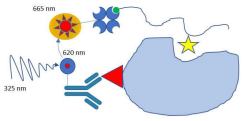
Apr 25, 2024 Version 3

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



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We use this protocol and it's working

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Disclaimer

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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 (Em/Em) 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
	Detection solution				
	Streptavidin-XL665 (SA-XL)	1	0.25	0.125	%
	MAb Anti-6HIS-Eu cryptate Gold	100	0.25	0.125	%
	Assay buffer				
	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 µL
Compounds Top Assay Concentration: 100 µM
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 µL
Compounds Assay Concentration: 100 µM and 50 µM
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, company of 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, company of 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, company of PerkinElmer Catalog #**61HI2KLA

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

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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 a clean plate lid (This confirms there are no bubbles in the Dispensing Cassette).

2.1 **DISPENSE** 4 µL 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 a clean plate lid (This confirms there are no bubbles in the Dispensing Cassette).

3.1 **DISPENSE** 4 µL [M] 50 nanomolar (nM) His-SARS COV2 MAC1 Enzyme to Columns **2-22** and **24** of assay plate

Note:

- [M] 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 [M] 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 [M] 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 [M] 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 a clean plate lid (This confirms there are no bubbles in the Dispensing Cassette).

7.1 **DISPENSE** 4 µL [M] 1600 nanomolar (nM) MAC1 Substrate (Biotin-ADPr) into Columns **1-24** (full plate)

Note:

- [M] 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 [M] 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 [M] 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 [M] 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 a clean plate lid (This confirms there are no bubbles in the Dispensing Cassette).

10.1 **DISPENSE** 8 µL [M] 0.25 % volume Detection Solution into full plate

Note:

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

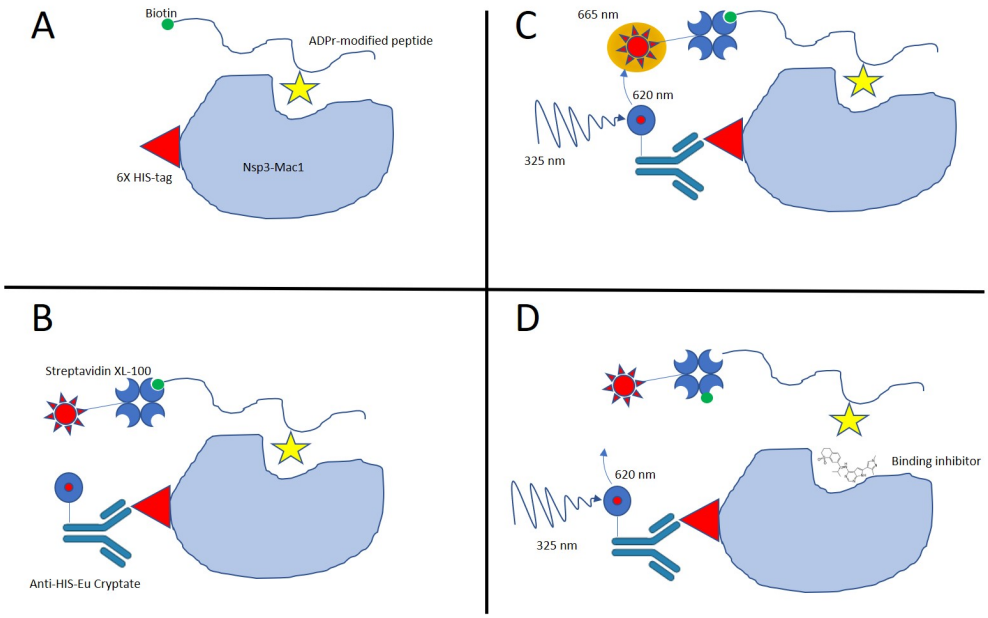
https://www.bmglabtech.com/en/pherastar-fsx/?utm_term=pherastar%20plate%20reader&utm_campaign=usa.roi.products&utm_source=adwords&utm_medium&gcFwOoFR_5EUHUaAlkREALw_wcB

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

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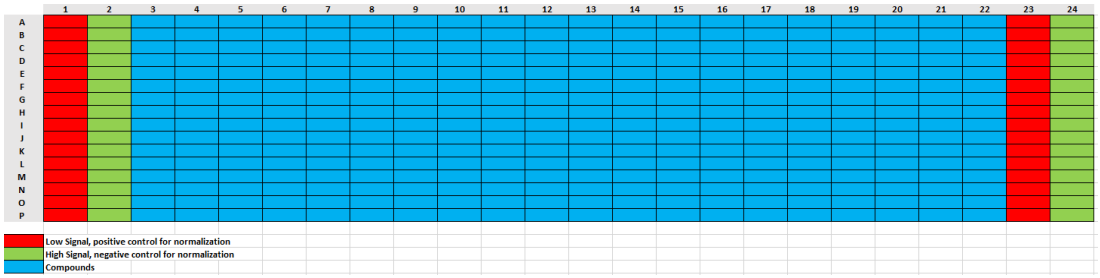

384 plate layout

Keywords

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

Protocol references

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