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Highly Sensitive Assays for Sumoylation and SUMO-Dependent Protein-Protein Interactions

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Abstract

Small ubiquitin-like proteins (SUMO) are recently discovered post-translational modifiers that regulate protein functions and intracellular trafficking. In this study, we are describing two chemoluminescence-based assays, one for SUMOylation and another one for SUMO-mediated protein-protein interactions. These assays can be used to characterize the activity and kinetics of the enzymes that catalyze SUMOylation, and in high-throughput screening for inhibitors of SUMOylation and SUMO-dependent protein-protein interactions. These novel assays represent the most sensitive assays for ubiquitin-like systems published to date. Similar strategies can be used to develop assays for other ubiquitin-like modification systems.

SUMOylation plays critical roles in essential cellular functions, such as gene transcription, DNA repair, RNA splicing, viral infection, and intracellular trafficking [1;2;3]. Four SUMO paralogues, SUMO-1, -2, -3 and -4, have been identified that appear to have distinct functions, but their functional differences are not well understood [4;5]. Inhibitors of SUMOylation as well as SUMO-mediated protein-protein interactions will be useful research tools to help dissect the roles of SUMOylation in various cellular pathways, but they have not yet been reported. Moreover, because of the critical roles of SUMO modifications, SUMO-dependent processes are potential targets for developing therapeutics, such as anti-cancer drugs [6;7]. Two SUMO-related assays have been reported to date using fluorescence-resonance-energy-transfer (FRET) with yellow fluorescent protein- and cyan fluorescent protein-tags [8;9]. In this study, we have developed different assays based on chemoluminescence using the AlphaScreen technology. The assays described here are the most sensitive assays for ubiquitin-like systems published to date. Similar strategies can be used to develop assays for other ubiquitin-like modification systems to characterize their enzymatic activities and to discover small molecule inhibitors in both low and high throughput formats.

The SUMO proteins are covalently attached to target proteins through the concerted actions of three enzymes, known generally as activation enzyme (E1), conjugation enzyme (E2) and ligase (E3) [2]. Available data suggest that SUMO modification, like ubiquitination, provides a platform for interactions with other proteins [1;2;10]. We have recently identified a SUMO-

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binding motif (SBM or SIM) that serves as a receptor for the SUMO moiety of SUMO-modified proteins in mediating SUMO-dependent protein-protein interactions [11]. This motif is different from the SUMO-1 modification consensus sequence (Φ KxE) found in SUMO-1 substrate proteins [12], which does not bind to SUMO-1 non-covalently, but binds Ubc9 in the substrate binding site for covalent SUMO modifications. In this study, we have designed two highly sensitive assays using the AlphaScreen technology, one for SUMOylation, and another one for SUMO-mediated protein-protein interactions. These assays require low sample consumption (in the nanogram range) and small assay volumes (25 μ L or less). They are not only suitable for routine analysis, but minimize the cost of high throughput screening.

AlphaScreen is a bead-based technology that was chosen because of its inherent high sensitivity. In this assay, the Donor beads contain a photosensitizer which converts ambient oxygen to an excited form of singlet oxygen, upon illumination at 680 nm. Singlet oxygen diffuses up to 200 nm in solution before it decays. Thus, if a biomolecular interaction brings the Donor beads in close proximity to Acceptor beads, singlet oxygen will activate thioxene derivatives in the Acceptor beads, leading to the emission of light between 520 and 620 nm. In the absence of Acceptor beads in proximity, the singlet oxygen falls to ground state with no light emission. Donor beads can release up to 60,000 singlet oxygen molecules per second resulting in signal amplification. Since signal detection is performed in a time resolved manner and at a lower wavelength than excitation, background interference is very low.

Materials required for the assays were obtained as follows. The E1 enzyme (SAE1/SAE2) was purchased from Boston Biochem (Cambridge, MA). Tris and HEPES are from MP Biomedicals (Solon, OH) and Tween-20 from Pierce (Rockford, IL). $MgCl_2$, DTT and ATP were obtained from Sigma (St-Louis, MO). AlphaScreen Glutathione (GSH) Acceptor and Donor beads, streptavidin Donor beads, and Histidine (Nickel Chelate) detection kit were from PerkinElmer (Waltham, MA). All other protein samples were expressed in *E. coli* and purified by affinity columns using either GSH Sepharose beads or Ni-NTA beads as described previously [13; 14]. The biotinylated-SIM peptide was synthesized and purified at the Peptide/DNA/RNA Synthesis Core facility at the City of Hope, and verified by Mass spectrometry analysis. All assays were performed in triplicates, using white opaque Optiplates-384 (PerkinElmer, Waltham) in a final detection volume of 25 μ L.

The overall strategy for SUMOylation assay is illustrated in Figure 1A. Glutathione S-transferase (GST)-fusion SUMO proteins were used to bind SUMO to the Donor beads linked with GSH. His-tagged substrate RanGAP1 was used to bind to the nickel chelate Acceptor beads. SUMOylation reactions were performed in 50 mM Tris HCl pH 7.4, 10 mM $MgCl_2$, and 0.2 mM DTT. The AlphaScreen detection reagents (beads) were added in the same buffer. To each well, 2.5 μ L of a mix of E1 and E2 was added followed by 2.5 μ L of GST-SUMO-1, 2.5 μ L of His-RanGAP1, and 2.5 μ L ATP (see Fig. 1 for final concentrations). Plates were sealed and incubated at room temperature for one hour. After incubation, 10 μ L of Ni-NTA coated Acceptor beads and 5 μ L of GSH coated Donor beads (20 μ g/mL of final concentration in 25 μ L) were added. The plates were read on an EnVision Multilabel Reader with the AlphaScreen option.

Specificity of the reaction was demonstrated by inspecting the dependence of the reaction on the concentrations of E1 and E2 enzymes (Fig. 1B & 1C). To the wells of a 384-Optiplate plate, the following components were added sequentially: 2.5 μ L of GST-SUMO-1 (0.3 μ M), 2.5 μ L of E1 or E2 serial dilutions, 2.5 μ L of mix of E1 or E2 (15 nM) / RanGap1 (1 μ M), and 2.5 μ L 10 μ M ATP. Plates were covered and incubated for two hours at room temperature. Then the product of the reaction was incubated with 15 μ L of AlphaScreen beads for one more hour while sealed in the dark. For both E1 and E2, signal increased as the enzyme concentrations

increased. However, in order to reduce protein consumption in a high throughput screen, 15 nM of the enzymes was used for subsequent assay optimization.

Specificity of the reaction was also demonstrated by examining the dependence on ATP concentration (Fig. 1D). To the wells of a 384-Optiplate, each component was sequentially added in the following order: 2.5 μ L of GST-SUMO-1 (0.1 μ M), 2.5 μ L of E1 (15 nM), 2.5 μ L of the mixture of E2 and RanGap1 (15 nM and 0.5 μ M, respectively), and 2.5 μ L of ATP serial dilutions. Plates were covered and incubated for two hours at room temperature. The product of the reaction was detected as described above. Signal increased linearly up to 10 μ M ATP, which was used for all subsequent optimization. The decrease in signal at higher concentrations of ATP may reflect saturation of one of the beads by the product.

Specificity of the reaction was further demonstrated by inspecting the dependence of the reaction on the substrate concentrations (Fig. 1E & 1F). To the wells of a 384-Optiplate plate, the following components were sequentially added: 2.5 μ L of GST-SUMO-1 serial dilutions, 2.5 μ L of E1 (15 nM), the 2.5 μ L of E2 (15 nM) and RanGap1 (0.5 μ M) mix, and 2.5 μ L of 10 μ M ATP. Then the plates were covered and incubated for two hours at room temperature. The product of the reaction was detected as described above. Optimal results were obtained using 0.1 μ M of GST-SUMO-1, and this concentration of GST-SUMO-1 was used for further assay optimization (Fig. 1E). Optimal RanGap1 concentration was determined to be around 0.5 μ M (Fig. 1F). At higher concentrations of the substrates (particularly SUMO), signal decreases were observed. This is most likely attributed to the saturation of one of the AlphaScreen beads at high product concentrations.

Another assay was designed for detecting the interaction between SUMO and SIM. The SIM of the protein PIASX was chosen, because it binds all SUMO paralogues with dissociation constants for SUMO-1 and -3 being approximately 9 and 5 μ M, respectively [11;15]. Such affinity is typical among protein interactions in signal transduction. Despite the moderate affinity between SUMO and SIM, AlphaScreen can artificially create conditions that produce high local concentrations of a given molecule. Using nM concentration binding partners, local concentrations in the μ M range can be reached as illustrated in Fig. 2A. Biotin was incorporated into the peptide through solid phase synthesis for binding to the Streptavidin-coated AlphaScreen Donor beads. GST-tagged SUMO proteins bind to the GSH Acceptor beads. The protein-peptide association will bring the Donor and Acceptor beads into close proximity. If a compound inhibits such a complex, the emission signal will be reduced or eliminated.

The assays were performed as follows. All reagents were diluted in a buffer containing 25 mM Hepes pH 7.4, 100 mM NaCl, and 0.1 % Tween-20. To each well, 5 μ L of GST-SUMO-1 or GST-SUMO-3 was added followed by 5 μ L of biotinylated-SIM. The two binding partners were incubated together for one hour at room temperature, and then 5 μ L of Streptavidin-coated Donor beads and 10 μ L of GSH coated Donor beads were added to the wells. Plates were sealed and incubated in the dark at room temperature for one additional hour before signal detection (Fig. 2B and 2C). GST-SUMO-1 was titrated (60 nM to 20 μ M) using fixed concentration of biotin-SIM (200 nM). Sufficient AlphaScreen signal were detected at approximately 6 μ M concentration of GST-SUMO-1 (Fig. 2B). Similarly, assays with various concentrations of GST-SUMO-3 were titrated by increasing concentrations of biotinylated SIM peptide (Fig. 2C). In all experiments, the assay volumes were 25 μ L. Sufficient signal was detected with 20 nM peptide.

In summary, we have provided very reproducible and highly sensitive assays using the AlphaScreen technology. Minimal protein amounts are needed (in the nanogram range). Thus, these assays are not only useful for routine analysis, but also well suited for minimal-cost high

throughput screening. Similar strategies can be used to develop assays for other SUMO substrates and other ubiquitin-like modification systems.

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Figure 1. Detection of SUMOylation using the AlphaScreen technology

(A) Scheme of the AlphaScreen assay that detects SUMOylation. GSH coated Donor beads and Ni²⁺ coated Acceptor beads are added to the reaction in order to capture the SUMOylated substrate. When beads are brought into proximity by the SUMOylation reaction, irradiation of the Donor beads at 680 nm will result in the transfer of singlet oxygen to the Acceptor beads that will subsequently emit light at 520–620 nm.

(B–F) Assay development. All assays were performed in white opaque Optiplates-384 in a volume of 25 μL using a buffer containing 50 mM Tris HCl pH 7.4, 10 mM MgCl₂, and 0.2 mM DTT. To each well, 2.5 μL of a mix of E1 and E2 was added followed by 2.5 μL of GST-SUMO-1, 2.5 μL of His-RanGap1, and 2.5 μL ATP. Plates were sealed and incubated at room

temperature for one hour. Then, 10 μL of Ni^{2+} coated Acceptor beads and 5 μL of GSH coated Donor beads (20 $\mu\text{g/mL}$ final concentrations in 25 μL). Plates were sealed and incubated in the dark at room temperature for one additional hour. The plates were read on an EnVision Multilabel Reader with the AlphaScreen option.

Figure 2. Detection of the interaction between the SUMO binding motif (SIM) and SUMO
 (A) Scheme of the AlphaScreen protein-protein interaction assay where the interaction between biotinylated-SIM and His-tagged SUMO is detected following the addition of streptavidin Donor beads and GSH Acceptor beads.
 (B–C) Assay development. All reagents were diluted in a buffer containing 25 mM Hepes pH 7.4, 100 mM NaCl, and 0.1 % Tween-20. To each well, 5 μ L of GST-SUMO-1 or GSTSUMO-3 was added followed by 5 μ L of biotinylated-SIM. The two binding partners were incubated together for one hour at room temperature, and then 5 μ L of GSH Acceptor beads and 10 μ L of streptavidin coated Donor beads were added to the wells. Plates were sealed and incubated in the dark at room temperature for an additional hour before reading.