



LabChip Assay: Off Chip Incubation, Mobility Shift

NAD⁺-Dependent Protein Deacetylase (Sirtuins, Class III) Assay

I. Introduction

The off-chip incubation, mobility shift assay uses a microfluidic chip to measure the conversion of fluorescent acetylated peptide substrate to a deacetylated peptide product. The reaction mixture from a microtiter plate well is introduced through a capillary sipper onto the chip, where the product and substrate are separated by electrophoresis and detected via laser-induced fluorescence. This application note describes assay condition for human Silent Information Regulator family of NAD⁺-dependent protein deacetylases (SIRT1, SIRT2 and SIRT3), also known as a Class III Histone Deacetylases or Sirtuins). Reversible protein acetylation/deacetylation is the one of the critical post-translational modifications involved in the regulation of many biological processes. SIRTs play a pivotal role in regulating chromatin architecture, gene silencing, DNA repair and aging. An imbalance in the equilibrium of histon acetylation has been associated with carcinogenesis. The sensitive and rapid off-chip mobility shift assay provides a novel means of screening compounds on the activities of SIRTs.

II. Methods

Substrate

Acetylated peptide FL-TGGK(Ac)APR-COOH (MW 1230) which corresponds to the histone H3 sequence surrounding Lys-14 was used in the deacetylation reaction.

III. Results

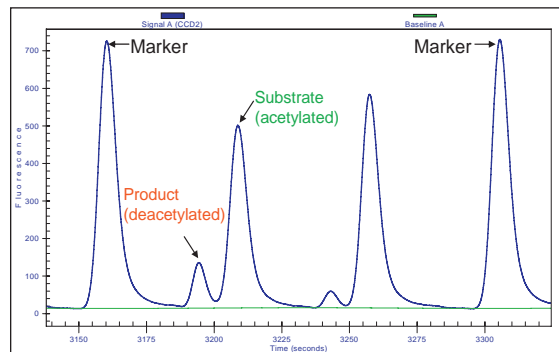


Figure 1. Caliper 3000 System Data Signature. Representative product and substrate peaks for SIRT assays. The electropherogram illustrates the fluorescent signal detected from a signal channel of a 12-sipper chip during 2 consecutive sips from different microtiter plate wells containing SIRT reactions.

Substrate/Product

Figure 1 shows the separation of product and substrate on a 12-sipper chip using the parameters shown in Table 1. Marker dye is sipped between rows to enable well assignments by the data analysis software (HTSWA). Acetylated substrate and deacetylated product are separated on a chip and appear as a distinctive peaks. The HTSWA software determines peak heights from which the ratio of product to the peak sum P/(P+S) is calculated. The P/(P+S) value x 100 = % product formed.

SIRT Assay condition (Final in reaction)	70 μ L reaction	Reaction Buffer (Final Concentration in reaction)	Stop Reaction Buffer	Chip/Trough Buffer
SIRT1 1 Unit	1 μ L Compound	50 mM Tris, pH 8.0	100 mM HEPES pH 7.5	100 mM HEPES pH 7.5
SIRT2 10 Units	35 μ L Enzyme	137 mM NaCl	20 mM EDTA	10 mM EDTA
SIRT3 0.85 Units	35 μ L Substrate	2.7 mM KCl	0.1% CR-3	0.1% CR-3
1 μ L compound in 100% DMSO	60 min reaction at 20° C	1 mM MgCl ₂	0.0015% Brij-3	0.0015% Brij-3
Substrate 1 μ M		500 μ M NAD ⁺ (for SIRT1 and SIRT2)		
		2500 μ M NAD ⁺ (for SIRT3)		
	Separation Condition	4-Sipper	Separation Condition	12-Sipper
	Pressure (psi)	-1.7	Pressure (psi)	-1.5
	Upstream Voltage (V)	-500	Upstream Voltage (V)	-500
	Downstream Voltage (V)	-2100	Downstream Voltage (V)	-2100
	Sample Sip Time (sec)	0.2	Sample Sip Time (sec)	0.2
	Post-sample Buffer Sip Time (sec)	40	Post-sample Buffer Sip Time (sec)	40

Table 1. Assay and separation conditions.

Enzyme Titrations

The initial titrations for SIRT1, SIRT2 and SIRT3 are shown on Figure 2. Reactions containing 70 μ L total volume with 1 μ M substrate and 5 different enzyme concentrations were assembled in duplicate on a 384-well microtiter plate. The plate was placed immediately onto the LabChip 3000 system and samples were introduced onto a 12-sipper chip every 3 minutes for 60 minutes. Temperature and humidity in the reaction chamber were maintained at 20° C and 50%, respectively. These environmental parameters were used for all kinetic experiments described in this application note.

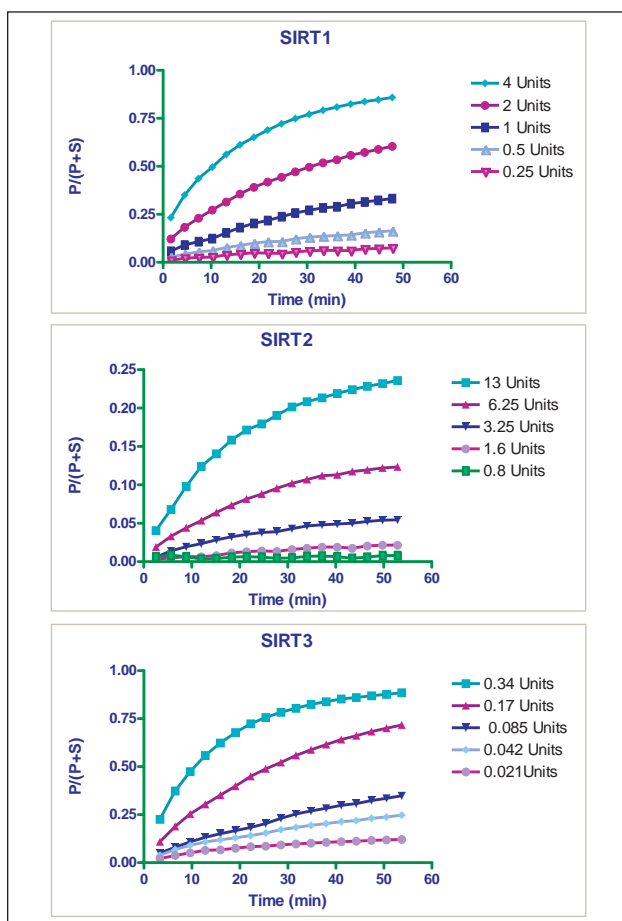


Figure 2. Enzyme titrations. Real time kinetics of SIRT1, SIRT2 and SIRT3 deacetylation reactions containing varying enzyme concentrations. Data represents averages from duplicate reactions.

Substrate and phosphorylated product were separated and detected on a chip. The enzyme concentration resulting in 30% product formed after 60 min incubation was extrapolated (SIRT1: 1 Unit; SIRT2: 10 Units and SIRT3: 0.08 Units) and chosen for further assay development studies.

Reaction Linearity

Real time kinetics were used to show that deacetylase reactions remained linear for at least 60 min. Reactions containing 70 μ L total volume with 1 μ M substrate and SIRT 1-1 Units, SIRT2 - 13 Units and SIRT3 - 0.08 Units were assembled in duplicate on a 384-well microtiter plate. The plate was placed immediately onto the LabChip 3000 system and samples were introduced onto a 12-sipper chip every 5 minutes for 60 minutes. Substrate and deacetylated product were separated and detected on the chip. The lines represent the linear regression from data points collected during 60 minutes of reaction time. Linearity is maintained over the course of at least the 60 minutes of the enzyme assay.

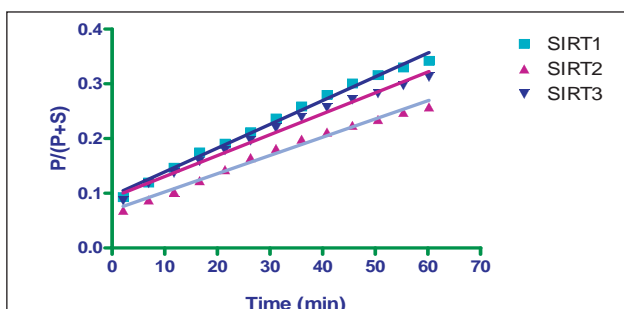


Figure 3. Linearity of the SIRT reaction rates.

Km Determinations

NAD⁺ Km values were determined for each enzyme using real-time kinetics (Figure 4). Initial reaction rates were determined by assembling 70 μ L reactions containing increasing concentrations of NAD⁺ with SIRT1 (1 Unit), SIRT2 (10 Units) and SIRT3 (0.08 Units). The plate was immediately placed on the LabChip 3000 system and samples were introduced onto 4-sipper chip every 5 minutes for 60 min. Substrate and deacetylated product were separated and detected on the chip. Initial rates V (pmol/min) were calculated for each NAD⁺ concentration by finding the slopes of product formed vs time during the first 30 minutes of the reaction. Km values were determined by plotting V (pmol/min) vs NAD⁺ concentration [S] (μ mol/L) and applying non-linear regression analysis using the Michaelis-Menten equation. The NAD⁺ Km values for SIRT1, SIRT2 and SIRT3 were found to be 0.56 mM, 1.3 mM and 3.2 mM, respectively.

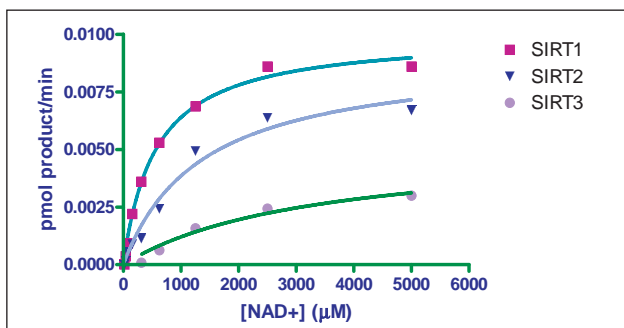


Figure 4. NAD⁺ Km determinations for SIRT1, SIRT2 and SIRT3. The plots show Michaelis-Menten non-linear regression analysis of initial reaction rates vs NAD⁺.

DMSO Tolerance

The effect of DMSO on SIRT activity was determined by running 70 μ L kinase reactions with increasing amounts of added DMSO (Figure 5). 100% DMSO (0.5 μ L to 7 μ L) was added to wells of a microtiter plate, mimicking the addition of compounds dissolved in DMSO. Reactions were then assembled in the wells incubated as described in Methods section.

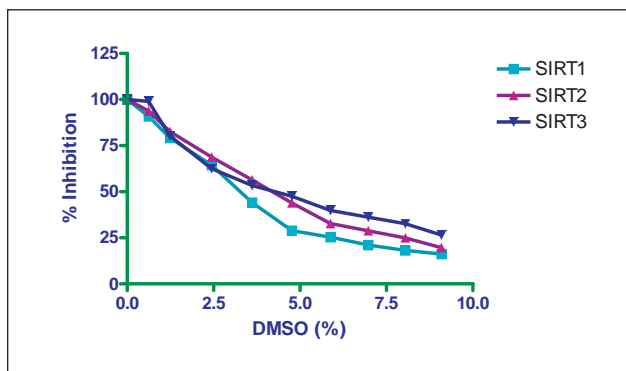


Figure 5. DMSO tolerance. Increasing DMSO concentration has significant effect on the SIRT1, SIRT2 and SIRT3 activities.

Substrate and deacetylated product in the stopped reaction were separated and detected on a 4-sipper chip. As shown in Figure 5, increasing DMSO concentration has a significant effect on the activity of either SIRT. Based on this DMSO profile, it is recommended to include up to 1.5 % DMSO in the SIRT reaction.

Inhibitor IC₅₀ Determinations

Known SIRT1 and SIRT2 inhibitor – Suramin was selected for inhibitor analysis. Reactions were assembled and incubated as described in Methods. Substrate and deacetylated product were separated and detected on a 12-sipper chip. IC₅₀ values were calculated using non-linear regression analysis of the fraction of product formed (P/P+S) vs Log of inhibitor concentration. The inhibition curves for enzymes are shown on Figure 6. As expected from the literature, suramin does not have any inhibitory effect on SIRT3, while it affects activity of SIRT1 and SIRT2 enzymes. The IC₅₀ values for suramin were 2.9 μ M for SIRT1 and 10.5 μ M for SIRT2

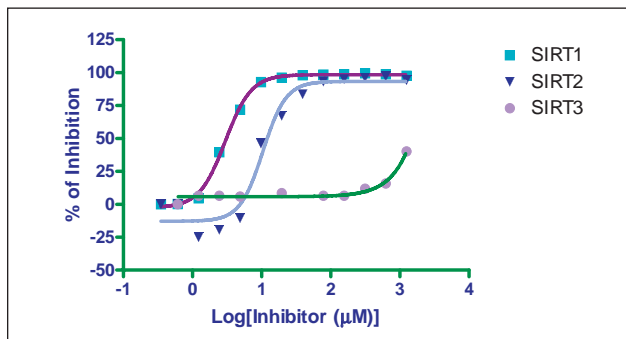


Figure 6. Inhibition curves showing the effects of Suramin on the activities of SIRT1, SIRT2 and SIRT3.

Data stability

For high throughput screening applications, it is necessary to assemble, incubate and terminate enzymatic reaction in microtiter plates during a single run of the assay. The LabChip 3000 system screening system may be programmed to read results from up to 60 plates in a single run. But the quality of the results will depend on the stability of substrate in the terminated reaction. As shown in Figure 7, peptide substrate and deacetylated product show a high degree of stability over 6 hours. The plate remained in the LabChip 3000 system chamber with temperature and humidity maintained at 20° C and 50%, and the reaction wells were sampled repeatedly over the course of 6 hours. The data from the first and last analysis are nearly identical in terms of substrate and product peak integrity, separation and relative intensity.

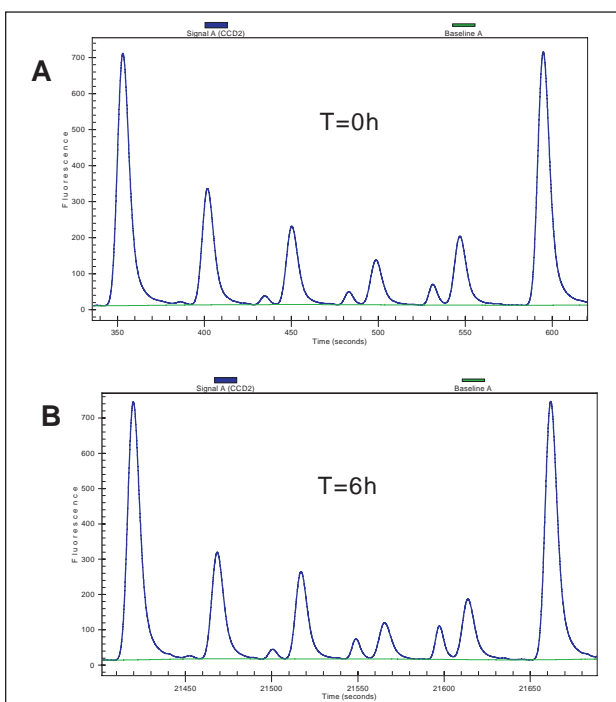


Figure 7. Stability of the substrate and deacetylated product in the terminated reaction mixture. Data collected immediately after reaction termination (A) and collected from the same reaction wells after 6 hours in LabChip 3000 chamber (B).

Summary

A highly sensitive assay for the SIRT class of HDAC's has been developed for use on the Caliper LabChip 3000 platform. This assay directly detects both the acetylated substrate as well as the deacetylated product and has the additional benefits common to all off-chip mobility shift assays - ease of assay development, extremely limited false positives or negatives, and extremely precise data quality.

IV. Materials

ITEM	ITEM NAME	MANUFACTURER	CATALOG #
Microfluidic System Components	LabChip 3000 Drug Discovery System	Caliper Life Sciences	
	Caliper Off-chip Mobility Shift Chip, 4-Sipper with Coating reagent	Caliper Life Sciences	761043-0266R
	Caliper Off-Chip Mobility Shift Chip, 12-Sipper with Coating reagent	Caliper Life Sciences	761037-0372R
Assay Components	SIRT1	Biomol	SE-239
	SIRT2	Biomol	SE-231
	SIRT3	Biomol	SE-270
	FL-TGGK-(Ac)APR-COOH	Intonation	Custom
	HEPES, Free Acid, ULTROL	Calbiochem	391338
	HEPES, Sodium salt, ULTROL	Calbiochem	391333
	Tris	Sigma	T1503
	Potassium Chloride	Sigma	P9333
	Sodium Chloride	Sigma	S3014
	Magnesium Chloride	Sigma	M2670
	Coating Reagent-3	Caliper Life Sciences	760050
	EDTA, disodium salt, 0.5 M	Sigma	E7889
	Suramin	Biomol	G-430
	β -Nicotinamide adenine dinucleotide oxidized form	Sigma	N1511

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LC3000-AP-208 12/06