# Design of Novel Fluorogenic and FRET Substrates for the Detection of Sirtuin Activity

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## Abstract

Sirtuins are a family of enzymes having $NAD^+$-dependent deacetylation activity. Based on their ability for histone deacetylation, sirtuins belong to class III histone deacetylases (HDAC). However, substrates of sirtuins are not limited to histones, but also include various transcription factors and co-regulators. Since sirtuins recognize a wide variety of substrates, they are implicated in many important cellular processes, including genomic stability and DNA repair, apoptosis, adipogenesis, and aging.

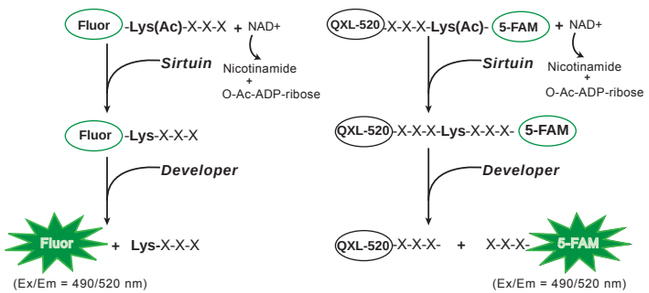
The use of fluorogenic peptide substrates has greatly facilitated assay development for sirtuins, despite results showing fluorophore-dependent increase of enzyme activity observed for sirtuin activation by the anti-aging compound, resveratrol. We developed and screened a novel series of fluorogenic and FRET sirtuin substrates with the resulting signal monitored at excitation/emission = 490/520 nm. Sirtuin 1 (SIRT1) and Sirtuin 2 (SIRT2) peptide fluorogenic substrates were derived from p53 sequences. These substrates release a green fluorophore after incubation with sirtuin and a developer. The SIRT1 and SIRT2 FRET peptide substrates were derived from human FOXO3 and tubulin sequences, respectively. In these FRET peptides, the fluorescence of 5-carboxyfluorescein (5-FAM) is quenched by QXL™ 520 and recovered upon reaction of the peptide with active sirtuin and the developer. The use of green fluorescence provides improved kinetic parameters, higher sensitivity and signal/background ratio in assay. SIRT1 fluorogenic and FRET substrates gave a signal increase of at least 12- and 5-fold, respectively compared to a previously described peptide coupled with a blue fluorophore, AMC (7-amino-4-methylcoumarin). The new substrates were tested with sirtuin inhibitors and the activator, resveratrol. $IC_{50}$ for inhibitors was consistent with previously described values. The results of activator tests varied for different substrates. Both SIRT2 fluorogenic and FRET substrates did not show any signal increase for enzyme after incubation with resveratrol. The same results were obtained for SIRT1 FRET substrate. When resveratrol was tested with a fluorogenic SIRT1 substrate coupled with a green fluorophore, the activation of enzyme was observed only at low concentrations of $NAD^+$. A previously described fluorogenic AMC-based substrate showed an increase of sirtuin activity after incubation with resveratrol at the broad range of $NAD^+$. Although new SIRT substrates were optimized for specific sirtuins (e.g. SIRT1 substrates were deacetylated by SIRT1 at highest degree), we observed cross-reaction between SIRT1 and SIRT2. Deacetylation of both substrates with SIRT3 was negligible. FRET substrates did not show deacetylation after incubation with HeLa nuclear extract (the source of HDACs), whereas fluorogenic substrates were easily deacetylated at the same conditions.

## Materials and Methods

- SensoLyte® Green SIRT1 and SensoLyte® Green SIRT2 Assay Kits
  √ Fluorogenic Green SIRT1 and SIRT2 substrates, Ex/Em=490/520 nm upon cleavage
- SensoLyte® 520 SIRT1 and SensoLyte® 520 SIRT2 Assay Kits
  √ FRET SIRT1 and SIRT2 substrates, Ex/Em=490/520 nm upon cleavage
- Ro-31-8220 and Suramin, sirtuin inhibitors (EMD Chemicals, San Diego, CA)
- Resveratrol (Enzo Life Sciences, Plymouth Meeting, PA)
- Recombinant Human Sirtuin 3 (BPS Bioscience, San Diego, CA)
- Commercial AMC-based fluorogenic SIRT1 and SIRT2 substrates, Ex/Em=350 nm/440 nm upon cleavage (Enzo Life Sciences, Plymouth Meeting, PA)

All incubations were performed at 37°C in 96-well black opaque plates. Fluorescence was measured using FlexStation 384II (Molecular Devices, Sunnyvale, CA)

## Assay Principle




**Scheme 1**. In the first step, substrate is incubated with sirtuin containing sample in presence of $NAD^+$. In the second step, after deacetylation the treatment of substrate with a developer releases the fluorophore.

## Results

**Table 1.** Comparison of SIRT1 substrates.

| | Km for Fluorogenic Substrates | | Km for FRET Substrate | |
|---|---|---|---|---|
| Substrates | Green SIRT1 | Competitor AMC SIRT1 | 520 FRET SIRT1 | Competitor NMA/DNP FRET SIRT1 |
| Peptide with fluorophore | 10 μM | 64 μM | 15 μM | 34 μM |
| $NAD^+$ | 300 μM | 558 μM | 300 μM | 57 μM |

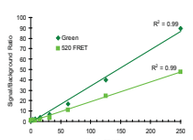



**Figure 1**. **Sensitivity of SensoLyte® Green and 520 FRET SIRT1 Assay Kits**. Fluorescence was measured at 60 min. after incubation of 5 μM peptide substrates and 100 μM of $NAD^+$ with serial dilutions of enzyme, followed by 10 min. incubation with the developer. Sensitivity for both assays at these conditions was 2 ng of enzyme/well or 13 ng/mL.

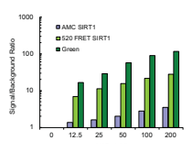



**Figure 2**. **The Green and 520 FRET SIRT1 substrates provide better signal/background ratio than a previously used commercial substrate.** AnaSpec's substrates at a final concentration of 5 μM were incubated with 0.4 μg of recombinant SIRT1 enzyme and the indicated titrations of $NAD^+$, followed by an additional 15 min. incubation with a developer. For the AMC-based commercial substrate, the final concentration was 100 μM, as recommended by the manufacturer.

**Table 2.** Comparison of SIRT2 substrates.

| | Km for Fluorogenic Substrates | | | Km for FRET Substrate |
|---|---|---|---|---|
| Substrates | Green SIRT2 | Competitor 1 AMC SIRT2 | Competitor 2 AMC SIRT2 | 520 FRET SIRT2 |
| Peptide with fluorophore | 9 μM | 186 μM | 51 μM | 4.4 μM |
| $NAD^+$ | 400 μM | 547 μM | 213 μM | 200 μM |

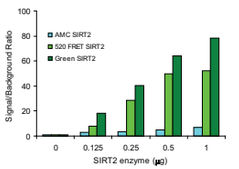



**Figure 3**. **Enzyme titration with SIRT2 substrates.** AMC, Green and FRET SIRT2 substrates were incubated with sirtuin 2 enzyme and signal/background ratio was calculated at the 60 min. time point.

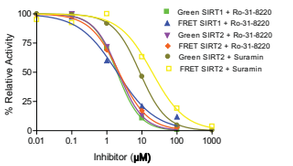



**Figure 4**. **Inhibitor studies.** The calculated $IC_{50}$ for sirtuin inhibitor Ro-31-8220 was ~2 μM for both enzymes using the fluorogenic and FRET assays. SIRT2 was also tested with suramin. The calculated $IC_{50}$ were 8.7 and 19.7 μM for fluorogenic and FRET substrates, respectively.

**Table 3. Substrates specificity.** Signal/background ratio was measured for HeLa nuclear extract (HDACs) and SIRT3 after 1 hour incubation with SIRT1 and SIRT2

| Enzyme \ Substrates | Green SIRT1 | FRET SIRT1 | Green SIRT2 | FRET SIRT2 |
|---|---|---|---|---|
| HeLa, 4 μg | 109 | 1.4 | 27 | 1 |
| SIRT3, 1 μg | 7.5 | 2 | 10 | 5 |

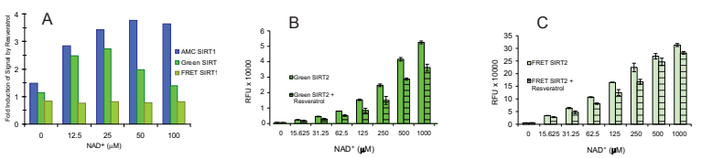



**Figure 5. Effect of resveratrol on sirtuin activity is substrate specific.** AMC, Green and FRET SIRT1 substrates (A) were incubated with 0.4 μg of SIRT1 enzyme in the presence of 100 μM of resveratrol. The effect of resveratrol was also tested for SIRT2 fluorogenic (B) and SIRT2 FRET (C) substrates incubated with 1 μg of SIRT2 enzyme.

## Conclusions

- We have developed a series of sirtuin fluorogenic and FRET substrates. These new substrates generate green fluorophores with longer excitation/emission, resulting in minimized interference from test compounds and cell components.
- Green fluorogenic and FRET substrates can detect few nanogram of SIRT1 or SIRT2 and provide higher signal/background ratio than traditionally used AMC-fluorogenic substrate.
- Choice of substrate can affect results of activation tests. Treatment of sirtuin 1 with resveratrol gives signal increase only in fluorogenic assays with AMC and Green SIRT1 substrates, but not in the FRET assay.