Published in final edited form as:

Methods Mol Biol. 2016 ; 1360: 87–95. doi:10.1007/978-1-4939-3073-9_7.

A high-throughput radiometric kinase assay

Krisna C. Duong-Ly and Jeffrey R. Peterson*

Cancer Biology Program, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111

Abstract

Aberrant kinase signaling has been implicated in a number of diseases. While kinases have become attractive drug targets, only a small fraction of human protein kinases have validated inhibitors. Screening libraries of compounds against a kinase or kinases of interest is routinely performed during kinase inhibitor development to identify promising scaffolds for a particular target and to identify kinase targets for compounds of interest. Screening of more focused compound libraries may also be conducted in the later stages of inhibitor development to improve potency and optimize selectivity. The dot blot kinase assay is a robust, high-throughput kinase assay that can be used to screen a number of small molecule compounds against one kinase of interest or several kinases. Here, a protocol for a dot blot kinase assay used for measuring insulin receptor kinase activity is presented. This protocol can be readily adapted for use with other protein kinases.

Keywords

kinase; kinase assay; kinase inhibitor; high throughput screen; dot blot kinase assay; insulin receptor

1. Introduction

The human kinome encodes 518 protein kinases that regulate a number of processes including cell growth, proliferation, apoptosis, metabolism, and differentiation (1). Even though these signaling molecules are often deregulated in diseases such as cancer, few of the human kinases have approved inhibitors. One challenge in the development of any inhibitor is achieving adequate potency. Often, libraries containing diverse molecular scaffolds must be screened to determine what scaffolds result in high potency against the kinase of interest. Achieving high potency *in vitro* is a necessary step in developing an inhibitor that is effective at clinically achievable concentrations. Improving potency is also important for increasing the likelihood of selective activity against the target of interest. Selectivity is a major challenge in the development of kinase inhibitors since most target the highly conserved ATP binding pocket. Due the conservation of this pocket among all kinases, compounds that bind to this region will often inhibit other kinases as well. Inhibitors that

*CORRESPONDING AUTHOR: jeffrey.peterson@fccc.edu.

³Store in a properly shielded container. Using the value for the number of Ci per μ L on the day of delivery and the half-life of ³²P, calculate the number of Ci per μ L on the day of the experiment.

have low potency must be administered at high concentrations to be effective—at these high concentrations, off-target effects are more likely to occur, potentially resulting in dose-limiting toxicities.

One approach to circumvent these challenges is to screen large libraries of compounds against large libraries of kinases in an unbiased, target-blind manner (2). The information extracted from such large screens can identify molecular scaffolds that potentially inhibit previously unidentified targets, suggest avenues for the repurposing of existing small molecule compounds, and provide critical information about inhibitor selectivity. Such screening efforts necessitate robust and high-throughput methods for carrying out kinase assays. These high-throughput kinase assays are often classified as either binding assays or functional assays. Binding assays measure the physical interaction between a compound and a kinase. Functional assays directly measure the change in kinase catalytic activity in the presence of an inhibitor. These approaches may not yield identical results because, for example, compounds can bind a kinase but not inhibit its catalytic activity. We recently reviewed various kinase assays that fall into these two classes (1) and both types of assays have been used in recent large-scale screens of small molecule libraries against collections of recombinant kinases (3–6).

In this protocol, we describe a high-throughput functional assay, commonly referred to as a “dot blot kinase assay,” that can be readily carried out in a biochemistry laboratory that is equipped and approved for the use of radioisotopes. This radiometric assay involves performing a kinase reaction in the presence of ATP containing a radiolabeled terminal (gamma) phosphate and a peptide substrate (Figure 1A). The products of the reaction are spotted on phosphocellulose (P81) paper to which the peptide substrate binds. The radiolabeled phosphate group that has been transferred to the bound peptide can be visualized and quantified using a phosphorimager (Figure 1B). In addition to being used for screening, this technique can also be used to carry out dose-response measurements to determine the potency of a particular compound against a kinase of interest.

The dot blot kinase assay is considered a “gold standard” for assaying kinase functional activity as it directly measures catalytic activity using conventional ATP as the phosphate donor. Thus, unlike many binding assays, the dot blot kinase assay closely approximates the *in vivo* reaction. A key advantage of the dot blot kinase assay is the efficiency with which many kinase assays can be performed in parallel; hundreds of kinase reactions could be readily performed in a single day by a single person assuming that the kinase(s) of interest are either commercially available or can be readily expressed and purified. The assay, as presented in this protocol, is best adapted to a screen in which multiple compounds are screened against a single kinase. However, this protocol can also be readily adapted for screens in which many kinases are screened against a single compound if purified kinases are available. For screens in which many kinases are screened against a library of compounds, automation may be necessary to provide adequate throughput. Commercially available screening services (3) may be more ideal in these cases.

Limitations of the assay include that the anionic P81 paper does not bind to all protein or peptide substrates equally well. The affinity of a peptide for the P81 paper depends on the

number and distribution of positive charges on the peptide. To wash away unincorporated radiolabeled ATP while stabilizing the binding of substrate to the phosphocellulose matrix, sequential washes with phosphoric acid are performed. If the peptide substrate does not have sufficient positive charge in phosphoric acid, the addition of positively charged groups at the termini may be necessary during peptide synthesis (7) and must be tested empirically.

The use of a phosphorimager facilitates quantitative analysis of phosphate transfer. Autoradiography in conjunction with densitometry can be used as an alternative, however, the dynamic range and linear range of the assay in this case is much more limited (8, 9). Several mathematical models have been developed to address this issue (10) but the implementation of these models is less convenient than phosphorimager analysis.

The specific methods outlined in this protocol for the dot blot kinase assay were used to examine small-molecule inhibition of the insulin receptor kinase domain (11) but these methods can be generalized for examining inhibition of other kinase targets. Reaction conditions such as buffer components, concentration and type of substrates, as well as temperature and time may need to be optimized for kinases other than the insulin receptor kinase. Literature specific to these kinases may provide optimized reaction conditions. Another issue of critical importance that must be considered beforehand is that any kinases tested must be catalytically active and purified to a high degree (>90% purity) to decrease the likelihood that contaminating kinases will obscure the results of the assay. One option to circumvent these difficulties is to purchase the kinase(s) of interest from a commercial source that guarantees purity and catalytic activity.

2. Materials

Prepare all buffers with ultrapure water (deionized water purified to attain a resistivity of 18 M Ω at 25°C). All buffers may be prepared in advance and stored at the indicated temperature. The laboratory must be licensed and personnel must be trained in the proper use and disposal of radioisotopes.

2.1 Kinase reaction

1. Compound(s) of interest: 50 μ M working stock of each compound in DMSO. (see Notes ¹ and ²).
2. 10 mM ATP: 1 mL to be stored in 50 μ L aliquots at -20°C .
3. (γ -³²P)-ATP: ATP (γ -³²P)-3000 Ci/mmol, 5 mCi/mL EasyTide, 500 μ Ci (Perkin Elmer #BLU502H500UC).
4. 5x IR buffer: 100 mM Hepes pH 7.4, 5 mM EGTA, 500 μ M Na₃VO₄, 5 mM DTT, 10 mM MnCl₂, 0.1 mg/mL BSA, 50 mM MgCl₂. Prepare 10 mL and store 1 mL aliquots at -20°C .

¹Compounds should be solubilized in DMSO at a concentration of at least 1 mM in small aliquots. Note that DMSO stocks can take >30 minutes to thaw. Frozen compound stocks should be stored at -20°C .

²DMSO is hygroscopic, meaning that it can absorb water over time. This can interfere with compound solubility. Consider purchasing small aliquots of DMSO for use in preparing stock solutions of compounds instead of continuously using DMSO from a large stock bottle.

5. 1 mM peptide substrate: Poly(Glu, Tyr) 4:1 peptide, MW 5000–20000.
6. Purified insulin receptor kinase. Thaw one aliquot of insulin receptor kinase. (See Note ⁴)

2.2 Transfer of kinase reaction products to filter paper

1. Aluminum foil.
2. Whatman Grade P81 ion exchange chromatography paper.
3. Pencil and ruler for marking P81 paper.
4. 0.5% phosphoric acid. Prepare 1 L and store at 4°C. Use chilled solution for washes.
5. Acetone.

2.3 Visualization and quantification

1. Phosphorimager, screen, and cassette.
2. Light box for blanking phosphorimager screen.
3. Phosphorimager quantification software.

3. Methods

3.1 Kinase reaction

The components of a kinase reaction include buffer, ATP, a protein/peptide substrate (in the case of a protein kinase), and the kinase itself (Figure 1A). Other components may be added to optimize the kinase reaction (see Note ⁵). Each assay should be performed in duplicate. We recommend using a reaction volume of 20 μ L, which consists of 3 components:

0.2 μ L compound dissolved in DMSO or DMSO alone

17.8 μ L buffer/substrate/kinase mixture

2 μ L ATP mixture

For initial screening of compounds, we recommend a final compound concentration of 500 nM. Thus, 50 μ M working stocks of each compound in DMSO should be prepared in advance. Here, we have chosen to use a generic peptide substrate, the poly(Glu,Tyr)4:1 peptide (see Note ⁶). This generic peptide is a random polymer of glutamate and tyrosine residues at a ratio of 4 glutamate residues to 1 tyrosine residues, an optimal ratio for

⁴For insulin receptor kinase, it is necessary to use a baculovirus/insect cell expression system in order to produce a catalytically active kinase domain. We perform anion exchange chromatography followed by gel filtration chromatography in order to obtain kinase that is >90% pure by SDS-PAGE. Details of the expression and purification of the insulin receptor kinase can be found in Duong-Ly et al. (11) and Wei et al. (13). After purification, we store the kinase in small one-time use aliquots at –80°C to preserve catalytic activity.

⁵Phosphatase inhibitors (sodium orthovanadate for tyrosine kinases; sodium fluoride and β -glycerol phosphate for serine/threonine kinases) are often added since the reaction components may be contaminated with small amounts of phosphatases. Salt may also be necessary for kinase solubility, however, it is best to avoid using a total salt concentration of greater than 50 mM since salt can inhibit the kinase reaction.

⁶Generic peptides such as poly(Glu,Tyr) and poly(Glu,Ala,Tyr) are often used as substrates for tyrosine kinases. The user should refer to literature on the kinase of interest to determine the optimal substrate to use in this assay.

measuring insulin receptor kinase activity (12). The concentration of unlabeled ATP should be greater than or equal to the K_M of the kinase for ATP to ensure robust activity; here, 200 μM is used. The unlabeled ATP is mixed with radioactively labeled ATP. We recommend using 0.5 μCi of (γ - ^{32}P)-ATP for each reaction. At the specific activity used here, the concentration of the radioactively labeled ATP is negligible compared to the concentration of the unlabeled ATP and does not need to be computed in the final ATP concentration.

1. Preheat a 30°C heat block with sufficient space for the number of kinase assays to be performed.
2. Use one microcentrifuge tube per compound to be tested. To each, add 0.2 μL of the appropriate 50 μM compound stock. Also, prepare a tube for the control reaction containing 0.2 μL DMSO.
3. Calculate the volumes of buffer, substrate, and kinase necessary per 20 μL reaction. For insulin receptor kinase, we use final concentrations of substrate and kinase of 80 μM and 50 nM, respectively. Calculate the volume of water required to bring the volume of the buffer/substrate/kinase mix for each reaction to 17.8 μL . Prepare sufficient buffer/substrate/kinase mix for the number of kinase assays that will be performed (see Note ⁷).
4. Add 17.8 μL buffer/substrate/kinase mixture to each tube.
5. Calculate the volumes of unlabeled ATP and radioactively labeled ATP necessary for each 20 μL reaction. For insulin receptor kinase, a final ATP concentration of 200 μM is used. Calculate the volume of water required to bring the volume of the ATP mixture to 2 μL . Prepare sufficient ATP mixture for the number of kinase assays that will be performed.
6. Move the experimental setup to the designated radioactivity area of the lab. Prepare the ATP mixture and add 2 μL ATP mixture to initiate each reaction.
7. Incubate the reactions at 30°C for 30 minutes.
8. Freeze products of each reaction on dry ice.
9. Store reaction products at -20°C.

3.2 Transfer of kinase reaction products to filter paper

Take care to transfer radioactive wastes to appropriate vessels for disposal.

1. Thaw the reaction products at room temperature.
2. Using a pencil, draw a grid on the P81 filter paper consisting of 1.5 cm squares. Make sure there are at least as many squares as there are reactions to analyze.

⁷Determine the concentrations of substrate and kinase to be used in the assay. The concentration of substrate must be in vast excess so that it is not depleted by more than 10% by the end of the reaction. The concentration of kinase must be determined empirically and should be the minimal concentration that yields adequate signal.

3. Place a piece of aluminum foil underneath the P81 paper. Spot 3 μ L of each reaction in the appropriate square.
4. Allow to air dry. This takes about 15 minutes in a laminar flow hood.
5. Wash the P81 paper 4 times with chilled 0.5% phosphoric acid for 5 minutes per wash to remove free radiolabeled ATP.
6. Wash the P81 paper with acetone for 5 minutes. Allow the filter paper to air dry for about 20 minutes in the laminar flow hood.

3.3 Visualization and quantification

Refer to the user manual for the phosphorimager and associated quantification software for detailed instructions.

1. Blank a phosphorimager screen on a white light source for at least 10 minutes.
2. Expose the dried P81 paper to the sensitive surface of the phosphorimager screen for 3 hours.
3. Scan the screen using the phosphorimager. If the signal is weak, re-blank the screen and re-expose for a longer period of time. If the signal is so high that it is no longer in the linear range, re-blank the screen and re-expose for a shorter period of time.
4. Measure the intensity of the spots using the phosphorimager software. Determine the percentage inhibition by dividing the intensity of an inhibited reaction by the intensity for the DMSO control reaction.

Acknowledgments

We would like to thank Dr. Jinhua Wu and Dr. Stevan Hubbard for providing insulin receptor kinase baculovirus and for advice on expression and purification of the kinase. The authors' work is supported by NIH R21 AI099929 (J.R.P.) and NIH T32 CA009035-36 (K.C.D.).

References

1. Duong-Ly KC, Peterson JR. The human kinome and kinase inhibition. *Curr Protoc Pharmacol*. 2013; doi: 10.1002/0471141755.ph0209s60
2. Goldstein DM, Gray NS, Zarrinkar PP. High-throughput kinase profiling as a platform for drug discovery. *Nat Rev Drug Discov*. 2008; doi: 10.1038/nrd2541
3. Anastassiadis T, Deacon SW, Devarajan K, et al. Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity. *Nat Biotechnol*. 2011; doi: 10.1038/nbt.2017
4. Davis MI, Hunt JP, Herrgard S, et al. Comprehensive analysis of kinase inhibitor selectivity. *Nat Biotechnol*. 2011; doi: 10.1038/nbt.1990
5. Karaman MW, Herrgard S, Treiber DK, et al. A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol*. 2008; doi: 10.1038/nbt1358
6. Fabian MA, Biggs WH, Treiber DK, et al. A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat Biotechnol*. 2005; doi: 10.1038/nbt1068
7. Hastie CJ, McLauchlan HJ, Cohen P. Assay of protein kinases using radiolabeled ATP: a protocol. *Nat Protoc*. 2006; doi: 10.1038/nprot.2006.149

8. Huebner VD, Matthews HR. Autoradiography of gels containing (32)p. *Methods Mol Biol.* 1985; doi: 10.1385/0-89603-064-4:51
9. Zouboulis CC, Tavakkol A. Storage phosphor imaging technique improves the accuracy of RNA quantitation using 32P-labeled cDNA probes. *Biotechniques.* 1994; 16:290–2, 294. [PubMed: 7514007]
10. Palfi A, Hatvani L, Gulya K. A New Quantitative Film Autoradiographic Method of Quantifying mRNA Transcripts for In Situ Hybridization. *J Histochem Cytochem.* 1998; doi: 10.1177/002215549804601006
11. Anastassiadis T, Duong-Ly KC, Deacon SW, et al. A highly selective dual insulin receptor (IR)/insulin-like growth factor 1 receptor (IGF-1R) inhibitor derived from an extracellular signal-regulated kinase (ERK) inhibitor. *J Biol Chem.* 2013; doi: 10.1074/jbc.M113.505032
12. Braun S, Raymond WE, Racker E. Synthetic tyrosine polymers as substrates and inhibitors of tyrosine-specific protein kinases. *J Biol Chem.* 1984; 259:2051–4. [PubMed: 6538195]
13. Wei L, Hubbard SR, Hendrickson WA, Ellis L. Expression, characterization, and crystallization of the catalytic core of the human insulin receptor protein-tyrosine kinase domain. *J Biol Chem.* 1995; 270:8122–30. [PubMed: 7713916]

Figure 1.

(A) Radiometric kinase assay. In addition to conventional (unlabeled) ATP, ATP containing a radiolabeled terminal phosphate is included in the reaction mixture. Once the kinase reaction is completed, the radiolabeled phosphate is transferred to the peptide. (B) Dot blot capture and visualization of radiolabeled kinase reaction products. Kinase reaction products are spotted on filter paper and washed, removing excess radiolabeled ATP and facilitating

peptide binding. A phosphorimager is used to visualize and quantify the counts on the filter paper for each kinase reaction.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript