Published in final edited form as:

Methods Mol Biol. 2016 ; 1363: 189–197. doi:10.1007/978-1-4939-3115-6_15.

Assessing Kinase Activity in Plants with In-Gel Kinase Assays

Pengcheng Wang and Jian-Kang Zhu

Abstract

The in-gel protein kinase assay is a powerful method to measure the protein phosphorylation activity of specific protein kinases. Any protein substrate can be embedded in polyacrylamide gels where they can be phosphorylated by protein kinases that are separated in the gel under denaturing conditions and then renatured. The kinase activity can be visualized in situ in the gels by autoradiography. This method has been used to compare the activities of protein kinases in parallel samples or to identify their potential substrates. Here, we describe in detail an in-gel kinase assay to measure the activity of some protein kinases in plants.

Keywords

Protein kinase; Substrate; Autoradiography; MAPK; CDPK; SnRK2; Casein kinase

1 Introduction

The in-gel kinase assay is a powerful method for directly measuring kinase activity and for characterizing the phosphorylation of a known or unknown protein substrate. The assay relies on co- polymerization of a substrate protein or peptide with a sodium dodecyl sulfate (SDS)-polyacrylamide gel matrix. Cell lysate, immunoprecipitation products, or recombinant proteins are separated in the gel by electrophoresis under denaturing conditions. After electrophoresis, SDS in the gel matrix is removed, and fractionated kinase samples are renatured in situ. The gel is then incubated with [γ - ^{32} P]-labeled adenosine triphosphate (ATP), and autoradiography is used to assess the level of phosphorylation of the gel-incorporated substrate [1].

Plant researchers have often used the in-gel kinase assay to monitor the activities of specific kinases and to determine their potential to phosphorylate specific substrates. The activity of at least three subfamilies of kinase can be detected by this method, and these are mitogen-activated protein kinases (MAPKs) [2, 3], sucrose non-fermenting-1 (SNF1)-related protein kinase 2 s (SnRK2s) [4–7], and calcium-dependent protein kinases (CDPKs) [8, 9]. Some commercially available proteins, including myelin basic protein (MBP), histone, and α -casein, are commonly used as substrates to detect the activity of these kinases. Some recombinant peptides have also been used to detect the activity of MAPKs and SnRK2s [4, 10]. Here, we describe in detail an in-gel kinase assay that we have successfully used to identify the activities of casein kinases and SnRK2s in response to abscisic acid treatment.

2 Materials

2.1 Chemicals and Solutions

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 M Ω cm at 25 °C) and analytical grade reagents.

1. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8. Add 800 mL water to 181.7 g Tris base, mix and adjust pH to 8.8 with HCl. Make up to 1 L with distilled water. Store at 4 °C.
2. Stacking gel buffer: 1 M Tris–HCl, pH 6.8. Add 800 mL water to 121.1 g Tris, mix and adjust pH to 6.8 with HCl. Increase volume to 1 L with distilled water. Store at 4 °C.
3. Acrylamide/Bis solution (37.5:1) 30 % (Bio-Rad, Hercules, CA, USA) (*see Note 1*). Store at 4 °C
4. Ammonium persulfate: 10 % solution in water. Store at 4 °C (*see Note 2*).
5. *N,N,N,N'*-tetramethyl-ethylenediamine (TEMED) (Bio-Rad, Hercules, CA, USA). Store at 4 °C.
6. SDS-PAGE running buffer: 0.025 M Tris–HCl, pH 8.3, 0.192 M glycine, 0.1 % SDS (*see Note 3*).
7. Protein sample buffer (4 $\times$ SDS): 0.25 M Tris–HCl, pH 6.8, 8 % SDS, 20 % glycerol, 0.04 % bromophenol blue, 5 % β -mercaptoethanol (*see Note 4*).
8. EDTA 0.5 M, pH 8.0: Add 186.1 g disodium ethylenediamine tetraacetate $\cdot$ 2H₂O to 800 mL of distilled water, add NaOH solution to adjust the pH to 8.0. Increase volume to 1 L with distilled water. Store at 4 °C.
9. EGTA 0.5 M, pH 7.0: Add 38.04 g EGTA powder to 160 mL distilled water, add NaOH slowly to adjust the pH to 7.0. Increase volume to 200 mL with distilled water. Store at 4 °C.
10. HEPES 0.5 M, pH 7.5: Add 119.15 g HEPES powder to 800 mL distilled water, adjust pH to 7.5 with NaOH. Increase volume to 1 L with distilled water. Store at 4 °C.
11. Tris–HCl 1 M, pH 7.5: Add 121.14 g Tris base powder to 800 mL distilled water, adjust pH to 7.5 with HCl. Increase volume to 1 L with distilled water. Store at 4 °C.
12. Activated Na₃VO₄ 200 mM. Freeze at –20 °C (*see Note 5*).

¹The commercial acrylamide/Bis solution is recommended for the in-gel kinase assay because obtaining the best results requires the co-polymerization of substrates in the gel.

²The 10 % APS can be stored at 4 °C for 1 month.

³We always prepare 10 $\times$ SDS running buffer by dissolving 30.3 g Tris, 144.0 g glycine, and 10.0 g SDS in water to a final volume of 1 L. Dilute 100 mL 10 $\times$ SDS running buffer to 1 L with distilled water to make 1 $\times$ SDS running buffer.

⁴We always prepare 4 $\times$ SDS sample buffer without β -mercaptoethanol, and we add 50 μ L β -mercaptoethanol to 1 mL before use. The 4 $\times$ SDS sample buffer with β -mercaptoethanol can be stored at room temperature for 1 month.

⁵Prepare the activated Na₃VO₄: Dissolve 1.84 g of sodium orthovanadate in 40 mL, adjust the pH to 10 using 1 M NaOH (the solution will be yellow). Boil the solution until it becomes colorless (approximately 10 min). Cool to room temperature. Readjust the

13. NaF 0.5 M: Dissolve 5.25 g NaF in 250 mL distilled water. Store at 4 °C.
14. β -Glycerophosphate disodium 1 M: Dissolve 2.16 g β -glycero-phosphate disodium in 10 mL distilled water. Store at 4 °C.
15. Phenylmethylsulfonyl fluoride (PMSF) solution 100 mM: Add 17.4 mg of PMSF per milliliter of isopropanol. Store at -20 °C (*see* Note 6).
16. ATP solution 1 mM: Dilute adenosine triphosphate, 100 mM (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) to 1 mM in 16 mM Tris-HCl, pH 7.5. Store at -80 °C.
17. [γ -³²P]-ATP, 3000 Ci/mmol (Perkin-Elmer, Waltham, MA, USA) (*see* Note 7).
18. Myelin basic protein (MBP) solution 2.5 mg/mL: Dissolve 10 mg MBP (Sigma-Aldrich, St Louis, MO, USA, M-1891) in 4 mL distilled water. Store as 1-mL aliquots at -20 °C.
19. Histone solution 10 mg/mL: Dissolve 100 mg histone type III-S (Sigma-Aldrich, St Louis, MO, USA, H-5505) in 10 mL distilled water. Store as 1-mL aliquots at -20 °C.
20. α -Casein solution 10 mg/mL: Dissolve 100 mg dephosphorylated α -casein (Sigma-Aldrich, St Louis, MO, USA, C-8032) in 10 mL distilled water. Store as 1-mL aliquots at -20 °C.
21. Leupeptin: 10 mg/mL in distilled water. Store as 100- μ L aliquots at -20 °C.
22. Antipain: 10 mg/mL in distilled water. Store as 100- μ L aliquots at -20 °C.
23. Aprotinin: 10 mg/mL in distilled water. Store as 100- μ L aliquots at -20 °C.
24. Quick Start Bradford 1 $\times$ Dye reagent (Bio-Rad, Hercules, CA, USA). Store at 4 °C.
25. Bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA).
26. Triton X-100.
27. Trichloroacetic acid (TCA) (Sigma-Aldrich, St Louis, MO, USA).
28. Sodium pyrophosphate (NaPPi) (Sigma-Aldrich, St Louis, MO, USA).

2.2 Equipments and Other Materials

1. Mini PROTEAN 3 Tetra vertical electrophoresis cell, casting stands, casting frames, companion running module, 0.75-mm- thick glass plates (*see* Note 8), 10- or 15-well combs (0.75-mm- thick) (Bio-Rad, Hercules, CA, USA).

pH to 10 and boil until the solution remains colorless and the pH stabilizes at 10. Adjust the final volume to 50 mL with distilled water. Store the activated Na₃VO₄ as 500- μ L aliquots at -20 °C.

⁶PMSF is inactivated in aqueous solutions. The half-life of a 20 mM aqueous solution of PMSF is ~35 min at pH 8.0. This short half-life means that aqueous solutions of PMSF can be safely discarded after they have been rendered alkaline (pH >8.6) and stored for several hours at room temperature.

⁷Radioactive materials. Please follow your facility's regulations regarding the use and disposal of radioactive materials.

⁸To obtain the best results, the 0.75-mm gel is recommended.

2. Plastic container or Petri dishes, 150 × 15 mm.
3. Orbital shaker.
4. Prestained protein ladder (Thermo Fisher Scientific, Waltham, MA, USA).
5. Cover glass forceps, straight (VWR, Radnor, PA, USA, 82027-396).
6. Spectrophotometer.
7. Gel dryer.

3 Methods

3.1 Samples Preparation

1. Prepare the kinase buffer premix: Mix 20 mL 0.5 M HEPES, pH 7.5, 1 mL 0.5 M EGTA, pH 8.0, 1 mL 0.5 M EDTA, pH 7.0, 2 mL 0.5 M NaF, 5 mL 1 M β -glycerophosphate disodium, 5 mL glycerol, add distilled water to a final volume of 100 mL (*see* Note 9).
2. Add 100 μ L 0.5 M dithiothreitol (DTT), 5 μ L 10 μ g/ μ L leupeptin, 5 μ L 10 μ g/ μ L aprotinin, 5 μ L 10 μ g/ μ L antipain, 50 μ L 100 mM PMSF, and 250 μ L 200 mM Na_3VO_4 to 4.585 mL kinase buffer premix. Keep the buffer on ice (*see* Note 10). The final components of kinase extract buffer are 100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4 , 10 mM NaF, 50 mM β -glycerophosphate, 1 mM PMSF, 5 μ g/mL leupeptin, 5 μ g/mL aprotinin, 5 % glycerol (*see* Note 11).
3. Place about 50 mg of plant tissue in a 1.5-mL centrifuge tube with liquid nitrogen and grind to a fine powder with a pestle. Add 50 μ L kinase extract buffer to the powder. After the preparations are mixed and centrifuged at 4 °C and 18,000 × *g* for 40 min, transfer the supernatants into clean tubes.
4. Add 5 μ L protein extract aliquots to 1 mL Bradford reagent, and determine the concentration of the protein extract using a spectrophotometer.
5. For each sample, transfer 80 μ g of total proteins to a new, clean tube; 10 μ L SDS sample buffer and kinase extract buffer are added to a total of 40 μ L (adjust the protein concentration to 2 μ g/ μ L). Boil the samples for 1 min. The samples can be then subjected to electrophoresis or frozen in liquid nitrogen and stored at −70 °C.

3.2 Gel Preparation and Electrophoresis

1. Prepare 3.5 mL of substrate-containing resolving gel for each 0.75-mm-space mini gel cast. Mix 0.875 mL of resolving buffer (1.5 M Tris–HCl, pH8.8), 1.17

⁹We prepare 100 mL kinase buffer premix and store it at 4 °C.

¹⁰The freshly prepared buffer should be light yellow. It should be discarded if it turns to green after several hours on ice. We always prepare the kinase extract buffer freshly before use. However, the freshly prepared kinase extract buffer can be dispensed into aliquots and stored at −70 °C for several months.

¹¹Although 7.5 % polyvinylpyrrolidone in kinase extract buffer is suggested by some protocols, it is not necessary for *Arabidopsis*.

mL of 30 % acrylamide/bis, 1.03 mL water, 0.35 mL MBP, histone or α -casein, 35 μ L 10 % APS, 35 μ L 10 % SDS, and 3 μ L TEMED, and cast gel within a 0.75-mm mini gel cassette. Overlay the gel with ethanol and allow the gel to polymerize for 1 h at room temperature or 30 min at 37 °C (*see* Note 12).

2. After the gel has polymerized, drain the ethanol.
3. Prepare the stacking gel by mixing 0.5 mL of stacking gel buffer (1.0 M Tris–HCl, pH 6.8), 0.33 mL of 30 % acrylamide/bis, 1.07 mL water, 20 μ L 10 % APS, 20 μ L 10 % SDS, and 2 μ L TEMED. Pour the stacking gel, insert a 10- or 15-well comb, and allow the gel to polymerize.
4. Load the sample (10 μ L/lane); also load one lane with prestained molecular weight markers.
5. Run the gel at 140 V. Increase the voltage to 170 V when the dye runs into the resolving gel, and continue running the gel for about 1.5 h. Turn off the power when the 25 kDa marker runs off the gel.

3.3 Gel Washing and Kinase Assay

1. Prepare 300 mL SDS removal buffer for each gel by mixing 7.5 mL 1 M Tris–HCl, pH 7.5, 3 mL 0.5 M NaF, 0.1 mL 200 mM Na₃VO₄, 0.15 g BSA, and 0.3 mL Triton X-100 with distilled water to a final volume of 300 mL. Add 0.15 mL DTT before use.
2. Remove the gel from the plate and place each gel in a separate, labeled container (*see* Note 13).
3. Add 100 mL SDS removal buffer to the container and agitate gently on an orbital platform shaker. After 20 min of washing, transfer the gel to a new clean container using a cover glass forceps.
4. Repeat **step 3** two times. Prepare 300 mL of renaturation buffer for each gel by adding 7.5 mL 1 M Tris–HCl, pH 7.5, 3 mL 0.5 M NaF, and 0.1 mL 200 mM Na₃VO₄ to a final volume of 300 mL. Add 0.15 mL DTT before use.
5. After the gel is washed three times in SDS removal buffer, transfer the gel to a new, clean container containing 100 mL renaturation buffer and wash the gel for 1 h by agitating gently at room temperature.
6. Replace the 100 mL of renaturation buffer with fresh renaturation buffer and agitate gently for 16 h (or overnight) at 4 °C (*see* Note 14).

¹²The recommended concentration of substrates is 1 mg/mL α -casein, 1 mg/mL histone, and 0.25 mg/mL of myelin basic protein. A lower concentration of substrates has worked well in some studies. For example, as low as 0.02 mg/mL of ACS6 recombinant protein was sufficient for detection of MAPK activity [11]. For detection of MAPK activity, MBP can be used as substrate [2]. For detection of SnRK2 and CDPK, his-tone can be used as a substrate [5, 9]. Some studies also used MBP as a substrate to detect the activity of SnRK2 [7]. α -casein can be used to measure the kinase activity of casein kinase I or II [12].

¹³Do not cut the stacking gel, because doing so may make small rifts on the gel. After several hours of continuous shaking, any small rifts may be enlarged and break the gel.

¹⁴We always do the washing overnight at 4 °C and continue washing into the next day.

7. Replace the 100 mL of renaturation buffer with fresh renaturation buffer and agitate gently for 1 h at room temperature. Prepare 150 mL of kinase reaction buffer for each gel by adding 12 mL 0.5 M HEPES, pH 7.5, 2 mL 1 M MgCl₂, 0.6 mL 0.5 M EGTA, pH 7.0, and 75 μ L 200 mM Na₃VO₄ to distilled water to a final volume of 150 mL (*see* Note 15). Add 0.15 mL DTT before use.
8. Transfer the gel to 100 mL of kinase reaction buffer and agitate gently for 30 min at room temperature.
9. Transfer the gel to 30 mL kinase reaction buffer, add 3 μ L [γ -³²P]-ATP, and agitate gently for 5 min at room temperature (*see* Notes 16 and 17).
10. Add 4.5 μ L 1 mM nonradioactive ATP to the reaction (*see* Note 18), incubate at room temperature for 1–2 h with gentle agitation. Prepare 500 mL gel wash solution for each gel by dissolving 25 g TCA and 5 g NaPPi in 500 mL distilled water (*see* Note 17).
11. Transfer the gel to a new, clean container containing 100 mL gel wash solution. Wash the gel for 1 h at room temperature with gentle agitation (*see* Note 17).
12. Repeat **step 11** four times.
13. Put the gel on filter paper, cover the gel with plastic wrap, and dry the gel with a gel dryer (*see* Note 17).
14. The radioactive bands can be detected with an autoradiographic film or with a phosphorscreen and phosphorimager (*see* Fig. 1).

Acknowledgments

This work was supported by NIH grant R01GM059138 to J-KZ.

References

1. Wooten MW. In-gel kinase assay as a method to identify kinase substrates. *Sci STKE*. 2002; 2002:pl15. [PubMed: 12372853]
2. Zhang S, Klessig DF. Salicylic acid activates a 48-kD MAP kinase in tobacco. *Plant Cell*. 1997; 9:809–824. [PubMed: 9165755]
3. Asai T, Tena G, Plotnikova J, et al. MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature*. 2002; 415:977–983. [PubMed: 11875555]
4. Fujii H, Verslues PE, Zhu JK. Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis. *Plant Cell*. 2007; 19:485–494. [PubMed: 17307925]

¹⁵For detection of Ca²⁺ independent protein kinases, EGTA is added to inhibit calcium-dependent kinases by chelating Ca²⁺. If the in-gel kinase is used to detect the CDPK activity, a lower concentration (0.1 μ M) of EGTA should be used [8].

¹⁶For freshly ordered radioactive ATP, 3 μ L [γ -³²P]-ATP is recommended. The volume of [γ -³²P]-ATP can be increased up to 9 μ L to get stronger phosphorylation signal, if one or two half-lives of ³²P have passed. Radioactive [γ -³²P]-ATP that has been stored for more than 1 month is not recommended for the in-gel kinase assay.

¹⁷All of the solutions and materials may contain radioactivity. Please follow your institution's procedures regarding disposal of solutions and solid materials.

¹⁸Isotope label-free ATP is added to reduce the nonspecific signal and background. However, it is not recommended when using in-gel kinase assay to detect the CDPK activity [9].

5. Boudsocq M, Barbier-Brygoo H, Laurière C. Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. *J Biol Chem*. 2004; 279:41758–41766. [PubMed: 15292193]
6. Yoshida R, Hobo T, Ichimura K, et al. ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in *Arabidopsis*. *Plant Cell Physiol*. 2002; 43:1473–1483. [PubMed: 12514244]
7. Mori IC, Muto S. Absciscic acid activates a 48-kilodalton protein kinase in guard cell protoplasts. *Plant Physiol*. 1997; 113:833–839. [PubMed: 12223647]
8. Romeis T, Piedras P, Jones JDG. Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defense response. *Plant Cell*. 2000; 12:803–815. [PubMed: 10810151]
9. Boudsocq M, Willmann MR, McCormack M, et al. Differential innate immune signalling via Ca^{2+} sensor protein kinases. *Nature*. 2010; 464:418–422. [PubMed: 20164835]
10. Mori IC, Uozumi N, Muto S. Phosphorylation of the inward-rectifying potassium channel KAT1 by ABR kinase in *Vicia* guard cells. *Plant Cell Physiol*. 2000; 41:850–856. [PubMed: 10965941]
11. Liu Y, Zhang S. Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in *Arabidopsis*. *Plant Cell*. 2004; 16:3386–3399. [PubMed: 15539472]
12. Kissmehl R, Treptau T, Hauser K, et al. A novel, calcium-inhibitable casein kinase in *Paramecium* cells. *FEBS Lett*. 1997; 402:227–235. [PubMed: 9037201]

Fig. 1.

Effects of ABA on the protein phosphorylation activities of SnRK2s and casein kinases in *Arabidopsis*. Gels contain α -casein (*left panel*) and histone (*right panel*) as substrates. Total protein extracts from control and ABA-treated seedlings were subjected to the in-gel kinase assay. The data show that SnRK2s are activated by ABA treatment while casein kinases are not