HTRF SIRT1 DETECTION KIT

1,000 TESTS

PROTOCOL

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

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Product information:

Storage temperature: ≤-16°C (frozen set) & 2-8°C (buffer set)

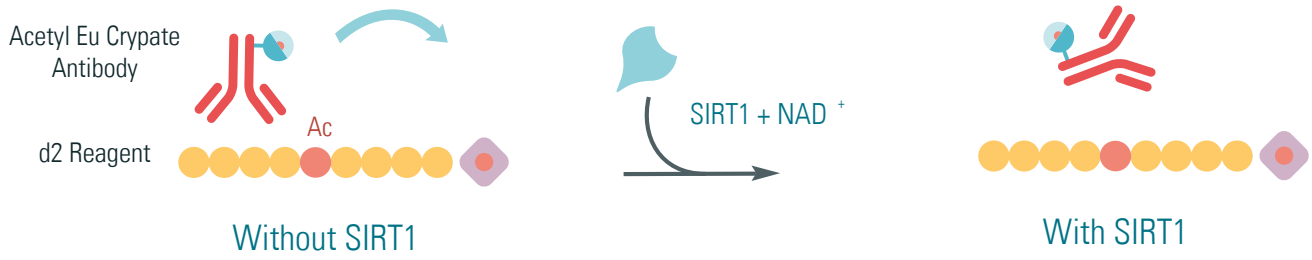
Packaging details :

Document reference: 64SI1PEB rev 07 (January 2022)

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.

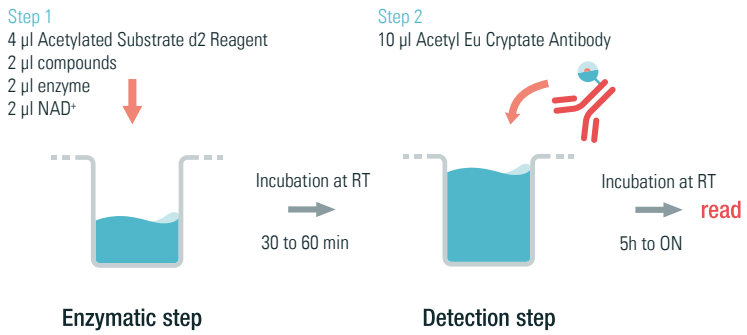
Active SIRT1 enzyme deacetylates the substrate, causing the loss of FRET and a subsequent reduction in assay signal.



The HTRF SIRT1 assay applies to SIRT1 activity assessment, and involves the two steps: the enzymatic step followed by detection.

During the enzymatic step, the Acetylated Substrate d2 Reagent containing a single acetylated lysine is incubated with the SIRT1 enzyme. Then deacetylation process is quantified using an anti-acetyl MAb labelled with Eu^{3+} cryptate.

2. ASSAY PROTOCOL FOR 384 WELL LOW-VOLUME PLATE (20µL)



Add assay components (working solutions) in the following order:

The enzymatic reaction is stopped by the addition of the Acetyl Eu Cryptate Antibody prepared in detection buffer containing EDTA and Nicotinamide (SIRT1 inhibitor).

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

3. REAGENT DESCRIPTION-STORAGE

Each kit is designed to run 1,000 tests at 20µL final volume.

Components	Stock Solution	Number of vial	Volume	Storage
Acetylated substrate d2 Reagent	1.5µM - to dilute	1	40µL	≤-16°C
Acetyl Eu Cryptate Antibody	to dilute	1	50µL	≤-16°C
NAD+ (SIRT1 cofactor)	75mM - to dilute	1	270µL	≤-16°C
DTT	150mM - to dilute	1	270µL	≤-16°C
HTRF SIRT enzymatic buffer	ready-to-use	1	40mL	2-8°C or below
HTRF SIRT detection buffer	ready-to-use	1	40mL	2-8°C or below

Storage: Enzymatic and detection buffers can be stored at 2-8°C, others components must be stored at ≤-16°C before use.
To avoid freeze/thaw cycles, aliquot stock solutions into disposable plastic vials for storage at ≤-16°C.
Reagents may be frozen and thawed once.

4. ADDITIONAL MATERIAL REQUIRED (NOT PROVIDED)

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

5. PREPARATION OF THE WORKING SOLUTIONS AND PRECAUTIONS

Allow the reagents to come to room temperature before use.

5.1. The working solutions are prepared from stock solutions (§ 3) following the instructions below:

Supplemented enzymatic buffer (enzymatic buffer supplemented with 1mM DTT)	Dilute 150 fold DTT stock solution with enzymatic buffer. e.g. take 100 µL of DTT stock solution and add it to 14.9 mL of enzymatic buffer. Mix gently.
Compounds	Dilute compound stock solution with supplemented enzymatic buffer to prepare a working solution which has 5X the required final concentration for the enzymatic step.
Enzyme	Dilute the enzyme stock solution with supplemented enzymatic buffer to prepare a working solution which has 5X the required final concentration for the enzymatic step.
NAD+ solution Depending on the assay, the NAD+ concentration should be optimized	For inhibitor screening* – recommended [NAD+ concentration] init: 2500µM. ilute NAD+ 30 fold in the supplemented enzymatic buffer. e.g. take 100 µL NAD+ stock solution and add it to 2900 µL of supplemented enzymatic buffer. Mix gently. For activator screening* – recommended [NAD+ concentration] init: 750µM. Dilute NAD+ 100 fold in the supplemented enzymatic buffer. e.g. take 30 µL NAD+ stock solution and add it to 2970 µL of supplemented enzymatic buffer. Mix gently.
Acetylated Substrate d2 Reagent	Dilute 100 fold with supplemented enzymatic buffer. e.g. take 20 µL of stock solution and add it to 1980 µL of supplemented enzymatic buffer. Mix gently.
Acetyl Eu Cryptate Antibody	Dilute 200 fold with detection buffer. e.g. take 10 µL of stock solution and add it to 1990 µL of detection buffer. Mix gently.

5.2. Precautions

- DTT and NAD+ are not stable at RT or at 2-8°C, Working solutions should be prepared just before the assay and cannot be stored
- 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 in an ice bath for the time of the experiment to avoid degradation
- Depending on the assay, NAD+ concentration should be optimized
- Enzyme concentration should be optimized
- Compounds should be diluted in supplemented enzymatic buffer. DMSO concentration must be kept <1% during the enzymatic step. For compounds containing DMSO, the concentration of DMSO must be the same in each well. This concentration must be identical for controls
- For an inhibitor screening assay, it is recommended to work with a NAD+ concentration of 500 µM final (enzymatic step) 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 NAD+ concentration of 150 µM final (enzymatic step) and to reach 10-20% of substrate deacetylation (without adding compounds) to obtain suitable assay windows.

6. SIRT ASSAY AND CONTROLS

6.1. Reagent concentrations

Name		Working concentration	Recommended concentrations	
			Concentration in the enzymatic step	Concentration in the final assay volume
Acetylated Substrate d2 Reagent		15 nM	6 nM	3nM
Compounds		5X	1 X	-
Enzyme		5X	1 X	-
NAD+	Inhibitors screening*	2500 µM	500 µM	-
	Activators screening*	750 µM	150 µM	-
Acetyl Eu Cryptate Antibody			-	1X

*recommended concentration of NAD+ in the assay.

6.2. Controls

The SIRT assay is performed as described below, using four different controls

No enzyme control (0% Deacetylation): used to define the upper limit of the assay window.

Negative control: used to calculate the specific signal (delta F) and 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.

	SIRT Assay	Controls			
Enzymatic step (10 µL)	Sample	Negative	No enzyme	Cryptate	Buffer
Acetylated Substrate d2 Reagent	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 www.cisbio.com/readers					

7. OPTIMIZATION OF THE SIRT ASSAY AND DATA REDUCTION

7.1. Data reduction

The fluorescence is measured at 620 nm (Cryptate) and 665 nm (XL665). A ratio is calculated (665/620) 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 %: $(Ratio_{sample} - Ratio_{Negative\ control}) / Ratio_{Negative\ control} \times 100$

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

7.2. 7.2 Enzyme titration

This step allows the optimal enzyme concentration (for which the Acetylated Substrate d2 Reagent reaches 80% of Deacetylation) to be determined. A compromise may be found between high assay window and enzyme consumption.

A fixed concentration of the Acetylated Substrate d2 Reagent (6 nM) and the required NAD+ concentration should be tested with the following enzyme concentrations: 0.1, 1, 5, 10, 20, 50, 100, 200, 400 mU / well. Allow the enzymatic reaction to run for 30 min* at room temperature.

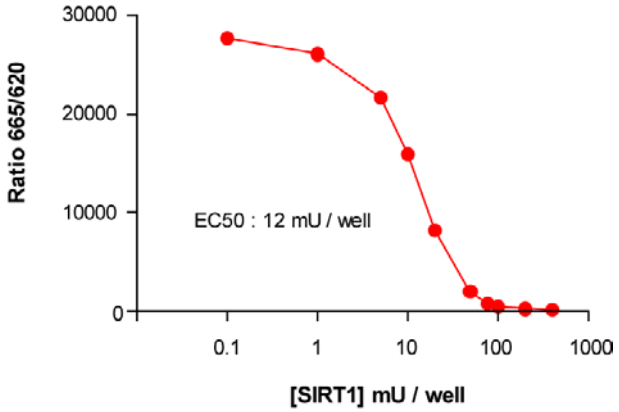
The signal is then plotted versus the different enzyme concentrations. Signal decrease is directly correlated to substrate deacetylation (i.e. enzyme activity).

*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: As SIRT1 enzymes can have different specific activities for the same molar concentration, we recommend expressing SIRT1 concentrations in specific activity (U):

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

SIRT1 Titration curve from 0.01 to 400 mU / well

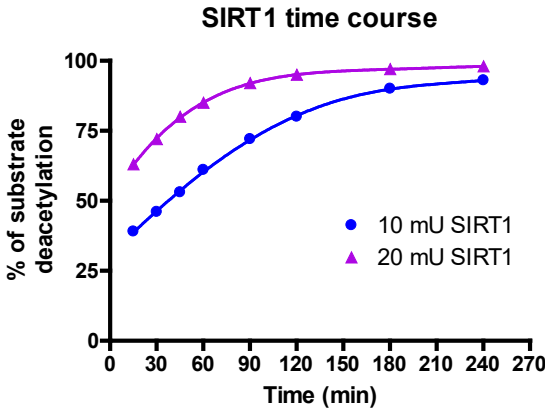


Titration curves are performed as described previously with 500 µM NAD+.
Enzymatic reaction time: 60 min

7.3. Kinetics study

A time course study is performed using the constant concentrations of SIRT1 determined in the SIRT1 titration, NAD+ and substrate (6 nM). The reaction is stopped at different end points by the addition of the detection reagents (0, 15, 30, 45, 60, 90, 120, 180, 240 min).

The signal is then plotted versus the different end points. Determine the linear part of the time course (correlation coefficient R2 > 0.99) and from this section, select the optimal incubation time to use for the next experiments.



SIRT1 kinetic study is performed as described previously.
NAD+ was fixed at 500 µM