

# Kinase-Catalyzed Crosslinking and Immunoprecipitation (K-CLIP) to Explore Kinase-Substrate Pairs

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Kinases are responsible for phosphorylation of proteins and are involved in many biological processes, including cell signaling. Identifying the kinases that phosphorylate specific phosphoproteins is critical to augment the current understanding of cellular events. Herein, we report a general protocol to study the kinases of a target substrate phosphoprotein using kinase-catalyzed crosslinking and immunoprecipitation (K-CLIP). K-CLIP uses a photocrosslinking  $\gamma$ -phosphoryl-modified ATP analog, such as ATP-arylazide, to covalently crosslink substrates to kinases with UV irradiation. Crosslinked kinase-substrate complexes can then be enriched by immunoprecipitating the target substrate phosphoprotein, with bound kinase(s) identified using Western blot or mass spectrometry analysis. K-CLIP is an adaptable chemical tool to investigate and discover kinase-substrate pairs, which will promote characterization of complex phosphorylation-mediated cell biology. © 2022 Wiley Periodicals LLC.

**Basic Protocol 1:** Kinase-catalyzed crosslinking of lysates

**Basic Protocol 2:** Kinase-catalyzed crosslinking and immunoprecipitation (K-CLIP)

Keywords: ATP-arylazide • kinase • kinase-substrate identification • photocrosslinking

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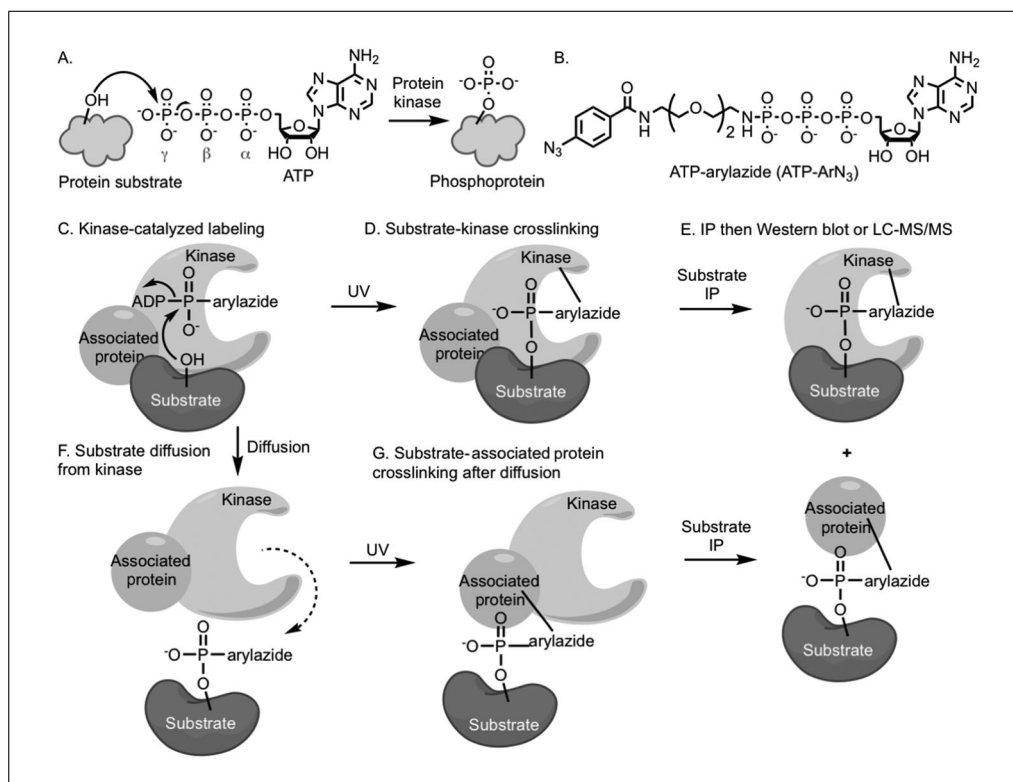
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## INTRODUCTION

Phosphorylation is a key protein post-translational modification that controls interactions, conformations, and/or functions of the modified protein (Wang & Cole, 2014). By governing protein activity, phosphorylation regulates myriad biological processes, including cell signaling (Krupa, Preethi, & Srinivasan, 2004). As a critical mediator of cellular events, protein phosphorylation is highly regulated, and diseases such as cancer and Alzheimer's disease develop when phosphorylation events become dysregulated (Lu, 2004). Protein kinases catalyze phosphorylation by transferring the  $\gamma$ -phosphoryl of adenosine 5'-triphosphate (ATP) onto a serine, threonine, or tyrosine of substrates to generate phosphoproteins (Fig. 1A). With a significant role in disease, kinases have been effective targets for pharmaceutical drug development, with many kinase inhibitors in clinical use (Gross, Rahal, Stransky, Lengauer, & Hoefflich, 2015). To thoroughly

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**Figure 1** Kinase-catalyzed crosslinking and immunoprecipitation (K-CLIP). **(A)** General scheme for phosphorylation of a protein substrate by a kinase using ATP as the cosubstrate. **(B)** Chemical structure of photocrosslinking ATP analog ATP-arylazide (ATP-ArN<sub>3</sub>). **(C)** Kinase-catalyzed labeling with ATP-ArN<sub>3</sub> covalently attaches the arylazide photocrosslinker to the substrate. **(D)** UV irradiation results in the formation of crosslinked kinase-substrate complexes through the arylazide group. **(E)** Immunoprecipitation (IP) of the substrate will isolate substrate-bound complexes, which then can be characterized by Western blotting after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) separation or liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. **(F)** Due to the transient nature of kinase-substrate interactions, a significant amount of substrate will diffuse away from the kinase before UV irradiation and crosslinking. **(G)** Upon UV irradiation after diffusion, the arylazide group will crosslink the substrate to nearby proteins, which will include associated proteins (Garre et al., 2018).

characterize the role kinases play in cellular events and disease states, identification of upstream kinases of a phosphoprotein and downstream substrates of a kinase is essential. Currently, the characterization of kinase-substrate pairs is often difficult due to the weak and transient interaction between kinases and their substrates (Shaffer & Adams, 1999). Development of chemical tools to identify the kinases that phosphorylate a specific phosphoprotein is vital for deciphering the intricate network of protein-protein interactions in cellular processes.

To create effective chemical tools to study cellular events,  $\gamma$ -phosphoryl-modified ATP analogs have been established to study kinases, substrates, and protein phosphorylation. A variety of  $\gamma$ -phosphoryl modifications—such as biotin (Green & Pflum, 2007), arylazide (Suwal & Pflum, 2010), benzophenone (Garre, Senevirathne, & Pflum, 2014), and methylacrylamide (Fouda, Gamage, & Pflum, 2021)—have been attached to ATP. These ATP analogs are accepted by kinases as cosubstrates whereby the modified  $\gamma$ -phosphoryl is transferred onto the hydroxyl of serine, threonine, or tyrosine amino acids of substrate proteins (Fig. 1C), which is known as kinase-catalyzed labeling (Senevirathne, Embogama, Anthony, Fouda, & Pflum, 2016). After labeling of the phosphoprotein, a variety of methods have been developed to study the interactions between a kinase and substrate to advance the current understanding of cellular events (Dedigama-Arachchige &

Pflum, 2016; Embogama & Pflum, 2017; Garre, Gamage, Faner, Dedigama-Arachchige, & Pflum, 2018; Ramanayake-Mudiyanselage, Embogama, & Pflum, 2021).

This protocol focuses on one specific kinase-catalyzed labeling reaction involving covalent crosslinking to study substrate-kinase interactions (Suwal & Pflum, 2010). Specifically, kinase-catalyzed crosslinking covalently joins kinases and substrates to overcome their normally weak and transient interaction, which facilitates subsequent enrichment and identification. Although multiple crosslinking ATP analogs have been developed for kinase-catalyzed crosslinking (Fouda et al., 2021; Garre et al., 2014; Suwal & Pflum, 2010), we focus here on the photocrosslinking ATP-arylazide analog (Fig. 1B), given its prior use toward kinase-substrate identification (Dedigama-Arachchige & Pflum, 2016; Garre et al., 2018). In kinase-catalyzed crosslinking with ATP-arylazide, the photoreactive arylazide group is transferred to the substrate after kinase-catalyzed labeling (Fig. 1C). Upon UV irradiation, a highly reactive nitrene species is generated from the arylazide (Wilson, Miyata, Erecińska, & Vanderkooi, 1975) that can covalently attach to the backbone or amino acid side chains of the kinase (Fig. 1D). The kinase-catalyzed crosslinking reaction results in a stable kinase-substrate complex for subsequent analysis.

To use kinase-catalyzed crosslinking for kinase-substrate monitoring or discovery, an enrichment step is necessary to isolate the substrate and kinase of interest after crosslinking. In this article, the kinase-catalyzed crosslinking and immunoprecipitation (K-CLIP) method (Garre et al., 2018) is highlighted where a primary antibody is used in an immunoprecipitation step to enrich the crosslinked kinase-substrate complex (Fig. 1E). Depending on whether the immunoprecipitation enrichment step uses a primary antibody that targets a substrate or kinase of interest, the K-CLIP method can identify the upstream kinases of a phosphoprotein substrate or the downstream substrates of a kinase. Given our prior published work applying K-CLIP to the identification of kinases of a target substrate phosphoprotein (Garre et al., 2018), this article focuses on substrate immunoprecipitation (Fig. 1E) and the analysis of upstream kinases of a phosphoprotein. We note that application of K-CLIP to the identification of substrates of a target kinase is currently ongoing in the Pflum laboratory. Finally, to analyze the enriched crosslinked complexes, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) separation and Western blot analysis can be used to probe for the presence of suspected kinase-substrate pairs, which provides a helpful lysate-based confirmation tool to complement *in vitro* kinase assays with recombinant proteins. Alternatively, unanticipated kinase-substrate pairs in the enriched crosslinked complexes can be identified using liquid chromatography–tandem mass spectrometry (LC-MS/MS), which makes K-CLIP a powerful discovery tool. Overall, K-CLIP is a versatile method capable of monitoring the active phosphorylation of any kinase or substrate of interest.

In prior work, K-CLIP was used to identify kinase(s) of the p53 protein (Garre et al., 2018). Because p53 is robustly phosphorylated by many known kinases (Kruse & Gu, 2009; MacLaine & Hupp, 2009), p53 was an ideal substrate model to establish the method. K-CLIP combined with LC-MS/MS analysis successfully identified two known (DNA-PK and PKR) kinases and one unknown (MRCK $\beta$ ) kinase of p53 (Garre et al., 2018). In addition, K-CLIP followed by SDS-PAGE separation and Western blot analysis confirmed known kinase-substrate pairs (Garre et al., 2018). The p53 model study established the K-CLIP method to both discover and validate the kinases of a target substrate in a complex cellular mixture.

Interestingly, the p53 K-CLIP study identified many proteins in addition to kinases, including known associated proteins of p53. Based on the data, we rationalized that associated proteins are crosslinked and identified by K-CLIP due to the transient interaction

between kinase and substrate that allows the substrate to diffuse away from the kinase before UV irradiation (Fig. 1F). In this case, diffusion of the modified substrate results in crosslinking to associated proteins that are near the substrate and kinase (Fig. 1G). In fact, other work using kinase-catalyzed crosslinking and a substrate peptide similarly observed both kinases and associated proteins after LC-MS/MS analysis (Dedigama-Arachchige & Pflum, 2016). In total, the data document that K-CLIP is useful to study the associated proteins of kinases and substrates during the phosphorylation event in addition to kinase-substrate pairs.

To facilitate future use of K-CLIP for both confirmation and discovery of kinase-substrate pairs and associated proteins, detailed protocols are provided here for kinase-catalyzed crosslinking reactions and the K-CLIP method using ATP-arylazide. Given the prior published p53 example, the protocols are focused on phosphoprotein substrate enrichment to identify kinases, with specific details for p53 as a model system. First, Basic Protocol 1 is provided to observe kinase-catalyzed crosslinking in complex lysate mixtures using SDS-PAGE separation and Western blot analysis, with p53 as the example. For application of K-CLIP to new phosphoprotein substrates, Basic Protocol 1 provides a necessary first step to ensure that ATP-arylazide and kinase-catalyzed crosslinking are compatible with the target substrate. Basic Protocol 2 describes the K-CLIP method using substrate immunoprecipitation enrichment, with p53 as the model system. Basic Protocol 2 is suitable for either confirmation of suspected kinase-substrate pairs using SDS-PAGE separation and Western blot analysis or discovery of unanticipated kinases and associated proteins using LC-MS/MS analysis. In total, the two protocols will assist in applying K-CLIP to any substrate phosphoprotein of interest to either confirm or discover kinases and associated proteins, which has the anticipated outcome of improving the current understanding of phosphorylation-mediated cell signaling and disease states.

## **BASIC PROTOCOL 1**

### **KINASE-CATALYZED CROSSLINKING OF LYSATES**

As a prerequisite of K-CLIP, the compatibility of kinase-catalyzed crosslinking and ATP-arylazide with the kinase-substrate pair of interest must be verified. Basic Protocol 1 outlines kinase-catalyzed crosslinking with Western blot analysis to observe high-molecular-weight crosslinked complexes, which confirm crosslinking of cellular proteins with the substrate of interest within the desired lysate. When applying Basic Protocol 1 to a target kinase-substrate pair, an important consideration is the selection of the lysate containing the substrate and kinases of interest. The versatility of the K-CLIP method allows for the use of almost any lysate or protein mixture, such as mammalian cell, bacterial or yeast lysates, or tissue homogenates. See the Critical Parameters for a discussion on lysate selection.

For Basic Protocol 1, several control reactions should be included in addition to the crosslinking reaction. Where the crosslinking reaction contains lysate and ATP-arylazide with UV irradiation (Table 1; crosslinking reaction), a critical negative control reaction should be performed containing lysate and ATP-arylazide without UV light (Table 1; negative control 1) to show the UV dependence of crosslinking. Other optional negative control samples include reactions incubated without ATP-arylazide (Table 1; optional negative control 2), with ATP in place of ATP-arylazide (Table 1; optional negative control 3), or with kinase inhibitor-treated lysates (Table 1; optional negative control 4), which establish the ATP analog and kinase activity dependence of crosslinking. The reactions are typically incubated for 2 hr at 31°C, but the reaction time may vary depending on the lysates and proteins involved.

In Basic Protocol 1, the samples that undergo kinase-catalyzed crosslinking are separated by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and

**Table 1** Suggested General Kinase-Catalyzed Crosslinking Reaction Conditions

|                          | Crosslinking reaction | Negative control 1 | Optional negative control 2 | Optional negative control 3 | Optional negative control 4 |
|--------------------------|-----------------------|--------------------|-----------------------------|-----------------------------|-----------------------------|
| Lysate                   | +                     | +                  | +                           | +                           | -                           |
| Inhibitor-treated lysate | -                     | -                  | -                           | -                           | +                           |
| ATP                      | -                     | -                  | -                           | +                           | -                           |
| ATP-arylazide            | +                     | +                  | -                           | -                           | +                           |
| UV                       | +                     | -                  | +                           | +                           | +                           |

visualized by Western blotting. For Western blotting, the primary antibody should be specific for the substrate of interest, and the secondary antibody can be conjugated to either a fluorophore or horseradish peroxidase (HRP) depending on the level of sensitivity desired. Crosslinking is validated by observing high-molecular-weight protein bands in the crosslinking reaction, which are reduced in the negative control reactions (Table 1). Depending on the efficiency of crosslinking, the higher-molecular-weight complexes might appear smeared without distinct banding. See Understanding Results for an explanation of the example gel image in the associated figure.

### Materials

10× kinase reaction buffer (see recipe)

Cell lysates: HEK293 or other user-provided lysate prepared in HEPES lysis buffer (see recipe)

ATP-arylazide (synthesized from Suwal & Pflum, 2010; see recipe)

Optional: ATP (e.g., MP Biomedicals Inc, cat. no. ICN15026605)

4× gel loading buffer: 4× Laemmli Protein sample buffer (e.g., Bio-Rad, cat. no. 1610747) containing β-mercaptoethanol (10%), as specified by the manufacturer

10% to 16% SDS-polyacrylamide gel (e.g., as described in He, 2011)

PVDF membrane (e.g., Millipore Sigma, cat. no. IPVH00010)

Blocking solution: 5% (w/v) bovine serum albumin (BSA) or nonfat milk in 1× phosphate-buffered saline with Tween (PBST; see recipe)

1× PBST (see recipe)

Primary antibody: anti-p53 (e.g., Santa Cruz, cat. no. sc-126)

Secondary antibody: HRP- or fluorophore-conjugated goat anti-mouse immunoglobulin G (IgG) H&L (e.g., Abcam, cat. no. ab97040)

Optional: enhanced chemiluminescence substrate for HRP visualization (e.g., SuperSignal West Dura Extended Duration Substrate; Thermo Fisher Scientific, cat. no. 34075)

1.5-ml microcentrifuge tubes (e.g., Fisher Scientific, cat. no. 05-408-129)

Benchtop microcentrifuge (e.g., Fisher Scientific accuSpin Micro 17)

Thermomixer (e.g., Benchmark Scientific MultiTherm Shaker)

UV lamp (e.g., UVP 3UV lamp 254/302/365 nm)

Electrophoresis apparatus (e.g., Bio-Rad Mini-Protean Tetra Cell)

Electrotransfer apparatus (e.g., Bio-Rad Mini-Transblot Module)

Rocking platform (e.g., VWR 200 Rocking Platform)

Gel imaging instrument (e.g., Protein Simple Alpha View-Fluorochem Q or GE FLA 9500 imager)

### Kinase-catalyzed crosslinking of cell lysates

1. Label microcentrifuge tubes with reaction numbers (Table 2).

**Table 2** Kinase-Catalyzed Crosslinking Reaction Conditions for p53<sup>a</sup>

|                        | Negative control 1 | Negative control 2 | Crosslinking reaction |
|------------------------|--------------------|--------------------|-----------------------|
| HEK293 lysate (125 µg) | 6 µl               | 6 µl               | 6 µl                  |
| Kinase buffer (1×)     | 2 µl               | 2 µl               | 2 µl                  |
| ATP (5 mM)             | 2 µl               | -                  | -                     |
| ATP-arylazide (5 mM)   | -                  | 2 µl               | 2 µl                  |
| Water                  | 10 µl              | 10 µl              | 10 µl                 |
| UV                     | +                  | -                  | +                     |

<sup>a</sup> In the example experiment for Basic Protocol 1, p53 was used as the substrate of interest, and the stock concentrations were 21 mg/ml HEK293 lysate, 50 mM ATP-arylazide, and 50 mM ATP. Final concentrations are indicated in parentheses next to each reagent.

2. Add enough 10× kinase buffer to obtain a final concentration of 1× in the labeled microcentrifuge tube(s), and dilute with enough deionized purified water such that all samples have the same final volume. In this example with p53, add 2 µl of 10× kinase buffer and 10 µl water to obtain a final volume of 20 µl.

*Typically we use a HEPES buffer. Buffers containing primary amines, such as Tris, are incompatible because of competition with crosslinking.*

*The final volume for the kinase reactions may vary depending on the initial concentration of proteins in the lysates (see step 3 for more information).*

*As shown in Table 1, inclusion of controls is highly recommended. In this example protocol with p53, the negative controls include omitting UV irradiation (Table 2; negative control 2) and replacing ATP-arylazide with ATP (Table 2; negative control 2), which are the minimum controls recommended for Basic Protocol 1.*

3. Thaw lysates on ice, and once thawed add to the tube(s). In the p53 example, add 6 µl of 21 mg/ml lysate (125 µg total protein) to the tube(s).

*Lyse cells in a buffer that does not contain free amines, such as HEPES, to avoid competition with crosslinking.*

*To avoid degradation of lysate proteins from freeze-thaw cycles, store as single-use aliquots. Pipetting must be performed on ice or in a cold room with prechilled microcentrifuge tube(s).*

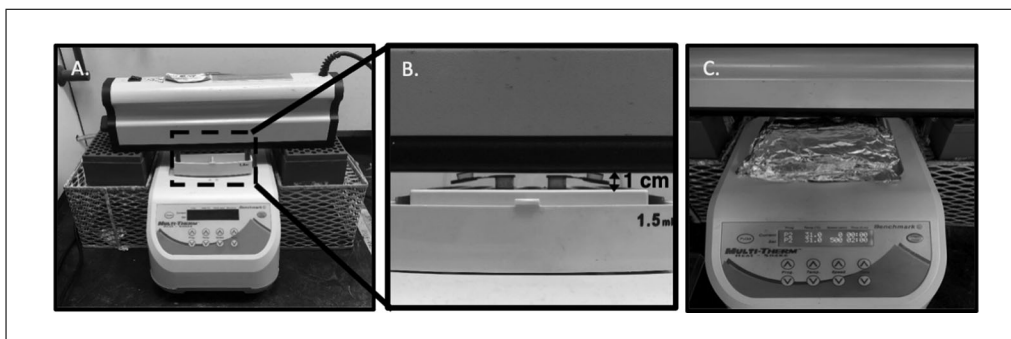
*For this protocol, 125 µg total protein in the lysate was used, but crosslinking may require more or less lysate depending on the abundance of the substrate of interest. Most kinase-catalyzed crosslinking reactions use 30 to 800 µg lysate. Testing multiple lysate amounts before crosslinking experiments is recommended to consider substrate abundance.*

*Lysate stock concentrations used previously have ranged from 5 to 45 mg/ml. The concentration of lysate will impact the total volume of the reaction. Depending on the lysate concentration, the total volume of lysates can range from 5 to 190 µl, which results in the total reaction volume ranging from 15 to 250 µl. Using a more-concentrated lysate is recommended, when possible, to have a more concentrated total reaction volume, which can promote more interactions between the kinases and substrates.*

4. Thaw ATP-arylazide (and optional ATP aliquots) on ice. Initiate kinase reaction(s) by adding ATP-arylazide (or ATP) to a final concentration of 5 mM. In the p53 example, add 2 µl of 50 mM ATP-arylazide (or ATP). To ensure all reagents are combined, spin down reactions in a microcentrifuge for 5 to 10 s.

*To avoid degradation of ATP and the ATP analogs from freeze-thaw cycles, store as single-use aliquots. Pipetting must be performed on ice or in a cold room with prechilled microcentrifuge tube(s).*

*The final concentration of ATP-arylazide in the kinase reactions might need to be optimized. The concentration of ATP-arylazide used previously varied from 2 to 10 mM.*



**Figure 2** Thermomixer setup with UV lamp for crosslinking. **(A)** The general setup of the UV irradiation area is shown, which includes the thermomixer, UV lamp, and support stands. **(B)** The samples to be UV irradiated are placed open in the thermomixer with a UV lamp placed 0.2 to 0.3 cm above the top of the microcentrifuge tubes, which is a total of 1 cm from the top of the thermomixer platform. The microcentrifuge tubes used are about 4 cm in length, which results in the UV light being a total of 3.8 to 4 cm away from the sample reaction. **(C)** Samples that do not require UV irradiation are covered with foil and placed in the thermomixer as far away as possible from the UV lamp.

5. Incubate reaction sample(s) immediately at 31°C with (Fig. 2A and 2B) or without (Fig. 2C) UV irradiation (365 nm) with shaking at 300 rpm for 2 hr using a thermomixer.

*To promote efficient crosslinking, the UV lamp should be as close to the sample tubes as possible to activate arylazide (see Fig. 2B). The distance of the UV lamp from the tube is critical to induce crosslinking, which might need to be optimized. The temperature of the reaction might also need optimizing.*

*UV light is harmful; avoid direct contact of the light with the eyes and skin.*

### **SDS-PAGE and Western blotting**

6. After the reaction incubation, add 4× gel loading buffer to a final concentration of 1× to the kinase reactions, and incubate sample(s) at 95°C for 5 min to heat denature the proteins. For this p53 example protocol, add 6.6 μl of 4× gel loading buffer to each sample.
7. Prepare SDS-polyacrylamide gels following established protocols (He, 2011), or use commercial gels. For the p53 example, which is 53 kDa, use a 10% gel.

*The percentage gel used depends on the size of the protein of interest. Use a higher percentage gel (i.e., 16%) to observe lower-molecular-weight proteins (molecular weight <30 kDa) and a lower percentage gel (i.e., 8%) for higher-molecular-weight proteins (molecular weight >150 kDa). The ideal gel percentage chosen should allow for visualization of the substrate of interest and higher-molecular-weight complexes.*

8. Load gel with the reaction samples, and separate proteins using 110 V for 15 min (or until the dye front is through the stacking layer). Then increase voltage to 180 V for about 45 min (or until the dye front runs off the gel).

*SDS-PAGE protocols have been published elsewhere (see Current Protocols article: Manns, 2011).*

*The run time for separation depends on the size of the substrate. For larger protein substrates, the gel may be run longer to ensure visualization of higher-molecular-weight crosslinked complexes. For smaller protein substrates, the gel may be run shorter than 1 hr to ensure the substrate of interest remains on the gel.*

9. Transfer separated proteins in the gel onto a PVDF membrane at 90 V for 2 hr using an electroblotting apparatus (see Current Protocols article: Goldman, Ursitti, Mozdzanowski, & Speicher, 2015).

10. After protein transfer, block membrane with blocking solution. In the p53 example, block membrane with 10 ml of 5% (w/v) nonfat milk in 1 × PBST for 1 hr at room temperature.

*The blocking step may need to be optimized to minimize background. Blocking may also be performed using BSA or a blocking solution such as SuperBlock (e.g., Thermo Fisher Scientific).*

11. Remove blocking buffer, and wash membrane three times with 30 ml of 1 × PBST for 3 min with rocking.
12. Incubate membrane with the manufacturer-recommended dilution of primary antibody in 5% (w/v) BSA or nonfat milk in 1 × PBST for 1 hr or overnight at 4°C. For the p53 example, add 10 ml of a 1:1000 dilution of p53 primary antibody in 5% (w/v) BSA in 1 × PBST to the membrane, and incubate overnight at 4°C.

*The primary antibody, dilutions, and incubation time should be used according to manufacturer's recommendation or optimized to obtain the best-quality images.*

13. After primary antibody incubation, remove solution and wash membrane three times with 30 ml of 1 × PBST for 3 min with rocking.
14. Incubate membrane with the manufacturer's recommended dilution of HRP- or fluorophore-conjugated secondary antibody in 5% (w/v) nonfat milk or BSA in 1 × PBST for 1 hr at room temperature. For p53, incubate membrane with 10 ml of a 1:10,000 dilution of an HRP-conjugated secondary antibody in 5% (w/v) nonfat milk in 1 × PBST for 1 hr at room temperature.

*The secondary antibody and dilutions used should be optimized to obtain the best-quality images.*

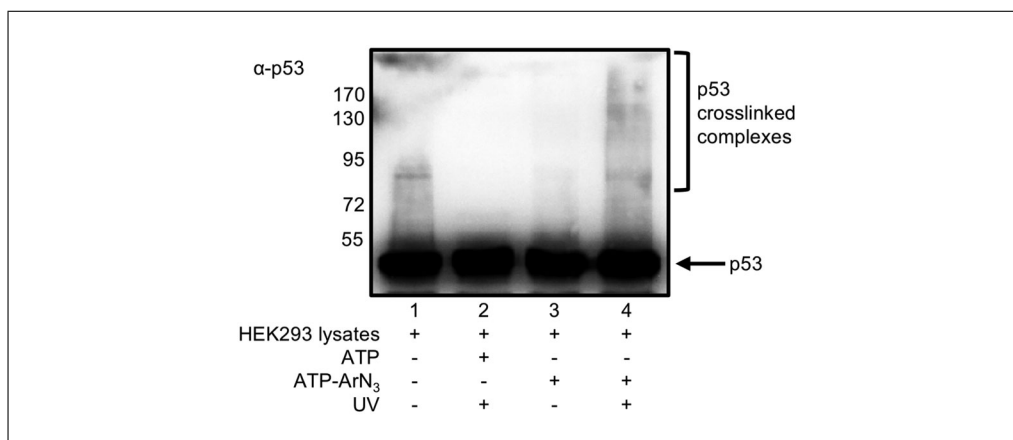
15. Remove secondary antibody, and wash membrane again three times with 30 ml of 1 × PBST for 3 min with rocking.
16. After washing, directly visualize fluorophore-labeled secondary antibody, or incubate membrane with 1 ml enhanced chemiluminescence reagent when using secondary HRP antibody, according to manufacturer's protocol (<https://assets.thermofisher.com/TFS-Assets/BID/Application-Notes/TR0067-Chemi-Western-guide.pdf>). For the p53 example, incubate with 1 ml enhanced chemiluminescence reagent (500 µl luminol/enhancer + 500 µl stable peroxide buffer). Visualize membrane using a chemiluminescent scanner.

*A gel image for the p53 kinase-catalyzed crosslinking example is shown in Figure 3.*

## **BASIC PROTOCOL 2**

### **KINASE-CATALYZED CROSSLINKING AND IMMUNOPRECIPITATION (K-CLIP)**

After observing crosslinked complexes with the desired substrate and lysate mixture (Basic Protocol 1), the next step is to isolate the crosslinked complexes to identify the kinase-substrate or substrate-associated protein pairs either by Western blot or LC-MS/MS analysis. Because kinase-catalyzed crosslinking will covalently conjugate all kinases and substrates in a lysate mixture, the immunoprecipitation enrichment step of K-CLIP (Fig. 1E) is required to identify only the proteins that are crosslinked to the substrate of interest. We provide in this protocol two methods to perform the immunoprecipitation enrichment step, which differ in how the substrate-antibody-agarose bead conjugate is generated. Method A (steps 7 to 18) involves incubating the primary antibody with agarose beads before adding lysates containing the crosslinked substrate, whereas Method B (steps 19 to 23) involves incubating the primary antibody with crosslinked lysates before addition of the agarose bead. Method A is more commonly



**Figure 3** Kinase-catalyzed crosslinking of endogenous p53 in HEK293 cell lysates. HEK293 cell lysates (125  $\mu$ g; all lanes) were incubated with ATP (lane 2) or ATP-arylazide (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of UV irradiation. Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene fluoride membrane. Western blotting with a p53 primary antibody ( $\alpha$ -p53) visualized p53 (arrow) and p53 crosslinked complexes (bracket). The molecular weight marker bands are indicated in kDa to the left of the gel image.

used for immunoprecipitation. However, in cases where (1) the abundance of the substrate of interest is low, (2) the binding affinity of the primary antibody for the substrate of interest is weak, and/or (3) the binding kinetics of the primary antibody for the substrate of interest is slow, Method B is recommended (Bates, 2012; <https://tools.thermofisher.com/content/sfs/brochures/TR0064-Immunoprecipitation-guide.pdf>). Alternatively, other enrichment methods can be used that are compatible with the substrate of interest. See the Critical Parameters for more information on alternative enrichment methods.

Basic Protocol 2 focuses on the immunoprecipitation enrichment step after kinase-catalyzed crosslinking (Basic Protocol 1). The bound proteins after immunoprecipitation can subsequently be separated by SDS-PAGE, electrotransferred to a PVDF membrane, and analyzed by Western blot analysis as previously described (Basic Protocol 1). In addition to probing the target substrate, antibodies to a suspected kinase or associated protein can also be tested, if interested. After confirmation of high-molecular-weight complexes in the enriched sample by Western blot analysis, the K-CLIP experiment can be repeated for proteomics analysis as published elsewhere (Garre et al., 2018; Shevchenko, Wilm, Vorm, & Mann, 1996).

#### **Additional Materials** (also see *Basic Protocol 1*)

- Protein A/G PLUS-agarose beads (e.g., Santa Cruz Biotechnology, cat. no. sc-2003)
- 1  $\times$  Tris-buffered saline (TBS; see recipe)
- HEPES lysis buffer (see recipe)
- Lysis buffer (see recipe)
- 2  $\times$  gel loading buffer: 2  $\times$  Laemmli Protein sample buffer (e.g., Bio-Rad cat. no. 1610747) containing  $\beta$ -mercaptoethanol (10%), as specified by the manufacturer
- Optional: light chain–specific secondary antibody: goat anti-mouse HRP light chain specific (e.g., Airgo Biolaboratories, cat. no. ARG21551).
- Tube rotator (e.g., Labnet Mini LabRoller Rotator)

#### **Kinase-catalyzed crosslinking with lysates**

1. Follow Basic Protocol 1 to initially perform kinase-catalyzed crosslinking of cell lysates. For Western blot analysis, use 500  $\mu$ g lysate in the kinase-catalyzed crosslinking reactions (Table 3). For subsequent proteomics analysis, use  $\geq 1$  mg lysate.

**Table 3** K-CLIP Reaction Conditions for p53 and Subsequent Western Blot Analysis<sup>a</sup>

|                        | Negative control 1 | Negative control 2 | Crosslinking reaction |
|------------------------|--------------------|--------------------|-----------------------|
| HEK293 lysate (500 µg) | 13.5 µl            | 13.5 µl            | 13.5 µl               |
| Kinase buffer (1×)     | 4 µl               | 4 µl               | 4 µl                  |
| ATP (5 mM)             | 4 µl               | -                  | -                     |
| ATP-arylazide (5 mM)   | -                  | 4 µl               | 4 µl                  |
| Water                  | 18.5 µl            | 18.5 µl            | 18.5 µl               |
| UV                     | +                  | -                  | +                     |

K-CLIP, kinase-catalyzed crosslinking and immunoprecipitation.

<sup>a</sup>For Basic Protocol 2, the stock concentrations are 37 mg/ml HEK293 lysate, 50 mM ATP-arylazide, and 50 mM ATP. Final concentrations are indicated in parentheses next to each reagent. The HEK293 lysate endogenously contains kinases and the p53 substrate.

**Table 4** Setup for IP of p53 Samples<sup>a</sup>

|                                          | Negative control 1 | Negative control 2 | Crosslinking reaction | IP negative control 1 | Optional IP negative control 2 |
|------------------------------------------|--------------------|--------------------|-----------------------|-----------------------|--------------------------------|
| Basic Protocol 1 kinase reaction samples | 40 µl              | 40 µl              | 40 µl                 | 40 µl                 | -                              |
| p53 antibody (1:1000)                    | 10 µl              | 10 µl              | 10 µl                 | -                     | 10 µl                          |
| Agarose beads                            | 20 µl              | 20 µl              | 20 µl                 | 20 µl                 | 20 µl                          |

IP, immunoprecipitation.

<sup>a</sup>For the p53 example, Method A was used for IP.

*In the case of Western blot analysis, an amount of lysate sufficient for visualizing crosslinking is needed. A larger amount of lysate is typically used for proteomics analysis to ensure sufficient peptide levels after trypsin digestion for LC-MS/MS. In this example, only K-CLIP for Western blot analysis is described here.*

### Immunoprecipitation

- In addition to the reaction controls from Basic Protocol 1 (Table 4), prepare negative control containing the crosslinked lysate and beads, but no antibody, which will identify nonspecifically bead-bound proteins (Table 4; immunoprecipitation negative control 1). Also prepare as a negative control an antibody-agarose bead sample to distinguish the bands of the antibody from the substrate of interest (Table 4; immunoprecipitation optional negative control 2), which is particularly helpful if the target substrate has a molecular weight similar to the light (25 kDa) or heavy (50 kDa) chain. For the antibody-agarose bead control, include primary antibody and agarose beads, but no lysates.

*Inclusion of controls are recommended for immunoprecipitation (see Table 4).*

### Preparation of protein A/G plus agarose beads

- Label microcentrifuge tubes with reaction numbers (Table 4).
- Add 20 µl bead slurry to the labeled microcentrifuge tubes.

*Vortex the stock of beads to make a homogenous mixture before dispensing, and use a pipette tip that has the end cut to make a wider opening that avoids damaging the beads. Keep all bead-containing tubes on ice to avoid degradation throughout the immunoprecipitation procedure.*

- Wash beads by adding 500 µl of 1× TBS, inverting three times, and centrifuging 1 min at 5000 × g, 4°C, to collect beads. Gently remove and discard 1× TBS using a pipette.

*Remove sample tubes carefully from the microcentrifuge to avoid disrupting the bead pellet.*

6. Repeat step 3 once more to wash beads a second time. Use washed beads immediately.

#### **Immunoprecipitation Method A**

7. After washing the beads twice, add primary antibody for the target protein. For this example, add 10  $\mu$ l p53 primary antibody, and dilute to 500  $\mu$ l with HEPES lysis buffer.

*Typically, 1 to 10  $\mu$ l (0.2 to 2  $\mu$ g) primary antibody is used for immunoprecipitation. The antibody manufacturer will often give advice on the optimal antibody amount for immunoprecipitation. However, optimization of antibody quantity might be needed.*

8. Rock bead-antibody mixture for 1 hr to overnight at 4°C with end-over-end rotation. For the p53 example, incubate antibody with beads for 1 hr.
9. After incubation of antibody and beads, centrifuge mixture 1 min at 5000  $\times$  g, 4°C. Remove and discard HEPES lysis buffer carefully using a pipette to avoid disrupting the beads.
10. Add 500  $\mu$ l HEPES lysis buffer, and invert beads three times. Centrifuge 1 min at 5000 $\times$  g, 4°C, and carefully remove buffer without disrupting the beads to remove unbound antibody.
11. Repeat step 10 a total of three times to wash the beads.
12. After the third wash, store washed beads on ice until the crosslinking reactions from Basic Protocol 1 are complete.

*The beads should not be stored for a long period of time while waiting for the crosslinking reactions. We suggest washing the beads when there is about 10 min left of the 2-hr crosslinking reactions.*

13. Add crosslinking reaction(s) to the separate tubes of washed beads, and dilute with HEPES lysis buffer to a total volume of 1 ml.
14. Rock reaction(s) overnight at 4°C or for the manufacturer's suggested amount of time with end-over-end rotations. For the p53 example, incubate samples overnight at 4°C.

*The incubation time with the crosslinking reactions and the antibody-conjugated agarose beads should be optimized for the antibody being used.*

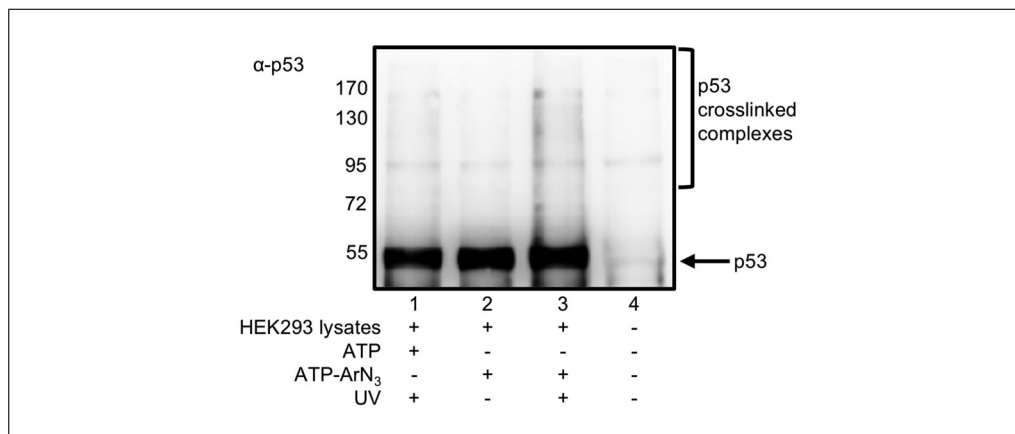
15. After incubation, centrifuge reactions 1 min at 5000  $\times$  g, 4°C, and carefully remove 1 ml supernatant without disrupting the beads.
16. Add 1 ml lysis buffer, and invert tubes three times. Centrifuge 1 min at 5000  $\times$  g, 4°C. Remove and discard lysis buffer.
17. Repeat step 16 a total of three times to wash the beads. Proceed to step 24.

#### **Immunoprecipitation Method B**

18. To the crosslinking reaction(s) from Basic Protocol 1, add manufacturer's recommended amount of primary antibody for immunoprecipitation, and rock overnight at 4°C or for manufacturer's recommended time with end-over-end rotations.

*The dilution of primary antibody and the amount of time to incubate the antibody with the reaction samples may need to be optimized.*

19. Prepare washed agarose beads as described previously (see steps 3 to 6).



**Figure 4** Kinase-catalyzed crosslinking and immunoprecipitation (K-CLIP) with endogenous p53 in HEK293 cell lysates. HEK293 cell lysates were incubated with ATP (lane 1) or ATP-arylazide (lane 2 and 3) in the presence (lane 1 and 3) or absence (lane 2) of UV irradiation. After immunoprecipitation of p53 from the lysates, bound proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by transfer to polyvinylidene fluoride membrane. Western blotting with a p53 primary antibody ( $\alpha$ -p53) visualized p53 (arrow) and p53 crosslinked complexes (bracket). A sample containing only the p53 primary antibody and protein A/G agarose beads used for immunoprecipitation was also included (lane 4). Molecular weight marker bands are indicated in kDa to the left of the gel image.

20. Add each antibody-containing reaction mixture to a tube of washed beads, and dilute to 1 ml with HEPES lysis buffer. Rock at 4°C with end-over-end rotations for 3 hr.

*The incubation time of the kinase-antibody mixture with the washed agarose beads might also need to be optimized for the chosen substrate of interest.*

21. After incubation of the antibody-containing reaction samples with the beads, centrifuge 1 min at  $5000 \times g$ , 4°C, and then carefully remove and discard supernatant without disrupting the beads.
22. Add 1 ml lysis buffer to the beads, and invert three times. Centrifuge 1 min at  $5000 \times g$ , 4°C, and then remove and discard lysis buffer.
23. Repeat step 22 a total of three times to wash the beads. Proceed to step 24.

#### **Preparation of samples for gel analysis**

24. To bound and washed beads from Method A or B, add 20  $\mu$ l of  $2\times$  gel loading buffer to each tube.
25. Heat bound beads at 95°C for 5 min to elute and denature proteins from the beads.
26. Prepare SDS-polyacrylamide gels following established protocols (see He, 2011), or use commercial gels. For the p53 example, use a 10% gel.
27. Centrifuge samples 1 min at  $5000 \times g$ , room temperature, to collect the samples.
28. Run SDS-PAGE and Western blot analysis as described in Basic Protocol 1.

*A gel image for the p53 K-CLIP example is shown in Figure 4.*

*Use a light chain–specific secondary antibody after immunoprecipitation to visualize the protein bands if the substrate of interest has a similar molecular weight as the heavy chain (50 kDa).*

## **REAGENTS AND SOLUTIONS**

### **ATP-arylazide stock solution**

Dissolve ATP-arylazide powder in 100  $\mu$ l of 100 mM HEPES, pH 7.4. Determine absorbance via UV-Vis spectrophotometer, and calculate concentration using Beer's

law ( $A = \epsilon bc$ ), where  $A$  is the absorbance at 254 nm,  $\epsilon$  is  $15.4 \times 10^3$  L/mol/cm,  $b$  is the path length (cm), and  $c$  is the concentration of ATP-arylazide. Make 5- or 10- $\mu$ l single-use aliquots, and store at  $-80^\circ\text{C}$  for up to 8 months. Avoid freeze-thaw cycles to prevent degradation.

*The concentration after resuspending solid ATP-arylazide ranges from 30 to 200 mM, which may need to be diluted before use in kinase reactions.*

*Dry ATP-arylazide powder can be stored at  $-80^\circ\text{C}$  for up to 1 year.*

*The ATP-arylazide should show minimal degradation by thin-layer chromatography (silica; 3:1.5:0.5 isopropanol:ammonium hydroxide:water;  $R_f = 0.4$ ).*

### **HEPES lysis buffer**

50 mM HEPES  
150 mM NaCl  
10% (w/v) glycerol  
0.5% (w/v) Triton X-100  
Adjust pH to 8.0 using HCl or NaOH  
Store at  $4^\circ\text{C}$  for up to 6 months

### **Lysis buffer**

50 mM Tris-HCl  
150 mM NaCl  
10% (w/v) glycerol  
0.5% (w/v) Triton X-100  
Adjust pH to 8.0 using NaOH or HCl  
Store at  $4^\circ\text{C}$  for up to 6 months

### **PBST, 1×**

137 mM NaCl  
2.7 mM KCl  
10 mM  $\text{Na}_2\text{HPO}_4$   
2 mM  $\text{KH}_2\text{PO}_4$   
Adjust pH to 7.4 with HCl or NaOH  
Add 0.1% (v/v) Tween-20  
Store at room temperature for up to 1 year

### **Reaction buffer, 10×**

250 mM HEPES  
500 mM KCl  
100 mM  $\text{MgCl}_2$ , pH 7.5  
Store at room temperature for up to 1 year

### **TBS, 1×**

20 mM Tris base  
150 mM NaCl  
Adjust pH to 7.6 using HCl or NaOH  
Store at  $4^\circ\text{C}$  up to 1 year

## **COMMENTARY**

### **Background Information**

Protein phosphorylation is an important post-translational modification that plays a major role in cellular biology by influencing protein activity. To better understand pro-

tein phosphorylation and the roles that phosphoproteins play in cellular events, a key challenge is to monitor and identify the kinases that phosphorylate cellular proteins. One method available for kinase-substrate

identification uses databases that predict substrates based on the fact that kinases recognize the primary sequence of amino acid residues surrounding a phosphosite, also called a substrate motif or consensus site, to initiate phosphorylation (Miller & Turk, 2016; Miller et al., 2008). Databases such as NetPhorest (Miller et al., 2008), Scansite (Obenauer, Cantley, & Yaffe, 2003), and KinasePhos (Ma et al., 2021) predict kinase-substrate pairs by comparing known substrate motif logos of the kinase to the primary sequence of the phosphoprotein. Although substrate motif predictions are fast and easy to use, without the need for experiments, a challenge is that substrate motif logos are only well characterized for a small subset of the 535 known kinases (Watson et al., 2020). As a result, the prediction of kinase-substrate pairs by substrate motif databases is biased toward characterized kinases. A second challenge is that known substrate motifs were often determined using arrays of short peptide substrates *in vitro*, which might not faithfully represent the preferences of kinases toward full-length substrate proteins under cellular conditions (Fujii et al., 2004; Miller & Turk, 2016). Due to the lack of available knowledge, using substrate motif databases is helpful but limited.

One method to identify kinase-substrate pairs experimentally using full-length protein substrates in a cellular context involves allele-sensitive kinases from the Shokat laboratory. In this chemical approach, an engineered kinase uses a base-modified ATP $\gamma$ S analog to selectively thiophosphorylate direct substrates in a lysate mixture (Allen et al., 2007). Upon chemical alkylation of the thiophosphoryl group on the substrate protein, an epitope for a thiophosphate ester-specific antibody is generated, which is then used for immunoprecipitation of the substrates. The allele-sensitive kinase method requires engineered kinases that tolerate the bio-orthogonal ATP $\gamma$ S analog. To date, among the nearly 535 known human kinases (Watson et al., 2020), ~40 mammalian kinases have been engineered to accept the ATP analog (Allen, 2008; Lopez, Kliegman, & Shokat, 2014), which limits the use of this method for general substrate identification.

The K-CLIP method represents a helpful alternative for kinase-substrate identification. K-CLIP is compatible with full-length proteins in a variety of complex cellular mixtures. In addition, K-CLIP avoids the need for kinase mutagenesis by using a  $\gamma$ -phosphoryl-modified ATP analog that is accepted by

native kinases. Due to the reliance on native kinases, K-CLIP can be applied to a variety of different kinases and biological systems. One limitation of the K-CLIP method is the need for a good-quality antibody against the kinase or substrate of interest for the immunoprecipitation step, although alternatives exist to overcome this limitation, including use of a tagged kinase or substrate (see Critical Parameters). The availability of various complementary methods to identify kinase-substrate pairs will ultimately help improve current knowledge of the roles kinases and their phosphoprotein substrates play in cellular events.

## Critical Parameters

### *Selection of lysate mixture*

The choice of lysate mixture is an important factor for application of Basic Protocols 1 and 2 to a new target phosphoprotein substrate in a complex mixture. Three key variables must be considered when choosing the lysate mixture and/or the target phosphoprotein substrate: (1) substrate abundance, (2) active phosphorylation, and (3) biological context. When considering abundance, the lysate mixture must contain the substrate of interest in sufficient quantity to visualize crosslinking by gel methods. Using lysates with sufficient endogenous levels of substrate is ideal by mimicking normal physiologic conditions. In cases where the endogenous abundance of the substrates is low, the lysate mixture can be modified to obtain sufficient quantities of substrate for crosslinking. Previously in work involving p53 as the substrate of interest, the mammalian cell lysate did not contain sufficient p53, and stabilization of p53 was achieved by inhibiting the binding of MDM2, which mediates degradation (Garre et al., 2018). We also have had success using a variety of modified lysate mixtures, including lysates with the substrate of interest overexpressed with a tag or homogenates with the tagged substrate of interest exogenously added. In addition to having sufficient substrate levels, kinase(s) in the lysate must be actively phosphorylating the substrate of interest to form crosslinked substrate-kinase complexes. Before applying Basic Protocols 1 and 2 to a target substrate, phosphopeptide-specific antibodies or Phos-tag SDS-PAGE (Fuji Film) can be used to establish kinase activity and phosphorylation states of the target substrate in the lysate mixture (Sugiyama et al., 2015). The last variable to consider is the biological context. Selecting an

appropriate complex lysate mixture that well represents the natural environment of the target substrate protein will help ensure that the isolated crosslinked kinase-substrate pairs are relevant to the biological system. For example, if studying a substrate target related to cancer, a lysate from a cancer-derived cell line should be used. Overall, taking time to consider these three key factors for the selection of the lysate is a critical step before performing crosslinking reactions.

### ***Selection of enrichment methods***

In order for K-CLIP (Basic Protocol 2) to be successful, a method to enrich the substrate of interest must be available. If the substrate of interest has a good quality antibody appropriate for immunoprecipitation, then crosslinked complexes can be isolated via direct immunoprecipitation. However, if a good quality antibody compatible with immunoprecipitation is unavailable for the substrate of interest, then an alternative enrichment method should be considered. One such method would be to express a tagged version of the substrate of interest and use an antibody specific for the tag. For example, a fusion protein tagged with FLAG can be expressed and subsequently enriched using FLAG-conjugated agarose beads (Gerace & Moazed, 2015). As another solution, particularly when expression of a tagged substrate is not possible, recombinantly expressed and tagged substrate can be exogenously added to a lysate mixture for subsequent enrichment. For example, a hexahistidine-tagged recombinant substrate can be added to a tissue homogenate for the crosslinking reaction, followed by enrichment using ion metal affinity chromatography (Spriestersbach, Kubicek, Schäfer, Block, & Maertens, 2015). Overall, K-CLIP is compatible with a variety of enrichment methods, which can be designed for the most appropriate lysate or complex mixture for the substrate of interest.

### ***Storage conditions and handling***

The quality of ATP-arylazide is crucial for successful kinase-catalyzed crosslinking experiments. Triphosphate-containing compounds, such as ATP and ATP-arylazide, are prone to hydrolysis and degradation. In the case of ATP-arylazide, degradation will generate unmodified ATP or ADP, along with the arylazide linker byproduct. The ATP and ADP degradation impurities can compete with ATP-arylazide in the kinase reactions, resulting in low levels of crosslinking and poor results. More critically, the arylazide linker byprod-

uct can participate in kinase-independent labeling (Arora & Boon, 2013), which could compromise the method by generating false positive hits. To minimize hydrolysis of ATP-arylazide, the analog should be stored as a solid at  $-80^{\circ}\text{C}$  for long-term storage (up to 12 months). Once dissolved in aqueous buffer, the analog should be stored at  $-80^{\circ}\text{C}$  as small single-use aliquots to avoid freeze-thaw cycles. In addition, ATP-arylazide should be kept as cold as possible while thawing and be used immediately after thawing in the kinase reaction to minimize degradation. All sample preparation and transfers should be performed on ice or in a cold room with prechilled microcentrifuge tubes. Even with storage at  $-80^{\circ}\text{C}$ , ATP-arylazide stored in aqueous buffer will degrade overtime and should be used within 6 months.

### **Troubleshooting**

Potential problems and solutions are described in Table 5.

## **Understanding Results**

### ***Basic Protocol 1***

The expected result after kinase-catalyzed crosslinking is to see both the substrate of interest and high-molecular-weight crosslinked bands in the membrane after visualization of the substrate by Western blot analysis. Bands for the substrate of interest should be at equal levels in all samples, which assures equal protein loading of the reactions into each lane. However, if needed, a separate gel can be used to assess a different loading control such as GAPDH, tubulin, or actin. Higher-molecular-weight bands or smears indicate that the kinase-catalyzed crosslinking was successful at generating complexes between the substrate of interest and kinases or associated proteins. In total, the uncrosslinked target substrate in all samples, along with higher-molecular-weight crosslinked complexes in the experimental lane, confirms successful crosslinking.

As an illustrative example and useful control to practice the method, p53 crosslinking is shown in Figure 3. In the p53 example, the kinase-catalyzed crosslinking experiment was performed with HEK293 lysate, which contains endogenous p53. HEK293 lysates were incubated with or without ATP-arylazide in the presence or absence of UV irradiation. Proteins in the lysate samples were separated by SDS-PAGE, followed by Western blotting to identify uncrosslinked p53 and p53 crosslinked complexes. As a loading

**Table 5** Troubleshooting Possible Problems

| Basic Protocol | Problem                                                                                                                                                          | Possible Cause                                                                                                  | Solution                                                                                                                                                                                            |
|----------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1 and 2        | Low levels or undetectable levels of substrate of interest by Western blot                                                                                       | Low abundance of substrate of interest                                                                          | Use more lysate or substrate of interest; if using endogenous substrate, consider use of overexpressed-tagged substrate                                                                             |
| 1 and 2        | Substrate protein bands observed, but no high-molecular-weight crosslinking bands or smearing observed in experimental lane compared with negative control lanes | Poor antibody sensitivity                                                                                       | Optimize Western blot protocol by testing different primary or secondary antibody dilutions or using different primary antibody                                                                     |
|                |                                                                                                                                                                  | Low lysate concentration in crosslinking reaction                                                               | Use a more concentrated lysate in the crosslinking reaction to increase likelihood for kinase-substrate interactions                                                                                |
|                |                                                                                                                                                                  | Low ATP-arylazide concentration in crosslinking reaction                                                        | Increase final concentration of ATP-arylazide in the kinase reactions                                                                                                                               |
|                |                                                                                                                                                                  | ATP-arylazide degradation, especially if older than 6 months                                                    | Use freshly dissolved stock of ATP-arylazide                                                                                                                                                        |
|                |                                                                                                                                                                  | Expired kinase buffer                                                                                           | Make fresh 10× kinase buffer                                                                                                                                                                        |
| 1 and 2        | High background in negative control lanes, which could mask crosslinked bands in crosslinking samples                                                            | Substrate of interest is highly abundant in lysate                                                              | Decrease amount of lysate used for kinase-catalyzed crosslinking experiments                                                                                                                        |
|                |                                                                                                                                                                  | Too much HRP developer used                                                                                     | Dilute HRP developer before adding to membrane or use less-sensitive fluorescence detection                                                                                                         |
|                |                                                                                                                                                                  | Too long of an exposure time on imager                                                                          | Shorten length of exposure                                                                                                                                                                          |
|                |                                                                                                                                                                  | Blocking step was unsuccessful                                                                                  | Optimize blocking step by trying alternative blocking reagents and incubation times                                                                                                                 |
| 2              | Low or undetectable levels of substrate of interest by Western blot after immunoprecipitation                                                                    | Immunoprecipitation step was unsuccessful                                                                       | Use larger quantity of primary antibody or different immunoprecipitation-compatible primary antibody; express substrate with an immunoprecipitation-compatible tag; use different enrichment method |
| 2              | Unequal substrate protein levels visualized by Western blot after immunoprecipitation                                                                            | Accidentally removed beads when performing washing steps                                                        | During washing steps, remove small amounts of buffer sequentially to avoid removing beads                                                                                                           |
| 2              | Unable to distinguish substrate of interest from antibody bands on Western blot after immunoprecipitation                                                        | Substrate of interest and heavy (50 kDa) or light (25 kDa) chain of the antibody have similar molecular weights | Use either light chain-specific or heavy chain-specific secondary antibody to visualize Western blot                                                                                                |

HRP, horseradish peroxidase.

control, equal levels of uncrosslinked p53 were observed in all lanes (Fig. 3; lanes 1 to 4). High-molecular-weight crosslinked bands containing p53 were observed in the presence of ATP-arylazide with UV (Fig. 3; lane 4). In contrast, no crosslinked complexes were visualized in the absence of UV light (Fig. 3; lane 3) or ATP-arylazide (Fig. 3; lane 2), which indicates that the crosslinking is dependent on the ATP analog and UV irradiation. In kinase-catalyzed crosslinking gels, the appearance of smeared instead of distinct bands for the crosslinked complexes is common.

### Basic Protocol 2

After performing K-CLIP with gel analysis, the expected result is to see both the uncrosslinked substrate of interest and the crosslinked complexes containing the substrate by Western blot analysis after immunoprecipitation. As a load control, the substrate of interest should have equal levels in all lanes containing lysate to show that the immunoprecipitation step was successful. In the crosslinked sample with ATP-arylazide in the presence of UV, high-molecular-weight crosslinked complexes containing the substrate of interest should be observed.

Again, as an illustrative example, K-CLIP with endogenous p53 in HEK293 lysates is shown in Figure 4. For the p53 K-CLIP example, HEK293 lysates were incubated with or without ATP-arylazide in the absence or presence of UV irradiation, followed by p53 immunoprecipitation. Enriched proteins were separated by SDS-PAGE and electrotransferred to PVDF membrane to visualize p53 and p53 crosslinked complexes by Western blotting. The levels of p53 were equal in the samples (Fig. 4; lanes 1 to 3), which indicates successful immunoprecipitation. High-molecular-weight crosslinked complexes specific to p53 were observed in the presence of ATP-arylazide with UV irradiation (Fig. 4; lane 3). In contrast, crosslinked complexes were absent with the omission of UV light (Fig. 4; lane 2) or ATP-arylazide (Fig. 4; lane 1). Because p53 (53 kDa) migrates similarly to the heavy chain of the IgG antibody (50 kDa), included in the analysis was an agarose bead antibody control to distinguish p53 from the IgG bands of the antibody used for immunoprecipitation (Fig. 4; lane 4). Also, as shown in the p53 example, high-molecular-weight crosslinked smearing is common with photocrosslinking.

## Time Considerations

### Basic Protocol 1

Kinase-catalyzed crosslinking and gel analysis can be completed in roughly 2 days. Day 1 requires roughly 6 hr for kinase reaction, gel separation, electrotransfer, and incubation with primary antibody overnight, whereas day 2 requires 2 hr for Western blotting and gel visualization.

### Basic Protocol 2

K-CLIP with gel analysis requires 3 days. Day 1 will require 3 hr for kinase reaction and overnight incubation for immunoprecipitation, and day 2 will require 5 hr for eluting proteins from the beads, gel separation, electrotransfer, and incubation with primary antibody overnight. Day 3 will take 2 hr for Western blotting and gel visualization.

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## Author Contributions

**Rachel J. Beltman:** conceptualization, formal analysis, investigation, validation, visualization, writing—original draft; **Mary Kay H. Pflum:** conceptualization, funding acquisition, methodology, project administration, resources, supervision, visualization, writing—review and editing.

## Conflict of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

The data that support the findings of this article are available from the corresponding author upon reasonable request.

## Literature Cited

- Allen, J. J. (2008). *Development and application of technologies to study individual kinase substrate relationships* (Publication no. 3328096) [Doctoral dissertation, University of California San Francisco]. ProQuest Dissertations Publishing.
- Allen, J. J., Li, M., Brinkworth, C. S., Paulson, J. L., Wang, D., Hübner, A., ... Shokat, K. M. (2007). A semisynthetic epitope for kinase substrates. *Nature Methods*, 4, 511–516. doi: 10.1038/nmeth1048
- Arora, D. P., & Boon, E. M. (2013). Unexpected biotinylation using ATP- $\gamma$ -biotin-LC-PEO-amine as a kinase substrate. *Biochemical and*

- Biophysical Research Communications*, 432, 287–290. doi: 10.1016/j.bbrc.2013.01.115
- Bates, D. (2012). Immunoprecipitation (IP). In P. Langton (Ed.), *Essential guide to reading biomedical papers* (pp. 129–136). Wiley-Blackwell. doi: 10.1002/9781118402184.ch14
- Dedigama-Arachchige, P. M., & Pflum, M. K. (2016). K-CLASP: A tool to identify phosphosite specific kinases and interacting proteins. *ACS Chemical Biology*, 11, 3251–3255. doi: 10.1021/acschembio.6b00289
- Embogama, D. M., & Pflum, M. K. (2017). K-BILDS: A kinase substrate discovery tool. *ChemBioChem*, 18, 136–141. doi: 10.1002/cbic.201600511
- Fouda, A. E., Gamage, A. K., & Pflum, M. K. H. (2021). An affinity-based, cysteine-specific ATP analog for kinase-catalyzed crosslinking. *Angewandte Chemie International Edition*, 60, 9859–9862. doi: 10.1002/anie.202014047
- Fujii, K., Zhu, G., Liu, Y., Hallam, J., Chen, L., Herrero, J., & Shaw, S. (2004). Kinase peptide specificity: Improved determination and relevance to protein phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 13744–13749. doi: 10.1073/pnas.0401881101
- Garre, S., Gamage, A. K., Faner, T. R., Dedigama-Arachchige, P., & Pflum, M. K. H. (2018). Identification of kinases and interactors of p53 using kinase-catalyzed cross-linking and immunoprecipitation. *Journal of the American Chemical Society*, 140, 16299–16310. doi: 10.1021/jacs.8b10160
- Garre, S., Senevirathne, C., & Pflum, M. K. H. (2014). A comparative study of ATP analogs for phosphorylation-dependent kinase–substrate crosslinking. *Bioorganic & Medicinal Chemistry*, 22, 1620–1625. doi: 10.1016/j.bmc.2014.01.034
- Gerace, E., & Moazed, D. (2015). Affinity pull-down of proteins using anti-FLAG M2 agarose beads. *Methods in Enzymology*, 559, 99–110. doi: 10.1016/bs.mie.2014.11.010
- Goldman, A., Ursitti, J. A., Mozdzanowski, J., & Speicher, D. W. (2015). Electrophoretic transfer from polyacrylamide gels. *Current Protocols in Protein Science*, 82, 10.17.11–10.17.16. doi: 10.1002/0471140864.ps1007s82
- Green, K. D., & Pflum, M. K. H. (2007). Kinase-catalyzed biotinylation for phosphoprotein detection. *Journal of the American Chemical Society*, 129, 10–11. doi: 10.1021/ja066828o
- Gross, S., Rahal, R., Stransky, N., Lengauer, C., & Hoefflich, K. P. (2015). Targeting cancer with kinase inhibitors. *Journal of Clinical Investigation*, 125, 1780–1789. doi: 10.1172/JCI76094
- He, F. (2011). Laemmli-SDS-PAGE. *Bio-Protocol*, 1, e80. doi: 10.21769/BioProtoc.80
- Krupa, A., Preethi, G., & Srinivasan, N. (2004). Structural modes of stabilization of permissive phosphorylation sites in protein kinases: Distinct strategies in Ser/Thr and Tyr kinases. *Journal of Molecular Biology*, 339, 1025–1039. doi: 10.1016/j.jmb.2004.04.043
- Kruse, J. P., & Gu, W. (2009). Modes of p53 regulation. *Cell*, 137, 609–622. doi: 10.1016/j.cell.2009.04.050
- Lopez, M. S., Kliegman, J. I., & Shokat, K. M. (2014). The logic and design of analog-sensitive kinases and their small molecule inhibitors. *Methods in Enzymology*, 548, 189–213. doi: 10.1016/B978-0-12-397918-6.00008-2
- Lu, K. P. (2004). Pinning down cell signaling, cancer and Alzheimer's disease. *Trends in Biochemical Sciences*, 29, 200–209. doi: 10.1016/j.tibs.2004.02.002
- Ma, R., Li, S., Li, W., Yao, L., Huang, H.-D., & Lee, T.-Y. (2021). KinasePhos 3.0: Redesign and expansion of the prediction on kinase-specific phosphorylation sites. *Genomics, Proteomics & Bioinformatics*, [Epub ahead of print]. doi: 10.1016/j.gpb.2022.06.004
- MacLaine, N. J., & Hupp, T. R. (2009). The regulation of p53 by phosphorylation: A model for how distinct signals integrate into the p53 pathway. *Aging*, 1, 490–502. doi: 10.18632/aging.100047
- Manns, J. M. (2011). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins. *Current Protocols in Microbiology*, 22, A.3M.1–A.3M.13. doi: 10.1002/9780471729259.mca03ms22
- Miller, C. J., & Turk, B. E. (2016). Rapid identification of protein kinase phosphorylation site motifs using combinatorial peptide libraries. *Methods in Molecular Biology*, 1360, 203–216. doi: 10.1007/978-1-4939-3073-9\_15
- Miller, M. L., Jensen, L. J., Diella, F., Jørgensen, C., Tinti, M., Li, L., ... Linding, R. (2008). Linear motif atlas for phosphorylation-dependent signaling. *Science Signaling*, 1, ra2. doi: 10.1126/scisignal.1159433
- Obenauer, J. C., Cantley, L. C., & Yaffe, M. B. (2003). Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Research*, 31, 3635–3641. doi: 10.1093/nar/gkg584
- Ramanayake-Mudiyanselage, V., Embogama, D. M., & Pflum, M. K. H. (2021). Kinase-catalyzed biotinylation to map cell signaling pathways: Application to epidermal growth factor signaling. *Journal of Proteome Research*, 20, 4852–4861. doi: 10.1021/acs.jproteome.1c00562
- Senevirathne, C., Embogama, D. M., Anthony, T. A., Fouda, A. E., & Pflum, M. K. H. (2016). The generality of kinase-catalyzed biotinylation. *Bioorganic & Medicinal Chemistry*, 24, 12–19. doi: 10.1016/j.bmc.2015.11.029
- Shaffer, J., & Adams, J. A. (1999). Detection of conformational changes along the kinetic pathway of protein kinase a using a catalytic trapping technique. *Biochemistry*, 38, 12072–12079. doi: 10.1021/bi991109q
- Shevchenko, A., Wilm, M., Vorm, O., & Mann, M. (1996). Mass spectrometric sequencing of

- proteins from silver-stained polyacrylamide gels. *Analytical Chemistry*, 68, 850–858. doi: 10.1021/ac950914h
- Spiestersbach, A., Kubicek, J., Schäfer, F., Block, H., & Maertens, B. (2015). Purification of his-tagged proteins. *Methods in Enzymology*, 559, 1–15. doi: 10.1016/bs.mie.2014.11.003
- Sugiyama, Y., Katayama, S., Kameshita, I., Morisawa, K., Higuchi, T., Todaka, H., ... Sakamoto, S. (2015). Expression and phosphorylation state analysis of intracellular protein kinases using Multi-PK antibody and Phos-tag SDS-PAGE. *MethodsX*, 2, 469–474. doi: 10.1016/j.mex.2015.11.007
- Suwal, S., & Pflum, M. K. H. (2010). Phosphorylation-dependent kinase–substrate cross-linking. *Angewandte Chemie International Edition*, 49, 1627–1630. doi: 10.1002/anie.200905244
- Wang, Z., & Cole, P. A. (2014). Catalytic mechanisms and regulation of protein kinases. *Methods in Enzymology*, 548, 1–21. doi: 10.1016/B978-0-12-397918-6.00001-X
- Watson, N. A., Cartwright, T. N., Lawless, C., Cámara-Donoso, M., Sen, O., Sako, K., ... Higgins, J. M. G. (2020). Kinase inhibition profiles as a tool to identify kinases for specific phosphorylation sites. *Nature Communications*, 11, 1684. doi: 10.1038/s41467-020-15428-0
- Wilson, D. F., Miyata, Y., Erecińska, M., & Vanderkooi, J. M. (1975). An aryl azide suitable for photoaffinity labeling of amine groups in proteins. *Archives of Biochemistry and Biophysics*, 171, 104–107. doi: 10.1016/0003-9861(75)90012-0