylation of the FLAG-MAPK in the ELISA (Figure 1, upper panel), while the expression level of the total FLAG-MAPK is comparable in each lysate as seen when probed by Western analysis with the ANTI-FLAG M2 antibody (Figure 1, lower panel). Phosphorylation was transient, where phosphorylated FLAG-MAPK level increased after 5 min of treatment, reached maximum at 15 min of PMA treatment, and decreased towards baseline after longer periods of stimulation. The time course for phosphorylation correlated closely with the activation of MAPK in several cell lines (13,14). PMA stimulation was carried out for 15 min throughout the rest of the studies. These data clearly demonstrate the utility of ANTI-FLAG M2 plate as an effective device for quantitative phosphorylation analysis.

An important aspect of the use of the ANTI-FLAG M2 plate for kinase analysis is to determine not only the ability to capture the kinase but also the functional consequence of capturing. To this end, we developed an “In-Well” in vitro kinase assay, in which the activity of the captured phospho-FLAG-MAPK can be analyzed by incubating the substrate GST-Elk-1 in the same well used to capture the phospho-MAPK. It has been well documented that the p44/42-MAPK pathway inhibitor PD098059 inhibits MEK1/2, upstream activators of p42/44-MAPK, and therefore blocks p42/44-MAPK phosphorylation and activation (7). To evaluate the effect of the inhibitor on the level of the phosphorylation of FLAG-MAPK expressed in the COS-7 cells, we used the ANTI-FLAG M2 plate to capture total FLAG-MAPK and subsequently detect the phospho-FLAG-MAPK on the plate using Anti-MAPK, Activated Alkaline Phosphatase Conjugate in an ELISA. As shown in Figure 2 (upper panel), phosphorylation of FLAG-MAPK was substantially increased upon PMA stimulation as compared to the cell lysate prepared from the cells transfected with the empty vector, whereas phospho-FLAG-MAPK was significantly decreased in cells pretreated with PD098056. To validate the effect of the inhibition by PD098059 on the phosphorylation of FLAG-MAPK observed on the ANTI-FLAG M2 plate, we performed Western analysis of the same whole-cell lysates used for the ELISA using Anti-MAPK, Activated (Diphosphorylated ERK-1&2) Unconjugated antibody. As shown in Figure 2 (middle panel), the results correlate very well with the ELISA analysis. Figure 2 (lower panel) shows equivalent expression of FLAG-MAPK as revealed by Western analysis of the treated whole-cell lysates using the ANTI-FLAG M2 antibody.

We then examined whether or not the captured phospho-FLAG-MAPK was capable of catalyzing the phosphorylation of the substrate GST-Elk-1 fusion protein in an in vitro kinase reaction. To this end, the same amount of cell lysate prepared from the transfected cells with or without pretreatment of PD098056 used in Figure 2 were incubated with the ANTI-FLAG M2 plate. A cell lysate prepared from the cells transfected with the empty vector was included as a control. The same amount of substrate GST-Elk-1 was then added to each well to initiate the kinase reaction. Western analysis of the entire kinase reaction content using anti-phospho-Elk-1 antibody reveals that the kinase reaction is specific, as demonstrated by the failure of phosphorylation of GST-Elk-1 in the well incubated with cells transfected with the empty vector,

even though the same amount of GST-Elk-1 was added to the well (Figure 3, upper panel, lane 1). Figure 3 (upper panel, lane 2) shows that captured phospho-FLAG-MPAK was able to catalyze the phosphorylation of GST-Elk-1. Because very little FLAG-MAPK activation was detected after PD098059 treatment (Figure 2), the phosphorylation of GST-Elk-1 was significantly decreased (Figure 3, upper panel, lane 3). These results indicate that the captured phosphorylated FLAG-MAPK still retains its functionality and was able to catalyze the phosphorylation of its substrate on the plate.

To maintain the microplate format throughout the whole kinase assay process and thus further enhance the utility of the ANTI-FLAG M2 plate for high-throughput applications, we performed an ELISA as an alternative to Western analysis to detect phosphorylated substrate GST-Elk-1. To this end, the entire kinase reaction content was transferred from the ANTI-FLAG M2 plate to a GSH plate to capture total substrate GST-Elk-1. Then, detection of phospho-ELK-1 was accomplished on the GSH plate in an ELISA using the anti-phospho-Elk-1 antibody. As expected, the level of phosphorylation of GST-Elk-1 correlated with the activation of the FLAG-MAPK captured on the ANTI-FLAG M2 plate, a result that is in line with the results using Western analysis (Figure 3, lower panel). On the whole, these results clearly indi-

cate that captured phosphorylated FLAG-MAPK is functionally active. More importantly, by using the GSH plate to capture the substrate followed by the detection of the level of phosphorylation of the substrate using a specific anti-phospho-antibody, we further extended the microplate format into the detection step of the kinase activity assay, which eliminated the laborious Western analysis.

To determine whether captured, non-activated FLAG-MAPK can be phosphorylated by its upstream regulators in an *in vitro* reaction, FLAG-MAPK expressed in COS-7 cells was first captured on ANTI-FLAG M2 plate using varying amounts of transfected cell lysate. Then, the captured FLAG-MAPK was phosphorylated *in vitro* with PMA-stimulated, non-transfected COS-7 cell lysate at a fixed ATP concentration of 200 μ M in the same wells. Detection of the phospho-FLAG-MAPK was performed with Anti-MAPK, Activated (Diphosphorylated ERK-1&2) Alkaline Phosphatase Conjugate. To monitor the basal level of FLAG-MAPK phosphorylation, an extra well was incubated with the highest amount of FLAG-MAPK-COS-7 cell lysate used (without PMA stimulation) for the phosphorylation reaction (160 μ g). The specificity of the assay is remarkable since no measurable basal level of FLAG-MAPK phosphorylation was observed in the wells in the absence of stimulated COS-7 cell lysate (data not shown). High signal-to-noise ratio was observed between the wells incubated with pFLAG-CMV-2-MAPK-transfected cell

Figure 2. Effect of inhibition of FLAG-MAPK and "In-Well" kinase assay of FLAG-MAPK on the ANTI-FLAG M2 plate. Upper panel, ELISA of phosphorylation of FLAG-MAPK after incubating the plate with equal amounts of cell lysate (15 μ g) prepared from COS-7 cell transfected with empty vector pFLAG-CMV-2 (control), PMA-stimulated COS-7 cells transfected with pFLAG-CMV-2-FLAG-MAPK (PMA), and PMA-stimulated COS-7 cells transfected with pFLAG-CMV-2-FLAG-MAPK pretreated with PD098059 (PMA+PD). Each bar represents the mean $\pm$ SD from triplicate wells. Middle panel, Western analysis of identical samples with Anti-MAPK, Activated (Diphosphorylated ERK-1&2) Unconjugated antibody (1:10 000). Lower panel, total FLAG-MAPK corresponding to lanes 2 and 3 in the upper panel, detected with the ANTI-FLAG M2 antibody.

Figure 3. "In-Well" analysis of FLAG-MAPK activity captured on ANTI-FLAG M2 plate. Capturing and *in vitro* MAPK reaction was carried out using anti-phospho-Elk-1 antibody as described in the Materials and Methods section. Phosphorylation of GST-Elk-1 was determined either by Western analysis or ELISA (upper and lower panel, respectively). Lane 1 and control (upper and lower panel, respectively), COS-7 cell transfected with empty vector pFLAG-CMV-2; lane 2 and PMA (upper and lower panel, respectively), PMA-stimulated COS-7 cells transfected with pFLAG-CMV-2-FLAG-MAPK; lane 3 and PMA+PD (upper and lower panel, respectively), PMA-stimulated COS-7 cells transfected with pFLAG-CMV-2-FLAG-MAPK pretreated with PD098059. Bars represent the means $\pm$ SD of triplicate wells.

lysate and empty vector-transfected cell lysates, as shown in Figure 4. In addition, the assay is very sensitive, as the level of the phospho-MAPK can be detected in as little as 5 μ g of the cell lysates. The degree of FLAG-MAPK phosphorylation increased with the increasing amount of the FLAG-MAPK captured on the plate, in which a linear range between 5 and 50 μ g of transfected COS-7 cell lysates is observed. The degree of FLAG-MAPK phosphorylation increased only slightly when a higher amount (>50 μ g) of transfected-COS-7 cell lysate was used, suggesting that the plate either failed to absorb all FLAG fusion protein from the cell lysate or an insufficient amount of PMA-stimulated, non-transfected cell lysates that provided the upstream regulators for the kinase reaction was used. Based on the finding that the maximal amount of FLAG-fusion protein to saturate the plate was close to 400 ng (data not shown) and the expression level of the FLAG-MAPK was approximately 7 ng/ μ L of the transfected cell lysate (data not shown), we hypothesized that the observed smaller degree of increased phospho-FLAG-MAPK level for the wells incubated with more than 50 μ g of the transfected cell lysate was most likely due to the fact that the plate was close to the saturation level. This experiment was performed on at least three occasions, and similar results were obtained each time. Taken together, these results indicate that the *in vitro* kinase assay on the ANTI-FLAG M2 plate is sensitive, specific, quantitative, and far less cumbersome and time consuming than the resin-based immunoprecipitation/kinase assay.

In conclusion, we demonstrate the feasibility of the ANTI-FLAG M2 plate-based kinase analysis. The format and protocol of this quantitative MAPK assay can be easily adapted to other kinases. With some minor modification, the plate could allow for the high-throughput screening of substrates of kinases and for the rapid and parallel identification of upstream regulators of protein kinase. Although we believe the sensitivity and binding capacity of the ANTI-FLAG M2 plate is adequate for most kinase assays, further improvements are in progress that aim to enhance the binding capacity of the ANTI-FLAG M2 plate.

Figure 4. "In-Well" phosphorylation of FLAG-MAPK by PMA-stimulated, non-transfected COS-7 cells lysate in the wells of the ANTI-FLAG M2 plate. Capturing, *in vitro* phosphorylation of FLAG-MAPK reaction, and detection of the phosphorylated FLAG-MAPK were described in the Materials and Methods section. (●) COS-7 cells transfected with pFLAG-CMV-2-MAPK; (▼) COS-7 cells transfected with empty vector pFLAG-CMV-2.

ACKNOWLEDGMENTS

We would like to thank Drs. Bill Kappel and John Dapron for their technical input on development of the ANTI-FLAG M2 plate.

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Address correspondence to Dr. Lin Zhang, Research and Development, Sigma-Aldrich/Biotechnology Division, P.O. Box 14508, St. Louis, MO, USA. e-mail: lzhang@sial.com