

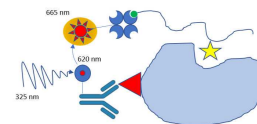
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Version 3

# SARS-CoV-2 nsp3 macrodomain Time-Resolved FRET peptide displacement assay V.3

DOI

<https://dx.doi.org/10.17504/protocols.io.eq2ly7r2mlx9/v3>



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ASAP Discovery



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**Protocol status:** Working

**We use this protocol and it's working**

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**Keywords:** Coronaviridae, Time-Resolved FRET assay, Fluorescence assay, resolved fret peptide displacement assay, fret peptide displacement assay this protocol detail, peptide, modified peptide, binding of adenosine, assay for sar, sars cov2 mac183000, activity of mac1, resolved fret, adenosine, tagged mac1 form

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## Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

## Abstract

This protocol details the Time-Resolved FRET (TR-FRET) assay for SARS-CoV-2 nsp3 macrodomain (Mac1) binding of adenosine diphosphate (ADP)-ribosylated (ADPr) peptide. This method is intended to measure the activity of Mac1 by using a specific ADPr-modified peptide that allows the detection of binding. When bound, the biotinylated-peptide and the HIS-tagged Mac1 form a proximity complex that is detected by TR-FRET using Streptavidin-Eu Cryptate and anti-HIS-XL665 as a donor/acceptor pair. Excitation of the Eu Cryptate complex at 325 nm emits a resonant energy of 625 nm which in turn excites the XL665 to emit fluorescence at 665 nm. This energy transfer occurs only when ADPr modified peptide is in sufficient proximity to Mac1 and inhibitors which displace the peptide will prevent energy transfer. Binding activity is reported as the ratio of Acceptor/Donor ( $E_m/E_m$ ) X 10,000.

### Experiment Concentrations (From Stock to Assay)

|  | A                                  | B        | C                 | D                    | E     |
|--|------------------------------------|----------|-------------------|----------------------|-------|
|  | Reagent                            | Stock    | Loaded into Combi | Final in assay plate | Units |
|  | His-SARS CoV-2 MAC1                | 183000   | 50                | 12.5                 | nM    |
|  | Substrate (Biotin-ADPr)            | 10000000 | 1600              | 400                  | nM    |
|  | <b>Detection solution</b>          |          |                   |                      |       |
|  | Streptavidin-XL665 (SA-XL)         | 1        | 0.25              | 0.125                | %     |
|  | MAB Anti-6HIS-Eu cryptate Gold     | 100      | 0.25              | 0.125                | %     |
|  | <b>Assay buffer</b>                |          |                   |                      |       |
|  | HEPES pH=7.0                       | 250      | 25                | 25                   | mM    |
|  | NaCl                               | 200      | 20                | 20                   | mM    |
|  | BSA                                | 0.5      | 0.05              | 0.05                 | mg/ml |
|  | Tween 20                           | 0.5      | 0.05              | 0.05                 | %     |
|  | HTRF PPI Europium Detection Buffer | 100      | 10                | 10                   | %     |

*For more information, please check out the "Materials" Section*

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**Compound Plate Design for Dose Response:**

**Total Assay Volume:** 16  $\mu$ L

**Compounds Top Assay Concentration:** 100  $\mu$ M

**Dilution Factor:** 3

**Dose Response Points:** 10

**Number of Replicates:** 2

**Backfill with DMSO:** Yes

**Compounds Plate Design for 2-Point Assay:**

**Total Assay Volume:** 16  $\mu$ L

**Compounds Assay Concentration:** 100  $\mu$ M and 50  $\mu$ M

**Dilution Factor:** 2

**Dose Response Points:** 2

**Number of Replicates:** 2

**Backfill with DMSO:** Yes

## Materials

### Assay Buffer Reagents (Concentration listed are from Stock Solutions)

1. [M] 250 millimolar (mM) ☒ HEPES 0.5M buffer soln. pH 7.0 Fisher Scientific Catalog #AAJ60064AE (or similar)
2. [M] 200 millimolar (mM) ☒ Sodium chloride Merck MilliporeSigma (Sigma-Aldrich) Catalog #S9888 (or similar)
3. [M] 0.5 mg/mL ☒ Bovine Serum Albumin (BSA) Merck MilliporeSigma (Sigma-Aldrich) Catalog #A7030
4. [M] 0.5 % volume ☒ TWEEN® 20 Merck MilliporeSigma (Sigma-Aldrich) Catalog #P9416
5. [M] 100 % volume ☒ HTRF PPI Europium Detection Buffer CISBIO BIOASSAYS (PerkinElmer) Catalog #61DB9RDF

**\*Note:** There are several forms of the Assay Buffer in this experiment. The **Assay Buffer** is the final, active buffer used throughout the experiment and has all of the five above reagents included. **HTRF PPI Europium Detection Buffer** needs to be added fresh before each experiment. Thus, there was an intermediate Buffer called **Mac1 Buffer** that contained HEPES, NaCl, BSA, and Tween only. Mac1 Buffer was filtered and stored at 4°C. **HTRF PPI buffer** was then added to Mac1 Buffer fresh (to a final concentration of 10%) prior to performing the experiment--creating the **active Assay Buffer**.

### Detection Solution Reagents (Concentration listed are from Stock Solutions)

[M] 1 % volume ☒ Streptavidin-XL665 CISBIO BIOASSAYS (PerkinElmer) Catalog #610SAXAC

- **Note:** Streptavidin-XL665 was dissolved in triply distilled water and diluted with HTRF PPI buffer to its stock concentration and then was aliquoted into 1.5mL sterile conical tubes

[M] 100 Mass Percent

☒ MAb Anti-6HIS-Eu cryptate Gold CISBIO BIOASSAYS (PerkinElmer) Catalog #61HI2KLA

**Note:** MAb Anti-6HIS-Eu cryptate Gold was dissolved in triple distilled water and then aliquoted into 1.5mL sterile conical tubes

### Additional Reagents:

[M] 183000 nanomolar (nM) **His-SARS COV2 MAC1 Enzyme**

- The Enzyme stock was originally [M] 183000 nanomolar (nM) and was diluted to [M] 50 nanomolar (nM) before every experiment in **freshly made Assay Buffer**. The final assay concentration is

[M] 12.5 nanomolar (nM)

[M] 10000000 nanomolar (nM) **Substrate (Biotin-ADPr) MAC1**

- Substrate stock (ARTK(Bio)QTARK(Aoa-RADP)S) was dissolved in DMSO to the stock concentration. Before each experiment, the substrate stock was diluted to [M] 1600 nanomolar (nM) in freshly made Assay Buffer.

## Safety warnings

 Please be sure to wear proper Personal Protective Equipment (PPE) while performing this experiment.

## Before start

**Note:** Inhibitor compounds stock concentration is `[M] 20 millimolar (mM)` . Compounds are pre-dispensed into 384 plates and stored at -20°C until use.



## Prepare Reagents

- 1 **PREPARE** all of the reagents/buffers required for this experiment.

### Assay Buffer

|  | A                                  | B     | C                 | D                    | E     |
|--|------------------------------------|-------|-------------------|----------------------|-------|
|  | Reagent                            | Stock | Loaded into Combi | Final in assay plate | Units |
|  | HEPES pH=7.0                       | 250   | 25                | 25                   | mM    |
|  | NaCl                               | 200   | 20                | 20                   | mM    |
|  | BSA                                | 0.5   | 0.05              | 0.05                 | mg/ml |
|  | Tween 20                           | 0.5   | 0.05              | 0.05                 | %     |
|  | HTRF PPI Europium detection buffer | 100   | 10                | 10                   | %     |

### Reagents (dilute reagents in assay buffer for required volume)

|  | A                       | B        | C                 | D                    | E     |
|--|-------------------------|----------|-------------------|----------------------|-------|
|  | Reagent                 | Stock    | Loaded into Combi | Final in assay plate | Units |
|  | His-SARS-CoV-2 MAC1     | 183000   | 50                | 12.5                 | nM    |
|  | Substrate (Biotin-ADPr) | 10000000 | 1600              | 400                  | nM    |

### Detection Solution (dilute reagents in assay buffer for required volume)

|  | A                              | B     | C                 | D                    | E     |
|--|--------------------------------|-------|-------------------|----------------------|-------|
|  | Reagent                        | Stock | Loaded into Combi | Final in assay plate | Units |
|  | Streptavidin-XL665 (SA-XL)     | 1     | 0.25              | 0.125                | %     |
|  | MAb Anti-6HIS-Eu cryptate Gold | 100   | 0.25              | 0.125                | %     |

## Prepare 384-well Plate

16m

- 2 **PRIME** Multi-Drop Combi Tube Dispensing Cassette with **Assay Buffer** by selecting the **PRIME** button on the Combi Dispenser until the tubes are filled completely.
  - **Note:** Be sure to cycle dispensing several times on a clean plate lid (This confirms there are no bubbles in the Dispensing Cassette).
- 2.1 **DISPENSE**  4  $\mu$ L Mac1 Buffer to Columns **1 and 23** of assay plate
  - **Note:** These will represent the ***inhibitor control columns***
- 2.2 **EMPTY** Multi-Drop Combi Tube Dispensing Cassette (by selecting the **EMPTY** button on the Combi Dispenser until the tubes of the cassette are emptied). **Discard** the **Assay Buffer** discharged from the cassette.
- 3 **PRIME** Multi-Drop Combi Tube Dispensing Cassette with **His-SARS COV2 MAC1 Enzyme** by selecting the **PRIME** button on the Combi Dispenser until the tubes are filled completely.
  - **Note:** Be sure to cycle dispensing several times on a clean plate lid (This confirms there are no bubbles in the Dispensing Cassette).
- 3.1 **DISPENSE**  4  $\mu$ L  50 nanomolar (nM) His-SARS COV2 MAC1 Enzyme to Columns **2-22** and **24** of assay plate  
**Note:**
  -  50 nanomolar (nM) His-SARS COV2 MAC1 is four times the final concentration for the assay. It will be diluted to be a final concentration of  12.5 nanomolar (nM) His-SARS COV2 MAC1 Enzyme
  - Column **2** and Column **24** are ***neutral control columns*** (Contain: Enzyme, Substrate, DMSO; **no experimental compounds**)
- 3.2 **EMPTY** Multi-Drop Combi Tube Dispensing Cassette (by selecting the **EMPTY** button on the Combi Dispenser until the tubes of the cassette are emptied). **Discard** the  50 nanomolar (nM) His-SARS COV2 MAC1 Enzyme discharged from the cassette.
- 4 **PRIME** Multi-Drop Combi Tube Dispensing Cassette with **Assay Buffer** by selecting the **PRIME** button on the Combi Dispenser until the tubes are filled completely. Then, **EMPTY** the Multi-Drop Combi Tube Dispensing Cassette (by selecting the **EMPTY** button on the Combi Dispenser until the tubes of the cassette are emptied). **Discard the Assay Buffer discharged from the cassette.**

- 5 **CENTRIFUGE** plate  1500 rpm, Room temperature, 00:01:00 to remove bubbles 1m
- 6 **INCUBATE** plate for  00:15:00 at  Room temperature 15m
- 7 **PRIME** Multi-Drop Combi Tube Dispensing Cassette with  1600 nanomolar (nM) MAC1 Substrate (Biotin-ADPr) by selecting the **PRIME** button on the Combi Dispenser until the tubes are filled completely.
- **Note:** Be sure to cycle dispensing several times on a clean plate lid (This confirms there are no bubbles in the Dispensing Cassette).
- 7.1 **DISPENSE**  4  $\mu$ L  1600 nanomolar (nM) MAC1 Substrate (Biotin-ADPr) into Columns **1-24** (full plate)
- Note:**
-  1600 nanomolar (nM) MAC1 Substrate (Biotin-ADPr) is four times the final concentration for the assay. It will be diluted to be a final concentration of  400 nanomolar (nM) MAC1 Substrate (Biotin-ADPr)
- 7.2 **EMPTY** Multi-Drop Combi Tube Dispensing Cassette (by selecting the **EMPTY** button on the Combi Dispenser until the tubes of the cassette are emptied). **Discard** the  1600 nanomolar (nM) MAC1 Substrate (Biotin-ADPr) discharged from the cassette.
- 8 **CENTRIFUGE** plate  1500 rpm, Room temperature, 00:01:00 to remove bubbles
- 9 **PRIME** Multi-Drop Combi Tube Dispensing Cassette with **Assay Buffer** by selecting the **PRIME** button on the Combi Dispenser until the tubes are filled completely. Then, **EMPTY** the Multi-Drop Combi Tube Dispensing Cassette (by selecting the **EMPTY** button on the Combi Dispenser until the tubes of the cassette are emptied). **Discard the Assay Buffer discharged from the cassette.**
- 10 **PRIME** Multi-Drop Combi Tube Dispensing Cassette with  0.25 % volume Detection Solution by selecting the **PRIME** button on the Combi Dispenser until the tubes are filled completely.
- **Note:** Be sure to cycle dispensing several times on a clean plate lid (This confirms there are no bubbles in the Dispensing Cassette).



10.1 **DISPENSE**  8  $\mu$ L  0.25 % volume Detection Solution into full plate

**Note:**

-  0.25 % volume Detection Solution is two times the final concentration for the assay. It will be diluted to be a final concentration of  0.125 % volume Detection Solution

10.2 **EMPTY** Multi-Drop Combi Tube Dispensing Cassette (by selecting the **EMPTY** button on the Combi Dispenser until the tubes of the cassette are emptied). **Discard** the  1600 nanomolar (nM) MAC1 Substrate (Biotin-ADPr) discharged from the cassette.

11 **CENTRIFUGE**  1500 rpm, Room temperature, 00:01:00 plate to remove bubbles

12 **INCUBATE** plate for  01:00:00 at  Room temperature

1h

**Recommended:** Clean/Empty the Multi-Drop Combi Reagent Dispenser and Dispensing Cassette during this incubation step

## Read Plate Fluorescence

13 **READ** and **RECORD** the plate Relative fluorescence units (RFU) via the "**Mac1 Protocol**" on the PHERAstar FS Control Software.

### Equipment

PHERAstar FS

Microplate reader

BMG LABTECH

0471B0001A

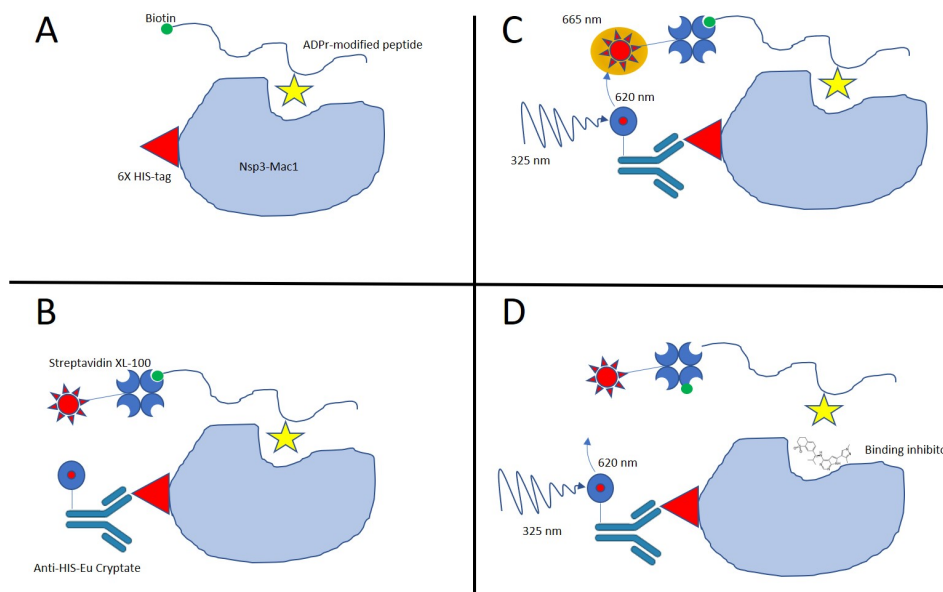
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## Expected result

Donor 325/620 ex/em should be ~ 5000 . Acceptor ~ 3000

## Diagram of assay

14 Figure 1 graphical depiction of assay principal and its use in screening campaign

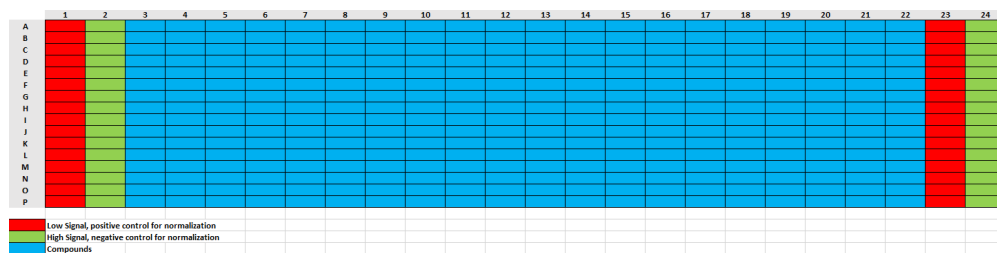


### Principal of ADPr peptide displacement assay

**A** - Binding of ADPr-modified peptide to Mac1 protein **B**- Detection reagents added to protein+peptide complex **C**- TR-FRET detects binding of peptide to Mac1 if proximity of donor and acceptor detection reagents is sufficient to enable resonant energy transfer **D**- Inhibitor compounds are detected by reduced TR-FRET signal when inhibitor displaces/prevents binding of ADPr-peptide to Mac1

## Experimental Design

15



## Keywords

16 Mac1, Nsp3, TR-FRET, HTRF, ADPr, Displacement, Screening, Assay, Inhibitor, Fragment, Binding, Macrodomain

## Protocol references

<https://doi.org/10.1126/sciadv.abf8711>