Megazyme

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α-GLUCURONIDASE

ASSAY PROCEDURE

K-AGLUA 02/19

FOR THE MEASUREMENT OF α-GLUCURONIDASE

(50 Manual Assays per Kit) or
(200 Microplate Assays per Kit)

INTRODUCTION:

After cellulose, xylan is the second most abundant polysaccharide found in nature. Xylan is a hemicellulose comprising a β-(1,4)-linked xylan back bone substituted with various side chains such as acetyl groups, α-(1-2/3)-linked L-arabinofuranose and α-(1-2)-linked 4-*O*-methylglucuronic acid. A repertoire of enzymes is required to effect the complete degradation of plant cell wall polysaccharides in the utilisation of plant biomass. Hence complete degradation of xylan requires the concerted action of various enzymes, including *endo*-β-(1,4)-xylanases, β-(1,4)-xylosidases, acetylxylan esterases, α-L-arabinofuranosidases and α-glucuronidases.

The importance of carbohydrate degrading enzymes has increased over recent years due to the growing focus on obtaining renewable energy from plant biomass in the form of biofuels such as cellulosic and hemicellulosic ethanol. Various research programmes are striving to develop optimised enzyme mixtures or modified microorganisms to increase the efficiency of biomass conversion and, as such, it is important that the various enzyme activities can be easily measured. Simple methods of measuring enzyme activity for some of the well studied carbohydrate degrading enzymes such as *endo*-β-(1,4)-cellulases, *endo*-β-(1,4)-xylanases, β-(1,4)-xylosidases exist. However, for some enzymes such as α-glucuronidases, current available methods are cumbersome.

This kit (**K-AGLUA**) provides a simple method suitable for the specific measurement of α-D-glucuronidase activity (α-D-glucosiduronate glucuronohydrolase; EC 3.2.1.139 and xylan α-1,2-glucuronosidase; EC 3.2.1.131). This kit contains highly purified, borohydride reduced aldotetrauronic acid with a terminal α-D-glucuronic acid substitution, which is an excellent substrate for α-D-glucuronidases from CAZy family GH67. This substrate can be also used for the measurement of α-D-glucuronidases from CAZy family GH115, however the rate of hydrolysis compared to GH67 is reduced.

PRINCIPLE:

Hydrolysis of aldotetrauronic acid (terminal substitution, borohydride reduced) by the enzyme α-glucuronidase releases the α-(1-2)-linked 4-*O*-methylglucuronic acid (1). In the second reaction D-glucuronic acid undergoes oxidation, catalysed by the enzyme uronate dehydrogenase, in the presence of nicotinamide-adenine dinucleotide (NAD^+) to D-glucarate with the formation of reduced nicotinamide-adenine dinucleotide (NADH) (2).

(1) Aldotetrauronic acid ($NaBH_4$ reduced) + H_2O $\xrightarrow{\text{(α-D-Glucuronidase)}}$ β-(1,4)-D-xylo-oligosaccharides + D-glucuronic acid

(Uronate dehydrogenase; UDH)

(2) D-Glucuronic acid + NAD^+ + H_2O ⟶ D-glucarate + NADH + H^+

The amount of NADH formed in this reaction is stoichiometric with the amount of free D-glucuronic acid. It is the NADH which is measured by the increase in absorbance at 340 nm.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 50 assays in manual format (or 200 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Aldotetrauronic acid (terminal substitution, borohydride reduced) (36 mg).
Stable for > 5 years below -10°C.

Bottle 2: α-D-Glucuronidase solution (2 mL; ~ 1.7 U/mL, see label on bottle).
Stable for > 2 years below -10°C.

Bottle 3: Stop Buffer (28 mL, pH 10) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 4: NAD^+ freeze dried powder.
Stable for > 5 years bellow -10°C.

Bottle 5: Uronate dehydrogenase suspension (1.1 mL).
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS (SUPPLIED):

1. Dissolve the contents of bottle 1 in 6 mL of distilled water (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).
Stable for > 5 years below -10°C.

2. Dilute an appropriate amount of enzyme (bottle 2) 20-fold in water **on the day of use**. Store at 4°C while in use.

NOTE: The activity of the α-D-glucuronidase solution (bottle 2) may reduce gradually by ~ 10% over 2 years, when stored appropriately.

3. Use the contents of bottle 3 as supplied.
Stable for > 2 years at 4°C.

4. Dissolve the contents of bottle 4 in 11 mL of distilled water. To avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes.
Stable for > 2 years below -10°C.

5. Use the contents of bottle 5 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently store the bottle in an upright position. **Swirl the bottle to mix contents before use.**
Stable for > 2 years at 4°C.

RECOMMENDED EXTRACTION/DILUTION BUFFERS:

NOTE: It is recommended that the selection of the extraction buffer is based upon the pH optima of the α-D-glucuronidase being tested so that the pH optima is within the buffering range of the extraction buffer.

1. Buffer A (pH 4.0-5.5)

[Sodium acetate (100 mM) containing 0.5 mg/mL BSA plus 0.02% (w/v) sodium azide]

Add 5.9 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 4.0-5.5 by the addition of 1 M sodium hydroxide. Add 0.5 g BSA (Sigma cat. no. A2153) and 0.2 g of sodium azide (Sigma cat. no. S2002) and readjust the pH to 4.0-5.5. Adjust the volume to 1 L. Store at 4°C.

2. Buffer B (pH 5.5-6.7)

[MES (100 mM) containing 0.5 mg/mL BSA plus 0.02% (w/v) sodium azide]

Add 21.3 g of MES buffer salt (acid form; Megazyme cat. no. **B-MES250**) to 900 mL of distilled water. Adjust the pH to 5.5-6.7 by the addition of 1 M sodium hydroxide. Add 0.5 g BSA (Sigma cat. no. A2153) and 0.2 g of sodium azide (Sigma cat. no. S2002) and readjust the pH to 5.5-6.7. Adjust the volume to 1 L. Store at 4°C.

3. Buffer C (pH 6.5-7.9)

[MOPS (100 mM) containing 0.5 mg/mL BSA plus 0.02% (w/v) sodium azide]

Add 20.9 g of MOPS buffer salt (acid form; Megazyme cat. no. **B-MOPS250**) to 900 mL of distilled water. Adjust the pH to 6.5-7.9 by the addition of 1 M sodium hydroxide. Add 0.5 g BSA (Sigma cat. no. A2153) and 0.2 g of sodium azide (Sigma cat. no. S2002) and readjust the pH to 6.5-7.9. Adjust the volume to 1 L. Store at 4°C.

4. Buffer D (pH 6.8-8.2)

[HEPES (100 mM) containing 0.5 mg/mL BSA plus 0.02% (w/v) sodium azide]

Add 23.8 g of HEPES buffer salt (acid form; Megazyme cat. no. **B-HEPES250**) to 900 mL of distilled water. Adjust the pH to 6.8-8.2 by the addition of 1 M sodium hydroxide. Add 0.5 g BSA (Sigma cat. no. A2153) and 0.2 g of sodium azide (Sigma cat. no. S2002) and readjust the pH to 6.8-8.2. Adjust the volume to 1 L. Store at 4°C.

NOTE: Sodium azide should not be added until the pH is adjusted. Acidification of sodium azide releases a poisonous gas.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 120 mm).
2. Disposable plastic or glass semi-microcuvettes (1 cm light path, 1.5 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 200 µL and 1 mL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette® - with 25 mL Combitip® (to dispense 0.5 mL aliquots of reagent solutions) [Extraction Buffer and Stop Buffer (solution 3)].
5. Stop clock.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8 Thermostated water bath.

A. MANUAL ASSAY PROCEDURE:

NOTE: In the standard procedure the α-D-glucuronidase reaction in Step 1 is performed at 40°C. However, if the temperature optimum of the enzyme sample being tested is different to this the reaction temperature can be altered accordingly.

Dilute the α-D-glucuronidase sample to **~ 17.4-174 mU/mL** in the appropriate extraction buffer and **pre-incubate at 40°C for 5 min** (pre-incubate 0.15 mL of enzyme for each assay to be performed).

STEP 1:

Pipette into test tubes	Sample Blank	Sample	Standard Blank	Standard
solution 1 (Aldotetrauronic acid)	0.10 mL	0.10 mL	0.10 mL	0.10 mL
extraction buffer	0.50 mL	0.50 mL	0.50 mL	0.50 mL
solution 3 (stop buffer)	0.50 mL	-	0.50 mL	-
Mix by vortex after each component addition and **equilibrate to the appropriate reaction temperature for at least 5 min.** Start the reactions by addition of:				
enzyme sample	0.10 mL	0.10 mL	-	-
solution 2 (α-D-Glucuronidase)	-	-	0.10 mL	0.10 mL
Immediately mix by vortex and incubate the reactions at 40°C. **After exactly 10 min**, terminate the reactions by addition of:				
solution 3 (stop buffer)	-	0.50 mL	-	0.50 mL
Immediately mix by vortex and allow the tubes to cool to room temperature for approx. 10 min. **Carefully pipette 1.0 mL for use in Step 2.**				

STEP 2:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic; 1.5 mL semi-micro)
Temperature: ~ 25°C or 37°C
Final volume: 1.22 mL
Sample solution: α-D-Glucuronidase reaction from Step 1
Read against air (without cuvette in the light path) or against water

Pipette into cuvettes	Sample
sample (from Step 1) solution 4 (NAD^+)	1.00 mL 0.20 mL
Mix*, read absorbances of the solutions (A_1) after approx. 3 min and start the reaction by addition of:	
suspension 5 (UDH)	0.02 mL
Mix* and read the absorbance of the solutions (A_2) at the end of the reaction (approx. 5 min at 25°C or approx. 5 min at 37°C). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same.	

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

CALCULATION (Manual Assay Procedure):

Determine the absorbance difference (A_2-A_1) for sample blank, standard blank, sample and standard. Subtract the absorbance difference of the sample blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{alpha-gluc sample}}$. Subtract the absorbance difference of the standard blank from the absorbance difference of the standard, thereby obtaining $\Delta A_{\text{alpha-gluc std}}$.

The value of $\Delta A_{\text{alpha-gluc sample}}$ or $\Delta A_{\text{alpha-gluc std}}$ should be at least 0.100 absorbance units to achieve sufficiently accurate results.

The activity of α-D-glucuronidase can be calculated as follows:

$$c = \frac{1\times10^6 \times V1 \times V2 \times D}{\varepsilon \times d \times v1 \times v2 \times 1000 \times t} \times \Delta A_{\text{alpha-gluc}} \quad [U/mL]$$

where:

1×10^6	=	conversion of extinction coefficient [l x µmol^{-1} x cm^{-1}]
V1	=	final volume from Step 1 [mL]
V2	=	final volume from Step 2 [mL]
D	=	dilution factor (e.g. if sample is diluted 10-fold, D = 10)
ε	=	extinction coefficient of NADH at 340 nm
	=	6300 [l x mol^{-1} x cm^{-1}]
d	=	light path [cm]
v1	=	sample volume from Step 1 [mL]
v2	=	sample volume from Step 2 [mL]
1000	=	conversion from L to mL
t	=	reaction time from Step 1 [min]

It follows for α-D-glucuronidase:

$$c = \frac{1\times10^6 \times 1.20 \times 1.22 \times D}{6300 \times 1 \times 0.1 \times 1 \times 1000 \times 10} \times \Delta A_{\text{alpha-gluc}} \quad [U/mL]$$

$$= \frac{2.3238 \times \Delta A_{\text{alpha-gluc}}}{10} \times D \quad [U/mL]$$

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (U/mg) is calculated from the amount weighed as follows:

Content of α-D-glucuronidase:

$$= \frac{c_{\text{alpha-gluc}}\ [\text{U/mL sample solution}]}{\text{weight}_{\text{sample}}\ [\text{mg/mL sample solution}]} \quad [U/mg]$$

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

B. MICROPLATE ASSAY PROCEDURE:

NOTE: For each batch of samples that is applied to the determination of α-D-glucuronidase either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C or 37°C
Final volume: 0.355 mL
Linearity: ~ 17.4-174 mU/mL α-D-Glucuronidase (in 0.025 mL sample volume)

Pipette into test tubes	Sample Blank	Sample	Standard Blank	Standard
solution 1 (Aldotetrauronic acid)	0.025 mL	0.025 mL	0.025 mL	0.025 mL
extraction buffer	0.125 mL	0.125 mL	0.125 mL	0.125 mL
solution 3 (stop buffer)	0.125 mL	-	0.125 mL	-
Mix* after each component addition, **seal the plate and equilibrate to the appropriate reaction temperature for at least 5 min.** Start the reactions by addition of:				
enzyme sample	0.025 mL	0.025 mL	-	-
solution 2 (α-D-Glucuronidase)	-	-	0.025 mL	0.025 mL
Immediately mix* and seal the plate and incubate the reactions at the appropriate temperature. **After exactly 10 min** stop the reactions by addition of:				
solution 3 (stop buffer)	-	0.125 mL	-	0.125 mL
Immediately mix* and allow the reactions to cool to room temperature for approx. 10 min. **Then add:**				
solution 4 (NAD^+)	0.050 mL	0.050 mL	0.050 mL	0.050 mL
Mix* and read absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:				
suspension 5 (UDH)	0.005 mL	0.005 mL	0.005 mL	0.005 mL
Mix* and read the absorbance of the solutions (A_2) at the end of the reaction (approx. 5 min at 25°C or 37°C). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same.				

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

CALCULATION (Microplate Assay Procedure):

$$U/mL = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times U/mL\ standard \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

EQUIPMENT FOR MICROPLATE ASSAY PROCEDURE (RECOMMENDED):

1. Disposable 96 well polystyrene clear, flat bottom microplates, e.g. Matrix Technologies Corp. cat. no. 4915 (www.matrixtechcorp.com).
2. Disposable 25 mL reagent reservoirs, e.g. Matrix Technologies Corp. cat. no. 8093-11 (www.matrixtechcorp.com).
3. Micro-pipettors, e.g. Gilson Pipetman® (200 µL and 1 mL) and Multichannel Micro-pipettors, e.g. Gilson Pipetman® Ultra 8-channel (1-20 µL and 20-300 µL).
4. Stop clock.
5. Microplate shaker, e.g. Heidolph Titramax 100 or 1000 (www.heidolph-instruments.com).
6. Microplate reader set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).

SAMPLE PREPARATION EXAMPLES:

Determination of α-D-glucuronidase in enzyme preparations:

Accurately weigh approx. 100 mg of α-D-glucuronidase preparation into a 100 mL beaker. Add 50 mL of extraction buffer (Extraction Buffer A, B, C or D) and gently stir the preparation on a magnetic stirrer until the enzyme completely dissolves. Dilute this solution by transferring 1.0 mL into a 100 mL volumetric flask and adjusting to volume with extraction buffer. Mix thoroughly by inversion and dilute further if necessary to obtain an enzyme concentration suitable for assay (~ 17.4-174 mU/mL).

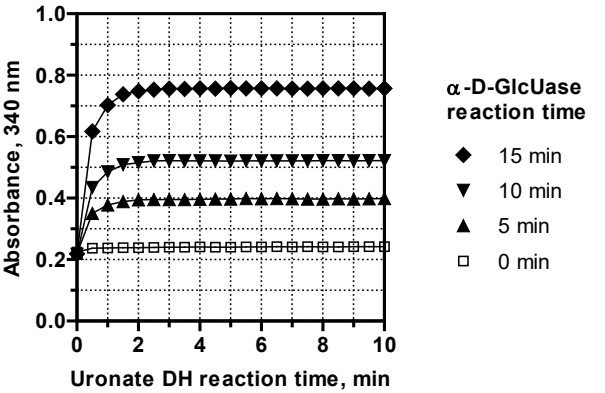

Figure 1. Increase in absorbance at 340 nm on incubation of α-D-glucuronidase time-course reactions with uronate dehydrogenase in the presence of NAD^+ at 25°C using 1 cm path-length cuvettes. The α-D-glucuronidase reactions used to generate this calibration curve were performed at 40°C, pH 6 for 0, 5, 10 and 15 min.

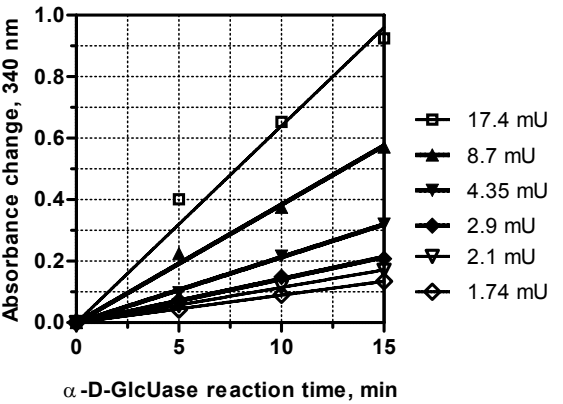

Figure 2. Linearity showing time-course reactions of α-D-glucuronidase with aldotetrauronic acid performed at 40°C, pH 6 for 0, 5, 10 and 15 min. Incubation of α-D-glucuronidase time-course reactions with uronate dehydrogenase were performed in the presence of NAD^+ at 25°C using 1 cm path-length cuvettes.

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