

A Rapid and Sensitive Quantitative Kinase Activity Assay Using a Convenient 96-Well Format

Anand R. Asthagiri,* Alan F. Horwitz,† and Douglas A. Lauffenburger*¹

*Department of Chemical Engineering and Division of Bioengineering & Environmental Health, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; and †Department of Cell & Structural Biology, University of Illinois, Urbana-Champaign, Illinois 61801

Received November 24, 1998

Activation of protein kinases in response to growth factor and extracellular matrix stimulation has been implicated in regulating a number of cell functions including differentiation, gene expression, migration, and proliferation. An improved quantitative assay for measuring protein kinase activity is crucial to the detailed study of this important category of signaling proteins and their role in regulating cell behavior. We describe a modified *in vitro* kinase activity assay that is both sensitive and quantitative. It offers several advantages when compared to the traditional immunoprecipitation/kinase assay: (i) high sensitivity that reduces the required amount of cell lysate by an order of magnitude, (ii) an immunoseparation technique utilizing antibody immobilization onto the surface of microtiter wells that replaces the cumbersome immunoprecipitation method, (iii) a 96-well plate configuration that eases handling of multiple samples and increases throughput of the assay, and (iv) the use of 96-well filter plates that greatly reduces radioactive liquid waste generation. While we implement this technique in a case study for measuring the activity of extracellular signal-regulated kinase 2 (ERK2), this assay can be extended to studying other protein kinases by using an appropriate antibody and *in vitro* substrate for the kinase of interest. © 1999 Academic Press

Key Words: extracellular signal-regulated kinase (ERK); immunocomplex kinase assay; immunoprecipitation; immunoseparation; kinase activity; mitogen-activated protein kinase (MAPK).

Growth factors and extracellular matrix proteins are known to initiate signaling cascades which lead to the

activation of various kinases, including the mitogen-activated protein (MAP)² kinase family of serine/threonine protein kinases. Upon activation by dual phosphorylation at a threonine and tyrosine residue, MAP kinases activate downstream targets that have been implicated in controlling gene expression, cell differentiation, and proliferation (1). To study the role of MAP kinases and other protein kinases in regulating these cell functions, a rapid and efficient technique for measuring kinase activity has been developed. While we highlight the applicability of this technique with a case study of ERK2, a particular member of the MAP kinase family, this technique can be adapted readily to the quantitative study of other kinases.

A functional assay of kinase activity generally follows three basic steps: (i) separation of the kinase from other cell lysate proteins, (ii) *in vitro* reaction catalyzed by this isolated kinase, and (iii) determination of the extent of this reaction as a measure of kinase activity. Typically, these steps are achieved by immunoprecipitating the kinase, performing an *in vitro* reaction with a protein substrate and radiolabeled [γ -³²P]ATP, and measuring the amount of ³²P incorporated into the substrate (2). Immunoprecipitations involve work with antibody-coated beads which pose certain disadvantages. In the wash steps, repeated centrifugations make it cumbersome to handle a large number of samples, and aspirations designed to remove the wash buffer can also result in loss of beads and the kinase. Following the immunoprecipitation and *in vitro* kinase

¹ To whom correspondence should be addressed. Fax: 617-258-0248. E-mail: lauffen@mit.edu.

² Abbreviations used: ATP, adenosine triphosphate; BSA, bovine serum albumin; CHO cells, Chinese hamster ovary cells; DEAE, diethylaminoethyl; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline.

reaction, the degree of ^{32}P incorporation into the substrate is measured typically by spotting a sample from the reaction onto a phosphocellulose membrane, which binds efficiently to proteins but minimally to ATP. The membrane is washed several times in large amounts of buffer, thereby generating a significant amount of ^{32}P -containing liquid waste.

Several groups have offered improvements to particular aspects of this traditional immunocomplex kinase assay. Non-radioactive techniques utilize substrate-immobilized microwells into which the kinase and ATP are supplied to initiate the reaction (3–5). The degree of substrate phosphorylation is measured using an anti-phosphotyrosine antibody. While this approach eliminates the use of radioisotopes, it does not propose an alternative to the cumbersome immunoprecipitation technique for isolating the kinase from the other cell lysate proteins. Therefore, the microwell format is not maintained through all three steps of the kinase activity assay, thereby disallowing high throughput and easy handling of samples. An alternate approach in a study of *cdc2* kinase activity made use of p13's high specificity for binding *cdc2* (6). Instead of immobilizing the substrate, p13-coated microwells were used to capture *cdc2*, and the kinase reaction was initiated by adding a protein substrate and [^{32}P]ATP. However, the degree of ^{32}P incorporation was measured using the traditional dot-blotting method that generates large amounts of radioactive waste and is not amenable to handling a large number of samples. Others have addressed this problem of liquid waste generation by using microplates which have filters fitted into each well (7, 8). These assays were optimized to perform the kinase reaction within these wells and subsequently to filter the contents leaving just the phosphorylated protein substrate on the filter. While this method reduces the amount of radioactive liquid waste, the kinases in each work were either isolated from cell lysate by tedious DEAE-cellulose chromatography (7) or a crude form was obtained directly from a vendor (8). Thus, while these alternative techniques offer simplifications to the traditional method, each approach addresses only a subset of the three general steps that comprise a kinase activity assay.

Here we have developed a method that utilizes a convenient 96-well format through all three steps of a kinase activity assay, from the initial isolation of the kinase from the cell lysate to the final quantification of the progress of the *in vitro* reaction catalyzed by this kinase. Maintaining this microtiter format through the entire assay permits high throughput and easy handling of multiple samples. In addition, this technique offers greater sensitivity than the traditional immunocomplex kinase assay. To demonstrate its applicability, the assay was utilized in measuring the activity of the kinase ERK2. An alternative to immunoprecipitation

is provided for isolating ERK2. Instead of coating the antibody on protein A/G-coated beads, anti-ERK2 antibody is immobilized to a protein A-coated microtiter well surface. When lysate is incubated in this well, ERK2 binds to the antibody and becomes associated with the well surface (Fig. 1, panel 1). Washes with fresh buffer successfully remove the undesired lysate proteins from the well leaving behind the bound ERK2. These quick and simple washes eliminate the cumbersome centrifugation steps required in an immunoprecipitation. Furthermore, this microtiter well immunoseparation can be generalized to isolating other proteins of interest by utilizing the appropriate antibody.

By performing this immunoseparation in protein A-coated 8-well strips that fit into a 96-well template plate, multiple samples can be handled with ease at one time. This 96-well format is extended into the second step of the assay in which an *in vitro* reaction with a substrate protein and [γ - ^{32}P]ATP is carried out in the same well used to purify ERK2 (Fig. 1, panel 2). Upon quenching the reaction, samples are transferred to a 96-well filter plate, in which each well contains a phosphocellulose membrane (Fig. 1, panel 3). After filtering the samples under vacuum, the phosphorylated substrate protein is captured on the filter, while the solution containing the unreacted [^{32}P]ATP flows through the filter. This filtration method greatly reduces the amount of radioactive liquid waste compared to the traditional spotting-and-washing technique. The radioactivity associated with each filter is quantified by scintillation counting and corresponds to the activity of the recovered ERK2. Taken together, this technique allows for easy handling of samples and high-throughput quantitative measurements of kinase activity.

MATERIALS AND METHODS

Antibodies and Reagents

The sc-154 anti-ERK2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Activated MAPK from Stratagene (La Jolla, CA) was used as a positive control.

Cell Maintenance

CHO cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 4 mM L-glutamine, 1 mM sodium pyruvate, and 1% (v/v) 100 \times nonessential amino acids.

EGF Stimulation and Lysate Preparation

Prior to EGF stimulation, cells were serum-starved on 100-mm tissue culture dishes for 18 h in serum-free media containing 25 mM HEPES-based DMEM, 4 mM

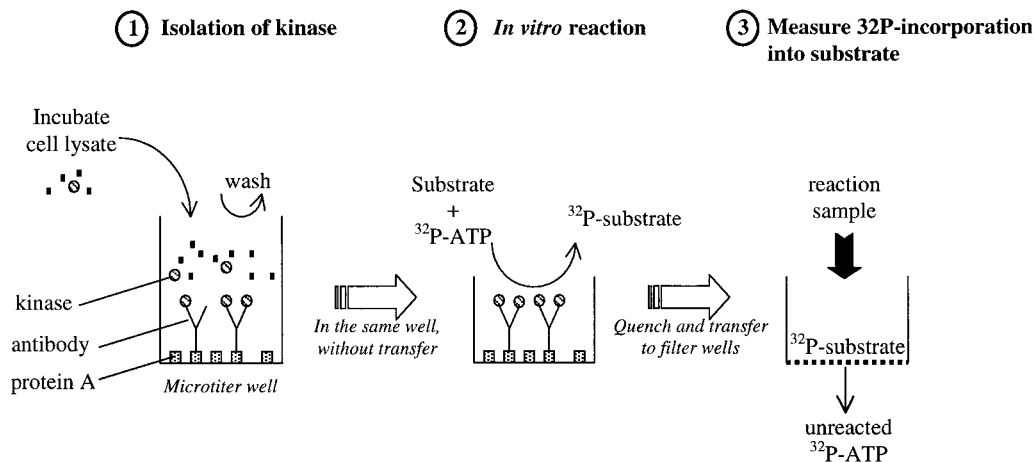


FIG. 1. This kinase activity assay preserves a 96-well format through the entire procedure allowing high throughput. A kinase activity assay can be partitioned into three general steps: (i) the isolation of the kinase from the other cell lysate proteins, (ii) an *in vitro* reaction in which the protein substrate is phosphorylated by the kinase, and (iii) the recovery of the phosphorylated substrate and quantification of the degree of phosphorylation as a measure of kinase activity. In the first step of this assay, an antibody that recognizes the kinase is used to isolate it. The antibody is coated in the well in the proper orientation through its interaction with protein A, which is covalently bound to the well surface. The second reaction step is performed in the same microtiter well in which the kinase was isolated. After quenching the reaction, the third step of recovering the phosphorylated substrate is performed by filtering a sample from the reaction through a 96-well plate fitted with a phosphocellulose membrane. This membrane captures the phosphorylated substrate and allows unreacted ATP to pass through. The filters are then punched out and scintillation counted to determine the amount of ^{32}P incorporation into the protein substrate.

L-glutamine, 1 mM sodium pyruvate, 1% (v/v) 100 \times nonessential amino acids, and 2 mg/ml bovine serum albumin (BSA). Dishes were washed twice with 2 ml PBS before adding 15 nM mouse EGF (Gibco BRL, Grand Island, NY) in serum-free media to begin stimulation. After incubation at 37°C for desired stimulation times, the EGF-containing media was aspirated and dishes were washed twice with cold PBS. While keeping dishes on ice, cells were lysed in cold lysis buffer containing 50 mM Tris, pH 7.5, 150 mM sodium chloride, 50 mM β -glycerophosphate, pH 7.3, 10 mM sodium pyrophosphate, 30 mM sodium fluoride, 1% Triton X-100, 1 mM benzamide, 2 mM EGTA, 100 μM sodium orthovanadate, 1 mM DTT, 10 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ pepstatin, and 1 mM PMSF. Cells were scraped into the buffer and allowed to lyse for approximately 15 min. Lysates were centrifuged at 14,000 rpm for 15 min, and the supernatant was collected. Micro BCA protein determination (Pierce) was used to determine total protein concentration in lysates.

ERK2 Activity Assay

Immunoseparation in microtiter wells. To coat anti-ERK2 antibody onto a microtiter well surface, Reacti-Bind protein A-coated wells (Pierce, Rockford, IL) were incubated overnight at 4°C with 50 μl of 10 $\mu\text{g}/\text{ml}$ sc-154 antibody in blocking buffer containing 1% BSA, 50 mM Tris (pH 7.5), 150 mM sodium chloride, and 0.05% Triton. Wells were then washed three times with blocking buffer. Cell lysate diluted in lysis buffer

to a total volume of 50 μl was incubated for 3 h at 4°C to allow sc-154 antibody binding of ERK2 to reach equilibrium. To measure background, an extra well was incubated with just lysis buffer and was handled throughout the assay in the same manner as other samples. Each well was then washed twice with 200 μl wash buffer containing 50 mM Tris (pH 7.5) and 150 mM sodium chloride and twice more with 200 μl kinase wash buffer containing 20 mM Tris (pH 7.5), 15 mM magnesium chloride, 5 mM β -glycerophosphate (pH 7.3), 1 mM EGTA, 0.2 mM sodium orthovanadate, and 0.2 mM DTT. The contents of the well were then resuspended in 20 μl kinase wash buffer.

***In vitro* kinase reaction.** To each well, 20 μl of 2 mg/ml MBP (Sigma, St. Louis, MO) was added as the substrate for ERK2. To initiate the *in vitro* reaction, 20 μl kinase assay buffer containing 20 mM Tris (pH 7.5), 15 mM magnesium chloride, 5 mM β -glycerophosphate (pH 7.3), 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM DTT, 0.4 μM protein kinase A inhibitor peptide (Upstate Biotech, Lake Placid, NY), 4 μM protein kinase C inhibitor peptide (Upstate Biotech), 4 μM calmidazolium (Upstate Biotech), 25 μM ATP, and 6 μCi [γ - ^{32}P]ATP was added. Reaction contents were maintained under agitation at 37°C with the Jitterbug (Boekel, Feasterville, PA). After 10 min, reactions were quenched with 60 μl of 75 mM phosphoric acid.

Measuring ^{32}P incorporation into MBP. [^{32}P]MBP was separated from unreacted [^{32}P]ATP by filtering 40 μl of the quenched reaction contents through a phos-

phocellulose filter using the Millipore Multiscreen system (Millipore, Bedford, MA). Each filter was washed five times with 200 μ l 75 mM phosphoric acid and three times with 200 μ l 70% ethanol. The filters were allowed to dry before punching out the filters into scintillation vials. 32 P amounts on the filter paper were quantified using CytoScint (ICN Biomedicals, Costa Mesa, CA) scintillation fluid and a RackBeta (Wallac, Gaithersburg, MD) scintillation counter. 32 P measurements are adjusted by subtracting the radioactivity associated with the background sample.

Western Blots

ERK2 was immunoseparated using microtiter wells as described above. After the final wash with kinase buffer, equal amounts of lysis buffer and 2 \times SDS sample buffer containing 120 mM Tris (pH 6.8), 4% SDS, 200 mM DTT, 20% glycerol, and 0.01% bromphenol blue were added to each well. Samples were boiled for 5 min and loaded onto a 10% polyacrylamide gel for electrophoresis (Bio-Rad, Hercules, CA). Following electrophoresis, proteins were transferred onto a Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Membrane blots were blocked in blocking buffer made of 5% dry milk in TBS overnight at 4°C. To probe for ERK2, membranes were incubated with 1 μ g/ml anti-ERK2 sc-154 antibody in blocking buffer for 1 h at room temperature. After three 5-min washes, the blot was incubated with a 1:50,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Pierce) for 1 h. Finally, membranes were washed and incubated with SuperSignal Ultra (Pierce) for 5 min prior to imaging the bands with the Molecular Imager system (Bio-Rad). Analysis and quantification were performed with the Multi-Analyst (Bio-Rad) software.

RESULTS AND DISCUSSION

Traditional kinase activity assays isolate the kinase of interest by immunoprecipitation and determine its functional activity to catalyze an *in vitro* reaction. This traditional technique has several drawbacks including difficulty in handling multiple samples. While some methods have addressed this by introducing a 96-well format for portions of the kinase activity assay, these methods do not maintain this convenient format through the entire assay (2–9). The portions of the assay that do not conform to the 96-well scheme limit the potentially easy and efficient processing of multiple samples. Our modified kinase activity assay eliminates this limitation by implementing a 96-well format to all three steps of the assay: (i) immunoseparation of the kinase from other lysate proteins, (ii) *in vitro* kinase reaction, and (iii) quantitation of the extent of this

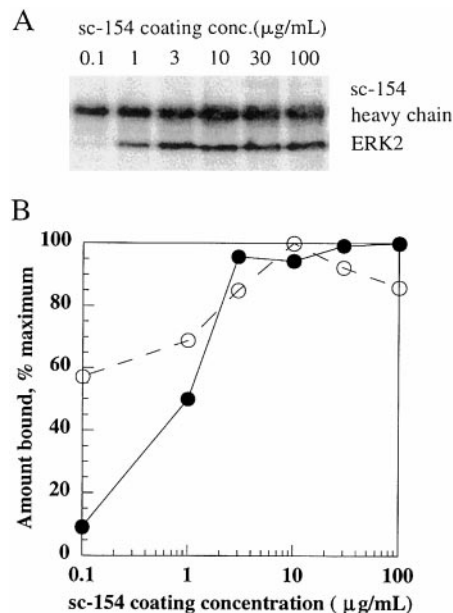


FIG. 2. Maximal amounts of immobilized anti-ERK2 sc-154 antibody and ERK2 recovery are achieved when wells are coated with 10 μ g/ml anti-ERK2 antibody. Wells were coated with solutions containing various concentrations of sc-154 antibody. Each well was then incubated with the same 20 μ g CHO cell lysate. (A) After washing, the contents of the well were determined by Western blot. (B) The intensities of ERK2 (●) and sc-154 antibody (○) bands were quantified and are shown as a percentage of maximal intensity of each protein.

reaction as a measure of kinase activity (Fig. 1). We have validated this assay for measuring the kinase activity of ERK2 in CHO cells.

Anti-ERK2 antibody was immobilized via its Fc domain interaction with the protein A coated on the bottom of a microtiter well. This ensures that the majority of the antibody is oriented with the antigen-binding Fab fragment directed into solution, thereby optimizing the binding of ERK2 to the antibody. To determine the quantity of antibody that can be applied to each well, solutions of varying antibody concentrations were incubated in the well overnight. Then, these same wells were incubated with 20 μ g of cell lysate to determine how the amount of immobilized antibody affects ERK2 recovery. Western blot analysis of the contents of the well following lysate incubation revealed that the maximal antibody coating amount was achieved by coating with a solution of 10 μ g/ml antibody concentration (Fig. 2). Furthermore, ERK2 recovery reached its maximum when the amount of immobilized antibody was at its highest level.

To determine the appropriate amount of cell lysate to utilize in the assay, the immunoseparation was performed with different amounts of CHO cell lysate, and the proteins remaining bound to the well were analyzed by Western blot (Fig. 3A). The blot shows that

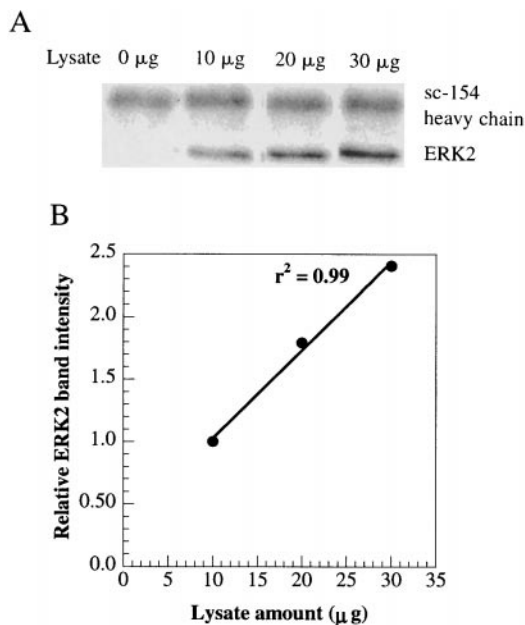


FIG. 3. ERK2 purification by immunoseparation in microtiter wells is linear with respect to total cell lysate incubated. (A) Various lysate amounts were incubated for 3 h in anti-ERK2 antibody-coated microtiter wells. After washing, ERK2 content in these wells was assayed by Western blot. (B) ERK2 band intensity was quantified and normalized to the ERK2 band intensity for the 10- μ g lysate case.

ERK2 was captured in a cell lysate amount-dependent manner. More ERK2 was recovered when higher amounts of cell lysate were used, suggesting that the antibody coating was sufficiently in excess. Furthermore, the amount of recovered ERK2 was linear with respect to lysate amount used (Fig. 3B). This implies that the ERK2 activity assay also should be linear for this same range of lysate amounts.

Following ERK2 binding, an *in vitro* reaction was carried out in the same well, using MBP and [γ - 32 P]ATP as the substrate. The initial rate of this reaction is a measure of the total activity of the ERK2 enzyme bound in each well. To determine a suitable time for sampling the reaction, reaction progress was monitored as a function of time. As shown in Fig. 4 for an *in vitro* reaction, 32 P incorporation into MBP increased linearly with time for at least 15 min. This shows that MBP and [32 P]ATP were in excess and were not depleted during this time. Therefore, quenching and sampling the reaction mixture for [32 P]MBP after 10 min provide a measure of ERK2 activity.

Upon quenching the reaction at 10 min, the reaction contents were filtered using a 96-well phosphocellulose filter plate, which efficiently binds proteins such as MBP but not unreacted [32 P]ATP. To determine the MBP-binding capacity of these filters, different volumes of reaction mixture were filtered, and the amount of [32 P]MBP was quantified. After correcting for back-

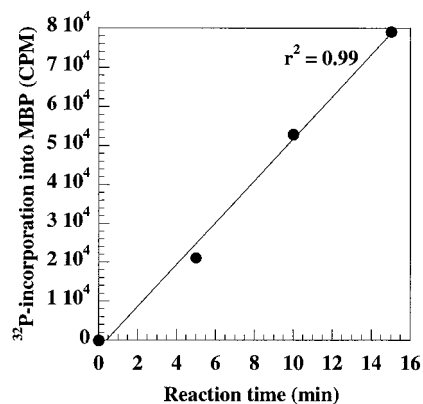


FIG. 4. *In vitro* reaction substrates are in excess yielding a linear reaction progress. CHO cells were serum starved for 18 h and lysed after 5 min of exposure to 15 nM EGF. Twenty micrograms of lysate was used in the immunoseparation of ERK2 from other lysate proteins in microtiter wells. Reactions were started by adding [32 P]ATP and MBP and were quenched at 5, 10, and 15 min. The amount of 32 P incorporation into MBP was measured as a function of reaction time.

ground, it is evident from Fig. 5 that the filter was not saturated in its ability to bind MBP for sample volumes up to 60 μ l. Therefore, in this assay, we take a 40- μ l sample from each reaction mixture, which is well within the protein-binding capacity of the filter.

Following the experimental validation of each step of the ERK2 activity assay, the overall performance of the assay was tested for its sensitivity and linear range. ERK2 activity was measured using different amounts of total lysate. As shown in Fig. 6, the measure of ERK2 activity is linear over a range of 5–50 μ g of CHO

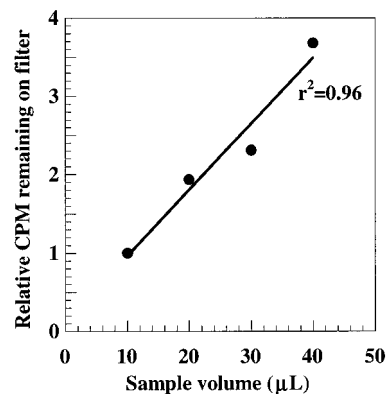


FIG. 5. MBP binding capacity of phosphocellulose filters is not saturated. Test tubes with or without 25 ng of active MAPK were supplied with [32 P]ATP and MBP. After 10 min of reaction at 37°C, both reactions were quenched with phosphoric acid. Different volumes of samples were taken from each quenched reaction and filtered through the phosphocellulose filter plate. After washing the filters, the amount of 32 P on the membrane was quantified by scintillation counting. The radioactivity associated with the filter for the case without active MAPK was treated as background and was subtracted from the measurements for the case with active MAPK.

cell lysate. This is consistent with the fact that the ERK2 purification from total cell lysate is also linear in this regime. Furthermore, this method is highly sensitive, as it can measure ERK2 activity in as little as 5 μg of cell lysate, which is at least an order of magnitude less than that used in typical immunoprecipitation/*in vitro* kinase assays.

Growth factors are well known to mediate the activation of ERK2. We used this modified method to determine the ERK2 response in CHO cells to EGF. A time course of ERK2 activity was measured in response to 15 nM EGF stimulation. As shown in Fig. 7, the ERK2 response increases to ~ 30 -fold above basal activity by 5 min and returns to a basal level by 20 min.

Thus, we have verified the performance of this rapid and efficient technique for measuring ERK2 activity and shown its applicability in measuring the ERK2 response of CHO cells to EGF stimulation. With slight modifications, this technique can be generalized to the study of other protein kinases. An antibody that binds to the protein kinase of interest must be used in the immunoseparation. In addition, a suitable protein substrate for the kinase must be supplied in the *in vitro* reaction. Changing the antibody and the protein substrate could alter the sensitivity and linear range of the activity assay. Validation experiments, such as those described here, should be performed to determine the optimal parameters for the activity assay. When compared to traditional immunoprecipitation/kinase assays, the advantages in implementing this method for measuring kinase activity are significant. They include (i) an immunoseparation technique that replaces the

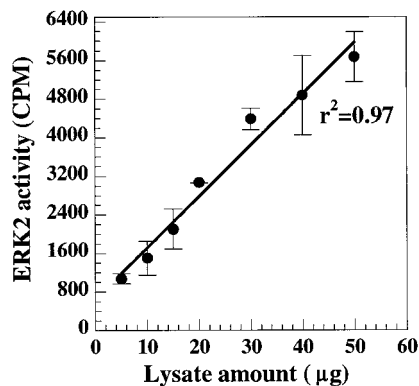


FIG. 6. ERK2 activity measurement is sensitive enough to measure activity in as little as 5 μg of cell lysate and is linear up to 50 μg of lysate. CHO cells were lysed after 5 min of stimulation with 15 nM EGF. Varying amounts of lysate were assayed for ERK2 activity using the modified assay.

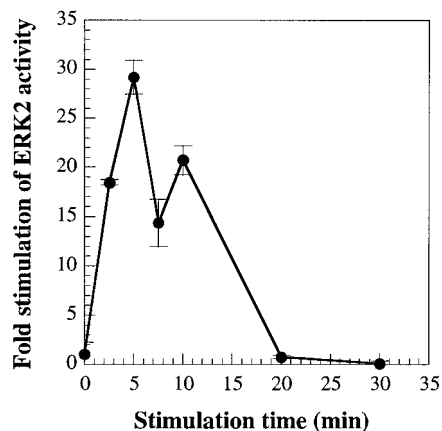


FIG. 7. Time-course of ERK2 activity in CHO cells in response to 15 nM EGF stimulation. CHO cells were stimulated with 15 nM EGF and were lysed at different time points. ERK2 activity in each cell lysate was measured and subtracted from background activity measured in plain lysis buffer. The background-adjusted ERK2 activity was normalized to the ERK2 activity immediately following serum starvation (i.e., the zero time point).

cumbersome immunoprecipitation method, (ii) a 96-well plate configuration that eases handling of multiple samples and increases throughput of the assay, and (iii) 96-well filter plates that greatly reduce radioactive liquid waste generation.

ACKNOWLEDGMENTS

This work was funded by NIH Grant NIGMS 53905 to D.A.L. and A.F.H., along with NIH training grant support to A.R.A.

REFERENCES

- Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) *Adv. Cancer Res.*, 49–139.
- Alessi, D. R., Cohen, P., Ashworth, A., Cowley, S., Leever, S. J., and Marshall, C. J. (1995) *Methods Enzymol.* **255**, 279–290.
- Angeles, T. S., Steffler, C., Bartlett, B. A., Hudkins, R. L., Stephens, R. M., Kaplan, D. R., and Dionne, C. A. (1996) *Anal. Biochem.* **236**, 49–55.
- Braunwalder, A. F., Yarwood, D. R., Sills, M. A., and Lipson, K. E. (1996) *Anal. Biochem.* **238**, 159–164.
- Lehel, C., Daniel-Issakani, S., Brasseur, M., and Strulovici, B. (1997) *Anal. Biochem.* **244**, 340–346.
- Ducommun, B., and David, B. (1990) *Anal. Biochem.* **187**, 94–97.
- Gopalakrishna, R., Chen, Z. H., Gundimeda, U., Wilson, J. C., and Anderson, W. B. (1992) *Anal. Biochem.* **206**, 24–35.
- Pitt, A. M., and Lee, C. (1996) *J. Biomol. Screening* **1**, 47–51.
- Hochstrasser, M., and Nelson, D. L. (1988) *Anal. Biochem.* **174**, 300–307.