Journal of Visualized Experiments

Direct Measurement of KDM1A Target Engagement Using Chemoprobe-based Immunoassays

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59390R3
Full Title:	Direct Measurement of KDM1A Target Engagement Using Chemoprobe-based Immunoassays
Keywords:	KDM1A; ORY-1001; Target Engagement; Chemoprobe; epigenetics; drug development
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Cornella de Llobregat, Barcelona, Spain

Cover Letter

From: Tamara Maes
C.S.O. Oryzon Genomics S.A.

Barcelona, 11/01/2018

To: JoVE

Dear Dr. Meyers,

Please receive our manuscript:

"Direct Measurement of KDM1A Target Engagement Using Chemoprobe-based Immunoassays", by Mascaró et al.

A number of years back, we selected KDM1A, an enzyme involved in the control of histone methylation status and transcription, as a potential target for treatment of oncological and neurodegenerative disease. It was a bold choice, and we soon found how challenging it can be to perform drug development when no proper tools are available. Although we identified potent tool compounds quite early, the biology was a challenge and more than once, potential partners voiced disbelieve on our statements related to the potency of the compounds we had been designing. Our compounds were good, our evidence needed to be improved.

The relevance of proper assessment of the drug-target engagement to drug development can't be sufficiently underscored; and the use of the right assays is key. The desired biological effect should go hand in hand with the target engagement. This simple fact is ignored all too often by academic researchers, who have both reported biological effects in absence of any target engagement, or vice versa, have reported biological effects that were produced only at doses hundreds of time above target saturation. The error has also been committed by industrial promoters who admittedly finalized clinical trials without having a clue on whether their drug actually hit the target.

This manuscript provides a protocol to assess engagement to KDM1A, a hot target being explored in multiple indications including oncology and neurodegenerative disease. But it also reflects a general strategy and the reminder that robust assays are indispensible to move forward on solid ground in basic biology and drug development. We would be honored if our manuscript would be considered for publication in JoVE.

Best regards,

Tamara Maes. PhD
CSO Oryzon Genomics S.A.

TITLE:

Direct Measurement of KDM1A Target Engagement using Chemoprobe-based Immunoassays

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KEYWORDS:

Target Engagement, Histone Modifications, Histone Demethylases, KDM1A, Chemoprobe, ELISA

SUMMARY:

Here, we present a protocol to measure KDM1A target engagement in a human or animal cell, tissue or blood samples treated with KDM1A inhibitors. The protocol employs chemoprobe tagging of the free KDM1A enzyme and direct quantification of the target occupation using chemoprobe-based immunoassays and can be used in preclinical and clinical studies.

ABSTRACT:

The assessment of the target engagement, defined as the interaction of a drug with the protein it was designed for, is a basic requirement for the interpretation of the biological activity of any compound in drug development or in basic research projects. In epigenetics, target engagement is most often assessed by the analysis of proxy markers instead of measuring the union of the compound to the target. Downstream biological readouts that have been analyzed include the histone mark modulation or gene expression changes. KDM1A is a lysine demethylase that removes methyl groups from mono- and dimethylated H3K4, a modification associated with the silencing of gene expression. Modulation of the proxy markers is dependent on the cell type and function of the genetic make-up of the cells investigated, which can make interpretation and cross-case comparison quite difficult. To circumvent these problems, a versatile protocol is presented to assess the dose effects and dynamics of the direct KDM1A target engagement. The assay described makes use of a KDM1A chemoprobe to capture and quantify uninhibited enzyme, can be broadly applied to cells or tissue samples without the need for genetic modification, has an excellent window of detection, and can be used both for basic research and analysis of clinical samples.

INTRODUCTION:

Lysine specific demethylase 1 (KDM1A)[1] is a demethylase involved in the control of gene transcription. This protein has emerged as a candidate pharmacological target[2] in oncology;

including Acute Myeloid Leukemia[3] (AML), Myelodysplasia Syndrome (MDS)[4], Myelofibrosis (MF)[5,6], Small Cell Lung Cancer (SCLC)[7]; in Sickle Cell Disease (SCD)[8,9], and in central nervous system diseases including Alzheimer's Disease (AD), Multiple Sclerosis (MS); and in aggression[10].

Most of the KDM1A inhibiting compounds in clinical development are cyclopropylamine derivatives and inhibit the protein by covalent binding to its flavin adenine dinucleotide (FAD) cofactor[11]. Inhibition of KDM1A induces gene expression changes, but these changes vary enormously across tissues, cell types, or disease cases. Inhibition of KDM1A also changes histone marks[12], yet these changes are generally produced locally at a specific site in the genome, and are again, highly tissue and cell specific.

The protocol was developed to directly measure KDM1A target engagement in biological samples and has been optimized for the use with cyclopropylamine derived inhibitors. The assay is based on ELISA technology and analyzes, in parallel, Total and Free (i.e. unbound by inhibitor) KDM1A in a native protein extract from a biological sample in a solid phase assay. As a first step, the biological sample is lysed in the presence of the biotinylated KDM1A selective chemoprobe OG-881[13,14], derived from the selective KDM1A inhibitor ORY-1001, a potent inhibitor of KDM1A in clinical development for the treatment of oncological disease. The chemoprobe has an IC_{50} for KDM1A of 120 nM and includes a FAD binding moiety linked to a biotinylated polyethylene glycol (PEG)-tail. The chemoprobe exclusively binds to the free KDM1A, but not to the inhibitor-bound KDM1A in the sample. After the chemoprobe binding, the KDM1A containing complexes in the sample are captured on microtiter plates with streptavidin coated surface to determine free KDM1A, or on plates coated with a monoclonal anti-KDM1A capture antibody to determine total KDM1A. After washing, both plates are incubated with an anti-KDM1A detection antibody, washed again, and incubated with a secondary HRP-conjugated donkey anti-rabbit IgG antibody for detection using a luminescent substrate and quantification by measuring relative light units (RLU) in a luminometer (**Figure 1**).

[Place **Figure 1** here]

A standard curve is included in both ELISA plates to verify the linearity of each assay. The determination of KDM1A target engagement in each sample is then calculated as a relative value to the pre-dose or vehicle treated sample.

PROTOCOL:

Blood samples were obtained from the *Instituto de Investigación Biomédica Sant Pau* Biobank according to Spanish legislation (Real Decreto de Biobancos 1716/2011) and approval of the local ethics committees. Studies with animal tissues were performed in accordance with the institutional guidelines for the care and use of laboratory animals (European Communities Council Directive 86/609/EEC) established by the Ethical Committee for Animal Experimentation at the PRAAL-PCB.

1. Preparation of biological samples for the assay.

CAUTION: This protocol involves manipulation of biological samples which may be subjected to the Occupational Safety and Health Administration (OSHA) Blood Borne Pathogens standard (29 CFR 1910.1030), Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 or equivalent regulations. In addition, the biological samples may contain traces of biologically active investigational chemical compounds and the protocol may involve further manipulation of such compounds. Review the safety data sheet (SDS) of the compounds used prior to the initiation of the experiment and strictly observe all applicable safety measures established in the research center, including the use of adequate personal protective equipment (PPE). Wear proper protective clothing and use proper shielding during the course of the experiment. Discard residues in the appropriate waste containers (biological/cytotoxic waste).

NOTE: This protocol starts with cells or samples of subjects treated with a KDM1A inhibitor and their untreated or vehicle/placebo treated controls[3].

1.1. Cells treated with vehicle or KDM1A inhibitor *in vitro*

1.1.1. For the cells grown in suspension, as 10 mL cultures, transfer the suspensions into clean 15 mL conical tubes and proceed to 1.1.3.

1.1.2. For the adherent cells (grown in 75 cm^2 flasks), remove the medium from the flask and wash briefly using 4 mL PBS. Detach the cells from their vessels using 1.5 mL of 0.5% Trypsin-EDTA during 2 - 5 min (trypsinization conditions may vary, follow provider recommendations for the cell line), add 4 mL PBS and transfer the cells into clean 15 mL conical tubes.

1.1.3. Insert the tubes in a bench top centrifuge and collect the cells by centrifugation for 5 min at 400 x g at 4 °C. Remove the supernatant, resuspend the pellet in 1 mL of PBS dispensed using a micropipette and transfer the suspension into a 1.5 mL microcentrifuge tube.

1.1.4. Insert the samples in a microtube centrifuge and centrifuge them for 5 min at 400 x g at 4 °C. Remove the PBS by aspiration with a micropipette and either keep the pellets on the ice and proceed to Step 2; or freeze the pellets on dry ice and store them at -80 °C until Step 3.

1.2. Samples from subjects or animals treated with vehicle/placebo or KDM1A inhibitor

1.2.1. Tissues: Cut the tissue in small, ≈1 cm^3 pieces using a scalpel. Freeze the tissues pieces in liquid N_2 in a Dewar container and store them at -80 °C until Step 3.

1.2.2. Polymorphic blood mononuclear cells (PBMCs): Dilute 10 mL of fresh blood (process maximum 2 h after blood withdrawal) collected in K_2-EDTA tubes with 2 volumes of PBS in a 50 mL conical tube. Isolate the PBMCs from blood using commercially obtained PBMC separation tubes according to the manufacturer's instructions. Keep pellets on the ice and proceed to Step 3.2; or freeze the pellets on dry ice and store them at -80 °C until Step 3.

NOTE: A wet cell pellet of 20 to 50 µL contains ≈ 1 x 10^7 cells, depending on the cell size. A wet PBMC pellet obtained from 10 mL of healthy human blood has a volume of ≈ 20 µL and contains ≈ 1 x 10^7 PBMCs. Tissues or cell pellets can be stored at -80 ºC for up to 6 months.

2. Solution preparation

2.1. Prepare 2 µM OG-881 working solution: Take a 10 µL single-use aliquot of the 20 mM biotinylated probe OG-881 stock solution out of the 4 °C fridge and leave it at room temperature (RT) for 10 min. Prepare the 2 µM working solution by serial dilution of the OG-881 stock solution in PBS, using a micropipette with filter tips and changing the tip between the different dilution steps.

2.2. Prepare 10x Protease inhibitor: dissolve 1 tablet in 1 mL PBS in a microcentrifuge tube.

2.3. Prepare the desired volume of 1x Cell lysis buffer with 25 nM OG-881 chemoprobe. For each mL, mix 100 µL commercially obtained 10x Cell lysis buffer, 150 µL of 10x Protease Inhibitor, 12.5 µL of 2 µM OG-881, and 737.5 µL of Type 1 double distilled water.

2.4. Optionally prepare the desired volume of 1x Cell lysis buffer but with 25 nM ORY-1001 instead of OG-881 as in Step 2.3. Less potent inhibitors may be used but may require higher concentrations, for use in the positive control with 100% inhibition (see Step 3.5).

NOTE: Take appropriate measures to avoid any unintended contamination of solutions or samples with the OG-881 or KDM1A inhibitor stock solutions. To calculate the desired volume of 1x Cell lysis buffer with 25 nM OG-881, assume that 400 µL is required per 40 mg of pulverized tissue, or 200 µL per wet pellet of 10^7 cells.

3. Native protein extraction

3.1. From tissues:

3.1.1. Pulverize and homogenize a ≈ 1 cm^3 cube of frozen tissue with a mortar and pestle chilled on dry ice. Aliquot the samples in single-use vials containing ≈ 40 mg of tissue powder, avoid thawing at all times. Proceed to step 3.1.2. for immediate processing or store at -80 °C.

3.1.2. Resuspend 40 mg of powdered tissue in 400 µL of 1x Cell Lysis buffer with 25 nM OG-881, vortex for 10 s, and force the sample at least five times through an 18-gauge blunt syringe needle until lysis of the tissue is achieved and a turbid light yellow to orange suspension is obtained. Avoid bubble formation.

3.1.3. Continue to Step 3.3

3.2. From cell pellets (PBMCs and cell lines):

3.2.1. Resuspend a pellet of ≈ 1 x 10^7 cells in 200 µL of 1x cell lysis buffer containing 25 nM OG-881. Vortex the samples briefly and keep them on ice for 5 min.

3.2.2. Sonicate the samples in a sonicator using 3 pulses of 20 s each at 45 kHz; place them on ice for 20 s between pulses.

NOTE: As soon as the biological samples have been resuspended in the 1x cell lysis buffer, keep them on the ice during the rest of the process.

3.3. Keep the samples on ice for an additional 5 min, vortex briefly and centrifuge the samples for 10 min at 14 000 x g in a pre-cooled centrifuge at 4 °C.

3.4. Using a 1 mL micropipette, transfer the supernatants into fresh 1.5 mL microcentrifuge tubes and leave them on the ice during 2 h. Continue to Step 4.

3.5. Optionally, a positive control to simulate 100% target engagement may be prepared as follows:

3.5.1. Resuspend the cell pellet or powdered tissue from a vehicle or untreated (predose) sample in the required volume of 1x Cell Lysis buffer with 25 nM ORY-1001 and process as outlined in Step 3.1 to 3.3.

3.5.2. Transfer the supernatants of the positive control into fresh 1.5 mL microcentrifuge tubes and leave them on ice for 1 h. ORY-1001 willfully inhibit KDM1A and block chemoprobe binding.

3.5.3. Add 5 µL of 2 µM OG-881 working solution to the positive control supernatant (volume for positive control generated from a 40 mg tissue sample) or 2.5 µL of 2 µM OG-881 working solution (volume for positive control generated from a 10^7-cell sample) to obtain the same OG-881 concentration as the other samples and leave on ice during 2 h. Continue to Step 4.

4. Quantification of native protein using Bradford assay

4.1. Dilute the commercially sourced Bradford Protein Assay reagent 5 times with H_2O Type 1 double distilled water. Calculate the volume of the reagent required for the total amount of samples and standards (1 mL per sample or standard + 5 mL excess volume).

4.2. For the Bovine Serum Albumin (BSA) standard curve, prepare one microcentrifuge tube with 1 mL diluted Bradford Protein Assay solution (blank) and seven microcentrifuge tubes with 995 µL of the diluted Bradford Protein Assay solution. Add 5 µL of each of the BSA Standards (concentration ranging from 125 to 2 000 µg/mL) to each of the 7 microcentrifuge tubes and mix them by gently inverting the tubes several times. Incubate for 5 min at RT.

4.3. Transfer the diluted Standards to cuvettes and read the OD of the blank and Bovine Serum Albumin Standard samples in a spectrophotometer at 280 nm.

4.4. For the biological samples, prepare one microcentrifuge tube with 1 mL diluted Bradford Protein Assay solution (blank) and as many microcentrifuge tubes with 999 µL of the diluted Bradford Protein Assay reagent as samples that need to be quantified. Using an automatic P2 micropipette, add 1 µL of native protein extract prepared in Step 3 to each microcentrifuge tube and mix by gently inverting the tubes several times. Incubate the samples 5 min at RT.

4.5. Transfer the volumes to cuvettes and read the OD of the samples in a spectrophotometer at 280 nm.

4.6. Preferentially, proceed immediately to Step 5. Alternatively, store the native protein extracts at -80 °C until Step 5. Avoid freeze thaw cycles.

5. Luminescent ELISAs for Total and Free KDM1A determination

NOTE: Keep the lab temperature constant at 23-24 °C (RT).

5.1. Coating of microtiter plates with capture KDM1A antibody or Streptavidin

5.1.1. Total KDM1A ELISA: for each plate, prepare 10 mL of KDM1A capture antibody to a final concentration of 2 µg/mL in PBS. Transfer 100 µL into each well of the plate.

5.1.2. Free KDM1A ELISA: for each plate, prepare 10 mL of streptavidin at 10 µg/mL in PBS. Transfer 100 µL into each well of the plate.

5.1.3. Top-seal the Total and Free KDM1A ELISA plates with adhesive film and incubate the plates overnight at 4 °C in the refrigerator.

5.2. Washing and Blocking the plates

5.2.1. Take the plates out of the refrigerator and let them equilibrate for around 45 min at RT before use.

5.2.2. Prepare 1000 mL wash buffer (0.1% Tween in PBS) and 50 mL blocking buffer (1% BSA in PBS) per plate.

5.2.3. Wash the plates 3 times with wash buffer. In this and subsequent steps, tap the plate on paper towels after every washing step to remove residual solution.

5.2.4. Add 200 µL of blocking buffer per well to both plates, top-seal both plates with an adhesive film and incubate 2 h at RT.

5.3. Biological sample preparation

5.3.1. Dilute the native protein extracts obtained at the end of Step 3 to the appropriate concentration using PBS. The recommended concentration will vary in function of the level of KDM1A expression in the biological sample. Examples of appropriate ranges are (1) Cell pellets: 0.5 - 10 µg per well. (2) PBMCs: 5 - 30 µg per well. (3) Pulverized tissue (brain, lung, skin): 20 - 100 µg per well. Keep the samples on the ice during the preparation. When possible, run technical triplicate sample analyses.

5.3.2. Prepare a Standard Curve using human rKDM1A:

5.3.2.1. To prepare the KDM1A Standard working solution, pipet the appropriate volume of rKDM1A for a final concentration of 25 pg/µL, add 75 µL of 2 µM OG-881, and complete with 1 x PBS to a total volume of 6 mL in a 15 mL falcon tube. Keep the KDM1A Standard working solution on ice during 1 h and mix the solution gently by inverting the 15 mL falcon tube several times every 20 min.

5.3.2.2. Prepare the KDM1A Standard Dilution Series in 1.5 mL microcentrifuge tubes according to **Table 1** (Standard Preparation), in volume enough for the triplicate analysis of two 96 well microtiter plates.

[**Table 1:** Standard Preparation]

5.4. ELISA

5.4.1. (Continued from Step 5.2.4.) After 2 h of incubation, discard the blocking buffer and wash the plates with wash buffer.

5.4.2. Transfer the appropriately diluted samples (native protein extracts and standard curve from Step 5.3.) to a refrigerated 96 deep well storage block following the plate distribution shown in **Table 2** (Deep Well Plate Design).

[**Table 2:** Deep Well Plate Design]

5.4.3. Keep this block on ice until pipetting 100 µL sample / well in the Total and Free ELISA plates following the plate distribution shown in **Table 3** (ELISA Plate Design).

[**Table 3:** ELISA Plate Design]

5.4.4. Incubate for 1 h at RT, discard the samples and wash the plates 5 times with wash buffer.

5.4.5. Prepare 20 mL of rabbit anti-KDM1A detection antibody at 0.125 µg/mL in blocking buffer, add 100 µL per well in each plate of the assay, except in wells corresponding to the negative controls C-. Top-seal the plate and incubate 1 h at RT.

5.4.6. Discard the detection antibody solution and wash the plates 6 times with wash buffer.

5.4.7. Prepare 25 mL of secondary goat anti-rabbit antibody HRP to a dilution 1:5 000 in blocking buffer, add 100 µL per well to the microtiter plates; and incubate 1 h at RT.

5.5. Chemiluminescent Detection

5.5.1. 30 min before the end of Step 5.4.7. and under soft light conditions, mix equal parts of Luminol-Enhancer and Peroxide Solution (10.5 mL: 10.5 mL, for 2 plates) in an amber bottle and leave it at RT.

NOTE: Keep the Luminol Working Solution in an amber bottle and avoid prolonged exposure to any intense light. Short-term exposure to typical laboratory lighting will not harm the working solution.

5.5.2. At least 20 min before measuring the luminescence, switch on the microplate reader at 25 °C and set up readouts to 1000 ms integration time and 150 ms settle time. Parameter settings may require optimization in function of the instrument.

5.5.3. After 1 h of incubation in Step 5.4.7., discard the secondary antibody solution and wash the plates 6 times with wash buffer.

5.5.4. Pipette 100 µL per well of the Luminol Working Solution (Chemiluminescent Substrate) prepared in Step 5.5.1. Pipette very slowly and avoid bubble formation. Use a timer to control the time between the addition of the addition of the solution and the luminescence measurement of the plates and keep this time constant to achieve a good inter assay reproducibility.

5.5.5. Top-seal the plates and centrifuge to 500 x g at RT for 45 s in a plate centrifuge to eliminate any remaining bubbles. Incubate the plates for 1 min on a plate shaker at 100 rpm.

5.5.6. Insert the plate inside the reader and leave it for 3 min to stabilize the temperature at 25 °C (without adhesive film). Always start with the Free ELISA plate.

5.5.7. Read the relative luminescence units (RLU) of each ELISA plate assay (free and total KDM1A).

5.5.8. Save and copy the Raw RLU values from the Raw Data excel files for further analysis of the results.

6. Calculation of the target engagement

6.1. In a spreadsheet software, calculate the RLU Free and RLU Total values of samples S_X and reference samples REF (untreated, vehicle or pre-dose sample) from their technical replicate Raw data as detailed below:

6.1.1. Enter the individual Raw RLUi Total and Raw RLUi Free data from blanks, standard curve, negative controls C- and biological samples (S_X and REF) into the analysis datasheet (e.g., Excel). Also, enter the amounts (in pg) of KDM1A from the Standard Curve in the datasheet.

6.1.2. Calculate the Raw mean RLU, standard deviations σ_{RLU}, and coefficient of variation CV_{RLU} from the individual Raw Total and Raw Free RLUi data for each technical replicate datapoint.

6.1.3. Apply the outlier elimination (example for triplicates): for each individual Raw RLU Total and Raw RLU Free data point RLUi from a technical triplicate datapoint, apply Grubbs criteria when the CV for the triplicate > 0.15, and reject single suspect Raw RLU value when

$$\left|RLU_i\text{-}RLU_m\right| > Z \cdot \sigma_{RLU} ,$$

whereby Z = 1.148 for n = 3 and 90 % confidence interval (CI).

6.1.4. If outlier elimination was applied, re-calculate the Raw mean RLU, standard deviation σ_{RLU} and CV_{RLU} from the non-rejected (nr) Raw RLUi Total and Raw RLUi Free values for each datapoint.

6.1.5. Apply the background correction: Calculate the mean RLU Free and RLU Total values for each standard sample, and each sample S_X and reference sample REF as:

$$RLU_{Free} = \overline{Raw\ RLU_{Free,nr}} - \overline{Raw\ RLU_{Free,blank,nr}}$$

$$RLU_{Total} = \overline{Raw\ RLU_{Total,nr}} - \overline{Raw\ RLU_{Total,blank,nr}}$$

6.2. Graphically represent the data as follows:

6.2.1. Plot the RLU_{Free} and RLU_{Total} values (Y-axis) relative to their sample identification (X-axis) in a bar graph.

6.2.2. Also plot the RLU values (Y-axis) of the standards in a scatter plot relative to their amount of pg of rKDM1A protein (X-axis) for Free and Total measurements, as well as the corresponding lineal trendlines and calculate the r^2 (the square of the linear correlation coefficient) values.

6.3. Calculate the target engagement (TE); i.e. the percentage of KDM1A bound by the KDM1A inhibitor in each sample S_X relative to a reference sample REF (untreated, vehicle or pre-dose sample) as follows:

6.3.1. Calculate the ratio R of the mean RLU Free to Total values for the SX and REF samples as:

$$R_{SX}\,(\%) = \frac{RLU_{\,Free,SX,nr}}{RLU_{\,Total,SX,nr}} \times 100$$

$$R_{REF}\,(\%) = \frac{RLU_{Free,REF,nr}}{RLU_{Total,REF,nr}} \times 100$$

6.3.2. Then calculate the target engagement (TE) of the sample S_X as:

$$TE_{SX}\,(\%) = 100 - (\left(\frac{R_{SX}\,(\%)}{R_{REF}\,(\%)}\right) \times 100)$$

Optional: (1) If N biological replicate experiments were conducted, each with n technical replicates; first calculate the TE_{SX} for the technical replicate sets. Subsequently, calculate the mean TE, SD and the CV values for the biological replicate set.

6.4. Revise whether the assay acceptance criteria are met: Verify that (1) the assay background is acceptable and the mean Blank < 0.05 x 10^7 RLU; (2) the sample auto-luminescence is absent and the RLUs of the negative controls C- are below the Lower Limit of Quantification (LLOQ = Mean Blank + 10x SD); (3) the rKDM1A standard curve is linear and r2 ≥ 0.98; (4) the biological samples have RLU values that fall in the dynamic and linear range of the assay i.e. between LLOQ and 2500 pg/well.

NOTE: Steps 6.1. to 6.4. can be readily automated in a calculus datasheet.

6.5. Export the TE data to an open source or commercially obtained statistics software of choice for the graphical representation of the TE values and additional statistical evaluations.

REPRESENTATIVE RESULTS:

The Linearity of Total and Free KDM1A determination.

A Standard Series was prepared as described in step 5.3.2., using 0 to 2500 pg of full-length human recombinant KDM1A enzyme. The RLU values of Total and Free rKDM1A were assessed to verify the linearity (**Figure 2A** and **2B**). Data are represented as mean from 3 experiments with 3 technical replicates (n) ± SD. The RLU values of Total and Free KDM1A detected in human PBMCs from the blood of 3 independent volunteers are superposed on the Standard curve in **Figure 2C and 2D**. Blood samples were obtained from the *Instituto de Investigación Biomédica Sant Pau* Biobank according to Spanish legislation (Real Decreto de Biobancos 1716/2011) and approval of the local ethics committees.

[Place **Figure 2** here]

Analysis of KDM1A target engagement in cells

AML cells were cultured following provider recommendations. Cells were treated with vehicle or ORY-1001 at different concentrations (0.25; 0.5; 1; 5 and 25 nM) (**Figure 3**). The native protein extracts were obtained in presence of 25 nM OG-881 chemoprobe. 0.5 µg of total protein was used to perform the target engagement analysis as described previously. Total and free KDM1A were determined, and the percentage of target engagement of ORY-1001 to KDM1A was calculated relative to the vehicle as described.

[Place **Figure 3** here]

Analysis of *in vivo* KDM1A target engagement

The objective of this experiment was to characterize the target engagement of ORY-1001 in different rat tissues, in function of the dose level. To achieve this goal, 15 Sprague-Dawley rats (200-250 g) were housed in a cytostatic security room to avoid potential contamination by the tested compound. A maximum of 3 rats/cage were randomly assigned to 5 study groups. The 5 different study groups received, respectively, vehicle; 1; 3; 10 or 30 µg / kg of ORY-1001 by oral administration for 4 consecutive days. Compound stock solutions were prepared daily. Animals were weighed before each administration to adjust the required volume. All animals were housed at constant room temperature (20 – 24 ºC) and relative humidity (45 – 65 %) under a 12 h light-dark cycle (lights on at 6:00 AM). Food and water were available *ad libitum*. Blood samples were collected 2 h after last administration in K_2EDTA tubes and PBMCs were isolated according to the procedure described previously in step 1.2.2. and preserved at -80 °C until native protein extraction. Lung samples were also collected 2 h after the last drug administration, frozen immediately in liquid nitrogen, and stored at -80 °C. Studies were performed in accordance with the institutional guidelines for the care and use of laboratory animals (European Communities Council Directive 86/609/EEC) established by the Ethical Committee for Animal Experimentation at the PRAAL-PCB.

After pulverization, the native protein extracts from lung were obtained as described and quantified. 5 µg of total protein from pooled PBMCs or 7.5 µg of total protein from lung from 3 animals were used per dose group to run the KDM1A target engagement assay.

The dose-response of KDM1A target engagement in PBMCs and in lung treatment of rats with ORY-1001 by oral gavage, calculated relative to the vehicle group is shown in **Figure 4A and 4B**. As can see in **Figure 4C**, the *ex-vivo* incubation with 25 nM ORY-1001 of lung protein extracts from the vehicle treated animals yields full TE yet but does not further increase TE in samples from rats treated for 4 days with 30 µg/kg ORY-1001, confirming KDM1A was already fully inhibited *in viv*o.

[Place **Figure 4** here]

FIGURE AND TABLE LEGENDS:

Figure 1. Schema of ELISA Enzyme linked chemoprobe immunoabsorbent assay for KDM1A target engagement: A) Determination of total KDM1A using sandwich ELISA and B) Determination of free KDM1A using chemoprobe ELISA.

Table 1: Standard Preparation. To prepare the Standard Series of KDM1A protein, pipette the indicated volumes of KDM1A Standard working solution and PBS into eight properly labeled 1.5 mL microcentrifuge tubes.

Table 2: Deep Well Plate Design. Standards (blue) and samples (yellow) from step 5.4.2. were pipetted into the reflected positions of the Deep Well Plate to facilitate loading into the ELISA plates following the direction of the blue (standard) and yellow (samples) arrows.

Table 3: ELISA Plate Design. The assay plate includes the standard curve with decreasing amounts of recombinant KDM1A target (in blue); the biological samples (S) in yellow; and corresponding negative controls (contain the samples but not the primary detection antibody) in white, to be loaded on the ELISA plates from the Deep Well Plate. The blank (0 in standard curve) contains all capture and detection reagents but no sample.

Figure 2. Determination of Total and Free rKDM1A in PBMCs of healthy volunteers. RLU values of Total rKDM1A assessed by ELISA (A) and of Free rKDM1A assessed by chemoprobe capture ELISA (B). Data were obtained from 3 replicate experiments, each analyzed in triplicate (N = 3; n = 3). RLU values of Total KDM1A assessed by ELISA (C) and of Free KDM1A (D) for the PBMCs of 3 independent untreated volunteers (red, blue and green squares) superposed on Standard Curve. Data were obtained from one experiment analyzed in triplicate (N = 1; n = 3). Values represented are the means ± SD.

Figure 3. Dose-response of KDM1A Target Engagement in a human AML cell line. Cells were treated with vehicle or ORY-1001 at different concentrations (0.25; 0.5; 1; 5 and 25 nM) and used for determination of target engagement as described. Data were obtained from 3 replicate experiments, each analyzed in triplicate (N = 3, n = 3). Values represented are the means ± SD.

Figure 4. *In vivo* and *ex vivo* native KDM1A target engagement. Dose-response of KDM1A target engagement in PBMCs (A) and lung samples (B) from rats treated with ORY-1001 for 4 consecutive days (p.o). Data were obtained from pooled PBMCs extracts from 3 animals per cohort, analyzed in duplicate (N = 1, n = 2) or from the lungs from 3 individual animals per cohort, analyzed in triplicate (N = 3, n = 3). C. Comparison of TE in pooled lung protein extracts of rats treated with vehicle (left) or 30 µg/kg ORY-1001; after 1 h *ex vivo* incubation of the extracts without (grey bars) or with 25 nM ORY-1001 (blue bars) (N = 3, n = 3). All data are represented as means ± SD.

DISCUSSION:

The protocol presented here was developed to directly measure KDM1A target engagement using a novel KDM1A chemoprobe capture based ELISA. The method has been validated on cultured human cell lines and ex vivo samples from human, rat and mouse and baboon (including PBMCs, lung, brain, skin, tumors), but can be readily applied to other species in which the KDM1A antibody target epitopes and catalytic center are conserved. As OG-881 is an activity based chemoprobe, the sample quality is important and proper manipulation and conservation of samples should be pursued especially during the initial steps of the protocol, to ensure the KDM1A activity is conserved.

The current experimental protocol was optimized to analyze KDM1A target engagement by covalent FAD targeting inhibitors. It can also be used with reversible inhibitors that block the access to the FAD cofactor of KDM1A. Potent reversible inhibitors with long residence times may employ the unmodified protocol.

The OG-881 chemoprobe may not be suitable for low potency reversible inhibitors with high off-rates. The particular chemoprobe used in this manuscript is not cell penetrant and therefore analyses are performed *ex vivo* on lysed samples.

The method can be run on instruments that are broadly available in research and analytical laboratories; it does not require genetic modifications to be introduced into cells, and it can easily be applied to different sample types. Another advantage is that it can be used on samples derived from different species that are frequently used in the preclinical proof of concept studies and in toxicology models and that it has successfully been translated to analyze clinical samples.

Other methods have been used for analysis of KDM1A target engagement. Many of these methods use proxy markers like changes of the H3K4me2 histone mark, using AlphaLisa[15]; or induction of expression markers using qRT-PCR or FACS analysis[16]. However, in cells or tissues, the histone marks are controlled by multiple factors, and assays that measure changes in the histone mark do not always provide a good dynamic range for analysis. KDM1A inhibition can induce potent changes in gene and protein expression, but the response tends to very heterogeneous and highly cell context dependent, which can complicate analyses of dose response[3,7].

Direct assessment of the occupation of the target is, therefore, the best option to measure target engagement. One assay that has been proposed for this is the cellular thermal shift assay (CETSA), based on the increase of thermal stability of target proteins upon binding of inhibitors. This method may, in principle, be applied to unmodified cells and different tissue types and has recently been used to assess the cellular activity of KDM1A inhibitors in cultivated cells[17]. However, this technology has rarely been used for *in vivo* pharmacodynamics studies[18] and to the best of our knowledge; its use has not been reported in clinical trials.

The protocol provided here describes a fully validated chemoprobe based method which has been used to determine KDM1A target engagement in cells and tissue samples. The method

has been successfully translated to analyze samples of human subjects treated with a KDM1A inhibitor[19] and will be of great use to model PK/PD responses in clinical trials.

ACKNOWLEDGMENTS:

This study was financed by Oryzon Genomics. S.A., Hoffman-La Roche, and partially supported by the CIIP-20152001 and RETOS collaboration program RTC-2015-3332-1.

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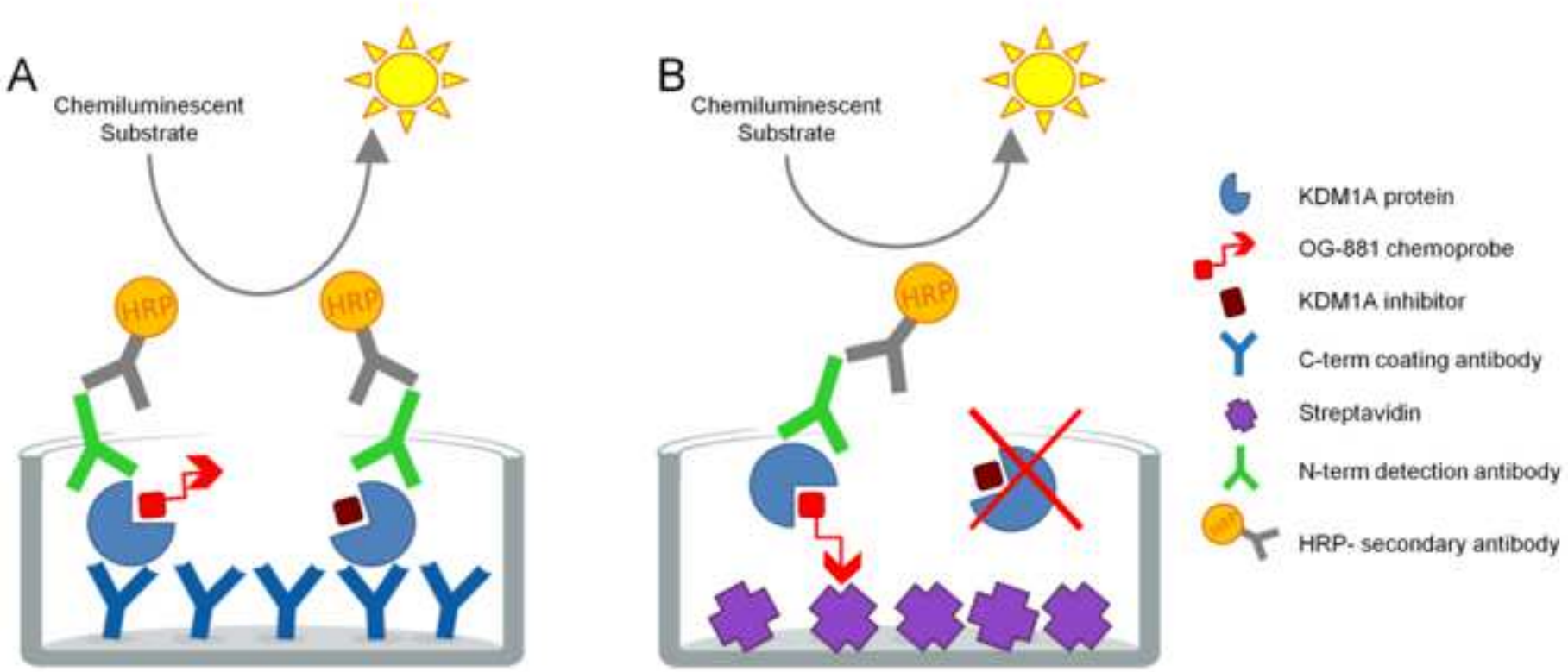

Figure 2

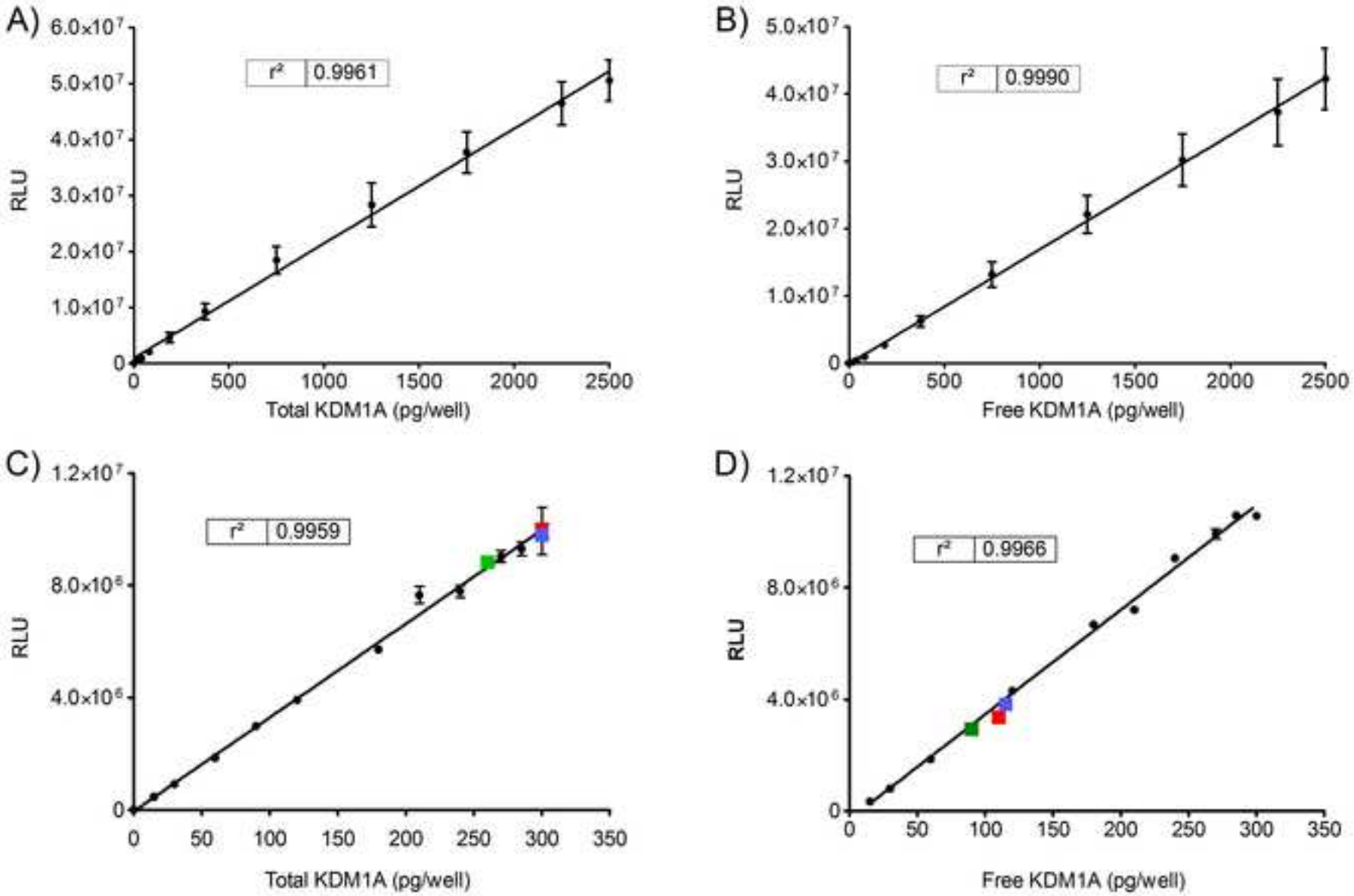

Figure 3

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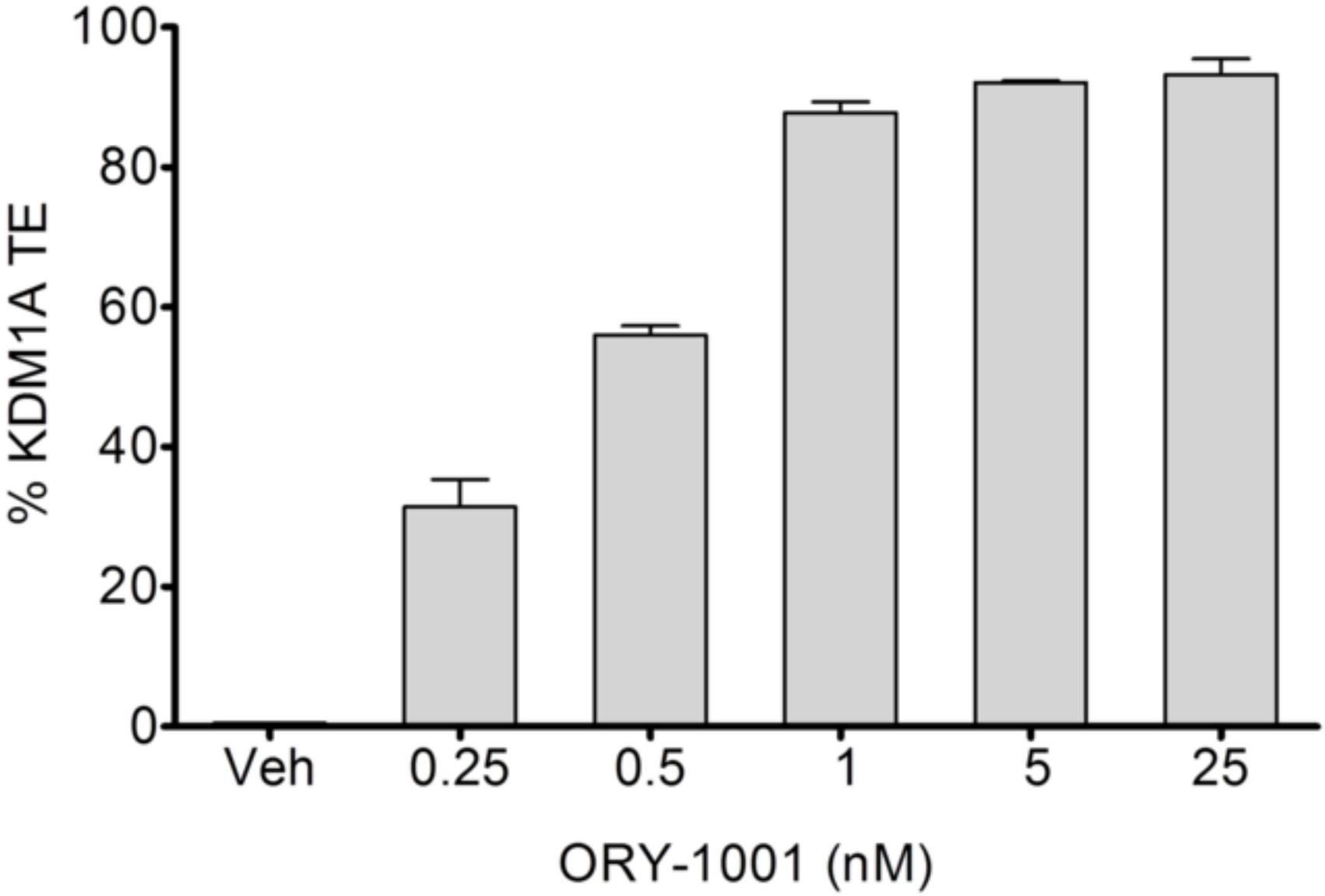

Figure 4

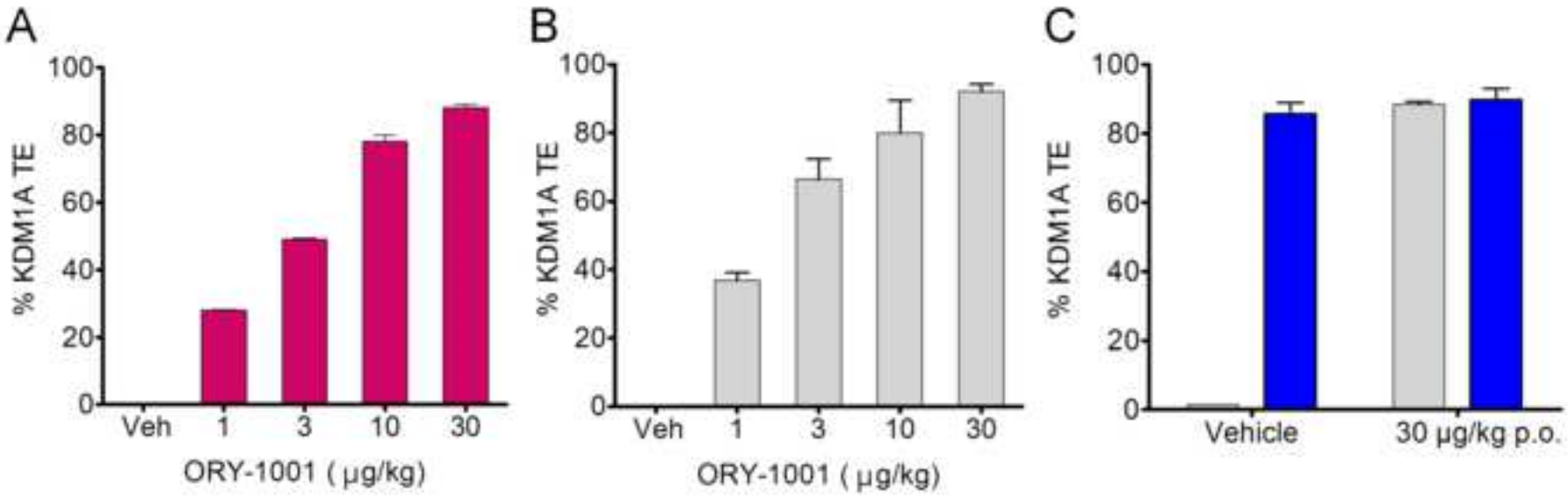

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TABLE FOR DILUTIONS STANDARD KDM1A PROTEIN PREPARATION

STANDARD SERIES (pg KDM1A/well)	KDM1A Standard working solution (µL)	PBS (µL)
2500 for C^-*	800	-
2500	800	-
1750	560	240
1250	400	400
750	240	560
250	80	720
25	8	792
0	0	800

NOTE:

(1) The volume prepared of each dilution is enough to run in triplicate 2 plates of assay.

(2) The recommended range is between 2.5 and 5000 pg / well

* For the negative control C^-, without KDM1A detection antibody

Table 2

Click here to access/download;Table;Table 2_Deep Well Plate Design_Mod.xlsx

Deep Well Plate Design

	1	2	3	4	5
A	2500 pg	1750 pg	1250 pg	750 pg	250 pg
B					
C					
D	S1				S6
E	S2				S7
F	S3				S8
G	S4				S9
H	S5				S10

6	7	8	9	10	11	12
25 pg	0 (Blank)	2500 pg	S11			
			S12			
			S13			
			S14			
			S15			
			S16			
			S17			
			S18			

Table 3

Click here to access/download;Table;Table 3_ELISA Plate Design_Mod.xlsx

ELISA PLATE DESIGN

HRP mAb					0.08 µg / mL Secor
Detection mAb					0.125 µg / mL Rabbit anti-KD
Coating		1	2	3	4
2.00 µg / mL Mouse anti-KDM1A Capture mAb O/N 4ºC	A	2500 pg	1750 pg	1250 pg	750 pg
	B	2500 pg	1750 pg	1250 pg	750 pg
	C	2500 pg	1750 pg	1250 pg	750 pg
	D	S1	S1	S1	S1
	E	S2	S2	S2	S2
	F	S3	S3	S3	S3
	G	S4	S4	S4	S4
	H	S5	S5	S5	S5

ıdary Antibody: anti -rabbit pAb

)M1A detection mAb (except in negative controls)

5	6	7	8	9	10	11
250 pg	25 pg	0 (Blank)	2500 pg	S11	S11	S11
250 pg	25 pg	0 (Blank)	2500 pg	S12	S12	S12
250 pg	25 pg	0 (Blank)	2500 pg	S13	S13	S13
S6	S6	S6	S6	S14	S14	S14
S7	S7	S7	S7	S15	S15	S15
S8	S8	S8	S8	S16	S16	S16
S9	S9	S9	S9	S17	S17	S17
S10	S10	S10	S10	S18	S18	S18

12
S11
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S18

Table of Materials

Click here to access/download;Table of Materials;Table_Equipments, Materials and Reagents_Mod.xlsx

Name of reagent	Company	Catalog Number
0,05% Trypsin-EDTA (1X)	Thermo Scientific	#25300-062
10 X Protease Inhibitor Tablets	Roche	#11836153001
96 deep well storage block	VWR	#734-1679
96 well ELISA plates	Nunc	#436110
Adhesive black Film	Perkin Elmer	#6050173
Adhesive transparent Film	VWR	#60941-062
Biotinylated KDM1A probe OG-881	Oryzon Genomics S.A.	NA
Bovine Serum Albumin	Sigma	# 3117057001
Bovine Serum Albumin Standard	Thermo Scientific	#23208)
Bradford Protein Assay	BioRad	#500-0001
Cell lysis buffer 10X	Cell Signaling	#9803
Centrifuge for 96- well plates	Hettich	Rotina 420R
Flask	Thermo Scientific	#156499
Full length, enzymatically active human Recombinant LSD1 / KDM1A	Active Motif	#31426
Graphpad Prism 5 Project	GraphPad Software	NA
Luminol-Enhacer and Peroxide Solution (Chemiluminescent Substrate)	Thermo Scientific	#37074
Micro Centrifuge	Eppendorf	5415 R
Microplate reader Infinite 200-Tecan	Tecan	Infinite 200
Mouse monoclonal capture antibody Anti-KDM1A (N-terminal epitope)	Abcam	#ab53269
Needle G18 gauge blunt	BD	#303129
ORY-1001 (iadademstat)	Oryzon Genomics S.A.	NA
PBMC separation tubes 10 ml	Greiner bio-one	#163288
PBMC separation tubes 50 ml	Greiner bio-one	#227288
PBS 1x	Sigma	#D8537
Plate shaker	Heidolph Instruments	Rotamax 120
Polysorbate 20	Sigma	#P7949
Rabbit monoclonal detection antibody Anti-KDM1A (C-terminal epitope)	Cell Signaling	#672184BF-100
Secondary antibody Peroxidase-conjugated Donkey Anti-rabbit IgG	Thermo Scientific	#31458
Spectrophotometer cuvette 1.5	Deltalab	#302100
Spectrophotometer for cuvette	GE Healthcare	GeneQuant 1300
Streptavidin	Promega	#Z704A
Syringe	BD	#303172
Type 1 ultrapure water	Millipore	Milli-Q Advantage A10
Ultrasonic cleaner	VWR	USC200T

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Dear JoVE Editor Dr. Bajaj,

Please receive our revised Manuscript “Direct Measurement of KDM1A Target Engagement Using Chemoprobe-based Immunoassays¨. As well as follow up on the editorial comments and replies to the reviewers, We are thrilled to proceed with the steps towards publication of this protocol in JoVE, and hope the edits are to your liking.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues

Thoroughly revised

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Does not apply

3. Please remove the embedded figure(s) from the manuscript.

Done. (Still visible with change track)

4. Please remove the embedded tables (lines 245-249, 255-258) from the manuscript.

All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Done. Four excel files replace the embedded tables:

Materials and Reagents.xlsx: List of the equipment’s, materials and reagents used in KDM1A TE assay

Deep Well Design.xlsx: Explain the distribution in the deep well plate.

ELISA Plate Design.xlsx: Show the ELISA plate design used in our experiments.

Standard Preparation.xlsx: Explain the preparation of dilution Standard Protein KDM1A

5. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Done. Included in table corresponding Materials and Reagents.xlsx

6. Please provide an email address for each author.

Was already provided

7. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”
Rephrased

8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Revised.

9. Please abbreviate liters to L to avoid confusion. Please use the micro symbol µ instead of u.

Revised and replaced.

10. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

Examples of commercial sounding language in your manuscript are: Greiner bio-one, SIGMA, Cell Signaling, ROCHE, Oryzon Genomics S.A., BioRad, Thermo Scientific, Active Motif, ABCAM, Luminunc, Tween, PROMEGA, Leucosep, Eppendorf, milliQ, etc.

Revised.

11. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Revised.

12. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol.

Checked.

Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Revised

13. Lines 69-97: The Protocol should contain only action items that direct the reader to do something. Please move the solutions, materials and equipment information to the Materials Table.

Suppress but verify that list of items in protocol is correct and non commercial. The table should include the non commercial, commercial name, + ref and manufacturer

Lines 69-97 removed and transferred to table. Done. The excel file corresponding to Material and Reagents Table include all of these information.

14. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Done

15. 1.1.1: Please specify the cell type and describe the trypsination conditions.

We have used this protocol with the following cell types: Leukemic cell lines such as THP1, MV(4;11) and MOLM13 (ref. ACC16, ACC102 and ACC554 respectively from DMSZ), prostate cancer cell line such as LNCap cells (ref.: CRL-1740 from ATCC) and Small Cell Lung Cancer such as NCI-H-510A (ref. ACC578 from DMSZ). All the cell lines were cultured and detached following provider recommendations. The specific conditions included are for LNCap cells.

16. 1.1.2: What volume of PBS is used?

Included in the protocol.

17. 1.2.1: Please specify the tissue type and the size of small pieces. What is used to cut?

We have used the following tissue types: skin, lung, brain and tumor. However, is useful for any type of tissue that expresses sufficient KDM1A. The size and amount are described in the protocol. We have validated the assay in different species such as human, rat, mouse and baboon. Other species can be used if the target epitopes of the antibodies and catalytic site are sufficiently conserved. LSD1 is a highly conserved enzyme, and modifies the tail of H3, a highly conserved protein.

18. 2.9: Please describe how to perform Bradford assay. Specific details are required for filming.

Included

19. 2.11.1, 3.3.1: Please provide some guidance on the desired volume/appropriate concentration.

Added a note.

20. 2.11.6: Please ensure that the step referenced here is correct.

OK

21. Lines 306-342: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

Calculation of target engagement rewritten as discrete imperative steps.

22. Lines 345-371: JoVE articles do not have an Abbreviations / definitions section. Please remove and define all abbreviations before use.

Done

23. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

OK

24. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Done

25. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

OK

26. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

OK

27. References: Please do not abbreviate journal titles.

Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes an ELISA based measurement of inhibitor-bound and -free KDM1A in tissue and cell extracts. Performance of the assay appears to be excellent and therefore the assay will be an interest of other researchers working on LSD1 chemical probes.

Major Concerns:

none.

Minor Concerns:

1. Initially I thought that this assay would be only applicable if the inhibitor-KDM1A binding affinity is way higher than that of OG-881-KDM1A interaction. If inhibitor-KDM1A affinity is low, then OG881 would compete out the inhibitor and bind to KDM1A. But then I realized that the author described cyclopropylamine derivatives covalently binds to FAD. Do all KDM1A inhibitors covalently bind to FAD? If not, the assay should still be limited to a subset of KDM1A inhibitors that act in this way. Discussion on this point will be appreciated for potential user of the assay.

The reviewer realized well. The method works perfectly for all the cyclopropylamine derivatives that covalently bind FAD, and there is no need for the inhibitor binding affinity to be higher than that of the OG-881-KDM1A interaction. We initially developed the method for use with the highly potent KDM1A inhibitor ORY-1001. Therefore, we designed a potent chemoprobe, and, taking into account that some loss of potency was inevitable due to the attachment of a “fishing line” , we add an excess of chemoprobe during the lysis procedure (see also below, in response to questions of Reviewer 3). But the assay works equally well with tranylcypromine, which is 1000 x less potent.

The chemoprobe can also be used to assess reversible inhibitors that prevent access of the chemoprobe to FAD. Reversible LSD1 inhibitors have been designed by different pharmaceutical companies. However, it is obvious that in the case of a reversible inhibitor, the balance in potency as well as the kinetics becomes important. It is convenient to characterize reversible inhibitors in an SPR or jump dilution assay to characterize the off-rate of the compound. We have included the following paragraph into the discussion.

The current protocol was optimized to analyze KDM1A target engagement by covalent FAD targeting inhibitors. It can also be used with reversible inhibitors that block the access of to the FAD cofactor of KDM1A. Potent reversible inhibitors with long residence times may employ the unmodified protocol. The OG-881 chemoprobe may not be suitable for low potency reversible inhibitors with high off-rates.

On the other hand, these compounds may not be the best candidates to be developed as (oral) drugs. Finally, we did find some compounds that were described by others to

inhibit KDM1A but for which we could not detect target engagement. However, we could not get them to work properly in the most basic biochemical activity assays either, so we guessed that figured.

2. I found the term 'target engagement' confusing. Molecular biologists like myself would think 'target' is KDM1A-target genomic loci/genes.

The term “target engagement” is very broadly used in drug development in the exact sense we employed it in the manuscript. In drug discovery or development, the target is the protein the drug is designed for, and “target engagement” is defined as the interaction of a drug with its target biomolecules. The demonstration of target engagement is key in almost any modern drug development program, and the methods used for demonstration are not always direct.

However it also must be said that KDM1A is involved in transcription regulation and is indeed recruited by zinc finger TFs to specific “target loci” in the genome. In addition, the impact of KDM1A inhibitors on the presence or histone methylation status at some of these loci has been used *as a proxy* to demonstrate “target engagement”, which doesn’t make things any easier…

To avoid confusion and make things clear from the start we have made a small modification to the first line of the abstract:

“The assessment of target engagement, *defined as the interaction of a drug with the protein it was designed for*, is a basic requirement for the interpretation of the biological activity of any compound in drug development or in basic research projects.”

In addition we have scanned the text for any use of target not in line with this meaning and have modified the following phrase for clarity of the use of the word target:

“Can be readily applied to other species in which the KDM1A *antibody* target epitopes and catalytic center are conserved”.

Reviewer #2:

Manuscript JoVE59390 Invited Methods Article

Cristina Mascaró, Raquel Ruiz Rodriguez,Tamara Maes

Direct Measurement of KDM1A Target Engagement Using Chemoprobe-based Immunoassay Methods refers to a KDM1A chemoprobes that can be used to assess KDM1A target engagement in cells and tissues. This chemoprobe can also be used to identify and quantify KDM1 in clinical samples and is able to compare in parallel with ELISA technology Total and Free enzyme if the sample has been treated with KDM1 inhibitor ORY-1001.KDM1 enzyme

* title and abstract appropriate for this methods article

* materials and equipment are described properly as well steps for the described protocol are highlighted?

Overall I think that protocol could be useful for readers and method easily applied to different samples . References are described exaustively to follow the rationale of the method and its applications.

Reviewer #3:

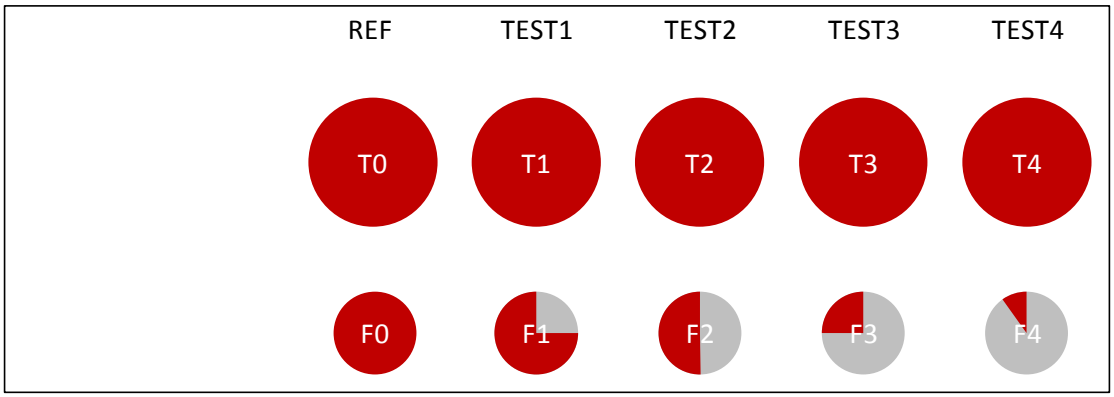
Manuscript Summary:

Nicely described assay that is developed to examine dose effects of KDM1A target engagement by KDM1A-like inhibitors in a 96 well format. A chemoprobe was developed that targets free and active KDM1A but not inhibitor-hound KDM1A. A better description of how the assay differentiates between free and active KDM1A would be helpful.

The target engagement is calculated as described. Below however, a more graphical representation of how the TE is calculated (means, blank subtraction and eventual outlier elimination is omitted for simplicity), if considered useful it can be incorporated in the figures.

Say the target engagement is evaluated in a series of samples treated with increasing dose of inhibitor TEST1 to TEST4 relative to a reference sample REF, untreated.

Between samples, there is little variation in the RLU Total (T) of KDM1A protein, but they are nevertheless measured and used to normalize the samples REF sample. The efficiency of detection of total KDM1A is represented by the size of the T circles. Samples TEST1 to TEST 4 have been treated with inhibitor and therefore produce a decreasing amount of signal in the RLU Free (F) KDM1A assay. The efficiency of detection of the free KDM1A is represented by the F circles. The efficiency may be lower, but that doesn't matter, as the signal will be normalized to the REF sample.



	REF	SX1	SX2	SX2	SX4
RLU_{Total}	100	100	100	100	100
RLU_{Free}	80	60	40	20	8
$R = RLU_{Free}/RLU_{Total}$ (%)	80%	60%	40%	20%	8%
R_{SX}/R_{REF} (%)	100%	75%	50%	25%	10%
TE (%) = 100 - R_{SX}/R_{REF} (%)	0%	25%	50%	75%	90%

We do not differentiate between free and active KDM1A.

It is however a requirement for KDM1A to be detected in the chemoprobe based ELISA that the protein is *both free and active* (ie not bound by the inhibitor and enzymatically active). If you kill the enzyme (pe by heating or denaturing the sample), the chemoprobe won't bind and there is no assay (also if the sample is not properly obtained/stored, activity can be lost).

Since this appears to be confusing, we will substitute “free and active” by free; a phrase in the discussion reflecting that the sample (and the KDM1A in it) has to be in good condition and the sample properly conserved or the method won’t work.

In addition, this is a relative quantitative method (luminescent-based ELISA) and authors provide a detailed protocol that should enable reproducible results.

One minor concern is that the authors state that the assay could be used in any biological sample yet only show results for a few cell types. This is okay as the manuscript focuses on method development but caution is warranted as to how generalizable this approach is for "any biological sample".

Point taken. We have indeed have not tested all tissue types in all species, but some diverse ones: skin, lung, brain, PBMC, tumor tissue, AML, PCa, SCLC, … cells. And we have used samples from rat, mice, humans, baboon.

“Any” was used in a comparative context, after finding out the great limitations to alternative methods we used in our research. Especially, “any” contrasts to methods that require genetic modification of cells.

We have eliminated “any” or substituted it by can be broadly used to…

In addition, it would be nice to know more details on the specificity of the chemoprobe, why it was chosen, and, during the protocol, why it was added when it was added?

The characteristics of the chemoprobe, its development, synthesis, specificity etc are described in a method development manuscript by Mascaró et al. that was submitted elsewhere; we have included an extra reference that will most likely be complement as we received the reviewers comments. The characteristics of the chemoprobe are briefly resumed in the introduction. *“OG-881[15,16], derived from the selective KDM1A inhibitor ORY-1001. The chemoprobe has an IC_{50} for KDM1A of 120 nM, and includes a FAD binding moiety linked to a biotinylated PEG-tail.”*). When contacted by the editor to submit a protocol manuscript, we discussed this and were specifically informed this was not a problem.

Summarized, OG-881 is about 6-7 x less potent than ORY-1001 (but more potent than many of the other KDM1A inhibitors reported in the field), and has a similar selectivity profile as ORY-1001 i.e. doesn’t inhibit related enzymes at the concentrations used. The chemoprobe OG-881 is a biotin-PEG tailed derivative of the highly potent inhibitor ORY-1001 (iadademstat). It was expected that the tailing would have some toll on potency, but we aimed for less than 10 x difference in potency with ORY-1001. It is added in excess at the time of lysis (Step 2.2**)** to drive the rapid engagement to any free KDM1A protein i.e KDM1A not inhibited by ORY-1001 (or another LSD1 inhibitor).

Overall, the manuscript is acceptable, and requires only minor revisions. It offers a succinct account into a KDM1A binding ex vivo approach.

Major Concerns:

no major concerns

Minor Concerns:

1) This actually binding (target engagement) is a relative quantitative method.... The description of DAta processing could more detailed and the ELISA (as described) is only semi-quantitative. A discussion of these potential drawbacks should be included in the discussion.

The formulae for data processing are provided, have been clarified a bit (I hope) by reflecting the stepwise calculation.

We use relative quantification but that doesn't mean the method is semi-quantitative. First of all, please consider that "target engagement" is inherently a relative value, unlike the plasma level of a protein, as we have to compare the free to the total KDM1A in the sample to determine the degree of target occupation.

During the development of the method we evaluated both direct quantification of the Total and Free KDM1A, i.e. calculating the amount of Total and Free KDM1A relative to the respective standard curves included on each plate, and posterior calculation of the TE from these data; as the relative method as described here. The latter was performing in a clearly superior fashion. We suspected variation /decline in the specific activity of the recombinant KDM1A standard or difference in the cellular context (KDM1A in multiprotein complex) relative to the recombinant pure KDM1A standard could affect the Free KDM1A readout.

We have fully validated the relative quantification method for use in clinical trials, and determined linearity, dynamic range and intra and inter-experimental % CV for the target engagement assay.

2) As noted, KDM1A can behave in a very heterogeneous manner in different cell types. Authors look at PBMCs and lung samples from rats. One cell type is likely sufficient for this manuscript but it would be much more convincing to obtain complete data from additional tissue or cell-lines for immunoassay experiments. On a related note, to what degree does the assay's success depend on the purity/source of the antibodies? Additional description of the versatility (or lack of generalizability) should be addressed in the discussion.

Admittedly, checking **all** tissue types was beyond our scope of research but we did use: skin, lung, brain, and PBMCs, as well as AML, PCa and SCLC cell lines, from different species. It was not our intention to demonstrate of all of these, but rather to reflect examples of typical uses of the protocol, parting from the idea that editorial objective of JoVE is not so much the process of the method development (object of the probe and method development manuscript); but providing a solid "executable" protocol.

But the reviewer is right; we indeed invested time in the development of an appropriate chemoprobe, and in the selection of an appropriate antibody pair compatible with the chemoprobe. The binding of the chemoprobe was verified not to interfere with the binding of the antibodies, and the binding of one antibody was not to interfere with the other.

Antibodies chosen were monoclonal and directed against the carboxy and aminoterminal region (target epitopes selected based on 3D structure) and raised in

different species. The selectivity of the secondary antibody to the detection antibody was also verified. In this respect, we have found that overall, commercial human protein directed antibody quality has clearly improved over time, but the same quality may not be available for all proteins in all species. In the case of LSD1, sequence conservation is high however and the antibodies chosen worked well in different species.

3) Can the authors truly distinguish between free and total KDM1A in this largely ex-vivo approach?

We don't use the word distinguish, as this would suggest KDM1A is either free or total and while if anything can be distinguished it is the "free" from "occupied" KDM1A.

When we developed and tested the chemoprobe, we first verified we could quantitively pull-down KDM1A from cell lysate (ie all KDM1A could be pulled down with the chemoprobe) using traditional western blotting. Admittedly, this requires pretty efficient binding. In this case, it was achieved by adding an excess of probe that binds covalently to the FAD cofactor. The probe's biotinylated tail can be captured by another highly efficient interaction: the biotin/streptavidin bond.

What could affect the binding of the probe to KDM1A?

a) Sample quality. If the enzyme is inactivated, the probe will not bind anymore. This was explicitly reflected.

b) Previous binding of inhibitors, especially, previous binding of covalent LSD1 inhibitors. If an LSD1 protein is already bound by an inhibitor, the chemoprobe can not bind anymore, LSD1 is not captured or detected. This is not a problem of course, it is what the assay is all about.

minor editing:

Long Abstract: line 27, remove "here" OK

p.14, line 488, insert "require" "it does not require genetic modifications...." OK
p.14, line 501, remove" on the other side" and replace with "In contrast," OK
p.14, line509-510, reconsider as "and to the best of our knowledge, it has not been reported in clinical trials." OK

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same. Confirmed

2. Please address all the specific comments marked in the manuscript. Confirmed

3. Presently the language is not publication grade. The manuscript needs thorough proofreading. Confirmed

4. Please remove the redundancy in the protocol and make it crisp. Have tried, although I had to balance this with the petition for additional explanations in some steps.

5. Please ensure you answer how question - how is this step performed. This will include providing hard experimental steps, graphical user interface, button clicks, the knob turns etc.

6. Once done please ensure that the highlight is no more than 2.75 pages including headings and spacings.

7. The references 11 and 16 (also answer to your response obtaining copyright permission), are these parent publications? If yes, then do you want us to wait on publishing this article? We cannot have submitted articles in the reference section.

Reference 11 *Maes T., et al. A dual inhibitor of Lysine Specific Demethylase 1 and Monoamine Oxidase B for the treatment of cognitive defects and behavioral alterations in neurodegenerative diseases. Submitted for publication.*

Reference 12. *Fernando Cavalcanti, et al. Vafidemstat reduces lymphocyte egress and demyelination in experimental autoimmune encephalomyelitis. Submitted for publication.*

These 2 references are not parent publications. The method is used to assess TE (in some of many experiments) of Reference 11. These manuscripts focus on the pharmacological and biological characterization of our dual KDM1A/MAOB inhibitor ORY-2001. They are still under revision, and we do not have a good view on the timelines for publication, so we have suppressed the references.

Reference 15 (now Reference 13): *WO2017158136, Oryzon Genomics, Methods to determine kdm1a target engagement and chemoprobes useful therefor.*
This is not a parent publication in the academic sense of the word: it is a patent application. No copyright applies.

Reference 16 (now Reference 14): *Mascaró et al. submitted for publication.* This publication is related to the patent application and relates the design, structure and characterization of the KDM1A chemoprobe, as well as some examples of use, and could be viewed as a parent

publication. It has been reviewed by the target journal and came back with three positive reviews and recommendation for publication, *we hope to submit the revised document this week and will complement the reference list for JoVE asap, and would propose to include it if we can provide the full information in time, but without holding back on the publication.As publication in JoVE still requires the video production, we feel that we will probably be able to provide this information prior to publication, but I must admit: you have the fastest review process I have come across (congrats).* We think it would be beneficial for the JoVE publication to include the reference in the JoVE manuscript as it would provide background information on the probe design, and we think it would be beneficial to the readers of the probe development manuscript to read and view in detail how the TE protocol is performed. Obviously, the other journal is aware that we have a protocol article under review.

Copyright: The data used to exemplify the working of the protocol included in the manuscript submitted to JoVE have not been submitted as part of another formal manuscript and are not subject to copyright.