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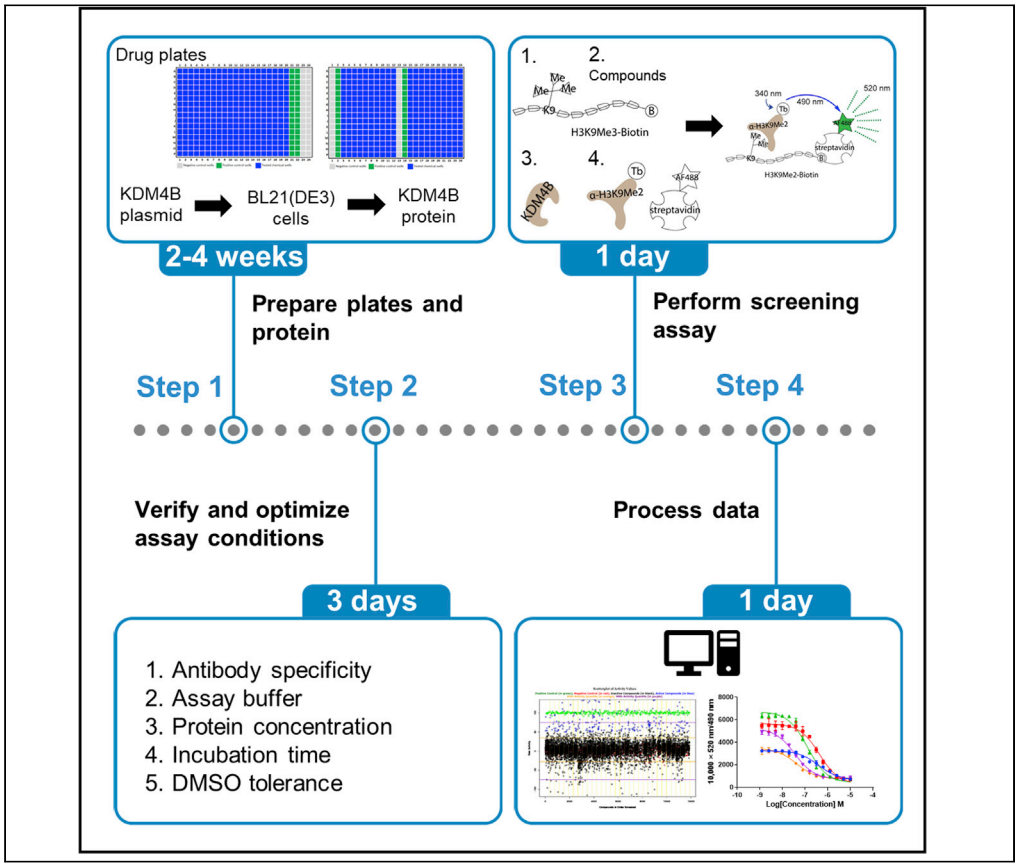
A protocol for high-throughput screening of histone lysine demethylase 4 inhibitors using TR-FRET assay



Identification of diverse chemotypes of selective KDM4 inhibitors is important for exploring and validating the roles of KDM4s in the pathogenesis of human disease and for developing therapies. Here, we report a protocol for high-throughput screening of KDM4 inhibitors using TR-FRET demethylation functional assay. We describe this protocol for screen of KDM4B inhibitors, which can be modified to screen inhibitors of other JmjC-domain-containing KDMs.

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Highlights

Describes protein expression and purification of KDM4B catalytic domain

Describes preparation and optimization of KDM4B TR-FRET reagents and conditions

Describes high-throughput KDM4B TR-FRET screening procedure

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A protocol for high-throughput screening of histone lysine demethylase 4 inhibitors using TR-FRET assay

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SUMMARY

Identification of diverse chemotypes of selective KDM4 inhibitors is important for exploring and validating the roles of KDM4s in the pathogenesis of human disease and for developing therapies. Here, we report a protocol for high-throughput screening of KDM4 inhibitors using TR-FRET demethylation functional assay. We describe this protocol for screen of KDM4B inhibitors, which can be modified to screen inhibitors of other JmjC-domain-containing KDMs. For complete details on the use and execution of this protocol, please refer to Singh et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps for using KDM4B. However, we have also used KDM4A in this protocol.

Prepare compound plates, proteins, and buffers.

Timing: [1 week]

1. Make compound stock solution plates.
   a. For compounds in powders, dissolve them in DMSO to prepare stock solutions at 10 mM.
   b. For primary single dose screen, compound stock solutions in DMSO are pre-arrayed in barcoded 384-well compound plates (Corning 3656) (Cui et al., 2011; Lin et al., 2021) at 10 μL/well in areas of columns 3-to-12 and 15-to-24; wells in columns 1, 2, 13 and 14 are empty (Figure 1A).
   c. For dose response test, a series of 1-to-3 dilutions of compound stock solutions (10 concentration levels with the highest concentration at 10 mM) are arrayed in barcoded 384-well compound plates (Corning 3656) at 6.6 μL/well in areas of columns 1-to-20 (Yang et al., 2020) (Figure 1B).
   d. For long term storage, stock solutions are stored at −20°C.
2. Perform Liquid chromatography–mass spectrometry (LC-MS) analysis to detect purity of the compounds to be tested.

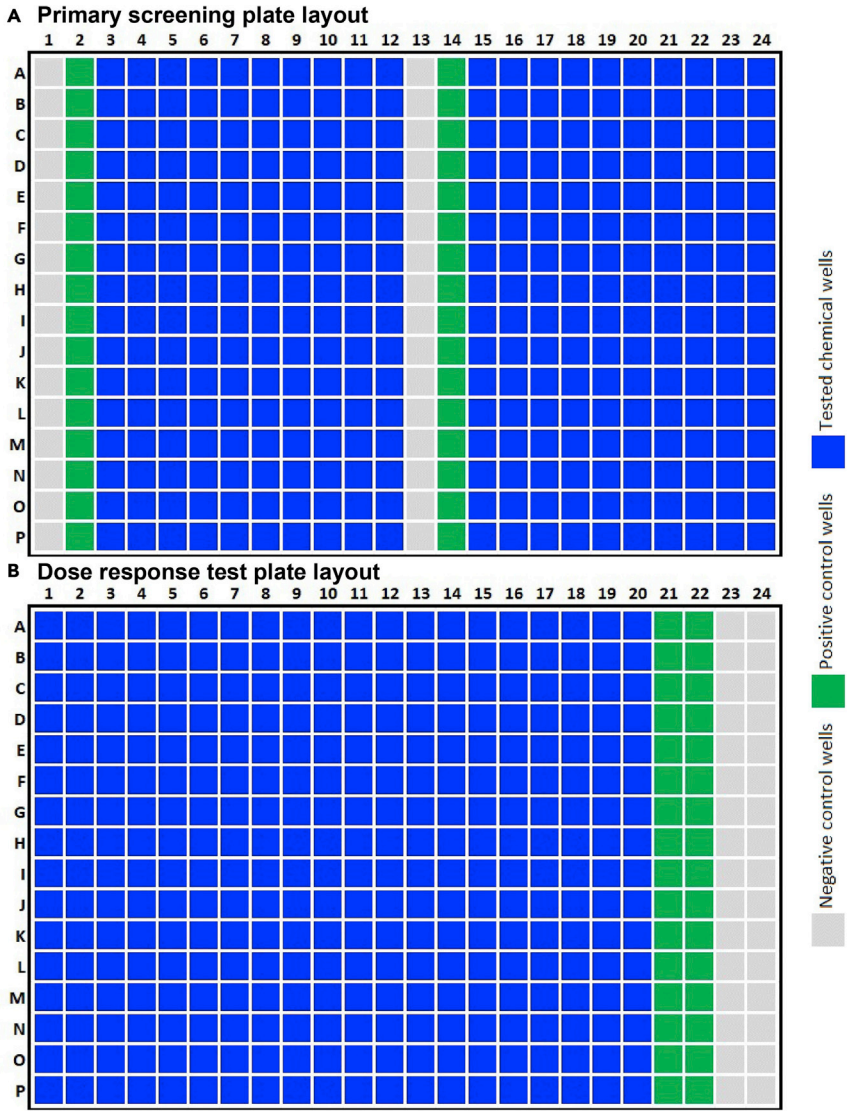

Figure 1. Plate layouts for primary single dose screen and for dose response test

(A) Plate layout for primary single dose screen.

(B) Plate layout for dose response test.

Grey indicates negative control wells. Green indicates positive control wells. Blue indicates tested chemical wells.

Note: Compounds stored for a long time may suffer stability problem, and thus the purity and concentration of compound could vary. We used HPLC with a chemiluminescent nitrogen detector (HPLC-CLND) to test the concentrations of the compounds. For more details about the management and quality control of St Jude compound library, see (Nishiguchi et al., 2021).

3. Prepare a control plate with DMSO (10 μL/well) dispensed into wells in columns 1, 2, 13 and 14 for the primary screen, or into wells of columns 21-to-24 for dose response test.

Prepare KDM4B protein

Timing: [2–4 weeks]

4. Express KDM4B protein

a. Subclone the catalytic domain of KDM4B (1–348) into pET28a(+) to produce the N-terminal 6xHis-tagged KDM4B protein.
b. Transform KDM4B plasmid into BL21(DE3) cells and culture the cells in LB media containing 100 μg/mL kanamycin at 37°C until A600 reaches 0.6.
c. Induce KDM4B protein expression using 0.1 mM IPTG at 16°C for 20 h.

5. Purify KDM4B protein
   a. Collect cells by centrifugation and re-suspend them in lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl). Cells are disrupted by a microfluidizer and cell debris is removed by centrifugation.
   b. The supernatant is loaded onto a Ni affinity column in lysis buffer and eluted with a linear gradient of imidazole from 50 to 300 mM.
   c. The A280 peak containing KDM4B is collected and further purified with size-exclusion chromatography on an AKTA system (running buffer: 50 mM Tris-HCl, pH 7.5, 500 mM NaCl).
   d. Concentrate the purified protein to 4 mg/mL, flash freeze it in liquid N_2, and stored at - 80°C.

Note: 3. Purify KDM4A protein

The procedure is very similar to KDM4B. Lysis buffer, 50 mM Tris-HCl, pH 8.2, 300 mM NaCl, 10% glycerol, 1 mM TCEP; size exclusion running buffer, 20 mM Tris-HCl, pH 8.2, 300 mM NaCl, 10% glycerol, 1 mM TCEP; storage 0.9–1.3 mg/mL at - 80°C.

Prepare assay buffers

6. Prepare buffers and solutions according to the tables in materials and equipment section

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Terbium-conjugated (Tb)-anti-Histone H3K9Me2 antibody (Tb-anti-H3K9Me2)	Invitrogen	A14151
Bacterial and virus strains		
BL21(DE3)	Thermo Fisher Scientific	EC0114
Chemicals, peptides, and recombinant proteins		
Tris-HCl pH 8.0, 1 M	Invitrogen	15568025
α-Ketoglutarate	Sigma	75890-100G
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	Sigma	FX0245-1
L-Ascorbic acid	Sigma	A4544-25G
H3K9Me3-Biotin	AnaSpec, Inc.	AS-64628
H3K9Me2-Biotin	AnaSpec, Inc.	AS-64627
H3K9Me1-Biotin	AnaSpec, Inc.	AS-64626
H3K9Me0-Biotin	AnaSpec, Inc.	AS-61702
AF488-streptavidin	Invitrogen	S11223
Bovine serum albumin (BSA), 30%	Sigma	A7284-50ML
LB media	Fisher	1.10285.0500
Kanamycin	EMD Millipore Corporation	BP906-5
Recombinant DNA		
KDM4B (1–348) construct	?	?
Software and algorithms		
GraphPad Prism 8.0	GraphPad Software	San Diego, CA

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
384-Well compound plates	Corning Incorporated Life Sciences	3656
Black low volume 384-well assay plates	Corning Incorporated Life Sciences	3821BC
V&P 384-well pintool	V&P Scientific, Inc.	San Diego, CA
PHERAstar *FS* plate reader	BMG LABTECH Inc., Corning Incorporated Life Sciences	Durham, NC 3656
Wellmate	Matrix Technologies Corp.	Hudson, NH 03051
HisTrap HP, 5 mL	Amersham Biosciences	17-5248-01
HiLoad 26/60 Superdex 75 prep	Amersham Biosciences	17-1070-01

MATERIALS AND EQUIPMENT

Assay Buffer		
Reagent	Final concentration	Amount
Tris-HCl (pH 8.0), 1 M	50 mM	2.5 mL
α-ketoglutarate, 500 mM	1 mM	100 μL
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 400 mM	80 μM	10 μL
L-Ascorbic acid, 100 mM	2 mM	1 mL
BSA, 10%	0.01%	50 μL
H_2O	n/a	Up to 50 mL
Total	**n/a**	**50 mL**

Note: Tris-HCl (pH 8.0, 1 M) is stored under 18°C–22°C. BSA (10%) is diluted from the commercial 30% BSA with in-house Milli-Q water and stored at 4°C. Stock solution of 500 mM α-ketoglutarate, 400 mM $Fe(NH_4)_2(SO_4)_2$ and 100 mM L-Ascorbic acid are prepared freshly from powder each time before assay. The buffer pH has not been readjusted after the buffer prepared based on the formula.

H3K9Me3-Biotin solution	
1.5 μM H3K9Me3-Biotin	75 μL of 1 mM stock solution, fill up to 50 mL with double distilled H_2O

H3K9Me3-Biotin is prepared as a 1 mM stock solution in DMSO and is stored at −80°C. An aliquot is thawed for each assay. Prepare 1.5 μM of working solution by mixing stock solution with assay buffer described above.

KDM4B protein solution	
KDM4B protein solution	34mg KDM4B protein in 1 mL storage buffer = 750 nM

KDM4B protein is stored at −80°C in the storage buffer (50 mM HEPES (pH 7.5), 500 mM NaCl, 15% Glycerol). An aliquot is thawed for each assay. Prepare 750 nM of working solution by mixing stock solution with assay buffer described above.

Detection reagent		
Reagent	Final concentration	Amount
AF488-streptavidin, 10 μM	80 nM	0.4 mL
Tb-anti-H3K9Me2, 1 μM	8 nM	0.4 mL
Assay buffer	n/a	49.2 mL
Total	n/a	50 mL

AF488-streptavidin (1 mg/unit) is reconstituted with 1.8 mL Milli-Q water to make a 10 μM stock solution. AF488-streptavidin and Tb-anti-H3K9Me2 (1 μM) are stored at −80°C. Aliquots of Tb-anti-H3K9Me2 (1 μM) and AF488-streptavidin (10 μM) are thawed for each assay. Prepare 8 nM of Tb-anti-H3K9Me2 and 80 nM of AF488-streptavidin working solution by mixing stock solution with assay buffer described above.

Note: AF488-streptavidin is light-sensitive and should be stored in an opaque container to minimize from light exposure.

STEP-BY-STEP METHOD DETAILS

Verify and optimize assay conditions

Timing: [3 days]

1. Verify the specificity of the Tb-anti-H3K9Me2 antibody against H3K9 peptides with 4 different methylation status (H3K9Me0, H3K9Me1, H3K9Me2, H3K9Me3)
   a. In a 384-well black low volume assay plate, 10 μL/well of H3K9Me0-Biotin, H3K9Me1-Biotin, H3K9Me2-Biotin or H3K9Me3-Biotin at 200 nM or 500 nM is first dispensed, followed by 10 μL/well of Tb-anti-H3K9Me2 (4 nM) and AF488-Streptavidin (40 nM).
   b. The mixture is set still and incubated for 30 min under room temperature (18°C–22°C).
   c. Detection of the TR-FRET signals (fluorescence emission ratio of 10,000 × 520 nm/490 nm) of each group using a PHERAstar FS plate reader.

   Note: This test is to demonstrate the specificity of Tb-anti-H3K9Me2 antibody to the H3K9Me2-Biotin, but not others. (Supplementary Figure 1A in (Singh et al., 2021))
   d. In a 384-well black low volume assay plate, 2 nM Tb-anti-H3K9Me2 and 20 nM AF488-Streptavidin at 20 μL/well are incubated with 1:2 dilutions of the substrate peptide H3K9Me3-Biotin or the resulting product peptide H3K9Me2-Biotin for 30 min.
   e. Detection of the TR-FRET signals of each group.

   Note: This test is to demonstrate the specificity of Tb-anti-H3K9Me2 antibody to the resulting product peptide H3K9Me2-Biotin at all tested concentrations but not to the substrate peptide H3K9Me3-Biotin. (Supplementary Figure 1B in (Singh et al., 2021))

   Note: Specific detection of the newly generated H3K9Me2 peptide by the Tb-anti-H3K9Me2 antibody confers the assay with great specificity.

2. To identify an optimal assay buffer, three buffers (buffer 1 to 3) are tested. The presence of KCl and $MgCl_2$ could negatively impact the KDM4B enzymatic activity, whereas the presence of Tris-HCl (pH 8.0) could positively increase KDM4B enzymatic activity (Supplementary Figure 1E and 1F in (Singh et al., 2021)). These observations indicate that salt and pH have significant impact on KDM4B activity in TR-FRET assay, and the mechanisms need to be further investigated. **The optimal assay buffer (buffer 4)** is then formulated based on the results from the initial three buffers (Supplementary Figures 1E and 1F in (Singh et al., 2021)).

Buffer 1	50 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 1 mM α-ketoglutarate, 80 μM $Fe(NH_4)_2(SO_4)_2$, 2 mM L-ascorbic acid and 0.01% BSA (Yang et al., 2017).
Buffer 2	50 mM HEPES (pH 7.5), 1 mM α-ketoglutarate, 80 μM $Fe(NH_4)_2(SO_4)_2$, 2 mM L-ascorbic acid and 0.01% BSA (Chu et al., 2014).
Buffer 3	50 mM HEPES (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM α-ketoglutarate, 80 μM $Fe(NH_4)_2(SO_4)2$, 2 mM L-ascorbic acid and 0.01% BSA.
Buffer 4	50 mM Tris-HCl (pH 8.0), 1 mM α-ketoglutarate, 80 μM $Fe(NH_4)_2(SO_4)2$, 2 mM L-ascorbic acid and 0.01% BSA.

3. To optimize the concentration of KDM4B protein and incubation time of the demethylation assay
   a. 10 μL/well of H3K9Me3-Biotin (1.5 μM) is first dispensed in 384-well assay plate, followed by 5 μL/well of KDM4B at 0 nM, 750 nM, 1.5 μM, 2.25 μM or 3 μM.
   b. The plate is incubated for 15, 30, 45, 60, 75 or 90-min under 18°C–22°C.
   c. The reaction mixtures are then dispensed with 5 μL/well 8 nM Tb-anti-H3K9Me2 antibody and 80 nM AF488-Streptavidin, incubated for an additional 15-min before the detection of TR-FRET signals.

   Note: The final KDM4B concentration at 250 nM with a 30-min incubation time is optimal (Supplementary Figure 1C and 1D in (Singh et al., 2021)). KDM4B continues removing methyl group(s) from the newly generated H3K9Me2 peptide, generating the H3K9Me1 peptide, which may consequently reduce the sensitivity of the Tb-anti-H3K9Me2 antibody over time. One alternative solution is to add a known KDM4B inhibitor at high concentration to all wells after incubation but before addition of the H3K9Me2 antibody. This would stop the reaction and make the assay less sensitive to different development times.

   ⚠ CRITICAL: It's critical to detect the reaction at an optimal incubation time in which the presence of the maximal H3K9Me2 peptide allows the highest detection sensitivity.

4. DMSO tolerance test for the KDM4B-mediated demethylation assay.
   a. In the presence of DMSO at 0.0, 0.1%, 0.2%, 0.5%, 1%, 2%, 5%, and 10%, 250 nM KDM4B is incubated with 1 μM H3K9Me3-Biotin in 15 μL/well for 30 min.
   b. 8 nM Tb-anti-H3K9Me2 antibody and 80 nM AF488-Streptavidin (5 μL/well) are dispensed for an additional 15-min incubation.
   c. Detection of TR-FRET signals.

   Note: DMSO has minimal negative impact under the tested concentration range from 0 to 10% (Supplementary Figure 1G in (Singh et al., 2021)).

Perform screening assay

Timing: [1 day]

5. Dispense 10 μL/well of 1.5 μM H3K9Me3-Biotin peptide solution in an assay buffer [50 mM Tris-HCl (pH 8.0), 1 mM α-ketoglutarate, 80 μM $Fe(NH_4)_2(SO_4)_2$, 2 mM L-ascorbic acid and 0.01% BSA] to black low volume 384-well assay plates by a Wellmate.
6. Briefly spin down the assay plates.
7. Dispense stock compound solutions from both compound plate (step 1) and control plate (step 3) using a robotic pintool at 30 nL/well (primary single dose screen) or 140 nL/well (dose response test) to an assay plate with 10 μL/well 1.5 μM H3K9Me3-Biotin peptide. Each compound is assayed in triplicates.

   Note: The 30 nL pintool is used to achieve final concentration of 20 μM for the primary screen. To obtain IC_{50} value for the compounds chosen from primary screen, we used higher volume of pintool (140 nl) in dose response assay to achieve a concentration range of 4.7 nM to 93.3 μM in a 1-to-3 dilutions for 10 concentration levels.

8. Dispense 5 μL/well assay buffer to columns 1 and 13, 5 μL/well KDM4B (750 nM) in an assay buffer to columns 2-to-12 and 14-to-24 in the primary single dose screen, or 5 μL/well assay buffer to columns 23-to-24, 5 μL/well KDM4B (750 nM) in an assay buffer to columns 1-to-22 in the dose response test.

9. The contents in each assay plate are mixed by shaking.
10. Each assay plate is spun down briefly, followed by 30 min incubation without shaking under 18°C–22°C.

Note: The final tested compound concentration was 20 μM with 0.2% DMSO in the primary screen. The compound concentration range was 4.7 nM to 93.3 μM in a 1-to-3 serial dilution with 0.7% DMSO in the dose response test.

11. Dispense 5 μL/well of 8 nM Tb-anti-H3K9Me2 antibody and 80 nM AF488-Streptavidin to the incubated assay plates. The assay plates are then shaken, spun down briefly and followed by 15-min incubation under 18°C–22°C protected from light.
12. TR-FRET signal (fluorescence emission ratio of 10,000 × 520 nm/490 nm) of each well in assay plates is detected by a PHERAstar *FS* plate reader.
13. Analyze activity of each tested compound by normalizing its TR-FRET signal to that of positive and negative controls in each plate for the primary screen and dose response test. For dose response test, the activities of each chemical at different concentration levels are analyzed by the GraphPad PRISM software by fitting into a sigmoidal dose–response equation to derive dose-response curve and IC_{50} value, if applicable. For representative primary screen figure, see Figure 1C in (Singh et al., 2021). For representative dose–response curve, see Figure 1F in (Singh et al., 2021).

Note: The DMSO control wells with KDM4B protein and those without KDM4B protein are used as negative (0% inhibition) and positive (100% inhibition) controls, respectively; the dose response test was performed in triplicates.

EXPECTED OUTCOMES

Our TR-FRET assay displayed predictability and reproducibility of responses to known KDM4B inhibitors and showed a clear threshold between positive and negative responses. The high-throughput screening (HTS) statistical parameter Z′ (Z-prime) had an average value of 0.73 (0.55–0.87) from our pilot screen. In the research paper associated with this protocol, we performed a pilot screening of 3262 FDA-approved drugs and bioactive molecules and obtained 60 compounds as KDM4B inhibitors (hit rate 1.84%) with a 50% inhibition cutoff.

QUANTIFICATION AND STATISTICAL ANALYSIS

% inhibition = 100% × (compound - negative)/ (positive - negative).

The Z-prime factor value is calculated using the Equation 1 for each plate based on the method reported in the literature (Zhang et al., 1999).

$$Z' = 1 - \frac{3Std_{Negative} + 3Std_{Positive}}{|Mean_{Negative} - Mean_{Positive}|} \quad \text{(Equation 1)}$$

LIMITATIONS

Since the concentration of KDM4B in the assay is of 250 nM, the maximum potency that can be measured in this assay (minimum IC_{50}) would be 125 nM (assuming all the enzyme is active). However, the His-Tagged KDM4B may not be all active. The observed IC_{50} value of Geldanamycin is 50.1 nM (Singh et al., 2021), which is lower than the theoretical lowest IC_{50} value of 125 nM.

His-Tagged KDM4B purification with Nickel may cause potential problems, as residue Ni may replace iron in the catalytic domain of JmjC domains of KDMs, resulting in reduced catalytic activity. However, other sources of protein purification strategy by using GST-tagged KDM may overcome this problem. Dialysis of purified protein may also help to reduce the levels of Ni.

Previous studies showed that KDM4s may form homo- or hetero-dimers(Levin et al., 2018). While it is unknown if this will cause any changes of catalytic activities of KDM4, it certainly has such possibility.

This protocol only detects the product of the KDM4B-mediated demethylation reaction, the dimethylated histone peptide (H3K9Me2). It cannot simultaneously monitor both the substrate (H3K9Me3) and the product (H3K9Me2), which makes an optimal incubation time critical as less dimethylated histone peptide (H3K9Me2) is detected under longer incubation times.

Quenching and autofluorescence compounds can interfere with the TR-FRET result.

TROUBLESHOOTING

Problem 1

No change in TR-FRET signal (step 12)

Potential solution

We recommend the purity and stability of KDM4B protein to be tested before performing the screening. The low purity and/or degradation of protein due to mishandling (like excessive freeze-thaw circles) could lead to low activity of protein tested at the concentration previously optimized.

Problem 2

TR-FRET signal is low (step 12)

Potential solution

384-well black low volume assay plates are required for this assay.

Make sure the detection reagent is stored in an opaque container, and assay plates are protected with black lids after addition of detection reagent (step 8) from light exposure.

We recommend optimizing the assay conditions including buffer, pH, protein concentration, and incubation time.

Problem 3

Tb-anti-H3K9Me2 antibody is sensitive only to the H3K9Me2 peptide. However, KDM4B can further demethylate H3K9Me2 to H3K9Me1 if the assay mixture incubated for an extended period of time, thus reducing the sensitivity of this assay (step 10).

Potential solution

Optimize the incubation time by reading the plates at different incubation time points (15, 30, 45, 60, 75, and 90 min) using the optimized conditions (protein concentration and buffer, etc.). The incubation time giving the highest reading signal should be chosen as the optimal incubation time. One alternative solution is to add a known KDM4B inhibitor at high concentration to all wells after incubation but before addition of the H3K9Me2 antibody. This would stop the reaction and make the assay less sensitive to different development times.

Problem 4

Interference by intrinsically fluorescent compounds leading to false positive signals (step 11).

Potential solution

Replace the fluorophore AF488 to AF647, which switches the light detection wavelength from 520 nm to 665 nm to avoid interference by intrinsically fluorescent compounds and reduce false positives.

Problem 5

Assay has low performance (Z′-factor value is small (negative or close to zero)) (step 13).

Potential solution

Our protocol has excellent screening window coefficient with Z′ score above 0.5. However, if Z′ score is low then the assay conditions and instrument need to be optimized or corrected.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jun Yang (Jun.Yang2@stjude.org).

Materials availability

The KDM4B (1–348) construct is available upon request by contacting the lead contact.

Data and code availability

This study did not generate datasets/codes.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Q.W. and W.L. wrote the manuscript. W.L. developed assay. Z.-M.L. purified KDM4B protein. Z.R., S.W.W., T.C., and J.Y. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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