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GLUCURONOYL ESTERASE ASSAY PROCEDURE

(GEUX3 METHOD)

K-GEUX3 10/19

FOR THE MEASUREMENT OF GLUCURONOYL ESTERASE

(100 Assays per Kit)

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INTRODUCTION:

Glucuronoyl esterases (GEs) are a recently discovered class of enzymes first isolated and described in 2006.[1] These esterase enzymes are phylogenetically distant from other carbohydrate esterases and fall into the CE15 CaZY family.[2] Glucuronoyl esterases play a key role in plant cell wall degradation and are involved in lignin hydrolysis by cleaving the ester linkages between lignin polyphenols and the glucuronoxylan present in the hemicellulose fraction of lignocellulosic biomass. Given that these linkages are believed to contribute to the recalcitrant nature of lignocellulose, the discovery of new and improved glucuronoyl esterases is an important target for the biofuel industry and biorefineries responsible for the conversion of lignocellulosics. A number of substrates to measure GE activity have previously been described in the literature. These substrates are either oligosaccharide fragments derived from the native glucuronoxylan polysaccharide linked to lignin or synthetic molecules whose structures have some degree of similarity to glucuronoxylan. Most of the substrates described are used to measure glucuronoyl esterase activity when coupled to chromatographic techniques.[3] Qualitative TLC-based assays and quantitative-HPLC assays have been described. However, they both have serious limitations mainly in terms of substrate availability, assay sensitivity and the fact that they do not lend themselves easily to automation (desirable for high throughputs screenings and enzyme discovery).

Recently, an enzyme-coupled spectrophotometric GE assay was reported.[4] This innovative approach to GE analysis, however, also has limitations in that the substrate employed in the assay is not commercially available and lacks a 4-O-methyl-group on the glucuronic acid residue which is a key feature for optimal substrate recognition by GEs.

To address the limitations of existing methods, Megazyme has developed a practical assay kit (**K-GEUX3**) for the quantitative measurement of glucuronoyl esterases. This enzyme-coupled colourimetric assay is based on a novel soluble chromogenic substrate which is combined with two ultra-pure ancillary enzymes: a GH67 α-glucuronidase and a GH43 β-xylosidase. The colourimetric substrate contained in the GEUX3 assay kit is a *p*-nitrophenyl-linked aldotriouronic acid which contains the key 4-O-methyl substitution and features a methyl ester on the carboxylate group of the glucuronic acid residue. The assay scheme is shown in Figure 1 (Appendix, page 7). Upon hydrolysis of the methyl ester group by a glucuronoyl esterase, the sequential hydrolytic activity by the ancillary enzymes α-glucuronidase and β-xylosidase results in the release of the colourimetric group *p*-nitrophenol. The rate of

release of *p*-nitrophenol relates directly to the rate of hydrolysis of GEUX3 by the glucuronoyl esterase being analysed. The substrate is water-soluble and the solution-based assay format makes it easily automatable for high throughput screening. The methyl ester group on the glucuronic acid terminal residue acts both as the 'recognition group' hydrolysed by the GE enzyme and as a 'blocking group' preventing hydrolysis by the ancillary α-glucuronidase. The reagent can be used at temperatures up to 50°C and within the pH range of 5.0-7.5. Assay linearity and repeatability data are shown in Figures 2-4 (Pages 7 and 8).

PRINCIPLE:

The GEUX3 Reagent contains three components: 1) the GEUX3 colourimetric substrate, 2) β-xylosidase and 3) α-glucuronidase. Upon hydrolysis of the colourimetric substrate by glucuronoyl esterase, the sequential hydrolytic activity by the ancillary enzymes β-xylosidase (2) and α-glucuronidase (3) results in the release of the *p*-nitrophenol colourimetric group. The reaction is terminated and the phenolate colour is developed by addition of tri-sodium phosphate solution (pH 12). Absorbance is measured at 400 nm. The rate of release of *p*-nitrophenol relates directly to the rate of hydrolysis of GEUX3 by the glucuronoyl esterase being analysed.

ACCURACY:

Standard errors of less than 5% are achieved routinely.

SPECIFICITY:

GEUX3 is not hydrolysed by enzymes other than glucuronoyl esterase, so it can be used for the specific assay of this enzyme in fermentation broths and industrial enzyme preparations.

KITS:

Kits suitable for performing 100 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: GEUX3 Substrate powder
p-nitrophenyl-2^2-(methyl 4-O-methyl-α-D-glucopyranosyluronate)-β-D-xylobioside plus α-glucuronidase and β-xylosidase. Lyophilised powder.
Stable for > 4 years; store sealed below -10°C.

Bottle 2: Glucuronoyl esterase control solution
~ 2.2 U/mL on GEUX3 substrate (exact activity stated on vial label) in 30% v/v glycerol and sodium azide (0.02% w/v).
Stable for > 4 years; store sealed below -10°C.

PREPARATION OF REAGENT SOLUTIONS:

1. Dissolve the contents of Bottle 1 in 5 mL of Buffer B. This is the **GEUX3 Reagent Solution**. Divide into appropriately sized aliquots and store in polypropylene tubes below -10°C between use and on ice during use. Once dissolved, the reagent is stable for > 2 years below -10°C.

2. Allow this bottle to equilibrate to room temperature and mix thoroughly before use in order to ensure that any ice present in the solution has dissolved fully. Using a positive displacement pipettor, dispense 0.5 mL of the contents of Bottle 2 into a polypropylene tube. Add 9.5 mL of **Buffer A** and mix thoroughly. Use immediately and discard after use.

PREPARATION OF ADDITIONAL REAGENTS (not supplied):

(A) Extraction/dilution buffer

(Sodium Phosphate, 0.1 M, pH 6.5 containing sodium azide, 0.02% w/v)
Add 15.6 g of sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$) to 800 mL of distilled water. Adjust the pH to 6.5 with NaOH and adjust the volume to 1 L. Add 0.2 g of sodium azide and dissolve,
Stable for > 1 year at 4°C.

(B) Substrate dissolution buffer

(Sodium phosphate, 0.01 mM, pH 6.5 containing sodium azide, 0.02% w/v)
Add 1.56 g of sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$) to 800 mL of distilled water. Adjust the pH to 6.5 with NaOH and adjust the volume to 1 L. Add 0.2 g of sodium azide and dissolve,

(C) Stopping Reagent

(2% w/v Tri-sodium Phosphate solution, pH 12.0)
Dissolve 20 g of tri-sodium phosphate in 1 L of distilled water and adjust the pH to approx. 12.0.
Stable at room temperature for at least 3 months.

CAUTION

If either buffer is prepared without addition of sodium azide as a preservative, then they should be stored at 4°C and used within 1 week.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (12 mL and 20 mL capacity).
2. Disposable polypropylene tubes (13 mL capacity)
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip®
   - with 25 mL Combitip®
5. Analytical balance.
6. Top-pan balance.
7. Spectrophotometer set at 400 nm.
8. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
9. Thermostated water bath set at 40°C.
10. Stop Clock.

CONTROLS AND PRECAUTIONS:

1. When dissolved the freeze-dried substrate should be stored at 0-5°C during use and below -10°C between uses. If the number of assays performed at any one time is expected to be limited, it is recommended that the substrate solution be divided into 1mL aliquots and stored in the frozen state.
2. On storage at 0-5°C, the blank absorbance values will increase by < 0.1 absorbance units over a one week period. This does not affect the performance of the substrate but these values must be determined at the same time as the assay is performed. Blank absorbance values as high as 0.2 do not affect the reliability or accuracy of the assay.
3. The assay format should be standardised with the enclosed control glucuronyl esterase preparation. The activity of this solution is shown on the enclosed vial.
4. The time of incubation of the assay (i.e. exactly 10 min) and incubation temperature (i.e. 40°C) must be accurately controlled.
5. If the absorbance values for a particular assay are greater than 1.20, the enzyme extract should be diluted with the appropriate buffer and re-assayed.

NOTE:
A single Reaction Blank is normally sufficient for each batch of samples being analysed. To obtain this blank value for glucuronoyl esterase samples, 1.5 mL of stopping reagent should be added to 0.05 mL of substrate solution. Then add 0.05 mL of enzyme extract.

ENZYME EXTRACTION:

1. **Liquid Preparations**

   Add 0.5 mL of liquid enzyme preparation (using a positive displacement dispenser) to 9.5 mL of Buffer A and mix thoroughly. This is termed the **Original Extract (liquid)**.

2. **Powder Preparations**

   Add 0.5 g of powdered enzyme preparation to 10 mL of Buffer A and gently stir the slurry over a period of approx. 15 min or until the sample is completely dispersed or dissolved. Clarify this solution (if required) by centrifugation (1,000 *g*, 10 min) or filtration through Whatman No. 1 (9 cm) filter circles. This is termed the **Original Extract (powder)**.

3. Add 1.0 mL of the **Original Extract (liquid or powder)** to 9.0 mL of the Buffer A (10-fold dilution) and mix thoroughly. This dilution process is repeated until a suitable concentration of glucuronoyl esterase for assay is achieved.

ASSAY PROCEDURE:

1. Dispense 0.05 mL aliquots of GEUX3 Reagent Solution into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate glucuronoyl esterase solution at 40°C for 5 min.
3. To each tube containing GEUX3 Reagent Solution (0.05 mL), add 0.05 mL of pre-equilibrated glucoronoyl esterase extract directly to the bottom of the tube. Incubate at 40°C for exactly 10 min (from time of addition).
4. At the end of the 10 min incubation period, add exactly 1.5 mL of Stopping Reagent and stir the tube contents vigorously.
5. Read the absorbance of the solutions and the reaction blank at 400 nm against distilled water.

CALCULATION OF ACTIVITY:

One Unit of activity is defined as the amount of enzyme, in the presence of excess α-glucuronidase and β-xylosidase, required to release one micromole of *p*-nitrophenol from GEUX3 per minute under the defined assay conditions, and is termed a **GEUX3 Unit** or **GEU**.

GEU/g or GEU/mL of original preparation

$$= \frac{\Delta A_{400}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\varepsilon_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight/Vol}} \times \text{Dilution}$$

where:

ΔA_{400} = Absorbance (reaction) - Absorbance (blank)

Incubation Time = 10 min

Total Volume in Cell = 1.6 mL

Aliquot Assayed = 0.05 mL

ε_{mM} of *p*-nitrophenol (at 400 nm) in 2% Tri-sodium solution = 18.1

Extraction Volume = 0.5 mL or 0.5 g per 10 mL (Original extract)

Dilution = Dilution of the original extract

APPENDIX:

A. Assay Principle

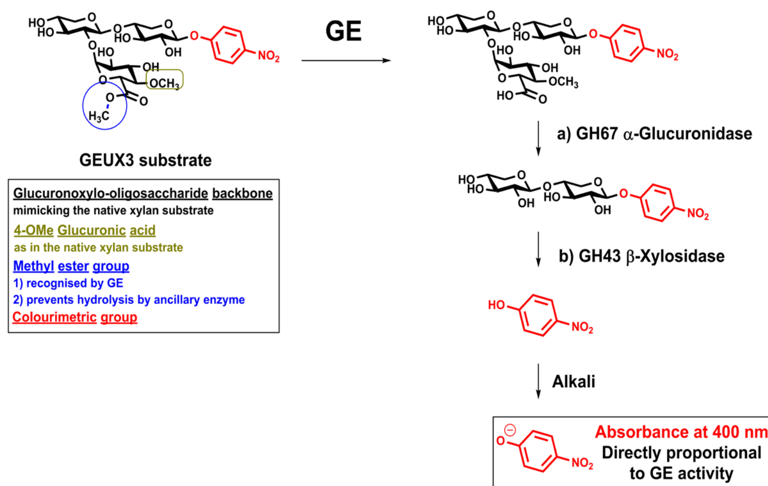

Figure 1. Theoretical basis of the GEUX3 assay procedure.

B. Linearity of the assay

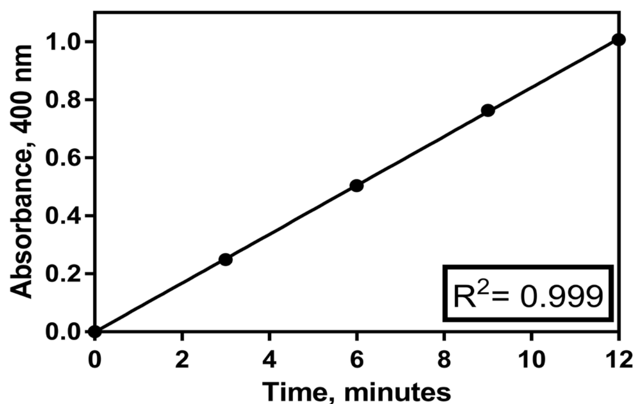

Figure 2. Linearity of the GEUX3 assay over time with glucuronoyl esterase (*R. flavefaciens*) (Megazyme cat. no. **E-GERF**) in sodium phosphate buffer (pH 6.5), containing BSA (1 mg/mL).

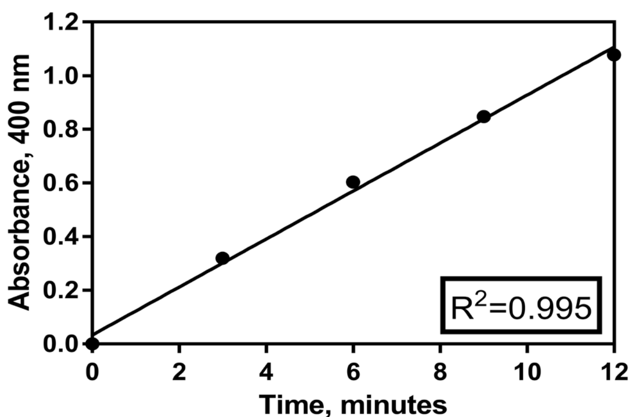

Figure 3. Linearity of the GEUX3 assay over time with an industrial enzyme preparation (Novozymes Cellic® CTec) in sodium phosphate buffer (pH 6.5).

C. Stability of the substrate

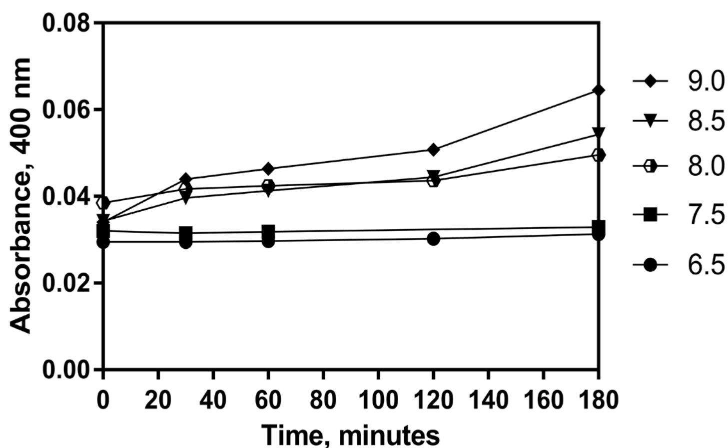

Figure 4. Stability of the GEUX3 substrate over time in various buffers across a range of pH points (pH 6.5-9.0).

D. Repeatability of the assay

	ΔAbs 400 nm		
Analysis	199 mU/mL	75 mU/mL	38.5 mU/mL
Day 1 A (i)	1.127	0.426	0.218
Day 1 A (ii)	1.102	0.418	0.217
Day 2 A (i)	1.083	0.416	0.216
Day 2 A (ii)	1.092	0.416	0.215
Day 1 B (i)	1.081	0.412	0.217
Day 1 B (ii)	1.091	0.405	0.21
Day 2 B (i)	1.102	0.415	0.212
Day 2 B (ii)	1.098	0.42	0.213
Std Dev.	0.014	0.006	0.003
% CV	**1.3**	**1.5**	**1.3**

Note: A = Analyst 1, B = Analyst 2, (i) = Extract 1, (ii) = Extract 2

Figure 5. Repeatability of GEUX3 assay with an industrial enzyme preparation (Novozymes Cellic® CTec) across a range of enzyme dilutions in sodium phosphate buffer (pH 6.5).

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