TECHNICAL NOTE

AlphaLISA SIRT1 p53 Lysine 382 Deacetylase Assay

AlphaLISA #10

AlphaLISA®

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This AlphaLISA immunodetection assay measures the deacetylation of a biotinylated p53 (368-393) peptide acetylated at lysine 382.

Anti-acetyl-p53 Lysine 382 (p53K382ac) AlphaLISA® Acceptor Beads

- AL124C: 250 µg, 500 assay points*
- AL124M: 5 mg, 10,000 assay points*
- AL124R: 25 mg, 50,000 assay points*

**0.5 µg/assay point*

Peptidic Substrate Sequence:

(Biotin)K-GG-HLKSKKGQSTSRHK**K(ac)**LMFKTEGPDSD-NH_2

AlphaLISA Assays

AlphaLISA technology is a powerful and versatile platform that offers highly sensitive, no-wash immunoassays using Alpha Streptavidin Donor and AlphaLISA Acceptor beads. In this technical note, we present the optimization of a signal decrease SIRT1 assay using as substrate a biotinylated p53-derived peptide acetylated at lysine 382. In the absence of enzyme or cofactor, the anti-p53K382ac Acceptor beads bind the acetylated residue on the peptide. Upon laser irradiation of the beads-target complexes at 680 nm, short-lived singlet oxygen molecules produced by the Donor beads can reach the Acceptor beads in proximity to generate maximal AlphaLISA signal at 615 nm (left panel). When enzyme and cofactor are added to the reaction, the peptide substrate is deacetylated and the anti-p53K382ac Acceptor beads do not recognize the biotinylated peptide anymore, leading to a signal decrease (right panel). This signal decrease is proportional to the deacetylation activity of the SIRT1 enzyme.

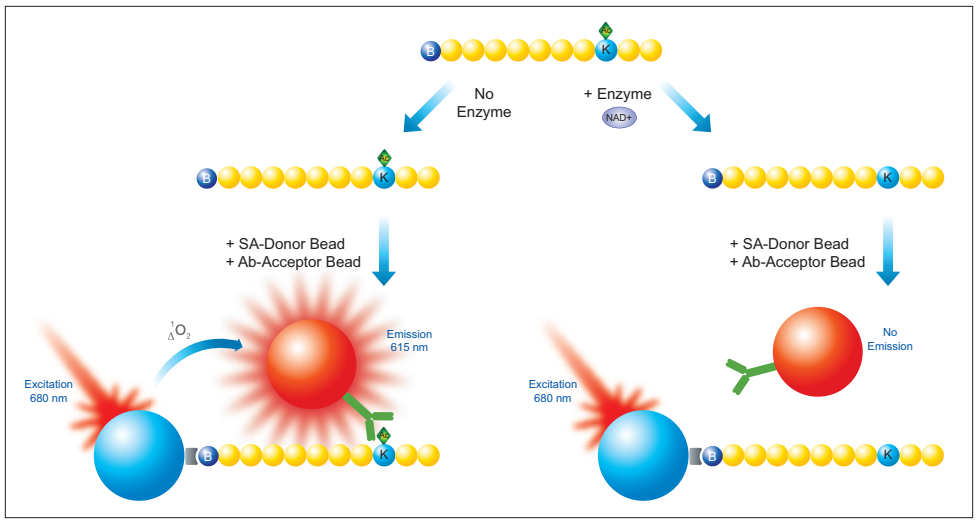

Figure 1. Schematic representation of the AlphaLISA detection of a modified p53-derived peptide.

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Development of a SIRT1 p53 Lysine 382 Deacetylase Assay:

Reagents needed for the assay:

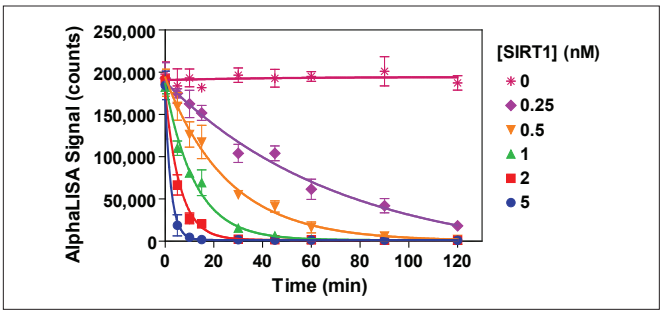
Anti-acetyl-p53 Lysine 382 AlphaLISA Acceptor beads	PerkinElmer # AL124
p53 (368-393) acetyl-lysine 382 peptide (p53K382ac), biotinylated	AnaSpec # 64869
Alpha Streptavidin Donor beads	PerkinElmer # 6760002
AlphaLISA 5X Epigenetics Buffer 1 Kit	PerkinElmer # AL008
Sirtuin 1 (human), recombinant	BPS BioScience # 50012
EX-527	Tocris # 2780
Suramin	Calbiochem # 574625
SIRT1 inhibitor III	Calbiochem # 566322
Nicotinamide	Sigma # N3376
ß-Nicotinamide adenine dinucleotide hydrate (NAD^+)	Sigma # N1636
White opaque OptiPlate™-384	PerkinElmer # 6007299
TopSeal™-A films	PerkinElmer # 6005185

Assay Buffer: 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT, 0.01% Tween-20 and 0.01% BSA.

Standard Protocol

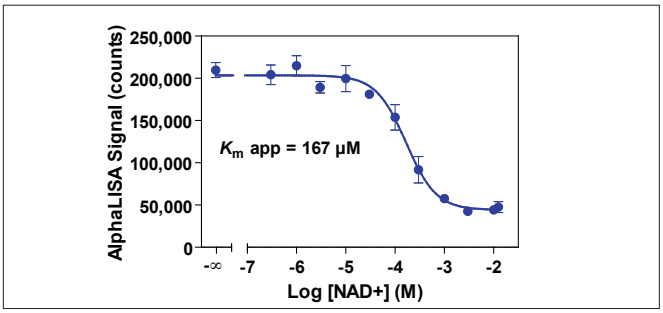
- Dilute SIRT1 enzyme, inhibitors, biotinylated p53K382ac peptide substrate and NAD^+ in Assay Buffer just before use.
- Add to the wells of a white OptiPlate-384:
  - 2.5 µL of enzyme (4X)
  - 2.5 µL of inhibitor (4X) or Assay buffer
  - Incubate 5 min at RT
  - 2.5 µL of biotinylated p53K382ac peptide substrate (4X)
  - 2.5 µL of NAD^+ (4X)
- Cover the plate with TopSeal-A film and incubate at room temperature (RT).
- Prepare 1X Epigenetics Buffer 1 as recommended in the buffer technical data sheet.
- Prepare a 5X Stop Solution Mix containing 250 µM of EX-527 and 100 µg/mL of Acceptor Beads in 1X Epigenetics Buffer 1 (final concentration of 50 µM EX-527 and 20 µg/mL Acceptor Beads in 25 µL total assay volume).
  - 5 µL of Stop Solution Mix
- Cover with TopSeal-A film and incubate for 60 min at RT.
- Prepare a 2.5X Streptavidin Donor beads solution at 50 µg/mL in 1X Epigenetics Buffer 1 (final concentration of 20 µg/mL in 25 µL total assay volume) in subdued light.
  - 10 µL of Streptavidin Donor beads
- Cover with TopSeal-A film and incubate in subdued light for 30 min at RT.
- Read signal in Alpha mode with the EnVision® or EnSpire® reader.

Experiment 1: Enzyme Titration and Time-Course



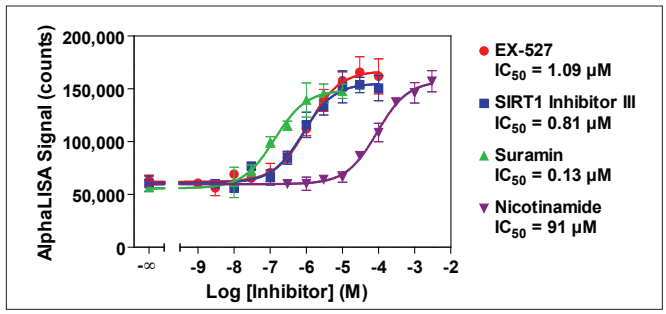
Enzymatic progress curves were performed by incubating SIRT1 at concentrations ranging from 0.25 to 5 nM with 3 nM biotinylated p53K382ac peptide substrate and 2 mM NAD^+. A mix of Acceptor beads and EX-527 was added to stop the reaction at the indicated times. Streptavidin Donor beads were added 60 min later and signal was read after 30 min. A 0.5 nM enzyme was selected for all subsequent experiments.

Experiment 2: NAD^+ Titration



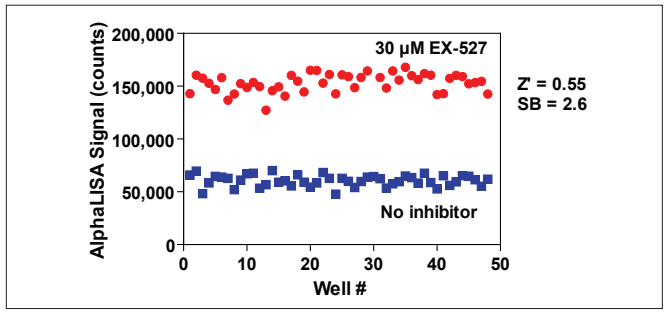
Serial dilutions of NAD^+ ranging from 300 nM to 12.5 mM were added to 0.5 nM SIRT1 and 3 nM biotinylated p53K382ac peptide substrate. Enzymatic reactions were incubated for 30 min. A 200 µM NAD^+ concentration was selected for subsequent experiments.

Experiment 3: Enzyme Inhibition



Serial dilutions of inhibitors ranging from 1 nM to 100 µM (EX-527), 3 nM to 100 µM (SIRT1 Inhibitor III), 10 nM to 10 µM (suramin) and 300 nM to 3 mM (nicotinamide) were pre-incubated for 5 min with 0.5 nM of SIRT1. Enzymatic reactions were initiated by the addition of 3 nM biotinylated p53K382ac peptide substrate and 200 µM NAD^+. Enzymatic reactions contained 1% DMSO and proceeded for 60 min.

Experiment 4: Z'-factor Determination



SIRT1 (0.5 nM) was pre-incubated with or without 30 µM EX-527 for 5 min. Enzymatic reactions were initiated by the addition of 3 nM biotinylated p53K382ac peptide substrate and 200 µM NAD^+. Enzymatic reactions contained 1% DMSO and proceeded for 60 min.

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009615_01 Printed in USA Apr. 2011