May 23, 2022

In Vitro B-galactosidase and B-hexosaminidase Activity Assay (Total Cell Lysate)

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dx.doi.org/10.17504/protocols.io.kqdg3p8r7l25/v1

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Synthetic substrate can be used at acidic pH (pH 4.1) to assess activity of lysosomal hydrolases, β-Galactosidase and β-Hexosaminidase. The substrate is cleaved by β-Galactosidase and β-Hexosaminidase and produces a fluorescent product proportional to activity.

DOI

dx.doi.org/10.17504/protocols.io.kqdg3p8r7l25/v1

Laura Smith 2022. In Vitro B-galactosidase and B-hexosaminidase Activity Assay (Total Cell Lysate). **protocols.io** https://dx.doi.org/10.17504/protocols.io.kqdg3p8r7l25/v1

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Apr 28, 2022

May 23, 2022

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Reagents

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

Substrates:
- β-Galactosidase: 4-Methylumbelliferyl β-D-galactopyranoside (Sigma M1633)
- β-hexosaminidase: 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma M2133):

Standard:4–methylumbelliferone (MWt. 176)

Stopping solution:0.25 M glycine buffer pH 10.4 reagent.

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

Preparation of reagents

2 McIlvaine citrate–phosphate (MV):

A	B
	pH 4.1
0.1 M citric acid	30 ml
0.2 M Na2HPO4	20 ml, then 1:1

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:

3 **β-Galactosidase: 4-Methylumbelliferyl β-D-galactopyranoside (Sigma M1633):**

Take 3.4 mg mg in 10 mL dH2O and heat at 80'C until dissolved (0.34mg/ml)
Make up in two bijou tubes; vortex to dissolve or sonnicate and store at -20.
For each experiment, heat at 60-80'C in oven to ensure all powder is solubilised.

4 **β-hexosaminidase: 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma M2133):**

Take 27 mg in 10 ml dH2O and heat at 80'C until dissolved (2.71mg/ml)
Make up in two bijou tubes of 5 ml and store at -20'C.
For each experiment, heat at 60-80'C in oven to ensure all powder is solubilised.

5 **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.

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6 **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.

Apparatus / Instrumentation

7 37^{o}C water bath
Plate reader, excitation 360 nm, emission 460 nm, sensitivity=50

Sample preparation

8 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

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

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

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

B-gal:
20µl B-gal/HEX buffer pH 4.1
10µl B-gal substrate solution

B-hex:
20µl B-gal/HEX buffer pH 4.1
10µl B-gal substrate solution

11 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).

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

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blanks.

13 Add 30 µl of reaction mixture to each well

14 Incubate at 37°C for 30 minutes.

15 Add 200 µL stopping solution to each well

16 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.

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

18 Calculate activity in nmol / hr / mg protein

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