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Lysosomal GCase (glucocerebrosidase) activity assay V.1

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We use this protocol and it's working

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Abstract

Here we report a method to measure enzyme activity of lysosomal glucocerebrosidase (GBA1, GCase) by monitoring the hydrolysis of the fluorescent substrate 4-methylumbelliferyl-β-D-glucopyranoside. The assay is performed at low pH, at which non-lysosomal glucocerebrosidase activity is expected to be low. This is consistent with the abolishment of 4-MUG hydrolysis in the presence of the GBA1 inhibitor CBE. Our data shows that GBA1 activity is significantly increased in purified lysosomes compared to the whole cell extract.

Materials

REAGENTS

- Citric acid (Sigma cat. # 251275)
- Sodium phosphate dibasic (Sigma cat. #567547)
- Sodium taurocholate (Sigma cat. #86339)
- Ethylenediaminetetraacetic acid (EDTA; Sigma cat. #E6511)
- Glycine (Sigma cat. #50046)
- Bovine serum albumin (BSA; Sigma cat. # A7906)
- 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG; Sigma cat. #M3633)
- 4-methylumbelliferone (4-MU; Sigma cat. #M1381)
- Conduritol B epoxide (CBE; Sigma cat. #C5424)
- Dimethyl sulfoxide (DMSO; Sigma cat. #D8418)

BUFFERS

- 0.1M citric acid
- 0.2M sodium phosphate
- Citrate-phosphate buffer, pH 5.4
- 0.5M EDTA, pH 8
- Assay buffer: Citrate-phosphate buffer with 0.25% (w/v) sodium taurocholate, 1mM EDTA, 1% (w/v) BSA
- Stop buffer: 1M Glycine, pH 12.5

EQUIPMENT

- PHERAstar® FS plate reader (Ex/Em = 350/460 FI optical module)

CONSUMMABLES

- FLUOTAC flat bottom black 96-well plate (Greiner cat. #655076)
- Standard 1mL and 200µL Pipette tips (Greiner cat. #686271 and #685261 respectively).

Troubleshooting

Buffer preparation

1 **0.1M citric acid:** dissolve 19.2 g citric acid in 1 L dH_2O

2 **0.2M sodium phosphate:** dissolve 28.4 g sodium phosphate dibasic in 1 L dH_2O .

3 **Citrate-phosphate buffer, pH 5.4:** mix 44.2 mL 0.1M citric acid with 56.8 mL 0.2M sodium phosphate to make 100mL citrate-phosphate buffer, pH 5.4 .

4 **0.5M EDTA:** dissolve 20.8 g EDTA in 80 mL dH_2O Adjust to pH 8 and top up to 100 mL .

5 **Assay buffer:** to make 500 mL assay buffer, add 1.25 g sodium taurocholate , 5 g BSA , and 1 mL 0.5M EDTA to 500 mL citrate-phosphate buffer .

6 **Stop buffer:** dissolve 37.5 g Glycine in 400 mL dH_2O . Adjust to pH 12.5 and top-up volume to 500 mL .

7 **10mM 4-MU calibrator stock solution:** dissolve 17.6 mg 4-MU in 10 mL Stop buffer . Aliquot and store at -20 °C, protected from light.

8 **25 mM CBE:** dissolve 5 mg CBE in 1.23 mL DMSO . Aliquot and store at -20°C.

Substrate preparation

9 Dissolve 4.2 mg 4-MUG in 2.5 mL assay buffer (final concentration = [M] 5 millimolar (mM)). A sonicator water bath may be used to facilitate dissolution.

Note

Ensure the solution is protected from light. Prepare fresh 4-MUG solution before each assay.

Sample preparation

10 Add 5 µg of protein from whole cell extracts or 1 µg of protein from Lyso-IP samples into the wells of a flat bottom black 96-well plate in duplicate.

Note

If CBE treatment is desired, make sure to allocate 2 extra wells per sample for the treatment.

11 Top up volume to 80 µL with assay buffer.

12 Add 1.2 µL of DMSO or 25mM CBE to each sample well.

13 Prepare blank samples in duplicate: add 80 µL to two empty wells.

14 Prepare calibrator wells: designate 24 empty wells for the calibrators and add 100 µL assay buffer to each of these wells.

Enzymatic reaction

1h

15 Add 20 µL of the 5mM 4-MUG solution prepared in **step 9** to each of the sample and blank wells.

16 Cover the plate and incubate at 37 °C for 01:00:00 . 1h

> Note
>
> Ensure the plate is protected from light throughout the incubation.

Preparation of calibrator serial dilutions

17 During the incubation, thaw an aliquot of 10mM 4-MU.

18 Label 11 1.5mL microcentrifuge tubes with numbers 1-11.

19 Add 1 mL stop buffer to tube 1.

20 Add 500 µL stop buffer to tubes 2-11.

21 Add 2 µL 10 mM 4-MU to tube 1.

22 Mix by pipetting up-and-down and transfer 500 µL from tube 1 to tube 2.

23 Repeat **step 22** sequentially for the remaining tubes. At the end, only tube 11 should contain 1 mL.

	A	B	C	D
	Tube	**Volume of Stop buffer (µL)**	**Volume and source of 4-MU (µL)**	**Final 4-MU concentration (nM)**
	1	1000	2 of 10mM stock	20 000

	A	B	C	D
	2	500	500 of tube 1	10 000
	3	500	500 of tube 2	5 000
	4	500	500 of tube 3	2 500
	5	500	500 of tube 4	1 250
	6	500	500 of tube 5	625
	7	500	500 of tube 6	312.5
	8	500	500 of tube 7	156.25
	9	500	500 of tube 8	78.12
	10	500	500 of tube 9	39.06
	11	500	500 of tube 10	19.53

Calibrator concentrations.

Stop reaction and fluorescence measurement

24 Add 100 µL stop buffer to each sample and blank well.

25 Add 100 µL stop buffer to 2 of the calibrator wells. These will be the blanks for the calibration curve.

26 Add 100 µL of each calibrator solution prepared in step 23 to 2 of the calibrator wells.

27 Measure fluorescence intensity in a plate reader (Ex/Em = 350/460).

Note

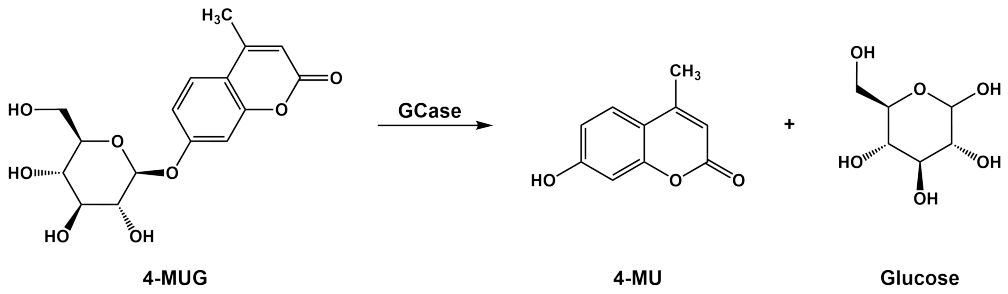
If immediate measurement of fluorescence intensity is not feasible, the plate can be stored at 4 °C, protected from light, for a up to 2 hours.

Data analysis

28 Plot the fluorescence intensity of the calibrator against the corresponding amounts of 4-MU in picomoles (pmol). Determine the linear equation representing this relationship.

29 Using the calibration curve equation, estimate the amount of released 4-MU in picomoles for the samples.

30 Divide the estimated amount of released 4-MU by the amount of protein lysate in milligrams (mg) and the incubation time in minutes. This yields the GCase activity, expressed in terms of released 4-MU in picomoles per milligram per minute (pmol/mg/min).



Hydrolysis of 4-MUG by GCase.

Protocol references

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