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GCase assay - Resorufin β-D-glucopyranoside V.1

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We use this protocol and it's working

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Abstract

We present a method for assessing the enzyme activity of lysosomal glucocerebrosidase (GBA1, GCase) in protein lysates by monitoring the hydrolysis of the fluorescent substrate Resorufin β-D-glucopyranoside. The assay is conducted at low pH, where non-lysosomal glucocerebrosidase activity is expected to be minimal. This approach is supported by the complete loss of the fluorescent signal in GBA1 knockout (KO) cells. The assay is compatible with cell and tissue lysates, as well as organellar immunoprecipitations. Given the variable expression of GBA1 across different tissues and cell types, the amount of protein used in the assay can be adjusted accordingly.

Materials

Reagents

- Resorufin β-D-glucopyranoside (Sigma, R4758)
- Sodium taurocholate (Sigma, 86339)
- Citric acid (Sigma, 251275)
- Sodium phosphate dibasic (Sigma, S9763)
- Dimethyl sulfoxide (DMSO, Sigma, D2650)
- Ethylenediaminetetraacetic acid (EDTA,Sigma, E6511)
- Bovine serum albumin (BSA, Sigma, A7906)

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

Equipment and consumables

- Fluoresce platereader with Ex = 571 nm and Em = 584 nm
- FLUOTAC flat bottom black 96-well plate (Greiner, 655076)

Before start

The protocols provide instructions to perform GCase assay in protein lysates. Since the same quantity of protein is used, we recommend to quantify protein content before the assay is performed using any protein quantification method (e.g., BCA, Bradford, ...).

Buffers preparation

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

2 **0.2 M sodium phospate**
Dissolve 28.4 g of sodium phosphate dibasic in 1 L of dH_2O

3 **0.5 M EDTA**
Dissolve 10.4 g of EDTA in 40 mL of dH_2O. Bring the pH 8 and top up to 50 mL with dH_2O

4 Prepare a fresh amount of assay buffer: Citrate-phosphate buffer with 0.25% (w/v) sodium taurocholate, 1mM EDTA. Approximately 200 µL are needed for each well of a 96 well plate.
For 20 ml of **Assay Buffer:**

- 8.85 mL of 0.1 M citric acid
- 11.15 mL of 0.2 M sodium phosphate
- 50 mg of sodium taurocholate
- 40 µL of 0.1 M EDTA
- Check pH 5.4

5 Prepare **10% BSA** in **Assay buffer**. A total of 20 µL per well are needed.
For 2ml:

- Weight 200 mg of BSA
- Dissolve in 2 mL of assay buffer

6 **Resorufin β-D-glucopyranoside substrate:**
Prepare a [M] 3.75 millimolar (mM) of Resorufin β-D-glucopyranoside in DMSO. Dilute this stock in assay buffer to obtain a working solution of [M] 187.5 micromolar (µM) (5x). A total of 40 µL of the 5x working solution is needed for each sample.
For a full 96 plate, so approx 4 ml:

- Weight the substrate and dissolve in DMSO to obtain the [M] 3.75 millimolar (mM) solution. We normally weight around 1 mg.

- Take 200 µL of the DMSO stock and dilute 1:20 in 3800 µL of assay buffer to obtain the 5x working solution [M] 187.5 micromolar (µM)

Note

Keep the diluted substrate in the dark.

Samples preparation

7 Defrost the protein lysates on ice and the lysis buffer used to generate the samples.

Note

Include GBA1 KO samples if possible.

8 In each well of a black 96 well-plate:
Pipette 15 µg of cell/tissue lysate. For Lyso-IP samples pipette 0.5 µg instead.

Note

We recommend running the samples in triplicates, or at least duplicates. Samples can be pipette directly into the wells of the 96-well plate, or a mastermix for each replicate can be done.

Protein amounts can be adjusted depending of the tissue and cell type used in the assay.

9 Top up volume to 20 µL with Lysis Buffer.

Note

If the samples are too diluted, the volume of lysis buffer to top up can be increased accordingly and the volume of assay buffer in the next step decreased of the same amount.

10 Add 120 µL of **Assay Buffer.**

> Note
>
> If more than 20 µL of samples have been used, reduce the amount of assay buffer added in this step accordingly.

11 Add 20 µL of **10% BSA.**

> Note
>
> Include background control wells with:
> 20 µL of lysis buffer
> 120 µL of assay buffer
> 20 µL of BSA

12 - Add 40 µL of the **Resorufin β-D-glucopyranoside substrate** working solution a [M] 187.5 micromolar (µM) (5x).

Fluorescence Measurement and analysis 4h 2m

13 The fluorescence of each well was measured every 00:02:00 s for 04:00:00 using a platereader with **excitation: 571 nm** and **emission: 584 nm.** 4h 2m

14 The raw RFU data were exported as a .csv file.

15 For each time point, average the background wells RFU.

16 Subtract the average background RFU for each time point from the samples RFU.

17 Average the technical replicates values (after background subtraction) for each time point.

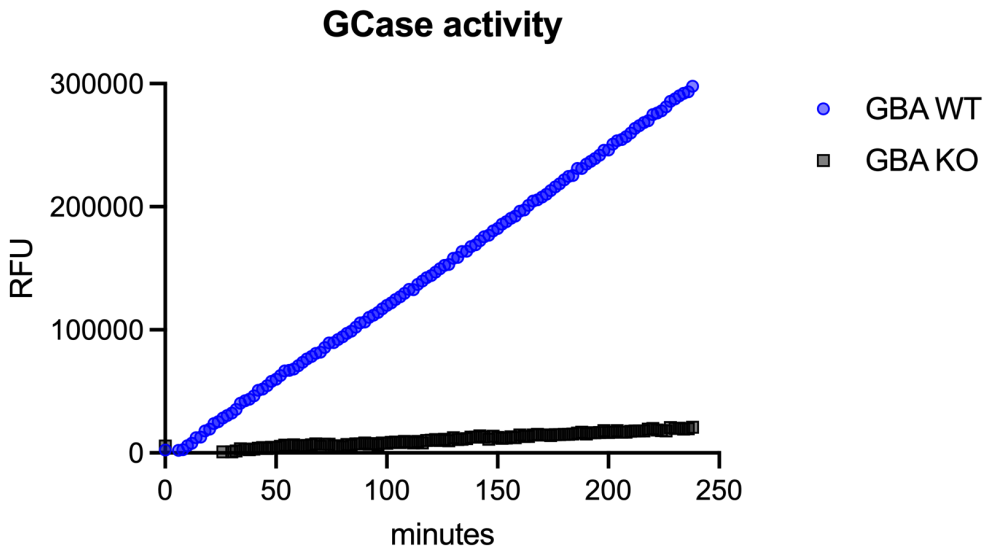
Note

If GBA1 KO control is included it is possible to subtract also the signal from the GBA1 KO wells from the average of the samples technicals replicates.

18 Either plot the RFU over time for each sample or alternatively the end-point RFU as shown in the expected results section.

19 Calculate statistical significance using the appropriate test.

Expected result



GCase activity from 10 ug of lysates from A549 GBA1 KO after GBA1 rescue (blue) or rescue with emty vector (black), after background substraction.

Protocol references

Deen, Matthew C., Proceviat, Cameron, Shan, Xiaoyang et al. (2020) Selective Fluorogenic β-Glucocerebrosidase Substrates for Convenient Analysis of Enzyme Activity in Cell and Tissue Homogenates. ACS Chemical Biology. ISSN 1554-8937 https://doi.org/10.1021/acschembio.9b01044