

# Development of an AlphaScreen homogenous assay to monitor protein ubiquitination

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# 1

## Summary

The Ubiquitin Proteasome Pathway (UPP) plays a pivotal role in protein catabolism. The UPP degradation pathway is initiated by the addition of polyubiquitin tails to target proteins. Ubiquitin is a small molecule of approximately 8.6 KDa that is conjugated to proteins by the sequential activity of three enzymes (E1, E2, and E3). Ubiquitin is attached to lysine residues of the target proteins as a polyubiquitin chain. Recognition of these ubiquitin tails by the proteasome will result in the proteolytic degradation of these tagged proteins.

Ubiquitination has been shown to have significant modulation effect of key cellular processes such as DNA repair, cell cycle control, oncogenesis, and cellular differentiation (Bendjennat *et al.*, 2003; Bashir T. *et al.*, 2003). In cancer treatment, the recent FDA approval of Bortezomib (Velcade, Millenium), a 26S proteasome inhibitor, highlights the biomedical importance of this pathway.

Using AlphaScreen™, we have developed a sensitive in vitro homogenous assay to monitor the ubiquitination of recombinant GST-fusion proteins. Compared to the most commonly used approach to detect this post-translational modification (western blot), the AlphaScreen assay is quantitative, fast and easy to perform, and requires very limited reagents. Moreover, the assay has low intra and inter assay variability and a Z' value of 0.6 suggesting that the assay should easily integrate to a high throughput screening protocol.

# 2

## Materials and methods

### Assay description

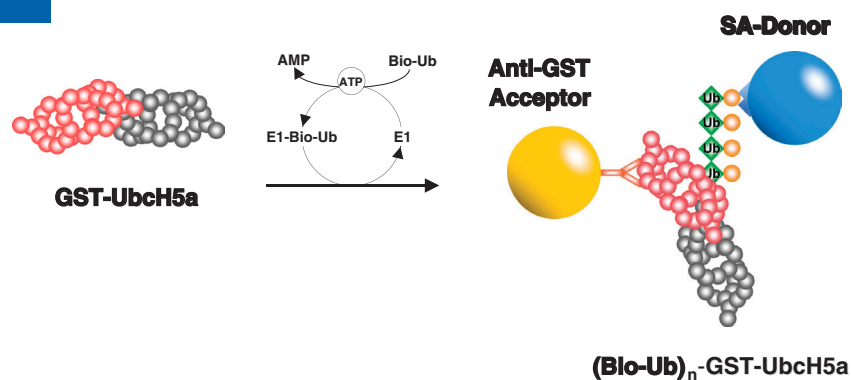


Fig. 1. In this model, the GST moiety of a GST-UbcH5a fusion protein is ubiquitinated using biotin-Ubiquitin (bio-Ub). Following ubiquitin activation by E1, in the presence of ATP, bio-Ub is transferred to UbcH5a. In this reaction, UbcH5a acts as the carrier to transfer the bio-Ub to its tagged GST moiety. The protein which becomes biotinylated and ubiquitinated is then captured by anti-GST Acceptor and streptavidin Donor beads resulting in signal generation. No signal will be generated in the absence of ubiquitination.

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## Materials and methods

### Materials

Product name	Supplier	Cat #	Lot #
Rabbit E1	Boston Biochem	E-302	1200044
GST-UbcH5a	Boston Biochem	E2-615	799HA
AlphaScreen GST detection kit	PerkinElmer	6760603C	4D1
Biotin-Ubiquitin	Boston Biochem	U-560	199873

### Assay protocol

The assays were performed in white 384 microtiter plates in a final volume of 25  $\mu$ l.

1. 7.5  $\mu$ l of ATP /rabbit E1 mix
2. The mix is incubated for 30 min at 23°C
3. 7.5  $\mu$ l bio-Ub / GST-UbcH5a mix is then added to the ATP/E1 mix
4. Reaction is incubated for 1 h at 23°C
5. 10  $\mu$ l anti-GST Acceptor / Streptavidin Donor beads mix (20  $\mu$ g/ml final concentration) is added
6. Reaction is incubated for 4h at 23°C
7. The plate is read using the AlphaQuest® instrument

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## Results of assay development

### Rabbit E1 titration

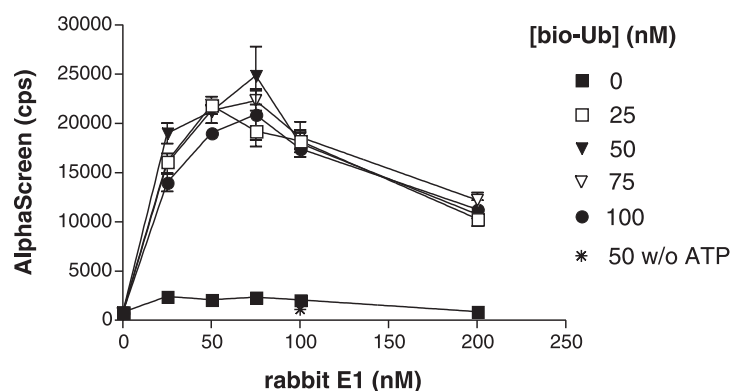


Fig. 2. Rabbit E1 was titrated in parallel with bio-Ub using 3.3 mM of ATP and 100 nM of GST-UbcH5a as the ubiquitinated substrate. Plate was read using the AlphaQuest after 4h of incubation at room temperature. We observed that maximum signal was generated using 50 to 75 nM of rabbit E1 in an ATP-dependent fashion.

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## Results of assay development

## GST-UbcH5a titration

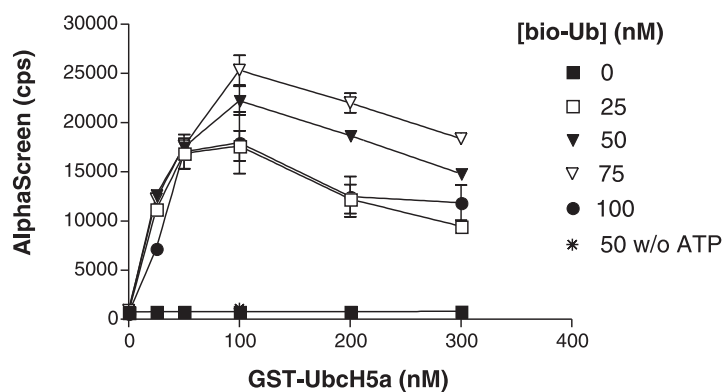


Fig. 3. GST-UbcH5a was titrated in parallel with bio-Ub using 3.3 mM of ATP and 100 nM of rabbit E1 enzyme. Plate was read using the AlphaQuest after 4 h of incubation at room temperature. The results demonstrate that for all different bio-Ub concentrations tested, maximum signal was reached at 100 nM of GST-UbcH5a, in an ATP-dependent fashion.

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## Results of assay development

## ATP titration

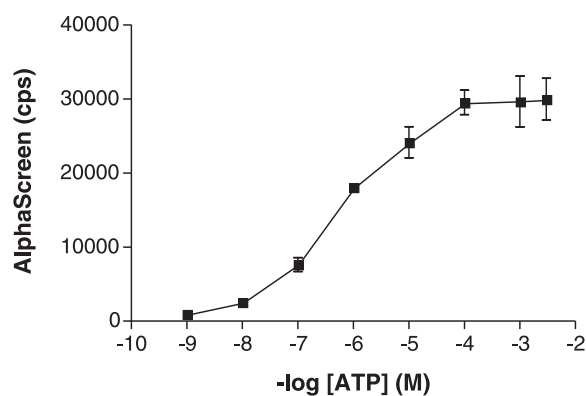


Fig. 4. Increasing concentrations of ATP were added to the ubiquitination reaction performed using 100 nM of rabbit E1, 100 nM of GST-UbcH5a, and 50 nM of bio-Ub. Saturation was observed at around 300  $\mu$ M of ATP. No signal was detected in the absence of ATP.

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## Results of assay development

### Time course for the detection of GST-Ubch5a ubiquitination

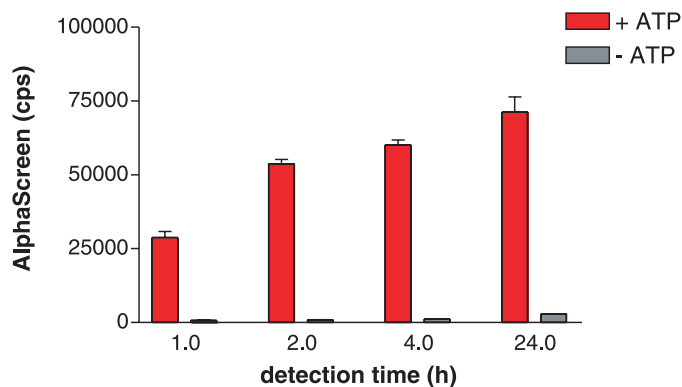


Fig. 5. Time course of the assay was performed by incubating the ubiquitination reaction for 1 h followed by the addition of the AlphaScreen beads (detection reaction). Reactions were then led to proceed for different periods of time. We observed that the signal doubled between 1 and 2 h followed by a slow increase up to 24 h (overnight).

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## Results of assay development

### Titration of biotin-Ubiquitin

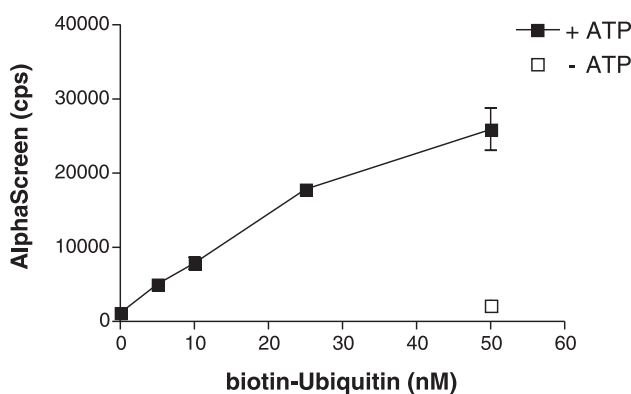


Fig. 6. Bio-Ub titration was performed using 50 nM of rabbit E1, 100 nM of GST-Ubch5a, and 3.3 mM of ATP. Detection using the AlphaQuest was done after 4 h incubation at room temperature. The sensitivity of the assay allowed the detection of protein ubiquitination using very low concentrations of bio-Ub. At 25 nM of bio-Ub, signal was almost 20 000 cps with a signal to background (-ATP) of 14.

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## Results of assay development

### Variations in biotin-Ubiquitin requirement (comparison with a different ubiquitination machinery)

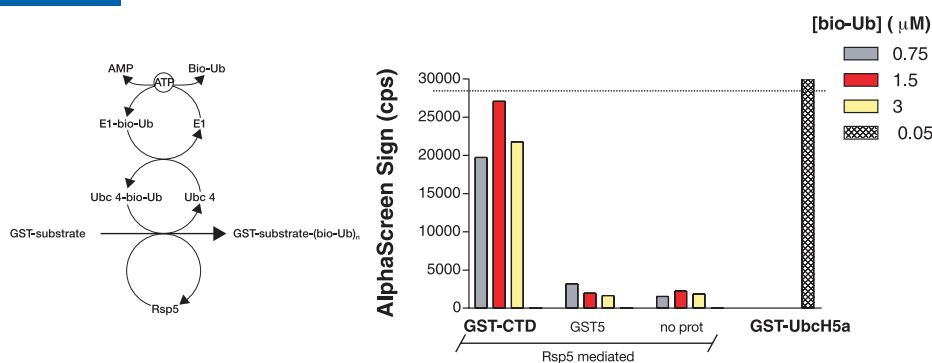


Fig. 7. Titration of bio-Ub was performed using a different ubiquitination machinery composed of yeast E1, Ubc 1 and 4 (mix), and the Rsp5 ligase. Enzymes were incubated with different GST-substrates at different concentrations of bio-Ub. We observed that this ubiquitination system required around 30-fold more bio-Ub (300 ng/well) than the rabbit E1/GST-UbcH5a system (10 ng/well). No signal was generated when using a protein known to not be ubiquitinated by Rsp5 (GST5) and when no substrate was added to the reaction (no prot) (Kus *et al.*, 2004).

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## Results of assay development

### Inter assay variability (n=3)

[bio-Ub (nM)]	average +ATP	average -ATP	STDEV		cv (%)	
			+ATP	-ATP	+ATP	-ATP
0	719	-	26	-	3.6	-
25	22748	-	4558	-	20.0	-
50	38756	-	2593	-	6.7	-
75	50977	-	3290	-	6.5	-
100	51863	-	8615	-	16.6	-
150	54216	1802	3377	306.1	6.2	17.0

Table II. Inter assay variability was assessed by performing three-independent titrations of bio-Ub (by the same operator) using 50 nM of rabbit E1, 100 nM of GST-UbcH5a, and 3.3 mM of ATP. The reactions were read after 4 h of incubation at room temperature. We observed an inter assay variability under 10% for most of the concentrations.

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## Results of assay development

### Performance of the assay in a screening context (n=24)

experiments		average (cps)	S:B	Intra assay (%)	z'
n=1	+ATP	31529	35	14	0.63
	-ATP	902		25	
n=2	+ATP	26672	29	13	0.59
	-ATP	928		11	

Table III. Intra plate variability was performed using 50 nM rabbit E1, 100 nM, GST-UbcH5a, 50 nM of bio-Ub, and 3.3 mM of ATP. Two-independent experiments were performed which included 24 data points / plate in the presence of ATP compared to the same amount of points in the absence of ATP. Results demonstrate that using manual dispensing, Z' value of 0.6 could be generated with these reagents, with intra plate variability around 15% and signal to background around 30.

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## Conclusion

- ▶ An homogenous and sensitive assay was developed to monitor protein ubiquitination using the AlphaScreen technology.
- ▶ In the course of assay development, we demonstrated the specificity of the detection by showing the dose-dependence of rabbit E1, GST-UbcH5a, ATP, and ubiquitin for signal generation.
- ▶ Around 300 ng/well of enzymes and as low as 10 ng/well of bio-Ub derivative were required to generate around 30 000 cps with signal to background (in absence of ATP) between 30 and 40. Kus *et al.*, reported that AlphaScreen was 270-fold more sensitive than western blot to monitor Rsp5 ligase mediated ubiquitination (manuscript in preparation).
- ▶ Since Rsp5 mediated ubiquitination of GST-tagged substrates required a larger amount of bio-Ub than the assay involving UbcH5a, we recommend that determination of the optimal bio-Ub concentration be an integral part of new assay development.
- ▶ The inter assay variability observed (10% and less) demonstrates the reproducibility of the assay. Generation of Z' values above 0.5 suggest that the assay is suitable for transfer to HTS.
- ▶ Because of its sensitivity and its compatibility to automation, the AlphaScreen Ubiquitination detection assay will allow for rapid identification of new ubiquitin ligases and novel inhibitors of well characterized ubiquitination pathways.

## **References**

Bashir, T., and Pagano, M. 2003. Aberrant ubiquitin-mediated proteolysis of cell cycle regulatory proteins and oncogenesis. *Adv Cancer Res.* **88**:101-144.

Bendjennat, M., *et al.* 2003. UV irradiation triggers ubiquitin-dependent degradation of p21(WAF1) to promote DNA repair. *Cell.* **114**(5): 599-610.

Kus, B., *et al.* 2004. A novel assay for high-throughput detection of in vitro ubiquitination using AlphaScreen Technology. (in preparation).

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