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AlphaScreen™ to Monitor Protein Ubiquitination on Proteome Scale

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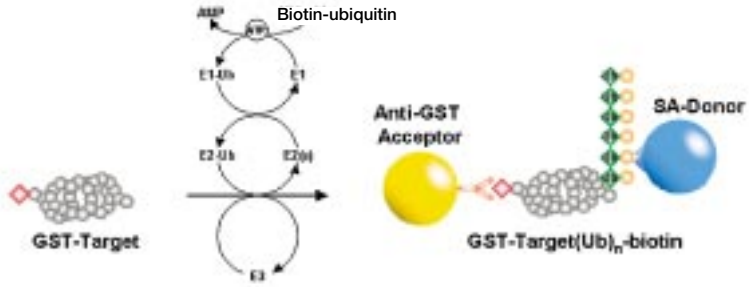
1 Abstract

The Ubiquitin Proteasome Pathway (UPP) is the principal mechanism for protein catabolism. The UPP has been shown to have significant involvement in the modulation of key cellular processes including: DNA repair, cell cycle control, oncogenesis, cellular differentiation, etc. Proteins degraded by the UPP are covalently tagged with the 8.6 kDa protein ubiquitin (Ub). Conjugation of Ub involves three sequential enzyme reactions that binds the C-terminal Gly of Ub on the ε-amine group of a Lys residue found within the substrate protein sequence. A polyUb chain is then elongated on the protein backbone through the ligation of additional monomers of Ub in successive steps. Proteins modified by polyUb chains are recognized by the proteasome and undergo simultaneous proteolytic degradation and recycling of Ub monomers by deubiquitinating enzymes.

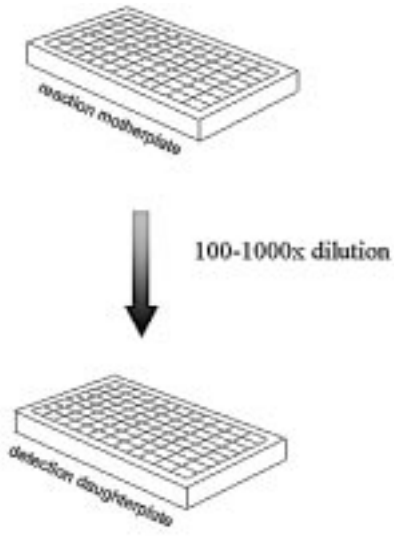
Using AlphaScreen, we have developed a highly sensitive assay to detect the ubiquitination of GST-fusion proteins. Compared to most common detection approaches involving Western-Blots, AlphaScreen detects 100-1000x lower concentrations of ubiquitinated end-product typically. Two strategies are possible: 1) transfer assay and 2) all-in-one-well assay. We are presenting the results obtained with the transfer assay. The assay is ATP and RSP5 dependent when tested with GST-tagged C-terminal domain of Rpb1 (large subunit of the yeast RNA polymerase II) used a positive control. The benefits of this new AlphaScreen assay allow for studying protein ubiquitination on a proteome-scale.

2 Assay description



GST fusion targets are ubiquitinated by the ubiquitination machinery involving RSP5 as the terminal ubiquitin ligase enzyme (E3). Biotinylated-ubiquitin is used as a substrate. Ubiquitinated GST-target produced is simultaneously captured by Anti-GST Acceptor and Streptavidin Donor beads. This proximity of Acceptor and Donor beads induced by the production of Ubiquitinated GST-target allows for the generation of the AlphaScreen signal.

3 Typical Assay Protocol



Reaction

- Add ubiquitination machinery
  - E1; 0.15 µM final
  - E2; 1 µM final
  - E3 (RSP5); 3 µM final
  - biotin-ubiquitin; 3 µM final
- Add GST-target; 3 µM final
- Incubate 3 hours at 23°C

Detection

- Dilute samples (100-1000x fold)
- Transfer to detection daughter plates
- Add Acceptor and Donor beads (20 µg/ml final each)
- Incubate 1 hour at RT
- Read on AlphaQuest/Fusion α

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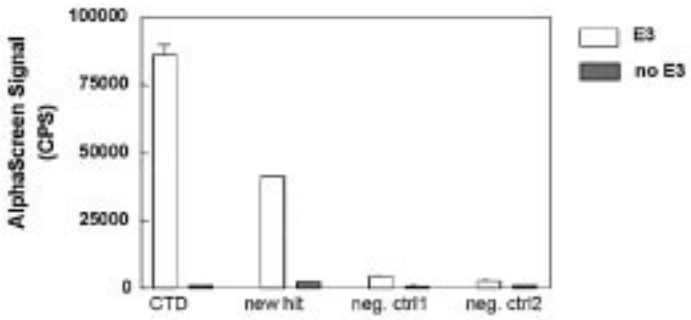
Assay validation: Specificity of E3 (RSP5)



The assay was validated by assessing the specificity of the terminal ubiquitin-ligase enzyme RSP5 (E3). Ubiquitination of the positive control CTD was abolished in the absence of E3. A similar observation was made with the new RSP5 target protein found during the present study (new hit). Proteins known not to be targets of E3 (negative control 1, negative control 2) were not significantly affected by the presence or absence of E3.

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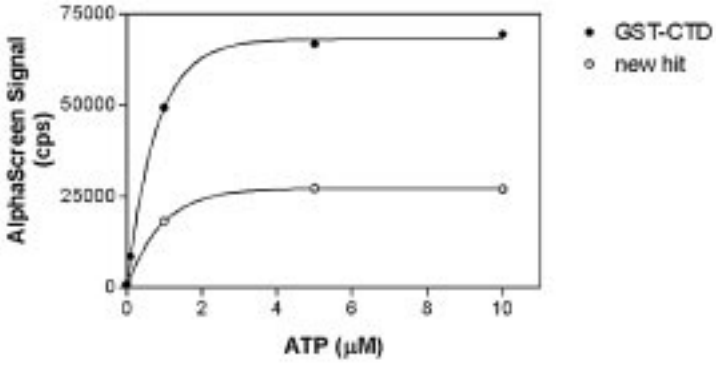
ATP Titration



Ubiquitination of both GST-CTD and the "new hit" was ATP dependent. For both proteins, reaction rate was maximum at approximately 3 µM ATP. K_{cat} values for ATP were similar with both substrates: 0.6 µM.

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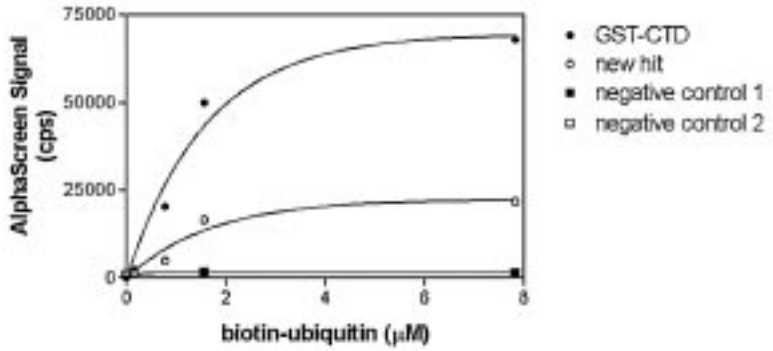
Biotin-ubiquitin Titration



Ubiquitination of GST-CTD and that of "new hit" was maximum at approximately 5 μM biotin-ubiquitin. Apparent biotin-ubiquitin Km value was similar with both GST-CTD and "new hit": 1.2 μM. Both negative control proteins used in this study were not significantly ubiquitinated.

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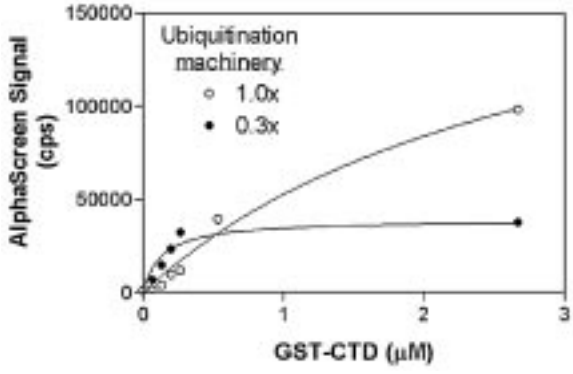
CTD Titration



The magnitude of GST-CTD ubiquitination was proportional to the concentration of ubiquitination machinery used during this study. Less than 1 mM GST-CTD was enough to reach a maximum response when 0.3x machinery was used. Increasing the machinery 3 fold extended the range of GST-CTD concentrations over which the reaction was linear.

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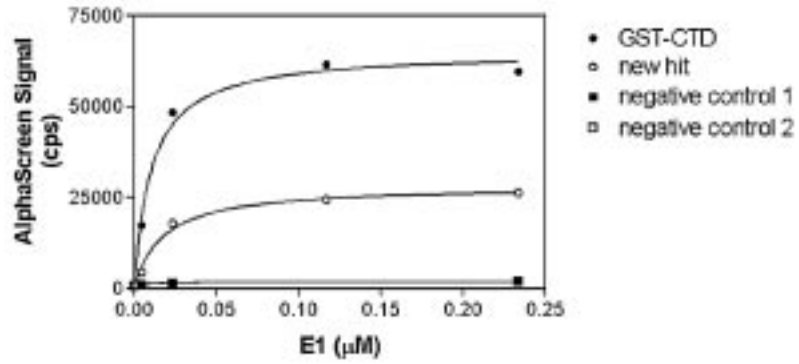
E1 Titration



Ubiquitination of GST-CTD as well as that of "new hit" was maximum when 0.1 μM E1 was used. Both negative control proteins used in this study were not significantly affected by levels of E1 used up to 0.23 μM.

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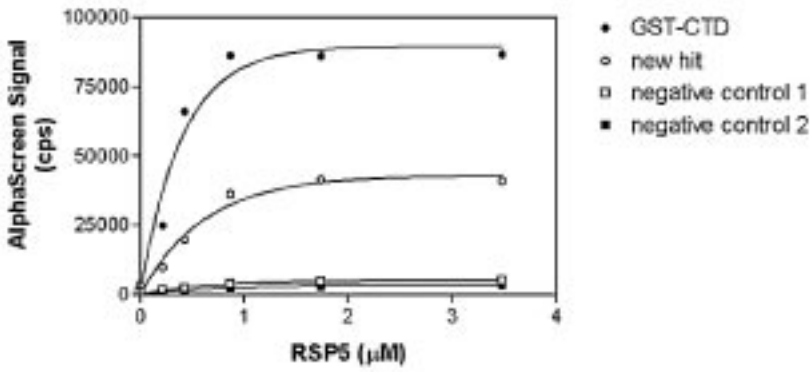
RSP5 (E3) Titration



Ubiquitination of CTD as well as "new hit" was maximum when 1 μM RSP5 (E3) was used. Both negative control proteins used in this study were not significantly affected by RSP5 used up to 3.5 μM.

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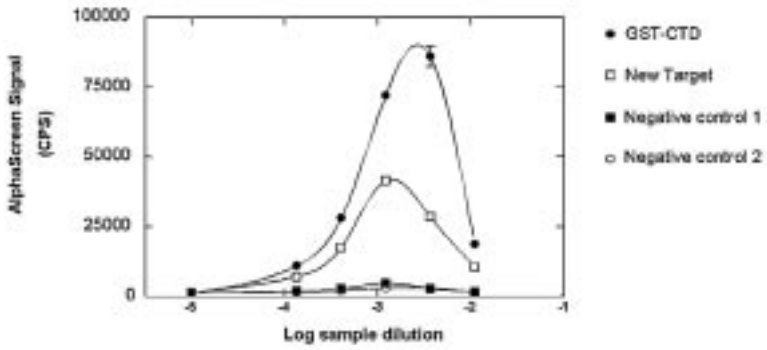
Sensitivity of the AlphaScreen Assay



Samples used to perform Western Blotting were diluted from 100 to 10,000 fold in detection buffer (HEPES 25 mM, 100 mM NaCl, 0.01% Tween-20). Detection was optimal between 300-1000x dilution. Below 300x dilution, a signal decrease is observed since samples are too concentrated and saturate both the Acceptor and Donor beads simultaneously (hooking effect).

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Conclusion

- A very sensitive assay to detect protein ubiquitination was developed using AlphaScreen.
- Compared to traditional Western Blotting, the AlphaScreen assay can detect up to 1000 fold less concentrated ubiquitinated proteins.
- Ubiquitination reaction was shown to be specific for the terminal ubiquitin ligase enzyme RSP5 (E3).
- Ubiquitination was ATP dependent with an apparent Kcat of 0.6 µM.
- Magnitude of ubiquitination was proportional to the levels of E1, E3 and biotin-ubiquitin.
- Apparent Km value of biotin-ubiquitin was 1.2 µM.
- GST-CTD produced a signal twice as strong as that obtained with the “new hit” protein. Lower levels of “new hit” ubiquitination compared to poly-ubiquitinated CTD may be responsible for the difference observed. This hypothesis is currently investigated.
- The all-in-one well assay is currently being developed.
- Because of its sensitivity and its compatibility with automation, AlphaScreen will allow for detecting ubiquitination of GST fusion targets on a proteome scale.

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