MANUAL

MDA5 AGGREGATION KITS

Part # 63ADK111PEG & 63ADK111PEH

Test size#: 500 tests (63ADK111PEG), 10,000 tests (63ADK111PEH) - assay volume: 20 µL

Revision: #04 of March 2024

Store at: ≤- 60°C (63ADK111PEG); ≤- 60°C (63ADK111PEH)

For research use only. Not for use in diagnostic procedures.

ASSAY PRINCIPLE

Revvity MDA5 aggregation assay is only intended for quantitative measurement of MDA5 aggregation in cells using HTRF® technology.

MDA5 aggregation is detected in a sandwich assay format using the same specific antibody, labeled with Europium Cryptate (donor) and with d2 (acceptor).

The principle of detection is based on HTRF® technology. When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). The donor & acceptor labeled antibodies bind to the MDA5 aggregated present in the sample, thereby generating FRET. Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the MDA5 aggregation concentration (Fig. 1).

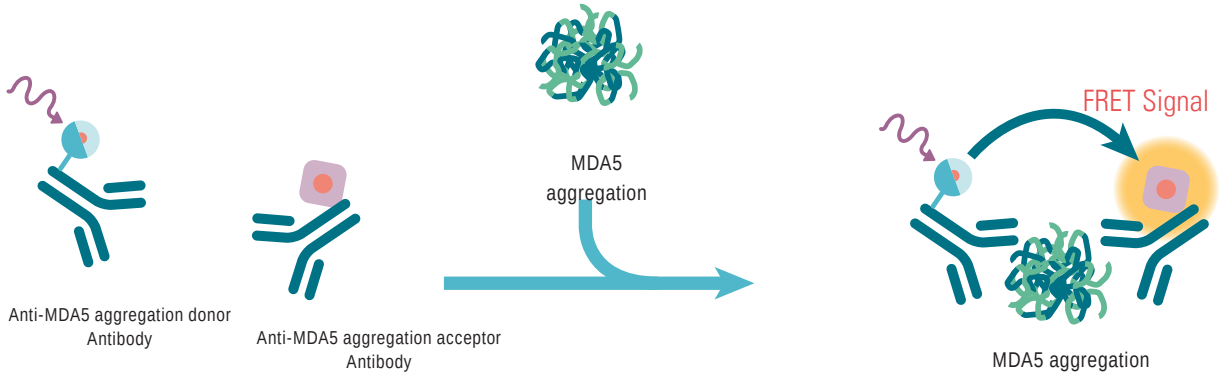
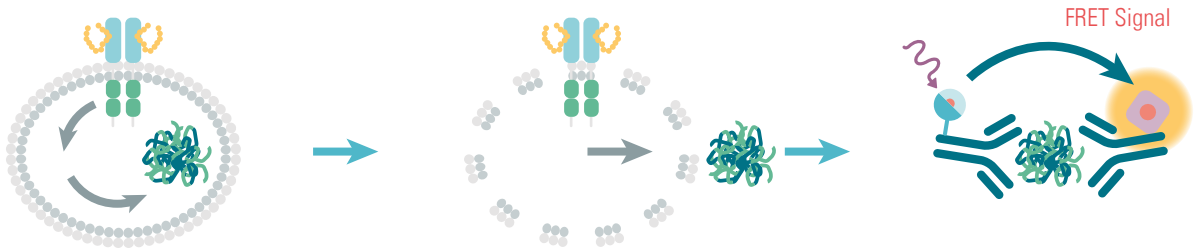


Figure 1: Principle of HTRF MDA5 aggregation sandwich assay.

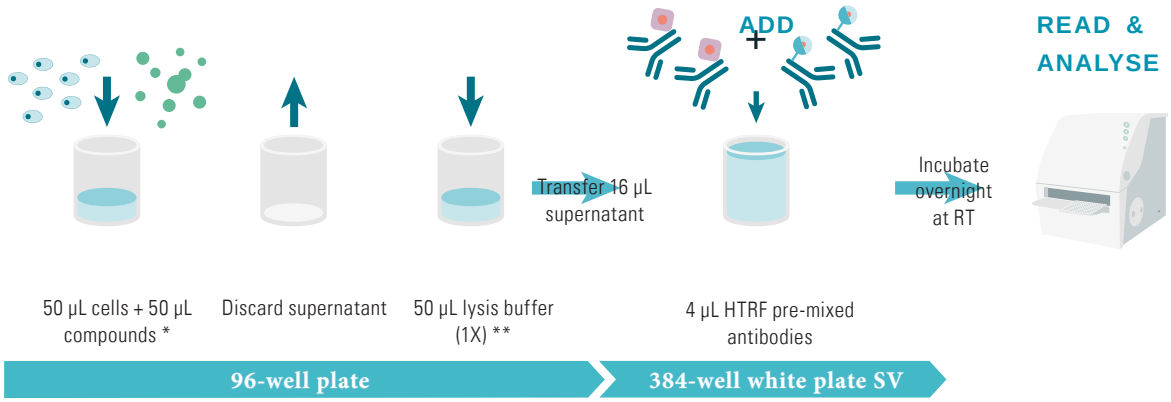
The assay is run under a two-plate assay manual, where cells are plated, stimulated and lysed in the same culture plate. Lysates are then transferred to the assay plate for the detection of MDA5 aggregation by HTRF® reagents. This manual gives the cells viability and confluence to be monitored.

Technical support team can help you to set-up this manual or another one.
Please contact us at www.revvity.com

MANUAL AT A GLANCE



TWO-PLATE ASSAY MANUAL (FOR ADHERENT CELLS):



* Note that concentration above 0.5% DMSO will impair assay performances.

** Depending on cell lines used, volume of lysis should be optimized, it can also be necessary to dilute the cell lysate to ensure samples are within the assay linear range.

MATERIALS PROVIDED:

Kit components	500 tests Cat # 63ADK111PEG	10,000 tests Cat # 63ADK111PEH
Control lysate Frozen/ready-to-use	1 vial - 150 µL	2 vials - 150µL
Anti-MDA5 aggregation-Eu Cryptate Antibody	1 vial - 20 µL Frozen - 50 X	1 vial - 400µL Frozen - 50 X
Anti-MDA5 aggregation-d2 Antibody	1 vial - 20 µL Frozen - 50 X	1 vial - 400 µL Frozen - 50 X
Lysis buffer #3 * stock solution 4X	4 vials - 2 mL Frozen	1 vial - 130 mL Frozen
Detection Buffer #8 ** ready-to-use	1 vial - 2 mL Frozen	1 vial - 50 mL Frozen

* Amounts of reagents provided are sufficient for generating 50 µL of cell lysate per well.
** The Detection Buffer is used to prepare working solutions of acceptor and donor reagents.

PURCHASE SEPARATELY:

- HTRF®-Certified Reader**. Make sure the setup for Eu Cryptate is used
- For a list of HTRF-compatible readers and set-up recommendations, please visit www.revvity.com
- Small volume (SV) detection microplates - Use white plate only.
- For more information about microplate recommendations, please visit our website at: www.revvity.com

STORAGE AND STABILITY

Antibodies, control lysate and buffers should be stored frozen until use.
Thawed detection buffer can be stored at 2-8°C in your premises. Thawed antibodies are stable 48 hours at 2-8°C; they can be refrozen (at -20°C or below) and thawed at least one more time. Control lysate must be stored frozen at -60°C or below. Thawed control lysate can be refrozen (at -60°C or below) and thawed one more time.

REAGENT PREPARATION

Allow all reagents to thaw before use.
We recommend centrifuging the vials gently after thawing, before pipeting the stock solutions.
Prepare the working solutions from stock solutions by following the instructions below.

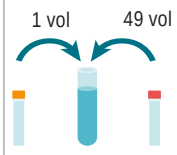
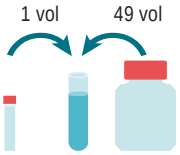
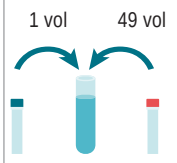
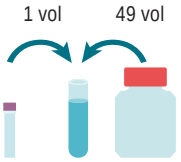
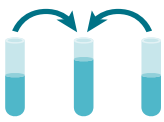
POSITIVE CONTROL SOLUTION: READY-TO-USE

The control cell lysate is only provided as an internal assay control to check the quality of the results obtained. The window between control lysate and negative control should be greater than 2.

TO PREPARE WORKING ANTIBODY SOLUTIONS:

HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the d2 and Cryptate-antibodies will impair the assay's quality. Be careful, as working solution preparation for antibodies may differ between the 500 and 10,000 tests data point kit.

Antibody working solutions are stable for 2 days at 4°C. Dilute the antibodies with detection buffer #8.

500 TESTS KIT - 63ADK111PEG		10,000 TESTS KIT - 63ADK111PEH
Anti-MDA5 aggregation- Cryptate antibody		
Dilute 50-fold the frozen stock solution with Detection buffer #8: e.g. add 2.45 mL of detection buffer to the 0.05 mL of Cryptate-antibody stock solution.	 	Dilute 50-fold the frozen stock solution with Detection buffer #8: e.g. add 49 mL of detection buffer to the 1mL of Cryptate antibody stock solution.
Anti-MDA5 aggregation-d2 antibody		
Dilute 50-fold the frozen stock solution with Detection buffer #8: e.g. add 2.45 mL of detection buffer to the 0.05 mL of d2- antibody stock solution.	 	Dilute 50-fold the frozen stock solution with Detection buffer #8: e.g. add 49 mL of detection buffer to the 1mL of d2- antibody stock solution.
Antibody mix		
It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2-antibody solution to 1 volume of Cryptate-antibody solution.		It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2-antibody solution to 1 volume of Cryptate-antibody solution.

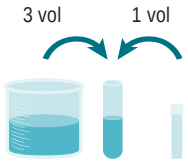
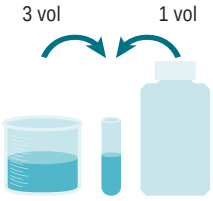
TO PREPARE LYSIS BUFFER:

Make sure that the lysate has been generated by using the kit reagents.

Prepare the required amount of lysis buffer before running the assay, working solutions are stable for 2 days at 2-8°C.

Lysis buffer 1X:

Determine the amount of lysis buffer needed for the experiment. Each well requires generally 50 µL of lysis buffer. Prepare a lysis buffer solution 1X by diluting 4-fold the lysis buffer 4X with distilled water.

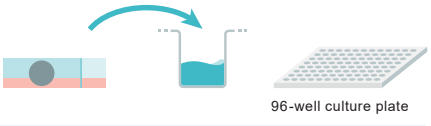
500 TESTS KIT - 63ADK111PEG		10,000 TESTS KIT - 63ADK111PEH	
Preparation of lysis buffer 1X			
Dilute the "lysis buffer 4X" 4-fold with distilled water to prepare lysis buffer 1X. e.g. take 1.25 mL of lysis buffer 4X and add it to 3.75 mL of distilled water. Mix gently.			Dilute the "lysis buffer 4X" 4-fold with distilled water to prepare lysis buffer 1X. e.g. take 1.25 mL of lysis buffer 4X and add it to 3.75 mL of distilled water. Mix gently.

TWO PLATE ASSAY MANUAL

FOR ADHERENT CELLS

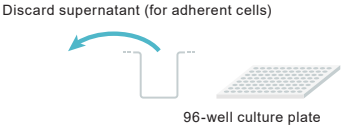
GENERAL LAB WORK PRIOR USING REVVITY KIT: CELLS PREPARATION

1. Plate 50 µL of cells in 96-well tissue-culture treated plate in appropriate growth medium and incubate overnight, at 37°C in CO2 atmosphere.
   Cell seeding densities of 200 K cells/well are generally sufficient for most cell lines, but optimization of cell seeding densities is recommended.
   Depending on receptor a starving step with serum-free medium could be essential.



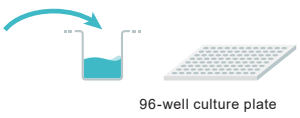
2. Dispense 50 µL of compounds (2X) diluted in cell culture medium
   For most compound, incubation time should be above 24 hours at 37°C.
   We recommend a time course study to determine the optimal stimulation time.
   Note that concentration above 0.5% DMSO will impair assay performances.
   Same final concentration of DMSO must be used for each compound dilutions.

3. Remove carefully cell supernatant either by aspirating supernatant or by flicking the plate.

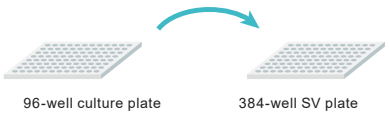


MDA5 AGGREGATION DETECTION USING REVVITY KIT

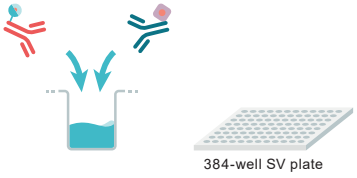
4. Immediately add 50 µL of lysis buffer #3 (1X) and incubate for at least 30 minutes at room temperature under shaking.
   Use the appropriate lysis buffer and incubate at room temperature with shaking.
   We recommend a time course study to determine the optimal lysis incubation time. Lysis volume can be decreased down to 25 µL.



5. After homogenization by pipeting up and down, transfer 16 µL of cell lysate from the 96-well cell-culture plate to a 384-well small volume white plate.
   Depending on cell lines used, it can be necessary to dilute the cell lysate to ensure samples are within the assay linear range



6. Add 4 µL of premixed antibody solutions (vol/vol) prepared in the detection buffer #8.
   Cover the plate with a plate sealer.
   Incubate overnight at RT.
   Set up your reader for Eu Cryptate and read the fluorescence emission at two different wavelenghts (665nm and 620nm) on a compatible HTRF® reader*.



* For more information about HTRF® compatible readers and for set-up recommendations, please visit our website at: www.revvity.com

Standard manual for two-plate assay manual in 20 µL final volume (after lysis step)

	Non treated cell lysate	Treated cell lysate	Positive control	Negative control	Blank control
Step 1 	Dispense 16 µL of non treated cell lysate	Dispense 16 µL of treated cell lysate	Dispense 16 µL of control lysate	Dispense 16 µL of lysis buffer 1X	Dispense 16 µL of non treated cell lysate
Step 2 	Add 2 µL of Anti-MDA5 aggregation-d2 Antibody working solution to all wells				Add 2 µL of detection buffer
Step 3 	Add 2 µL of Anti MDA5 aggregation-Eu Cryptate Antibody working solution to all wells				
Step 4 	Cover the plate with a plate sealer. Incubate overnight at room temperature.				
Step 5	Remove the plate sealer and read on an HTRF® compatible reader				

The blank control is used to check the Cryptate signal at 620 nm.

The Negative control is used to check the non-specific signal. The ratio between control lysate signal / non-specific signal should be greater than 2.

DATA REDUCTION & INTERPRETATION

1. Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

2. Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

3. Calculate the % delta F which reflects the signal to background of the assay. The negative control plays the role of an internal assay control. Delta F is used for the comparison of day to day runs of the same assay.

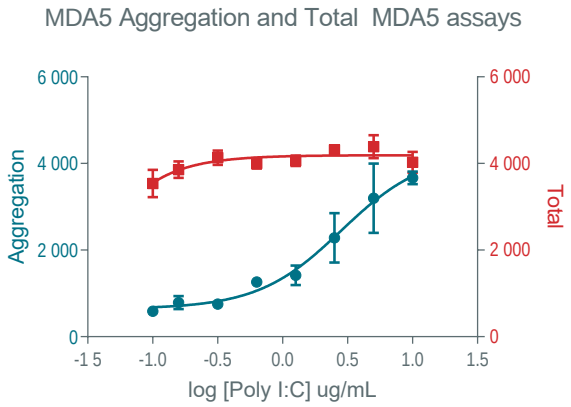
$$\text{delta F (\%)} = \frac{\text{Ratio Standard or sample - Ratio Negative Control}}{\text{Ratio Negative Control}} \times 100$$

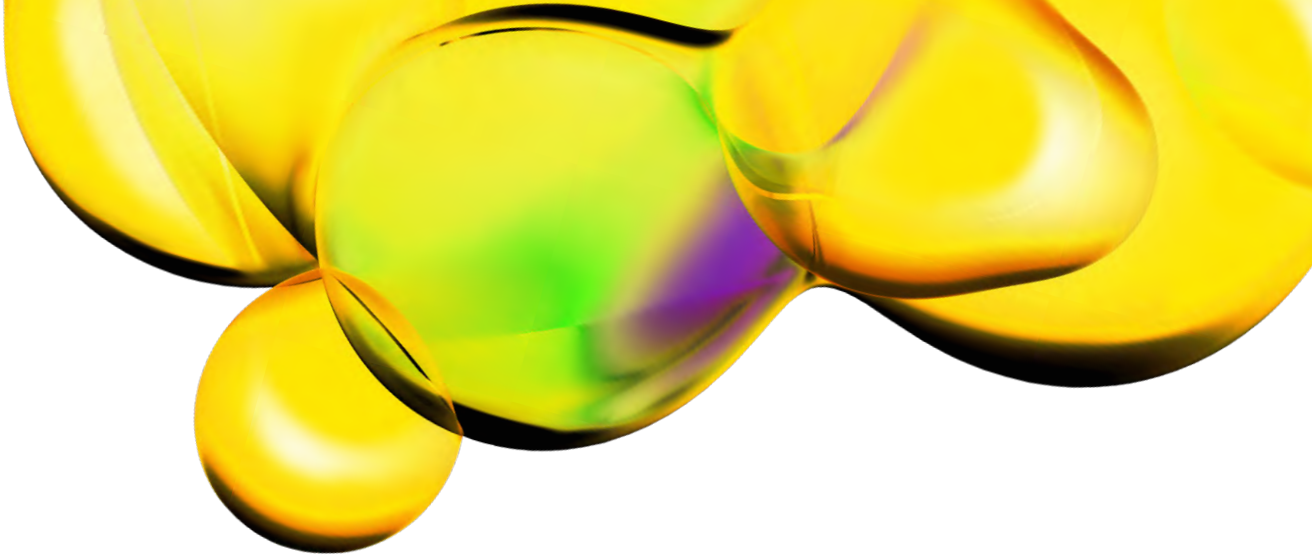
For more information about data reduction, please visit www.revvity.com

RESULTS

These data should be considered only as an example. Results may vary from one HTRF® compatible reader to another.

PHERAstarFS with flash lamp (BMG) was used for reading.





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