In-Gel Kinase Assay as a Method to Identify Kinase Substrates

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Abstract

Phosphorylation of proteins by kinases is central to many cellular processes, including signal transduction. Thus, assays to identify or characterize kinases are a key tool for research in this area. Kinase substrates can be incorporated into polyacrylamide gels and used to characterize kinase activity in mixed samples. This methodology can be adapted for the identification of novel kinase-substrates or kinase-kinases that participate in the regulation of cell signaling. Here, I review the rationale and principles of an in-gel kinase assay. This strategy relies on co-polymerization of a substrate within the gel matrix, followed by detection of enzymatic activity in situ. The following Protocol provides a detailed method for performing the in-gel kinase assay and discusses the uses of the assay to evaluate kinase activity in the context of proliferation, differentiation, and survival pathways.

Introduction

The in-gel kinase assay is a powerful method whereby one can directly assess the substrate potential and characterize the phosphorylation of a known or unknown target. The assay relies on co-polymerization of a kinase substrate (for example, a protein or another kinase) with a sodium dodecyl sulfate (SDS)-polyacrylamide gel matrix. A protein, enzyme, or cell lysate sample is then separated by electrophoresis under denaturing conditions. After electrophoresis, SDS is removed and the substrate and fractionated sample are renatured in situ. The assay is performed when the gel is incubated with [γ^{32}P]-labeled adenosine triphosphate (ATP) and autoradiography performed to evaluate the phosphorylation activity of the kinase on the gel-incorporated substrate (*1*).

Numerous studies have employed the in-gel kinase assay. For example, the assay has been used as a means to monitor column fractions for kinase activities and their potential to phosphorylate substrate (*2*, *3*), hence aiding in both the purification and identification of the kinase. Alternatively, the assay has been used as a confirming step to validate a particular kinase's ability to phosphorylate a specific substrate (*4*-*8*). If the target substrate is a kinase, the assay can be used to identify kinase-kinases. Cells or tissues that have different genetic backgrounds can be used to examine the pathways leading to phosphorylation of a particular substrate (*9*); for example, kinase-kinase activation pathways depend on upstream elements, such as Ras, Src, or particular second messengers, which in turn are influenced by genetic elements that can vary. Thus, in-gel kinase assays are a powerful method that can be used in combination with other approaches to explore regulation of cellular signaling pathways.

Typically, in-gel kinase assays have proved to be difficult to conduct in a reproducible manner. Herein, I describe in detail an in-gel assay that was successfully used to identify novel kinases that phosphorylate the atypical protein kinase C (*9*).

Materials

SDS-Polyacrylamide Gel Electrophoresis

Acrylamide (Bio-Rad)

Ammonium persulfate (APS) (Bio-Rad)

Bis-acrylamide (Bio-Rad)

Pre-stained Broad-Range SDS-polyacrylamide gel electrophoresis (PAGE) protein markers (Bio-Rad)

SDS (Bio-Rad)

N,N,N′,N′-Tetramethyl-ethylenediamine (TEMED) (Bio-Rad)

Tris-Ultra Pure (United States Biochemicals)

In-Gel Kinase Assay

Sample containing kinase activity

Substrate: peptide, myelin basic protein, poly Glu-Tyr, or a purified protein

β-mercaptoethanol (2-ME) [Sigma-Aldrich (http://www.sigma-aldrich.com)]

β–glycerophosphate (Sigma-Aldrich)

Acetic acid

[γ^{32}P]-ATP 10 mCi/ml (3000 Ci/mmol)

Aprotinin (10 mg, Sigma-Aldrich)

ATP (Sigma-Aldrich)

Dithiothreitol (DTT) (Sigma-Aldrich)

Ethylene diaminetetra-acetic acid (EDTA) (Sigma-Aldrich)

3MW Gel Blot Paper (Midwest Scientific)

Glycerol (Sigma-Aldrich)

Guanidine hydrochloride (Calbiochem)

Hepes (Sigma-Aldrich)

Leupeptin (10 mg, Sigma-Aldrich)

Methanol

$MgCl_2$

$MnCl_2$

Na_3OV_4 (Sigma-Aldrich)

NaCl (Sigma-Aldrich)

NaF (Sigma-Aldrich)

Nonidet P 40 (NP-40) (Sigma-Aldrich)

Phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich)

p-Nitrophenylphosphate (PnPP) (Sigma-Aldrich)

Sodium pyrophosphate (Sigma-Aldrich)

Trichloroacetic acid (TCA) (Sigma-Aldrich)

Tris base (such as Trizma [Sigma-Aldrich])

Tween-20 (Sigma-Aldrich)

Autoradiography

Beta-Max Film (Kodak)

Equipment

Film-developing machine or tanks (for Beta-Max film)

Gel Dryer (Bio-Rad Model 583)

Incubator that will accommodate a small platform shaker (Fisher Isotemp)

Minigel apparatus and gel plates with 1-mm spacers and 10-well combs (Hoeffer Mighty Small SE245)

Orbital platform shaker (Labline Orbital Shaker)

Power supply for electrophoresis (EC600-90, Bio-Rad)

Roto-Mix Slow Shake (Thermodyne)

Small plastic containers for minigels [12 cm (L) × 8 cm (W) × 3 cm (H) with a tight lid]

Recipes

Recipe 1: Lysis Buffer

Final Concentration	*Stock*	*Volume*
20 mM Tris-HCl, pH 7.5	1.0 M	2.0 ml
137 mM NaCl	5.0 M	2.74 ml
1 mM $MgCl_2$	100 mM	1.0 ml
1 mM Na_3OV_4	100 mM	1.0 ml
100 μM NaF	10 mM	1.0 ml
1.0% NP-40	Pure	1.0 ml
10 mM β-glycerophosphate	1.0 M	1.0 ml
10% glycerol	10%	10.0 ml

Add deionized, distilled water (ddH_2O) to a final volume of 100 ml.

Recipe 2: Lysis Buffer with Antiproteases

Final Concentration	*Stock*	*Volume*
1 mM PMSF	100 mM	250 μl
10 μg/ml leupeptin	2 mg/ml	125 μl
5 μg/ml aprotinin	2 mg/ml	62.5 μl
2.5 μg/ml PnPP	5 mg/ml	12.5 μl

Prepare in 25 ml of fresh Lysis Buffer (Recipe 1).

Recipe 3: Buffer 1

Dissolve 181.65 g of Tris base in 750 ml of ddH_2O. Adjust pH to 8.8 with concentrated HCl. Add H_2O to a final volume of 1 l.

Recipe 4: Buffer 2

Dissolve 60.55 g of Tris base in 800 ml of ddH_2O. Adjust pH to 6.8 with concentrated HCl. Add H_2O to a final volume of 1 l.

Recipe 5: SDS Sample Buffer

Buffer 2 (Recipe 4)	25 ml
SDS	4.0 g
ddH_2O	45 ml
2-ME	10 ml
Glycerol	20 ml

Final volume of solution should be 100 ml.

Recipe 6: Acrylamide

146 g acrylamide

4.0 g bis-acrylamide

Add to 250 ml of ddH_2O and stir to dissolve. Add ddH_2O to a final volume of 500 ml.

Recipe 7: 50% Glycerol

Add 50 ml of glycerol to 50 ml of ddH_2O.

Recipe 8: 10% SDS

Add 10 g of SDS to ddH_2O for a final volume of 100 ml.

Recipe 9: 10% APS

Add 1 g of APS to ddH_2O for a final volume of 10 ml.

Recipe 10: Resolving Gel

Water + substrate (100 to 500 μg/ml)	2.36 ml
Buffer 1 (Recipe 3)	1.24 ml
Acrylamide (Recipe 6)	1.30 ml
50% glycerol (Recipe 7)	0.10 ml
10% SDS (Recipe 8)	0.05 ml
10% APS (Recipe 9)	0.17 ml
TEMED	2.5 μl

Total volume for one gel is 5.22 ml.

Note: If the substrate is dissolved in a glycerol-containing solution, the volume of glycerol must be adjusted appropriately in the resolving gel. The amount of substrate depends on the purity of the kinase being tested and may need to be determined empirically. Add catalysts (APS and TEMED) just before casting the gel. Extra APS has been added to speed polymerization.

Recipe 11: Stacking Gel

ddH_2O	2.4 ml
Buffer 2 (Recipe 4)	0.95 ml
Acrylamide (Recipe 6)	0.375 ml
10% SDS (Recipe 8)	37.5 μl
10% APS (Recipe 9)	43.75 μl
TEMED	3 μl

Total volume for one gel is 3.81 ml.

Recipe 12: Run Buffer

Tris base	12.0 g
Glycine	57.6 g
SDS	4.0 g

Add ddH_2O to final volume of 4 liters.

Note: This solution should have a pH of 8.3 without adjustment.

Recipe 13: SDS-Removal Solution I

Final Concentration	*Stock*	*Amount*
20% (v/v) 2-propanol	Pure	50 ml
50 mM Tris-HCl, pH 8.0	1 M	12.5 ml

Add ddH_2O to a final volume of 250 ml. This volume is adequate for one minigel.

Recipe 14: SDS-Removal Solution II

Final Concentration	*Stock*	*Amount*
50 mM Tris-HCl, pH 8.0	1 M	12.5 ml
1 mM DTT	Powder	0.039 g

Add ddH_2O to a final volume of 250 ml. This volume is adequate for one minigel.

Recipe 15: Denaturation Buffer

Final Concentration	*Stock*	*Amount*
50 mM Tris-HCl, pH 8.0	1 M	12.5 ml
20 mM DTT	Powder	0.771 g
6 M Guanidine hydrochloride	Crystal	143.30 g

Add ddH_2O to a final volume of 250 ml; 50 to 100 ml is adequate for one minigel.

Recipe 16: Renaturation Buffer

Final Concentration	*Stock*	*Amount*
50 mM Tris-HCl, pH 8.0	1 M	12.5 ml
5 mM DTT	Powder	0.195 g
0.04% Tween-20	Pure	0.1 ml
100 mM NaCl	5 M	5 ml
5 mM $MgCl_2$	100 mM	12.5 ml

Add ddH_2O to a final volume of 250 ml. This volume is adequate for one minigel.

Recipe 17: Kinase Assay Buffer

Final Concentration	*Stock*	*Amount*
25 mM Hepes, pH 7.4	1.0 M	5 ml
20 mM $MgCl_2$	1.0 M	4 ml
1 mM $MnCl_2$	100 mM	2 ml
5 mM NaF	0.5 M	2 ml
100 μM Na_3OV_4	100 mM	200 μl
2.5 μg/ml PnPP	5 mg/ml	250 μl
1 mM DTT	Powder	31.2 mg

Add ddH_2O to a final volume of 200 ml.

Recipe 18: Hot Kinase Buffer

Final Concentration	*Stock*	*Amount*
50 μM ATP	10 mM	75 μl
20 Ci/ml [γ^{32}P]-ATP	5 μCi/μl	60 μl

Add to 15 ml of Kinase Assay Buffer (Recipe 17).

Recipe 19: Gel Wash Solution

Final Concentration	*Stock*	*Amount*
5% TCA	100%	50 ml
1% Sodium pyrophosphate	Crystal	10 g

Add ddH_2O to a final volume of 1 liter.

Recipe 20: Coomassie Stain

Dissolve 0.25 g of Coomassie Brilliant Blue R-250 in 400 ml of ddH_2O. Add 800 ml of methanol and 70 ml of acetic acid. Adjust volume to 1 liter with H_2O.

Instructions

Below, a step-by-step method for an in-gel kinase assay is described. The strategy consists of co-polymerizing a kinase substrate within the gel matrix, then detecting kinase activity by an in-gel (in situ) kinase assay. By this method, we identified three putative kinases that phosphorylate the atypical protein kinase C (*9*) (Fig. 1). Minigels cast with 1-mm spacers are ideal for this assay. In my experience, the thickness of the gel is critical to success of the assay.

In addition, the success of the assay is highly dependent on the quality of the samples containing the enzymatic activity. Therefore, the gels should be made and ready to load before the samples are prepared and the samples should be used immediately whenever possible.

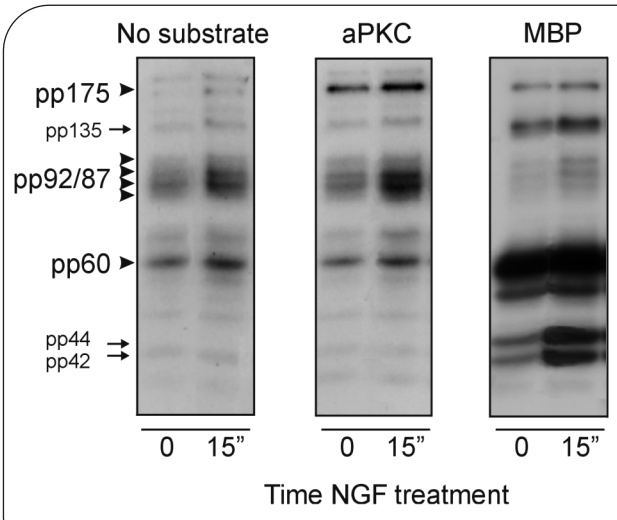

Fig. 1. Testing for substrate specificity of a sample. Gels contain different substrates. Lysates from cells stimulated with nerve growth factor (100 ng/ml) for 15 min were subjected to the in-gel assay with no substrate, purified atypical protein kinase C (aPKC) (100 μg/ml), or myelin basic protein (MBP) (100 μg/ml). Note the stimulation of autophosphorylation of pp92/87 and pp69 kinase activity by nerve growth factor (NGF). The activity of pp175 is specific to aPKC as a substrate, whereas pp44/42 is specific to MBP as a substrate. [Reprinted with permission from Elsevier Science (*9*)]

Sample Preparation

The samples are prepared in lysis buffer, SDS-containing sample buffer is added, and the mixture is boiled to denature the proteins. Samples should be as fresh as possible, and freezing and thawing of samples should be avoided.

When using the in-gel assay for detecting activity of kinases in column fractions, it might also be necessary to concentrate the activity of the fractions, depending on the relative abundance of the kinase. UltraFreeMC (Millipore) centrifugal filtration units fitted with 10 kD nominal molecular weight cutoff membranes can be used for this purpose (*3*).

1. Prepare samples containing kinase in Lysis Buffer with Antiproteases (Recipe 2).

   Note: Samples should have 40 to 60 μg of protein per 25 μl sample volume.

2. Add SDS Sample Buffer (Recipe 5) to sample in a 1:1 ratio (vol/vol), using microcentrifuge tubes with locking caps to prevent them from "popping" open when boiling.

   Note: Maximum sample volume is ~25 μl, because of the size of the comb used to cast the stacking gel.

3. Boil samples for 3 min in tubes with locking caps to prevent loss of the samples. Samples should be immediately loaded onto the gel and are not stored.

In-Gel Kinase Assay

Day I

1. Pour a 7-cm substrate-containing Resolving Gel (Recipe 10) in a Hoeffer minigel cast with 1-mm spacers.

   Note: If the substrate is dissolved in a glycerol-containing solution, the amount of glycerol must be adjusted appropriately in the resolving gel. Typically, we use 100 to 500 μg/ml of substrate, depending on the purity of the kinase to be tested.

2. Overlay the gel with butanol.
3. Place in an incubator at 37° C to speed polymerization. Polymerization generally takes ~45 min.
4. After the gel has polymerized, drain the butanol, wash the gel in with ~5 mls of Run Buffer (Recipe 12), and drain.
5. With the minigel sandwich set in the electrophoresis unit, pour a Stacking Gel (Recipe 11) with a 10-well comb and allow to polymerize.

   Note: For best results, the gel should be used within 2 hours of casting the stacker. The gels are cast and used the same day and are not typically stored.

6. Wash the wells with Run Buffer (Recipe 12) to remove unpolymerized acrylamide, and add Run Buffer (Recipe 12) to the upper and lower tanks of the electrophoresis chamber.

7. Load the samples and a lane with prestained molecular weight markers.
8. Run the gel at 12.5 mA/gel until the dye runs off the gel (about 2.5 hours).
9. Turn off the power, remove the gel from the apparatus, and disassemble the gel plates.
10. Cut off the stacking gel and discard.
11. Remove the resolving gel from the plate and place each resolving gel in an individual, labeled container.

    Note: If you are analyzing more than one sample or substrate on more than one gel, make sure to label each container in order to identify the different gels.

12. Remove the SDS from the gel by washing it in 50 ml of SDS Removal Solution I (Recipe 13), agitating gently on an orbital platform shaker. Wash for 1 hour, replacing the solution at least four times during that period.

    Note: This and all subsequent washes are done at room temperature unless specified otherwise. We have observed that an orbital platform shaker, rather than a rocking platform, performs best for these assays. The gels must be gently agitated to ensure removal of the SDS.

13. Wash gel for 1 hour with SDS-Removal Solution II (Recipe 14) with agitation; change the solution twice.
14. Denature the proteins by incubating the gel in 50 ml of Denaturation Buffer (Recipe 15) for 30 min with very gentle agitation.
15. Replace with 50 ml of Denaturation Buffer (Recipe 15) and agitate gently for 30 min.
16. Replace with 50 ml of Denaturation Buffer (Recipe 15) and agitate gently for 1 hour.
17. Renature the proteins by incubating gel in 50 ml of Renaturation Buffer (Recipe 16) for 40 min, replacing the Renaturation Buffer at 10-min intervals to ensure complete removal the guanidine hydrochloride.
18. Place the box containing the gel and 50 ml of fresh Renaturation Buffer (Recipe 16) in the cold at 4°C for 3 hours.

    Note: The gel is not agitated during this step.

19. Replace the solution with 50 ml of fresh Renaturation Buffer (Recipe 16) and incubate the gel for 15 hours (overnight) at 4°C.

    Note: The gel is not agitated during this step.

Day 2

The next steps require the use of radioactive ^{32}P-labeled ATP. Care should be taken once the radioactive solutions are prepared and the gel is radioactive. Use appropriate shielding and follow your facility's regulations with regard to use and disposal of radioactive materials. We typically place the smaller in-gel assay boxes inside a Plexiglas box to minimize our exposure to radioactivity.

1. Wash the gel twice, 10 min each wash, with 50 ml of SDS-Removal Solution II (Recipe 13) at room temperature with gentle agitation on the orbital shaker.
2. Incubate the gel in 15 ml of Kinase Assay Buffer (Recipe 17) at room temperature with gentle agitation on the orbital shaker
3. Prepare Hot Kinase Assay Buffer (Recipe 18) and place in an incubator at 30°C for 10 min.
4. Replace the Kinase Assay Buffer (Recipe 17) on the gel with 15 ml Hot Kinase Buffer (Recipe 18) and incubate for 1 to 2 hours at 30°C with gentle agitation on the orbital shaker.

    Note: Make sure that the minigel does not fold back on itself. During the assay period, the assay buffer must evenly cover the gel surface. The length of the incubation required will depend on the activity of the kinase and its purity, and will need to be determined empirically.

5. Remove the radioactive assay solution and dispose according to proper procedures for your institution.
6. Wash the gel extensively with Gel Wash Solution (Recipe 19) for 45 min at room temperature with gentle agitation. Change the wash solution three or four times during the wash period.

    Note: The gels may be stained and destained with Coomassie Stain (Recipe 20) at this point, if one wishes to observe particular protein bands. However, the relative molecular weight of protein(s) of interest can also be determined using the positions of the prestained molecular weight markers.

7. Dry the gel onto two sheets of filter paper, covering the gel with plastic wrap. Typically, mini-gels dry quickly (10 to 20 min).

    Note: The glycerol in the gel will make the gel surface tacky

8. Expose the gel to autoradiographic film.

Note: We find that use of Beta-max film (Kodak) increases the sensitivity of the signal.

Related Techniques

If the phosphorylation signal is strong enough, the site of phosphorylation may be identified. We have had some success at cutting the radioactive band from the dried gel, rehydrating the gel in water to remove the paper used in the drying process, and conducting phospho-amino acid analysis to identify the phosphorylated amino acid.

One method that can be used to identify the kinase is immunodepletion. To immunodeplete, the samples are incubated with an antibody (or antibodies) against the potential target. The antibody is then removed from the sample, along with any bound target kinase by incubation with Protein-A Sepharose. The samples should be subjected to at least two rounds of immunodepletion, with an antibody to the potential target. The immunodepleted sample can then be compared to a nondepleted sample as a way to initiate identification of the putative kinase (*2*). This method is a powerful tool for characterization of kinase-kinases that regulate many aspects of cell signaling, including proliferation, apoptosis, survival, and differentiation.

Notes and Remarks

In-gel assays rely on active kinases. Thus, one must use conditions that minimize the degradation of activity of the kinase under study. Appropriate protease and phosphatase inhibitors must be included in the lysis buffer. With whole-cell lysates, we typically use the lysates immediately, if possible. We never freeze-thaw the samples because we find that enzyme activity often disappears afterward. The reproducibility of results depends on consistency in preparation of the samples. If signal strength is low, the length of the incubation of the gel with the Hot Kinase Buffer can be increased. Additionally, other factors may be required for activation of the kinase under study, and these additional factors, such as cations or lipids, should be included in the in-gel Kinase Assay Buffer. Another potential problem is insufficient quantity of substrate. This can be especially problematic for the study of kinase-kinases. We have successfully used glutathione-*S*-transferase (GST)- or His-tagging of the target protein to enrich for the target protein by standard purification methods (*9*).

It is also important to use the highest purity or molecular biology grade chemicals for the assay and electrophoresis. Some investigators use 2-ME instead of DTT in the gel solutions. We find that, when compared side by side, assays conducted with DTT are superior to those containing 2-ME in terms of kinase activity. The same is true when comparing guanidine hydrochloride to urea. We find that renaturation with guanidine hydrochloride results in improved kinase activity for the kinases that we have studied to date.

A negative control should be included in the assay. This consists of a gel with no substrate included, allowing one to assess the autophosphorylation potential of the kinase(s) under study. In addition, different substrates can be copolymerized in the gel matrix to examine the substrate specificity of the kinase (*2*, *9*).

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