Megazyme

endo-CELLULASE

ASSAY PROCEDURE

(CELLG3 METHOD)

K-CELLG3 01/13

(180 / 360 Assays per Kit)

(EPA Number 12195471.3)

INTRODUCTION:

Cellulase (*endo*-1,4-β-glucanase) plays a key role in the hydrolysis of cellulosic biomass to fermentable sugars. This enzyme also finds widespread industrial applications in the modification of cellulosic materials and in the degradation of mixed linkage 1,3;1,4-β-glucans.

Numerous methods are available for the measurement of cellulase, including those based on increase in reducing sugar levels on hydrolysis of CM-cellulose or 1,3:1,4-β-glucan[1]. *endo*-Cellulase can be specifically assayed using viscometric methods with soluble CM-cellulose 7M as substrate, or by employing soluble or insoluble (crosslinked) dyed cellulose or mixed-linkage β-glucan. In general, assays based on the use of dyed polysaccharides are standardised against a reducing sugar method that employs either CM-cellulose or β-glucan as substrate. The problem here is that these polysaccharide substrates are not completely defined and thus lead to some variation in the values obtained.

The Megazyme **CellG3,** cellulase test reagent, employs high purity β-glucosidase and benzylidene blocked, 2-chloro-4-nitrophenyl-β-D-cellotrioside (BClPNPβ-G3). The level of β-glucosidase used ensures maximum sensitivity of the assay. On hydrolysis of BClPNPβ-G3 to benzylidene blocked cellobiose and 2-Cl-4-nitrophenyl-β-D-glucose by cellulase, the 2-Cl-4-nitrophenyl-β-D-glucose is immediately cleaved to D-glucose and free 2-Cl-4- nitrophenol (ClPNP) by the β-glucosidase present in the substrate mixture (Scheme 1). Thus, the rate of release of ClPNP relates directly to the rate of hydrolysis of BClPNPβ-G3 by cellulase. The reaction is stopped, and the phenolate colour is developed, on addition of Trizma base solution (pH 9). Standard curves relating enzyme activity to increase in absorbance at 400 nm on hydrolysis of BClPNPβ-G3 by *Trichoderma* and *A. niger* cellulases are shown in Figures 1 and 2. A standard curve relating enzyme activity on CM-cellulose 4M (Nelson/Somogyi reducing sugar assay[2]) to absorbance increase at 400 nm on incubation of *Trichoderma* cellulase with BClPNPβ-G3 is shown in Figure 3. Cellulase enzymes from different sources vary in their ability to hydrolyse BClPNPβ-G3, so it is necessary to establish a specific standard curve for each cellulase to allow accurate quantitation. The assay can be used at temperatures up to 80°C and in the pH range of 4.5 to 8.0. With cellulase enzymes with activity at high pH values, it is necessary to terminate the reaction with tri-sodium phosphate (pH 11.0).

Of the cellulases evaluated in the current study, that with the lowest rate of hydrolysis of BClPNPβ-G3 was the cellulase from *A. niger* (E-CELAN, Megazyme). BClPNPβ-G3 substrate solution as supplied in dimethyl sulphoxide (DMSO) is very stable when stored at 4°C or

-20°C. The substrate, **CellG3** is stable for at least 7 days at 4°C and for > 2 years at - 20°C.

ACCURACY:

Standard errors of less than 3% are readily achieved.

SPECIFICITY:

The assay is specific for cellulase. The substrate is not hydrolysed by β-glucosidase, cellobiohydrolase or any other enzymes tested.

KITS:

Kits suitable for performing 180 / 360 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: (x2) Benzylidene blocked, 2-chloro-4-nitrophenyl-β-D-cellotrioside (BClPNPβ-G3; 3 mL) in DMSO. Stable for > 2 years at -20°C.

Bottle 2: KCl solution (15 mL, 100 mM) plus sodium azide (0.02% w/v). Stable for approx. 4 years at 4°C.

Bottle 3: Thermostable β-glucosidase (0.40 mL, 400 U/mL) in 50% w/v ammonium sulphate solution plus sodium azide (0.02 % w/v). Stable for approx. 4 years at 4°C.

Bottle 4: *Trichoderma* cellulase standard solution (5 mL, ~ 1.6 U/mL; actual value stated on the vial label) in 50% aqueous glycerol plus sodium azide (0.02% w/v). Stable for approx. 4 years at -20°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Transfer 6 mL of the contents of bottle 2 to bottle 1, cap the bottle and mix the contents well. Transfer 3 mL aliquots of this solution to each of three 13 mL polypropylene tubes (16 x 120 mm) and store these at -20°C (stable for > 2 years). Before use of this substrate, add 50 µL of the contents of bottle 3 (after gentle swirling of contents) to the contents of one of the polypropylene tubes and mix well. This is termed **CellG3** substrate solution. Store at 4°C during use. Warm to 40°C for 5 min before dispensing to ensure complete dissolution.
Stable for > 2 weeks at 4°C. Stable for > 2 years at -20°C.

2. Using a positive displacement dispenser, accurately transfer 1 mL of the contents of bottle 4 to 9 mL of 100 mM sodium acetate buffer, pH 4.5 containing sodium azide (0.02% w/v). Stable for 2 days at 4°C. Stable for ~ 1 year at - 20°C. Stable to repeated freezing and thawing.

BUFFERS:

(A) Concentrated Acetate Buffer

(Sodium acetate buffer, 1 M, pH 4.5)

Add 60.0 g of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water. Adjust the pH of this solution to pH 4.5 by the addition of 5 M (20 g/100 mL) NaOH solution. Adjust the volume to 1 L. Stable for > 2 years at room temperature.

(B) Acetate Extraction/Dilution Buffer

(Sodium acetate buffer, 100 mM, pH 4.5 containing sodium azide, 0.0% w/v)

Add 100 mL of concentrated acetate buffer (A) to 850 mL of distilled water. Adjust the pH to pH 4.5 by dropwise addition of 2 M HCl or 2 M NaOH and adjust the volume to 1 L. Add 0.2 g of sodium azide and dissolve.
Stable for > 2 years at room temperature.

(C) Concentrated Phosphate Buffer

(Sodium phosphate buffer, 0.5 M, pH 6.0)

Add 156 g of sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) to 1.5 L of distilled water. Adjust the pH to 6.0 with 4 M NaOH and adjust the volume to 2 L. Add 0.2 g of sodium azide and dissolve. Stable for > 2 years at 4°C.

(D) Phosphate Extraction/Dilution Buffer

(Sodium phosphate buffer, 100 mM, pH 6.0)

Add 200 mL of concentrated phosphate buffer (C) to 750 mL of distilled water. Adjust the pH to 6.0 with 1 M HCl or 1 M NaOH and adjust the volume to 1 L. Add 0.2 g of sodium azide and dissolve. Stable for ~ 1 year at 4°C.

NOTE:

1. Do not add the sodium azide to the buffer until it has been adjusted to pH 4.5 or pH 6.0. Adding sodium azide to an acidic solution results in the release of a poisonous gas.
2. If diluted buffer is prepared without adding sodium azide as a preservative, then it should be stored on ice and used within a week. Alternatively, this can be stabilised against microbial contamination by storing the buffer in a well sealed Duran® bottle and adding 1 drop of toluene.

STOPPING REAGENT:

2% (w/v) Trizma base (pH 9.0)

Dissolve 20 g of Trizma base (Sigma cat. no. T-1503) in 900 mL of distilled water. Adjust the pH to 9.0 with 1 M NaOH the volume to 1 L. Stable for > 2 years at room temperature.

EQUIPMENT (RECOMMENDED):

1. Disposable 13 mL polypropylene tubes, e.g. Sarstedt cat. no. 60.541.685 PP (www.sarstedt.com).
2. Disposable plastic micro-cuvettes (1 cm light path, 3.0 or 1.5 mL), e.g. Plastibrand®, semi-micro, PMMA; Brand cat. no. 7591 15 (www.brand.de).
3. Micro-pipettors, e.g. Gilson Pipetman® (50 µL, 100 µL and 150 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip®
   - with 25 mL Combitip® (to dispense extraction buffer and 3.0 mL of Stopping Reagent).
5. Analytical balance.
6. Spectrophotometer set at 400 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Bench centrifuge or Whatman GF/A glass fibre filter paper circles.

CONTROLS AND PRECAUTIONS:

1. For each set of assays, a reagent blank value should be determined. To obtain this value, add 3.0 mL of Stopping Reagent to 0.10 mL of pre-equilibrated CellG3 reagent solution and then add 0.10 mL of diluted enzyme preparation. A single reagent blank determination is sufficient for each batch of assays.
3. If reagent blank absorbance value exceeds 0.2, then the CellG3 substrate should be discarded.
4. If reaction values exceed 1.2, then the enzyme preparation should be diluted in the appropriate buffer and re-assayed. Appropriate corrections to the calculations should then be made.
5. CellG3 is not hydrolysed by enzymes other than *endo*-cellulase (and *endo*-xylanase enzymes which have an *endo*-cellulase secondary activity), so it can be used to specifically assay for this enzyme in fermentation broths and industrial enzyme preparations. Different cellulases have different abilities to hydrolyse CellG3, so for accurate determination of enzyme activity, it is necessary to have a specific standard curve for the particular cellulase.

USEFUL HINTS:

1. The substrate should be stored frozen between use and on ice after thawing. In the form supplied (DMSO solution), the substrate mixture is stable for > 4 years at -20°C.
2. The number of assays which can be performed per kit can be doubled by halving the volumes of all the reagents used and by employing semi-micro spectrophotometer cuvettes. Do not alter the concentration of substrate in the final reaction mixture.

ASSAY PROCEDURE:

Enzyme Extraction and Dilution:

1. Add 1.0 mL of liquid enzyme preparation to 49 mL of Extraction/Dilution buffer (100 mM, pH 4.5 or 6.0) using a positive displacement dispenser (these solutions can be very viscous), and mix thoroughly. This is termed the Original Extract.
2. Add 1.0 mL of the Original Extract to 9.0 mL of Extraction/Dilution buffer (100 mM, pH 4.5 or 6.0) (10-fold dilution) and mix thoroughly. This process of dilution is repeated until a concentration of cellulase suitable for assay is achieved.
3. Alternatively, add 1.0 g of powder enzyme sample to 50 mL of Extraction/Dilution buffer (100 mM, pH 4.5 or 6.0) and gently stir the slurry over a period of approx. 15 min or until the sample is completely dispersed or dissolved. Clarify this solution by centrifugation (1,000 *g*, 10 min) or by filtration through Whatman No. 1 (9 cm) filter circles. This is termed the Original Extract.
4. Add 1.0 mL of the Original Extract to 9.0 mL of Extraction/Dilution buffer (10-fold dilution) (100 mM, pH 4.5 or 6.0) and mix thoroughly. This process of dilution is repeated until a concentration of cellulase suitable for assay is achieved.

Assay of Cellulase:

1. Dispense 0.10 mL aliquots of CellG3 substrate solution directly to the bottom of 13 mL glass tubes and pre-incubate the tubes at 40°C for approx. 3 min.
2. Pre-incubate diluted cellulase solution (approx. 5 mL) at 40°C for 3 min.
3. To each tube containing CellG3 solution, add 0.1 mL of cellulase solution to the bottom of the tube, stir on a vortex mixer and incubate tubes at 40°C for exactly 10 min (from time of addition).

4. At the end of the 10 min incubation period, add 3.0 mL of Stopping Reagent and stir the tube contents.

5. Read the absorbance of the reaction solutions and the reagent blank at 400 nm against distilled water.

CALCULATION OF ACTIVITY:

Units of cellulase per mL or g of enzyme solution being assayed:
One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable β-glucosidase, required to release one micromole of *2-chloro-4*-nitrophenol from CellG3 in one minute under the defined assay conditions, and is termed a **CellG3 Unit.**

CellG3 Units / mL of solution being assayed:

$$= \frac{\Delta A_{400}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\varepsilon_{mM}}$$

$$= \frac{\Delta A_{400}}{10} \times \frac{3.2}{0.1} \times \frac{1}{16.6}$$

$$= \Delta A_{400} \times 0.1928$$

where:

ΔA_{400} = Absorbance (sample) - Absorbance (blank)
Incubation time = 10 min
Total volume in cell = 3.25 mL (or 1.625 mL)
Aliquot assayed = 0.1 mL (or 0.05 mL)
ε_{mM} 2-Cl-4-nitrophenol = 16.6 (at 400 nm) in 2% Trizma base (pH 9)

CellG3 Units / mL or g of original enzyme preparation:

$$= \Delta A_{400} \times 0.1928 \times \frac{\text{Extraction Volume}}{\text{Sample Weight or Volume}} \times \text{Dilution}$$

$$= \Delta A_{400} \times 0.1928 \times \frac{50}{1.0} \times \text{Dilution}$$

$$= \Delta A_{400} \times 9.64 \times \text{Dilution}$$

where:

Extraction volume = 50 mL per 1.0 mL or 1.0 g of original enzyme preparation.
Dilution = Further dilution of the Original Extract.

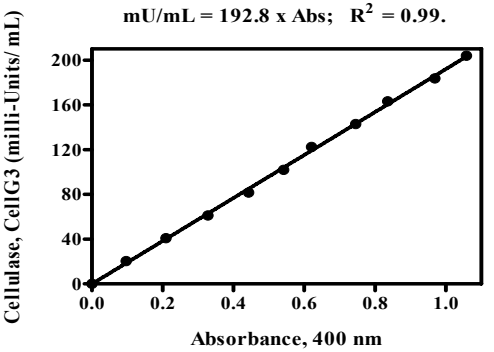


Figure 1. Standard curve relating cellulase activity on CellG3 substrate to absorbance increase at 400 nm on incubation of *Trichoderma* cellulase with CellG3. In fact, this curve relates to any cellulase which shows a linear relationship between cellulase activity (CellG3 Units) and absorbance increase at 400 nm.

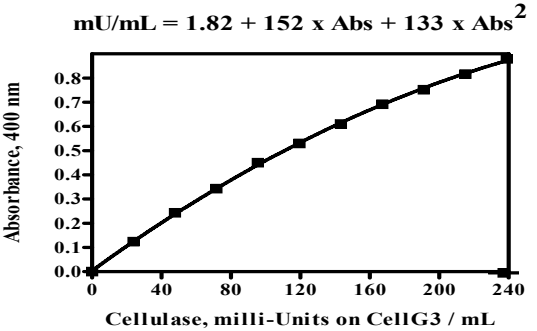


Figure 2. Standard curve relating cellulase activity on CellG3 substrate to absorbance increase at 400 nm on incubation of *Aspergillus niger* cellulase with CellG3.

For *A. niger* cellulase:
CellG3 Units / mL of solution being assayed:

$$= (0.002 + 0.152 \times \Delta A_{400} + 0.133 \times \Delta A_{400}^2) \times \frac{50}{1} \times \text{Dilution}$$

$$= (0.002 + 0.152 \times \Delta A_{400} + 0.133 \times \Delta A_{400}^2) \times 50 \times \text{Dilution}$$

where:

Extraction volume = 50 mL per 1.0 mL or 1.0 g of original enzyme preparation.

Dilution = Further dilution of the Original Extract.

NOTES ON CALCULATION OF ACTIVITY:

With cellulase enzymes that give a linear standard curve relating CellG3 Units to Absorbance increase at 400 nm, the curve and regression equation shown in Figure 1 can be universally used. Of the enzymes studied to date, this applies to cellulases from *Trichoderma longibrachiatum* (Megazyme cat. no. E-CELTR), *Bacillus amyloliquefaciens* (E-CELBA), *Thermatoga maritima* (E-CELTM) and *Talaromyces emersonii* (E-CELTE).

Producing a standard curve for a cellulase that gives a non-linear relationship between CellG3 Units and absorbance at 400 nm is more difficult. The first thing that is required is an accurate concentration of the enzyme being used. This can be done in one of two ways. If the standard curve is linear over a portion of the curve (i.e. at the lower enzyme concentrations), then the activity of the enzyme can be determined from this, and this can be used to establish the complete standard curve. In the case of *Aspergillus niger* cellulase (Megazyme cat. no. E-CELAN) for example, it can be seen from Figure 2 that the reaction is linear up to an absorbance of 0.24. This has been used to determine the concentration of the enzyme in CellG3 Units per mL of solution. This procedure can potentially be applied to other cellulases that give non-linear standard curves in the CellG3 assay.

A second approach to standardise cellulases that give non-linear CellG3 standard curves is to use an enzyme that has been standardised with another method, e.g. the Nelson-Somogyi reducing sugar method using CM-cellulose 4M as substrate.

The relative ability of a particular cellulase to hydrolyse CellG3 and CM-cellulose 4M varies considerably. Equations relating the action of several pure cellulases on CellG3 substrate and on CM-cellulose 4M (Nelson-Somogyi method) is shown below:

Trichoderma longibrachiatum (assays run at 40°C)
Units/mL on CMC-4M = 1.47 x CellG3 Units

Bacillus amyloliquefaciens (assays run at 40°C)
Units/mL on CMC-4M = 1.68 x CellG3 Units

Thermatoga maritima (assays run at 80°C)
Units/mL on CMC-4M = 1.84 x CellG3 Units

Talaromyces emersonii (assays run at 40°C)
Units/mL on CMC-4M = 23.9 x CellG3 Units

Aspergillus niger (assays run at 40°C)
Units/mL on CMC-4M = 29.8 x CellG3 Units

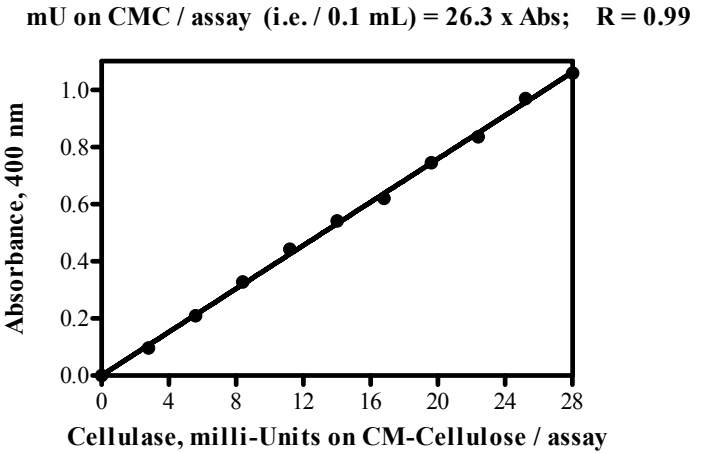
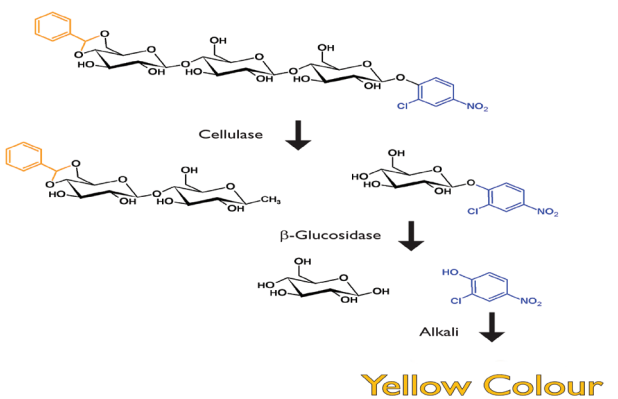


Figure 3. Standard curve relating the activity of *Trichoderma longibrachiatum* cellulase on CM-cellulose 4M (Nelson-Somogyi) to absorbance increase at 400 nm on hydrolysis of CellG3.



Scheme 1. Theoretical basis of the CellG3 cellulase assay procedure. Immediately cellulase cleaves a bond within BClPNPβ-G3, the non-blocked reaction product containing the 2-chloro-4-nitrophenyl substituent is instantly cleaved to D-glucose and free 2-chloro-4-nitrophenol. The reaction is terminated and the Cl-phenolate colour is developed on addition of Trizma base solution (pH ~ 9.0).

REFERENCES:

1. McCleary, B. V., McKie, V. and Draga, A. (2012). Measurment of *endo*-1,4-β glucanase. In **Methods in Enzymology** (Gilbert, H. J., Ed.), **Vol. 510**, pp 1-17.

2. Somogyi, M. (1952). Note on sugar determination. **J. Biol. Chem. 195**, 19-23.

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