Megazyme

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MALT β-GLUCANASE / LICHENASE

ASSAY PROCEDURE

(MBG4 METHOD)

K-MBG4 08/18

Manual
(100 β-Glucanase Assays per Kit) or
(100/200 Lichenase Assays per Kit)

Auto-Analyser
(400 β-Glucanase Assays per Kit) or
(330 Lichenase Assays per Kit)

INTRODUCTION:

Mixed linkage β-glucanase (*endo*-1,3:1,4-β-glucanase, EC 3.2.1.73) is the enzyme responsible for the degradation of β-glucan found in cereal cell walls. It is responsible for the hydrolysis of 1,4-β glycosidic bonds bearing a 1,3-β linked glucosyl residue on their non-reducing side. This enzyme is particularly important to the malting and brewing industries as elevated levels of β-glucan in wort can cause filtration problems.[1] Lichenase (EC 3.2.1.73) exhibits the same mode of action as mixed linkage β-glucanase but is found in microbial organisms. In recent years, the potential for the application of lichenase in the animal feed, detergent, paper and pulp industries has grown substantially.[2]

Numerous methods are available for the measurement of these enzymes, including those based on increase in reducing sugar levels[3] on hydrolysis of 1,3:1,4-β-glucan (e.g. **P-BGBM**). Dyed soluble or insoluble (crosslinked) mixed-linkage β-glucan[4,5] (**S-ABG100**, **I-AZBGL**, **T-BGZ**) can also be used for the convenient measurement of these enzymes. However, a defined substrate would be more desirable as any possibility of batch to batch variability would be eliminated. In addition, a soluble colourimetric substrate would be particularly useful for application in automated analysis assay systems where the use of dyed polysaccharide substrates is not possible due to the filtration step required in these assay procedures.

The **MBG4** test reagent described herein meets these requirements. Based on simple colourimetric substrates originally described by Planas *et al.,*[6] Megazyme has developed a novel substrate for the measurement of mixed linkage β-glucanase/lichenase, namely 4,6-*O*-benzylidene-2-chloro-4-nitrophenyl-β-(3[1]-β-D-cellotriosyl-glucoside) (BCNPBG4). The rate of release of CNP relates directly to the rate of hydrolysis of BCNPBG4 by mixed linkage β-glucanase/lichenase. The reaction is stopped and the phenolate colour is developed by addition of Tris buffer solution (pH 10). This substrate exhibits the distinct advantage over the previously described substrates in that the benzylidene acetal acts as a "blocking group" preventing hydrolysis by *exo*-acting enzymes including β-glucosidase and cellobiohydrolase which commonly occur in crude sample extracts. The reagent is stable up to 70°C.

Two distinct assay procedures are described here: (1) for malt β-glucanase, which includes the extraction of β-glucanase from malt flour and (2) for lichenase. Standard curves, assay linearity and reproducibility data are all shown in the appendices (Page 13).

ACCURACY:

Standard errors of less than 3% are readily achieved (see Tables 1-3 in Appendices F and H, page 17, 18, 20 and 21).

SPECIFICITY:

The assay is specific for β-glucanase/lichenase and the substrate is not hydrolysed by β-glucosidase or cellobiohydrolase. The substrate can be hydrolysed by certain *endo*-1,4-β-glucanases but this will not result in an increase in absorbance unless β-glucosidase is also present in the sample. In any case, the *endo*-1,4-β-glucanase activity in malt extracts is negligible.

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:

Assay kits are available from Megazyme containing:

Bottle 1: (x2)

Each vial contains 4,6-*O*-benzylidene-2-chloro-4-nitrophenyl-β-(3^1-β-D-cellotriosyl-glucose) (BCNPBG4) in 50% DMSO/H_2O (5 mL) plus sodium azide (0.02% w/v). Stable for > 4 years below -10°C.

Bottle 2:

Malt flour of standardised malt β-glucanase activity (~ 5 g, ~ 0.12 MBG4 U/g; actual value stated on the vial label). It is recommended that the kit user standardises at least one batch of user's own malt against the enclosed malt flour, to be employed as a secondary reference malt. Stable for > 4 years at room temperature.

Bottle 3:

Bacillus sp. lichenase standard solution (5 mL, ~ 1.34 MBG4 U/mL; actual value stated on the vial label) in 50% aqueous glycerol plus sodium azide (0.02% w/v) and BSA (0.05% w/v). Stable for > 2 years below -10°C.

PREPARATION OF REAGENT SOLUTIONS:

1. The MBG4 reagent solution is used as provided. Heat vial to 60°C for 10 minutes prior to use. Store in aliquots of 1 mL below -10°C. Stable for up to 72 h at room temperature.
2. Extraction of the β-glucanase present in the malt flour standard is performed as per the β-glucanase extraction procedure described on page 5. No further dilution is required.
3. With a positive displacement pipette, dispense 0.5 mL of the contents of bottle 3 to 9.5 mL of **Buffer D** and mix well. Once diluted, the standard is stable for at least 2 months below -10°C.

BUFFERS:

(A) Concentrated Acetate Buffer

(Sodium acetate buffer, 1 M, pH 4.5)

Add 60 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) Malt β-Glucanase Extraction/Dilution Buffer

(Sodium acetate buffer, 100 mM, pH 4.5 containing 0.02% w/v sodium azide)

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 > 1 year at 4°C.

(C) Concentrated Phosphate Buffer

(Sodium phosphate buffer, 0.5 M, pH 6.5)

Add 78 g of sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$) to 800 mL of distilled water. Adjust the pH to 6.5 with 4 M NaOH and adjust the volume to 1 L.
Stable for > 1 year at 4°C.

(D) Phosphate Extraction/Dilution Buffer

(Sodium phosphate buffer, 100 mM, pH 6.5 containing 0.02% sodium azide)

Add 200 mL of concentrated phosphate buffer (C) to 750 mL of distilled water. Adjust the pH to 6.5 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 6.5. Adding sodium azide to strongly acidic solutions can result 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 at 4°C 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 10.0)

Dissolve 20 g of Tris buffer salt (**B-TRIS500**) in 900 mL of distilled water. Adjust the pH to 10.0 with 1 M NaOH and adjust the volume to 1 L. Stable for > 2 years at room temperature.

EQUIPMENT (RECOMMENDED):

1. Disposable plastic micro-cuvettes (1 cm light path, 1.5 or 3.0 mL), e.g. Plastibrand®, semi-micro, PMMA; Brand cat. no. 759115 (www.brand.de).
2. Micro-pipettors, e.g. Gilson Pipetman® (50 µL and 100 µL).
3. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.1 mL substrate solutions).
   - with 25 mL Combitip® (to dispense extraction buffer and 3.0 mL of Stopping Reagent).
4. Analytical balance.
5. Spectrophotometer set at 400 nm.
6. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
7. Stop clock.
8. Bench centrifuge or Whatman GF/A glass fibre filter paper circles.

CONTROLS, PRECAUTIONS AND BLANKS:

General

1. Store the malt β-glucanase extract on ice if there is any delay prior to assay. The extract must be assayed within 1 h.
2. A control should be run with each set of assays using either the malt flour or lichenase standard provided as applicable.
3. If reaction absorbance values (after blank subtraction) exceed 1.2 in the malt β-glucanase/lichenase assay, then the enzyme preparation should be diluted in the appropriate buffer and re-assayed. Appropriate corrections to the calculations should then be made.

Malt β-glucanase blanks

1. For each set of assays, a single **reagent blank** value should be determined. To obtain the reagent blank value add 0.9 mL of Stopping Reagent to a solution of 0.1 mL of pre-equilibrated MBG4 reagent plus 0.5 mL Buffer B. Read absorbance at 400 nm.

2. If the reagent blank absorbance value exceeds 0.4, then the MBG4 substrate solution should be discarded.

3. Each individual sample also requires a **malt blank** to account for the colour present in the malt extract. To obtain the malt blank value, add 0.9 mL of Stopping Reagent to a solution of 0.1 mL of Buffer B plus 0.5 mL of the specific malt extract. Read absorbance at 400 nm.

4. The reagent blank value is added to the malt blank value to give the total blank to be subtracted from reaction absorbances.

Lichenase blanks

1. A single reaction blank determination is usually sufficient for each batch of lichenase assays. To obtain a reaction blank, the 3.0 mL of Stopping Reagent should be added to 0.2 mL of the lichenase solution to be assayed **prior to addition of 0.1 mL of MBG4 reagent**.

2. If reagent blank absorbance value exceeds 0.2, then the MBG4 substrate solution should be discarded.

USEFUL HINTS:

1. The substrate solution (Bottle 1) should be aliquoted into appropriately sized vials and stored below -10°C. Each of these vials can be thawed prior to use as required to maximise the long term stability of the reagent. Ensure that the solution has reached room temperature before use in the assay.

2. The total number of lichenase assays which can be performed can be doubled by halving the volumes of all of the reagents used and by employing 1.5 mL semi-micro spectrophotometer cuvettes.

A. MANUAL ASSAY PROCEDURE:

a. Assay of β-Glucanase Activity in Malt Flour

(i) Enzyme Extraction and Dilution:

1. Mill malt (approx. 20 g sample) to pass a 0.5 mm screen using a Tecator Cyclotec® mill or equivalent.

2. Accurately weigh 0.5 g samples of malt flour into glass centrifuge tubes (14 x 120 mm; 17 mL capacity).

3. Add 8.0 mL of Extraction Buffer Solution B (100 mM sodium acetate, pH 4.5) to each tube and stir the contents thoroughly on a vortex mixer.

4. Allow the enzyme to extract over a 15 min period at room temperature (less than 30°C) with occasional mixing.

5. a) Filter the turbid suspension through glass fiber filter paper to obtain the malt β-glucanase extract solution **or**
   b) centrifuge the tubes and contents at 3,000 x *g* for 5 min and **carefully** pipette off the malt β-glucanase extract solution.

(ii) Manual Assay of Malt β-Glucanase Extract:

1. Dispense 0.1 mL aliquots of MBG4 substrate solution directly to the bottom of 13 mL glass tubes and pre-incubate the tubes at 30°C for approx. 3 min.
2. Pre-incubate the malt β-glucanase extract solution at 30°C for 3 min.
3. To each tube containing MBG4 solution, add 0.5 mL of malt β-glucanase extract solution to the bottom of the tube, stir on a vortex mixer and incubate tubes at 30°C for exactly 20 min (from time of addition).
4. At the end of the 20 min incubation period, add 0.9 mL of Stopping Reagent and stir the tube contents.
5. Read the absorbance of the reaction solutions at 400 nm. Substract the total blank (reagent blank plus specific malt blank) to calculate the absorbance change produced by hydrolysis of MBG4.

CALCULATION OF ACTIVITY:

Units of β-glucanase per mL or g of enzyme solution being assayed:
One Unit of activity is defined as the amount of enzyme required to release one micromole of 2-chloro-4-nitrophenol (CNP) from MBG4 in one minute under the defined assay conditions, and is termed an **MBG4 Unit.**

MBG4 Units/mL or g of original enzyme preparation:

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

where:

ΔE_{400}	=	$E_{400(\text{Reaction})} - [E_{400(\text{Reagent blank})} + E_{400(\text{Malt blank})}]$
Total volume in cell	=	1.5 mL
Aliquot assayed	=	0.5 mL
ε_{mM} CNP	=	16.6 (at 400 nm) in 2% Tris buffer (pH 10)
Extraction volume	=	8 mL per 0.5 g of malt flour
Dilution	=	Dilution of the original extract (if required)

Thus:

MBG4 Units/g of malt flour:

$$= \frac{\Delta E_{400}}{20} \times \frac{1.5}{0.5} \times \frac{1}{16.6} \times \frac{8}{0.5} \times \text{Dilution}$$

$$= \Delta E_{400} \times 0.145 \times \text{Dilution}$$

Note that malt β-glucanase units on barley β-glucan can be converted to MBG4 Units or vice versa using:

Units on barley β-glucan = 3.98 x MBG4 Units

A standard curve is provided and this conversion discussed in Appendix D, Figure 3, page 15.

b. Assay of Lichenase

(i) Enzyme Extraction and Dilution:

1. Add 1.0 mL of liquid enzyme preparation to 49 mL of Phosphate Extraction/Dilution Buffer D (100 mM, pH 6.5) 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 Phosphate Extraction/Dilution Buffer D (100 mM, pH 6.5) (10-fold dilution) and mix thoroughly. This process of dilution is repeated until a suitable concentration of lichenase for assay is achieved.
3. Alternatively, add 1.0 g of powder enzyme sample to 50 mL of Phosphate Extraction/Dilution Buffer D (100 mM, pH 6.5) 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 Phosphate Extraction/Dilution Buffer D (10-fold dilution) (100 mM, pH 6.5) and mix thoroughly. This process of dilution is repeated until a suitable concentration of lichenase for assay is achieved.

(ii) Assay of Lichenase:

1. Dispense 0.10 mL aliquots of MBG4 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 lichenase solution at 40°C for 3 min.
3. To each tube containing MBG4 solution, add 0.2 mL of lichenase 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 lichenase per mL or g of enzyme solution being assayed:
One Unit of activity is defined as the amount of enzyme required to release one micromole of 2-chloro-4-nitrophenol (CNP) from MBG4 in one minute under the defined assay conditions, and is termed an **MBG4 Unit.**

MBG4 Units/mL or g of original enzyme preparation:

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

where:

Total volume in cell	=	3.3 mL
Aliquot assayed	=	0.2 mL
ε_{mM} CNP	=	16.6 (at 400 nm) in 2% Tris buffer (pH 10)
Extraction volume	=	50 mL per 1.0 mL or 1.0 g of original enzyme preparation
Dilution	=	Further dilution of the original extract (if required)

Thus:

MBG4 Units/mL or g of original enzyme preparation:

$$= \frac{\Delta E_{400}}{10} \times \frac{3.3}{0.2} \times \frac{1}{16.6} \times \frac{50}{1} \times \text{Dilution}$$

$$= \Delta E_{400} \times 4.97 \times \text{Dilution}$$

Note that MBG4 Units can be converted to lichenase Units on barley β-glucan or vice versa using:

Units on barley β-glucan = 32.61 x MBG4 Units (*Bacillus* sp.)

or

Units on barley β-glucan = 25.30 x MBG4 Units (*C. thermocellum*)

Standard curves are provided and these conversions discussed in Appendix E, Figures 4 and 5, page 16.

B. AUTO-ANALYSER ASSAY PROCEDURE:

EQUIPMENT (RECOMMENDED):

1. ChemWell®-T auto-analyser fitted with a 405 nm filter.
2. Polypropylene tubes (13 mL capacity).
3. Pipettors, 1 mL (e.g. Gilson Pipetman®) to dispense enzyme extract.
4. Adjustable-volume dispenser:
   - 0-10 mL (for Extraction Buffer).
5. Top-pan balance.
6 Vortex mixer (optional).
7. Stop Clock.
8. Microfuge or Whatman GF/A glass fibre filter paper circles (9 cm diameter).

PREPARATION OF REAGENTS:

All reagents are prepared as described above for the manual assay procedure.

a. Extraction and Assay of β-Glucanase Activity in Malt Flour

1. The β-glucanase extract of malt flour is prepared as described above for the manual assay procedure.
2. Perform the assay using the **K-MBG4 (SAMPLE), K-MBG4 (BLANK)** ChemWell®-T assay files and the **K-MBG4 (CALC)** ChemWell®-T indices file.

Automated Assay Parameters:

Assay volumes:	MBG4 Reagent:	0.025 mL
	Sample (extract):	0.125 mL
	Stopping Reagent:	0.250 mL
Reaction time:	10 min at 37°C	
Wavelength:	405 nm	

Assay type: Stopped reaction
Reaction direction: Increase

CALCULATION OF ACTIVITY (Automated Assay Procedure):

One Unit of activity is defined as the amount of enzyme required to release one micromole of 2-chloro-4-nitrophenol (CNP) from MBG4 in one minute under the defined assay conditions, and is termed an **MBG4 Unit.**

MBG4 Units/mL or g of original enzyme preparation:

$$= \frac{\Delta E_{405}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\varepsilon_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight}} \times \text{Dilution}$$

where:

ΔE_{405} = Absorbance (reaction) - Absorbance (blank)
Incubation Time = 10 min
Total Volume in Cell = 0.400 mL
Aliquot Assayed = 0.125 mL
Apparent ε_{mM} of CNP (at 405 nm) in 2% Tris buffer pH 10 = 12.456
Extraction volume = 8 mL per 0.5 g
Dilution = Dilution of the original extract (if required)

Thus:
MBG4 Units/mL or g of original enzyme preparation =

$$= \frac{\Delta E_{405}}{10} \times \frac{0.400}{0.125} \times \frac{1}{12.456} \times \frac{8}{0.5} \times \text{Dilution}$$

$$= \Delta E_{405} \times 0.411 \times \text{Dilution}$$

NOTE 1:
The absorption coefficient (ε_{mM}) of 12.456 was determined experimentally from the absorbance obtained using a 50 µM solution of 2-chloro-4-nitrophenol (CNP) in 2% Tris buffer pH 10 in a ChemWell®-T auto-analyser system.

NOTE 2:
A factor is required to convert the activity value in MBG4 units obtained on the ChemWell®-T auto-analyser system to MBG4 units obtained in the manual assay (which is the value stated on the control vial label). This arises from the incubation temperature difference in these assays.

Malt β-glucanase $MBG4_{CW}$ Units = MBG4 Units x 1.25

b. Assay and extraction of Lichenase Activity

1. The Original Extract is prepared as described above for the manual assay procedure.
2. Perform the assay using the **K-MBG4 (LICHENASE)** ChemWell®-T assay file.

Automated Assay Parameters:

Assay volumes:	MBG4 Reagent:	0.03 mL
	Sample (extract):	0.06 mL
	Stopping Reagent:	0.30 mL
Reaction time:	10 min at 37°C	
Wavelength:	405 nm	
Assay type:	Stopped reaction	
Reaction direction:	Increase	

CALCULATION OF ACTIVITY (Automated Assay Procedure):

One Unit of activity is defined as the amount of enzyme required to release one micromole of 2-chloro-4-nitrophenol (CNP) from MBG4 in one minute under the defined assay conditions, and is termed a **MBG4 Unit.**

MBG4 Units/mL or g of original enzyme preparation:

$$= \frac{\Delta E_{405}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\varepsilon_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight}} \times \text{Dilution}$$

where:

ΔE_{405} = Absorbance (reaction) - Absorbance (blank)

Incubation Time = 10 min

Total Volume in Cell = 0.39 mL

Aliquot Assayed = 0.06 mL

Apparent ε_{mM} of CNP (at 405 nm) in 2% Tris Buffer = 12.456

Extraction volume = 50 mL per 1 mL or 1 g

Dilution = Further dilution of the original extract (if required)

Thus:

MBG4 Units/mL or g of original enzyme preparation =

$$= \frac{\Delta E_{405}}{10} \times \frac{0.39}{0.06} \times \frac{1}{12.456} \times \frac{50}{1} \times \text{Dilution}$$

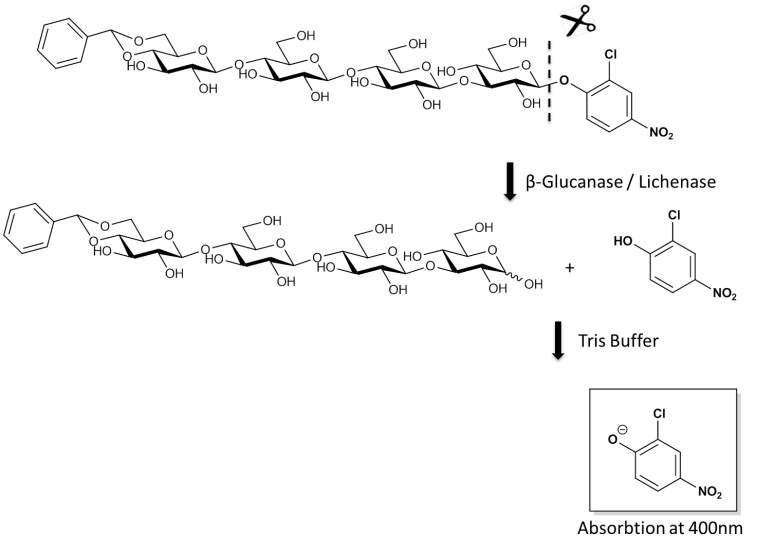
$$= \Delta E_{405} \times 2.609 \times \text{Dilution}$$

NOTE 1:
The absorption coefficient (ε_{mM}) of 12.456 was determined experimentally from the absorbance obtained using a 50 µM solution of 2-chloro-4-nitrophenol (CNP) in 2% Tris buffer in a ChemWell®-T auto-analyser system.

APPENDIX:

GENERAL INFORMATION

A. Principle of the assay



Scheme 1. Theoretical basis of the MBG4 cellulase assay procedure.

The MBG4 reagent contains a single substrate, namely 4,6-*O*-benzylidene-2-chloro-4-nitrophenyl-β-(3[1]-β-D-cellotriosyl-glucoside) (BCNPBG4). The benzylidene acetal group prevents any hydrolytic action by *exo*-acting hydrolytic enzymes such as β-glucosidase or cellobiohydrolase. Mixed linkage β-glucanase (*endo*-1,3:1,4)-β-glucanase)/lichenase (EC 3.2.1.73) acts specifically to release 2-chloro-4-nitrophenol (CNP) from this substrate. The rate of release of CNP is directly related to the β-glucanase/lichenase activity in a sample. The reaction is terminated and the phenolate colour is developed on addition of Tris buffer solution (pH = 10.0).

B. Linearity

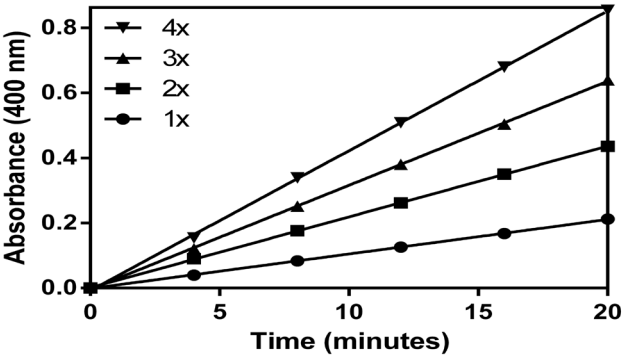


Figure 1. Linearity of MBG4 assay with malt β-glucanase in sodium acetate buffer (pH 4.5). Reaction was terminated at 4 min intervals by adding 2% w/v Tris buffer solution (0.9 mL, pH 10).

C. Thermal stability of MBG4 reagent

The stability of the MBG4 reagent solution was determined by incubating 0.1 mL aliquots of this solution at temperatures up to 70°C for 20 min before addition of 3 mL Tris buffer and reading the absorbance at 400 nm. The reagent can be considered completely stable up to 70°C. If performing assays above 70°C (e.g. on thermostable lichenases), it is recommended that a reagent blank is incubated under the desired assay conditions to allow for subtraction of the absorbance corresponding to spontaneous hydrolysis.

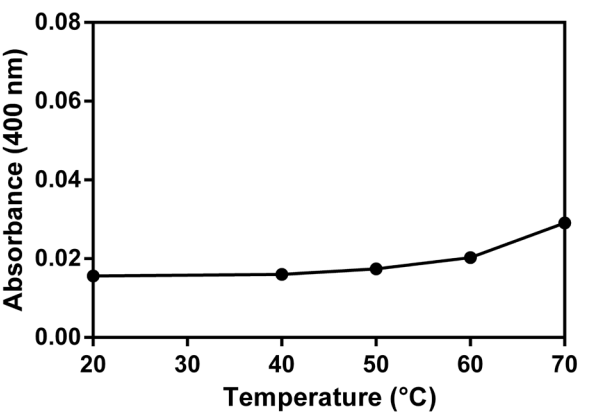


Figure 2. Investigation into the thermal stability of MBG4 reagent.

MANUAL ASSAYS

D. Activity relationship between hydrolysis of MBG4 reagent and barley β-glucan by malt β-glucanase

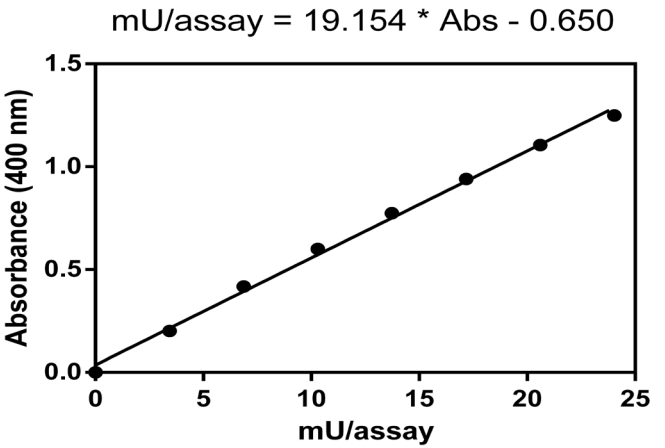


Figure 3. Standard curve relating the activity of malt β-glucanase on barley β-glucan to absorbance increase at 400 nm on hydrolysis of MBG4 reagent under the standard assay conditions.

Malt β-glucanase Units (barley β-glucan)/g:

$$= \text{mUnits/assay (i.e. per 0.5 mL)} \times \frac{1}{1000} \times \frac{8}{0.5} \times 2 \times \text{Dilution}$$

where:

mU/assay = 19.154 x (absorbance) - 0.650. (Figure 2)

1000 = conversion from milli-Units to Units.

$\frac{8}{0.5}$ = initial extraction volume (i.e. 8 mL per 0.5 g of solid).

2 = conversion from volume assayed (0.5 mL) to 1 mL of extract.

Dilution = further dilution of the initial extraction solution.

E. Activity relationship between hydrolysis of MBG4 reagent and barley β-glucan by lichenase

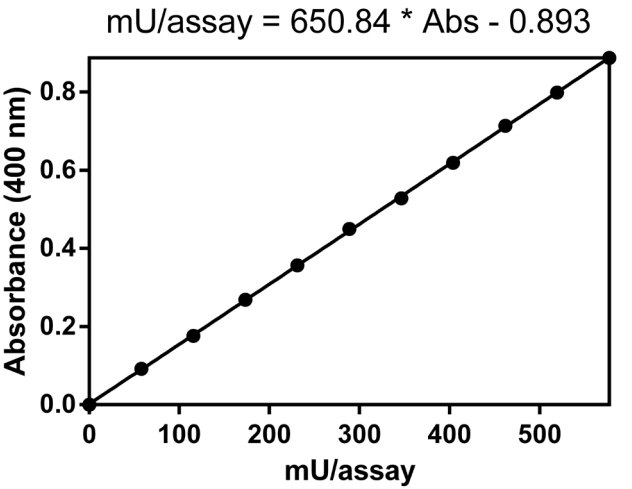


Figure 4. Standard curve relating the activity of *Bacillus* sp. lichenase (**E-LICHN**) on barley β-glucan to absorbance increase at 400 nm on hydrolysis of MBG4 reagent under the standard assay conditions.

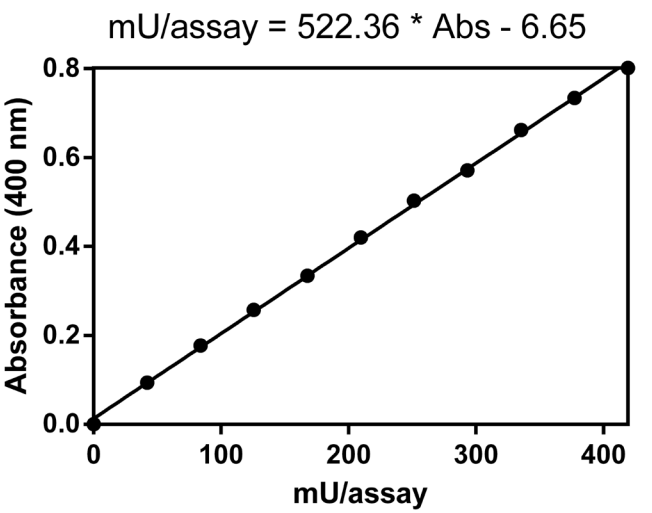


Figure 5. Standard curve relating the activity of *Clostridium thermocellum* non-specific *endo*-1,3(4)-β-glucanase (**E-LICACT**) on barley β-glucan to absorbance increase at 400 nm on hydrolysis of MBG4 reagent under the standard assay conditions.

Lichenase Units/mL on barley β-glucan:

$$= \text{mUnits/assay (i.e. per 0.2 mL)} \times \frac{1}{1000} \times 50 \times 5 \times \text{Dilution}$$

where:

mU/assay = 650.84 x (Absorbance) - 0.893 (Fig 4: *Bacillus* sp.)

or

mU/assay = 522.36 x (Absorbance) - 6.650 (Fig 5: *C. Thermocellum*)

1000 = conversion from milli-Units to Units.

50 = initial extraction volume (i.e. 50 mL per mL/g enzyme).

5 = conversion from volume assayed (0.2 mL) to 1 mL of extract.

Dilution = further dilution of the initial extraction solution.

F. Repeatability and Reproducibility

Malt β-glucanase

The repeatability and reproducibility of the malt β-glucanase assay was determined by having two analysts perform a series of assays on three different extract solutions ranging in activity from 26-102 MBG4 mU/g malt flour over two consecutive days. The results are outlined in Table 1.

Table 1.

	ΔA obtained for malt β-glucanase flour		
Analysis	**102 mU/g**	**51 mU/g**	**26 mU/g**
Day 1 A(i)	0.70865	0.3644	0.1834
Day 1 A(ii)	0.7081	0.36885	0.1781
Day 2 A(i)	0.70075	0.3527	0.17265
Day 2 A(ii)	0.70315	0.3524	0.1708
Day 1 B(i)	0.7105	0.3645	0.184
Day 1 B(ii)	0.7049	0.3629	0.1819
Day 2 B(i)	0.7126	0.3657	0.18335
Day 2 B(ii)	0.70315	0.3661	0.18485
Standard Dev	**0.004**	**0.006**	**0.005**
% CV	**0.9**	**2.7**	**4.7**

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

Note that the Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as 3 x σ of the blank sample solution absorbance and 10 x σ of the blank sample solution absorbance, respectively, using absorbance values from 10 replicates.

LOD = 4.3×10^{-4} U/mL (corresponding to an absorbance of 0.003)
LOQ = 1.4×10^{-3} U/mL (corresponding to an absorbance of 0.010)

Lichenase

The repeatability and reproducibility of the malt β-glucanase assay was determined by having two analysts perform a series of assays on three different extract solutions ranging in activity from 18-70 MBG4 mU/mL *Bacillus* sp. lichenase solution over two consecutive days. The results are outlined in Table 2.

Table 2.

	ΔA obtained for *Bacillus* sp. lichenase		
Analysis	**70 mU/mL**	**35 mU/mL**	**18 mU/mL**
Day 1 A	0.7059	0.3489	0.18115
Day 2 A	0.7047	0.3472	0.18085
Day 1 B	0.708	0.3564	0.1839
Day 2 B	0.70485	0.3651	0.18195
Standard Dev	**0.003**	**0.008**	**0.003**
% CV	**0.4**	**2.3**	**1.5**

Note: A=Analyst 1, B=Analyst 2

Note that the Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as 3 x σ of the blank sample solution absorbance and 10 x σ of the blank sample solution absorbance, respectively, using absorbance values from 10 replicates.

LOD = 9.1×10^{-5} U/mL (corresponding to an absorbance of 0.0009)
LOQ = 3.0×10^{-4} U/mL (corresponding to an absorbance of 0.003)

AUTO-ANALYSER ASSAYS

G. Standard curve for automated assay on Chemwell®-T Auto-Analyser

The Chemwell-T automatically converts the absorbance obtained in an assay into the β-glucanase/lichenase activity measurement (in $MBG4_{CW}$ units) in the sample solution. Shown below are standard curves for the automated MBG4 β-glucanase and lichenase assay formats respectively on a Chemwell®-T auto-analyser.

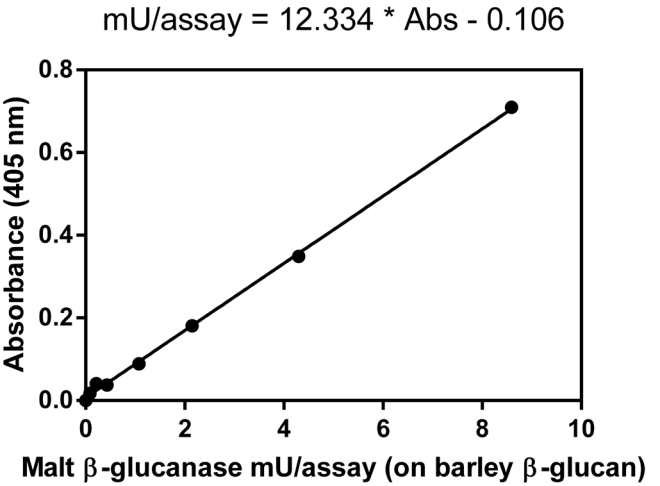


Figure 6. Standard curve relating the activity of malt β-glucanase on barley β-glucan to absorbance increase at 405 nm on hydrolysis of MBG4 reagent under the standard assay conditions.

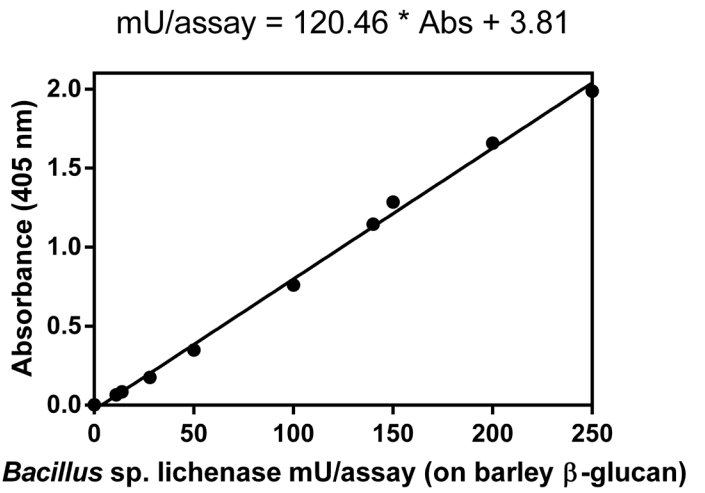


Figure 7. Standard curve relating the activity of *Bacillus* sp. lichenase (**E-LICHN**) on barley β-glucan to absorbance increase at 405 nm on hydrolysis of MBG4 reagent under the specified automated assay conditions.

H. Reproducibility

The reproducibility of the automated MBG4 assay format was assessed for malt β-glucanase only. To perform this study, four separate extractions (A-D) were performed on the malt β-glucanase control flour and all extracts were analysed in quadruplicate on two separate Chemwell-T instruments. The results showing the mean values for the quadruplicate analyses are outlined in Table 3.

Table 3.

Auto-analyser	Sample	Malt β-glucanase $MBG4_{CW}$ mU/g
Chemwell-T 1	Extract A	158
	Extract B	161
	Extract C	163
	Extract D	163
Chemwell-T 2	Extract A	162
	Extract B	164
	Extract C	164
	Extract D	163
Standard deviation (σ)		**1.854**
% CV		**1.1**

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