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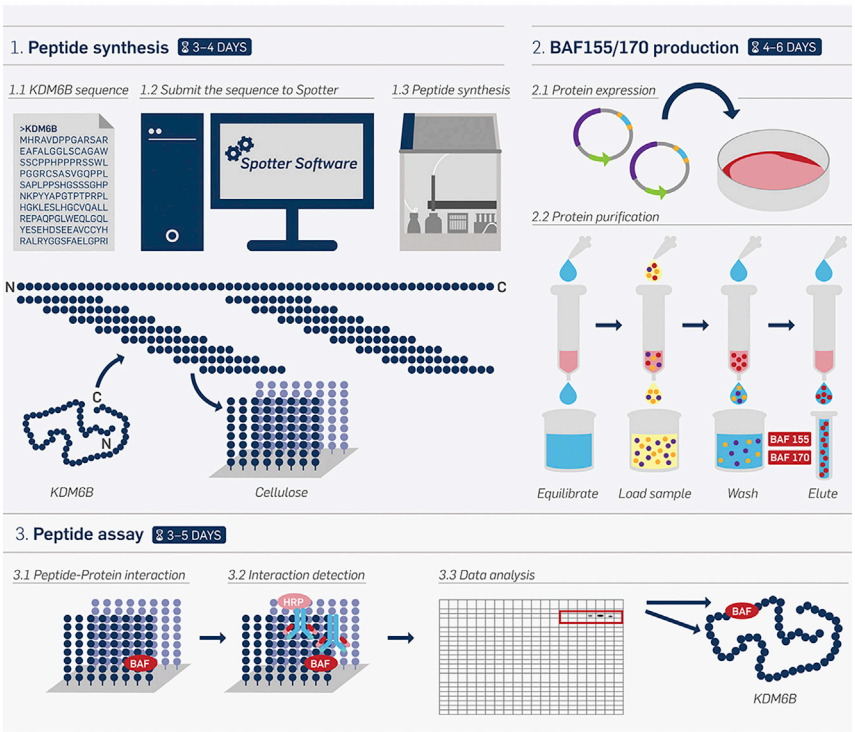
Mapping of domain-mediated protein-protein interaction by SPOT peptide assay



Identification of peptides mediating protein-protein interaction (PPI) is crucial for understanding the function of interlinked proteins in cellular processes and amino acid-associated diseases. Traditional PPI assays are laborious, involving the generation of many truncated proteins. SPOT peptide assay allows high-throughput detection of domains essential for PPI by synthesizing several hundred peptides on a cellulose membrane. Here, we present a rapid SPOT peptide protocol for identifying the binding motifs, which mediate interaction between the chromatin remodeling factors BAF155/BAF170 and the epigenetic factor Kdm6b.

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Highlights

Parallel synthesis of a large number of overlapping peptides using SPOT arrays

Rapid and efficient purification of a FLAG fusion protein from cultured cells

Simple technique to identify peptides mediating protein-protein interaction

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Mapping of domain-mediated protein-protein interaction by SPOT peptide assay

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SUMMARY

Identification of peptides mediating protein-protein interaction (PPI) is crucial for understanding the function of interlinked proteins in cellular processes and amino acid-associated diseases. Traditional PPI assays are laborious, involving the generation of many truncated proteins. SPOT peptide assay allows high-throughput detection of domains essential for PPI by synthesizing several hundred peptides on a cellulose membrane. Here, we present a rapid SPOT peptide protocol for identifying the binding motifs, which mediate interaction between the chromatin remodeling factors BAF155/BAF170 and the epigenetic factor Kdm6b.

For complete details on the use and execution of this protocol, please refer to Narayanan et al. (2015).

BEFORE YOU BEGIN

SPOT method preparation

Timing: [1 h]

Note: It is possible to synthesize up to 10 μmol of peptide per cm2 Cellulose (Hilper et al., 2007). In some cases, however, a high peptide density can cause difficulties in binding assays when assessing interactions with the molecules of interest. In this case, lowering the peptide concentration on the peptide spot apparently resolves this problem (Kramer et al., 1999). In general, 5 to 10 nmol of peptide for each SPOT is appropriate for the peptide assays (Cushman, 2008).

1. Find the sequence of the proteins of interest from a public database (e.g., www.uniprot.org) for peptide synthesis.
2. Pour 1 L dimethylformamide (DMF) into the tank of SPOT robot.
3. Prepare 4 L bottle of DMF and connect it to the peptide synthesizer. When using a MultiPep 1 equipment (Figure 1A), this will be connected as Dilutor 2 solvent 1)
4. Next, prepare 2 L of ethanol and appropriately attach the bottle to the synthesizer. This will be Dilutor 2 solvent 2 of the MultiPep 1 equipment.

Note: All analytical grade reagents should be prepared in a fume hood. Store the reagents at 20°C–25°C.

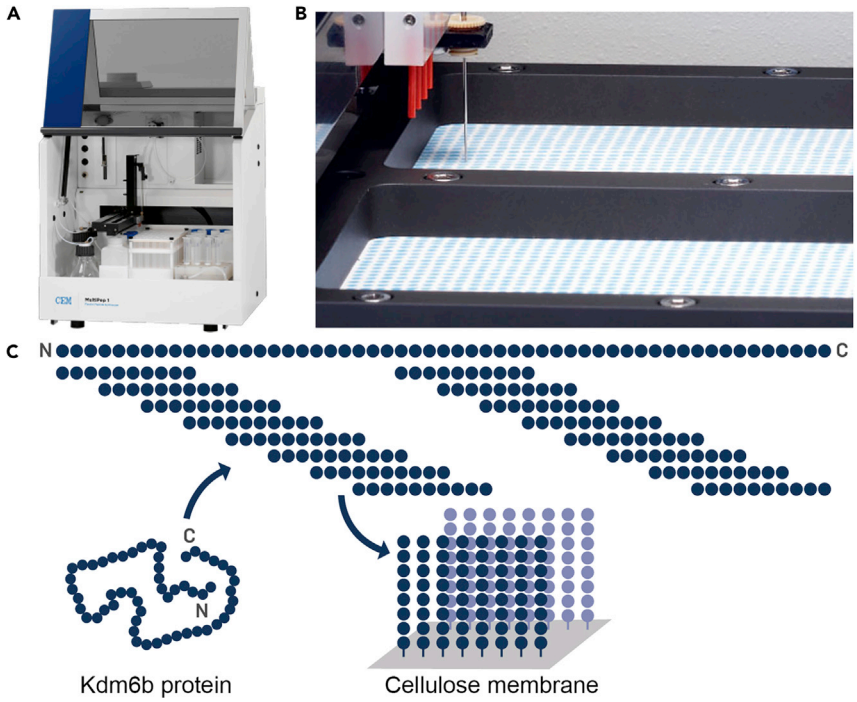

Figure 1. Synthesis of a large number of overlapping peptides using SPOT method

(A) CEM MultiPep 1 SPOT synthesizer was used to synthesize peptides.

(B) synthesized peptides were spotted on cellulose membrane for peptide assay.

(C) The entire sequence of a protein is synthesized as overlapping linear peptides covalently bound to a continuous cellulose membrane by the SPOT synthesis technique.

Note: The instructions refer to a CEM/Intavis MultiPep 1 SPOT synthesizer (Figure 1A), but may also be applied to other systems after modification (e.g., peptide synthesizer systems from CSBio, Protein Technologies or Biotage).

Peptide array synthesis

Timing: [1–2 days]

5. Thaw the amino acid (aa) derivatives of interest from −20°C to reach 20°C–25°C lasting for about 2 h. Prepare the appropriate amount of each aa derivative according to your peptide sequence (e.g., the amount of amino acids for KDM6B synthesis were prepared as follows: 3.9 mg Ala, 1.9 mg Cys, 2.8 mg Asp, 5.9 mg Glu, 3 mg Phe, 3 mg Gly, 3.3 mg His, 2.6 mg Ile, 3.7 mg Lys, 5 mg Leu, 1.3 mg Met, 2.6 mg Asn, 5.1 mg Pro, 4.3 mg Gln, 6.5 mg Arg, 4.7 mg Ser, 3.6 mg Thr, 3.8 mg Val, 2.3 mg Trp, 2.6 mg Tyr) and allocate them into 2 mL microcentrifuge tubes. Add N-methyl pyrrolidone (NMP) and dissolve the derivatives for 16 h with shaking in the fume hood.

   Note: When using the MultiPep software, the mass of aa needed for the preparation is calculated in the "REPORT" tab for 0.5 M solution per aa.

   Note: The volumes of aa derivatives solutions are calculated based on spot volume and the number of peptides for which the corresponding aa is required. Normally, the spot volume for an array of 20 spots/row is 0.2 μL.

6. To prepare the cellulose membrane, soak it in 30 mL of DMF in light-proof container for 1 h. Shake by gentle agitation every 15 min.

7. Weigh 2.973 g of hydroxybenzotriazole (HOBT•H_2O) and dissolve it into 20 mL of DMF to reach a final concentration of 1.1 M.
8. Add 6 mL of diisopropylcarbodiimide (DIC) to 29 mL DMF using a glass pipette to make a 1.1 M activator solution.
9. Prepare 20% of piperidine by diluting 64 mL of piperidine in 256 mL DMF to reach a total volume of 320 mL Fmoc deprotection solution.

   Note: The amount of Fmoc deprotection solution consumed is dependent on the cycle number.

10. Prepare 5% acetic anhydride as a capping reagent for amino acids. Mix 750 uL of acetic anhydride with 15 mL DMF in a conical tube with a glass pipette.

    Note: When using the MultiPep software, the amount of each reagent required is calculated in the "REPORT" tab. Reagents should be freshly prepared on the day of synthesis in a fume hood.

Side chain de-protection

Timing: [0.5 h]

11. Add 8.5 mL of trifluoroacetic acid (TFA) into the beaker using a glass pipette. Then add 200 μL of triisopropylsilane and 300 μL of ddH_2O sequentially to prepare the de-protection solution. 9 mL is required for each membrane.

    Note: Wear proper protective equipment and conduct all steps in a fume hood as TFA is very corrosive and volatile. The volatility of TFA makes it difficult to pipette without spillover. Place the de-protection mix beaker and the TFA bottle as close as possible to shorten the duration of TFA in the pipette. Using a manual pipette bulb is highly recommended to avoid the damage of mechanical pipettes caused by TFA.

12. Prepare 80 mL of Dichloromethane (DCM).
13. Prepare 80 mL of DMF.
14. Prepare 40 mL of 100% Ethanol.

HeLa cell culture

Timing: [2–3 days]

15. Culture the cells in 10 cm dishes with Dulbecco's modified eagle medium (DMEM) plus 5% FBS in an incubator at 37°C with 5% CO_2 supply.
16. On the day for transfection (day 0), cell density of around 3×10^6 cells/10 cm plate (85%–95% confluent) is suitable.

    Note: For large-scale purification or low-expression plasmids, 2–4 plates are required for each plasmid.

Cell lysis

Timing: [10 min]

17. We suggest adding protease inhibitor cocktail to the lysis buffer before use.

FLAG fusion protein immunoprecipitation solution

Timing: [20 min]

18. Mix 0.5 mL of 10× Wash Buffer with 4.5 mL ddH_2O for 1× Wash Buffer (5 mL/sample).
19. TBS containing 0.1% Tween-20 (TBST): Prepare 1 L of TBST wash buffer by adding 100 mL of 10× TBS and 1 mL Tween-20 detergent to 900 mL of MilliQ water.
20. Prepare blocking solution by dissolving 5% dried skim milk (w/v) in TBST. Keep at 4°C until needed.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Flag tag monoclonal antibody	Sigma	F2555 RRID:AB_796202
BAF155 Antibody	Santa Cruz	sc-48350X RRID:AB_671096
BAF170 Antibody	Santa Cruz	sc-17838 RRID:AB_2286337
HRP-conjugated goat anti-rabbit IgG	Abcam	ab6721 RRID:AB_955447
Chemicals, peptides, and recombinant proteins		
Dimethylformamide	Sigma	D4551
Ethanol	Supelco	1.00983
Fmoc-amino acids	Sigma	531480, 47349, 47672, 47618, 47695, 47674, 47625, 47627, 47639, 47628, 47633, 47624, 47634, 338338, 47636, 47619, 47622, 47561, 47623, 47638
N-Methyl pyrrolidone	Sigma	328634
Hydroxybenzotriazole	Sigma	157260
Diisopropylcarbodiimide	Sigma	D125407
Piperidine	Sigma	571261
Acetic anhydride	Sigma	320102
Trifluoroacetic acid	Sigma	T6508
Triisopropylsilane	Sigma	233781
Dichloromethane	Sigma	270997
Bromophenol blue	Sigma	B0126
Trizma® base	Sigma	T4661
NaCl	Sigma	S7653
Tween-20	Sigma	P9416
Milk powder	Roth	T145.3
Acylamid	Roth	3029.1
TEMED	Sigma	T8133
APS	Sigma	A3678
Critical commercial assays		
DMEM	Gibco	41966029
FBS	Gibco	10270106
Penicillin-streptomycin	Gibco	15070063
Opti-MEM™ I Reduced Serum Medium	Gibco	31985062
Lipofectamine 2000	Invitrogen	11668030
Cell culture PBS	Gibco	10010023
Trypsin-EDTA (0.25%)	Gibco	25200056
FLAG ® Immunoprecipitation Kit	Sigma	FLAGIPT1
Protease inhibitor cocktail	Sigma	P8340
Non-Reducing Sample Buffer	Thermo Scientific	39001
Protein standard	Bio-Rad	161-0374

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pierce™ ECL Plus Western Blotting Substrate	Thermo Scientific	32132
SuperSignal West substrate solution	Pierce	34080
Re-BlotWestern blot recycling kit	Chemicon	2060
BioMax film	Kodak	8701302
Experimental models: cell lines		
HeLa cells	N/A	N/A
Recombinant DNA		
pCAGGSBS-BAF155-Flag	(Tuoc et al., 2013b)	N/A
pCMV-BAF170-Flag	(Tuoc et al., 2013a)	N/A
Software and algorithms		
Multipep software	CEM	N/A
Other		
MultiPep 1 Automated Parallel Peptide Synthesizer	CEM	N/A
Cellulose membranes	Intavis AG	32.100
Microcentrifuge for 1.5–2.0 mL tubes	N/A	N/A
Benchtop centrifuge for 50 mL tubes	N/A	N/A
Vortex	N/A	N/A
Orbital shakers	N/A	N/A
SONOPULS HD2070 ultrasonic homogenizers	Bandelin	2451
FluorChem E System	ProteinSimple	92-14860-00
Vivaspin 20, 10,000 MWCO PES, 12pc	Sartorius	VS2001

MATERIALS AND EQUIPMENT

Alternatives: Cellulose membranes, containing synthesized peptides can be prepared by commercial companies (e.g., INTAVIS, JPT, PEPperPRINT). Stored the membranes at −20°C until ready for use.

Alternatives: Instead of using commercially available buffer for purification of FLAG-tagged protein, it is also possible to substitute it for self-made buffer.

Solutions for purification of FLAG-tagged protein

Lysis Buffer: 30 mL is stable for 24 months when stored at −20°C. Can be stored at 4°C for 1–2 weeks.		
Reagent	Final Concentration	Amount
Tris HCl; pH 7.4	50 mM	1.5 mL
NaCl	150 mM	0.263 *g*
EDTA	1 mM	60 µL
TRITON X-100	1%	300µL

2× Sample Buffer: 30 mL can be stored at 20°C–25°C for 24 months.		
Reagent	Final Concentration	Amount
Tris HCl; pH 6.8	125 mM	3.75 mL
SDS	4%	1.2 *g*
Glycerol	20% (v/v)	6 mL
Bromphenol blue	0.004%	0.804 mg

Wash Buffer and Elution Buffer: Can be stored at 2°C–8°C.	
Name	Reagent
10× Wash Buffer	0.5 M Tris HCl, pH 7.4; 1.5 M NaCl
Elution Buffer	0.1 M Glycine, pH 3.5

Solutions for electroblotting experiments: can be stored at 20°C–25°C for 24 months.	
Name	Reagents
Cathode Buffer	25 mM TRIS, 40 mM 6-Aminohexanoic acid, 20% Methanol adjust to pH 9.2
Anode Buffer I	30 mM Tris, 20% Methanol
Anode buffer II	300 mM Tris, 20% Methanol

Alternatives: The peptide membranes can be reused after stripping for new peptide analysis.

Solutions for stripping: can be stored at 2°C–8°C for 3 to 6 months.	
Name	Reagents
Stripping buffer I (10×)	Re-BlotWestern Blot Recycling kit (Chemicon, Cat# 2060)
Blocking Buffer (20×)	Re-BlotWestern Blot Recycling kit (Chemicon, Cat# 2060)
Stripping buffer IIIA	1% SDS; 8 M Urea; 0.1% 2-Mercaptoethanol
Stripping buffer IIIB	400 mL MilliQ Water; 100 mL Acetic acid; 500 mL Ethanol

Stripping buffer II: 30 mL is stable at 20°C–25°C for 24 months.		
Reagent	Final Concentration	Amount
6-aminohexanoic acid	40 mM	0.157 *g*
Tris	25 mM	0.091 *g*
Methanol adjusted to pH 9.2	20%	6 mL
2-Mercaptoethanol	0.14%	70 µL per 10 mL SDS buffer
SDS buffer	2%	6 mL

PBS Buffer (10× stock): 500ml is stable at 20°C–25°C for 12 months		
Reagent	Final Concentration	Amount
NaCl	1.37 M	40 *g*
KCl	27 mM	1 *g*
$Na_2HPO_4 \cdot 2H_2O$	76 mM	6.76 *g*
$NaH_2PO_4 \cdot 2H_2O$	6 mM	0.47 *g*
KH_2PO_4 adjust pH to 7.4 with HCl	15 mM	1.02 *g*

HeLa cell culture medium preparation: DMEM + 10% FBS. Can be stored at 4°C for 1 month.	
Reagent	Amount
DMEM	500 mL
Fetal Bovine Serum (FBS)	55 mL
Pen/Strep antibiotics	5.5 mL

Tris buffered saline (TBS) (10× stock): Dissolve 24 *g* Tris-base and 88 *g* NaCl in 900 mL MilliQ water and adjust to a pH of 7.6 with 12 N HCl before adjusting final volume to 1 L with MilliQ water. Stable for 24 months at 20°C–25°C.		
Reagent	Final Concentration	Amount
Tris-base	200 mM	24 *g*
NaCl	1500 mM	88 *g*
MilliQ water	NA	1 L

Coomassie staining buffer: stable for up to 12 months at 20°C–25°C.	
Reagent	Amount
Methanol	450 mL
ddH_2O	450 mL
CH_3COOH	100 mL
Coomassie Blue	1 *g*

Coomassie destaining buffer: stable for up to 12 months at 20°C–25°C.	
Reagent	Amount
Methanol	450 mL
ddH_2O	450 mL
CH_3COOH	100mL

STEP-BY-STEP METHOD DETAILS

Day 1: Designing the peptide array

Timing: [2 h]

Note: Multimedia presentation for procedure of peptide synthesis can be obtained from (Kudithipudi et al., 2014) and freely accessible at PubMed Central portal.

Note: Membranes are designed with the help of the spotter software.

1. Switch on the peptide synthesizer, its accessories, and start the instrument-controlling computer.
2. Run the peptide synthesis software on the computer. For example, perform the following steps (a–e) to run the spotter software when using the MultiPep 1 synthesizer from CEM (Figure 1A):
   a. From the "Edit sequence" function of the software, open the peptide sequence file and transform it into to the proper format, which is readable by MultiPep.
   b. Enter the protein name.
   c. 'peptide walk' is performed. Type the length of the peptides to be synthesized on the next line, and the number of overlaps between subsequent peptides (e.g., ".seq, 20,2" means each peptide contain 20 amino acids and shifted by two residues).
   d. Give the order: ".space." if you want to insert a single space between spots. The command ".newline." will leave the remaining row after your last peptide vacant.
   e. Enter ".end" to command the instrument to stop the peptide spot.
3. After inputting all the sequences, ensure all information are in the right format. For the MultiPep 1 program this is done by pressing the "clean sequence" tab.

Note: For the MultiPep software, 'SHOW PEPTIDES' will display the position of each peptide together with their designated number of lines or spaces, which have been inserted.

Note: A full-length purified protein can be used as positive control for the peptide-protein interaction.

⚠ CRITICAL: Prudently design your SPOT arrays to maximize yield and simplify use. Since the amount of consumables is irrelevant to the number of peptide spots, you can perform several parallel experiments in a single synthesis. Try to avoid separating one experiment over two blots.

Day 1–3: Peptide array synthesis

Timing: [3–4.5 Days]

Note: Peptide production is a repeated process, including a series of each aa adding. First, Fmoc is deprotected, and then washed with DMF, followed by an ethanol rinse, and air-dry of the membrane. Activated amino acids are subsequently spotted. A capping step can be added as an option. The final step is DMF wash. These procedures are cycled until final assembly of your desired peptides. After that, a side chain deprotection protocol will get rid of the side chain protection groups.

4. Put the aa derivatives into the loading tubes. Since we used the MultiPep 1 synthesizer (Figure 1A), we specifically did the following before loading the aa derivatives:
   a. Under the "EDIT METHODS" function, click "file"/"SPOTpreactiv."
   b. Calibrate and assemble the 1 mL syringe and the spotter needle.
   c. Prime the two dilutors to get rid of air bubbles.

   Note: For priming, switch on the vacuum pump and press 5 times to drain the waste reservoir and press the dilutor 2 solvent 1 prime button. Both DMF and ethanol in dilutor 2 require priming. The same goes for the solvent 2 after finishing solvent 1.
   d. Pipette the prepared aa derivatives into MultiPep 1 tubes in the fume hood.

   Note: Remember that each tube and cap should be well labeled.

5. Properly mount the membrane on the device frame in the fume hood. For instance, fix the frame into the MultiPep 1 machine and keep the vacuum tube attached to underneath of the frame.

   ⚠ CRITICAL: Avoid introducing any bubble or wrinkle on the membrane after placing it on the frame.

6. Wash the membrane in ethanol thrice.

   Note: If you find bubbles on the membrane or the membrane does not dry evenly, take the membrane off the frame and redo DMF washing.

7. Run the synthesis, for example, as in (a–d) when using MultiPep 1.
   a. Repeat the "File/SPOTpreactiv"step for peptide synthesis.
   b. Decap the reagents, which consists of 1.1 M DIC, 1.1 M HOBt•H_2O, 5% acetic anhydride solutions and 20% piperidine).
   c. Mount all reagents (1.1 M DIC, 1.1 M HOBt•H2O, 5% acetic anhydride solutions and 20% piperidine,) in the correct points on the substance stand of MultiPep 1 machine and place aa cylinders/tubes into the specified points on the stand.

   Note: Remove all the caps from the aa cylinders/tubes, before loading them into the stand.
   d. Unlid the 0.5 mL micro-centrifuge tubes and load into holders, which are beside the aa tubes.

   Note: Activation of amino acid derivatives is carried out in these mixing tubes. Activator is then mixed with amino acids in the 3:2 ratio.

   Note: Make sure reagents are in the correct place and the cabinet door is closed.
   e. Press the START button under "Run Synthesis" tab.

   Note: Check the reaction occasionally during the first cycle to make sure the spotting is running well. Usually it takes 3–4 days for a synthesis to complete, therefore you can keep the synthesis proceeding through the night. Keep track of the reagents over the course of the synthesis as the reagents may run out and require replenishment.

⚠ CRITICAL: Press the "PAUSE" button to pause the synthesis in order to refill reagents. The pause is only allowed, if the needle is disengaged from the reagent stand, the membrane, and aa tubes. It is suitable to halt during the countdown between deprotection steps.

8. Once the peptide synthesis is finished, discard all remaining reagents in proper waste containers.

Note: Collect the waste according to institutional hazardous waste rules. Connect a carboy to the synthesizer equipment during peptide synthesis.

9. Upon completion of the synthesis, mark (with pencil) the corners of the membrane and any other membrane position to be cut.
10. Take out the membrane and put it in a single solvent-resistant container slightly larger than the membrane.
11. Use 20 mL dH_2O to wash the membrane thrice for 5 min on a shaker.
12. Incubate the membrane in 0.02% bromophenol blue solution with gentle shaking at 20°C–25°C for about 1 h.
13. Rinse the membrane with dH_2O until background disappears. Peptide spots on the membrane should be observed.

Note: Weak signal for peptide spots at this step indicates synthesis problem.

14. Wash the membrane with ethanol and air-dry it at 20°C–25°C.
15. When the membrane is absolutely dry, use a pencil to draw a grid on the membrane which encircles each spot location.

⚠ CRITICAL: Do not use pen to draw the membrane, as pen marks would fade in deprotection phases.

Optional: Make either photocopy or scan the membrane for reference for spot map.

16. Transfer the membrane into a container labeled TFA in the fume hood, and incubate it in 15 mL of deprotection solution for 1.5 h.

⚠ CRITICAL: Cover the container with a lid. Agitate the container every 15 min.

17. Wash the membrane in 30 mL dichloromethane (DCM) four times for 10 min each.
18. Wash the membrane in 30 mL DMF four times for 2 min each.
19. Wash the membrane in 30 mL ethanol twice for 2 min each.

Caution: Waste deprotection and washing solutions in the deprotection steps must be collected in separate waste containers. TFA reacts strongly with DMF and can cause lesion to the skin when in contact. Usage of protective wears (e.g., glasses, nitrile gloves, coat) and working under the fume hood are strongly advised.

20. The membrane should be kept in the fume hood until it is thoroughly dry.
21. For a short-term storage for a month, the membrane can be loosely wrapped in tin foil and kept in a box at 4°C until peptide assay.

Pause point: For a long storage, the membrane can be kept at −20°C after being heat-sealed in a polyester bag.

Day 4: Transfection and cell culture

Timing: [2 days]

In many cases, especially for small proteins, the cDNA(s) encoding for the GST-fused protein could be cloned into a bacterial expression plasmid and the bacterially expressed GST-fused proteins then are purified for peptide assay as previously described (Luo et al., 2004). Compared with eukaryotes, prokaryotic cells lack protein folding system. Thus, bacteria-based expression and purification system might not be suitable for big proteins such as BAF155 (155 kDa) and BAF170 (170 kDa) as most large proteins form inclusion body. To avoid difficulty in protein purification, the exogenous BAF155-flag and BAF170-flag were overexpressed in mammalian cell system and were purified to examine their interaction with JMJD3 (Kdm6b).

Note: Cells are transfected with plasmids encoding for flag-tagged proteins.

22. Dilute 24 μg of the plasmid, for our purpose either CMV-BAF155-flag (Narayanan et al., 2018) or CMV-BAF170-flag plasmids (Xie et al., 2019), in 1.5 mL of Opti-MEM I medium and mix gently.
23. Dilute 60 μL of Lipofectamine 2000 in 1.5 mL of Opti-MEM I medium and mix thoroughly by inverting the tube.
24. After 5 min incubation, combine the Lipofectamine and DNA solutions (total volume is 3 mL).
25. Mix gently by slowly inverting the tube for a few times and incubate the solution for 20 min at 20°C–25°C, which allow DNA-Lipofectamine complex formation.

Note: Complexes maintain stability for 6 h at 20°C–25°C.

⚠ CRITICAL: Opti-MEM I Reduced (minimum 50%) Serum Medium, in resource table, must be used for plasmid transfection using Lipofectamine 2000.

26. Add the 3.0 mL of DNA-Lipofectamine solution to cell cultures and gently agitate the dish to dispense the complex evenly over the cells.
27. Place the dishes at 37°C in the CO_2 incubator for 24–48 h.

Note: Add the complex drop-wise and spread out to ensure even distribution.

Note: It is not necessary to remove the excess DNA-Lipofectamine complex by changing the medium.

Note: In order to avoid excessive cytotoxicity and maximize transfection efficiency, you can optimize transfection conditions by adjusting cell density along with DNA and Lipofectamine 2000 concentrations. Ensure that cells are greater than 90% confluent and modify Lipofectamine 2000 (μL): DNA (μg) ratios from 0.5:1 to 5:1.

⚠ CRITICAL: Do not add antibiotics to media during transfection as this will result in cell death.

Day 5: Cell lysis

Timing: [2.5 h]

Cells are first dissociated with trypsin and collected in a falcon. Cells are then lysed for subsequent protein extraction.

28. Wash cells by removing the medium and rinse twice with PBS buffer.

Note: Caution must be taken not to dislodge the cells.

29. Add 2.5 mL Trypsin for cell dissociation and incubate the cells for 5min at 37°C in the CO_2 incubator.

Note: Swirl the content gently for the Trypsin to cover the entire cell layer.

30. Add 7.5 mL medium to deactivate trypsin and gently dissociate cells by pipetting.
31. Then move cells to a 50 mL falcon.

Note: Pre-warm the growth medium and trypsin solution to 37°C.

⚠ CRITICAL: Avoid cell exposure to trypsin solution for more than 10 min, otherwise this will damage cell membranes and lead to cell death.

32. Centrifuge at 1000 rpm for 5 min.
33. Rinse with PBS twice.
34. Add 1–1.5 mL lysis buffer with protease inhibitor.
35. Sonicate the cell lysate (50% duty cycles, 5 output control, 3 pulsed)
36. Place the lysates on a shaker with agitation for 15–30 min.
37. Centrifuge the lysates for 10 min at 12000 × *g*.
38. Move the supernatant to a tube on ice.

Pause point: Lysates can be kept at −80°C for later use.

⚠ CRITICAL: If there are still clumped cells in lysates, the protein extracts should be filtered using a 0.45 μm strainer.

Optional: Save 1/30 volume of the lysate (sample S1, Figure 2) to test the expression of FLAG fusion protein by Western blotting or coomassie blue analysis.

Day 5 and 6: Enrichment of the FLAG-tagged protein

Timing: [6–7 h]

Flag-fused proteins in a large-scale could be affinity-immunoprecipitated and purified, using FLAG-M2 antibody. The immunoprecipitation is done with FLAG-M2-coupled affinity gel, an antibody-based purification resin. Subsequently elute the immunoprecipitated FLAG-fused proteins from the resin with acidic buffer or by competition with the FLAG peptide. Proteins are ultimately concentrated to remove solvents and undesirable molecules.

Optional: It is recommended to include two control reactions. The positive control is immunoprecipitation with a known FLAG- tagged protein and the negative control is an un-transfected sample.

Note: Using a column for the immunoprecipitation reaction is highly recommended to avoid loss of resin during the wash and elution steps. Therefore, we placed a spin column (with FLAG-M2- coupled resin) in a centrifuge tube in the following procedure.

Note: Multimedia presentation for procedure of ECL Plus detection can be obtained from (Ma et al., 2017, Chen et al., 2014) and freely accessible at PubMed Central portal.

39. Rinse the empty column with 1× washing buffer.
40. Load 0.8 mL of anti-FLAG M2 affinity gel on the column. Suspend the resin thoroughly. To achieve homogeneous suspension of the resin, use a 2:1 proportion of the suspension to packed gel volume.

Note: Enlarge plastic pipette tip by cutting to allow easy transfer of resin.

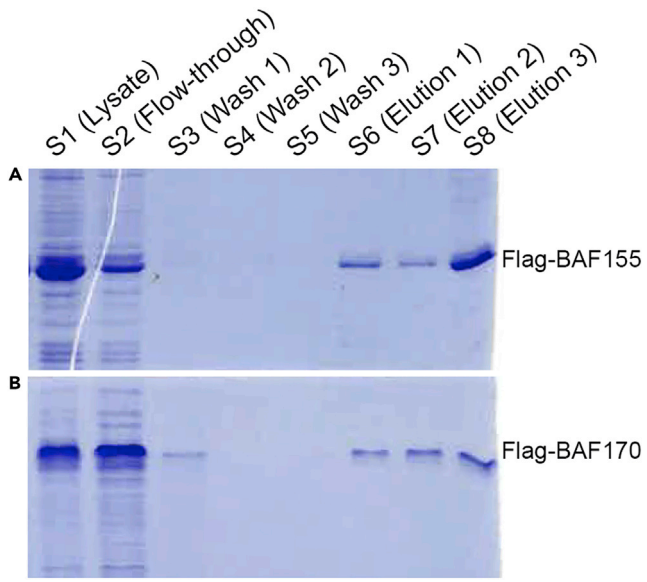

Figure 2. Expression and purification of Flag-BAF155 and Flag-BAF170 from mammalian culture system

(A and B) Coomassie blue staining was performed to check the expression and purity of Flag-BAF155 (A) and Flag-BAF170 (B). Different samples such as lysate (S1), flow-through (S2), in washing steps (S4 and S5) and in elution steps (S6–S8) during protein purification were collected for the staining.

Note: Calculate required reagent volumes based on the amount of samples to be processed.

41. Spin the resin for 30 s at 5000–8200 × *g*.
42. Discard the flow-through.
43. Rinse the column with 1 mL 1× washing buffer and centrifuge to remove the wash solutions.
44. Wash with 1 mL of elution buffer thrice, allowing each milliliter to pass through the column before making the next addition.
45. Pass 10 mL of 1× washing buffer through the column, pipetting the beads up and down to accelerate the process.
46. Add cell lysate to the washed resin.

⚠ CRITICAL: This is a slow process. Therefore, prevent beads from drying out during the process.

47. Agitate the sample gently on a shaker for 2 h.

Note: Passing cell lysates several times through the column may increase the amount of extracted proteins.

Note: Agitating the sample on shaker for up to 16 h might increase the binding efficiency of proteins to resin.

Optional: Collect the lysate that has passed through the column (sample S2, Figure 2) for Western blotting or coomassie blue analysis. The efficiency of the column in removing FLAG fusion protein from the cell lysate can be determined by how much remaining FLAG fusion protein is in S2 compared with the starting material (S1).

48. Spin the resin for 30 s at 5,000–8,200 × *g* and discard the flow-through.

49. Wash the beads with at least 3 column volumes of ice-cold washing buffer.

Optional: Collect the washing buffer that has passed through the column (sample S3–S5, Figure 2) for Western blotting or coomassie blue analysis.

50. Elute the proteins with 3× 2 mL of the elution buffer. Incubate the sample at 20°C–25°C with gentle shaking for 5 min. Then centrifuge the resin for 30 s at 5000–8200 × *g*. Allow the beads to drain between each addition and collect 1 mL of fractions in separate tubes which contain 50 μL of 1 M Tris (pH 8) (S3–S8).

⚠ CRITICAL: All steps should be carried out at 20°C–25°C. The resin should not be left in the elution buffer longer than 20 min.

Optional: Save 30 μL elution buffer that has passed through the column (sample S6–S8, Figure 2) for Western blotting or coomassie blue analysis.

Optional: Protein can also be eluted under native condition by competing with 3× FLAG peptide, which brings about high elution efficiency. Prepare 3× FLAG elution buffer by mixing 180 μL of 5 μg/μL 3× FLAG peptide solution with 6 mL of 1× wash buffer to make a final concentration of 150 ng/mL). Add 6 × 1 mL of 3× FLAG elution buffer to the resin. The sample is then incubated for 30 min at 4°C with gentle shaking. Collect the fractions by centrifuging the resin for 30 s at 5000–8200 × *g*.

51. Wash the beads with 2 column volumes of elution buffer, pipetting the beads up and down to accelerate the process.
52. Neutralize the beads with 2 column volumes of PBS.

Note: For long term storage of the column, add sodium azide (to 0.1% v/v final concentration) directly to the PBS in the column. Seal the column and store at 4°C.

Pause point: The eluted fractions can be stored at −20°C for long term storage.

53. Save 20 μL samples (S3 to S8) for blotting and coomassie blue staining to validate the purity of purified proteins (Figure 2).
    a. Prepare blotting samples by adding 10–50 μg of lysates or purified protein (above samples S1–8) into 12 μL total volume of Non-Reducing Sample Buffer (Cat#39001, Thermo Scientific).
    b. Load samples in SDS-PAGE gel (7.5%–12%).
    c. Run SDS-PAGE as standard protocol for blotting.
    d. After completing the SDS-PAGE running, move the gel into a staining box containing Coomassie staining buffer.
    e. Gel should be incubated in the buffer for 2 h with slow shaking.
    f. After staining, pour off the Coomassie staining buffer into a bottle marked "Used Coomassie staining buffer".

Note: Coomassie staining buffer can be re-used for several times, provided fresh 10% acetic acid is added.

    g. Add appropriate amount of the Coomassie destaining buffer to completely cover the gel and incubate the gel with slow shaking for some hours until protein bands are visible.
    h. After completing the destaining process, gel can be dried by using a gel dryer for documentation purpose. Coomassie blue staining analysis revealed a high purity of purified Flag-BAF155 and Flag-BAF170 proteins as their elution samples (elution 1–3, samples S6-S7, Figure 2) show only one band.

Note: The expression and purity of proteins in the samples can also be confirmed by Western blot analysis with anti-FLAG or antibodies against the protein(s) of interest, in our case anti-BAF155/BAF170.

Note: amount of purified proteins could be also measured by densitometry or standard BSA-based Bradford assay.

54. Pool the eluted fractions containing FLAG tagged protein as detected by the Western blot analysis.
55. Place the pooled fractions from the above steps in a Vivaspin centrifugal concentrator. Fill the concentrator to the maximum volume.

Note: In this protocol, we used VIVASPIN20, 10,000MWCO PES. The selection of the most MWCO depends on the molecular size of the sample. To obtain maximum recovery, choose a MWCO 1 to 2 the molecular size of the species of interest.

Note: Dilute the sample up to the maximum volume before the first centrifugation step for samples smaller than the maximum device volume. This will speed up the salt removal rate.

56. Centrifuge for 30 min at 1000 × *g*.
57. Empty the filtrate container. Refill the concentrator using fresh buffer to make concentrate back up to original volume.
58. Centrifuge again as before.
59. Empty the filtrate container. Repeat the process to achieve considerable reduction in the concentration of contaminating microsolute. Transfer the concentrated, de-salted sample from the bottom of the concentrate pocket to new tubes. Keep the sample on ice.

Note: Three wash cycles can typically remove 99% of initial salt content.

⚠ CRITICAL: Some proteins may stick to the ultrafiltration membrane. It is possible to take them off the membrane using blocking solution.

Pause point: For long storage, it is important to freeze the protein preparation. Freeze it rapidly with dry ice/ethanol mixture or using liquid nitrogen in an attempt to prevent denaturation. Additionally, it is suggested that small aliquots of the solution be frozen to prevent multiple thaw-freeze cycles that may impair the biological activity or alter the structure of proteins. Add stabilizing agents such as glycerol (50% [w/v]). Store the sample at −20°C.

Day 7–9: Peptide analysis

Timing: [3.5 days]

Membrane containing the synthesized JMJD3/Kdm6b peptides was washed and then incubated with the mixture of purified Flag-BAF155 and Flag-BAF170 proteins for 16 h. On the next day, membrane was washed and exposed to anti-Flag antibody, and a HRP-conjugated secondary antibody. Peptides on membranes were scanned for interaction between BAF155/BAF170 and synthesized peptides using enhanced chemiluminescence (ECL) reagents.

60. Soak the membrane in about 5–10 mL of methanol for 5–10 min.

Note: The above step helps to prevent the aggregation of hydrophobic peptides in the following TBST washing steps.

61. Rinse the membrane with TBST with four times shaking for 5 min each.
62. Block the membrane with the prepared blocking solution for 1 h at 20°C–25°C.
63. Move the membrane into new blocking, containing 5 μg/mL of purified Flag-BAF155 and Flag-BAF170 protein solution and incubate it for 16 h at 4°C.

Note: Different proteins may require different concentrations to achieve optimal interaction affinity. The purity of proteins is also a factor that influences the amount of protein used for the experiment. Thus, appropriate concentrations of protein should be used according to one's experience.

64. Rinse the membrane with TBST with shaking for four times, 5 min each.
65. Detect the protein-peptide binding by incubating the blocked membrane first with anti-Flag (dilution 1:1000) primary antibody in blocking solution.
66. Keep the membrane at 4°C with slow shaking for 16 h.
67. Rinse the membrane with TBST with shaking for four times, 5 min each.
68. Incubate the membrane in blocking solution, containing appropriate HRP-conjugated secondary antibody (dilution 1:5000) at 20°C–25°C rocking for 1–2 h.
69. Rinse the membrane with TBST with shacking for four times, 10–15 min each.
70. Detect signals of HRP reaction using ECL Plus detection reagents.
    a. Cover the membrane with 1 mL of SuperSignal west substrate solution and incubate for 1 min.
    b. Place the X-ray film against the membrane in a light-proof cassette and expose for an appropriate time period from 15 s to 10 min.
    c. Develop the X-ray film with an X-ray film developer.

Note: To obtain an optimal signal, several X-ray films should be exposed for different time periods (e.g., 15 s, 1 min, 3, min, 5 min and 10 min).

Note: Multimedia presentation for procedure of X-ray film signal detection with chemiluminescent reagents can be obtained from (Gallagher and Chakavarti, 2008) and freely accessible at PubMed Central portal.

Optional: If detection of the protein-peptide binding by anti-Flag primary antibody causes a high background or low signal on the X-ray film or for interaction detection of synthesized peptides with either BAF155 or BAF170, the membrane can be stripped (see below steps: 71–88) and should be incubated with either anti-BAF155 or anti-BAF170 antibody (dilution 1:200).

Note: The membrane can be re-used several times to detect the interaction of the spotted peptides and proteins of interest.

⚠ CRITICAL: If the membrane is not directly used in the next experiment, it should be wetted with TBST solution and kept in a heat-sealed polyester bag for a long-term moisture condition at 2°C–8°C. Avoid membrane dry out.

Stripping and re-probing

Stripping to remove transient PPI, primary and secondary antibodies, and re-probing of the membrane should be done for several purposes: (1) analysis of a given membrane using different purified protein and antibodies; (2) confirmation of the results with the same or different antibody; (3) correction of errors or improvement of exposing signal (e.g., high background, weak signal) by incubation of the membrane with new antibody or under new condition.

The below three protocols (stripping protocol 1–3) describe the stripping procedure with their increasing harshness. It should be noted that the stripping can be performed for several times for

new staining, although the signal on exposed X-ray films from the membrane may become weaker with higher background after additional stripping round(s). Generally, the membrane should be tripped with the milder protocol (stripping protocol 1) first. With this gentle condition, the stripping on membrane could be repeated for probing with several new proteins. If the signal is still detected after stripping with the protocol 1, then the membrane should be stripped with harsher conditions (protocol 2 or 3). Nevertheless, membrane can be stripped only limited number of times in these stringent conditions.

Stripping protocol 1

Timing: 1 h

This protocol uses Re-BlotWestern Blot Recycling kit (Chemicon, Cat# 2060), which contains 10× Stripping buffer I and 20× milk-based Blocking Buffer. By using this protocol, the membrane can be re-used for several peptide assays.

71. Incubate membrane in 1× stripping buffer I with gentle shaking for 10–15 min at 20°C–25°C.

Note: It may be necessary to increase the stripping incubation time (additional 5–10 min) when using membrane that have been stripped previously.

72. Add an equal volume of 1× blocking buffer.
73. Wash/rinse blots two times 5 min with PBS.
74. Check the success of the stripping by exposing the membrane with ECL Plus detection reagents and with X-ray film for about 10 min to confirm the complete removal of transiently interacting protein and bound antibodies.
75. Re-block for 10 min with 5% milk in PBS.
76. The blot is now ready for reprobing with protein (e.g., purified Flag-Brg1 protein) and antibodies (e.g., Flag or Brg1 antibody) as in the above steps: 63–70.

Stripping protocol 2

Timing: 2 h

77. Rinse the membrane thrice with ddH_2O for 10 min.
78. Rinse the membrane with stripping buffer II at least four times for 30 min at 50°C.

Note: Avoid temperatures more than 50°C since it will cause damage to the membrane and/or the peptides.

79. Wash the membrane with 10× PBS at least thrice for 20 min each at 20°C–25°C.
80. Wash thrice for 20 min with TBST at 20°C–25°C.
81. Wash thrice for 10 min with TBS at 20°C–25°C.
82. Check the success of the stripping by exposing the membrane with ECL Plus detection reagents and with *X-ray film* for about 10 min to confirm the complete removal of transiently interacting protein and bound antibodies.

Stripping protocol 3

Timing: 1 h

83. If signal is still observed in the above protocol 2, then rinse the membrane with ddH_20 twice for 10 min each.

84. Incubate the membrane with stripping buffer IIIA three times for 10 min each.
85. Incubate the membrane with stripping buffer IIIB three times for 10 min each.
86. Rinse the membrane with ddH20 twice for 10 min each.
87. Wash the membrane with TBST three times for 10 min each.
88. Check the success of the stripping by exposing the membrane with ECL Plus detection reagents and with *X-ray film* for about 10 min to confirm the complete removal of transiently interacting protein and bound antibodies.

Optional: If spots still remain repeat stripping protocol 3.

EXPECTED OUTCOMES

A successful peptide assay should show a strong signal on the membrane with low background. Each dark spot within the grid indicates binding of the protein to a specific peptide (Figures 3A and 3B). The intensity of each dot can be quantified by densitometry using ImageJ and Prism. An example of interaction between a purified Flag-fused BAF155 and BAF170 protein mixture and Kdm6b/JMJD3 peptides is shown in Figure 3. The peptides 462–463 within the conserved catalytic JmjC domain were identified to mediate the interaction between chromatin remodeling factors BAF155/BAF170 and H3K27 demethylases UTX/Kdm6a and JMJD3/Kdm6b (Narayanan et al., 2015). The experiment was easy-to-replicate. The data were reproducible with the customized Kdm6b peptide array, which was obtained from JPT Peptide Technologies. The study can be extended to identify key amino acid(s), which are indispensable for PPIs. For that purpose, similar peptide(s), however, with point mutations or with substitutions of amino acid(s), should be generated.

LIMITATIONS

Although peptide SPOT array is very much suitable for determining peptide binding domain within proteins that are challenging to generate and to purify in classical biochemical approaches, the method has some drawbacks. (1) In many cases, PPIs are mediated by their large peptide domain. Typically, peptides, which are synthesized on cellulose membrane (e.g., Intavis AG, 32.100) by SPOT method, are small (about 20 amino acids) and are not sufficient for interacting with the proteins of interest. (2) Interaction between proteins requires their three-dimensional structures. The synthesized peptides with their primary structure on cellulose membrane might not truly mimic the structure of their full-length protein.

TROUBLESHOOTING

Problem 1

Low yield of eluted protein (in Step 53g)

Potential solution

It may be caused by either low expression level, low binding efficiency, or low elution efficiency. To increase binding efficiency, we recommend increasing the amount of lysate (e.g., lysate from two 10 cm tissue-culture dishes) and/or prolonging the time for binding. Try any of the following to improved elution: (1) use more 3× FLAG in the elution solution, (2) supplement the buffer for elution with salt (0.1 M glycine, pH 3.5), (3) add a detergent, e.g., Sodium Cholate or CHAPS [(3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)].

Problem 2

No signals for peptide spots on the membrane (Step 13 in peptide array synthesis section)

Potential solution

If signal for peptide spots on the membrane is very weak or is not observed after washing to get rid of background during the synthesis procedure in Step 25, then it is likely due to synthesis problem. Therefore, it is better to synthesize a new peptide membrane.

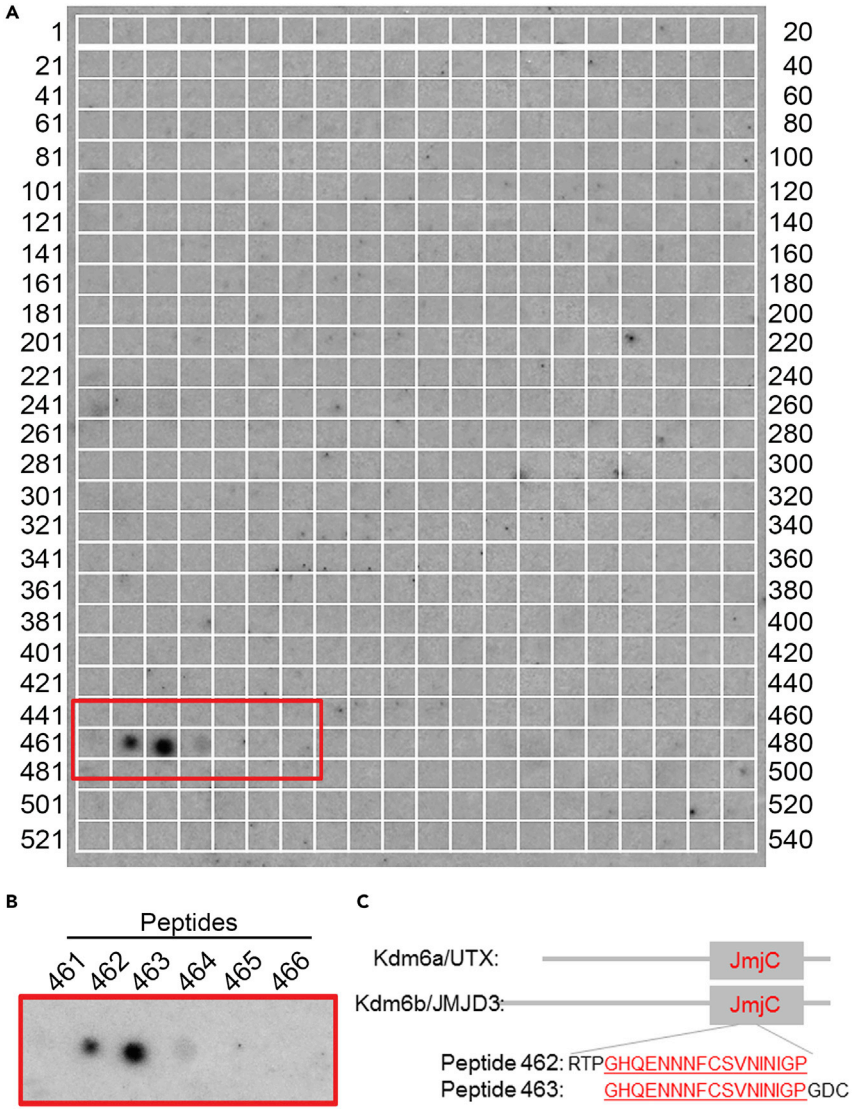

Figure 3. Peptide assay showing BAF155/BAF170-binding sequence of Kdm6b

(A and B) Fully developed film (A) and selected area (B) of a peptide array for a purified Flag-fused BAF155 and BAF170 protein mixture and spotted Kdm6b/Jmjd3 peptides. The membrane contains the synthesized peptides (peptide length: 20 aa, overlap: 17 aa) through protein sequence.

(C) The amino acid sequences of the binding area within conserved JmjC domain of Kdm6a/UTX and Kdm6b/Jmjd3 demethylases. Figure reprinted with permission from (Narayanan et al., 2015).

Problem 3

Weak signals on the exposed X-ray film (in Step 70c)

Potential solution

If the signals on X-ray film is weak after long enough exposure (about 15 min), you can improve the signal by following solutions:

a) Increase the antibody concentrations.
c) Shorten the washing times. Use TBS instead of TBST.
d) Try a supersensitive chemiluminescence substrate.
e) Use different antibodies.

Problem 4

High background on the exposed X-ray film (in Step 70c).

Potential solution

Increase the concentration of detergent in washing buffer, the washing duration, and/or the washing volumes. A reduced concentration of antibody or alternative ones should also be considered. The exposing time for X-ray film could be also reduced.

Problem 5

Clear spots on dark background (in Step 70c).

Potential solution

You can try reducing the primary and/or secondary antibody concentrations. A high amount of antibody conjugate leads to exhaustion of all the substrate before exposure. If this happens, consider increasing the washing times with TBST and redetect or simply stripe the membrane and re-incubate with lower concentrations of proteins.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tran Tuoc (Tran.Tuoc@ruhr-uni-bochum.de)

Materials availability

Materials are available upon request.

Data and code availability

The data used in this protocol are from our published study (Narayanan et al., 2015)

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AUTHOR CONTRIBUTIONS

X.M. and T.T. contributed to establishment of protocols. J.S. and H.P.N. provided research tools and contributed to discussions. X.M., G.S., and T.T. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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