Kinase-catalyzed Biotinylation with Inactivated Lysates for Discovery of Substrates (K-BILDS)

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Protein phosphorylation is catalyzed by kinases to regulate a large variety of cellular activities, including growth and signal transduction. Methods to identify kinase substrates are crucial to fully understand phosphorylation-mediated cellular events and disease states. Here, we report a set of protocols to identify substrates of a target kinase using Kinase-catalyzed Biotinylation with Inactivated Lysates for Discovery of Substrates (K-BILDS). As described in these protocols, K-BILDS involves inactivation of endogenous kinases in lysates, followed by addition of an active exogenous kinase and the γ-phosphate-modified ATP analog ATP-biotin for kinase-catalyzed biotinylation of cellular substrates. Avidin enrichment isolates biotinylated substrates of the active kinase, which can be monitored by western blot. Substrates of the target kinase can also be discovered using mass spectrometry analysis. Key advantages of K-BILDS include compatibility with any lysate, tissue homogenate, or complex mixture of biological relevance and any active kinase of interest. K-BILDS is a versatile method for studying or discovering substrates of a kinase of interest to characterize biological pathways thoroughly. © 2023 Wiley Periodicals LLC.

Basic Protocol 1: FSBA treatment of lysates to inactivate kinases
Basic Protocol 2: Kinase-catalyzed Biotinylation with Inactivated Lysates for Discovery of Substrates (K-BILDS)

Keywords: ATP-biotin • biotinylation • kinase • kinase substrate identification • phosphorylation

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INTRODUCTION

Phosphorylation is a key post-translational modification that alters protein function and localization to regulate a variety of cellular activities (Hunter, 1995; Wang & Cole, 2014). Kinases catalyze phosphorylation of protein substrates by transferring the γ-phosphoryl of ATP to amino acid residues, such as serine (Ser), threonine (Thr), tyrosine (Tyr), and histidine (His), to produce phosphoproteins (Fig. 1) (Kalagiri & Hunter, 2021; Wang & Cole, 2014). Phosphorylation is a dynamic modification, with removal catalyzed by protein phosphatases (Hunter, 1995). Unregulated protein phosphorylation can result in development of diseases, including cancers and diabetes (Cohen, 2001). The critical role that protein phosphorylation plays in disease has made kinases prime drug targets, with multiple clinically used therapeutics for cancer and other conditions (Gross et al., 2015). With roles in normal and disease states, protein phosphorylation must be thoroughly

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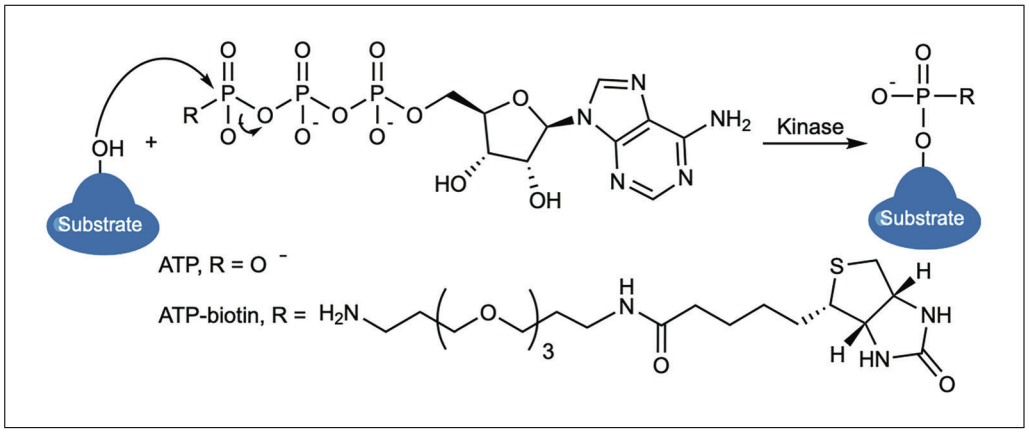

Figure 1 Kinase-catalyzed phosphorylation and biotinylation. The kinase phosphoryl transfer mechanism is shown, where ATP or a γ-phosphate-modified ATP analog, such as ATP-biotin, labels substrates at the hydroxyl group of a Ser, Thr, or Tyr residue. Kinases phosphorylate other residues, including His, which is not shown here.

studied to characterize the mechanisms dictating basic cell biology and disease (Kussmann et al., 1999; Manning et al., 2002).

To fully characterize the role of phosphorylation in biology and disease, kinase-substrate pair identification is essential. In fact, prior substrate discovery studies uncovered links between kinases and crucial biological processes. For example, cyclin-dependent kinase 1 (CDK1) was found to play a role in regulation of mitosis through studies on the phosphorylation of its substrate nucleoporin 53 (Nup53) (Blethrow et al., 2008; Vollmer et al., 2012). Unfortunately, the low stoichiometry of phosphosites and the dynamic nature of phosphorylation make substrate identification difficult (Olsen et al., 2010; Tsai et al., 2015). Tools to identify kinase-substrate pairs are limited but are crucial to decode the role of protein phosphorylation in human cell biology.

As one approach to monitor kinase-substrate pairs, ATP analogs with tags at the γ-phosphate, such as biotin (Green & Pflum, 2007; Senevirathne et al., 2012), dansyl (Green & Pflum, 2009), and aryl azide (Suwal & Pflum, 2010), act as kinase co-substrates to label substrates. In particular, the ATP analog ATP-biotin (Fig. 1) is accepted by kinases to label substrates with a biotin group, which allows subsequent phosphoprotein purification and visualization. Prior work established that ATP-biotin is a co-substrate for a variety of kinases (Senevirathne et al., 2016) and that the phosphoryl biotin tag is resistant to phosphatase activity (Senevirathne & Pflum, 2013), which makes ATP-biotin and kinase-catalyzed biotinylation ideal for phosphoprotein studies.

The protocols in this article highlight a strategy to identify kinase-substrate pairs using ATP-biotin, entitled Kinase-catalyzed Biotinylation with Inactivated Lysates for Discovery of Substrates, or K-BILDS (Embogama & Pflum, 2017). Because ATP-biotin is compatible with all kinases (Senevirathne et al., 2016), K-BILDS initially utilizes the irreversible pan-kinase inhibitor 5′-(4-fluorosulfonlbenzoyl)adenosine (FSBA) to inactivate endogenous kinases in lysates by reacting with an active-site lysine (Knight et al., 2012; Likos et al., 1980; Pal et al., 1975). An initial assessment of kinase inactivation by FSBA using gel methods (Fig. 2A) is required before proceeding to a full K-BILDS study. For K-BILDS (Fig. 2B), addition of an exogenous active kinase and ATP-biotin to the FSBA-inactivated lysates promotes biotinylation of substrates by the active kinase. Because FSBA might only partially inactivate some kinases, a control ATP-biotin reaction omitting exogenous active kinase is also prepared to account for background biotinylation. Avidin enrichment subsequently isolates biotinylated substrates from samples with and without active kinase. Substrates of the target kinase can then be monitored

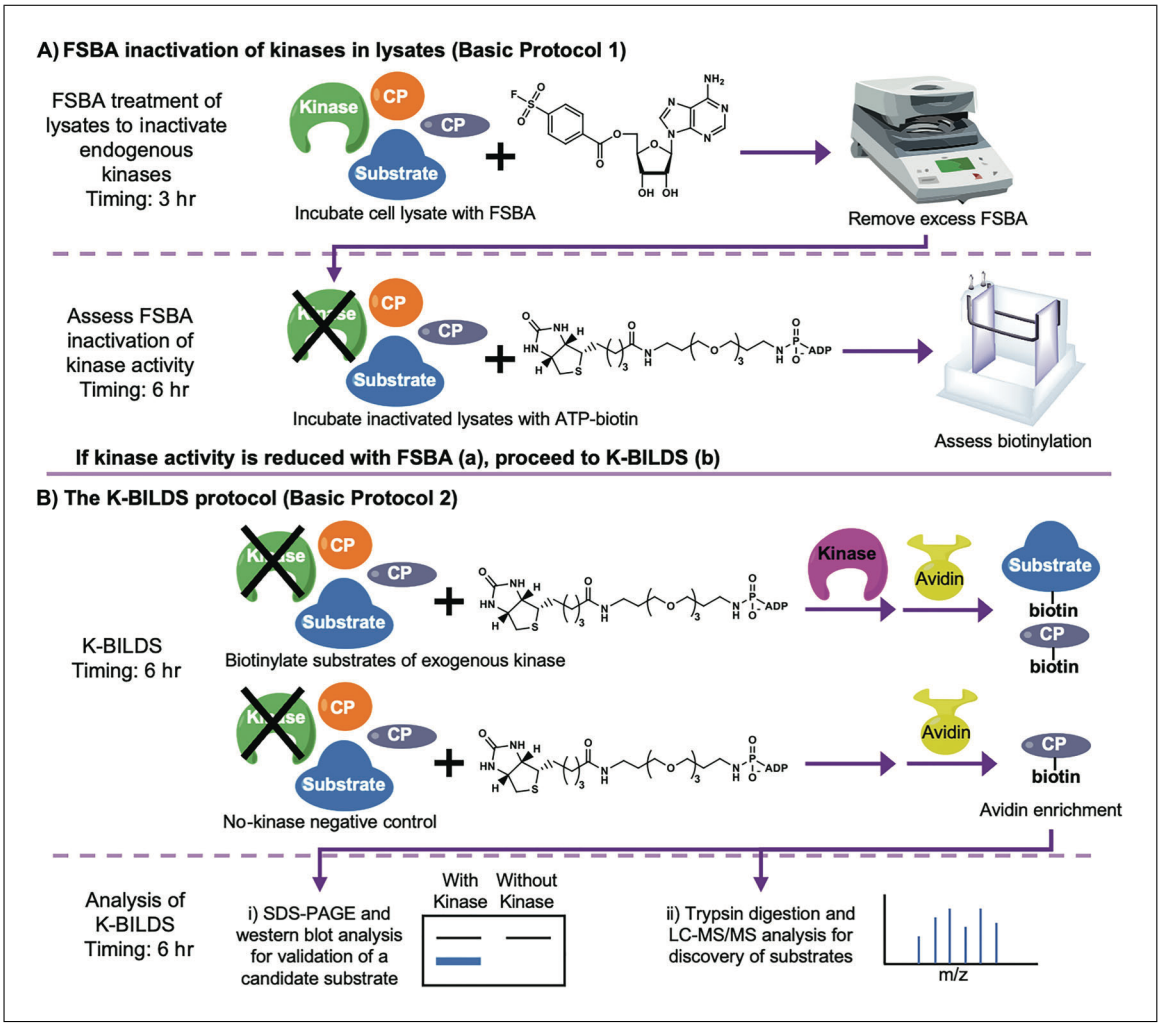

Figure 2 FSBA treatment of lysates to inactivate kinase activity and the K-BILDS workflow. (**A**) The K-BILDS method begins with FSBA treatment to inactivate kinases in cell lysates, which is followed by removal of excess FSBA by filtration (top panel). Successful inactivation of endogenous kinases must be confirmed by gel analysis before proceeding to a K-BILDS experiment (bottom panel). (**B**) FSBA-inactivated cell lysates are incubated with exogenous recombinant kinase and ATP-biotin to generate biotinylated substrates. A critical negative control omits exogenous kinase, which allows background biotinylated proteins to be distinguished from substrates. Avidin resin enriches biotinylated substrates that are then separated by SDS-PAGE and analyzed by either western blotting to validate candidate substrates or in-gel trypsin digestion with LC-MS/MS analysis to discover substrates. Proteins present in the kinase reaction, but not the negative control, are possible substrates. Dark green: endogenous kinase; blue: substrate; magenta: active exogenous kinase; orange and gray: cellular proteins (CP); yellow: avidin resin.

in two ways. If a substrate is known or suspected, proteins in the enriched samples are separated by SDS-PAGE, and candidate substrates are visualized by western blot analysis (Fig. 2Bi). In this way, K-BILDS is an effective substrate validation tool. Alternatively, to use K-BILDS as a substrate discovery method, proteins in the enriched samples are identified by LC-MS/MS analysis after trypsin digestion (Fig. 2Bii).

Prior work with K-BILDS identified substrates of the well-studied Ser/Thr kinase protein kinase A (PKA) from HeLa cell lysates (Embogama & Pflum, 2017). PKA regulates many cellular activities, including signaling, transcription, and homeostasis (Shabb, 2001), which has made substrate identification an active area of study. With numerous known substrates, PKA was used to validate K-BILDS as a substrate discovery tool. K-BILDS identified 56 previously known and over 200 candidate substrates of PKA in HeLa lysates (Embogama & Pflum, 2017). Additionally, three candidate K-BILDS hits were

validated *in vitro* as PKA substrates (Embogama & Pflum, 2017). This previous study with PKA successfully established K-BILDS as a substrate identification method. A valuable feature of K-BILDS is that the phosphoryl modification only need be preserved up to the enrichment step, but not for identification steps, such as gel or LC-MS/MS analyses (Embogama & Pflum, 2017), which makes K-BILDS compatible with identification of low-abundance or unstable modifications, such as phosphohistidine (pHis). In addition, K-BILDS can be used with any lysate or tissue homogenate and any kinase of interest to facilitate characterization of transient kinase-substrate interactions in any biological context. With these advantages, K-BILDS has wide application to study substrates of any target kinases in any complex mixture relevant to the biological system.

To encourage future application of K-BILDS to monitor kinase-substrate interactions, this set of protocols provides guidance on how to implement K-BILDS with any lysate or tissue homogenate and any kinase of interest. Basic Protocol 1 outlines steps to initially treat the chosen lysate or tissue homogenate with FSBA to irreversibly inactivate endogenous kinases (Fig. 2A). A low level of endogenous kinase activity is a prerequisite for the full K-BILDS method. With confirmation by gel analysis that FSBA effectively inactivated the kinase activity in the chosen lysate, Basic Protocol 2 details steps to complete the K-BILDS protocol using ATP-biotin and the selected active kinase (Fig. 2B). Given prior application of K-BILDS to PKA and HeLa cell lysates, specific experimental details are provided using PKA and HeLa cell lysates as a model system, which is an ideal learning tool before application to a new lysate or tissue homogenate and kinase. Taken together, the protocols will assist in applying K-BILDS to any kinase and complex mixture of interest to confirm or discover kinase-substrate relationships, which will augment studies on kinase-mediated cell biology and disease formation.

BASIC PROTOCOL 1

FSBA TREATMENT OF LYSATES TO INACTIVATE KINASES

Prior to performing K-BILDS (Basic Protocol 2), optimization of FSBA treatment of the lysate of choice is required to ensure effective inactivation of endogenous kinases (Fig. 2A). In addition, FSBA treatment can result in the precipitation of cellular proteins, which will confound K-BILDS analysis. To optimize the FSBA treatment conditions, the concentration of FSBA solubilized in dimethyl sulfoxide (DMSO) can be varied to establish conditions with maximal kinase inhibition and minimal protein loss. A higher concentration of DMSO can better solubilize FSBA for effective inactivation but must be tested to ensure minimal protein loss. In this example protocol here, two different concentrations of FSBA in DMSO (5 mM in 20% DMSO and 7 mM in 10% DMSO) were tested. Prior work used 1 to 20 mM FSBA in 3% to 20% DMSO, depending on the batch of FSBA and lysates employed (DeCamp & Colman, 1986; Embogama & Pflum, 2017; Knight et al., 2012; Müller et al., 2016). After treatment of lysates with varying FSBA and DMSO concentrations, the FSBA-treated lysates are incubated with ATP-biotin to monitor kinase activity via kinase-catalyzed biotinylation. For comparison, a positive-control reaction with ATP-biotin and untreated lysates is also included (Table 1). In addition, a negative-control reaction without FSBA or ATP-biotin establishes background biotinylation levels, including endogenously biotinylated proteins (Table 1). Then, the levels of

Table 1 Reactions Recommended to Assess FSBA Inactivation

	Negative control	Positive control	Kinase inactivation reaction
Lysate	+	+	+
FSBA in DMSO	−	−	+
DMSO	+	+	+
ATP-biotin	−	+	+

biotinylated and total proteins are compared in the FSBA-treated and untreated lysates using SDS-PAGE analysis (Fig. 2A). The expectation for successful kinase inactivation with FSBA treatment is a reduction in biotinylated proteins without loss of total protein compared to the positive-control reaction with untreated lysates (Embogama & Pflum, 2017). As a benchmark of success, the endogenous kinase activity in HeLa cell lysates was reduced by roughly 70% after FSBA treatment in the previous study with PKA (Embogama & Pflum, 2017). For application to any cell lysates of interest, FSBA inactivation of kinase activity in the chosen lysate should be tested and optimized to ensure success of the K-BILDS study.

NOTE: Another experiment necessary prior to a full K-BILDS experiment is to confirm the compatibility of ATP-biotin with the chosen kinase. Acceptance of ATP-biotin by a variety of kinases has already been established (Green & Pflum, 2007; Senevirathne et al., 2016; see Current Protocols article: Senevirathne et al., 2012). If a chosen kinase has not yet been tested, a detailed procedure to assess the compatibility of ATP-biotin with a recombinant kinase was described previously (see Current Protocols article: Senevirathne et al., 2012).

Materials

Cell lysates (e.g., HeLa S3 cell lysates; see recipe)
Lysis buffer (see recipe), 4°C
DMSO (ATCC, cat. no. 4-X)
200 mM FSBA stock (see recipe)
Tris/DTT buffer (see recipe; make fresh)
Distilled water
ATP-biotin stock (see recipe)
4× SDS-PAGE loading buffer (see recipe; make fresh)
Pre-stained protein ladder (EZ-Run™ Prestained Rec Protein Ladder, Fisher Scientific, cat. no. BP36031)
Gel-fixing solution (see recipe)
SYPRO™ Ruby Protein Gel Stain (Thermo Fisher Scientific Invitrogen™, cat. no. S12000)
Destaining solution (see recipe)
1× TBST (see recipe)
Membrane-blocking buffer (see recipe)
Streptavidin-Cy5 staining solution (see recipe; make fresh)

0.6- and 1.5-ml microcentrifuge tubes (Fisherbrand™, cat. no. 05-408-120 and 05-408-129)
Vortex
Microcentrifuge (Fisherbrand™ accuSpin™ Micro 17, cat. no. 13-100-675), room temperature and 4°C
31°C thermomixer (Eppendorf™ Thermomixer™ R, Fisher Scientific, 05-400-205) or MultiTherm shaker (Benchmark Scientific, cat. no. H5000-HC)
Amicon Ultra-0.5 ml 3-kDa centrifugal filter units (Amicon, cat. no. UFC5003)
95°C heating block filled with sand or water (VWR Standard Dry Block Heater)
Electrophoresis cell (Mini-PROTEAN® tetra vertical electrophoresis cell, Bio-Rad, cat. no. 1658004)
PVDF membrane (Immobilon®-PSQ membrane, Millipore Sigma, cat. no. IPVH00010)
Electrophoretic transfer cell (Bio-Rad Mini Trans-Blot® electrophoretic transfer cell, Bio-Rad, cat. no. 1703930)
Container (for fixing, staining, or western blotting; flat-bottom plastic or glass container of similar width and length as membrane or gel)
Rocking platform shaker (VWR, model no. 200)

Table 2 Samples for FSBA Treatment

Sample number[a]	1	2	3	4	5	6
Sample name	Negative control	Positive control	5 mM FSBA/ 20% DMSO	Negative control	Positive control	7 mM FSBA/ 10% DMSO
HeLa lysate[b]	7 µl	7 µl	7 µl	7 µl	7 µl	7 µl
Lysis buffer	73 µl	73 µl	73 µl	83 µl	83 µl	83 µl
DMSO	20 µl	20 µl	17.5 µl	10 µl	10 µl	6.5 µl
FSBA in DMSO[c]	-	-	2.5 µl	-	-	3.5 µl
Final volume	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl

[a]Duplicate samples (1 and 2; 3 and 4) generated here will later serve as negative and positive controls in biotinylation reactions with ATP-biotin to assess the efficiency of FSBA inactivation (see Table 3).
[b]In the example FSBA treatment, HeLa lysates with a stock concentration of 28 mg/ml were used to add 200 µg total protein to each tube.
[c]A stock solution of 200 mM FSBA in DMSO was used.

Chemiluminescence imaging system (FluorChem imager, ProteinSimple, model no. Q)
Fluorescence imaging system (Typhoon™ FLA imager, GE Healthcare, model no. 9500)

Additional reagents and equipment for preparing SDS-PAGE gels [10%-16%; purchased or made according to prior work (He, 2011)] and for SDS-PAGE (see Current Protocols article: Gallagher & Sasse, 2001; He, 2011) and immunoblotting (see Current Protocols article: Gallagher et al., 2008)

FSBA treatment of lysates to inactivate kinases

1. Label six 0.6- or 1.5-ml microcentrifuge tubes with sample numbers (Table 2).
2. Thaw cell lysates on ice (if frozen) and add 200 µg total protein to each tube on ice.

   With the HeLa lysate example, add 7 µl of 28 mg/ml stock (200 µg total protein) to each tube, as shown in Table 2. The protein concentration in lysates is determined by Bradford assay (Bradford, 1976).

   Perform sample preparation on ice or in a cold room.

   The concentration of total protein in the lysates will dictate the final sample volume (Table 2). Lysate amounts used previously varied from 200 µg to 1 mg total protein in a total reaction volume of 100 to 500 µl. If performing a larger-scale inactivation reaction, use the same final concentrations of reagents but with a larger scaled overall volume (for example, a 1-mg reaction would use a five times larger reaction volume than the 200 µg reaction in Table 2, or 500 µl).
3. Add cold lysis buffer to tubes, varying the volume depending on the desired DMSO percentage (see step 4).

   With the HeLa lysate example, add 73 or 83 µl lysis buffer to the appropriate samples, as shown in Table 2, to vary the DMSO percentage (10% or 20%).
4. Remove the tubes from ice and add DMSO at room temperature.

   Remove tubes containing DMSO from ice because DMSO has a freezing point of 19°C. DMSO should be added at room temperature to avoid freezing.

   With the HeLa lysate example, 20% and 10% DMSO concentrations are being tested. Because FSBA is dissolved in DMSO, 17.5 or 6.5 µl additional DMSO is added to the FSBA samples (Table 2) for a final concentration of 20% or 10%, respectively.

   Optimize the DMSO concentration to identify the percentage that solubilizes FSBA but avoids protein loss. The DMSO concentration ranged from 3% to 20% in prior experiments.

5. Thaw an aliquot of 200 mM FSBA stock (dissolved in DMSO) at room temperature or prepare fresh stock and add to tubes. Briefly vortex the samples and spin down 10 s at 1500 × *g* in a microcentrifuge to ensure that the reagents are combined at the bottom of the tube.

   With the HeLa lysate example, add 2.5 or 3.5 µl of the 200 mM stock to produce a final concentration of 5 or 7 mM, respectively (Table 2).

   FSBA degrades quickly so should be added last, after the lysis buffer and DMSO. Use a freshly prepared FSBA stock or a single-use aliquot stored immediately after resuspension in DMSO.

   Precipitation may appear upon adding FSBA and mixing the samples. Briefly vortex the samples once more if a pellet is observed at the bottom of the tube.

   More than one FSBA and DMSO concentration combination can be tested to reduce kinase-catalyzed biotinylation while minimizing protein loss. The FSBA and DMSO concentrations need to be retested and optimized with every batch of FSBA, different cell line, and new application. A range of 1 to 20 mM FSBA has typically been tested in prior work.

6. Incubate samples in a thermomixer or MultiTherm shaker at 31°C for 2 hr with shaking at 60 × *g*.

7. During the 2-hr FSBA treatment, label six new 1.5-ml microcentrifuge tubes and six Amicon Ultra-0.5 ml 3-kDa centrifugal filter units with sample numbers (Table 2). Place each filter unit into a microcentrifuge tube. Also label 12 microcentrifuge tubes supplied with the filter units (two tubes per sample).

   A 3-kDa filter is recommended for most applications to ensure removal of excess FSBA while minimizing protein loss during filtration.

8. About 15 min prior to completion of FSBA treatment (step 6), wash the filter units by filling to capacity with fresh Tris/DTT buffer, closing the cap, and centrifuging for 10 min at 14,200 × *g* at room temperature. Invert the filter unit into the same microcentrifuge tube and spin for 2 min at 1000 × *g* to completely remove the wash buffer. Discard the wash buffer and place the filter upward in preparation for addition of samples.

   According to the manufacturer's user guide (Millipore), the filter units contain a small amount of glycerin. We have found that the glycerin storage solution can influence the results of the biotinylation assessment in later steps. Therefore, this buffer wash is critical to prevent interference by glycerin.

   Do not let the filters dry out. Prepare for immediate use. Refer to the user guide for detailed figures on proper usage.

9. After the 2-hr incubation (step 6), centrifuge reaction samples for 2 min at 1000 × *g* to pellet any precipitation. Pipet each supernatant into new microcentrifuge tubes from step 7.

10. Dilute each supernatant with Tris/DTT buffer to a final volume of 500 µl (or the maximum-capacity volume of the centrifugal device).

    With the HeLa lysate example, add 400 µl Tris/DTT to generate a 500-µl volume. The Tris/DTT buffer both dilutes and quenches the FSBA (Ohnuma et al., 2011).

    If sample volumes >100 µl are used for FSBA treatment (due to larger-scale reactions or dilute lysates), then dilute with Tris/DTT to achieve the maximum-capacity volume of the filter unit. If the reaction volumes are greater than the maximum-capacity volume of the filter unit, steps 11 to 12 can be repeated an extra time to combine and concentrate the full sample, with the filtrate discarded. If sample volumes of significantly greater than

Table 3 Samples for Assessing Kinase Inactivation by FSBA

Sample number	1	2	3	4	5	6
Sample name	DMSO control	ATP-biotin control	5 mM FSBA/ 20% DMSO	DMSO control	ATP-biotin control	7 mM FSBA/ 10% DMSO
Lysate samples from Table 2[a]	20 µl of Sample 1	20 µl of Sample 2	20 µl of Sample 3	20 µl of Sample 4	20 µl of Sample 5	20 µl of Sample 6
ATP-biotin[b]	-	5 µl	5 µl	-	5 µl	5 µl
Water	5 µl	-	-	5 µl	-	-
Final volume	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl

[a]The lysates used for each sample here come from the lysate samples generated after FSBA treatment (see Table 2).
[b]A working ATP-biotin stock of 20 mM was used in the example here for a final concentration of 4 mM in the reactions.

500 µl are used for FSBA treatment, larger centrifugal filter units, with 2-ml (Amicon, cat no. UFC2003) or 4-ml (Amicon, cat no. UFC8003) capacities, can be employed.

11. Add the diluted samples from step 10 to their respective labeled, rinsed, and emptied filter units (see step 8) and close the caps.

    The same microcentrifuge collection tube that was used for pre-rinsing in step 8 can be used for this step.

12. Centrifuge for 30 min at 14,200 × *g*, 4°C.

13. Discard the filtrate collected at the bottom of each microcentrifuge tube. Place the top filter, containing the supernatant, back in the same emptied microcentrifuge tube and add enough Tris/DTT buffer to the top supernatant give a 500-µl final volume or to fill the unit to capacity. Close the cap and repeat step 12.

14. To collect FSBA-treated lysates, invert the top filter unit into a new labeled 1.5-ml microcentrifuge tube from step 7 and spin for 2 min at 1000 × *g*, 4°C, to collect the supernatant.

    Use lysates right away or store ≤1 year at −80°C.

    Roughly 30 µl is collected typically. Although similar volumes of FSBA-treated lysates from each sample should be collected after filtration, the volume could vary slightly. If different volumes are collected, then dilute the lower-volume samples to the same volume as the sample with the largest volume to ensure that equal quantities are compared in subsequent analysis steps.

Assessment of FSBA inactivation of kinase activity

15. Label six new 1.5-ml microcentrifuge tubes with sample numbers (Table 3).

16. If using frozen FSBA-treated lysates, thaw on ice and then add the lysates to the tubes.

    In the example reactions with HeLa lysates, 20 µl FSBA-treated lysate (or roughly two-thirds of the sample from step 14, or ∼130 µg) is used per reaction (Table 3).

17. Add distilled water to obtain the same final volume in all samples.

    With the HeLa lysate example, add 5 µl water to the tubes without ATP-biotin to obtain a final volume of 25 µl (Table 3).

18. Thaw the ATP-biotin stock on ice immediately before use. Initiate the reaction by adding enough volume to the appropriate sample (Table 3) to obtain a final ATP-biotin concentration of 4 mM. Briefly vortex the samples to mix and spin down 10 s at 1500 × *g* to combine all reagents.

    In the example reactions, 5 µl of a 20 mM ATP-biotin stock solution is used.

The ATP-biotin concentration might need to be optimized to obtain robust biotinylation. The typical final concentration ranges from 1 to 10 mM.

19. Incubate samples in a thermomixer or MultiTherm shaker at 31°C for 2 hr without shaking.

20. Following reaction incubation, add fresh 4× SDS-PAGE loading buffer to each sample to obtain a 1× concentration in the kinase reactions. Briefly vortex the samples to mix and spin down 10 s at 1500 × *g* to combine the reagents.

    With the HeLa lysate example, add 8.4 µl of 4× SDS-PAGE loading buffer to each sample to give a final volume of 33.4 µl and a 1× SDS-PAGE loading buffer concentration.

21. To prepare the samples for separation by SDS-PAGE, incubate samples for 2 min at 95°C in a heating block filled with sand or water to denature proteins. Briefly vortex the samples and centrifuge 10 s at 1500 × *g*.

22. Pour two SDS-PAGE gels based on prior protocols (He, 2011) or use two purchased gels.

    Two 16% gels are used with the HeLa lysate example.

    If preparing gels (He, 2011), make the gels ahead of time so that samples can be loaded immediately.

    The acrylamide gel percentage can range from 8% to 16%. The desired acrylamide percentage is selected depending on the molecular weight range of interest in the lysates (Rath et al., 2013).

23. Load the reaction samples and pre-stained protein ladder into separate wells of the two gels.

    For the HeLa lysate example, two-thirds to three-quarters of the sample is loaded on the gel for biotinylation visualization, whereas the remaining quarter to third of the sample is loaded on the gel for analysis of total protein levels.

24. Using an electrophoresis cell, electrophorese at 110 V for 10 to 15 min or until the dye front reaches the separating layer. Separate proteins at 200 V for 45 to 60 min or until the dye front is at the bottom of the gel.

    Alternative electrophoresis conditions have been previously published (He, 2011).

25. With the gel for biotin visualization, electro-transfer proteins in the gel to a PVDF membrane for 2 hr at 90 V on ice using an electrophoretic transfer cell (see Current Protocols article: Goldman et al., 2015). With the gel for observation of total protein levels, fix gel in 100 ml gel-fixing solution twice, for 15 to 30 min each, on a rocking platform shaker.

    The gel fixing time depends on the SYPRO™ Ruby procedure being performed. There are two possible procedures according to the manufacturer's protocols: the basic protocol requires a 30-min fixing time, whereas the rapid protocol only requires 15 min. Only the basic protocol is discussed here.

26. With the gel for observation of total protein levels, remove fixing solution, add 60 ml SYPRO™ Ruby Protein Gel Stain, and rock overnight at room temperature. Then, remove the SYPRO™ Ruby stain from gel, add 100 ml destaining solution, and destain for 30 min at room temperature with rocking. Finally, wash the gel three times with distilled water for 5 min each with rocking and then visualize SYPRO™ fluorescence using a chemiluminescence imaging system.

    Shield SYPRO™ Ruby Protein Gel Stain from light by covering the container with foil to avoid quenching the fluorescence.

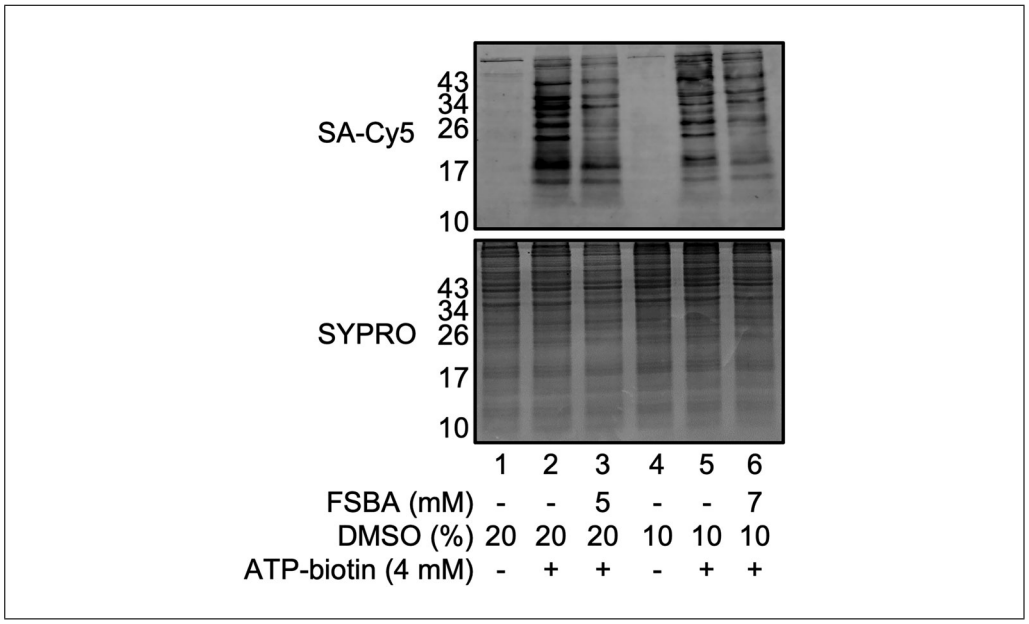

Figure 3 Kinase-catalyzed biotinylation of HeLa cell lysates to assess kinase activity after FSBA treatment. HeLa cell lysates were treated with either 5 or 7 mM FSBA in either 10% or 20% DMSO, as indicated (lanes 3 and 6). Positive controls were prepared with DMSO alone (lanes 1 and 2 and lanes 4 and 5). The lysates were then incubated without (lanes 1 and 4) or with (lanes 2 and 3 and lanes 5 and 6) ATP-biotin for 2 hr at 31°C. Proteins were separated by SDS-PAGE and visualized with streptavidin-Cy5 (SA-Cy5) to assess biotinylation and SYPRO™ Ruby stain to visualize total proteins. Molecular-weight markers (kDa) are indicated to the left of the gel.

27. With the membrane for biotin visualization (from step 25), dry following electro-transfer. Next, activate the membrane with methanol for 10 s without rocking and then wash three times with 10 ml of 1× TBST for 5 min each with rocking. Block the membrane with 10 ml membrane-blocking buffer for 1 hr at room temperature or overnight at 4°C with rocking. After removing the membrane-blocking buffer, incubate the membrane in 10 ml fresh streptavidin-Cy5 staining solution for 1 hr at room temperature in the dark with rocking. Then, remove the streptavidin-Cy5 staining solution and wash membrane with 10 ml of 1× TBST three times for 5 min each with rocking. Wash once with 10 ml water for 5 min with rocking.

    CAUTION: *Methanol can be toxic with skin contact and if inhaled.*

    Allowing the membrane to dry after electro-transfer can promote adherence of the proteins to the membrane. However, this drying step is optional.

    Shield streptavidin-Cy5 from light during staining by covering the container with foil to avoid quenching the fluorophore before imaging. A 1:1000 to 1:2000 dilution of the streptavidin-Cy5 staining solution is recommended for optimal biotinylation signal with ATP-biotin. Also see the manufacturer's recommendations (Thermo Fisher) for streptavidin-Cy5 use.

28. Directly visualize the Cy5 fluorophore to determine biotinylation levels using a fluorescence imaging system.

    An example gel image for HeLa lysate inactivation is shown in Figure 3.

BASIC PROTOCOL 2 KINASE-CATALYZED BIOTINYLATION WITH INACTIVATED LYSATES FOR DISCOVERY OF SUBSTRATES (K-BILDS)

With FSBA inactivation and the ATP-biotin compatibility of the chosen kinase established, as discussed in Basic Protocol 1, the K-BILDS method can be performed. The K-BILDS experiment is initiated by incubating FSBA-treated lysates with the active recombinant kinase of interest and ATP-biotin. A negative-control reaction without

kinase is needed to account for background biotinylation (Fig. 2B). Avidin enrichment then isolates biotinylated proteins. As one application of K-BILDS is substrate discovery, the enriched biotinylated proteins can be visualized with a streptavidin-based stain, such as streptavidin-Cy5, after separation by SDS-PAGE. When general biotinylation is observed, the expectation is the presence of biotinylated proteins in both samples with or without kinase addition but slightly elevated biotinylation in the kinase-treated samples. However, we note that biotinylated protein levels might only change subtly with and without the added kinase in this lysate-based experiment due to background biotinylation (by low levels of endogenous kinase activity), especially in a case where the chosen kinase has a small population of substrates. The example K-BILDS experiment with PKA presented here monitors biotinylated protein levels, similar to prior work (Embogama & Pflum, 2017). With the presence of biotinylated proteins in K-BILDS established, mass spectrometry (MS) analysis can be used subsequently to discover substrates of the chosen kinase, as previously described (Embogama & Pflum, 2017; Shevchenko et al., 2006).

As an alternative to monitoring of biotinylated proteins, a second application of K-BILDS is substrate validation. In this application, the enriched biotinylated proteins are separated by SDS-PAGE after K-BILDS, and a substrate of interest can be visualized by western blot analysis with a specific antibody. With western blot of a known or suspected substrate, the expectation is enriched levels of the substrate in sample with exogenous kinase compared to the control sample without exogenous kinase. K-BILDS can be used either to discover new substrates or to validate a putative kinase-substrate relationship, depending on the needs of the project.

Materials

FSBA-treated cell lysates (see Basic Protocol 1)
Recombinant kinase [e.g., cAMP-dependent Protein Kinase, catalytic subunit (PKA); New England Biolabs, cat. no. P6000]
Distilled water
ATP-biotin stock (see recipe)
Phosphate-binding buffer (PBB; see recipe)
Streptavidin resin (GenScript, cat. no. L00353)
2% (w/v) sodium dodecyl sulfate (SDS; Fisher Scientific, cat. no. BP166-500)
4× SDS-PAGE loading buffer (see recipe; make fresh)

0.6- and 1.5-ml microcentrifuge tubes (Fisherbrand™, cat. no. 05-408-120 and 05-408-129)
Vortex
Microcentrifuge (Fisherbrand™ accuSpin™ Micro 17, cat. no. 13-100-675), room temperature and 4°C
31°C thermomixer (Eppendorf™ Thermomixer™ R, Fisher Scientific, 05-400-205)
Amicon Ultra-0.5 ml 3-kDa centrifugal filter units (Amicon, cat. no. UFC5003)
Spin columns, with screw caps and plugs (Pierce™ Spin Columns - Screw Cap, Thermo Fisher Scientific, cat. no. 69705)
Rotator (Labquake®, model no. 415110)
95°C water bath
Floating test tube rack (Fisherbrand™, cat. no. 14-127-44)
Vacuum concentrator (Savant SpeedVac concentrator, Thermo Scientific™, cat. no. SPD131DDA)
95°C heating block filled with sand or water (VWR Standard Dry Block Heater)

Additional reagents and equipment for SDS-PAGE and immunoblotting (see Basic Protocol 1)

Table 4 K-BILDS Reactions[a]

Sample number	1	2
Sample name	ATP-biotin control	ATP-biotin + PKA reaction
FSBA-treated HeLa lysate[a]	36 µl	36 µl
PKA[b]	-	1 µl
ATP-biotin[c]	5 µl	5 µl
Water	19 µl	18 µl
Final volume	60 µl	60 µl

[a]In the example K-BILDS experiment, FSBA-treated HeLa lysates with a stock concentration of 28 mg/ml were used to add 1 mg total protein to each tube.
[b]A commercial PKA stock at a concentration of 2500 U/µl was used to add 2500 U to the reactions.
[c]A working ATP-biotin stock of 24 mM was used here for a final concentration of 2 mM in the reactions.

K-BILDS

1. Label two 0.6-ml microcentrifuge tubes with sample numbers (Table 4).
2. Thaw FSBA-treated lysates and recombinant kinase on ice (if frozen) and then add to the reaction tubes.

   For this example, 36 µl HeLa lysate after FSBA treatment (using steps 1 to 14 from Basic Protocol 1), or 1 mg total protein, and 2500 units (U) of PKA (Table 4) are added.

   The samples in the example experiment detailed here were used for both MS and gel analysis. As a result, 1 mg inactivated lysate was needed because high protein quantities are used for MS analysis. When first performing the K-BILDS experiment, it is highly recommended to use smaller quantities (200 µg, for example) of inactivated lysates per sample due to the high cost of FSBA. The sample volume can be scaled down to 15 to 30 µl for gel analysis to conserve reagents.

   The recombinant kinase amount used varies with enzyme activity. In this example, 2500 U PKA is sufficient to phosphorylate substrates with ATP-biotin. Alternative numbers of units can be tested for a chosen kinase by observing biotinylation of the kinase through autophosphorylation or biotinylation of a protein or peptide substrate using the previously published protocol (see Current Protocols article: see Current Protocols article: Senevirathne et al., 2012).
3. Add distilled water to samples to achieve an equal final volume.

   For this K-BILDS example, add 18 or 19 µl water (Table 4).
4. Thaw ATP-biotin on ice and then initiate the reaction by adding to the reaction tubes. Briefly vortex the samples to mix and spin down 10 s at 1500 × *g* in a microcentrifuge to combine the reagents.

   In the example K-BILDS experiment, add 5 µl of a 24 mM ATP-biotin stock solution to obtain a final concentration of 2 mM.

   The final concentration of ATP-biotin can be varied to achieve robust biotinylation. ATP-biotin final concentrations are typically 1 to 10 mM.
5. Incubate samples in a thermomixer at 31°C for 2 hr without shaking.
6. Label an Amicon Ultra-0.5 ml 3-kDa centrifugal filter unit for each sample. Additionally, label two microcentrifuge tubes supplied with the filter units for each sample. Add each filter unit to one microcentrifuge tube.
7. About 15 min prior to completion of kinase-catalyzed biotinylation (step 5), wash each filter unit by adding 400 µl PBB and closing the cap. Centrifuge 10 min at 14,200 × *g* at room temperature. Invert the filter unit into the same microcentrifuge tube and spin for 2 min at 1000 × *g* to fully remove the wash buffer. Discard eluate in the tube and place emptied filter unit upward in the emptied microcentrifuge tube.

Do not let the filters dry out. Prepare for immediate use. Refer to the user guide for detailed figures on proper usage.

8. Add kinase-catalyzed reaction samples from step 5 to their respective filter units and dilute to the maximum capacity (up to 500 µl) with PBB. Close the caps.

   For this example protocol, PBB was used to dilute to only 300 µl. Diluting to the maximum capacity of the filter would have also been acceptable.

9. Centrifuge 30 min at 14,200 × *g*, 4°C.

10. Discard filtrate collected at the bottom of the microcentrifuge tube but keep the supernatant in the filter unit. Place filter back in the same microcentrifuge tube and repeat the dilution in step 8 and the centrifugation in step 9 for a second time.

    The filtration in steps 8 and 9 serves to remove excess ATP-biotin that could interfere with subsequent enrichment with streptavidin (steps 17 to 31). Steps 8 and 9 can be repeated an additional time if larger quantities of ATP-biotin are used.

11. Collect reaction samples by inverting filter unit into a new microcentrifuge tube from step 6 and spinning 2 min at 1000 × *g*, 4°C.

    At this point, if desired, label new 0.6-ml microcentrifuge tubes for each sample and add 50 µg of each reaction to the tubes to serve as the input control for gel analysis. Store at −20°C until gel analysis is performed (steps 31 to 34). The input is used to show equal protein loading among samples.

12. During step 10, prepare the streptavidin resin by first labeling a spin column, screw cap, and plug with their respective sample numbers (Table 4). Label two 1.5-ml microcentrifuge tubes per sample. Set aside the caps and plugs. Add the spin columns to their respective microcentrifuge tubes.

13. Mix the stock solution of streptavidin resin by gentle manual inversion until the mixture is homogenous and immediately add 200 µl bead slurry to each spin column.

    Cut 1 cm off the pipet tip used to dispense the resin to avoid damaging the beads.

    The resin mixture settles quickly. Pipet the resin into the spin column immediately after mixing. Additionally, mix and then pipet the resin in the same manner for each sample and use a new pipet tip for each sample to ensure an equal amount of beads is dispensed each time.

    Bead type and slurry quantity can be optimized for the desired application. A quantity range of 200 to 400 µl has been successfully used with K-BILDS.

14. Centrifuge spin columns containing the bead slurry for 1 min at 500 × *g* at room temperature to remove the bead storage buffer.

15. Carefully remove spin columns and discard bottom filtrate in the microcentrifuge tubes.

16. Add 300 µl PBB to the resin in the spin columns, centrifuge 1 min at 500 × *g*, and discard filtrate. Repeat this wash step with PBB twice more. Then, insert plugs in the bottoms of the spin columns.

    Do not allow the beads to dry; keep resin in buffer until use. Keep resin on ice until all filtration steps are complete. As previously noted, 400 µl PBB or diluting to the maximum filter capacity could also be used to dilute the samples.

17. Add reaction samples from step 11 to the streptavidin beads in the spin columns and dilute to 300 µl using PBB.

    As previously mentioned, 400 µl PBB or the maximum filter capacity can be used to dilute the samples prior to avidin binding.

18. Screw the caps on top of the spin columns. Draw a line to mark the liquid level on the spin columns and invert to check for leaks.

    Leaks can be prevented at this step by tightly screwing on the cap and making sure the plug is secure. A sample that leaks will need to be discarded.

19. Place spin columns containing the resin and reaction in a rotator and mix for 1 hr at room temperature.

    The incubation time (10 min to 1 hr) might need to be optimized depending on sample type (e.g., lysates, peptide mixtures), sample amount, or resin used. Peptide mixtures or less concentrated lysate samples may not need a long incubation time.

20. Carefully remove cap and plug from the spin columns and immediately place into new labeled 1.5-ml microcentrifuge tubes from step 12. Set aside caps and plugs for step 24.

21. Centrifuge spin columns for 1 min at 500 × *g* to collect flow-through. If desired, store the flow-through on ice until sample concentration in step 30.

    Analysis of the flow-through by gel analysis (steps 31 to 34) is optional but was included in the example experiment.

22. To wash the beads, add 400 µl PBB to the bound streptavidin resin in the spin columns, centrifuge 1 min at 500 × *g*, and discard filtrate. Repeat this PBB wash step nine more times.

23. To wash the beads and remove buffer components, add 400 µl distilled water to the bound streptavidin resin in the spin columns, centrifuge 1 min at 500 × *g*, and discard the filtrate. Repeat this water wash step four more times.

24. Insert plugs into the bottoms of the spin columns. Add 200 µl of 2% SDS to each sample to elute the biotinylated proteins from the streptavidin resin. Loosely but securely screw caps on the spin columns.

    The caps are screwed on loosely so that pressure cannot build up in the spin columns during the heating process. Pressure build-up could cause the plug to come loose and leak or allow water to contaminate the sample. To ensure a stable sample volume without leaks or contamination while the spin columns are in the water bath in step 25, draw a line at the liquid level to monitor the sample volume.

25. Heat the spin columns in a 95°C water bath in a floating test tube rack for 7 min.

    To prepare a hot water bath for elution and denaturation, fill a 500-ml beaker with 200 ml water and heat to 95°C using a hot plate (Nuova, Thermolyne, model no. 2).

    Monitor samples to ensure that they do not get submerged in the bath, which can lead to sample dilution and possible contamination.

26. While the samples are heating, label a new 1.5-ml microcentrifuge tube for each sample, which will be used to collect the eluate.

27. Remove samples from the 95°C water bath and dry the outside of the spin columns. Check that the liquid level is still at the marked line.

    Samples with a change in liquid level should be discarded, as sample loss or contamination has occurred.

28. First, remove the top caps to relieve any pressure. Then, take the bottom plugs out and immediately place spin columns in the new labeled 1.5-ml microcentrifuge tubes from step 26.

29. Centrifuge 1 min at 500 × *g* to collect the eluate.

30. Remove water from the flow-through (from step 21, optional) and eluate (from step 29) samples in a vacuum concentrator to dryness.

Drying might take 1 to 2 hr, depending on the volume collected. Sample drying can also take place overnight. If samples are not analyzed immediately, store dried samples at −20°C.

K-BILDS can be analyzed using several analytical methods, including SDS-PAGE or LC-MS/MS. For example, as outlined below in steps 31 to 34, SDS-PAGE separation followed by staining with a streptavidin-fluorophore conjugate can determine levels of biotinylated substrate. Alternatively, SDS-PAGE followed by western blot analysis of a specific protein allows K-BILDS to be used as a substrate validation tool. Finally, further sample prep for MS analysis has been previously detailed (Shevchenko et al., 2006) and can be conducted to discover potential substrates of a chosen kinase, similar to prior work (Embogama & Pflum, 2017).

Analysis of K-BILDS

31. Retrieve dried flow-through and eluate samples (from step 30) and reconstitute with water by briefly vortexing.

With the HeLa lysate example, resuspend the samples in 30 µl water.

The volume of water used to resuspend dried samples can vary depending on the amount of protein contained in the samples. Enough volume of water should be used to resuspend the sample fully, which typically ranges from 30 to 45 µl.

If analyzing lysate input samples (see annotation to step 11), thaw samples on ice. Dilute the input samples to 30 µl with water or to the same volumes used with the flow-through and eluate samples. All samples should have the same volume.

32. Add fresh 4× SDS-PAGE loading buffer (with fresh BME) to the samples to obtain a 1× concentration.

For the PKA K-BILDS example, add 10 µl of 4× SDS-PAGE loading buffer with BME for a 40-µl total volume. Briefly vortex to mix the samples and spin down 10 s at 1500 × g.

33. Incubate samples for 2 min at 95°C in a heating block filled with sand or water to denature proteins. Briefly vortex the samples to mix and centrifuge 10 s at 1500 × *g* to combine the reagents.

34. Load samples into an SDS-PAGE gel and perform SDS-PAGE and membrane blot as described in steps 22 to 27 of Basic Protocol 1.

REAGENTS AND SOLUTIONS

ATP-biotin stock

ATP-biotin is synthesized as previously described (Senevirathne & Pflum, 2013) and stored as a dry solid for ≤1 year at −80°C. Prepare ATP-biotin stock solution by dissolving the solid in water and store as single-use aliquots for ≤6 months at −80°C. Determine the concentration after dissolving by diluting an aliquot from 100- to 5000-fold, measuring absorbance with a UV-Vis spectrophotometer, and calculating the concentration using Beer's law (A = εbC), where "A" is the highest absorbance observed around 260 nm; "ε" is the extinction coefficient of ATP, which is 15.4 × 10^3 M^{-1} cm^{-1}; "b" is the path length in centimeters; and "C" is the ATP-biotin concentration (which typically ranges from 100 to 300 mM). Determine the purity of the ATP-biotin stock by TLC analysis using silica gel plates (Millipore Sigma, cat. no. 1057150001) and a solvent system comprising isopropanol, ammonia, and water (3:1.5:0.5). ATP-biotin (0.5-07 R_f, (Senevirathne & Pflum, 2013) should be ≥80% pure, with minimal degradation products present.

Destaining solution

10% (v/v) methanol (Fisher Scientific, cat. no. A412-4)
7% (v/v) acetic acid (Fisher Scientific, cat. no. A38C-212)
Store ≤6 months at room temperature

CAUTION: *Methanol can be toxic with skin contact and if inhaled, and acetic acid can cause severe skin and eye damage.*

FSBA stock, 200 mM

Dissolve 5′-(4-fluorosulfonlbenzoyl)adenosine (FSBA, Millipore Sigma, cat. no. F9128) in DMSO to 200 mM. Use immediately or store immediately as single-use aliquots for ≤1 month at −20°C.

CAUTION: *FSBA can cause severe skin and eye damage.*

Gel-fixing solution

50% (v/v) methanol (Fisher Scientific, cat. no. A412-4)
7% (v/v) acetic acid (Fisher Scientific, cat. no. A38C-212)
Store ≤6 months at room temperature

CAUTION: *Methanol can be toxic with skin contact and if inhaled, and acetic acid can cause severe skin and eye damage.*

HeLa S3 cell lysates

Lysates from HeLa S3 cells (ATCC, cat. no. CCL-2) are generated at a protein concentration of 10 to 30 mg/ml in lysis buffer (see recipe) containing freshly added 1% (v/v) Xpert protease inhibitor cocktail (GenDEPOT). Store ≤1 year at −80°C in single-use aliquots to minimize freeze-thaw cycles.

K-BILDS can be adapted for use with different cell lines and tissues.

NOTE: *Relevant ethics guidelines and regulations should be followed when performing experiments with animal or human samples. Cell line authentication should be conducted regularly.*

Lysis buffer

50 mM Tris base (Fisher Scientific, cat. no. BP152-5)
150 mM sodium chloride (NaCl; Millipore Sigma, cat. no. SX0420)
10% (w/v) glycerol (Millipore Sigma, cat. no. G7757)
0.5% (w/v) Triton X-100
Adjust pH to 8.0 with HCl (Millipore Sigma, cat. no. 258148) and/or NaOH (Millipore Sigma, cat. no. SX0590)
Store ≤4-6 months at 4°C

CAUTION: *HCl and NaOH can cause severe skin and eye damage.*

Membrane-blocking buffer

Prepare a 5% (w/v) bovine serum albumin (BSA; GenDEPOT, cat. no. A0100) solution in 1× TBST (see recipe) or purchase a blocking solution such as SuperBlock™ (Thermo Fisher, cat no. 37515). Store ≤1 year at 4°C.

Nonfat milk is not recommended to block membranes, as milk contains biotin, which can lead to high background.

Phosphate-binding buffer (PBB)

28 mM sodium phosphate monobasic monohydrate ($H_2NaO_4P{\cdot}H_2O$, Millipore Sigma, cat. no. SX0710-1)

72 mM sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$, Millipore Sigma, cat. no. SX0715-1)
150 mM NaCl (Millipore Sigma, cat. no. SX0420)
Adjust pH to 7.2 with HCl (Millipore Sigma, cat. no. 258148) and/or NaOH (Millipore Sigma, cat. no. SX0590)
Store ≤1 year at room temperature

CAUTION: *HCl and NaOH can cause severe skin and eye damage.*

SDS-PAGE loading buffer, 4×

Mix 4× Laemmli sample buffer (Bio-Rad, cat. no. 1610747) with fresh 10% (w/v) β-mercaptoethanol (BME; Millipore Sigma, cat. no. M6250). Prepare fresh immediately before use.

CAUTION: *BME should be used in a fume hood, as it can be toxic if inhaled and fatal with skin contact.*

Streptavidin-Cy5 staining solution

Prepare 10 ml streptavidin-Cy5 stain (Thermo Fisher ZyMax Grade, cat. no. 438316) diluted 1:2000 in 1× TBST (see recipe) containing 2% (w/v) BSA (GenDEPOT, cat. no. A0100). Prepare fresh immediately before use.

TBST, 1×

Initially, prepare 10× TBS with 200 mM Tris base (Fisher Scientific, cat. no. BP152-5) and 1500 mM NaCl (Millipore Sigma, cat. no. SX0420). Adjust pH to 7.6 with HCl (Millipore Sigma, cat. no. 258148) and/or NaOH (Millipore Sigma, cat. no. SX0590). Store ≤1 year at room temperature. To make TBST, dilute 10× TBS with water to generate a 1× TBS solution and then add Tween (Thermo Fisher Scientific, cat. no. BP337) to 0.1% (w/v). Store ≤1 month at 4°C.

CAUTION: *HCl and NaOH can cause severe skin and eye damage.*

Tris buffer, 10×

Prepare 500 mM Tris base (Fisher Scientific, cat. no. BP152-5)
Adjust pH to 7.5 using HCl (Millipore Sigma, cat. no. 258148) and/or NaOH (Millipore Sigma, cat. no. SX0590)
Store ≤1 year at 4°C

CAUTION: *HCl and NaOH can cause severe skin and eye damage.*

Tris/DTT buffer

Dilute 1 ml of 10× Tris buffer (see recipe) and 100 µl fresh 200 mM dithiothreitol (DTT; Oakwood Chemical, cat. no. M02712) with water to a volume of 10 ml. Prepare fresh immediately before use.

CAUTION: *DTT can cause serious eye damage.*

COMMENTARY

Background Information

A variety of methods have been developed to identify kinase-substrate pairs, and these have been reviewed elsewhere (Shah & Kim, 2019). We focus here on methods involving ATP analogs, given the use of γ-phosphate-modified ATP in this article. Radiolabeled [γ-^{32}P]-ATP has been commonly used to study phosphoproteins, although it cannot alone monitor kinase-substrate pairs. Instead, [γ-^{32}P]-ATP and non-radioactive ATP-γS analogs are used in the analog-sensitive kinase alleles (ASKAs) method to identify kinase substrates (Allen et al., 2007; Lopez et al., 2014). With ASKAs, an engineered mutant kinase that accepts a base-modified [γ-^{32}P]-ATP or ATP-γS analog labels substrates in cell lysates for subsequent

immunoprecipitation and identification by MS analysis (Allen et al., 2007; Shah et al., 1997). Because ASKAs are only available for roughly 40 of the over 500 kinases (Lopez et al., 2014), alternative methods are needed. An alternative of particular relevance to this article is the heavy ATP kinase assay combined with quantitative MS (HAKA-MS) method, which relies on the non-radioactive ATP-γ^{16}O analog. In HAKA-MS, lysates are exposed to FSBA to inactivate endogenous kinase activity, like with K-BILDS. Subsequently, the FSBA-treated lysates are incubated with an active kinase of interest and ATP-γ^{16}O, followed by digestion with trypsin, immunoprecipitation of phosphotyrosine (pTyr)-containing peptides, and monitoring of the ^{16}O-phosphoryl-labeled proteins by quantitative MS (Müller et al., 2016; Shah & Kim, 2019). Unfortunately, only antibodies to pTyr are viable for immunoprecipitation, excluding use of HAKA-MS for proteins phosphorylated at Ser and Thr. We also note that the pHis modification is unstable under the heat and acid conditions required for traditional immunoprecipitation and MS, which limits the use of ASKAs and HAKA-MS (Fuhs et al., 2015; Makwana et al., 2022).

As an alternative to these methods, K-BILDS has several key advantages that allow wide application to any biological system. First, K-BILDS can involve any lysate, tissue homogenate, or complex mixture that contains biologically relevant kinases and substrates. Because ATP-biotin is accepted by wild-type kinases, cell treatment or expression of mutant or tagged proteins is unnecessary. Second, kinase-substrate identification by K-BILDS is compatible with full-length substrates in cell lysates. Because some phosphorylation events require a full-length protein substrate and associated cellular proteins, the presence of all cellular components necessary for kinase specificity promotes study of biologically relevant substrates (Wieland et al., 2010). Third, the phosphoryl biotin tag is resistant to phosphatase removal (Senevirathne & Pflum, 2013), allowing substrates, including low-abundance substrates, to be identified regardless of abundance. Finally, biotinylated proteins are enriched without use of acid or heat (Embogama & Pflum, 2017), which preserves labile modifications, such as pHis, during substrate capture. With these features, K-BILDS is a versatile method applicable to a variety of cell lysates and tissue homogenates in different biological contexts.

One limitation of K-BILDS is incomplete inactivation of endogenous kinases by FSBA (Likos et al., 1980). Endogenous kinase activity in HeLa cell lysates was reduced by roughly 70% upon FSBA treatment in prior work (Embogama & Pflum, 2017). Fortunately, addition of exogenous active kinase encourages substrate phosphorylation primarily by the target kinase. Moreover, background biotinylation from residual active kinases or endogenously biotinylated proteins is accounted for by including a negative control without addition of an active kinase (Fig. 2B). Another limitation of K-BILDS is that a source of exogenous active kinase is needed. Many recombinant kinases are commercially available. However, if a recombinant kinase is unavailable or has low activity, other strategies to obtain active kinases can be explored, such as overexpression and immunoprecipitation of the target kinase from cells (Jia & Jeon, 2016). Finally, we note that, as for all discovery methods, substrates identified by K-BILDS require follow-up validation using cell-based or *in vitro* assays to establish the kinase-substrate relationships.

Critical Parameters

Cell lysate or tissue homogenate selection

The lysate or tissue homogenate used in K-BILDS should be consistent with the biological context of the target kinase. For example, active phosphorylation by the endogenous kinase of interest in the selected lysate would show biological relevance, even if not required for K-BILDS. In addition, the presence of known substrate(s) in the chosen cell lysates can serve as a positive control for K-BILDS. Beyond selection of an appropriate lysate or tissue homogenate, lysis conditions can be varied to alter the composition of the proteins in the lysates, such as use of high salt or detergent concentrations. In particular, if membrane-bound proteins are of interest, then use of a higher Triton X-100 concentration [1% (w/v)] or a stronger detergent [0.5% to 4% (w/v) deoxycholate] in the lysis buffer is advised.

Kinase selection

A key advantage of K-BILDS is wide application to any active kinase of interest. In fact, a variety of kinases have been established as compatible with ATP-biotin (Green & Pflum, 2007; Senevirathne et al., 2016; see Current Protocols article: Senevirathne et al., 2012). Optimization might still be required to

identify ideal reaction conditions, including kinase concentration, buffer components, and incubation temperature. If not already known from prior work (Green & Pflum, 2007; Senevirathne et al., 2016; see Current Protocols article: Senevirathne et al., 2012), the compatibility of ATP-biotin with the kinase of interest should be tested prior to implementing K-BILDS. A previously published protocol paper details the procedures for determining kinase compatibility with ATP-biotin (see Current Protocols article: Senevirathne et al., 2012).

Avidin resin selection

Several different avidin beads can be used as alternatives to streptavidin resin. Streptavidin beads were previously used for K-BILDS (Embogama & Pflum, 2017). The high affinity of biotinylated proteins for streptavidin beads aids in the capture of low-abundance phosphorylated substrates. Alternatively, neutravidin is a deglycosylated derivative of avidin that maintains affinity for biotin with a reduction of nonspecific binding (Gaj et al., 2007). Neutravidin resin (Thermo Fisher Scientific, cat. no. 29201) can be useful if high background signal complicates analysis by gel methods.

Handling and storage conditions

ATP-biotin purity is critical, as degradation can generate ATP, ADP, and a biotin amine intermediate (Senevirathne et al., 2016). ATP and ADP could potentially lower biotinylation efficiency by competing with ATP-biotin. More critically, the biotin amine intermediate can impact results by causing nonspecific biotinylation independent of kinase activity (Senevirathne et al., 2016). FSBA purity is also crucial, and careful handling is required to maintain effective irreversible inactivation of kinases, including minimizing freeze-thaw cycles by storing in single-use aliquots. Additionally, FSBA is poorly soluble in water and is dissolved and stored in DMSO prior to addition to the K-BILDS reactions. However, the DMSO concentration in the K-BILDS reaction samples should be minimized to prevent protein precipitation and denaturation (Arakawa et al., 2007).

Troubleshooting

Common problems related to the protocols, along with possible causes and solutions, are provided in Table 5 to assist in troubleshooting.

Understanding Results

Basic Protocol 1: FSBA treatment of lysates to inactivate kinases

The expected result of FSBA-mediated kinase inactivation of a lysate, SDS-PAGE separation of proteins, and staining with streptavidin-Cy5 is a lower level of biotinylation in FSBA-treated samples compared to the untreated (DMSO) positive control. In addition, minimal protein loss in FSBA-treated samples compared to the untreated (DMSO) positive control should be observed in gels visualized with SYPRO™ Ruby total protein stain. Multiple combinations of FSBA and DMSO concentrations can be tested to ensure reduced biotinylation in FSBA compared to DMSO samples, with minimal protein loss.

An example experiment with FSBA treatment of HeLa lysates is shown here. Following the FSBA treatment of lysates and gel analysis, reduced biotinylation levels were observed in the presence of FSBA (Fig. 3, lanes 3 and 6) compared to the DMSO positive control (Fig. 3, lanes 2 and 5). Minimal biotinylation was observed in the absence of ATP-biotin (Fig. 3, lanes 1 and 4), which indicates few endogenously biotinylated proteins in HeLa lysates. All samples displayed similar total protein levels (Fig. 3, lanes 1 to 6), which assured minimal protein loss with DMSO and FSBA treatment. In this example, two FSBA/DMSO concentrations were tested, and both reduced kinase-mediated biotinylation (Fig. 3, lanes 3 and 6). Additionally, protein loss was not observed with either 10% or 20% DMSO (Fig. 3, lanes 2 and 5). Other combinations of FSBA/DMSO could also be successful; prior work used 10 mM FSBA in 20% DMSO (Embogama & Pflum, 2017), which gave about a 70% reduction in biotinylation. Although maximum reduction in biotinylation after FSBA treatment is desired, as little as a 50% decrease has been successful in K-BILDS. This protocol can be used to determine optimal FSBA/DMSO concentrations for kinase inactivation in any complex lysate mixture of interest.

Basic Protocol 2: K-BILDS

Once appropriate FSBA/DMSO concentrations have been established for kinase inactivation of lysates (Basic Protocol 1), the next step is to perform K-BILDS with gel analysis. With K-BILDS, FSBA-treated lysates are incubated with ATP-biotin in the absence and presence of exogenous kinase, and then biotinylated proteins are enriched with avidin

Table 5 Troubleshooting Guide for K-BILDS

Basic Protocol	Problem	Possible cause	Solution
1, 2	No or low biotin signal is observed by gel analysis of samples containing untreated lysate or FSBA-treated lysate with PKA and ATP-biotin	Low protein abundance	Increase lysate or recombinant protein kinase concentrations
		Expired lysis buffer	Make fresh buffer
		Centrifugal filter unit was insufficiently washed; glycerin storage buffer is reducing ATP-biotin labeling efficiency	Repeat wash of the filter unit with buffer or use 0.1 N NaOH prior to washing with buffer
		Low ATP-biotin concentration	Increase final ATP-biotin concentration in the reaction
		Degraded ATP-biotin	Dilute a new stock of ATP-biotin and avoid freeze-thaw cycles by storing as single-use aliquots
		Quenched Cy5 fluorophore due to light exposure prior to visualization	Protect streptavidin-Cy5 from light or purchase a new batch
1	Biotinylation from kinase activity is similar with and without FSBA treatment; high background biotinylation after inactivation	Degraded FSBA	Dissolve a fresh stock of FSBA
		FSBA concentration is not optimized	Increase or decrease the FSBA concentration to achieve optimal kinase inactivation
		DMSO concentration is not optimized	Increase or decrease the DMSO concentration to achieve optimal kinase inactivation
		Lysate protein concentration is too high	Decrease amount of lysate
		ATP-biotin concentration is too high	Decrease ATP-biotin concentration
		Degraded ATP-biotin	Resuspend a fresh stock of ATP-biotin and avoid freeze-thaw cycles by storing as single-use aliquots
		Membrane blocking is needed or was unsuccessful in western blot	Add a blocking step or use alternative blocking agents, but avoid nonfat milk, which contains biotin
		Long imager exposure time after western blot	Shorten imager exposure time
1	Significant protein loss with FSBA and/or DMSO treatment	FSBA concentration is not optimized	Increase or decrease the FSBA concentration to achieve optimal kinase inactivation
		DMSO concentration is not optimized	Increase or decrease the DMSO concentration to achieve optimal kinase inactivation

(*Continued*)

Table 5 Troubleshooting Guide for K-BILDS, *continued*

Basic Protocol	Problem	Possible cause	Solution
2	When using western blot analysis after K-BILDS, a known substrate is not enriched and observed with K-BILDS	Neutravidin is not binding to lower-abundance biotinylated substrates	Try streptavidin resin or an alternative
		Enrichment incubation time is too short for biotinylated protein to bind to avidin	Use a longer incubation time
		Insufficient avidin resin was used	Use more resin

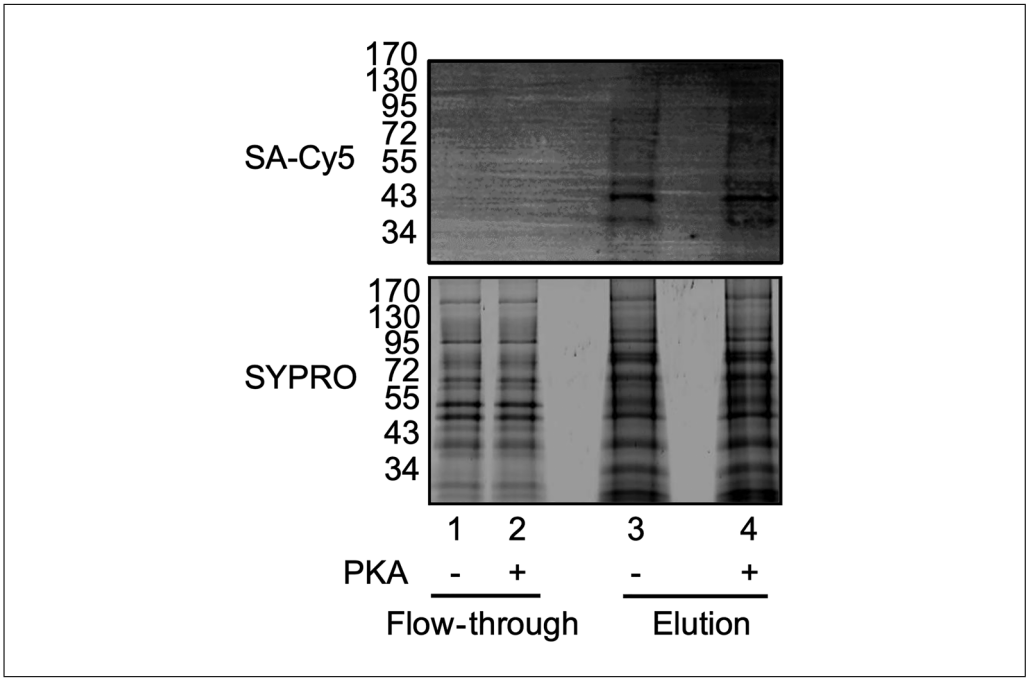

Figure 4 K-BILDS with PKA using FSBA-treated HeLa cell lysates. HeLa cell lysates (1 mg) were inactivated using FSBA, excess FSBA was removed, and treated lysates were incubated with ATP-biotin (2 mM) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of recombinant PKA (2500 U). Biotinylated proteins were enriched using streptavidin resin, and the unbound proteins in the flow-through (lanes 1 and 2) and bound proteins after elution (lanes 3 and 4) were separated by SDS-PAGE and visualized using SYPRO™ Ruby total protein stain or electro-transferred onto a membrane and visualized using streptavidin-Cy5 (SA-Cy5) to observe biotinylated proteins (Embogama & Pflum, 2017). Molecular-weight markers (kDa) are indicated to the left of the gel.

resin and separated by SDS-PAGE. The expected result is that more biotinylated proteins are observed in the eluted sample containing exogenous kinase compared to eluted sample without kinase. However, we note that biotinylation levels can appear similar with and without active kinase because the number of substrates of the chosen kinase might be a small fraction of the total proteins in the lysates. Flow-through samples should contain low levels of biotinylated proteins, which serves as a control to ensure effective streptavidin enrichment. As an alternative to monitoring biotinylation, western blotting for a substrate of interest in the enriched samples can be performed while using the input samples as a loading control. MS analysis can also be conducted to discover unanticipated substrates phosphorylated by a particular kinase.

As an example, K-BILDS with PKA was performed using FSBA-treated HeLa lysates as described. As expected, increased biotinylation was observed in the eluted sample with PKA (Fig. 4, lane 4) compared to the eluted sample without PKA (Fig. 4, lane 3). No biotinylated proteins were present in flow-through samples (Fig. 4, lanes 1 and 2), which indicated successful streptavidin enrichment.

In prior work with the K-BILDS workflow, similarly elevated biotinylation in the presence of PKA was observed by gel analysis prior to performing MS analysis to identify the substrates (Embogama & Pflum, 2017).

Time Considerations

Basic Protocol 1, steps 1 to 14: FSBA treatment of lysates to inactivate kinases: 3 hr.

Basic Protocol 1, steps 15 to 28: Assessment of FSBA inactivation of kinase activity: 6 hr.

Basic Protocol 2, steps 1 to 30: K-BILDS: 6 hr.

Basic Protocol 2, steps 31 to 34: Analysis of K-BILDS: 6 hr.

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Author Contributions

Chelsea R. Gary: Data curation, formal analysis, investigation, methodology, validation, writing—original draft, writing—review and editing; **Mary Kay H. Pflum:** Conceptualization, data curation, funding acquisition, project administration, supervision, writing—original draft, writing—review and editing.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data are available upon request from the authors.

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