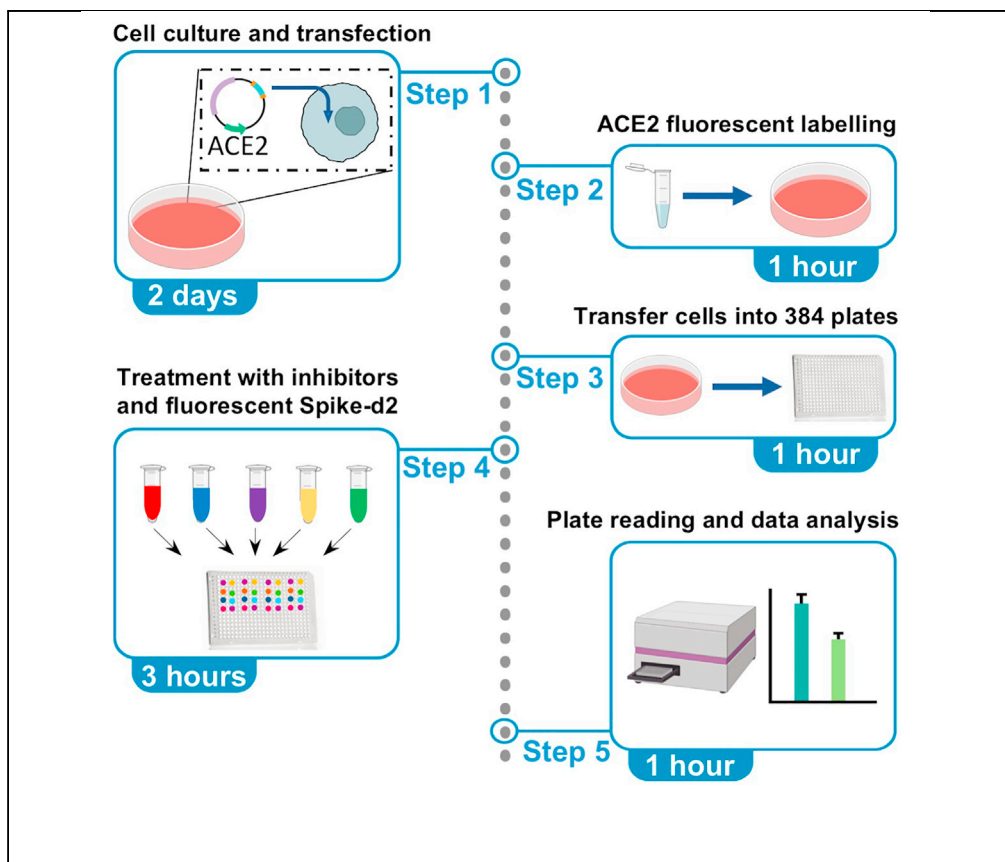


## Protocol

# Detection of SARS-CoV-2 spike protein binding to ACE2 in living cells by TR-FRET



The SARS-CoV-2 coronavirus infects human cells through the interaction of the viral envelope spike protein (IPR044366) with the human angiotensin-converting enzyme 2 (ACE2), expressed at the surface of target cells. Here, we describe a detailed protocol to measure the binding of the receptor binding domain (RBD) of spike to ACE2 by time-resolved fluorescence resonance energy transfer (TR-FRET). The assay detects the spike/ACE2 interaction in physiologically relevant cellular contexts and is suitable for high-throughput investigation of interfering small-molecule compounds and antibodies.

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### Highlights

Quantitative detection of SARS-CoV-2 spike interaction with ACE2 in living cells

High-throughput-compatible assay suitable for screening of inhibitory molecules

Suitable to investigate the impact of membrane components on ACE2/spike complex

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## Protocol

## Detection of SARS-CoV-2 spike protein binding to ACE2 in living cells by TR-FRET

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## SUMMARY

The SARS-CoV-2 coronavirus infects human cells through the interaction of the viral envelope spike protein (IPR044366) with the human angiotensin-converting enzyme 2 (ACE2), expressed at the surface of target cells. Here, we describe a detailed protocol to measure the binding of the receptor binding domain (RBD) of spike to ACE2 by time-resolved fluorescence resonance energy transfer (TR-FRET). The assay detects the spike/ACE2 interaction in physiologically relevant cellular contexts and is suitable for high-throughput investigation of interfering small-molecule compounds and antibodies.

For complete details on the use and execution of this protocol, please refer to Cecon et al. (2021).

## BEFORE YOU BEGIN

The protocol below describes the specific steps of the Spike/ACE2 binding assay in Human Embryonic Kidney 293 Cells (HEK293T) cells based on the TR-FRET technique to screen for inhibitory compounds. The assay is compatible with any cell type provided that the cells can be transfected to express the SNAP-tagged human ACE2 fusion protein. The TR-FRET assay is based on the energy transfer between an energy donor and energy acceptor that occurs if they are in close proximity to each other (less than 10 nm) (Mathis, 1995; Bazin et al., 2002; Degorce et al., 2009). Distinctively from classical FRET, the Time-Resolved (TR) aspect of the TR-FRET uses special donor fluorophores from the lanthanide family, displaying extended lifetime emission properties, which allows the introduction of a time delay between donor excitation and FRET signal measurement. This time-resolved feature results in measurement of the FRET signal at a time window of lowest background, following the decay of non-specific transient fluorescence signals, providing thus improved signal-to-noise ratio. This protocol uses the receptor binding domain (RBD) of the SARS-CoV-2 Spike protein labeled with the d2 fluorophore (energy acceptor, emission at 665 nm) and the human ACE2 labeled with the terbium-cryptate fluorophore (energy donor; excitation at 320-340 nm, Table 1) to monitor the proximity between RBD and ACE2 through the resulting TR-FRET signal.

The protocol describes a high-throughput(HT)-compatible assay format, but it can also be performed in a low-throughput format.

**Note:** The advantage of performing the TR-FRET assay in the low-throughput / adherent cell format is that it allows to treat the cells for long incubation time with compounds (*i.e.* 16–24 h incubation).

A list of all reagents, buffer and equipment for this protocol are described in the [key resources table](#) and [materials and equipment](#) sections. Make sure all materials required for the experiments are available and/or prepared in advance.



**Note:** The experimental design should be established in advance in order to calculate the precise volume needed of each reagent. Please refer to [Table 2](#) for the volumes required per well.

### Cell culture

⌚ Timing: 2–7 days

1. Thaw an aliquot of HEK293T cells and immediately plate the cells in 10 cm cell culture dishes containing complete culture medium.
2. HEK293T cells are cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose and supplemented with 1% Streptomycin/Penicillin and 10% fetal bovine serum, at 37°C, 5% CO<sub>2</sub>.
  - a. Cells are cultured in 10 cm cell culture dishes, and they are split in cell culture medium twice per week at a 1:10 ratio.
  - b. Culture cells for at least 5–7 days after thawing before using them for transfection to ensure they are healthy.

⚠ **CRITICAL:** Cells should be constantly inspected and tested for mycoplasma contamination. Several kits for mycoplasma detection are commercially available, such as **PlasmoTest** kit (cat. Rep-pt1, InvivoGen, France; <https://www.invivogen.com/plasmotest>) or **Mycoseq** kit (ThermoFisher Scientific, Waltham, USA; <https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FMSG%2Fmanuals%2F4465874.pdf>).

### Experimental design

⌚ Timing: 30 min

3. In order to calculate the amounts of reagents required for the experiment, the experimental plate design needs to be set in advance. The data points should be performed in triplicates and it **should always** include the following groups: i. background ("BKG" - absence of d2-labelled RBD); ii. total binding ("TB" - presence of d2-labelled RBD); iii. non-specific binding ("NSB" - presence of RBD-d2 AND excess of non-labelled RBD); iv. test (total binding of RBD-d2 in the presence of a compound to be tested for its effect on the RBD/ACE2 interaction). [Figure 1](#) shows an example of plate template.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
SARS-CoV-2 spike RBD protein	Sino Biological	Cat# 40592-V08H
SARS-CoV-2 spike S1-S2 protein	Sino Biological	Cat# 40589-V08B1
d2-labelled SARS-CoV-2 spike RBD protein	<a href="#">Cecon et al., 2021</a>	N/A
d2-labelled SARS-CoV-2 spike S1-S2 protein	<a href="#">Cecon et al., 2021</a>	N/A
Terbium cryptate Lumi4-Tb	Cisbio Bioassays	Cat# SSNPTBX
TagLite labelling medium	Cisbio Bioassays	Cat# LABMED
jetPEI transfection reagent	Polyplus-transfection	Cat# 101-10N
Cell Dissociation Solution Non-Enzymatic	Sigma-Aldrich	Cat# C5789
Dulbecco's modified Eagle's medium (DMEM)-Glutamax	Life Technologies	Cat# 31966047
TRYPsin 0.25% EDTA	Life Technologies	Cat# 25300096
Penicillin/streptomycin	Life Technologies	Cat# 15140130
Trypan blue solution	Sigma-Aldrich	Cat# T8154

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
PlasmoTest kit	InvivoGen	Cat# Rep-pt1
10× phosphate buffered saline (PBS)	Life Technologies	Cat# 14040174
<b>Experimental models: Cell lines</b>		
Human: Human Embryonic Kidney (HEK) 293 cells	Sigma-Aldrich	RRID:CVCL_0063
<b>Recombinant DNA</b>		
SNAP-ACE2 plasmid	Cecon et al., 2021	Addgene id:178592
<b>Software and algorithms</b>		
Image J	Schneider et al., 2012	RRID: SCR_003070 <a href="https://imagej.nih.gov/ij/download.html">https://imagej.nih.gov/ij/download.html</a>
GraphPad Prism 6	GraphPad Software Inc	RRID: SCR_002798 <a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a>
<b>Other</b>		
Cell culture incubator at 37°C, 5% CO <sub>2</sub>	N/A	N/A
Centrifuge for 1.5 mL microcentrifuge tubes	Eppendorf 5427 R	N/A
Tecan F500	Plate reader	N/A
384-well white Flat Bottom low-volume microplate	PerkinElmer	Cat# 6008280
Plate sealing polyester films	VWR	Cat# 391-1250

## MATERIALS AND EQUIPMENT

### 10× phosphate buffered saline (PBS)

Reagent	Final concentration	Amount
NaCl	1.4 M	81.8 g
KCl	27 mM	2.01 g
Na <sub>2</sub> HPO <sub>4</sub>	100 mM	14.2 g
KH <sub>2</sub> PO <sub>4</sub>	18 mM	2.45 g
ddH <sub>2</sub> O	n/a	To 1 L
<b>Total</b>	<b>n/a</b>	<b>1 L</b>

**Note:** The 10× PBS solution should be filtered using a 0.22 μm filter or autoclaved prior to use. Solution can be stored at 20°C–22°C for up to 1 month.

### 1× phosphate buffered saline (PBS)

Reagent	Final concentration	Amount
10× PBS solution	1×	100 mL
ddH <sub>2</sub> O	n/a	900 mL
<b>Total</b>	<b>n/a</b>	<b>1 L</b>

**Note:** 1× PBS solution can be stored at 4°C for up to 1 week.

### Complete DMEM cell culture medium

Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM)	n/a	445 mL
Fetal bovine serum	10 %	50 mL
Penicilin / streptomycin	1 %	5 mL
<b>Total</b>	<b>n/a</b>	<b>500 mL</b>

**Note:** Medium should be stored at 4°C for up to 2 months.

**Alternatives:** We usually buy sterile cell culture medium bottles, but the medium can also be prepared from powder. In this case, after preparing the medium it should be filtered using a 0.22 µm filter prior to use.

<b>Transfection solution</b>		
Reagent	Final concentration	Amount
NaCl (5 M)	150 mM	1.5 mL
ddH <sub>2</sub> O	n/a	48.5 mL
<b>Total</b>	<b>n/a</b>	<b>50 ML</b>

**Note:** Solution can be stored at stored at 4°C for up to 1 month.

<b>TagLite buffer 1 ×</b>		
Reagent	Final concentration	Amount
TagLite labelling medium 5 ×	1 ×	1 mL
ddH <sub>2</sub> O	n/a	4 mL
<b>Total</b>	<b>n/a</b>	<b>5 mL</b>

**Note:** Prepare fresh TagLite buffer before each experiment. The final volume to be prepared will depend on the number of data points and should be calculated in advance.

<b>SNAP substrate labeling solution</b>		
Reagent	Final concentration	Amount
Lumi4-tb substrate stock (0.1 mM)	100 nM	5 µL
TagLite labelling medium 1 ×	n/a	5 mL
<b>Total</b>	<b>n/a</b>	<b>5 mL</b>

**Note:** Prepare fresh SNAP substrate solution before each experiment. The final volume to be prepared will depend on the number of data points and should be calculated in advance.

<b>Spike(RBD) working solution</b>		
Reagent	Final concentration	Amount
Spike(RBD) stock (37.6 µM)	3.5 µM	4.7 µL
TagLite labelling medium 1 ×	n/a	45.3 µL
<b>Total</b>	<b>n/a</b>	<b>50 µL</b>

**Note:** Prepare fresh working solution before each experiment. The final volume to be prepared will depend on the number of data points and should be calculated in advance.

<b>Spike(RBD)-d2 working solution</b>		
Reagent	Final concentration	Amount
Spike(RBD)-d2 stock (11 µM)	35 nM	3.2 µL
TagLite labelling medium 1 ×	n/a	996.8 µL
<b>Total</b>	<b>n/a</b>	<b>1 mL</b>

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	BKG	BKG	BKG																					
B	TB	TB	TB																					
C	NSB	NSB	NSB																					
D	T1, C1	T1, C1	T1, C1	T2, C1	T2, C1	T2, C1	T3, C1	T3, C1	T3, C1	T4, C1	T4, C1	T4, C1	T5, C1	T5, C1	T5, C1	T6, C1	T6, C1	T6, C1	T7, C1	T7, C1	T7, C1	T8, C1	T8, C1	T8, C1
E	T1, C2	T1, C2	T1, C2	T2, C2	T2, C2	T2, C2	T3, C2	T3, C2	T3, C2	T4, C2	T4, C2	T4, C2	T5, C2	T5, C2	T5, C2	T6, C2	T6, C2	T6, C2	T7, C2	T7, C2	T7, C2	T8, C2	T8, C2	T8, C2
F	T1, C3	T1, C3	T1, C3	T2, C3	T2, C3	T2, C3	T3, C3	T3, C3	T3, C3	T4, C3	T4, C3	T4, C3	T5, C3	T5, C3	T5, C3	T6, C3	T6, C3	T6, C3	T7, C3	T7, C3	T7, C3	T8, C3	T8, C3	T8, C3
G	T1, C4	T1, C4	T1, C4	T2, C4	T2, C4	T2, C4	T3, C4	T3, C4	T3, C4	T4, C4	T4, C4	T4, C4	T5, C4	T5, C4	T5, C4	T6, C4	T6, C4	T6, C4	T7, C4	T7, C4	T7, C4	T8, C4	T8, C4	T8, C4
H	T1, C5	T1, C5	T1, C5	T2, C5	T2, C5	T2, C5	T3, C5	T3, C5	T3, C5	T4, C5	T4, C5	T4, C5	T5, C5	T5, C5	T5, C5	T6, C5	T6, C5	T6, C5	T7, C5	T7, C5	T7, C5	T8, C5	T8, C5	T8, C5
I	T1, C6	T1, C6	T1, C6	T2, C6	T2, C6	T2, C6	T3, C6	T3, C6	T3, C6	T4, C6	T4, C6	T4, C6	T5, C6	T5, C6	T5, C6	T6, C6	T6, C6	T6, C6	T7, C6	T7, C6	T7, C6	T8, C6	T8, C6	T8, C6
J	T9, C1	T9, C1	T9, C1	T10, C1	T10, C1	T10, C1	T11, C1	T11, C1	T11, C1	T12, C1	T12, C1	T12, C1	T13, C1	T13, C1	T13, C1	T14, C1	T14, C1	T14, C1	T15, C1	T15, C1	T15, C1	T16, C1	T16, C1	T16, C1
K	T9, C2	T9, C2	T9, C2	T10, C2	T10, C2	T10, C2	T11, C2	T11, C2	T11, C2	T12, C2	T12, C2	T12, C2	T13, C2	T13, C2	T13, C2	T14, C2	T14, C2	T14, C2	T15, C2	T15, C2	T15, C2	T16, C2	T16, C2	T16, C2
L	T9, C3	T9, C3	T9, C3	T10, C3	T10, C3	T10, C3	T11, C3	T11, C3	T11, C3	T12, C3	T12, C3	T12, C3	T13, C3	T13, C3	T13, C3	T14, C3	T14, C3	T14, C3	T15, C3	T15, C3	T15, C3	T16, C3	T16, C3	T16, C3
M	T9, C4	T9, C4	T9, C4	T10, C4	T10, C4	T10, C4	T11, C4	T11, C4	T11, C4	T12, C4	T12, C4	T12, C4	T13, C4	T13, C4	T13, C4	T14, C4	T14, C4	T14, C4	T15, C4	T15, C4	T15, C4	T16, C4	T16, C4	T16, C4
N	T9, C5	T9, C5	T9, C5	T10, C5	T10, C5	T10, C5	T11, C5	T11, C5	T11, C5	T12, C5	T12, C5	T12, C5	T13, C5	T13, C5	T13, C5	T14, C5	T14, C5	T14, C5	T15, C5	T15, C5	T15, C5	T16, C5	T16, C5	T16, C5
O	T9, C6	T9, C6	T9, C6	T10, C6	T10, C6	T10, C6	T11, C6	T11, C6	T11, C6	T12, C6	T12, C6	T12, C6	T13, C6	T13, C6	T13, C6	T14, C6	T14, C6	T14, C6	T15, C6	T15, C6	T15, C6	T16, C6	T16, C6	T16, C6
P																								

**Figure 1. Example of plate template**

Example of an experiment comprising the three mandatory groups background (“BKG”), total binding (“TB”) and non-specific binding (“NSB”), as well as 16 test compounds (T1-T16, distributed horizontally) at 6 different concentrations each (C1-C6, distributed vertically).

**Note:** Prepare fresh working solution before each experiment. The final volume to be prepared will depend on the number of data points and should be calculated in advance.

## STEP-BY-STEP METHOD DETAILS

### HEK293T cells seeding – Day 1

⌚ **Timing:** 30 min

To obtain HEK293T cells expressing SNAP-ACE2, cells are transiently transfected using transfection reagents.

**Alternatives:** stable cell lines expressing SNAP-ACE2 can be used.

1. From the stock culture of HEK293T cells, remove the cell culture medium and add 5 mL of sterile PBS to wash the cells.
2. Remove the PBS and add 1 mL trypsin/EDTA solution.
3. Incubate at 37°C in the cell culture incubator for 2–5 min.
4. Add 9 mL of complete DMEM medium and resuspend the cells.

**Note:** Pipette the medium with cells up-and-down using a 10 mL pipette to better detach and dissociate the cells.

5. Collect the cell suspension in a 15 mL conical tube.
6. Take an aliquot of 10 µL of the cell suspension in a microcentrifuge tube to count the number of cells.
7. Spin down the 15 mL tube containing the cells at 180×g for 5 min.

8. Meanwhile, mix the 10  $\mu\text{L}$  cell suspension aliquot with 10  $\mu\text{L}$  trypan blue solution and transfer the 20  $\mu\text{L}$  into a cell counting slide.
9. Count live cells manually with a hemocytometer or with an automated cell counter to determine the cell density.
  - a. Exclude from the counting trypan blue-stained dead cells.
  - b. Calculate the required volume of cell suspension to obtain  $2 \times 10^6$  cells / mL.
10. Following the centrifugation, aspirate the supernatant.
11. Resuspend the cell pellet in the proper volume of complete DMEM medium calculated to obtain  $2 \times 10^6$  cells / mL.
12. Seed 1 mL of cell suspension solution in a new 10 cm cell culture dish containing at least 9 mL of complete DMEM medium.
13. Incubate cells for 18–24 h at 37°C in a CO<sub>2</sub> (5%) incubator to allow cells to recover and attach to the plate.

**Note:** Transfection can also be done in cells seeded in smaller dishes (i.e. 6-well plates) if the experimental design does not require a high number of cells (low number of data points).

### Cell transfection – Day 2

⌚ Timing: 30 min

**Note:** This step is skipped if a stable cell line expressing SNAP-ACE2 is used.

**Alternatives:** The protocol shown here is for the JetPEI transfection reagent following the manufacturer’s instructions (Polyplus-transfection; Illkirch, France). Other transfection reagents can be used and the respective manufacturer’s protocol should be followed. [Troubleshooting 1](#)

14. Prepare the transfection solution containing the SNAP-ACE2 plasmid.
  - a. Prepare one microcentrifuge tube per well to be transfected and mix 250  $\mu\text{L}$  150 mM NaCl with 5  $\mu\text{g}$  plasmid DNA coding for SNAP-ACE2.
  - b. In another microcentrifuge tube, add 15  $\mu\text{L}$  of JetPEI reagent to 250  $\mu\text{L}$  NaCl (ratio 1:3 DNA: JetPEI). If several plates are to be transfected, multiply the volume by the number of plates.
  - c. Transfer 250  $\mu\text{L}$  of the JetPEI-NaCl solution into the microcentrifuge tube containing the DNA.
  - d. Incubate for 15 min, at 20°C–22°C.
15. Distribute the 500  $\mu\text{L}$  of transfection solution, dropwise, all over the cells that are to be transfected.
16. Place the plate back in the incubator (37°C, 5% CO<sub>2</sub>) and allow cells to incubate for 48 h.

**⚠ CRITICAL:** Cells should not exceed 70% confluency before transfecting to assure an optimal transfection rate. We found that an incubation time of 48 h (post-transfection) is optimal for the expression and plasma membrane translocation of membrane proteins.

**Note:** If performing the TR-FRET assay in the low-throughput format or with adherent cells, the transfected cells can be plated 24 h post-transfection (Day 3) into white 96-well plates at a density of  $0.5 \times 10^5$  cells/well. Cells are then incubated for another 24 h before starting the TR-FRET assay (Day 4).

### Fluorescent labelling of SNAP-ACE2 – Day 4

⌚ Timing: 1.5 h

**Table 1. Donor (Tb) reading settings using TECAN F500 plate reader**

Reading settings in Tecan F500 plate reader	
Excitation wavelength	340 nm
Emission wavelength	620 nm
Excitation bandwidth	20 nm
Emission bandwidth	10 nm
Gain	Optimal
Number of flashes	10
Integration time	500 $\mu$ s
Lag time	150 $\mu$ s

This step consists in the covalent labelling of the N-terminal SNAP-tagged human ACE2 expressed at the cell surface of transfected cells with the TR-FRET-compatible terbium (Tb) fluorophore. The Lumi4-Tb SNAP substrate is a suicide substrate of the SNAP enzyme labelled with a terbium fluorophore (Keppler et al., 2003).

17. Prepare Lumi4-Tb SNAP substrate solution at 100 nM by adding 5  $\mu$ L of the stock solution (0.1 mM) into 5 mL of Tag-Lite buffer 1 $\times$ .
18. Remove the cell culture medium and add 5 mL sterile PBS to wash the cells.
19. Remove the PBS and add the 5 mL Lumi4-Tb SNAP substrate solution.
20. Incubate the plate at 20°C–22°C for 1 h.
21. Remove the labeling solution and add 3 mL sterile PBS to wash the cells.

**Note:** PBS solution used for the washing steps can be supplemented with 1 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub> to prevent detaching the cells.

22. Remove the PBS and repeat the washing step with PBS.
23. Remove the PBS and repeat the washing step with TagLite buffer 1 $\times$ .
24. Remove TagLite buffer and add 1 mL of cell dissociation solution.
25. Detach and dissociate the cells by pipetting up-and-down and collect them in a 1.5 mL micro-centrifuge tube.
26. Spin down the 1.5 mL tube containing the cells at 1500 $\times$ g for 5 min.
27. Remove the supernatant and add 1 mL of TagLite buffer 1 $\times$  to resuspend the cell pellet.
28. Verify successful SNAP labeling
  - a. Add 10  $\mu$ L of the cell suspension per well in two wells (duplicate) of a white 384-well plate.
  - b. Read the plate in a plate reader using the filter configuration compatible with the excitation and emission spectra of the Terbium fluorophore. Settings in the TECAN F500 plate reader to read the Tb donor signal are shown in Table 1.

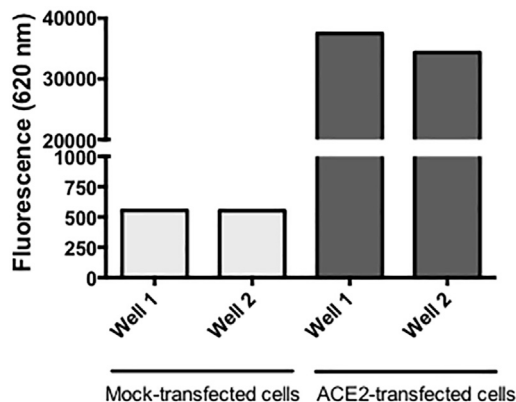
**Note:** The Tb fluorophore emits at multiple wavelengths and it is, therefore, suitable as a donor fluorophore also for acceptors emitting at the green spectrum (see Cisbio website - <https://fr.cisbio.eu/content/htrf-technology/>). In both cases, the Tb (donor) signal is measured at 620 nm.

**△ CRITICAL:** ACE2 labeling is essential for the TR-FRET assay. Only proceed to the next step if successful labeling of ACE2 is obtained. Use mock-transfected cells to access the background of Tb signal. From our experience, 5 to 10-fold signal-to-background ratio indicates successful SNAP labelling. An example of Tb donor signal in SNAP-ACE2-expressing cells and in mock-transfected cells is shown in Figure 2. Troubleshooting 2

### TR-FRET assay

⌚ Timing: 2–3 h





**Figure 2. Example of Tb donor signal**

Tb donor signal (at 620 nm) obtained in SNAP-ACE2-expressing cells and in mock-transfected cells, in two replicates of each group (well 1, well 2). In this experiment, HEK293T cells were transfected with 500 ug of SNAP-ACE2 construct in one well of a 6-well plate. Forty-eight hours post-transfection, cells were labelled with Lumi4-Tb substrate as described, and 10  $\mu$ L of the cell suspension was used to measure the resulting Tb donor signal in the Tecan F500 plate reader. Gain was set to “optimal” and corresponded to a value of 92 in this experiment.

29. Distribute 10  $\mu$ L per well of the Tb-labeled SNAP-ACE2 cells into a white 384-well plate. It is recommended to use an electronic pipette to increase speed and accuracy.

**Note:** Labelled cells can be diluted by a factor of 3–5 in TagLite buffer if high Tb signal is obtained. Optimal cell density and/or Tb signal will depend on the cell surface expression of SNAP-ACE2 and can be tested in advance to define the best conditions to obtain a low background signal and high signal-to-noise ratio. For example, in the experiment shown in [Figure 2](#), cells could be used at a dilution of 3.5 times, which would result in a Tb signal still 20-fold higher than background (i.e. mock-transfected cells).

**Optional:** Remaining labeled cells can be frozen and stored ( $-20^{\circ}\text{C}$ ) to be used later, within 2 weeks. Cell suspension solution should be divided into aliquots to avoid repeated freeze-thaw cycles.

30. Add 2  $\mu$ L of test compound at the desired concentration to the “test” wells.

**Note:** Working solution should be 7 $\times$  concentrated.

31. In the “NSB” wells, add 2  $\mu$ L of 3.5  $\mu$ M non-labeled RBD (final concentration of 0.5  $\mu$ M).
32. In the “BKG” and “TB” wells, add 2  $\mu$ L of 1 $\times$  TagLite buffer.
  - a. Seal the plate with a plate sealing film and incubate at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$  for 15 min.
33. Add 2  $\mu$ L of Tag-lite buffer to “BKG” wells.
34. Add 2  $\mu$ L of 35 nM RBD-d2 solution to all wells (final concentration of 5 nM), except in the “BKG” wells.
  - b. Incubate the plate at  $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$  for 2 h.

**Note:** An overview of the order of TR-FRET reagents to be added to the different wells is shown in [Table 2](#).

35. Read the plate in a TR-FRET-compatible plate reader. Settings in the TECAN F500 plate reader to read the TR-FRET signal are shown in [Table 3](#).

**△ CRITICAL:** Perform at least triplicates for each data point (see [Figure 1](#) for an example of plate template).

**Alternatives:** other TR-FRET-compatible plate readers, such as EnVision (PerkinElmer) can also be used.

**Table 2. Volume and order of reagent distribution at each step of the TR-FRET assay in a 384-well plate**

	Background ("BKG")	Total binding ("TB")	Non-specific binding ("NSB")	Test compounds ("Test")
Cells	10 $\mu$ L	10 $\mu$ L	10 $\mu$ L	10 $\mu$ L
TagLite buffer 1 $\times$	2 $\mu$ L	2 $\mu$ L	–	–
Test compounds (7 $\times$ )	–	–	–	2 $\mu$ L
Non-labeled RBD (7 $\times$ )	–	–	2 $\mu$ L	–
Seal the plate and incubate at 20°C–22°C for 15 min				
TagLite buffer 1 $\times$	2 $\mu$ L	–	–	–
RBD-d2 (7 $\times$ )	–	2 $\mu$ L	2 $\mu$ L	2 $\mu$ L
Seal the plate and incubate at 20°C–22°C for 2 h				
Read the plate in a TR-FRET-compatible plate reader				

**Optional:** The TR-FRET signal being stable, several reads can be performed at different time points, once every hour, for example, until equilibrium is reached. In this case, it is important to fix the "Gain" setting of the donor channel (620 nm) to a fixed value in order to be able to compare the different readings.

**Alternatives:** Kinetics parameters can also be determined with slight changes in the protocol, as previously shown (Cecon et al., 2021). To determine association kinetics parameters, read the TR-FRET signal continuously during 2 h, starting immediately after adding RBD-d2. To determine dissociation kinetics parameters, allow binding of RBD-d2 to reach equilibrium (2 h incubation) and only then add excess of non-labelled RBD to displace bound RBD-d2 and read the TR-FRET signal continuously during 2 h, starting immediately after adding non-labelled RBD.

## Data analysis

⌚ Timing: 30 min

- Once the reading is finished, export the results file as .csv file.
- Calculate the TR-FRET signal, which is the ratio of the acceptor (RBD-d2, measured at 665 nm) over the donor (Tb-ACE2, measured at 620 nm) signals for each well. For convenience, the ratio is multiplied by 10000. An example of a TR-FRET data sheet result is shown in Table 4. The specific signal for each well can be obtained by subtracting the NSB signal from the TR-FRET signal.

$$\text{TR-FRET signal} = \frac{\text{Signal } 665 \text{ nm}}{\text{Signal } 620 \text{ nm}} \times 10^4$$

$$\text{Specific Signal (for each well)} = \text{TR} - \text{FRET signal} - \text{NSB}$$

**Note:** By performing the ratio, the TR-FRET is normalized to the donor signal, counteracting thus any potential differences in the number of cells per well. Some plate readers allow to integrate the calculation equation, providing thus the final TR-FRET signal data.

**Note:** the raw data value are arbitrary units and, therefore, it is not possible to perform direct comparison of raw data between different plate readers. If such comparison is envisioned, the fold-change of the specific signal should be used.

**Table 3. TR-FRET reading settings using TECAN F500 plate reader**

Reading settings in Tecan F500 plate reader	
Fluorescence 1: d2	
Excitation wavelength	340 nm
Emission wavelength	665 nm
Excitation bandwidth	20 nm
Emission bandwidth	8 nm
Gain	Optimal
Number of flashes	10
Integration time	500 $\mu$ s
Lag time	150 $\mu$ s
Fluorescence 2: Tb	
Excitation wavelength	340 nm
Emission wavelength	620 nm
Excitation bandwidth	20 nm
Emission bandwidth	10 nm
Gain	Optimal*
Number of flashes	10
Integration time	500 $\mu$ s
Lag time	150 $\mu$ s

\*When reading TR-FRET multiple times (every hour or in a kinetics mode, for example) the Gain of the donor signal should be set to a fixed value corresponding to the value obtained in the first reading.

38. Use a spreadsheet or statistics software package to calculate the arithmetic mean of the triplicates.

**Note:** Check that the Tb signal is homogeneous between wells to identify potential outliers.

#### Troubleshooting 3

39. Normalize the data to 0–100% scale by setting the mean of the “TB” group as 100% and the mean of the “NSP” group as 0 % using a statistics software package like GraphPad Prism.

**Note:** As the raw values (fluorescence signal) are given in arbitrary units, the normalization to a percentage scale allows to pool together independent repetitions of the experiment.

40. Use a statistics software package like GraphPad Prism to calculate standard deviations and statistical significance and to plot the data in a graph.

## EXPECTED OUTCOMES

A successful TR-FRET experiment should show a clear difference between “TB” and “NSB” signals, with “NSB” signal being similar or very close to the “BKG” signal ([troubleshooting 4](#); [troubleshooting 5](#)). The amplitude of the signal depends on the distance between the two fluorophores. In the TR-FRET mode for ACE2/RBD, a high “TB” TR-FRET signal with a low BKG is observed, as it can be easily competed with excess of non-labelled RBD, conferring a high amplitude of the specific signal (“TB”-“NSB”). The ratio between the TR-FRET signals for “TB” and “NSB” (“TB”/“NSB”) can be expected to range between 5 (at Kd concentration of RBD-d2) to 30-fold (at saturating concentrations of RBD-d2). Of note, the “TB” TR-FRET signal is proportional to the concentration of RBD-d2, up to a saturation limit that we have detected to occur at approximately 100 nM of RBD-d2 ([Cecon et al., 2021](#)). In the case that the objective of the assay is to screen the efficacy of candidate compounds to interfere with the RBD-ACE2 interaction, the concentration of RBD-d2 used should be around its affinity (Kd), i.e., low nanomolar range (for example, 5 nM), as described in this protocol. Positive interfering compounds are the ones giving a specific TR-FRET signal significantly lower than the specific signal of RBD-d2 alone (i.e., without “test” compound). A

**Table 4. Example of raw fluorescence signal data (in triplicates) and TR-FRET ratio calculation**

	Signal 665 nm	Signal 620 nm	Ratio $\times 10^4$
Background ("BKG")	7083	91025	778
Background ("BKG")	7443	95990	775
Background ("BKG")	6256	82306	760
Total Binding ("TB")	131850	99294	13279
Total Binding ("TB")	135114	102504	13181
Total Binding ("TB")	131898	101650	12976
Non-specific binding ("NSB")	5486	58405	939
Non-specific binding ("NSB")	6842	94789	722
Non-specific binding ("NSB")	6369	83848	760

representative bar graph of an expected result of an experiment including all the groups mentioned in this protocol ("BKG", "TB", "NSB" and "Test") is shown in [Figure 3](#).

### LIMITATIONS

The protocol described here comprises an assay to detect the binding of RBD to ACE2. The choice of using the RBD domain of the spike protein was due to the reduced size of this peptide, compared to the full protein, which favors the proximity between the d2-fluorophore and the Tb-labelled ACE2. The assay has the advantage of providing a very high specific signal, being thus suitable to the identification of compounds interfering with this interaction. However, one cannot exclude that other regions of the Spike protein might be relevant for cell interaction and cell entry of SARS-CoV-2 virus. The use of d2-labeled full-length S1-S2 Spike protein for the TR-FRET assay is possible, as reported ([Cecon et al., 2021](#)), but the amplitude of the signal is significantly lower than that observed for the RBD-d2, reaching 2-fold the "NSB" values. Such difference is probably due to the bigger size of the S1-S2 spike protein, which might increase the distance between the fluorophores.

In addition, the use of recombinant proteins (RBD or S1-S2) may represent only a simplified model of SARS-CoV-2 interaction with cellular systems, as it has been described that the Spike protein is highly glycosylated and acts as a trimer ([Xu et al., 2021](#)). Therefore, inhibitory compounds identified using the TR-FRET assay described here need to be validated using additional techniques such as cellular or *in vivo* infection models with SARS-CoV-2 virus.

Finally, the TR-FRET signal is correlated to the distance between the acceptor and donor fluorophores. Therefore, a change in TR-FRET signal could reflect either a conformational change within the protein complex or an increase in association or dissociation of RBD and ACE2. Compounds of interest identified by this method can have an impact on those processes and would need to be characterized further for their exact properties.

### TROUBLESHOOTING

#### Problem 1

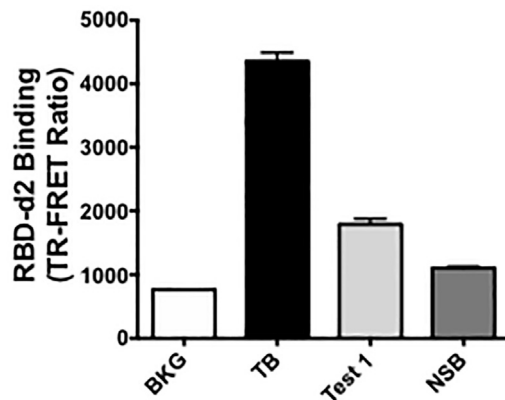
No detectable SNAP-ACE2 expression ([cell transfection – Day 2 step 14](#)).

#### Potential solution

Transfection efficiency can be indirectly accessed at step 28, when verifying the SNAP labelling. If low transfection rate is suspected to occur, transfection efficiency can be tested using different transfection reagents and verified by direct methods such as Western Blot (using antibodies against ACE2 or against the Flag-tag) or fluorescence imaging (using microscopy-compatible SNAP fluorophores for SNAP labeling).

#### Problem 2

No detectable SNAP-ACE2 labeling with Tb fluorophore ([fluorescent labelling of SNAP-ACE2 – Day 4 step 28](#)).



**Figure 3. Example of expected result**

Representative bar graph of an experiment testing the effect of a nanobody against RBD (10  $\mu\text{g}/\text{mL}$ ) on RBD-d2 binding to ACE2. Data are expressed as mean  $\pm$  SD of triplicates. See [Cecon et al. \(2021\)](#) for information on the nanobody used.

#### Potential solution

Verify that the cells were efficiently transfected with SNAP-ACE2 construct by using techniques like western blot or immunofluorescence imaging.

Make sure cells are not overconfluent at the moment of labelling and verify that they do not detach during the washing steps after labelling.

#### Problem 3

Too much variation between the wells for the Tb fluorescent signal ([data analysis](#) step 38).

#### Potential solution

Check the precision of the pipette used for the step of distribution of the cells in the 384-well plate. Make sure that the same pool of Lumi4-Tb-labelled cells is used for all wells in the experiment.

#### Problem 4

No detectable differences between BKG and TB signals in the d2 fluorescent channel ([expected outcomes](#)).

#### Potential solution

Decrease the cell density used in the assay in order to decrease BKG signal. Make sure RBD-d2 is freshly prepared and added to the corresponding wells. Increase the RBD-d2 concentration or perform a saturation curve to verify that RBD-d2 is working as expected (see saturation curve in [Cecon et al., 2021](#)).

#### Problem 5

No significant difference between TR-FRET signals for "TB" and "NSB" ([expected outcomes](#)).

#### Potential solution

Make sure that the concentration of non-labelled RBD is at least 100 $\times$  higher than the concentration of RBD-d2. Check (by visual inspection) for the presence of aggregates in the RBD stock solution, as the real concentration of the protein might be reduced if insoluble aggregates are present.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ralf Jockers ([ralf.jockers@inserm.fr](mailto:ralf.jockers@inserm.fr)).

## Materials availability

Plasmids generated in this study have been deposited to Addgene (SNAP-ACE2, id: 178592).

## Data and code availability

This paper does not report original datasets.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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## AUTHOR CONTRIBUTIONS

All authors contributed to the conceptualization and to the writing of the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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