In Vitro GCase Activity Assay (Total Cell Lysate)

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May 23, 2022

1

dx.doi.org/10.17504/protocols.io.n2bvj625nlk5/v1

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Taurocholate inhibits the cytosolic B–glucosidase (GBA2) and activates lysosomal B–glucosidase (GBA1, a.k.a glucocerebrosidase (GCase)).In cultured cells the pH optimum is increased in the presence of taurocholate.Cultured cells are assayed without taurocholate at pH 4.5 and with taurocholate at pH 5.4.In normal controls and heterozygotes the B-glucosidase activity in the presence of taurocholate is approximately 2-10 times higher than without taurocholate.In cases of Gaucher disease the β-glucosidase activity may be lower in the presence of the taurocholate.However, in fibroblasts there can be as much as a threefold increase in activity but the specific activity is very low compared with controls and heterozygotes.

At acid pH B–glucosidase hydrolyses the substrate 4–methylumbelliferyl–B–D–glucopyranoside to 4–methylumbelliferone and glucose.Adding alkaline buffer stops the enzyme reaction and causes 4–methylumbelliferone to fluoresce at a different wavelength from unhydrolysed substrate, thereby permitting its measurement in the presence of a vast excess of unhydrolysed substrate.

DOI

dx.doi.org/10.17504/protocols.io.n2bvj625nlk5/v1

Laura Smith 2022. In Vitro GCase Activity Assay (Total Cell Lysate). **protocols.io** https://dx.doi.org/10.17504/protocols.io.n2bvj625nlk5/v1

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Apr 28, 2022

May 23, 2022

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Principle

1 Taurocholate inhibits the cytosolic β–glucosidase (GBA2) and activates lysosomal β–glucosidase (GBA1, a.k.a glucocerebrosidase (GCase)).In cultured cells the pH optimum is increased in the presence of taurocholate.Cultured cells are assayed without taurocholate at pH 4.5 and with taurocholate at pH 5.4.In normal controls and heterozygotes the β-glucosidase activity in the presence of taurocholate is approximately 2-10 times higher than without taurocholate.In cases of Gaucher disease the β-glucosidase activity may be lower in the presence of the taurocholate.However, in fibroblasts there can be as much as a threefold increase in activity but the specific activity is very low compared with controls and heterozygotes.

At acid pH β–glucosidase hydrolyses the substrate 4–methylumbelliferyl–β–D–glucopyranoside to 4–methylumbelliferone and glucose.Adding alkaline buffer stops the enzyme reaction and causes 4–methylumbelliferone to fluoresce at a different wavelength from unhydrolysed substrate, thereby permitting its measurement in the presence of a vast excess of unhydrolysed substrate.

Reference

2 Clinical Genetics (1978) **13:**145 – 153.D.A. Wenger, C. Clark, M. Sattler and C. Wharton.Synthetic substrate β–glucosidase activity in leucocytes:A reproducible method for the identification of patients and carriers of Gaucher's disease.

Reagents

3

Buffers: McIlvaine citrate–phosphate (MV), pH 5.4

Substrates:
- GCase: 4–methylumbelliferyl–β–D–glucopyranoside (MWt. 338)

Standard:4–methylumbelliferone (MWt. 176)

NaT:Na taurocholate 80 mg / ml H_2O (149 mM) – make fresh

Stopping solution:0.25 M glycine buffer pH 10.4 reagent.

Inhibitors: CBE (Sigma 5424) and DNJ (Enzo BML-SL230-005).

Preparation of reagents

4 McIlvaine citrate–phosphate (MV):

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A	B	C	D
	GCase + NaT	**GCase**	
	pH 5.4	**pH 4.5**	
0.1 M citric acid	44 ml	54 ml	
0.2 M Na2HPO4	56 ml	46 ml	

0.1M citric acid monohydrate (Mwt = 210.14 g/mol) – 5.2535 g in 250 mL dH2O
0.2M Na2HPO4 (Mwt = 141.9 g/mol) – 7.098 g in 250 mL dH2O

Substrates:

5 GCase: 4–methylumbelliferyl–β–D–glucopyranoside (MWt. 338) (Sigma M3633)

Take 250 mg in 74 mL dH2O and heat at 80'C until dissolved (3.38mg/ml) to give 10 mM concentration.
Aliquot into bijou tubes and store at -20 indefinitely.
For each experiment, heat at 60-80'C in oven to ensure all powder is solubilised.

6 Standard: 4–methylumbelliferone (MWt. 176) (Sigma M1381):

Desired concentration is 1 nmol in 200 uL aliquots.
Take 2 mg in 1.5 mL dH2O, then do a 1:1000 dilution in dH2O to give a 0.2ug/200ul concentration.
Aliquot into 200 uls in eppendorfs.

When using in experiment, add 1 mL glycine buffer to Eppendorf and load 200 uL into each well.

7 Na taurocholate 80 mg / ml H_2O (149 mM):

Make fresh (measure NaT on scale then take x grams / 80 * 1000 to give uL of dH2O to resuspend).

8 Stopping buffer: 0.25 M glycine buffer pH 10.4 reagent:

Make up 64g NaOH in 200 mL dH2O.
Make up 150g glycine in 1600 mL dH2O.
When all mixed, add both together.
Ensure pH is 10.4 and make up to 2 L with dH2O.

9 Inhibitors:

When these arrive, re-constitute powder as follows:

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a.CBE (Sigma C5424, Mr=162): 5mg / 617ul dH2O = 50mM
b.DNJ (Enzo BML-SL230-005): 5mg / 195ul dH2O = 100mM

Apparatus / Instrumentation

10 37^{o}C water bath
Plate reader, excitation 360 nm, emission 460 nm, sensitivity=50

Sample preparation

11 Enzyme:

Samples are resuspended in water or 1% (v/v) TX-100 in PBS. For TX-100 lysis, cells incubated on ice for 15 mins and debris/nuclei removed by centrifugation at 17, 000 x g, 10 min, 4 °C. Supernatant containing GBA enzyme placed in fresh tube. All samples are sonicated in water bath for 1 minute. Protein concentration measured with BCA protein assay.

Method Protocol

12 Dilute in water a portion of the sonicate to give a protein concentration of 0.25 - 4 mg / ml.

13 Set up mix in eppendorf tubes for each well as follows:

Make a master mix e.g multiply by number of wells.

GCase at pH 5.4 + NaT:
5 µl MV buffer pH 5.4
3 µl Na taurocholate solution
10 µl MUG substrate solution
+/- 0.4 µL 50mM CBE

GCase at pH 4.5:
5 µl MV buffer pH 5.4
3 µl dH2O
10 µl MUG substrate solution
+/- 0.4 µL 100 mM DNJ

14 Add 10 µl diluted enzyme sample in to duplicate wells of a 96 well plate. (Note: may have to optimise volume of sample loaded depending on sensitivity of fluorescence machine used).

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15 Add 10 µl of lysis buffer used (water of TX-100 in PBS) to duplicate wells to serve as substrate blanks.

16 Add 18 µl of reaction mixture to each well (18.4 per well if inhibitors used).

17 Incubate at 37°C for 30 minutes.

18 Add 220 µL stopping solution to each well

19 Standard:

To 1 nmol 4–methylumbelliferone standard in 200 µl H_2O add
1.0 ml stopping reagent.Mix and 200 ml to empty wells to serve as a fluorescence standard.

20 Read fluorescence, excitation 360 nm, emission 460 nm.

21 Calculate activity in nmol / hr / mg protein

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