

# A Cell-based Screening Method Using an Intracellular Antibody for Discovering Small Molecules Targeting Hard-to-drug Proteins

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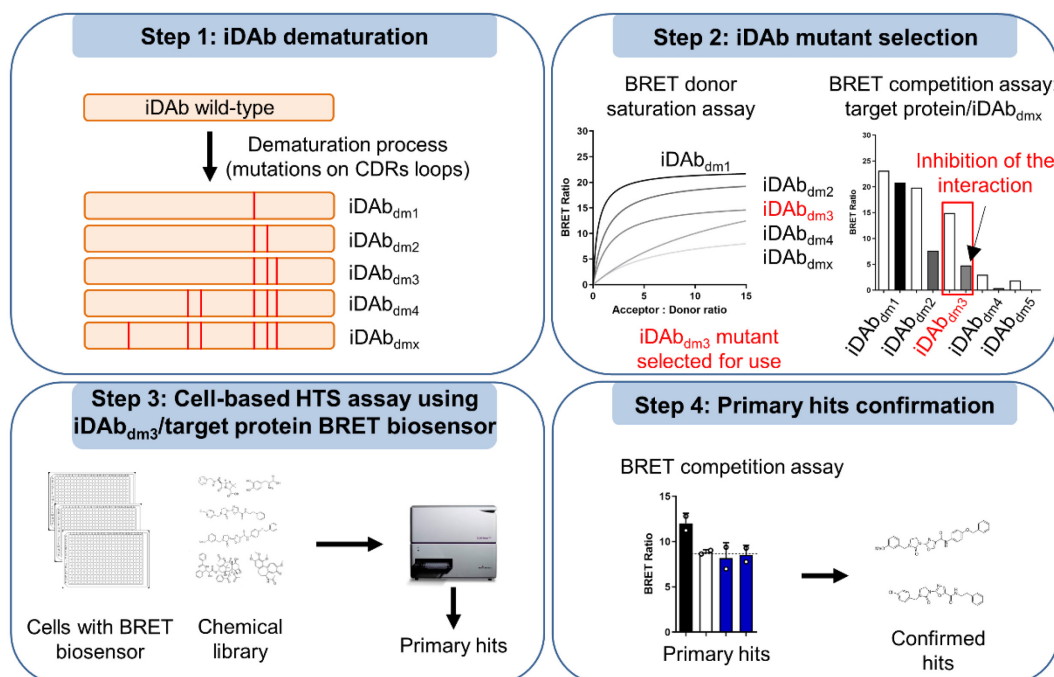
## Abstract

Targeting hard-to-drug proteins, such as proteins functioning by protein-protein interactions (PPIs) with small molecules, is difficult because of the lack of well-defined pockets. Fragment or computational-based methods are usually employed for the discovery of such compounds, but no generic method is available to quickly identify small molecules interfering with PPIs. Here, we provide a protocol describing a generic method to discover small molecules inhibiting the interaction between an intracellular antibody and its target, in particular for proteins that are hard to make in recombinant form. This protocol reports a versatile and generic method that can be applied to any target/intracellular antibody. Because it is a cell-based assay, it identifies chemical matters that are already displaying advantageous cell permeability properties.

**Keywords:** BRET, Intracellular antibody, Hard-to-drug targets, Cell-based screening, Antibody-derived compound, Chromosomal translocation proteins

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## Graphic abstract:



## Cell-based intracellular antibody-guided small molecule screening.

## Background

Targeting protein-protein interactions (PPIs) with small molecules is a challenging task, due to their relatively large interaction interface and the lack of well-defined pockets. Hence, implementing generic methods to discover such compounds would be highly valuable. Interestingly, PPIs can be inhibited by intracellular macromolecules, such as intracellular single domain antibodies (hereafter named iDABs) (Tanaka *et al.*, 2007 and 2011), or antibody mimetics (Guillard *et al.*, 2017; Bery *et al.*, 2019b). The advantages of such reagents are their high specificity for their target and their ability to discriminate isoforms of protein families (Bery *et al.*, 2019a and 2019b), which can enable the identification of novel targetable regions. Therefore, we developed an intracellular antibody-guided method to screen small molecules named antibody-derived compound (Abd). We first used this method in a competitive surface plasmon resonance (cSPR) set-up to identify anti-RAS small molecules that bind at the combining site of an anti-RAS iDAB (Quevedo *et al.*, 2018). However, favourable binding properties of the intracellular antibody with its target (very high affinity, high  $K_{on}$ , and low  $K_{off}$ ) and recombinant protein targets that are easily expressed are required for this biochemical Abd assay. Furthermore, the selected compounds should have advantageous properties in cellular uptake with this assay. Therefore, new versatile methods would be important to rapidly discover small molecules against difficult drug targets.

Accordingly, we implemented an Abd cell-based screening method. We developed bioluminescence resonance energy transfer 2 (BRET2)-based LIM domain only 2 (LMO2) biosensors, based on the strategy of RAS biosensors (Bery *et al.*, 2018), to monitor the interaction between the chromosomal translocation protein LMO2 and its iDAB. This technique enabled the selection of compounds that interfere with this interaction in cells (Bery *et al.*, 2021). The cell-based Abd assay is a versatile method, as the measured *in vitro* affinity of the iDABs for their target is not a limitation. High-affinity iDABs could be dematured to reduce their binding for use in the cell-based assay (Tanaka *et al.*, 2021). In addition, this method is not limited by proteins that are difficult to express and/or to purify in recombinant form. Finally, the intrinsic advantage of cell-based assays is that the compounds already have the

characteristic of cell entry, a highly relevant property for the use of small molecules as drugs.

This protocol could be implemented in any intracellular antibody (or antibody mimetic) targeting challenging proteins, such as transcription factors or chromosomal translocation proteins.

## Materials and Reagents

1. 6-well plates (Corning, catalog number: 3516)
2. Corning HYPERFlask M cell culture vessels (Corning, catalog number: 10030)
3. Corning® 250 mL PP centrifuge tubes (Corning, catalog number: 430776)
4. White 96-well plates, clear bottom (PerkinElmer, catalog number: 6005181)
5. White 384-well plates, clear bottom (PerkinElmer, catalog number: 6007480)
6. Aluminum foil
7. White adhesive bottom seal for 96 and 384-well plates (PerkinElmer, catalog number: 6005199)
8. HEK293T cells (ATCC# CRL-3216, RRID:CVCL\_0063)
9. Chemically competent DH5α cells (New England Biolabs (NEB), catalog number: C2987H)
10. Penicillin/streptomycin (PS) (Thermo Fisher, catalog number: 10378016)
11. Trypsin (Thermo Fisher, catalog number: 25300054)
12. DMEM (Thermo Fisher, catalog number: 10566016)
13. OptiMEM medium (Thermo Fisher, catalog number: 31985070)
14. Lipofectamine 2000 (Thermo Fisher, catalog number: 11668019)
15. Fetal bovine serum (FBS; Sigma, catalog number: F2442)
16. Phosphate-buffered saline (PBS; Thermo Fisher, catalog number: 10010023)
17. RLuc8 construct (see Table 1, sequence freely available in Bery *et al.*, 2018)
18. GFP<sup>2</sup> constructs (see Table 1, sequences freely available in Bery *et al.*, 2021)
19. LMO2 competitors' plasmids (see Table 1, sequences freely available in Bery *et al.*, 2018)
20. OptiMEM no red phenol (Thermo Fisher, catalog number: 11058021)
21. Coelenterazine 400a (Cayman Chemicals, catalog number: 16157). Store at -20°C if not reconstituted.
22. 100% ethanol (Sigma, catalog number: 32221-M54)
23. Dimethyl Sulfoxide (DMSO; Sigma, catalog number: D2650)
24. QIAquick Gel Extraction Kit (QIAGEN, catalog number: 28704)
25. Agarose (Sigma, catalog number: A9539-500G)
26. Restriction enzymes: NotI-HF, XbaI, PspOMI (NEB, catalog numbers: R3189S, R0145S and R0653S respectively)
27. Vent DNA polymerase (NEB, catalog number: M0254S)
28. T4 DNA Ligase (NEB, catalog number: M0202S)
29. Ampicillin (Sigma, catalog number: A0166)
30. pEF-myc-cyto, empty plasmid (Invitrogen, catalog number: V89120)
31. In-house library of 10,720 compounds (comprising 6991 compounds from BioFocus and 3729 from ChemBridge). Stored at -20°C
32. BRET medium (see Recipes)
33. BRET substrate (see Recipes)

**Table 1. List of donor and acceptors constructs available for BRET2-based LMO2 biosensors**

RLuc8 construct	Donor	GFP <sup>2</sup> Acceptors constructs	Competitors constructs
LMO2		iDAb LMO2 WT	iDAb LMO2-myc (positive control, Bery <i>et al.</i> , 2018)
		iDAb LMO2 S55A/T107A (iDAb LMO2 <sub>dm</sub> )	iDAb RAS-myc (negative control, Bery <i>et al.</i> , 2018)
		iDAb LMO2 S28G/H31G/S55A/T107A (iDAb LMO2 <sub>dm1</sub> )	
		iDAb LMO2 S55A/E102A/T107A (iDAb LMO2 <sub>dm2</sub> )	
		iDAb LMO2 S28G/H31G/S55A/E102A/T107A (iDAb LMO2 <sub>dm3</sub> )	
		iDAb LMO2 S28G/H31G/S55A/S103A/T107A (iDAb LMO2 <sub>dm4</sub> )	
		iDAb LMO2 S55A/E102A/S103A/T107A (iDAb LMO2 <sub>dm5</sub> )	
		iDAb LMO2 S28G/H31G/S55A/E102A/S103A/T107A (iDAb LMO2 <sub>dm6</sub> )	

## Equipment

1. Haemocytometer (Sigma, catalog number: Z359629)
2. SpeedVac instrument (any supplier)
3. Janus liquid handling workstation (PerkinElmer)
4. Echo Acoustic Dispenser (Labcyte)
5. CLARIOstar microplate reader including the luminescence module (BMG Labtech)
6. PHERAstar FSX microplate reader with the BRET2 optic filters (BMG Labtech)
7. Cell culture 37°C incubator (any supplier)
8. Centrifuge (any supplier)

## Software

1. MARS Data analysis (BMG Labtech, included with the microplate readers)

## Procedure

### A. Construction of iDAb LMO2 mutants (dematuration process)

To set up a BRET-based biosensor capable of monitoring the LMO2-iDAb LMO2 interaction that could be displaced by small molecules, we first constructed various mutants of iDAb LMO2. These mutations were on key residues involved in LMO2 binding and that were on the complementarity determining regions (CDRs) of the iDAb. Here, the aim is to lower the interaction sufficiently (*i.e.*, a dematuration process) to facilitate its inhibition by compounds from a chemical library. We used pEF-GFP<sup>2</sup>-iDAb LMO2<sub>dm</sub>-myc (*i.e.*, iDAb LMO2

S55A/T107A) as template (Bery *et al.*, 2018).

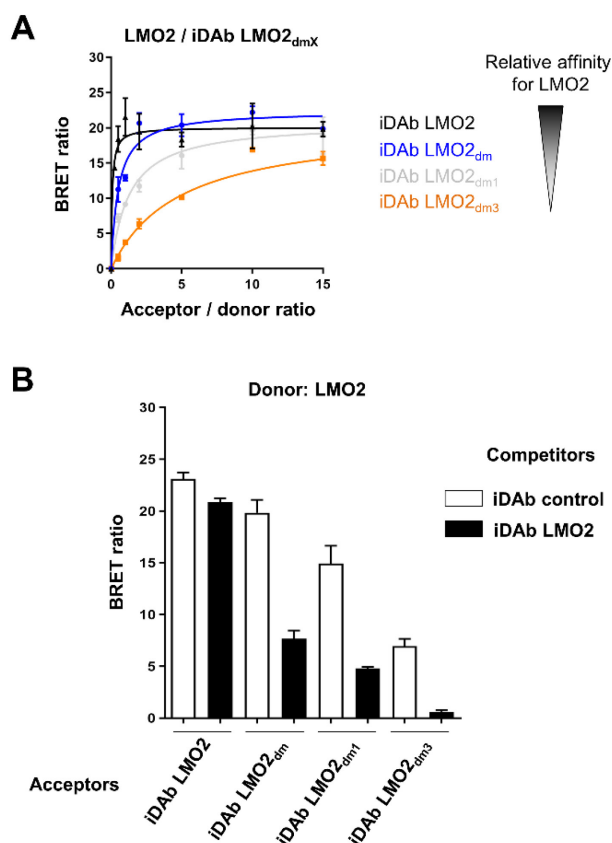
1. Generation of pEF-GFP<sup>2</sup>-iDAb LMO2 S55A/E102A/T107A-myc vector by polymerase chain reaction (PCR) site-directed mutagenesis:
  - a. PCR amplify the iDAb LMO2 S55A/T107A-myc with iDAbPspOMIFor and E102ARev primers and with E102AFor and MyctagstopXbaIRev primers (see Table 2), using Vent DNA polymerase [1  $\mu$ L of template (20 ng/ $\mu$ L), 5  $\mu$ L of 10 $\times$  PCR buffer, 2  $\mu$ L of dNTP mix (10 mM each), 0.4  $\mu$ L of MgSO<sub>4</sub>, 0.6  $\mu$ L of DMSO, 0.5  $\mu$ L of each F&R primers (50  $\mu$ M), 1  $\mu$ L of Vent DNA polymerase, and 39  $\mu$ L of dH<sub>2</sub>O].
  - b. PCR: Place at 95°C for 5 min, then 35 cycles of: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by a single elongation step of 72°C for 10 min. The annealing temperature varies depending on the primers. This can be checked online: <https://tmcalculator.neb.com/#/main>.
  - c. Run the two PCR products generated on a 1% agarose gel (120 V, 30 min).
  - d. Purify the PCR fragments using the QIAquick gel extraction kit, according to the manufacturer's instructions, and elute in 30  $\mu$ L of elution buffer.
  - e. PCR assembly of both PCR products following the same protocol from Step A1a-b, using iDAbPspOMIFor and MyctagstopXbaIRev primers.
  - f. Run the entire assembled PCR product on a 1% agarose gel (120 V, 30 min).
  - g. Purify the PCR fragment using the QIAquick gel extraction kit and elute in 30  $\mu$ L of elution buffer.
  - h. Digest the PCR fragment with PspOMI and XbaI at 37°C for 2 h (1.5  $\mu$ L of each enzyme, 5  $\mu$ L of 10 $\times$  Cutsmart, and 1  $\mu$ g of PCR product, H<sub>2</sub>O to 50  $\mu$ L final volume).
  - i. Heat inactivate the digestion at 65°C for 20 min.
  - j. Ligate at room temperature (RT: 22°C) for 2 h the digested PCR product (insert) into the pEF-GFP<sup>2</sup>-MCS vector (Bery and Rabbitts, 2019) [10 ng pEF-GFP<sup>2</sup>-MCS (NotI/XbaI), 40 ng iDAb LMO2 S55A/E102A/T107A-myc insert, 2  $\mu$ L of 10 $\times$  T4 DNA ligase buffer, 1  $\mu$ L of T4 DNA ligase, H<sub>2</sub>O to 20  $\mu$ L final volume].
  - k. Add 5  $\mu$ L of the ligation into 50  $\mu$ L of DH5 $\alpha$  competent cells and leave to transform on ice for 30 min. Heat shock at 42°C for 40 s and then place on ice for 10 min. Add 500  $\mu$ L of lysogeny broth (LB) medium and shake at 37°C for 1 h. Plate the cells on an LB agar plate with ampicillin (100  $\mu$ g/mL).
2. Generate all the other iDAb LMO2 mutants following the same procedure shown in Step A1 and using the primers in Table 2.

**Table 2. Primers used for the site-directed mutagenesis to demature iDAb LMO2**

<b>iDAbPspOMIFor</b>	5' TTATTCGGGCCCCGTATGGCCGAGGTGCAGCTGTTGG 3'
<b>MyctagstopXbaIRev</b>	5' TATTAATCTAGACTATGCGGCCCCATTCAGATCC 3'
<b>S28GHFor</b>	5' CCTCTGGATTTCGGCTTCAGTCATAGTCC 3'
<b>S28GHRev</b>	5' GGAATATGACTGAAGCCGAATCCAGAGG 3'
<b>H31GFor</b>	5' GGATTGAGCTTCAGTGGCAGTCCTATGAATTGG 3'
<b>H31GRev</b>	5' CCAATTCATAGGACTGCCACTGAAGCTGAATCC 3'
<b>E102AFor</b>	5' GAGAGGGTTGACGGCCTCTCTTGAGTTGGC 3'
<b>E102ARev</b>	5' GCCAACTCAAGAGAGGCCGTCAACCCTCTC 3'
<b>S103AFor</b>	5' GAGAGGGTTGACGGAGGCGCTTGAGTTGGC 3'
<b>S103ARev</b>	5' GCCAACTCAAGCGCCTCCGTCAACCCTCTC 3'

## B. BRET2 donor saturation of LMO2/iDAb LMO2 mutants

To check whether iDAb LMO2 mutants interact with LMO2, a donor saturation assay is performed (Figure 1A). This assay will also give insight into the relative affinity of each mutant towards LMO2, as it will be mandatory to determine the right acceptor quantity to express within the cells to avoid the titration of inhibitors in a competition assay (see section C). The whole protocol is performed in a tissue culture hood to maintain sterile conditions.



**Figure 1. Selection of the optimal iDAb LMO2 mutant for the chemical library screening.**

A. BRET donor saturation assay between LMO2 (donor) and various iDAb LMO2 mutants obtained after the dematuration process (acceptors). The mutations added within the CDR loops (dematuration) decrease the relative affinity of the corresponding iDAb, as shown by the donor saturation assay. B. BRET competition assay between LMO2 and the different iDAb LMO2 (mutants) to be tested. iDAb control is a non-relevant iDAb (iDAb RAS) and iDAb LMO2 is the wild-type iDAb binding LMO2 with high affinity (positive control).

- On day 1, plate 650,000 HEK293T cells in each well of a 6-well plate in DMEM medium supplemented with 10% FBS and 1% PS.
- On day 2, transfect the cells:
  - Add in 100  $\mu$ L of OptiMEM medium: 50 ng RLuc8 construct and varying amount of GFP<sup>2</sup> construct: 50, 100, 250, 500, 750 and 1,000 ng. The pEF-myc-cyto empty plasmid is used to equalize the total amount of transfected DNA between each condition (total DNA amount is 1.6  $\mu$ g).
  - Add 4  $\mu$ L of Lipofectamine 2000 transfection reagent in a separate tube with 100  $\mu$ L of OptiMEM medium.
  - Add the DNA mix from Step B2a into the Lipofectamine mix from Step B2b, mix, and leave for 20 min at RT.
  - Add 200  $\mu$ L of transfection mix into each well.
  - Incubate the cells for 24 h at 37°C (in a cell culture incubator with 5% CO<sub>2</sub>).
- On day 3, prepare the BRET plate:
  - Aspirate the medium and add 500  $\mu$ L of trypsin into each well to detach the cells (2 min at RT).
  - Neutralize the trypsin with 1 mL of complete DMEM (with 10% FBS).
  - Count the cells of one or two conditions (assuming that the cell number is equivalent between each condition after checking under the microscope).
  - Add 500,000 cells and 1 mL of PBS in a 15-mL Falcon tube.

- e. Centrifuge at  $240 \times g$  and  $20^{\circ}\text{C}$  for 5 min, and aspirate the supernatant.
- f. Add 1 mL of OptiMEM no phenol red supplemented with 4% FBS (BRET medium) and carefully resuspend the cells.
- g. Add 100  $\mu\text{L}$  of cell suspension per well of a white clear bottom 96-well plate (make quadruplicates for each condition).
- h. Leave the cells at  $37^{\circ}\text{C}$  (in a cell culture incubator with 5%  $\text{CO}_2$ ) for 20 h before performing the BRET reading.

### C. BRET2 competition assay

After the donor saturation assay described in section B, a competition assay is performed to (i) check the specificity of the interaction, and (ii) determine the optimal dematured iDAb that will be used for the chemical library screen. In that case, the optimal mutant iDAb displays a high BRET signal, but its interaction with LMO2 has to be almost fully inhibited by the wild-type iDAb LMO2 in a competition assay (Figure 1B). This protocol is performed in a tissue culture hood to maintain sterile conditions.

1. On day 1, plate 650,000 HEK293T cells in each well of a 6-well plate in DMEM medium supplemented with 10% FBS and 1% PS.
2. On day 2, transfect the cells:
  - a. Add in 100  $\mu\text{L}$  of OptiMEM medium: 50 ng LMO2-RLuc8 construct, 100 ng of GFP<sup>2</sup>-iDAb LMO2 mutants construct with 100 ng of pEF-iDAb competitor (always use a negative control (*i.e.*, iDAb RAS in a competition assay). The pEF-myc-cyto empty plasmid is used to equalize the total amount of transfected DNA between each condition (total DNA amount is 1.6  $\mu\text{g}$ ).
  - b. Add 4  $\mu\text{L}$  of Lipofectamine 2000 transfection reagent in a separate tube with 100  $\mu\text{L}$  of OptiMEM medium.
  - c. Add the DNA mix from Step C2a into the Lipofectamine mix from Step C2b, mix, and leave for 20 min at RT.
  - d. Add 200  $\mu\text{L}$  of transfection mix into each well.
  - e. Incubate the cells at  $37^{\circ}\text{C}$  (in a cell culture incubator with 5%  $\text{CO}_2$ ) for 24 h.
3. On day 3, prepare the BRET plate as described in Step B3.

### D. BRET2 measurements

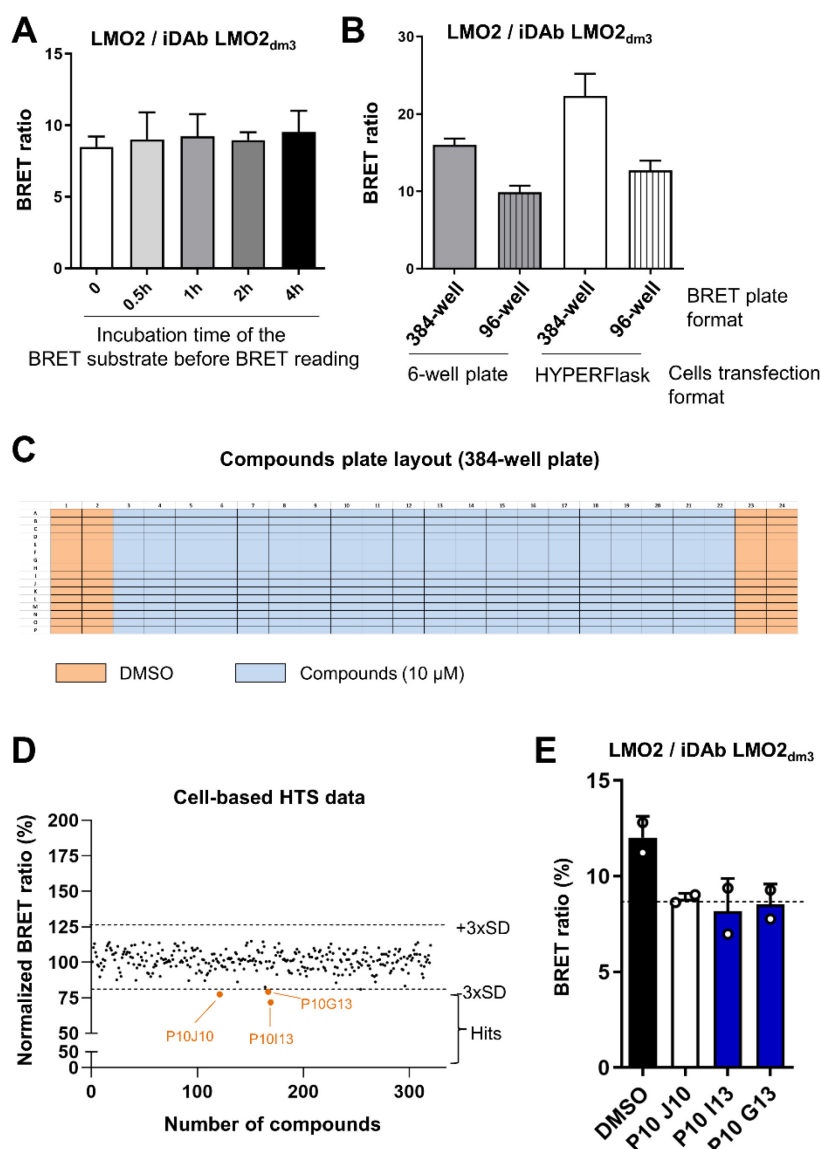
*Note: This step does not require sterile tissue culture hood use.*

1. Resuspend an aliquot of Coelenterazine 400a (BRET substrate) in 500  $\mu\text{L}$  of 100% ethanol.
2. Add 750  $\mu\text{L}$  of BRET medium to make 1.25 mL of BRET substrate at 100  $\mu\text{M}$ .
3. Read the GFP<sup>2</sup> fluorescence first on a CLARIOstar instrument with the following settings: excitation  $395 \pm 30$  nm and emission  $510 \pm 40$  nm.
4. Tape the bottom of the 96-well plate with a white tape before the BRET reading (otherwise a loss of signal is observed).
5. Link the resuspended BRET substrate with the pump/injector of the CLARIOstar.
6. Read the BRET signal with a CLARIOstar instrument equipped with a luminescence module. Set up a luminescence protocol with two multichromatics and use the following monochromator settings:  $410 \pm 40$  nm (emission 1) and  $515 \pm 15$  nm (emission 2), which are needed to determine the BRET signal (see data analysis section for BRET signal calculation). Read the plate in well mode, which is performed at RT: 10  $\mu\text{L}$  of substrate is injected in well A1, the BRET signal read in well A1, then the substrate is injected in well A2, and the BRET signal read in well A2, until the last selected well on your plate.
7. Read the RLuc8 signal at  $480 \pm 40$  nm.



## E. High throughput chemical screening (HTS) with LMO2/iDAb LMO2 mutant BRET biosensor

Once the mutant has been validated, it can be used for the HTS assay. Several parameters have to be optimized or tested before starting the HTS assay, such as the stability of the BRET substrate at RT, the miniaturization of the assay (from 96-well plate to 384-well plate format), and the scaling up of the transfection of HEK293T cells with the BRET pair to obtain a number of cells sufficient for the HTS assay (Figure 2A and 2B). The screen was performed with an in-house library of 10,720 compounds from BioFocus and Chembridge libraries compressed into 384-well plate format at 10  $\mu$ M. Two sessions of HTS, containing 5,360 compounds each, were performed in 68 assay plates (34 plates in duplicate) because of timing/handling purposes. The protocol for one session of 34 plates is described below.



**Figure 2. Optimisation and implementation of the cell-based high-throughput screening assay to identify inhibitors of LMO2/iDAb LMO2 PPI.**

A. BRET signal of LMO2/iDAb LMO2<sub>dm3</sub> interaction using the BRET substrate that was incubated at RT for the indicated time (protected from light). B. Impact on the BRET signal when scaling up the cell transfection

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format (from 6-well plate to HYPERFlask) and miniaturising the assay for HTS (from 96-well plate to 384-well plate format). 20,000 and 50,000 cells were plated per well of a 384-well plate and a 96-well plate respectively. C. HTS compounds 384-well plate layout. D. Representative data from the cell-based HTS. These data are from one 384-well plate that includes 3 potential hits indicated in orange [below the threshold of  $3 \times$  standard deviations (SD) of the  $BRET_{DMSO}$  (mean)]. E. Confirmation of the primary hits at  $10 \mu M$  by BRET competition assay with the LMO2/iDAb LMO2<sub>dm3</sub> pair. Two hits are confirmed to inhibit the LMO2/iDAb LMO2<sub>dm3</sub> interaction and are depicted by blue bars. The threshold [ $BRET_{DMSO}$  (mean)  $\pm 3 \times SD$ ] is shown with the dotted line.

1. On day 1, inoculate  $120 \times 10^6$  HEK293T cells per HYPERFlask. Two HYPERFlasks are needed for one HTS session (*i.e.*,  $240 \times 10^6$  HEK293T cells in total). The surface of one HYPERFlask corresponds to the surface of  $\sim 10 \times T175$ . Fill the HYPERFlask with medium (up to 560 mL, which is the maximal volume of an HYPERFlask).

*Note: Be careful when filling the HYPERFlask with medium, as it is easy to make lots of air bubbles that need to be avoided for proper cell growth. Always fill the HYPERFlask up to the lid to avoid air bubbles.*

2. On day 2, transfect the cells in the HYPERFlasks with the BRET pair pEF-LMO2-RLuc8 and pEF-GFP<sup>2</sup>-iDAb LMO2<sub>dm3</sub>. For one HYPERFlask:
  - a. In a 15 mL Falcon tube, add in 10 mL of OptiMEM: 19  $\mu g$  of pEF-LMO2-RLuc8, 37  $\mu g$  of pEF-GFP<sup>2</sup>-iDAb LMO2<sub>dm3</sub>, and 244  $\mu g$  of pEF-myc-cyto empty plasmids.
  - b. In a separate 50 mL Falcon tube, add 750  $\mu L$  of Lipofectamine 2000 in 10 mL of OptiMEM, and mix gently by vortexing for 5 s.
  - c. Add the DNA mix from Step E2a into the Lipofectamine mix from Step E2b, vortex gently for 5 s, and wait for 20 min.
  - d. Add this mix (DNA+Lipofectamine) into 500 mL of complete DMEM, gently mix the medium, and pour it into a sterile beaker. Remove the medium for the HYPERFlask and pour the transfection mix into the HYPERFlask carefully without any bubbles. Complete with medium until the top of the flask.

*Note: The use of a beaker avoids the formation of bubbles when pouring the medium into the HYPERFlask.*

3. On day 3 (morning, 24 h after the transfection), harvest the transfected cells:
  - a. Add 100 mL of trypsin per HYPERFlask (*i.e.*, 200 mL in total).
  - b. Incubate the flasks at  $37^\circ C$ , for 2 min.
  - c. Transfer the trypsinised cells into a sterile beaker already containing 100 mL of complete medium.
  - d. Wash each flask with 100 mL of complete medium and pour it into the beaker (final volume of 300 mL for one flask). Pull all the cells in one beaker (final volume of 600 mL).
  - e. Mix gently but thoroughly to ensure a good dissociation of the cells.
  - f. Count the cells.
  - g. Add  $90 \times 10^6$  cells per 250 mL Corning centrifuge tube, and prepare 4 centrifuge tubes, corresponding to  $360 \times 10^6$  transfected cells in total.
  - h. Centrifuge the cells at  $220 \times g$  and  $20^\circ C$  for 5 min.
  - i. Remove the supernatant carefully and resuspend each cell pellet with 200 mL of BRET medium supplemented with 1% PS (4 pellets in total). This corresponds to a concentration of  $0.45 \times 10^6$  cells/mL.
  - j. Seed 45  $\mu L$  of cell suspension per well of a white 384-well plate with a PerkinElmer Janus liquid handling workstation housed in a category 2 enclosure. It takes around 10 min to seed  $6 \times 384$ -well plates with this equipment.

*Note: Run an unused plate first to remove all the remaining air bubbles from the liquid handling workstation. Gently mix the cells with a magnetic stirbar while dispensing them into the 384-well plate to keep them dissociated, as HEK293T might have a tendency to form aggregates that could clog the workstation.*

- k. Leave the plates on a flat surface for 20 min before incubating them into a cell culture incubator at 37°C with 5% CO<sub>2</sub> for 24 h. This step will ensure a homogenous plating of the cells through each well.
4. On day 3 (beginning of the afternoon), dilute the chemical library (initial concentration of 10 mM) to 100 µM.  
Add 150 nL of each compound into 15 µL of BRET medium using an Echo Acoustic Dispenser (Labcyte).
5. On day 3 (late afternoon), add the compounds to the cells.
  - a. Add 5 µL of 1% DMSO (prepared in BRET medium) in the columns 1, 2, 23, and 24 as negative controls (Figure 2C).
  - b. Add 5 µL of compounds at a concentration of 100 µM to cells in each well using the PerkinElmer Janus liquid handling workstation (final concentration in each well: 10 µM, 0.1% DMSO).
  - c. Incubate the plate at 37°C (cell culture incubator with 5% CO<sub>2</sub>) for 20 h.
6. On day 4, plate reading (morning, for 20 h of incubation).
  - a. Read the GFP<sup>2</sup> signal of each plate before the BRET2 reading with a PHERAstar FSX plate reader.
  - b. After the GFP<sup>2</sup> reading, tape the bottom of the 384-well plate with a white tape (furnished with the plates).
  - c. Prepare 80 mL of 100 µM BRET substrate: dissolve 3 mg of coelenterazine 400a with 32 mL of 100% ethanol, then complete to 80 mL by adding 48 mL of BRET medium.
  - d. Read the BRET2 signal using a BRET2 optic module with the PHERAstar FSX plate reader: inject 5.5 µL of BRET substrate (final concentration of 10 µM) into a well, read the BRET2 signal 0.2 s after the injection for 1 s, inject into another well, and read the BRET2 signal with the same settings.
  - e. The reading time for one 384-well plate is around 8 min. It takes ~4.5 h to read 34 plates (see data analysis section for the HTS plates analysis and hits determination, Figure 2D).

*Note: All the steps described in the section E are done under a tissue culture hood to maintain sterility except steps 4 and 6.*

## F. Confirmation of primary hits

After HTS assay, we identified 34 primary hits that decreased the BRET ratio emanating from the interaction LMO2 / iDAb LMO2<sub>dm3</sub>. The next step is to re-test these compounds with the same BRET assay than the HTS to check if these are genuine hits (Figure 2E). Below is the protocol for performing a competition assay with the BRET pair LMO2/iDAb LMO2<sub>dm3</sub> in a full 96-well plate. It can be scaled up depending on the number of compounds to be tested.

1. On day 1, plate 650,000 HEK293T cells in each well of a 6-well plate as done in Step C1.
2. On day 2, transfect the cells:
  - a. In 100 µL of OptiMEM medium, add 50 ng LMO2-RLuc8 construct and 100 ng of GFP<sup>2</sup>-iDAb LMO2<sub>dm3</sub> construct with 1,450 ng of the pEF-myc-cyto empty plasmid (the total DNA amount is 1.6 µg).
  - b. Add 4 µL of Lipofectamine 2000 transfection reagent in a separate tube with 100 µL of OptiMEM medium.
  - c. Add the DNA mix from Step F2a into the Lipofectamine mix from Step F2b, mix, and leave for 20 min at RT.

- d. Add 200  $\mu$ L of transfection mix into each well.
- e. Incubate the cells for 24 h at 37°C (in a cell culture incubator with 5% CO<sub>2</sub>).

*Note: Four transfected wells of a 6-well plate typically yield enough cells to perform a competition assay in a full 96-well plate.*

3. On day 3, prepare the BRET plate:
  - a. Aspirate the medium and add 500  $\mu$ L of trypsin into each well to detach cells (2 min at RT).
  - b. Neutralize the trypsin with 1 mL of complete DMEM (with 10% FBS).
  - c. Pool the cells in a 15 mL Falcon and count the cells.
  - d. Add 5 million cells in a new 15 mL Falcon tube and add 5 mL of PBS.
  - e. Centrifuge at 240  $\times$  g and 20°C for 5 min, and aspirate the supernatant.
  - f. Add 12 mL of BRET medium and carefully resuspend the cells.
  - g. Seed 90  $\mu$ L of cells ( $\approx$  38,000 cells) per well of a white clear bottom 96-well plate.
  - h. Leave the cells for 4 h at 37°C in a cell culture incubator with 5% CO<sub>2</sub>, before the addition of the compounds.
4. On day 3, prepare the compounds:
  - a. Dilute 10 mM stock of the compounds in 100% DMSO in BRET medium to reach 100  $\mu$ M, 2% DMSO (10 $\times$  the assay concentration which is 10  $\mu$ M).
  - b. Add 10  $\mu$ L of 10 $\times$  compounds in each well of the 96-well plate including a DMSO negative control. The final concentration in the well is 10  $\mu$ M, 0.2% DMSO. Perform quadruplicates for each point.
  - c. Incubate the cells at 37°C for an additional 20 h before the BRET reading.
5. On day 4, read the BRET signal as described in section D.

*Note: All the steps described in the section F are done under a tissue culture hood to maintain sterility except step 5 (BRET reading).*

## Data analysis

### A. BRET ratio calculation for donor saturation and competition assays

The BRET signal or BRET ratio corresponds to the light emitted by the GFP<sup>2</sup> acceptor construct (515  $\pm$  15 nm) after addition of Coelenterazine 400a, divided by the light emitted by the RLuc8 donor construct (410  $\pm$  40 nm). The donor-only negative control where only the RLuc8 plasmid is transfected into the cells and gives a background signal that is subtracted from the BRET ratio. Using the CLARIOstar and PHERAstar instruments, we multiplied the value of the BRET ratio by 100 to get whole numbers (above 1). For the titration curves, the results are analyzed by nonlinear regression assuming a hyperbolic model.

### B. BRET ratio normalization for the competition assays

In a BRET competition assay, the data are normalized to a negative control (e.g., no competitor or non-LMO2 binder for iDabs and DMSO for small molecules) to compare the effect of a competitor (iDab or small molecules). The normalized BRET ratio is calculated as follows:

$$\text{BRET}_{\text{compound}} / \text{BRET}_{\text{DMSO}} \times 100$$

$$\text{BRET}_{\text{iDab LMO2}} / \text{BRET}_{\text{iDab control}} \times 100$$

$$\text{BRET}_{\text{iDAb LMO2}} / \text{BRET}_{\text{no competitor}} \times 100,$$

where  $\text{BRET}_{\text{compound}}$  corresponds to the BRET ratio for the compound-treated cells,  $\text{BRET}_{\text{DMSO}}$  corresponds to the BRET ratio for DMSO-treated cells,  $\text{BRET}_{\text{iDAb LMO2}}$  corresponds to the anti-LMO2 iDAb (mutants) transfected cells,  $\text{BRET}_{\text{iDAb control}}$  corresponds to the negative control iDAb transfected cells (non-LMO2 binder, e.g., anti-RAS iDAb), and  $\text{BRET}_{\text{no competitor}}$  corresponds to the cells not transfected with a competitor.

### C. HTS data calculation

1. Analyse the HTS data with MARS Data analysis software. The BRET ratio is calculated as indicated in A, without the removal of the donor only signal.
2. Analyse the hits for each plate:
  - a. Calculate the BRET ratio mean of all the DMSO treated wells (negative control) from one plate  $\pm$  SD. Therefore, one  $\text{BRET}_{\text{DMSO}}$  (mean) is calculated per plate.
  - b. Normalize the BRET ratio data of all the compounds to the  $\text{BRET}_{\text{DMSO}}$  (mean) of the same plate.
  - c. Because no positive hits were available, a hit was defined as showing a normalized BRET ratio below the  $\text{BRET}_{\text{DMSO}}$  (mean)  $- 3 \times \text{SD}$ .
  - d. A positive hit is a compound that shows a BRET ratio below the indicated threshold in both duplicate plates (Figure 2D).
  - e. From over 10,720 screened compounds, 34 hits were identified. A hit rate of 0.3% was obtained with this HTS.

## Recipes

### 1. BRET medium

OptiMEM no phenol red + 4% FBS (+ 1% PS, for the HTS assay).

### 2. BRET substrate

- a. Resuspend 1 mg of Coelenterazine 400a in 10.2 mL of 100% ethanol to make a 250  $\mu\text{M}$  solution.
- b. Divide this solution into 20 aliquots of 500  $\mu\text{L}$ .
- c. Evaporate the ethanol from the solution with a SpeedVac instrument and store aliquots at  $-80^\circ\text{C}$  (stable up to 1 year at  $-80^\circ\text{C}$  when dry).
- d. One aliquot is enough to read one 96-well plate. Coelenterazine 400a substrate is light sensitive, protect from light with aluminum foil.

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## Competing interests

The authors declare that they have no competing interests.

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