HTRF® SIRT1 HISTONE H3K9 DEACETYLATION ASSAY

APPLICATION NOTE

ABSTRACT SIRT1 Histone H3K9 deacetylation assay that measures the deacetylation of a biotinylated histone H3(1-21) peptide at lysine 9.

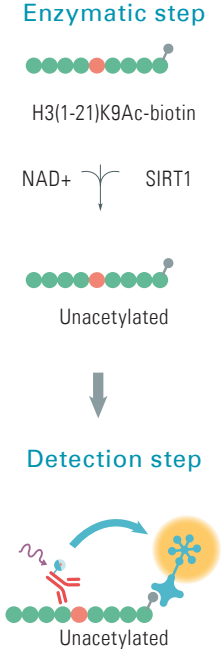
The HTRF SIRT1 Histone H3K9 deacetylation assay uses a H3(1-21) lysine 9 acetylated biotinylated peptide (substrate), a Eu^{3+}-cryptate labeled anti-H3K9me0 detection antibody and XL665-conjugated Streptavidin (SA-XL665).

The assay is performed in a single well and run in two steps: the enzymatic step and the detection step. HTRF signal is proportional to the concentration of Unacetylated H3(1-21) peptide. The assays within this technical note were performed in a 384-well plate in a 20 µL final volume.

Enzyme	SIRT1
Substrate	H3(1-21)K9Ac-biotin ARTKQTAR-K(Ac)-STGG-KAPRKQLA-GGK(Biotin)
Detection Antibody	Anti-H3K9me0-Eu(K)

SIRT1 HISTONE H3K9 DEACETYLATION ASSAY AND REAGENTS

H3K9me0-Eu(K) Ab.	Cisbio Bioassays	# 61KB0KAE
Streptavidin XL-665	Cisbio Bioassays	# 610SAXLA
Detection buffer	Cisbio Bioassays	# 62SDBRDD
SIRT1	Cyclex	# CY-E1151
Histone H3(1-21) lysine 9 acetylated biotinylated peptide	AnaSpec	# AS-64361
Nicotinamide	Sigma	# N3376
Suramin	Sigma	# S2671
NAD	Sigma	# N1636
Enzymatic buffer	50 mM HEPES pH 7.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 0.01% Tween20	



Data shown on this application note has been obtained using Greiner # 784075, 384-well white microplates. For more information on the white plates we recommend, please visit www.cisbio.com/htrf-microplate-recommendations.

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ASSAY PROTOCOL

ENZYMATIC STEP

- Prepare working solutions of enzyme, peptide substrate, cofactors and inhibitor in enzymatic buffer just prior to use.
- Add to a 384-well small volume plate in the following order:
  - 4 µL of inhibitor (2.5X) or enzymatic buffer
  - 2 µL of SIRT1 enzyme (5X)
  - Incubate for 5 min at room temperature
  - 4 µL of H3(1-21)K9Ac-biotin peptide / NAD+ mixture (2.5X)
- Cover the plate with a plate sealer and incubate at room temperature.

DETECTION STEP

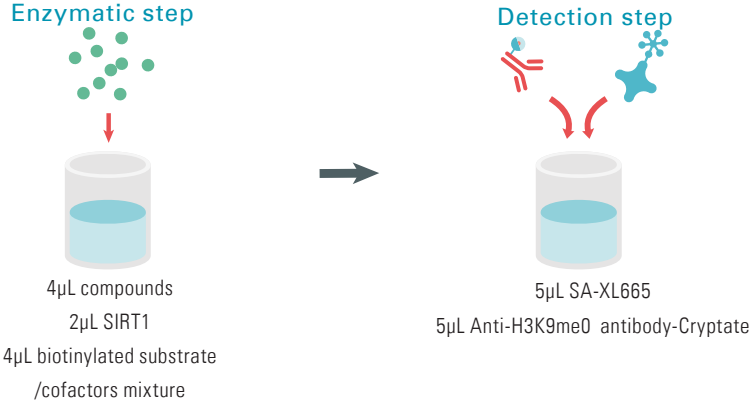
- The peptide-biotin / streptavidin-XL665 ratio must be equal to 4/1 final in the well (e.g. if peptide-biotin used at 4 nM final in the well, SA-XL665 must be used at 1 nM final in the well)
- Prepare detection mixture containing the anti-H3K9me0-Eu(K) 2X according to the product datasheet recommended final concentration and SA-XL665 at 2X the final concentration (hence 0.5X the final concentration of peptide-biotin in the well) in Detection buffer supplemented with 20mM Nicotinamide.
- Caution: Adjust SA-XL665 concentration according to peptide-biotin concentration used.
- Add 10 µL of detection mixture (2X) to the plate.
- Cover the plate with a plate sealer and incubate 1h at room temperature.
- Remove plate sealer and read fluorescence emission at 665nm and 620nm wavelengths on an HTRF compatible reader.

HTRF Ratio = (665nm/620nm)X10^4

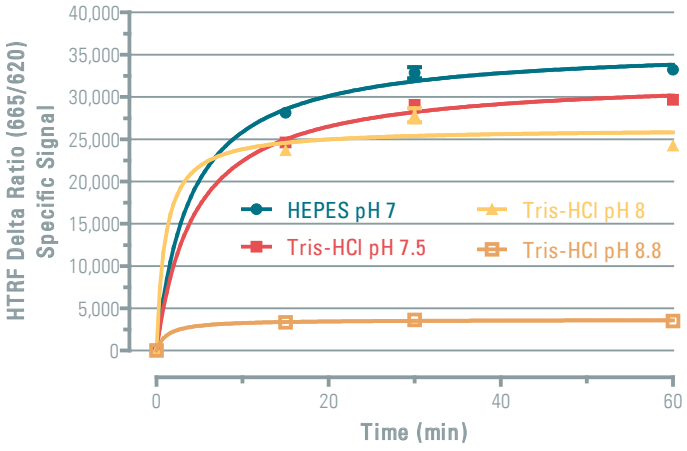
Delta Ratio = Sample Ratio - Ratio negative

DISTRIBUTION: ENZYME INHIBITION STUDY

	ENZYMATIC STEP				DETECTION STEP	
	ENZYMATIC BUFFER	INHIBITOR	SIRT1	COFACTOR/ SUBSTRATE MIXTURE	CRYPTATE-Ab	SA-XL 665
SAMPLE	-	4 µL	2 µL	4 µL	5 µL	5 µL
POSITIVE CONTROL	4 µL	-	2 µL	4 µL	5 µL	5 µL
NEGATIVE CONTROL	6 µL	-	-	4 µL	5 µL	5 µL

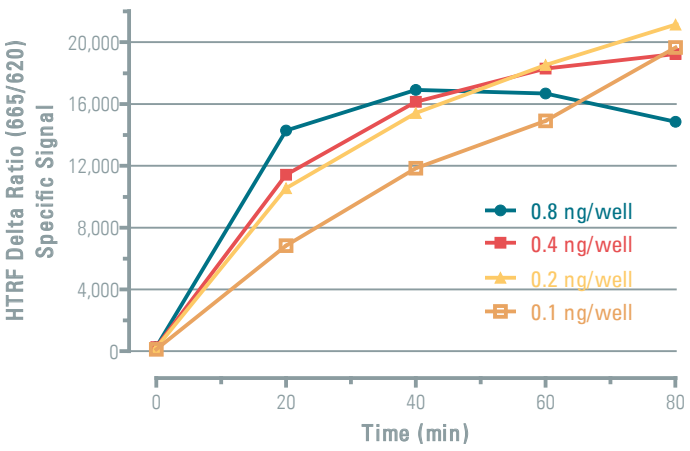


1. ENZYMATIC BUFFER OPTIMIZATION



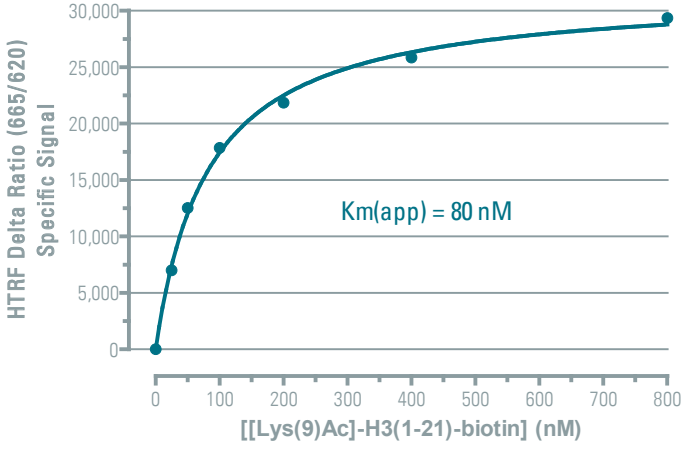
This step allows the optimal enzymatic buffer pH to be determined. The assay was carried out with 0.8 ng/well SIRT1, 200 nM biotinylated H3(1-21)K9Ac peptide substrate and 500 µM NAD+. The enzymatic reaction was carried out at RT and then stopped by adding H3K9me0-K Ab and SA-XL665 (detection reagents) after each time point (15, 30, 60 min). For further experiments 50 mM HEPES pH 7.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 0.01% Tween20 buffer was selected.

2. TIME COURSE AND ENZYME TITRATION



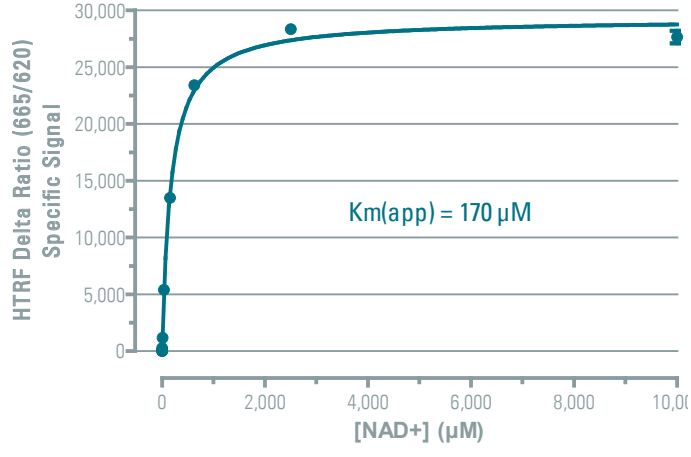
This step allows the optimal enzyme concentration and enzyme reaction time to be determined. Human recombinant SIRT1 was serially diluted to the concentrations indicated in the figure (0.1, 0.2, 0.4, 0.8 ng/well), and the assay was carried out with 100 nM biotinylated H3(1-21)K9Ac peptide substrate and 500 µM NAD+ cofactor. Enzyme kinetics depends on the SIRT1 specific activity and substrate concentrations. The enzymatic reaction was carried out at RT and then stopped by adding H3K9me0-K Ab and SA-XL665 (detection reagents) after each time point (20, 40, 60, 80 min). A 40 min reaction time using 0.2 ng/well SIRT1 was selected for other experiments.

3. PEPTIDE TITRATION



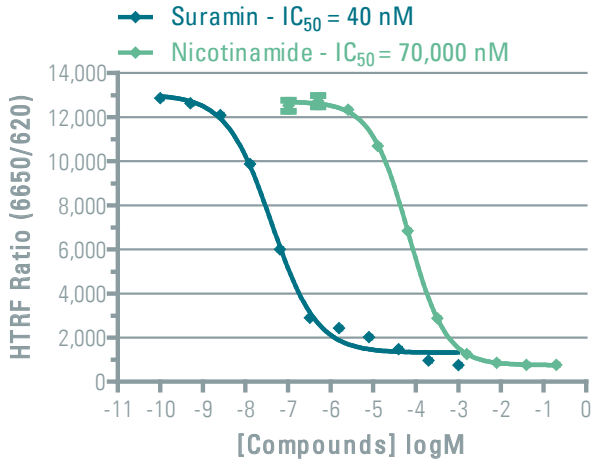
This step allows the determination of apparent Km for peptide. The Km value was determined with 0.4 ng/well SIRT1 and 500 µM NAD+ in the enzymatic step. We recommend testing biotinylated H3K9(1-21)Ac substrate concentrations ranging from 800 nM to 5 nM (serial dilutions). The streptavidin XL-665 concentration varie according to the peptide concentration keeping constant the ratio of 1/4 (Streptavidin XL-665 / peptide). For each concentration of peptide and streptavidin XL-665, a negative control is performed by removing the enzyme from the well. This negative control is used as non specific signal to calculate the HTRF delta ratio (hence specific signal). The enzyme reaction was stopped by adding the detection reagents at the optimal incubation period (RT, 20 min). The 80 nM apparent Km value for peptide was determined from this experiment using a Michaelis-Menten plot.

4. NAD TITRATION



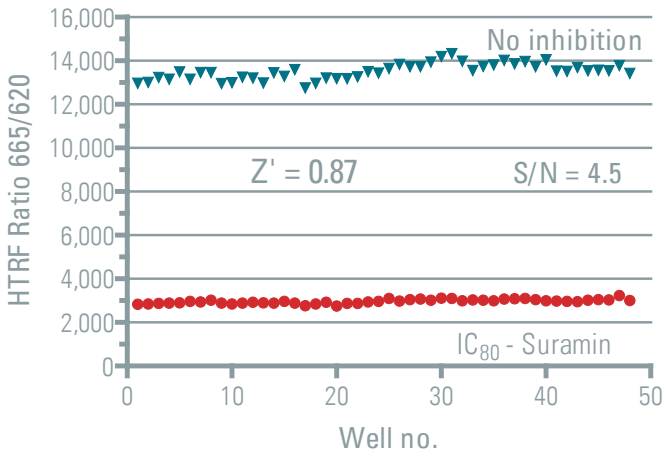
This step allows the determination of apparent Km for NAD+ cofactor. The Km value was determined with 0.2 ng/well SIRT1 and 100 nM H3(1-21)K9Ac peptide substrate in the enzymatic step. We recommend testing NAD+ concentrations ranging from 10,000 to 0.15 µM (serial dilutions). A negative control is performed by removing the enzyme from the well. This negative control is used as non specific signal to calculate the HTRF delta ratio (hence specific signal). The enzyme reaction was stopped by adding the detection reagents at the optimal incubation period (RT, 60 min). The 170 µM apparent Km value for peptide was determined from this experiment using a Michaelis-Menten plot.

5. ENZYME INHIBITION



SIRT1 H3K9 deacetylation inhibitor assay was validated by measuring the activity of reference inhibitors. This assay was performed using 0.2 ng/well SIRT1, 100nM peptide substrate and 200µM NAD+ cofactor. Serial dilutions of inhibitors were pre-incubated for 5 min with SIRT1 enzyme. Enzymatic reaction was initiated by the addition of 100 nM biotinylated H3(1-21)K9Ac peptide substrate. The enzyme reaction was stopped with the dectection conjugates after 40 min incubation at RT. IC50 values were calculated from inhibition curves.

6. Z' FACTOR DETERMINATION



The robustness of the assay was proven by performing a Z' determination with Suramin at IC80 (320 nM). The enzyme reaction was carried out with 0.2 ng/well SIRT1 enzyme, 100 nM H3(1-21)K9Ac substrate and 200µM NAD+ for 40 min at RT. Z' of 0.87 shows the robustness of the assay.

For more information, please visit us at www.cisbio.com/epigenetic-toolbox-reagents

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Adachi K, Tokuda C, Roux T, Trinquet E, Degorce F - Miptec 2013, Basel, Switzerland.

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Adachi K, Tokuda C, Chevallier F, Roux T, Gonzalez-Moya A, Degorce F. - Discovery on Target 2012, Boston, MA, USA.

Development of a panel of HTRF assay reagents for epigenetic targets.
Chevallier F, Jean A, Raynaldy D, Romier M, Servent F, Tokuda C, Adachi K. - Miptec 2011, Basel, Switzerland.

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Adachi K, Tokuda C, Chevallier F, Preaudat M. - SBS 2011, Orlando, USA.

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