

Megazyme

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endo-CELLULASE

ASSAY PROCEDURE

(CellG3 METHOD)

K-CellG3 08/18

(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 associated with these assays is that the polysaccharide substrates are not completely defined and thus lead to some variation in the activity values obtained.

The development of the Megazyme **CellG3** cellulase assay kit was reported in 2012.² It employs high purity β -glucosidase and benzylidene blocked 2-chloro-4-nitrophenyl- β -D-cellobioside (BCNPG3). The level of β -glucosidase used ensures maximum sensitivity of the assay. On hydrolysis of BCNPG3 to benzylidene blocked cellobiose and 2-chloro-4-nitrophenyl- β -D-glucose by cellulase, the 2-chloro-4-nitrophenyl- β -D-glucose is immediately cleaved to D-glucose and free 2-chloro-4-nitrophenol (CNP) by the β -glucosidase present in the substrate mixture (Scheme 1, page 11). The reaction is stopped, and the phenolate colour developed, on addition of Tris solution (pH 9.0). The rate of release of CPNP relates directly to the rate of hydrolysis of BCNPG3 by cellulase.

Since the launch of the CellG3 assay kit, Megazyme has developed a new assay kit based on the same principle, namely CellG5 (**K-CellG5**). The CellG5 reagent contains a cellopentaose core and exhibits vastly improved sensitivity for certain cellulases. In addition, the exchange of the benzylidene blocking group in CellG3 for 3-keto-butyridene in CellG5 which improves the substrate's water solubility significantly. This allows for a reduction in the concentration of DMSO required in the assay which is a relevant parameter as DMSO is known to inhibit certain cellulases. Megazyme now recommends the use of **K-CellG5** for all routine assays for the measurement of *endo*-cellulase.

Standard curves relating enzyme activity on CM-cellulose 4M (Nelson/Somogyi reducing sugar assay)³ to increase in absorbance at 400 nm on hydrolysis of CellG3 and CellG5 reagents by *Trichoderma* and *A. niger* cellulases are shown in Figure 1 (page 8). As cellulase enzymes

from different sources vary in their ability to hydrolyse CellG3, 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 60°C and in the pH range of 4.5 to 8.0. In the case of cellulase enzymes exhibiting activity at high pH values, it is necessary to terminate the reaction with Tris solution (pH 10.0).

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.

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 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-cellobioside (BCNPG3; 3 mL) in DMSO. Stable for > 2 years below -10°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* sp. 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 below -10°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 below -10°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 or stable for > 2 years below -10°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 or stable for ~ 1 year below -10°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 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, 0.02% w/v)

Add 100 mL of concentrated acetate buffer (A) to 850 mL of distilled water. Adjust the pH to 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 ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) 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 containing sodium azide)

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) Tris buffer (pH 9.0)

Dissolve 20 g of Tris buffer salt (**B-TRIS500**) in 900 mL of distilled water. Adjust the pH to 9.0 with 1 M NaOH and 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. 759115 (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 3.00 mL of Stopping Reagent to 0.10 mL of pre-equilibrated CellG3 reagent solution and then add 0.10 mL of diluted enzyme preparation.
2. If reagent blank absorbance value exceeds 0.2, then the CellG3 substrate should be discarded.
3. 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.
4. 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 below -10°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 suitable concentration of cellulase 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 suitable concentration of cellulase 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 0.10 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**.

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

$$= \frac{\Delta A_{400}}{10} \times \frac{3.2}{0.1} \times \frac{1}{16.6}$$
$$= \Delta A_{400} \times 0.1928$$

CellG3 Units/mL of solution being assayed:

where:

- ΔA_{400} = Absorbance (sample) - Absorbance (blank)
Incubation time = 10 min
Total volume in cell = 3.2 mL (or 1.6 mL)
Aliquot assayed = 0.1 mL (or 0.05 mL)
 ϵ_{mM} (CNP) = 16.6 (at 400 nm) in 2% Tris buffer (pH 9.0)

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

APPENDIX:

A. Comparison between CellG3 and CellG5 assays

A comparison between the absorbance obtained in the CellG3 and CellG5 assays for a range of cellulase activities (determined by Nelson/Somogyi reducing sugar assay³ on CM-cellulose 4M) is shown in Figure 1. The relative ability of a particular cellulase to hydrolyse CellG3 and CMC-4M varies considerably. *A. niger* cellulase for example displays relatively low hydrolytic activity on CellG3 compared to CMC-4M. This is expected as *A. niger* cellulase is known to hydrolyse short chain cellooligosaccharides (< DP5) at a very low rate.¹ As CellG5 contains a cellopentaose core, this problem is alleviated. In fact, the relative hydrolytic activity on CellG5 and CMC-4M is very similar for each of the cellulase enzymes (*Trichoderma longibrachiatum*, *Bacillus amyloliquefaciens*, *Aspergillus niger* and *Thermotoga maritima*) investigated to date. For this reason, Megazyme now recommends **K-CellG5** over K-CellG3 for all routine assays of *endo*-cellulase. Conversely, the large difference in relative hydrolytic activity observed between CellG3 and CMC-4M for different cellulases may prove useful for the screening, identification and characterisation of *endo*-cellulases of unknown origin.

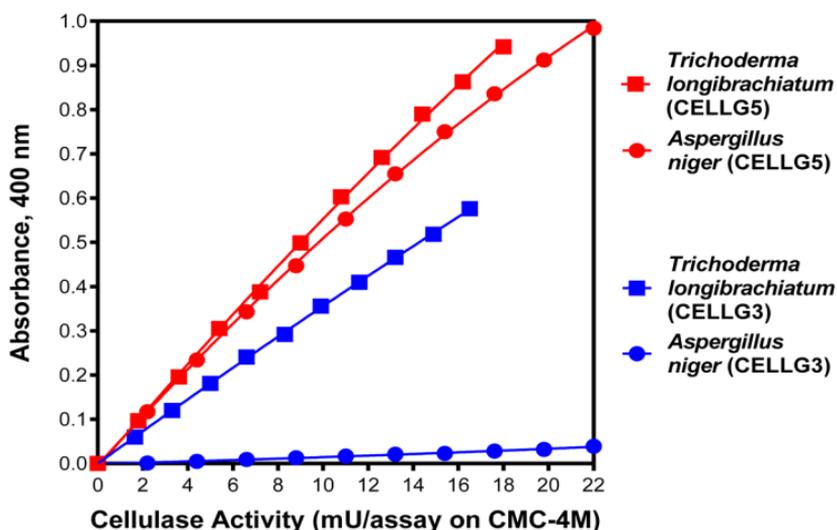


Figure 1. Standard curves relating cellulase activity on CM-cellulose to absorbance increase in CellG3 and CellG5 assays upon incubation with *Trichoderma longibrachiatum* and *Aspergillus niger* cellulases.

B. Notes on calculation of activity using CellG3

Figure 2 shows the standard curve relating *Trichoderma longibrachiatum* cellulase activity on CellG3 to absorbance obtained in the CellG3 assay.

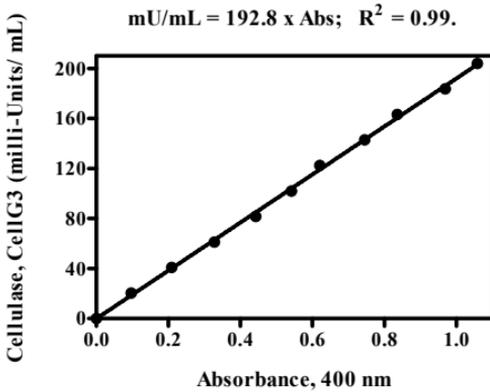


Figure 2. Standard curve relating cellulase activity on CellG3 substrate to absorbance increase at 400 nm on incubation of *Trichoderma* cellulase with CellG3. In fact, this curve relates to any cellulase which shows a linear relationship between cellulase activity (CellG3 Units) and absorbance increase at 400 nm.

Note that for *Aspergillus niger* cellulase, the relationship between cellulase activity (CellG3 Units) and absorbance at 400 nm is non-linear as shown in Figure 3.

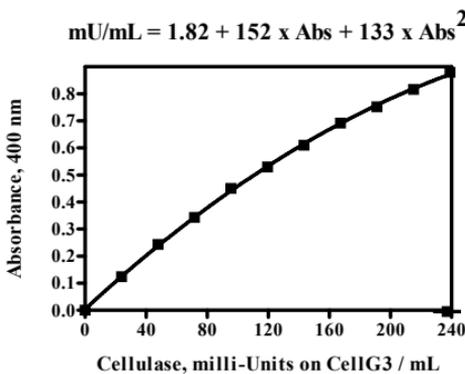


Figure 3. Standard curve relating cellulase activity on CellG3 substrate to absorbance increase at 400 nm on incubation of *Aspergillus niger* cellulase with CellG3.

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

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

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

where:

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

Dilution = Further dilution of the Original Extract

C. Notes on conversion of cellulase activity from CellG3 Units to Standard Units on CMC-4M

A standard curve relating the action of *Trichoderma longibrachiatum* cellulase on CMC-4M to absorbance obtained at 400 nm in the CellG3 assay is shown in Figure 4.

mU on CMC / assay (i.e. / 0.1 mL) = 26.3 x Abs; R = 0.99

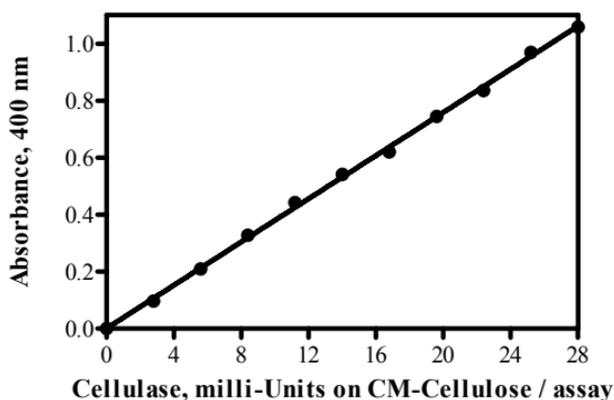


Figure 4. Standard curve relating the activity of *Trichoderma longibrachiatum* cellulase on CM-cellulose 4M (Nelson-Somogyi) to absorbance increase at 400 nm on hydrolysis of CellG3.

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) are 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

Thermotoga 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

D. Thermal stability of CellG3 reagent

Figure 5 shows the increase in the blank absorbance of CellG3 reagent solution (following addition of 3.0 mL Stopping Reagent) after incubation with 0.1 mL assay buffer (sodium acetate pH 5) for 10 min at a range of temperatures from 40°C to 90°C. The absorbance value increase of 0.075 obtained for the 90°C incubation corresponds to only 5% total substrate degradation. This experiment shows that the CellG3 assay can be used at temperatures up to at least 80°C provided that assay incubation times not longer than 10 min are employed.

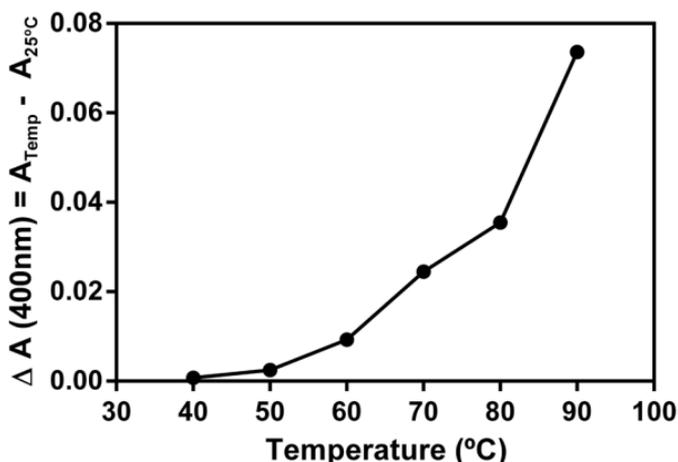
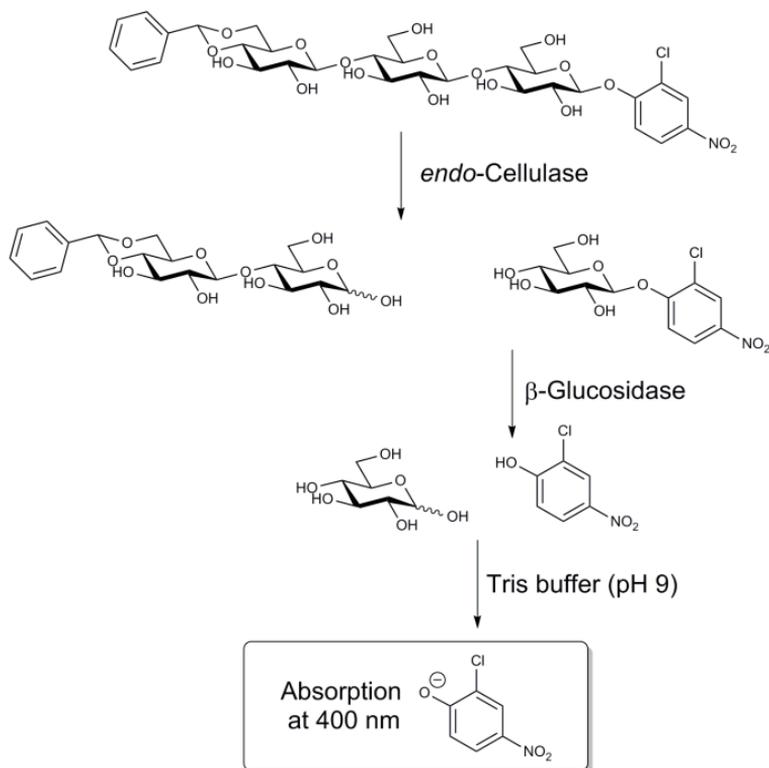


Figure 5. Substrate blank absorbance increase following 10 min incubation at a range of temperatures.

E. Principle of the assay



Scheme 1. The CellG3 assay reagent for the measurement of *endo*-cellulase (*endo*-1,4-β-glucanase) contains two components; 1) 4,6-O-benzylidene-2-chloro-4-nitrophenyl-β-D-cellobioside (BCNPG3) and 2) thermostable β-glucosidase. The benzylidene blocking group prevents any hydrolytic action by the β-glucosidase on BCNPG3. Incubation with an *endo*-cellulase generates a non-blocked colourimetric oligosaccharide that is rapidly hydrolysed by the ancillary β-glucosidase. The rate of formation of 2-chloro-4-nitrophenol is therefore directly related to the hydrolysis of BCNPG3 by the *endo*-cellulase. The reaction is terminated and the phenolate colour developed on addition of Tris buffer solution (pH 9.0).

REFERENCES:

1. McCleary, B. V., McKie, V. & Draga, A. (2012). Measurement of *endo*-1,4-β glucanase. "Methods in Enzymology" (Gilbert, H. J., Ed.), Vol. 510, pp 1-17.
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3. Somogyi, M. (1952). Note on sugar determination. *J. Biol. Chem.*, 195, 19-23.



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