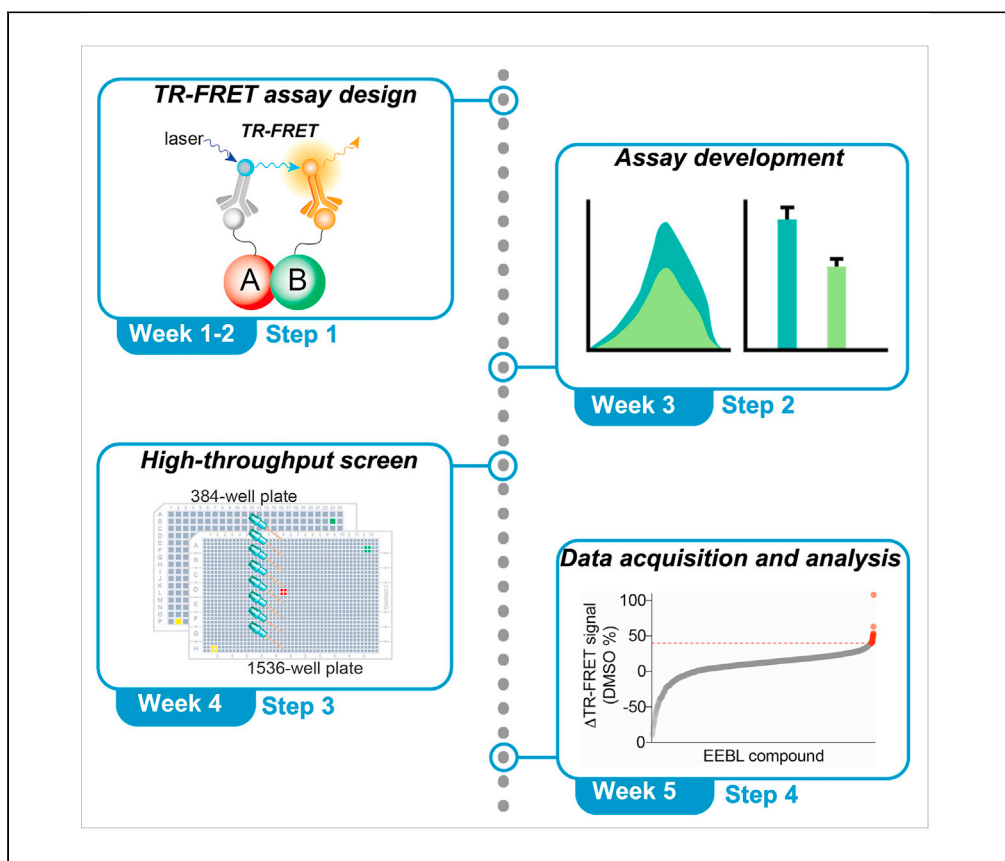


## Protocol

# A time-resolved fluorescence resonance energy transfer screening assay for discovery of protein-protein interaction modulators



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### Highlights

Protocol for high-throughput PPI inducer and inhibitor discovery

A TR-FRET assay recapitulates mutated SMAD4 interaction with SMAD3

A TR-FRET uHTS platform for variant-directed PPI inducer discovery

Protein-protein interactions (PPIs) have emerged as promising yet challenging therapeutic targets. A robust bioassay is required for rapid PPI modulator discovery. Here, we present a time-resolved Förster's (fluorescence) resonance energy transfer assay protocol for PPI modulator screening in a 1536-well plate format. We use hypomorph SMAD4<sup>R361H</sup>-SMAD3 PPI as an example to illustrate the application of the protocol for screening of variant-directed PPI inducers. This platform can be readily adapted for the discovery of both small-molecule PPI inducers and inhibitors.

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## Protocol

## A time-resolved fluorescence resonance energy transfer screening assay for discovery of protein-protein interaction modulators

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## SUMMARY

Protein-protein interactions (PPIs) have emerged as promising yet challenging therapeutic targets. A robust bioassay is required for rapid PPI modulator discovery. Here, we present a time-resolved Förster's (fluorescence) resonance energy transfer assay protocol for PPI modulator screening in a 1536-well plate format. We use hypomorph SMAD4<sup>R361H</sup>-SMAD3 PPI as an example to illustrate the application of the protocol for screening of variant-directed PPI inducers. This platform can be readily adapted for the discovery of both small-molecule PPI inducers and inhibitors.

For complete details on the use and execution of this protocol, please refer to Tang et al. (2020).

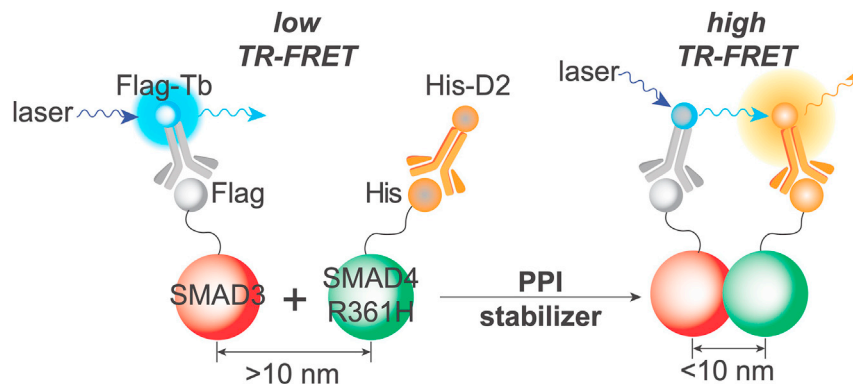
## BEFORE YOU BEGIN

Protein-protein interactions (PPI) are emerging therapeutic targets in diverse disease areas, such as cancer (Hahn et al., 2021). The discovery of an increasing number of oncogenic PPIs has significantly expanded the therapeutic target space (Boettcher et al., 2018; Grzeskowiak et al., 2018; Ivanov et al., 2017a, 2017b; Li et al., 2017; Mo et al., 2017; Rusnak et al., 2018). This development demands highly efficient experimental approaches for accelerated discovery of PPI modulators. The protocol below describes the specific steps of screening for small molecule inducers, molecular glues, for the target-of-interest PPI, SMAD4<sup>R361H</sup>-SMAD3 (Tang et al., 2020), by using a cell lysate-based TR-FRET HTS assay (Figure 1). This protocol does not require purified protein components for efficient assay development and screening implementation (Li et al., 2017). The "molecular glue" is a promising strategy to enhance protein-protein interactions, and re-activate the function of such protein-protein interactions (Hata and Lagna, 2021; Schreiber, 2021). High-throughput screening technology provides the potential to reveal small molecule modulators for targeted PPIs (Janzen, 2014; Macaron et al., 2011). We have also used a similar strategy to discover other PPI inhibitors or inducers (Du et al., 2013; Mo et al., 2019; Xiong et al., 2018).

## Molecular cloning

© Timing: 1–2 weeks





**Figure 1. Schematic illustration of the design of the TR-FRET assay for monitoring the SMAD4/SMAD3 PPI to discover small-molecule PPI inducers**

Anti-Flag-Tb coupled with Flag-SMAD3 serves as the TR-FRET donor and anti-His-D2 coupled with His-SMAD4<sup>R361H</sup> serves as the acceptor. At the basal level, R361H reduces SMAD4 interaction with SMAD3, yielding low TR-FRET signal. Upon treatment with a PPI inducer, the induced SMAD3/SMAD4<sup>R361H</sup> complex formation brings two fluorophores into close proximity (<10 nm), generating a high TR-FRET signal.

1. Generation of mammalian gene expression plasmids for expressing Flag- or His-tagged SMAD4 wild-type (WT), SMAD4-R361H mutant and SMAD3, respectively.
2. Sequencing verification of plasmids.
3. DNA plasmid purification.

**Note:** We generated plasmids to exogenously overexpress proteins with affinity tags for fluorophore-conjugated antibody coupling.

**Note:** We used Gateway cloning technology for plasmid construction by following manufacturer's protocols (<https://tools.thermofisher.com/content/sfs/manuals/gatewayman.pdf>). The SMAD4 point mutation R361H was introduced using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) by following manufacturer's protocols (<https://www.agilent.com/cs/library/usermanuals/public/210518.pdf>), and the SMAD4 pDONR221 plasmid as DNA template. Other molecular cloning technologies can also be used.

**Note:** We highly recommend using sequencing verified plasmid prepared from the same batch throughout the protocol.

**Alternatives:** Untagged endogenous proteins could also be used to couple authenticated fluorophore-conjugated primary antibodies to the proteins of interest (Cui et al., 2014). In this case, the molecular cloning step could be omitted. Purified proteins can be used for fluorophore coupling. In this case, exogenous expression of the protein of interest in cultured cells is not needed.

## Culture cell lines

⌚ Timing: 1 week

4. Culture human embryonic kidney 293T (HEK293T) cells in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin, and keep cells growing in a humid environment with 5% CO<sub>2</sub> at 37°C.

**Note:** We highly recommend following standard cell culture guideline to maintain cells within low passages at exponential growth phase without other biological contamination.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Tb cryptate-labeled anti-Flag M2 antibody (1:500~1:1000)	Cisbio Bioassays	Cat# 61FG2TLB
D2-labeled anti-His antibody (1:200~1:500)	Cisbio Bioassays	Cat# 61HISDLF
<b>Chemicals, peptides, and recombinant proteins</b>		
Emory Enriched Bioactive Library (EEBL)	ECBDC	N/A
Protease Inhibitor Cocktail	Sigma-Aldrich	Cat# P8340
PIC2 Phosphatase Inhibitor Cocktail	Sigma-Aldrich	Cat# P5726
PIC3 Phosphatase Inhibitor Cocktail	Sigma-Aldrich	Cat# P0044
Gateway™ LR Clonase™ II enzyme mix	Thermo Fisher	Cat# 11791100
Linear polyethylenimine (PEI)	Polysciences	Cat# 23966
<b>Recombinant DNA</b>		
SMAD3 WT in pDONR221	Human ORFeome Library	Clone# IOH27044
SMAD4 WT in pDONR221	Human ORFeome Library	Clone# IOH3638
Gateway™ pDEST™26 Vector for His-tag	Invitrogen	Cat# 11809019
Modified pcDNA3.2-V5-dest for Flag-tag	ECBDC	N/A
<b>Oligonucleotides</b>		
R361H forward primer (5'-CTTCTGGAGGAGATCACTTTTGGTCAAC-3')	Eurofins Genomics	N/A
R361H reverse complementary primer (5'-GTTGACCAAAACAAAAGTGATCTCCTCCAGAAG-3')	Eurofins Genomics	N/A
<b>Experimental models: Cell lines</b>		
HEK293T	ATCC	Cat# CRL-3216; RRID:CVCL_0063
Dulbecco's Modified Eagle's Medium	Corning	Cat# 10-013-CV
Opti-MEM Reduced Serum Medium	Gibco	Cat# 31985062
<b>Software and algorithms</b>		
GraphPad Prism	GraphPad; v7	<a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a>
Bioassay Software	CambridgeSoft	N/A
<b>Others</b>		
Biomek NXP Automated Workstation	Beckman	N/A
PHERASTAR FS reader	BMG Labtech	N/A
Assay plate 384 well, black with clear flat bottom tissue culture treated low flange, with lid	Corning	Cat# 3764
1536-Well black solid bottom microplate, with lid	Corning	Cat# 3724
MultiDrop Combi Dispenser	Thermo Scientific	N/A

## MATERIALS AND EQUIPMENT

**Alternatives:** Throughout this protocol, we refer to several specific robotic liquid handling equipment for streamlined operation. Other HTS compatible liquid handlers or multi-channel pipettes can also be used.

<b>FRET buffer</b>			
Reagent	Final concentration	Stock concentration	Add to 1000 mL
Tris-HCl (pH7.0)	20 mM	1M	20 mL
NaCl	50 mM	5M	10 mL
Nonidet P-40	0.01%	10%	1 mL
MilliQ water			969 mL

Store at 4°C.

#### 0.5% Triton X-100 Cell lysis buffer

Reagent	Final concentration	Stock concentration	Add to 400 mL
Tris-HCl (pH8.0)	20 mM	1M	8 mL
NaCl	137 mM	5M	10.96 mL
Glycerol	5%	50%	40 mL
Triton X-100	0.5%	10%	20 mL
EDTA	2 mM	500 mM	1.6 mL
MilliQ water			319.44 mL

Store at 4°C. Right before use, add protease inhibitor cocktail and PIC2 and PIC3 phosphatase inhibitor cocktail at 1:100 dilution.

#### 1% NP40

Reagent	Final concentration	Stock concentration	Add to 200 mL
Tris-HCl (pH8.0)	20 mM	1M	4 mL
NaCl	137 mM	5M	5.48 mL
Nonidet P-40 (NP40)	1%	10%	20 mL
Glycerol	5%	50%	20 mL
EDTA	2 mM	500 mM	800 µL
MilliQ water			150 mL

Store at 4°C.

#### PEI stock solution (1 mg/mL)

Reagent	Add to 100 mL	Note
Polyethylenimine powder	100 mg	1. Filter through 0.22 µm filter membrane. 2. Recommend tested for efficiency by transfecting cells with GFP at a 1:1–1:6 DNA:PEI ratio.
HCl	adjust to pH 7.0	
MilliQ water	100 mL	

Aliquot 0.5–1 mL to each 1.5 mL tube, and store at –20°C. Thawed solutions can be stored at 4°C for up to 2 months. Avoid frequent freeze-thaw cycles.

#### Cell culture medium

Reagent	Final concentration	Add to 500 mL
DMEM medium		440 mL
Fetal Bovine Serum (FBS)	10%	50 mL
2 mM glutamine	1%	5 mL
100 U/mL penicillin/streptomycin	1%	5 mL

Store at 4°C. Warm to 37°C right before use.

## STEP-BY-STEP METHOD DETAILS

### Protein expression and cell lysate stock preparation

⌚ **Timing: 4 days**

This major step describes the specific protocol to prepare cell lysate from HEK293T cells co-overexpressing Flag-SMAD3 and His-SMAD4 WT or R631H. We have also used this protocol for overexpressing proteins with various tag configurations in HEK293T cells or other protein expression systems (Du et al., 2013; Mo et al., 2019; Xiong et al., 2018).

1. Seeding HEK293T cells – Day 1

**Table 1. Combinations of constructs and TR-FRET antibody pairs**

No	DNA construction pairs	TR-FRET antibodies
1	Flag-SMAD4-WT + His-SMAD3	Anti-Flag-Tb (1:500 dilution to FRET buffer, final
2	Flag-SMAD4-R361H + His-SMAD3	1:1000 dilution in mixture with cell lysate) + Anti-His-D2
3	His-SMAD4-WT + Flag-SMAD3	(1:250 dilution to FRET buffer, final 1:500 dilution in
4	His-SMAD4-R361H + Flag-SMAD3	mixture with cell lysate)

- a. Before seeding cells, check cells visually and under microscope to make sure the cells are healthy indicated by observing 1) pinky orange culture media, 2) majority of cells are attached to the bottom of the flask, 3) cells are in plump or elongated shape, and 4) reached 70%–80% confluency in monolayer.
- b. Pre-warm cell culture medium, PBS and trypsin to 37°C in water bath.
- c. Carefully remove media from one 175 cm<sup>2</sup> flask of the required cells into a waste pot (containing laboratory disinfectant), taking care not to increase contamination risk with any drips.
- d. Wash the cells twice with PBS.
- e. Add 3 mL trypsin, incubate in CO<sub>2</sub> incubator at 37°C until cells are rounded up and detached from the bottom.
- f. Add 10 mL cell culture medium to cease trypsinization.
- g. Detach and resuspend cells by pipetting the medium with cells up and down against the flask bottom.
- h. Count viable cell numbers using TC20™ Automated Cell Counter by following manufacturer's protocols (<https://www.bio-rad.com/webroot/web/pdf/lsr/literature/10024423.pdf>).

**Note:** We recommend using cells with ≥90% viability.

**Alternatives:** Viable cell number can be counted automatically using other automated cell counters or manually using a hemocytometer under a microscope.

- i. Prepare cell suspensions at concentration 0.3–0.5 × 10<sup>6</sup> cells/mL by diluting cells in culture medium.
- j. Plate 2 mL cell suspension per well in 6-well plate and incubate in CO<sub>2</sub> incubator at 37°C for 24 h.

**Note:** We recommend testing the optimal plating density within the recommended range for the cells to reach 70%–80% confluency on the second day for transfection, and to reach >80% transfection efficiency using GFP plasmid as control (Jäger et al., 2013). [Troubleshooting 1](#)

**Alternatives:** Other cell culture plates or dish formats could also be used with cell numbers adjusted proportionally.

## 2. Plasmid transfection – Day 2

- a. Thaw PEI stock solution (1 mg/mL) at 25°C and mix by gently inverting tubes before use.

**Note:** Properly prepared PEI solution should be clear. We do not recommend using PEI solutions that are cloudy or with precipitation.

△ **CRITICAL:** To develop a robust TR-FRET assay for HTS, we recommend testing the combinations of various epitope-tagged constructs with corresponding TR-FRET fluorophore pairs (Table 1) (Blagg and Workman, 2017).

**Alternatives:** We used PEI as a cost-effective transfection reagent for large-scale HTS application and it produces very high transfection efficiencies (>70%) in HEK293T cells (Schirrmann

and Büssov, 2010). Other plasmid transfection reagents, such as FuGene® HD, can also be used by following manufacturer's protocol ([https://www.promega.com/products/luciferase-assays/transfection-reagents/fugene-hd-transfection-reagent/?catNum=E2311&gclid=Cj0KCQiA3NX\\_BRDQARIsALA3flKo0C-VsU8ps6GFihjTxZrYOPgzlevuV0g6XAz\\_O1hUwSvCl67bVlcaAjGrEALw\\_wcB#protocols](https://www.promega.com/products/luciferase-assays/transfection-reagents/fugene-hd-transfection-reagent/?catNum=E2311&gclid=Cj0KCQiA3NX_BRDQARIsALA3flKo0C-VsU8ps6GFihjTxZrYOPgzlevuV0g6XAz_O1hUwSvCl67bVlcaAjGrEALw_wcB#protocols))

b. Add 100  $\mu$ L transfection mixture (1.5  $\mu$ g Flag-SMAD3, 1.5  $\mu$ g His-SMAD4-R361H or WT, and 9  $\mu$ g PEI in Opti-MEM) per well and mix well by gently rocking the plate. Incubate plates in CO<sub>2</sub> incubator at 37°C for 48 h.

**Note:** We recommend gently vortexing the transfection mixture and incubating at 25°C for 15–30 min to allow the formation of stable DNA:PEI complex for efficient transfection. Plasmids expressing fluorescence protein can be used to rapidly estimate the transfection efficiency.

**Note:** Depending on the expression of each plasmid when co-expressed, the ratio of plasmids may need to be changed from 1:1 to ensure optimal expression of each encoded protein from the plasmid that gives desired assay performance. [Troubleshooting 2](#).

**△ CRITICAL:** Transfection efficiency >80% estimated from fluorescence protein are recommended for the following cell lysate preparation. To ensure the desired transfection efficiency specific to this protocol, we recommend i) removing 500  $\mu$ L growth medium from each well before adding transfection mixture to increase the DNA:PEI complex working concentration, ii) adding transfection mixture in a dropwise manner, and iii) supplementing 1 mL fresh pre-warmed medium per well 24 h after transfection.

### 3. Lysate stock preparation – Day 4

- Wash monolayer cell culture with 1  $\times$  PBS three times, followed by adding trypsin (0.25%, 200  $\mu$ L) and incubate in CO<sub>2</sub> incubator at 37°C for 1 min. Detach transfected HEK293T cells from cell culture plate by adding 1 mL of cell culture medium and pipetting up and down. Transfer cell suspension from each well to a pre-chilled microcentrifuge tube and pellet cells by centrifuge at 200  $\times$  g for 5 min. Discard the supernatant and wash cell pellet once with ice-cold PBS.
- Add 100  $\mu$ L lysis buffer per sample and vortex vigorously for 5 s to resuspend cell pellet. Lyse cells with rotation for 30 min at 4°C, followed by a centrifuge at 10,000  $\times$  g for 10 min at 4°C. Aspirate the supernatant and place in a fresh tube kept on ice. Combine lysates from wells transfected with the same plasmids to reduce variation.
- Measure the protein concentration using the Bradford assay by following manufacturer's instructions ([https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0011181\\_Coomassie\\_Bradford\\_Protein\\_Asy\\_UG.pdf&title=VXNlciBHdWlkZTogIENvb21hc3NpZSAoQnJhZGZvcmlFByb3RlaW4gQXNzYXkgS2l0](https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0011181_Coomassie_Bradford_Protein_Asy_UG.pdf&title=VXNlciBHdWlkZTogIENvb21hc3NpZSAoQnJhZGZvcmlFByb3RlaW4gQXNzYXkgS2l0)).

**Note:** If using cell lines which adhere well to plate surface, we recommend washing the cells with ice-cold PBS first, then adding lysis buffer directly to the well to detach cells from plates, and transferring them to tube. Cell pellets can be stored at –80°C for short-term or in liquid nitrogen for long-term storage. Both 0.5% Triton X-100 and 1% NP-40 lysis buffer work for cell lines used in this study. Other lysis buffer with different washing stringency could be used.

**Alternatives:** For determination of protein concentration, other assays, such as BCA assay, can also be used by following manufacturer's instructions [https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0011430\\_Pierce\\_BCA\\_Protein\\_Asy\\_UG.pdf&title=VXNlciBHdWlkZTogUGllcmNlIEJDQSBQcm90ZWlulEFzc2F5IEtpdA==](https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0011430_Pierce_BCA_Protein_Asy_UG.pdf&title=VXNlciBHdWlkZTogUGllcmNlIEJDQSBQcm90ZWlulEFzc2F5IEtpdA==)

**TR-FRET assay development and evaluation**

⌚ Timing: 1 day

This step describes how cell lysate-based Time-Resolved Fluorescence-Resonance-Energy-Transfer (TR-FRET) assay was designed, evaluated and optimized to monitor protein-protein interactions for high-throughput screening of compounds (Du et al., 2011; Fu, 2004).

4. Dose-dependent TR-FRET assay
  - a. Perform serial dilution of cell lysate in the FRET buffer.

**Note:** We recommend 2-fold serial dilution directly in a black 384-well plate with final volume of 15  $\mu$ L/well to reach 8–16 titrations in total. Empty FRET buffer without lysate will be used as background control.

- b. Add TR-FRET antibody mixture.

**Note:** We recommend adding 15  $\mu$ L/well antibody mixture containing 2 $\times$  fluorophore-conjugated antibodies (1:500 diluted anti-FLAG M2-Tb and 1:250 diluted anti-6xHIS-D2 antibodies in FRET buffer). The optimal dilution and ratio of conjugated antibodies may vary based on the expression levels of PPI. [Troubleshooting 3](#).

**⚠ CRITICAL:** Mix the reaction thoroughly by centrifugation of the plate at 200  $\times$  g for 5min.

- c. Measure TR-FRET signal using a BMG Labtech PHERAstar FSx reader by following manufacturer's manual.

**Note:** We recommend incubating the assay plate for 2 h at 4°C before the measurement. Unless necessary to read plate cold, let assay plate come to 25°C before reading so that there is no variation in signal due to temperature changes while reading. We recommend using the HTRF optic module (excitation at 337 nm, emission A at 665 nm, emission B at 620 nm) with following measurement settings: integration start at 50  $\mu$ s, integration time for 150  $\mu$ s and 8 flashes per well.

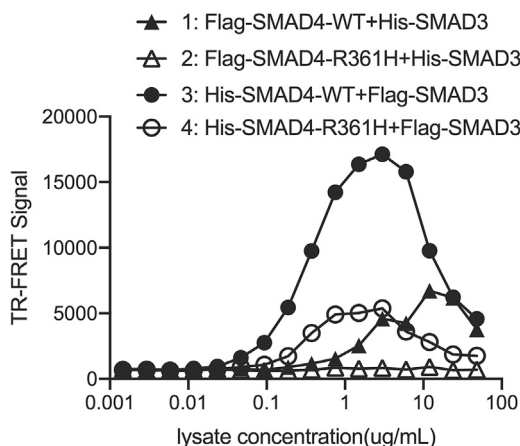
**⚠ CRITICAL:** We noticed that bubbles in the well may lead to inaccurate readings, and thus recommend eliminating bubbles before the measurement.

5. Estimation of an optimal dilution factor of cell lysate
  - a. The TR-FRET signal is calculated as the ratio of F665 nm/F620 nm  $\times$  10<sup>4</sup>, where F665 nm and F620 nm are the fluorescence intensity at 665 and 620 nm, respectively.
  - b. Plot the raw FRET signal against cell lysate concentration as shown in [Figure 2](#).
  - c. Estimate an optimal dilution factor of cell lysate with desired assay window. The chosen dilution factor should avoid ramifications of the hook effect (Ross et al., 2020). The selected dilution factor will be used for assay performance evaluation and primary HTS implementation.

**Note:** To maximize the opportunity to reveal inducers that may enhance mutant SMAD4/SMAD3 interaction, we selected the EC<sub>90</sub> (90% maximal effective concentration) condition of the SMAD4<sup>R361H</sup>/SMAD3 interaction, which corresponded to EC<sub>10</sub> (10% maximal effective concentration) of the SMAD4<sup>WT</sup>/SMAD3 PPI.

**⚠ CRITICAL:** Selection of optimal dilution factor should be in the upward-sloping phase, where PPI signal positively correlates with PPI concentration, but not in the downward-sloping phase, where PPI signal negatively correlates with PPI concentration due to the artifact from low antibody occupancy.





**Figure 2. Cell lysate dose-dependent TR-FRET curves showing different TR-FRET signals from lysate expressing different pairs of SMAD4-WT/R361H and SMAD3 constructs**

The TR-FRET signal from cell lysate expressing the plasmid combination indicated in 3&4 exhibit higher S/B and assay window than that in 1&2. The lysate concentration-dependent “bell curve” of 3&4 represents a typical “hook effect” in this antibody-based TR-FRET assay. Selection of optimal lysate concentration should be in the upward-sloping phase, where PPI signal positively correlates with PPI concentration, but not in the downward-sloping phase, where PPI signal negatively correlates with PPI concentration due to the artifact from low antibody occupancy. Data are presented as mean  $\pm$  SD from triplicate of a representative experiment.

## 6. Assay performance evaluation

- a. Prepare the working reaction mixture by diluting the cell lysate stock according to the optimal dilution factor identified in step 5 in FRET buffer (containing 1:1000 anti-FLAG M2-Tb and 1:500 anti-6xHIS-D2 antibodies) to the selected concentration.

**Note:** We recommend including cell lysate co-expressing SMAD4-WT and SMAD3 PPI as a known biological positive control in parallel for assay development and performance evaluation. Cell lysate expressing empty-vector controls are recommended as a technical negative control.

- b. Add 5  $\mu$ L/well working reaction mixture into 1536-well solid bottom plate.

**Note:** Multidrop™ Combi Reagent Dispenser was used to add the working cell lysate, which was also used for the similar steps during the subsequent HTS.

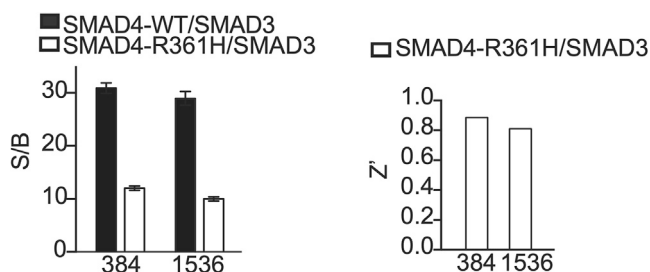
**Note:** To cost-effectively scale up the assay for HTS, we recommend miniaturizing the assay by scaling down proportionally the volume from 30  $\mu$ L in a 384-well plate to 5  $\mu$ L in a 1536-well plate format. We did not observe any significant changes of the TR-FRET signal between 384- and 1536-well plate format.

**Note:** We recommend centrifugation of the plate at 200  $\times$  g for 5 min and incubation at 4°C for 2 h.

**△ CRITICAL:** To mitigate the evaporation-induced edge effect, we recommend adding the working cell lysate in the center and dispensing FRET buffer in the empty surrounding wells.

- c. Measure TR-FRET signal using a BMG Labtech PHERAstar FSx reader by following manufacturer’s manual.
- d. Calculate the Signal-to-Background (S/B) ratio and Z’ for SMAD4-WT/SMAD3 and SMAD4-R361H/SMAD3 PPI, respectively (Figure 3), using equations as shown in Table 2.

**△ CRITICAL:** S/B ratio >4 and Z’ factor >0.5 suggests a robust assay condition for HTS (Zhang et al., 1999).



**Figure 3. Signal-to-Background (S/B) ratio and Z' factor for SMAD4-WT/SMAD3 and SMAD4-R361H/SMAD3 PPI respectively in both 384-well format and 1536-well format**

S > 4 and Z' factor > 0.5 suggests a robust assay condition for HTS. Data are presented as mean  $\pm$  SD from triplicate of a representative experiment.

- e. To determine the assay performance in terms of DMSO tolerance, repeat steps 3a–3d by comparing the assay performance with various DMSO added up to 10% (v/v) during step 3a. To determine the assay performance in terms of temporal stability, repeat step 3c–3d after various incubation time ranging from 2–24 h.

**Note:** Given that DMSO is a common solvent used in chemical libraries ([Wigglesworth et al., 2012](#); [Janzen and Popa-Burke, 2009](#)), we recommend evaluating the assay performance in the presence of DMSO prior to the HTS. Specific to this protocol, the final DMSO concentration is 2% (v/v) during the HTS. Our results show that our reaction system is stable and robust up to 10% DMSO ([Figure 4](#)). For confirmation studies in biological assays, DMSO concentration should be adjusted based on DMSO tolerance test results for a particular assay system, usually less than 1%. The stock concentration of re-ordered compounds should be prepared accordingly.

**△ CRITICAL:** A DMSO tolerant and temporally stable assay that maintains similar robustness (S/B ratio > 4 and Z' factor > 0.5) is recommended for HTS application.

### uHTS primary screening

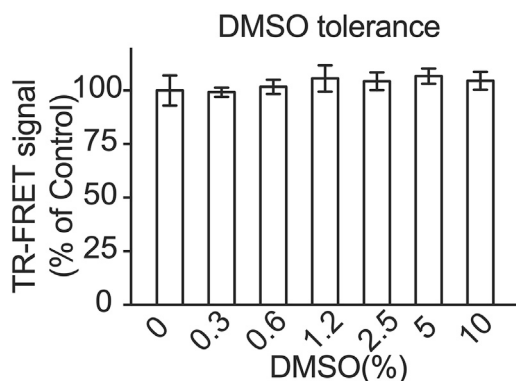
⌚ Timing: 2 days

This step describes primary screening process to identify small molecule PPI inducers of SMAD4-R361H/SMAD3 PPI by using the TR-FRET assay we detailed above in an ultrahigh-throughput 1536-well plate format. We have applied similar protocol to identify small molecule PPI inhibitors for other targets as well.

**△ CRITICAL:** With the established TR-FRET assay, starting with a pilot screening is highly recommended. The positive compounds successfully identified and validated will provide more confidence on the screening platform.

**Table 2. Z' factor and signal-to-background (S/B) equations**

Equations	Definition	Meaning
$S/B = FRET_{PPI} / FRET_{vector}$	$FRET_{PPI}$ and $FRET_{vector}$ are the TR-FRET signals from lysate containing His-SMAD4-R361H and Flag-SMAD3 or empty Flag-vector controls respectively.	Signal-to-background ratio suggests the signal window of the assay.
$Z' = 1 - \frac{3 \times SD_{PPI} + 3 \times SD_{vector}}{FRET_{PPI} - FRET_{vector}}$	$SD_{PPI}$ and $SD_{vector}$ are standard deviations of the TR-FRET signals from lysate containing His-SMAD4-R361H and Flag-SMAD3 or empty Flag-vector controls respectively.	Z' factor reflects the robustness of the assay for HTS. Z' factor between 0.5 and 1 indicates a robust assay, suitable for HTS.



**Figure 4. DMSO tolerance test**

TR-FRET PPI signal were measured using cell lysate containing various amount of DMSO as indicated. Data are presented as mean  $\pm$  SD from triplicate of a representative experiment.

7. Plate the working reaction mixture in black 1536-well solid bottom plates (5 $\mu$ L/well) using Multi-Drop Combi reagent dispenser following manufacturer's protocol ([https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLPD%2Fmanuals%2FN05616\\_ver1.7%2520Multidrop%2520Combi%2520User%2520Manual.pdf&title=TXVsdGikcm9wIENvbWJpIFVzZXIgdWFWudWFs](https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLPD%2Fmanuals%2FN05616_ver1.7%2520Multidrop%2520Combi%2520User%2520Manual.pdf&title=TXVsdGikcm9wIENvbWJpIFVzZXIgdWFWudWFs)).

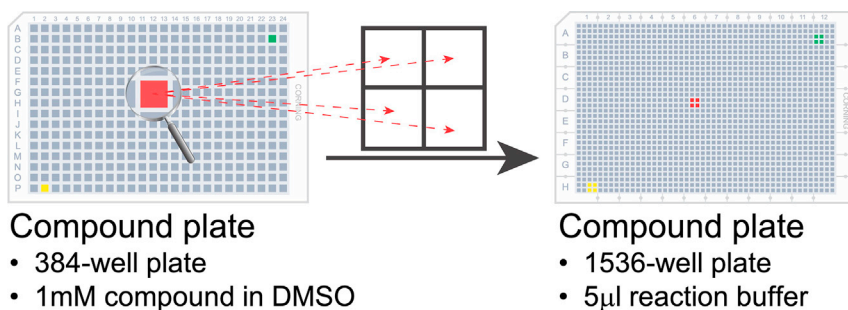
**Note:** Taking the dispenser dead volume into account, we recommend preparing 1–10 mL extra reaction mixture.

**Note:** We recommend adding a known negative biological control, such as cell lysate expressing empty vector control, and positive biological control, such as cell lysate expressing SMAD4-WT/SMAD3 PPI, in each plate for screening quality control.

**△ CRITICAL:** Even if some wells will not be used, we recommend adding working reaction mixture or control lysates to fill the whole plate to mitigate the evaporation-induced edge effect.

8. Add 0.1  $\mu$ L compounds from the 384-well compound plate by using a robotic liquid handler, such as Biomek NX<sup>P</sup> Automated Workstation with Pintool (VP Scientific) (Cleveland and Koutz, 2005) by following manufacturer's protocol (<https://www.beckman.com/techdocs/B20063AC/wsr-145922>).

**Note:** We selected our inhouse Emory Enriched Bioactive Library (EEBL), a library of 2036 bioactive compounds. The working stock of 1 mM was used. The final compound concentration is 20  $\mu$ M with 2% DMSO (v/v). Each compound is tested with single concentration point in four replicates (Figure 5).



**Figure 5. Schematic illustration of the compound adding from 384-well compound plate to 1536-well reaction buffer plate**

Each compound tested at one dose in four replicates.

**Table 3. Effect of compound calculation equation**

Equations	Definition
$\% \text{ of control} = 100 \times \frac{\text{FRET}_{\text{compound}} - \text{FRET}_{\text{DMSO}}}{\text{FRET}_{\text{DMSO}}}$	<p>FRET<sub>compound</sub> and FRET<sub>DMSO</sub> are the TR-FRET signals from PPI in the presence of compounds or DMSO with background FRET<sub>vector</sub> subtracted.</p>

**Note:** We recommend centrifugation of the plates at 200 × g for 5 min and incubation at 4°C for 16 h before measuring the TR-FRET signal.

**Alternatives:** Other chemical libraries with different stock concentrations can be used accordingly depending on the desired hit rate.

- Measure the TR-FRET signal using a BMG Labtech PHERAstar FSx reader by following manufacturer's manual.
- Data analysis to calculate the compound's effect on PPI modulation as the change of TR-FRET signal ( $\Delta$ TR-FRET) upon compound treatment using the equation as indicated (Table 3).

**Note:** We analyzed the screening data by using Bioassay software from CambridgeSoft (Cambridge, MA). Based on the PPI inducing effect with cutoff of  $\Delta$ TR-FRET  $\geq$  2SD<sub>PPI</sub> (40% in this case) compared with the DMSO control, we prioritized the top ranked 23 primary hits. These hits were then cherry-picked from the parental stock for dose-response studies.

**Note:** To further validate the cherry-picked compounds, a dose-response confirmatory assay is highly recommended to test whether those compounds can reproducibly induce a significant increase of the TR-FRET signal. [Troubleshooting 4](#).

**Pause point:** If the hit rate is lower than expected, based on the PPI and chemical libraries used, we recommend re-evaluating the whole procedure by [troubleshooting 5](#)

## EXPECTED OUTCOMES

A robust high-throughput screening platform should allow us to identify primary hit compounds which increase or decrease the PPI signal, suggesting the induction or inhibition of the PPI-of-interest, and thus can be further validated and developed into small molecule PPI modulators to interrogate the biological significance and towards therapeutic agents. By following the protocol detailed above, a TR-FRET PPI HTS assay with robust performance with  $S/B \geq 4.0$ ,  $Z' \geq 0.5$ , and DMSO tolerance up to 2% is expected. Depending on the nature of PPI and chemical library, hit rate varying from 0.01%–1% is expected. Orthogonal secondary assays with complimentary readouts are expected to confirm the primary hits. For further functional studies, hit compounds, or their derivatives, will be tested in cell or organisms to assess whether the compounds could engage the targeted protein(s) and induce the targeted PPI *in vivo* through biological experiments.

## LIMITATIONS

Ultrahigh-throughput screening is efficient and productive for large scale screening, however it requires advanced technology such as a robotic liquid handling system to ensure the robust assay performance and mitigate variations from human handling, as well as professional techniques or skills to perform such machine. Meanwhile high-throughput suggests a small volume system, which also requires precise and accurate operation. The scale of the screening also depends on the size and number of the compound library.

## TROUBLESHOOTING

### Problem 1

Low transfection efficiency (step 1).

### Potential solution

Transfection efficiency is a critical parameter to be optimized before assay development and screen. We recommend authenticating all the reagents and supplies for cell culture, plasmid preparation, and DNA transfection based on the concept of “trust but verify” for utilization. In this protocol, we used PEI as cost-effective transfection reagent for HTS applications. If transfection efficiency is low, we recommend utilizing the cationic lipid transfection reagents, such as Lipofectamine or FuGene HD, which usually has higher transfection efficiency for many cell lines. Also, we recommend optimizing cell density (50%–90%), plasmid amount (1–3  $\mu\text{g}/\text{well}$ ), DNA:PEI ratio (1:1–1:4), transfection complex formation time (10–40 min) and post-transfection expression time (24–72 h) to achieve desired transfection efficiency.

### Problem 2

Low TR-FRET assay window due to sub-optimal protein concentration, stoichiometry and tag-orientation (step 2).

### Potential solution

TR-FRET signals from protein-protein interactions highly depend on the protein concentrations. In a typical two antibody-based TR-FRET assay, a protein concentration-dependent “hook effect” of PPI signal is expected. Therefore, we highly recommend a cell lysate-titration to determine the dilution factors for optimal protein concentrations at the upward-sloping phase of the bell curve. If no bell curve was observed, check transfection efficiency and protein expression using western blot, or further optimize protein stoichiometry and tag-orientation as discussed below.

TR-FRET PPI signal also depends on protein stoichiometry. In this protocol, we recommend 1:1 ratio of donor (Flag-SMAD3) and acceptor (His-SMAD4 WT or R361H) which gives a robust assay window. We also recommend titrating the donor and acceptor plasmid ratio during transfection to adjust protein stoichiometry to further optimize the assay if needed.

Tag-orientation is an important parameter to be optimized during development of a TR-FRET PPI assay. Efficiency energy transfer requires an optimal tag-orientation that brings donor and acceptor fluorophores into close-proximity (Mo and Fu, 2016; Mo et al., 2016). We recommend performing tag-orientation testing as described in [Figure 2](#) to determine the optimal tag-orientation.

Comprehensive optimization by combining the test of protein concentration, stoichiometry and tag-orientation is highly recommended to solve the issue of low TR-FRET assay window (Doyle et al., 2021).

### Problem 3

Low TR-FRET assay window due to sub-optimal TR-FRET antibodies selection and combinations (step 4).

### Potential solution

We highly recommend testing combination of available TR-FRET antibodies, since the PPI signal directly depends on the affinity, configuration, and concentration of fluorophore-conjugated antibodies. After determining the optimal protein concentration, stoichiometry and tag-orientation, we recommend optimizing antibody concentration and combination ratios through a two-way titration of donor and acceptor antibody pairs.

### Problem 4

No positive hit compounds shown from pilot screening (step 10).

### Potential solution

Re-evaluate the assay performance including S/B ratio, Z' factor, and DMSO tolerance. We also recommend including a known positive control compound for screening if available. Use freshly prepared TR-FRET buffer.

## Problem 5

False positive hit compounds (step 10).

## Potential solution

It's inevitable that false positive compounds will show up through the screening due to a series of factors, including the interference of the compound itself, autofluorescence, or the signal interference caused by the non-specific binding of the target protein to the TR-FRET antibody (Busch et al., 2013; Du and Havel, 2012). We recommend validating all the selected compounds through different orthogonal methods, such as dose-response TR-FRET assay and GST-pulldown assay (Fu, 2004).

## RESOURCE AVAILABILITY

### Lead contact

Haian Fu (hfu@emory.edu).

### Materials availability

The plasmids generated in this study are available to qualified scientists upon request.

### Data and code availability

The primary screening data is available in Cancer Target Discovery and Development (CTD2) Data Portal.

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## AUTHOR CONTRIBUTIONS

Conceptualization, X.M. and H.F.; assay design and development and chemical screening, C.T., X.M., Q.N., and Y.D.; data analysis, C.T., X.M., Y.D., D.C., and H.F.; writing – original draft, C.T., X.M., and H.F.; writing – review & editing, all authors.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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