

HTRF[®] SIRT1 assay 20,000 tests Using HTRF Technology

Document reference : 64SI1PEC rev 00 (April 09)

HTRF[®] package insert

Conjugate and reagent sets

HTRF[®] SIRT1 frozen set 64SI1PEC
HTRF[®] SIRT buffer set 64SIRUDF

For in vitro research use only

Storage temperature : at - ≤-20°C
Storage temperature : 2-8°C

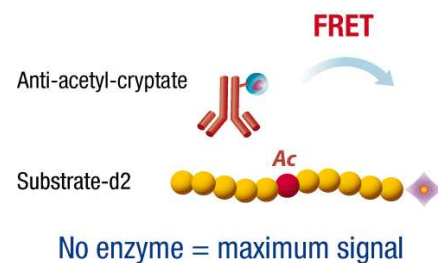
1. Assay description

The HTRF SIRT1 assay is a homogeneous method for directly measuring SIRT1's deacetylation activity. SIRT1 belongs to the class III family of HDACs, which are NAD⁺ dependant enzymes. It regulates a variety of biological processes, such as glucose homeostasis, mitochondrial biogenesis, adipogenesis, apoptosis, senescence and metabolism. The beneficial role of SIRT1 modulators in treating diseases such as cancer, diabetes, metabolic disease, and inflammation has been demonstrated, making them attractive targets for the development of new drugs.

The HTRF SIRT1 assay applies to SIRT1 activity assessment, and involves the two steps described below:

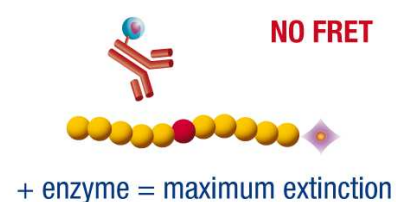
Enzymatic step:

During this step, the substrate-d2 containing a single acetylated lysine is incubated with the SIRT1 enzyme.



Detection step:

The deacetylation process is quantified using an anti-acetyl MAb labelled with Eu³⁺ cryptate.



2. Reagent description-Stock solutions

Each kit is designed to run 20,000 tests at 20 µl final volume.

	Components	Quantity	Stock solutions
Frozen set	Acetylated substrate-d2	1 vial of 0.8 ml Frozen	1.5µM
	Anti-acetyl MAb Cryptate	1 vial of 1 ml Frozen	400X
	NAD+ (SIRT1 cofactor)	1 vial of 1.6 ml Frozen	75 mM
	DTT	1 vial of 1.6 ml Frozen	150 mM
Buffer set	HTRF SIRT enzymatic buffer	1 vial of 240 ml Liquid	1X Other components must be added (§ 4.1).
	HTRF SIRT detection buffer	1 vial of 240 ml Liquid	1X Ready to use

Storage: All kit components must be stored at ≤ -20°C.

To avoid freezing/thawing cycles, aliquot stock solutions into disposable plastic vials for storage at ≤ -20°C. Reagents may be frozen and thawed once.

The two buffers (HTRF SIRT buffer set) must be stored at 2-8°C.

3. Additional material required (not provided)

Components	Recommended supplier	Stock solution to prepare
SIRT 1	Biomol (ref # SE-239)	Refer to supplier's recommendations

4. Preparation of the working solutions

The working solutions are prepared from stock solutions (§ 2) by following instructions below :

4.1. Preparation of the buffers

4.1.1. Enzymatic buffer:

Precaution :

It is recommended to prepare the required amount of enzymatic buffer just before running the assay. DTT is not stable at ambient temperature or 2-8°C. It should be added just before use.

Components to add just before use	DTT	Enzymatic buffer
Recommended dilution	1 volume of stock solution	149 volumes
For 20,000 tests	1.6 ml of stock solution	238.4 ml
Final concentration in the enzymatic buffer	1 mM	

4.1.2. Detection buffer

Ready to use

4.2. Working solutions to prepare

Precaution :

- Working solutions cannot be stored and must be used immediately.
- HTRF conjugate concentrations have been set for optimal assay performances. Note that any dilution or improper use of the detection reagents will impair assay quality.
- Enzyme working solution must be prepared just before use and must be kept on ice prior to launching the assay (to avoid the degradation of enzymatic activity).
- NAD⁺ working solution must be prepared just before, it is not stable at ambient temperature or 2-8°C.

	Volume of stock solution	Volume of buffer to add
Substrate-d2	1 volume	99 volumes of <i>enzymatic buffer</i>
For 20,000 tests	0.8 ml	79.2 ml of <i>enzymatic buffer</i>
Compounds	Determined by the user. Dilute in <i>enzymatic buffer</i> . Keep DMSO ≤1% in the enzymatic step.	
Enzyme*	Determined by the user . Dilute in <i>enzymatic buffer</i>	
NAD⁺	Determined by the user . Dilute in <i>enzymatic buffer</i>	
Anti-acetyl MAb Cryptate	1 volume	199 volumes of <i>detection buffer</i>
For 20,000 tests	1 ml	199 ml of <i>detection buffer</i>

*The enzyme concentration to apply depends on enzyme activity and the incubation time. Some recommendations are described (§ 6.1 and 6.2) using SIRT1 from Biomol.

Reagent concentrations :

Name		Working concentration	Recommended concentrations	
			Concentration in the enzymatic step	Concentration in the final assay volume
Substrate-d2		2.5 X 15 nM	1 X 6 nM	
Compounds		5 X -	1 X -	
Enzyme		5 X -	1 X -	
NAD ⁺	Inhibitors screening*	5 X 2500 µM	1X 500 µM	
	Activators screening*	5 X 750 µM	1X 150 µM	
Anti-acetyl MAb cryptate		2 X	- -	1 X

*recommended concentration of NAD⁺ in the assay.

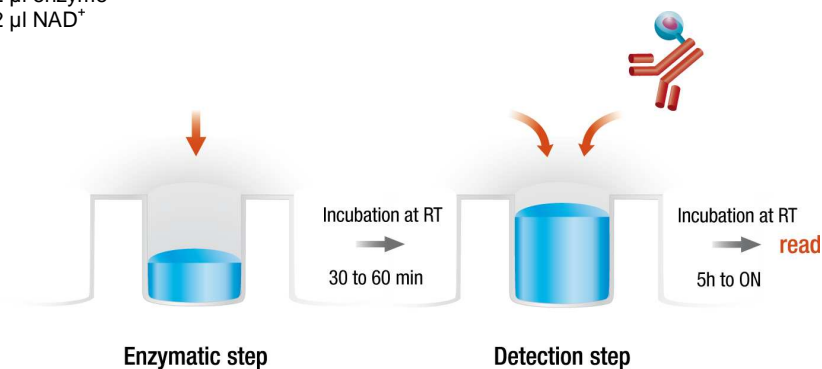
5. Assay protocol for 384 well low-volume plate (20 µl)

10 µl Enzymatic step:

- + 4 µl substrate-d2
- + 2 µl compounds (or enzymatic buffer)
- + 2 µl enzyme
- + 2 µl NAD⁺

20 µl Detection step:

- 10 µl Anti-acetyl MAB Cryptate



The enzymatic reaction (step 1) is performed by adding the assay components (working solutions) in the order presented above. The enzymatic reaction is stopped by the addition of the detection reagents which contain EDTA and a SIRT1 inhibitor (nicotinamide).

For a 384w low-volume plate, we recommend 10 µl for the enzymatic step and 10 µl for the detection step for a final assay volume of 20 µl.

Plate references : 384 small volume plate (# 784076 Greiner or equivalent).

Controls :

Appropriate controls must be prepared for each condition tested :

No enzyme control (0% deacetylation): this control consists of the anti-acetyl antibody-Cryptate in the presence of enzymatic reaction components (without enzyme). This control defines the upper limit of assay window.

Negative control : this control consists of the presence of all SIRT 1 assay reagents except the substrate. It is used for the calculation of Delta F and defines the lower limit of the assay window.

Buffer control : used to make sure that buffers are not contaminated by Cryptate and do not generate any background fluorescence.

Cryptate control : used to check the Cryptate signal at 620 nm

	Deacetylation Assay	Controls			
Enzymatic step (10 µl)	Sample	Negative	No enzyme	Cryptate	Buffer
Substrate-d2	4 µl	-	4 µl	-	-
Compounds	2 µl	2 µl	2 µl	-	-
Enzyme	2 µl	2 µl	-	-	-
NAD ⁺	2 µl	2 µl	2 µl		
Enzymatic buffer	-	4 µl	2 µl	10 µl	10 µl
Seal plate and incubate between 30 and 60 min at RT					
Detection step (10 µl)					
Anti-acetyl-cryptate	10 µl	10 µl	10 µl	10 µl	-
Detection buffer	-	-	-	-	10 µl
Seal plate and incubate from 5 hours to ON at RT (20°C)					
Remove plate and sealer and read on an HTRF compatible reader More information at http://www.htrf.com/technology/htrfmeasurement/compatible_readers/					

NB:

- For compounds containing DMSO, the concentration of DMSO must be the same in each well; also for controls.
- For low volume compound addition, adjust volume to 2 µl with 1X of enzymatic buffer.

Data calculation :

The cryptate is excited at 337 nm and the fluorescence is measured at 620nm (Cryptate emission wavelength) and 665nm (d2 emission wavelength). A ratio is calculated (665 nm /620 nm) for each well. Results are expressed as follows :

Specific signal : Ratio_{sample} – Ratio_{Negative control}

Ratio : (665 nm / 620 nm) X10⁴

Mean ratio : Σ ratio / 2 (when n=2)

CV% : (Std deviation / Mean ratio) X 100

Delta F % : (Ratio_{sample} – Ratio_{Negative control}) / Ratio_{Negative control} X 100

% Deacetylation of substrate : 100- (Ratio_{Sample} / Ratio_{No enzyme} X 100)

6. Optimization of the deacetylation assay

6.1. Enzyme titration

This step allows the optimal enzyme concentration (for which the substrate-d2 reaches 80% of deacetylation) to be determined. A compromise may be found between a high assay window and the enzyme consumption.

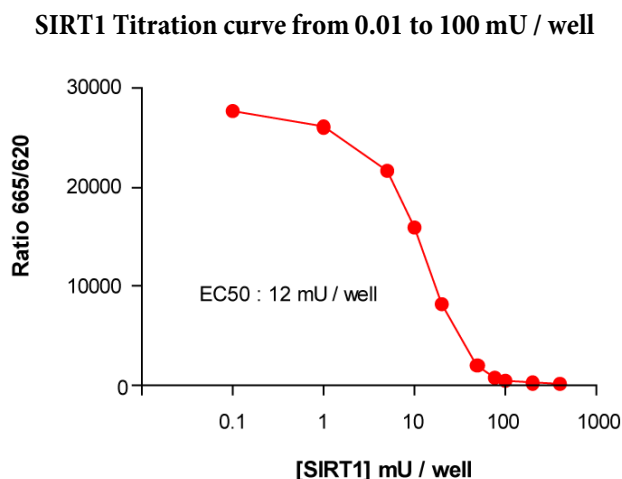
For this step, a fixed concentration of the substrate-d2 (6 nM) during the enzymatic step and NAD⁺ at the required concentration should be tested with the following enzyme concentrations: 0.5; 1; 5; 10; 20; 50; 100 mU / well (equal to 0.25; 0.5; 2.5; 5; 10; 25; 50 mU / μ l with 2 μ l of enzyme / well). Allow the enzymatic reaction to run for 30 min* at room temperature. After the addition of HTRF detection reagents, leave the plate to incubate from 5H to ON at room temperature, and then read the signal.

The signal is then plotted versus the different enzyme concentrations. Signal decrease is directly correlated to substrate deacetylation (i.e. enzyme activity).

*The reaction time should be at least 30 min. To select a lower enzyme concentration which gives 80% of substrate deacetylation, it is recommended to leave the plate to incubate from 30 min to 1H.

NB: Depending on their origins and batches, SIRT1 enzymes have different specific activities for the same molar concentration. For this reason, it is recommended to express SIRT1 concentration in specific activity: U

1U = 1pmol of substrate deacetylation /min (refer to supplier's enzyme characteristics).

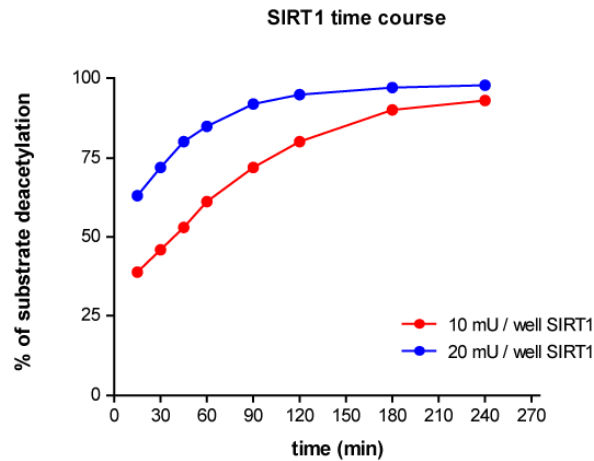


*Titration curves are performed as described previously with 500 μ M NAD⁺.
Enzymatic reaction time: 60 min*

6.2. Kinetic study

A time course study is performed using a constant concentration of SIRT1 (determined in the previous experiment: SIRT1 titration), NAD⁺ and substrate (6 nM). The reaction is stopped at different end points by the addition of the detection reagents (0; 5; 15; 30; 45; 60; 90; 120; 180 min).

The signal is then plotted versus the different end points. Determine the linear part of the time course and select the incubation time to use.



*SIRT1 kinetic is performed as described previously.
NAD⁺ was fixed at 500 μ M.*

6.3 Compounds screening:

For an inhibitor screening assay, it is recommended to work with a concentration of NAD⁺ of 500 μ M and to reach 80% of substrate deacetylation (without adding compounds) to ensure suitable assay windows.

For an activator screening assay, it is recommended to work with a concentration of NAD⁺ of 150 μ M and to reach 10-20% of substrate deacetylation (without adding compounds) to obtain suitable assay windows.