CLARIOstar® determines activity of a moss-produced human acid alpha-glucosidase (GAA) in a fluorescence-based assay

Birgit Berg[1], Anita Fischer[1], Fabian Hässler[1], Paulina Dabrowska-Schlepp[1], Franka Maurer[2] and Stefanie Tintelnot[2]
[1]Greenovation Biotech GmbH, Germany, [2]BMG LABTECH GmbH, Ortenberg, Germany

- **Enzymatic activity of moss-GAA - recombinantly expressed human alpha-glucosidase in moss *P.patens***
- **Microplate-based assay enables comparison to marketed alpha-glucosidase**
- **CLARIOstar® supports assay optimization and quick enzyme quantification in samples from moss cultivation**

Introduction

The lysosomal enzyme acid alpha-glucosidase (GAA, Uniprot: P10253) is a hydrolase responsible for degradation of lysosomal glycogen to glucose. Genetically inherited deficiency of the GAA is known as Pompe disease. Patients suffering from this disorder accumulate glycogen in various body tissues – especially in skeletal and respiratory muscles. Currently, Myozyme® and Lumizyme® (Sanofi Genzyme) - recombinant forms of human alfa glucosidase produced in Chinese Hamster Ovary (CHO) cells are the only approved treatments for Pompe disease.

Moss-GAA is an alternative recombinant version of human GAA produced in moss Physcomitrella patens. This proprietary expression system profits from advantages, such as: eliminated risk of contamination by zoonotic pathogens and easy manipulation of the glycosylation pathway. Additionally, moss-made proteins exhibit highly homogenous glycosylation profile, which can be beneficial for targeted uptake of lysosomal enzymes, such as GAA.

The GAA producing moss strains are cultured in illuminated bioreactors and secrete moss-GAA into the cultivation supernatant. To measure the activity and quantify the recombinantly produced enzyme in culture samples, we established a known fluorescence-based activity assay in a 96-well plate format, optimized its performance and conducted assay qualification and initial validation.

Assay principle

Fluorescence-based enzyme activity assay using 4-methylumbelliferyl-α-D-glucopyranosid has been extensively used for the measurements of GAA concentration and activity in different types of samples. In this assay GAA hydrolyses the substrate to release the 4-methylumbelliferone that can be measured fluorometrically. We used CHO-GAA-Myozyme® as a standard for assay development, optimization and its qualification. Subsequently the developed assay was adapted (examination of possible matrix effects) for use with culture media samples originating from the moss cultivation.

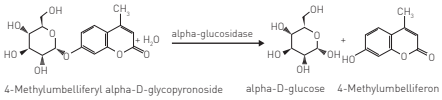

Fig. 1: Assay principle for the measurement of enzymatic activity of GAA

Materials & Methods

- CLARIOstar® microplate reader (BMG LABTECH)
- 4-methylumbelliferyl α D-glucopyranoside (Roth)
- CHO-GAA–Myozyme® (Sanofi Genzyme)
- Bovine Serum Albumin (Sigma)
- Black flat-bottomed 96-well microplates (Roth)
- Shaker Titramax 1000 (Heidolph)
- Incubator (Binder)

Experimental Procedure

20 µL of GAA containing sample was incubated with 80 µL assay buffer (56 mM citric acid, 88 mM Na2HPO4, 0,4% BSA, pH 4.4) containing 0.25 mM 4-MU α -D-glucopyranoside in a black 96-well microplate. The plate was agitated for 10 sec. at 900 rpm, covered with an adhesive microplate seal and incubated at 37°C protected from light for 60 min. The reaction was stopped by adding 200 µL of stop solution (0.1 M Glycine, 0.1 M NaOH) and fluorescence was measured as outlined below.

Instrument settings

Optic settings	Fluorescence intensity, endpoint	
	Monochromator	Excitation 360-20 Dichroic: auto 402,5 Emission 450-30
General settings	Number of flashes	40 per well
	Settling time	0.2 s

Results & Discussion

1. Linearity

Calibration curve fitting is a critical component of assay performance. We assessed linear regression and 4PL function for quality of the curve fit by comparison of recovery rates for known amounts of CHO-GAA within the range 39,06 – 4000 ng/mL. Significantly better fit of the calibration curve, based on the visual assessment as well as better recovery rates between 100% – 120% throughout the whole working range have been achieved with 4-PL fit, which was chosen as the assay's calibration function.

Rev. 09/2019
Keywords: 4-Methylumbelliferone; Moss; Glucosidase, Enzyme activity

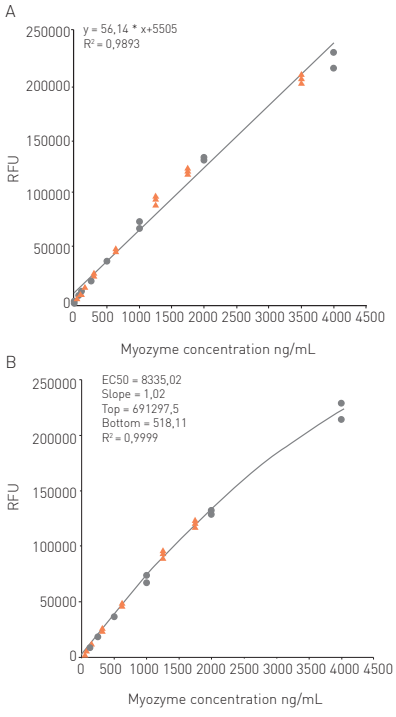

Fig. 2: Assessment of the calibration curve fit: A) linear regression; B) 4-PL. Red markers represent spike recovery values (n=4). Black markers represent data used for generation of the calibration curve (n=2).

2. Sensitivity (LOQ)

Limit of quantitation (LOQ) has been assessed based on the CV% of the CHO-GAA spike throughout the concentration range. The CV% increases above the 10% between the 31 and 39 ng/mL. This result corresponds well to the value based on customary signal -to-noise ratio 1:10 (interpolated from the mean absorbance of the blank + 10xSD of the blank), which was calculated to 36 ng/mL. The LOQ of the assay was set to 36 ng/mL.

3. Precision

Intra-assay precision was assessed by analysing three CHO-GAA samples in respective dilutions (2 dilutions per sample, each dilution in triplicate) covering the relevant working range. Intra-assay precision of %CV ⩽ 5% was found. For the estimation of inter-assay precision corresponding samples were measured in following experiment, performed on a different day, by another operator. Inter-assay precision of %CV ⩽ 8% was determined.

Table 1: Precision of GAA assay

Intra-assay precision				
CHO-GAA [ng/mL]	Dil. factor	Mean	SD	CV%
5000	10	4734,79	175,54	4%
	5			
2500	10	2354,01	31,32	1%
	5			
1250	10	1267,94	59,38	5%
	5			
Inter-assay precision				
CHO-GAA [ng/mL]	Dil. factor	Mean	SD	CV%
5000	10	5045,29	359,80	7%
	5			
2500	10	2501,84	157,43	6%
	5			
1250	10	1362,98	1135,51	8%
	5			

4. Specific enzyme activity

The described assay was subsequently used to measure specific enzyme activity of different GAA preparations. For this an appropriate standard curve with 4-methylumbelliferone has been included in the microplate layout. The specific activity of recombinant GAA versions: CHO-GAA and moss-GAA has been measured, showing comparable values for both enzymes.

Table 2: Comparison of specific enzyme activity between the marketed version of GAA and the moss product (n=4)

	Specific activity (µmol/mg/min)		
rhGAA	Mean	SD	CV%
CHO-GAA	9,9	1,5	15,1
Moss-GAA	9,8	1,4	13,9

Conclusion

This application note describes the adaptation, optimization and qualification of a fluorometric enzyme activity assay for acid alpha-glucosidase in 96 well plate. Microplate-based format enables fast and simultaneous quantification of moss-GAA concentrations in multiple in-process samples. Analyzed assay parameters: linearity, sensitivity and precision demonstrate satisfactory ranges for the intended assay use.

The filter-based microplate reader CLARIOstar® from BMG LABTECH provides an easy-to-use instrument, which measures this fluorometric assay quickly and accurately. Furthermore, the complementary MARS Analysis Software Module is extremely helpful for evaluating and analyzing assay results.