Within the realm of lysine methylation, the discovery of lysine methyltransferase (KMTs) substrates has been burgeoning because of established systematic substrate screening protocols. Here, we describe a protocol enabling the systematic identification of JmjC KDM substrate preference and in vitro substrates. Systematically designed peptide libraries containing methylated lysine residues are used to characterize enzyme-substrate preference and identify new candidate substrates in vitro.
Protocol

Evaluation of Jumonji C lysine demethylase substrate preference to guide identification of in vitro substrates

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SUMMARY
Within the realm of lysine methylation, the discovery of lysine methyltransferase (KMTs) substrates has been burgeoning because of established systematic substrate screening protocols. Here, we describe a protocol enabling the systematic identification of JmjC KDM substrate preference and in vitro substrates. Systematically designed peptide libraries containing methylated lysine residues are used to characterize enzyme-substrate preference and identify new candidate substrates in vitro.

BEFORE YOU BEGIN
The protocol below describes the specific steps for determining a substrate recognition motif for the KDM5A demethylase, as well as how to leverage this information for in vitro substrate discovery. Results for KDM3A are shown alongside KMD5A to highlight (1) how KDM substrate preference deviates between enzymes and (2) that the method is not solely applicable to a single enzyme.

Theoretical considerations
The procedure for determining JmjC KDM substrate recognition motifs involves monitoring enzyme activity towards a library of peptides representing systematic mutations from the canonical substrate sequence (i.e., peptide permutation library). Analogously, this approach has been established for KMTs (Dhayalan et al., 2011; Kudithipudi et al., 2014; Lanouette et al., 2015; Rathert et al., 2008a, 2008b). Thus, this methodology is limited to those JmjC KDMs with a known peptide substrate. Many methyl-modifying enzymes have established substrates within the histone code (Hyun et al., 2017). For example, the canonical histone substrate for the KDM5 family of enzymes is histone H3 trimethylated at lysine-4 (i.e., H3-K4me3).

Practical considerations
To note, the methodology begins assuming a pure source of recombinant enzyme is available. Recombinant KDM5A and KDM3A enzymes were expressed and purified as described (Krishnan and Trievel, 2016; Rose et al., 2012). Furthermore, although our example monitors JmjC KDM activity via detection of succinate (i.e., turnover of the 2-oxoglutarate cofactor), there are other methods for detecting in vitro JmjC KDM activity. This includes other techniques monitoring cofactor turnover and formaldehyde formation that would also be viable alternatives for detecting enzyme activity ((Hirsilä et al., 2003; Kivirikko and Myllylä, 1982; Krishnan et al., 2012; Luo et al., 2006).
Design permutated peptide substrate (PPS) library sequences

- **Timing**: 1–2 h

Permutation libraries consist of peptide sequences whereby a given residue position is mutated to all other naturally occurring amino acids while leaving the remainder of the sequence unaltered. Systematically performing this for multiple residue positions enables the assessment of amino acid specificity of peptide-protein interactions over a defined window. The specificity of numerous KMTs has been mapped via this approach and the epitope is generally defined by the residues occurring directly proximal to the modification site. Structural analysis of JMJD14, a plant KDM5 enzyme, found that residues directly proximal to the H3-K4me3 target site are important for substrate recognition (Yang et al., 2018). Thus, the permutated window consisted of residues -3 to +5 amino acids relative to the H3-K4me3 methylation site (**ART(Kme3)QTARKSTGGKA**; K4 position bold, permutated window underlined).

1. Retrieve peptide sequence known to permit demethylase activity (e.g., the H3-K4 sequence [**ART(Kme3)QTARKSTGGKA**] was used for KDM5A).
2. At each residue position within the desired window (e.g., –3 to +5 relative to the methylation site), generate 19 other sequences whereby the wild-type residue is exchanged to another naturally occurring amino acid (Table 1 shows an example for the -3 position only).

Optional: Add a tryptophan residue to the C-terminal end of the peptide sequence, separated from the peptide sequence by a flexible linker (e.g., 6-aminohexanoic acid [ahx]), to enable quantification of peptide concentration through tryptophan fluorescence.

### Preparation of peptides, cofactors, and buffer stocks

- **Timing**: 1 day

Due to the focus of the protocol described herein, the nonessential need to obtain peptides via the same methodology used, and the broad availability of peptide synthesis protocols, a detailed synthesis...
protocol is not described. To note, the peptides were synthesized following standard Fmoc (N-(9-fluorenylethoxycarbonyl) chemistry, on an automated ResPep SL peptide synthesizer (Intavis), at a scale of 2 µmol following procedures previously described (McKenna et al., 2021; Wei et al., 2018).

3. Dissolve dry peptides in 1x phosphate-buffered saline (PBS; pH 7), or another activity-compatible buffer), to a final concentration of 10 mM.

   **Note:** Given that peptides were synthesized at a scale of 2 µmol, dissolve the peptides in 200 µL of 1x PBS to obtain a concentration of 10 mM.

   **Note:** Peptides synthesized in-house may be highly acidic due to residue trifluoroacetic acid and may require pH to be adjusted to 7 by gradual addition of sodium hydroxide (a 5 M NaOH solution was used to add low volumes of 5–10 µL to peptides).

   △ **CRITICAL:** all buffers and reagents should be prepared in ATP-free water. This includes cofactors and buffers described below.

   **Optional:** If peptides are synthesized in-house, it is highly recommended to quantify the peptides to ensure proper concentration. If an ahx-Trp is added to the C-terminal ends of peptide sequences, concentration may be determined by absorbance at 280 nm and the extinction coefficient of the peptide (available using the Expasy ProtParam tool; https://web.expasy.org/protparam/).

4. Prepare 4x peptide stocks (40 µM) by diluting peptides in 1x PBS. Next, prepare a 1 mM peptide stock solution for the wild-type substrate peptide in 1x PBS.

   **Pause point:** Peptide stocks should be aliquoted and stored at −20°C until use.

5. Make 10 mM solutions of ascorbic acid, α-ketoglutarate, and Fe(II)SO₄ in a volume of 1 mL using ATP-free water, in separate 1.5 mL Eppendorf tubes.
   a. Dilute 10 mM cofactor stocks to 1 mM with ATP-free water.

6. Make a 40 mL solution of 0.5 M HEPES in a 50 mL conical tube. Dissolve HEPES in 30 mL of ATP-free water and adjust pH to 7.5 using 5 M NaOH. Top up volume to 40 mL to yield a final concentration of 0.5 M HEPES.

   **Note:** Stores HEPES solution at 4°C protected from light.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| KDM5A1301-His | SGC Oxford | n/a |
| KDM3A515-1317-His-Flag | (Rose et al., 2012) | n/a |
| Fmoc-Ala-OH | P3 Biosystems | Cat# 41004 |
| Fmoc-Arg(Orb)-OH | P3 Biosystems | Cat# 41002 |
| Fmoc-Asn(Trt)-OH | P3 Biosystems | Cat# 41007 |
| Fmoc-Asp(OtBu)-OH | P3 Biosystems | Cat# 41019 |
| Fmoc-Cys(Trt)-OH | P3 Biosystems | Cat# 41008 |
| Fmoc-Glu(OtBu)-OH | P3 Biosystems | Cat# 41005 |
| Fmoc-Gln(Trt)-OH | P3 Biosystems | Cat# 41011 |
| Fmoc-Gly-OH | P3 Biosystems | Cat# 41010 |
| Fmoc-His(Trt)-OH | P3 Biosystems | Cat# 41017 |
| Fmoc-Ile-OH | P3 Biosystems | Cat# 41018 |
| Fmoc-Leu-OH | P3 Biosystems | Cat# 41003 |

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Fmoc-Lys(Boc)-OH    | P3 Biosystems | Cat# 41001 |
| Fmoc-Met-OH         | P3 Biosystems | Cat# 41020 |
| Fmoc-Phe-OH         | P3 Biosystems | Cat# 41013 |
| Fmoc-Pro-OH         | P3 Biosystems | Cat# 41009 |
| Fmoc-Ser(tBu)-OH    | P3 Biosystems | Cat# 41016 |
| Fmoc-Thr(tBu)-OH    | P3 Biosystems | Cat# 41012 |
| Fmoc-Trp(Boc)-OH    | P3 Biosystems | Cat# 41014 |
| Fmoc-Val-OH         | P3 Biosystems | Cat# 41015 |
| Fmoc-Lys(Boc,Me)-OH | P3 Biosystems | Cat# 47238 |
| Fmoc-Lys(Me)2-OH, HCl | P3 Biosystems | Cat# 47237 |
| HBTU                | P3 Biosystems | Cat# 31001 |
| NMM                 | Alfa Aesar | Cat# A12158 |
| Rink Amide MBHA Resin | P3 Biosystems | Cat# 52002 |
| Acetic anhydride    | Fisher Scientific | Cat# 108-24-7 |
| Piperidine          | Sigma-Aldrich | Cat# 104094 |
| DMF                 | Fisher Scientific | Cat# D119-20 |
| Ethanol             | Commercial Alcohols | Cat# P016EAAN |
| DCM                 | Acros Organics | Cat# 354800025 |
| trifluoroacetic acid | Fisher Scientific | Cat# L06374 |
| TIPS                | Acros Organics | Cat# 214922500 |
| Ethyl Ether         | Fisher Scientific | Cat# E138-4 |
| HEPES               | BioShop | Cat# HEP005.1 |
| Fe(II)SO₄            | BDH Chemicals | Cat# B28400 |
| α-ketoglutarate     | Sigma-Aldrich | Cat# K-3752 |
| Ascorbic acid       | J.T. Baker Chemical Co. | Cat# BS81.5 |
| Tris(2-carboxyethyl)phosphine (TCEP) HCl salt | Sigma-Aldrich | Cat# 646547 |
| Bovine serum albumin | BioShop | Cat# ALB001.100 |
| Dimethyl sulfoxide  | Bio Basic Canada | Cat# D0231 |
| NaCl                | BioShop | Cat# SOD0002.1 |
| KCl                 | BioShop | Cat# POC308.500 |
| Sodium phosphate monobasic monohydrate | Sigma-Aldrich | Cat# 59638 |
| Sodium phosphate dibasic anhydrous | BioShop | Cat# SPD307.500 |
| Potassium phosphate monobasic | BioShop | Cat# PPM666.1 |
| Acetic acid         | Anachemia | Cat# 00598-463 |

Critical commercial assays

Succinate-Glo JmjC Demethylase/Hydroxylase Assay Promega Corporation (Alves et al., 2018) Cat# V7990

Software and algorithms

Peptide Specificity Analyst (PeSA) (Topcu and Biggar, 2019) https://doi.org/10.5281/zenodo.6323540

Other

BioTek Cytation 5 microplate reader BioTek Cat# BTCT5M
Greiner 384-well plate, white Greiner Bio-One Cat# 781075

MATERIALS AND EQUIPMENT

2x Reaction Buffer/Peptide Mix

| Reagent                              | Final concentration | Amount  |
|--------------------------------------|---------------------|---------|
| 0.5 M HEPES (pH 7.5)                 | 50 mM               | 50 μL   |
| 1 mM ascorbic acid                   | 200 μM              | 100 μL  |
| 1 mM Fe(II)SO₄                       | 20 μM               | 10 μL   |

(Continued on next page)
Alternatives: Here a peptide list comprising the known methylproteome was scored using JmjC KDM recognition motifs. This peptide list is provided (Table S1, accessed from PhosphoSitePlus on 12-03-2020), however any peptide list can be used (Hornbeck et al., 2012).

STEP-BY-STEP METHOD DETAILS

Determine non-saturating enzyme concentration

© Timing: 4 h

Given a fixed set of reaction parameters (e.g., cofactor concentration, pH, temperature, etc.), a user can determine the optimal concentration range of JmjC KDM yielding a non-saturated signal. Establishing a non-saturating enzyme concentration is key for downstream steps when comparing the effect of different peptides on JmjC KDM activity. This is achieved by dilution series of the given KDM from the highest concentration possible to a low or sub nanomolar concentration (e.g., <1–10 nM). In our prototypic example of KDM5A and KDM3A, by dilution series we determined enzyme concentrations yielding non-saturating signal (Figure 1).

Note: To maximize the signal-to-background ratio, we recommend users of this methodology establish optimal conditions (e.g., pH, temperature, etc.) prior to beginning this workflow. This may be done experimentally or by literature search.

Note: Commercially available Promega Succinate-Glo™ assay reagents are stored at –80°C. Begin thawing reagent components on ice ~1 h before use. For Acetoacetyl-CoA 100×, specifically, thaw at 22°C–24°C for 5 min before addition to Succinate-Glo™ Buffer.

1. Place a white 384-well microplate on a Peltier device and set temperature to 4°C.
2. Prepare 2× Reaction Buffer/Peptide Mix.
3. Add 2.5 μL of 2× Reaction Buffer/Peptide Mix to all experimental wells in the 384-well microplate.
4. Thaw JmjC KDM stock protein on ice.
5. Perform a 20 μL dilution series (e.g., 4× or 2×) of the JmjC KDM protein in 1.5 mL Eppendorf tubes on ice in the same buffer used initially for storage (see Table 2 for an example dilution range).

   **Note:** Save at least a 20 μL aliquot of storage buffer for the “no enzyme” control.

   **Alternatives:** Dilution series may be performed in higher or lower volumes depending on the number of replicate reactions performed.

6. Set Peltier device to 23°C.

   **Alternatives:** Other temperatures for the JmjC KDM reaction may be used (e.g., optimal temperature may be determined prior to this protocol or published in literature).

7. To initiate the reaction, add 2.5 μL of each JmjC KDM dilution to a specific microplate well containing the 2× Reaction Buffer/Peptide Mix (Table 2 shows an example plate layout).
   a. Set the 384-well microplate on a plate shaker for 2 min at 22°C–25°C.
   b. Centrifuge the plate at 240 × g for 1 min at 23°C.

   **Note:** For the no enzyme control (NEC), add 2.5 μL of storage buffer containing an absence of JmjC KDM protein.

8. Allow the demethylation reaction to occur for 60 min at 23°C.
9. Add 5 μL of Succinate Detection Reagent I to each assay well.
   a. Shake the 384-well microplate for 2 min at 22°C–25°C.
   b. Centrifuge the plate at 240 × g for 1 min at 23°C and incubate for 60 min at 23°C.

   **Note:** The time of JmjC reaction may be adjusted, however, the reaction time used in this step must be kept consistent when performing all downstream JmjC KDM reactions. For example, reactions with KDM3A occurred for 3 h.

10. Add 10 μL of Succinate Detection Reagent II.
    a. Shake the 384-well microplate for 2 min at 22°C–25°C.
    b. Centrifuge the plate at 240 × g for 1 min at 23°C and incubate for 10 min at 23°C.

11. Read the luminescence from each well using a microplate reader.

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**Figure 1. Determining non-saturating JmjC KDM concentration**

(A and B) Two-fold dilution series of recombinant (A) KDM5A and (B) KDM3A enzymes, observing activity towards H3-K4me3 and H3-K9me2 peptides, respectively. The data represents the average of three luminescent readings and error bars represent the SEM (n=3). Data for KDM5A was previously published (Hoekstra and Biggar, 2021).
12. Calculate the average luminescence signal across technical replicates.
13. For each condition, subtract the average signal from that of the average of the NEC to account for background luminescence.
14. Using data analysis/graphing software, such as GraphPad Prism, visualize the data to determine non-saturating and linear dose responsive JmjC KDM concentrations (Figure 1). Choose a concentration of protein within the mid-linear range of relative activity.
   a. See Troubleshooting 1 if JmjC KDM activity is difficult to observe.

### Validate KDM methyl-state preference

**Timing:** 4 h

This step aims to validate the methyl-state preference (i.e., mono-, di-, tri-methylation) of the recombinant JmjC KDM for an established substrate. This is important as many JmjC KDMs have been shown to be capable of discriminating between different methyl-states of the same substrate. As the peptide libraries used for this method are synthesized as one methyl-state, this validation should be performed prior to commercially ordering or synthesizing the full peptide permutation library. Given the optimal concentration of the JmjC KDM determined in the previous step, the JmjC KDM activity can be monitored by performed reactions with peptide substrates of different methylation states. For assay validation, it is important to include an unmethylated substrate and a ‘no peptide control’ (NPC) alongside the standard NEC.

△ CRITICAL: Some JmjC KDMs can convert 2-oxoglutarate to succinate in an appreciable extent in the absence of methylated peptide substrate. As a result, a ‘no peptide control’ (NPC) is critical to use going forward to assess the true level of relative demethylation activity (Table 3).

15. Thaw 40 μM wild-type substrate (including null-, mono-, di-, and tri-methyl state peptides) on ice.
16. Repeat steps 1–3, except using 2× Reaction Buffer (similar to 2× Reaction Buffer/Peptide Mix minus peptide substrate).
   a. Keep 2× Reaction Buffer on ice for same day use.
17. Add 1.25 μL of 40 μM wild-type peptide substrates, individually, to each experimental well containing 2× Reaction Buffer.
   a. For the NPC, use 1.25 μL of 1× PBS.

**Note:** If peptides were dissolved in a buffer other than 1× PBS, use the peptide buffer for the NPC.

18. Thaw JmjC KDM stock protein on ice and dilute to 4× the optimal concentration determined in step 14.

### Table 2. Example of plate layout for JmjC 2× dilution series

| Column | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|--------|---|---|---|---|---|---|---|---|---|----|----|----|----|
| Row A-C (n=3) | NEC | 10 | 5.0 | 2.5 | 1.3 | 0.63 | 0.31 | 0.16 | 0.078 | 0.039 | 0.020 | 0.01 | 0.005 |

Initial concentration of purified KDMSA was 20 μM, yielding a final reaction concentration of 10 μM. Concentrations listed are in μM.

### Table 3. Example of plate layout for testing JmjC KDM activity towards differentially methylated peptides (monomethylation [Kme1], demethylation [Kme2], and trimethylation [Kme3])

| Column | 1 | 2 | 3 | 4 | 5 | 6 |
|--------|---|---|---|---|---|---|
| Row A-C (n=3) | NEC | NPC | Null | Kme1 | Kme2 | Kme3 |
a. Considering the number of experimental conditions requiring enzyme (5 in total), the volume of JmjC KDM needed per reaction (1.25 mL), and the number of replicates (e.g., n=3), make at least 20.63 mL of 2× JmjC KDM (this is 10% excess of the minimum volume needed (18.75 mL)).

b. Perform the protein dilution with the JmjC storage buffer.

19. Set Peltier device to 23°C (or appropriate constant temperature).
20. Add 1.25 µL of 2× JmjC KDM dilution to all experimental wells, except for the NEC. Add 1.25 µL JmjC KDM storage buffer to the NEC.
21. Repeat steps 8–13.
22. For each condition, subtract the signal from that of the NPC to account for demethylation-uncoupled 2-oxoglutarate turnover.

Optional: Represent methyl-state preference as relative JmjC KDM activity; normalize subtracted luminescent signals to that of the peptide displaying the highest signal. Again, using data analysis/graphing software visualize the data to represent methyl-state preference (Figure 2).

Determine JmjC KDM substrate preference by permutated peptide substrate (PPS) library

© Timing: 4 h (per mutation position)

These steps aim to map JmjC KDM substrate preference by monitoring activity towards all peptides in the PPS library. At this point, all peptides within the library should be methylated according to the preferred methyl-state (e.g., H3-K4me3 and H3-K9me2 for KDM5A and KDM3A, respectively). The experimental set-up is performed in the same manner as the previous section (methyl-state validation), except using permuted peptides alongside the wild-type peptide and reaction controls (Table 4). In analyzing the data relative to the positive control (wild-type peptide) and negative controls (no peptide and no enzyme controls), one can determine the relative effect of individual amino acid mutations on JmjC KDM activity.

Note: To limit variability in the start of reaction time between experimental conditions; it is recommended to assess JmjC KDM activity towards peptides representing one residue position of the PPS library at a time.

23. Thaw 40 µM peptide stocks for the PPS library position being tested on ice.
24. Make fresh 2× Reaction Buffer (e.g., for each residue position, 242 μL of 2× Reaction Buffer is required (22 conditions × 4 replicates × 2.5 μL = 220 μL + 10% extra).

25. Repeat steps 1–3, using the freshly prepared 2× Reaction Buffer.

26. Add 1.25 μL of 4× (40 μM) peptide, individually, to experimental wells containing 2× Reaction Buffer. For the NPC, use 1.25 μL of 1× PBS.

**Note:** Again, if peptides were dissolved in a buffer other than 1× PBS, use that buffer for the NPC.

**△ CRITICAL:** Ensure each run of the experiment contains a reaction condition with a wild-type peptide. All mutation positions have a wild-type peptide. However, if mutation positions are not run in full, a wild-type peptide must be included.

27. Thaw JmjC KDM stock on ice and dilute to 4× the optimal concentration determined in the previous step in JmjC storage buffer.

**Note:** Consider the number of experimental conditions requiring enzyme (e.g., the volume of JmjC KDM needed per reaction well (1.25 μL), and the number of replicates (e.g., n=4), make 115.5 μL of 2× JmjC KDM (this is 10% excess of what is needed; 21 conditions × 4 replicates × 1.25 μL = 105 μL)).

28. Set Peltier device to 23°C (or appropriate constant temperature).

29. Add 1.25 μL of 4× JmjC KDM solution to all experimental peptide conditions (wild-type peptide and mutant peptides) and the NPC well. Add 1.25 μL JmjC KDM storage buffer to the NEC well.

30. Repeat steps 8–13.

31. For each condition, subtract the average signal of the NPC to account for demethylation-uncoupled 2-oxoglutarate turnover.

32. To assess the relative effect of mutations on JmjC KDM activity; normalize substrate luminescent signals to that of the wild-type peptide.

**Note:** Normalization is achieved from dividing the luminescent signals of reactions with experimental peptides by that of the WT peptide. This is critical to gauge the relative effect of individual mutations on enzyme activity.

33. Repeat steps 23–32 for each mutation position.

**Optional:** Represent relative activity data as a heat-map to visualize JmjC KDM substrate preference (Figure 3) using basic data analysis software (e.g., Microsoft Excel).

### JmjC KDM recognition motif generation, substrate prediction, and in vitro validation

**Timing:** 1–2 h

The major aim of this step is to use JmjC KDM substrate preference to prioritize peptides to be tested for in vitro KDM activity. Peptide Specificity Analyst (PeSA) software can be used to easily produce a
candidate KDM recognition motif (Topcu and Biggar, 2019). In turn, these candidate recognition motifs can then be used to score and prioritize the methyllysine proteome for candidate substrates.

34. Download and open PeSA software (Topcu and Biggar, 2019; also available at https://github.com/EmineTopcu/PeSA).

35. Select “Permutation Array” tab at the menu.

36. Click “Load from File” and upload your JmjC KDM quantification matrix.

**Note:** The quantification matrix can be uploaded with either the relative activity values or the background subtracted luminescent values. If the latter, normalization to the wild-type luminescent values in each position can be performed within the software to obtain relative activity values. Under “Normalized”, select “Per Row/Column” (see Table S2 for an example of the KDM5A quantification matrix).

**Note:** Experimentally, only the residue positions flanking the methylation site are assessed. However, to maintain the central target lysine within the visualization of the recognition motif; in the quantification matrix one can assign the central methylated K the relative value of “1” and all other mutations in this central position as “0”.

37. Set the “Threshold” to the desired the value.

**Note:** In this context the “Threshold” defines the minimum level of relative JmjC KDM activity for an amino acid substitution to be included within the candidate recognition motif. Keep in mind that stringency increases with higher threshold values.
Alternatives: Another threshold value may be used and should be considered when Troubleshooting problem 2. Figure 4 represents KDM5A and KDM3A recognition motifs set at various threshold values (e.g., 0.25, 0.5, 0.75, and 1.0).

38. Obtain the recognition motif under the “motif” tab on the left-hand side of the window.
39. Score peptides that possess windows which reflect known methylation sites in the methylproteome, using the PeSA generated candidate recognition motif.
   a. Each position in the queried sequence which shares an amino acid with the recognition motif contributes a value of 1 to the total score. An example of scoring is provided in Table 5.

Note: Given the recognition motif produced and Kme position, ensure that the queried windows are of the same length and Kme residue in question is in the correct position. The PeSA score reflects the number of residues in the queried peptide matching those in the recognition motif at their corresponding positions. As a result, the predictions were made based on a 9-residue recognition motif, and therefore, the highest possible score obtainable is a total PeSA score of 9 (i.e., for windows exactly matching the recognition motif) (Table 5).

40. Determine mean (μ) and standard deviation (σ) of PeSA scores across all queried peptide sequences.
41. To define high-ranking peptide sequences, determine the PeSA score corresponding to 2 standard deviations above the mean.
   a. For KDM5A, using the 0.5 relative activity recognition motif, a PeSA score of 9 reflects high-ranking peptide sequences (μ = 6.6, σ = 1.2) (Figure 5A).

42. Repeat steps 23–32 in the previous section, except using high-ranking peptides instead of PPS library peptides to validate the in vitro activity of substrate predictions/rankings (Figure 5B).

Table 5. Example of motif-based scoring

| Protein-site  | Queried sequence | Position and tolerable substitutions within motif | PeSA score |
|---------------|------------------|-----------------------------------------------|------------|
| H3-K9 (known) | QTARKSTGG        | -4 [CRGQ] -3 [RIQCVAKT] -2 [A] -1 [R] 0 [K] +1 [RTS] +2 [T] +3 [MG] +4 [G] | 9          |
| RPA2-K693 (unknown) | CQMKGQTMG       | C Q M G K Q T M G | 6          |

Example of motif-based scoring. Example of scoring 9-mer sequences with KDM3A motif (defined by amino acids maintaining at least 100% relative activity). Tolerable amino acids at each position are shown in brackets. Matching and mismatching residues, compared to the motif, are shown in green and red, respectively. PeSA score is defined by the number of matching residues.
a. See Troubleshooting 2 if JmjC KDM shows no activity to none, or few, of the predicted JmjC KDM substrates

Note: If the focus is purely on JmjC KDM substrate discovery, we recommend testing as many of the high-ranking peptides as possible, if not all. However, if the user is also interested in assessing whether the method accurately predicts or enriches in highly active in vitro substrates for the select JmjC KDM, one can assess a select number of random substrates with high-, medium-, and low-ranking PeSA scores (Figure 6 demonstrates this analysis for KDM5A).

EXPECTED OUTCOMES
In our experience, PeSA scores of large lists of peptide sequences (e.g., methylproteome; n=2,155 sequences) follow a Gaussian distribution. For example, Figure 5A shows the distribution of PeSA scores of methylproteome sequences, scored with the 0.5 relative activity KDM5A recognition motif. High- and low-ranking substrates are defined by PeSA scores occurring 2 standard deviations (σ = 1.2) above (or equal to) or below (or equal to) the population mean (μ = 6.6), respectively.

If testing relative JmjC KDM activity towards predicted substrates, we expect PeSA scores to correlate with in vitro JmjC KDM activity. For example, we sought to test KDM5A activity towards a handful of high, medium- and low-ranking substrates (Figure 5B). We were able to observe that none of the medium and low-ranking substrates displayed any significant KDM5A activity (activity defined as greater than 50% of H3-K4me3 activity). Furthermore, we observed KDM5A activity towards 90% of high-ranked peptides. This verifies that the 0.5 relative activity KDM5A recognition motif was accurate in predicting peptide sequences that would be amenable to KDM5A activity in vitro. Ultimately, this added step of validation does prioritize several potential substrates to refine and focus further validation efforts on.

LIMITATIONS
Limitation 1
A limitation to consider is that the relative importance of the individual amino acids at each position in the recognition motif is determined in the context of a fixed sequence and thus sequence bias may influence the results. This is a consequence of using permutation-based exploration of enzyme
specificity. It is certainly possible that the relative importance of a given amino acid is only observable when occurring in the presence of specific amino acids at other positions. Supporting this, and in the context of methyl-binding domains, JMJD2A-double Tudor domain binding to permutations of H3-K23me3, H3-K4me3, and H4-K20me3 show distinct specificities depending on wild-type sequence used (Liu et al., 2013).

Limitation 2
The protocol defined here assumes recognition of substrates by a given enzyme is specified by the residues directly proximal to the modification site (i.e., −4 to +5 positions). Although this has been shown to be the case for many KMT enzymes, and for Suv39H2 the +5 position also determines specificity (Schuhmacher et al., 2015), recognition of substrates may also be determined by more distant interactions. Additionally, on the H3 tail, more distal sequence elements were recently shown to be important for KDM5A-dependent demethylation of the H3-K4 site (Petronikolou et al., 2020).
Finally, as JmjC KDMs belong to the Fe(II)/2-oxoglutarate-dependent family of dioxygenases they are susceptible to product inhibition conferred by the formation of succinate. The potency of inhibition varies depending on the specific enzyme of interest. To attenuate this effect, the assay can be performed in a format such that any succinate formed is immediately converted during the demethylation reaction, rather than converting succinate at the end of the JmjC KDM reaction (e.g., incorporating coupling enzymes in the KDM reaction). To achieve this, Succinate-Glo Solution and Acetoacetyl-CoA, provided in the assay kit, may be incorporated in the demethylation reaction by ensuring each of these components are diluted 100-fold in the final assay volume. Although the materials in the kit utilized is proprietary, succinate may be directly converted to succinyl-CoA by 3-oxoacid-CoA-transferase in the presence of acetoacetyl-CoA. For KDM3A, specifically, we found that the modified protocol increases the luminescent signal (Figure 6).

Problem 2
The JmjC KDM assessed shows no activity to none, or few, of the predicted JmjC KDM substrates. This problem is referring to corresponding protocol step 42.

Potential solution
No activity towards any of the substrate predictions could be due to several factors, such as limitations inherent to permutation-based exploration of specificity. The latter cannot be addressed by adjusting variables within this protocol and would need to be resolved by assessing specificity in another manner or by using multiple substrate permutations. However, the adjustable variables include reducing the stringency of substrate predictions to be more permissive for substrate discovery (i.e., use recognition motif defined by a lower activity threshold). It may also be beneficial to test activity of the given JmjC KDM at multiple substrate concentrations. Finally, and related to Limitation 2, the critical positions defining specificity may exist outside of the permuted positions and thus it may be beneficial to expand this window and re-predicting substrates based on this expanded recognition motif.

RESOURCE AVAILABILITY
Lead contact
Further information and requests should be directed to and will be fulfilled by the lead contact Dr. Kyle K. Biggar (kyle_biggar@carleton.ca).

Materials availability
This protocol is not associated with any newly generated materials.

Data and code availability
The protocol includes the methylproteome peptide list (Table S1) accessed from PhosphoSitePlus on 12-03-2020 (Hornbeck et al., 2012).

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101271.

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AUTHOR CONTRIBUTIONS
Methodology, M.W. and A.C.; data curation, M.H. and A.C.; validation, M.H.; writing – original draft, M.H. and A.C.; writing – review & editing, A.C., K.K.B., and W.G.W.; conceptualization, K.K.B.; resources, K.K.B.; funding acquisition, K.K.B.; supervision, K.K.B. and W.G.W.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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