Development of an AlphaScreen Homogenous Assay to Monitor Protein Ubiquitination.

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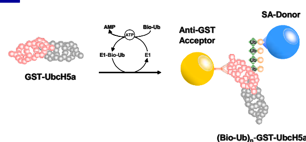
1 Introduction

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 high throughput screening protocol.

2 Assay format



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.

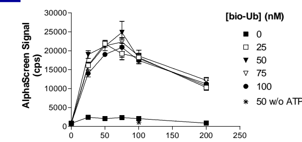
3 Materials and Methods

Product name	Supplier	Cat #	Lot #
Rabbit E1	Boston Biochem	E-302	1200044
GST-UbcH5a	Boston Biochem	E2-615	790HA
AlphaScreen GST detection kit	PerkinElmer	6760603C	4D1
Biotin-Ubiquitin	Boston Biochem	U-560	199873

Reagents were added as follows in white 384 microtiter plates (final volume of 25 µl):

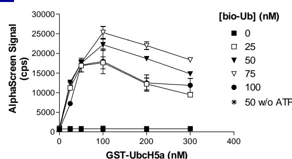
1. 7.5 µl of ATP /rabbit E1 mix
2. The mix is incubated for 30 min at 23ºC
2. 7.5 µl bio-Ub / GST-UbcH5a mix is then added to the ATP/E1 mix
3. Reaction is incubated for 1 h at 23ºC
4. 10 µl anti-GST Acceptor /Streptavidin Donor beads mix (20 µg/ml final concentration) is added
5. Reaction is incubated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

4 Rabbit E1 Titration



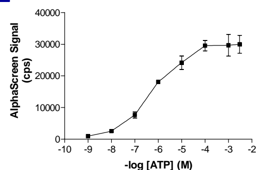
Rabbit E1 was titrated in parallel with bio-Ub using 3.3 mM of ATP and 100 nM of GST-UbcH5a as the ubiquitinated substrate. Plate was read using the AlphaQuest after 4h of incubation at room temperature. We observed that maximum signal was generated using 50 to 75 nM of rabbit E1 in an ATP-dependent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

5 GST-UbcH5a titration



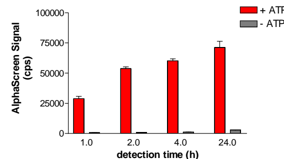
GST-UbcH5a was titrated in parallel with bio-Ub using 3.3 mM of ATP and 100 nM of rabbit E1 enzyme. Plate was read using the AlphaQuest after 4 h of incubation at room temperature. The results demonstrate that for all different bio-Ub concentrations tested, maximum signal was reached at 100 nM of GST-UbcH5a,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

6 ATP titration



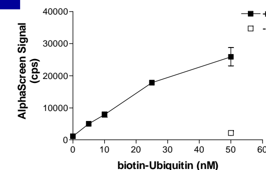
Increasing concentrations of ATP were added to the ubiquitination reaction performed using 100 nM of rabbit E1, 100 nM of GST-UbcH5a, and 50 nM of bio-Ub. Saturation was observed at around 300 µM of ATP. No signal was detected in the absence of ATP.

7 Time Course for the Detection of GST-UbcH5a Ubiquitination



Time course of product detection was performed by incubating the reaction for 1h 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. Maximum signal detection was nearly achieved after 4 hours.

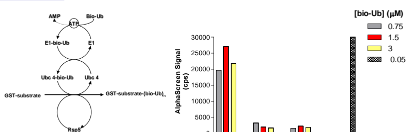
8 Titration of biotin-Ubiquitin



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.

9 Biotin-Ubiquitin requirement

(comparisons of different ubiquitination machineries)



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 concentration 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).

10 Inter-assay reproducibility (n=3)

[bio-Ub] (nM)	average +ATP	average -ATP	STDEV +ATP	STDEV -ATP	cv (%) +ATP	cv (%) -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

Inter-assay reproducibility 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.

11 Assay Automation

Performance in 25 ul

Experiments		Average (cps)	S:B	Intra assay CV (%)	Z'
N=1	+ATP	30 277	15	11	0.64
	-ATP	2018		10	
N=2	+ATP	33757	9	13	0.51
	-ATP	3667		11	
N=3	+ATP	27783	10	11	0.58
	-ATP	2306		15	

Performance in 10 ul

Experiments		Average (cps)	S:B	Intra assay CV (%)	Z'
N=1	+ATP	22497	12	13	0.54
	-ATP	1801		9	
N=2	+ATP	25920	7	11	0.52
	-ATP	3887		16	
N=3	+ATP	23521	7	7	0.69
	-ATP	3311		15	

The assay was performed in ProxiPlates using 75 nM of rabbit E1, 100 nM of GST-UbcH5a, and 3.3 mM of ATP. Detection using the **EnVision ALpha** was done following 4 h incubation at room temperature. Dispensing was performed using a the **PlateTrack.** Each plate was loaded with N=24 for each conditions.

12 Conclusion

- An homogenous and sensitive assay was developed to monitor protein ubiquitination using the AlphaScreen technology.
- Assay specificity was demonstrated by the dose-dependence of rabbit E1, GST-UbcH5a, ATP, and ubiquitin for signal generation.
- As low as 300 ng/well of enzymes and 10 ng/well of bio-Ub substrate were required to generate around 30 000 cps with signal to background (S:B) varying between 30 and 40. Kus *et al.*, reported that AlphaScreen was 270-fold more sensitive than western blot to monitor Rsp5 ligase mediated ubiquitination (unpublished data).
- We recommend that determination of the optimal bio-Ub concentration be an integral part of new assay development as demonstrate by the different assay tested.
- 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 should easily transfer to HTS.
- Because of its sensitivity and its compatibility with automation, the AlphaScreen Ubiquitination detection assay will allow for rapid identification of new ubiquitin ligases and novel inhibitors of well characterized ubiquitination pathways.

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