

Megazyme

MALT & BACTERIAL BETA-GLUCANASE & CELLULASE

ASSAY PROCEDURE

(AZO-BARLEY GLUCAN METHOD)

KMBGL 01/03



INTRODUCTION:

The need for an accurate and reliable assay for malt β -glucanase has become more urgent with modern brewing processes where rapid and efficient wort and beer filtration, and stability of the product are essential. A better knowledge of enzyme stability, the levels of activity in the malt and changes in activity during the mashing process may lead to a reduction or the elimination of problems caused by β -glucan.

PRINCIPLE:

Malt extract is incubated with Azo-Barley glucan substrate under defined conditions. The dyed substrate is depolymerised by malt β -glucanase to fragments which are soluble in the presence of precipitant solution.

On centrifugation of the precipitant-treated reaction mixture, the absorbance (at 590 nm) of the supernatant solution is directly related to the level of malt β -glucanase in the extracted malt. The principle of the assay is outlined in Scheme 1 (page 14).

Fifty malt samples can be extracted and analysed by a single operator in one day.

ACCURACY:

Interlaboratory evaluation of 5 malt samples (17 laboratories) indicated a coefficient of variation of less than 7%. As a result of this evaluation, this procedure became a Recommended Method of the Cereal Chemistry Division of the Royal Australian Chemical Institute.

KITS:

Kits suitable for carrying out 100 assays are available from Megazyme and consist of:

1. Full assay method
2. Azo-Barley Glucan Substrate
3. Standard Malt Flour
4. Concentrated Buffer Solution

SPECIFICITY:

Azo-Barley glucan is hydrolysed by both malt β -glucanases and cellulases, but since the level of cellulase in malt is extremely low, the assay is specifically measuring the level of mixed-linkage β -glucanase (malt β -glucanase) in these samples.

ENCLOSED SOLUTIONS:

(A) Azo-Barley glucan substrate:

Chemically modified (to increase solubility), dye-labelled barley β -glucan (1% w/v) in 0.02% sodium azide.

Store at 0-5°C between use and heat to 30°C before dispensing.

(B) Concentrated Buffer A:

800 mM sodium acetate, plus
800 mM sodium phosphate
stabilised in 0.02% sodium azide

Dilute one vial (25.0 mL) of the concentrate to 500.0 mL with distilled water before use. This diluted buffer (**Extraction Buffer Solution A**) contains 40 mM sodium acetate buffer plus 40 mM sodium phosphate buffer, pH 4.6.

Store at 0-5°C between use.

ENCLOSED MALT FLOUR:

Malt flour of standardised malt β -glucanase activity (as specified on the bottle 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.

OTHER REAGENTS REQUIRED:

1. Precipitant Solution A:

Dissolve 40.0 g sodium acetate ($\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$) and 4.0 g zinc acetate in 150 mL distilled water. Adjust pH to 5.0 with conc. HCl, adjust volume to 200.0 mL. To this solution add 800.0 mL of methyl cellosolve (methoxyethanol) and mix well.

Store at room temperature.

Methyl cellosolve is **poisonous** and should be treated accordingly.

2. Extractant Buffer Solution A: (40 mM sodium acetate buffer plus 40mM sodium phosphate buffer, pH 4.6)

If Concentrated **Buffer Solution A** (as supplied) is insufficient, more extractant buffer can be prepared as follows:

Dissolve 5.44 g of sodium acetate ($\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$) and 6.24 g of sodium dihydrogen orthophosphate ($\text{NaH}_2\text{PO}_4\cdot 2\text{H}_2\text{O}$) in 900 mL of distilled water. Adjust the pH to 4.6 by the addition of 1 N HCl. Add 0.2 g of sodium azide. Adjust the volume to 1.0 litre.

Store at 0-5°C.

EQUIPMENT (RECOMMENDED):

1. Centrifuge tubes (16 x 120 mm; 17 mL capacity).
2. Bench Centrifuge.
3. Positive displacement pipettor (e.g. Eppendorf Multipette[®] with 12.5mL Combitip[®]) to dispense the viscous substrate solution.
4. Pipettor, 0 to 1.0 mL (e.g. Gilson Pipetman[®]) to dispense malt extract.
5. Adjustable-volume dispensers: 0-5.0 mL (for precipitant solution)
0-10.0 mL (for acetate buffer)
6. Top-pan balance.
7. Spectrophotometer set at 590 nm.
8. Vortex mixer.
9. Thermostatted water bath set at **30.0°C** (or 40°C; Appendix C+D).
10. Stop clock.

CONTROLS AND PRECAUTIONS:

1. Assay the malt β -glucanase within one (1) hour of extraction.
2. Take care not to contaminate the Azo-Barley glucan substrate solution with malt extract. Blank absorbance values (read against distilled water) should be approximately 0.08 at 590 nm. If values are significantly higher than this value, or if they are increasing with time, discard the substrate.
3. With each set of determinations a **Reaction Blank** must be included. This is prepared by adding 3.0 mL of the Precipitant Solution to 0.5mL of the Azo-Barley glucan substrate and mixing thoroughly; then add 0.5 mL of malt extract, mix thoroughly, and centrifuge as in step 6 of the Assay Procedure.

A single Reaction Blank is normally sufficient for each batch of malt samples analysed, as the Blank absorbance value does not vary significantly from sample to sample.

4. If reaction absorbance values for a particular extract are greater than **0.9 absorbance units**, an aliquot of the extract should be diluted with an equal volume of the Extractant Buffer and reassayed. Appropriate corrections to the calculations should then be made.
5. With each batch of malt samples analysed, a malt flour of standardised activity must be included.

USEFUL HINT:

Warm the Azo-Barley glucan substrate solution to approximately **30°C** before dispensing (this gives a significant decrease in the viscosity).

ASSAY PROCEDURE:

Enzyme Extraction

1. Mill malt (approximately 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 x120 mm; 17 mL capacity).
3. Add 8.0 mL of Extraction Buffer Solution (40 mM acetate/phosphate, pH 4.6) to each tube and stir the contents thoroughly on a vortex mixer.
4. Allow the enzyme to extract over a 15 minute period at room temperature (less than 30°C), with occasional mixing.
5. Centrifuge the tubes and contents at 1,000g for 10 minutes.

Assay of Malt β -glucanase Activity

1. Dispense (with a positive displacement dispenser) **0.5 mL** aliquots of Azo-Barley glucan substrate solution (pre-warmed to **30°C**) into centrifuge tubes and pre-incubate the tubes and contents at **30°C** for 5 minutes.
2. Pre-incubate malt extracts (from step 5 above) at **30°C** for 5 minutes.
3. To each tube of 0.5 mL Azo-Barley glucan substrate add a **0.5mL** aliquot of malt extract, mix vigorously and then incubate at **30°C** for exactly **10 minutes** (from time of addition).
4. At the end of the 10 minute incubation period, add 3.0 mL of **Precipitant Solution A** and stir the tube contents vigorously.
5. Allow the tubes to stand at room temperature for 5 minutes and then stir them again.
6. Centrifuge the tubes and contents at 1,000g for 10 minutes.
7. Read the absorbance (at 590 nm) of the supernatant of each sample and the reaction blank against distilled water.

AZO-BARLEY GLUCAN SUBSTRATE (M and C values):

AZO-BARLEY GLUCAN

LOT 60602

$$Y = M X + C$$

M= 630; C = 4; X = Absorbance.

Thus: $Y = 630 \times \text{Abs.} + 4$

CALCULATION OF ACTIVITY:

1. Malt β -glucanase is calculated using the equation:

$$Y = MX + C$$

[EQUATION 1]

- where: Y = malt β -glucanase activity (in U/kg of malt)
M = slope of calibration graph
X = absorbance of reaction at 590 nm (minus blank)
C = intercept on Y-axis

Values for M and C vary slightly between batches of substrate.

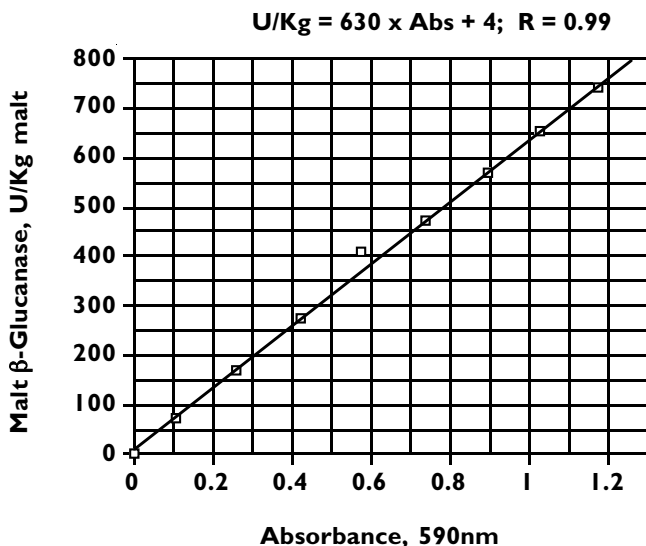


Figure 1. Malt β -Glucanase standard curve on Azo Barley Glucan (Lot 60602) at pH 4.6 and 30°C for 10 min, using 3 ml of EGME Precipitant Solution (**Precipitant Solution A**) .

2. Corrected Malt β -glucanase (U/kg malt):

$$\text{U/kg malt} = Y \times \frac{A}{B}$$

[EQUATION 2]

- where: Y = malt β -glucanase activity (in U/kg of malt) as calculated using Equation 1.
A = activity value for **control malt** as supplied; this value is provided on the label of the vial of control malt flour included in the kit.
B = activity value for **control malt** as calculated using Equation 1.

NOTE: Units of activity with the Azo-Barley Glucan method (substrate lot 60602) can be converted to IOB IRV Units using the equation:

$$\text{IRV Units} = 1.6 \times \text{Azo-Barley Glucan Units (per Kg malt)} - 14.$$

(note: this equation was previously presented incorrectly)

APPENDIX A: Preparation of Calibration Curve for Malt β -Glucanase

The calibration curve and the resultant equation relating absorbance (590nm) to malt- β -glucanase I activity were prepared using Azo-Barley glucan substrate and malt β -glucanase I (free of β -glucosidase).

The malt β -glucanase I activity was first standardised using barley β -glucan (5 mg/mL) in 40 mM sodium acetate/sodium phosphate buffer (pH 4.6) employing the Nelson/Somogyi reducing sugar assay.

The curve relating absorbance (at 590 nm) to activity of malt β -glucanase I in the volume of liquid assayed (0.5 mL) is obtained.

This is converted to activity per kg of malt as follows:

$$\begin{aligned} \text{U/kg of malt} &= \text{U}/0.5 \text{ mL of extract} \times 16 \times 2,000 \\ &= \text{U}/0.5 \text{ mL} \times 32,000 \end{aligned}$$

- U** **International Units** of enzyme activity; equals one micromole of glucose reducing sugar equivalent released per minute at 30°C and pH 4.6.
- 16** from a total extract of 8.0 mL, 0.5 mL is used in the assay.
- 2000** Weight correction factor; 0.5 g of malt was extracted but the results are expressed as units per kg of malt.

References:

- McCleary, B.V. and Shameer, I. (1987). "Assay of malt β -glucanase using Azo Barley Glucan : an improved precipitant" *J. Inst. Brew.* **93**, 87-90.
- Buch, G.J. (1986). "Malt β -glucanase : a collaborative test on a new rapid assay". *J. Inst. Brew.* **92**, 513- 514.
- Buckee, G.K. and Baker, C.D. (1988). "Collaborative trial on the determination of beta-glucanase in malt by viscometric and dye labelled method". *J. Inst. Brew.*, **96**, 387-390.
- Gill, A.A. and Haselmore, R.M. (1987). "A comparison of two methods for the estimation of β -glucanase activity in malt". *J. Inst. Brew.*, **89**, 34-37.
- Rotter, B.A., Marquardt, R.R., Guenter, W. and Crow, G.H. (1990). "Evaluation of three enzymic methods as predictors of *in vivo* response to enzyme supplementation of barley-based diets when fed to young chicks". *J. Sci. Fd. Agric.*, **50**, 19-27.

APPENDIX B:

Alternative Precipitant Solution (B).

Because methyl cellosolve is poisonous, some analysts would prefer to use an alternative precipitant. Such a precipitant can be prepared with industrial methylated spirits (IMS), **however the sensitivity of the assay is reduced.** (Note: IMS is ~ 95% ethanol and 5% methanol).

Precipitant Solution B:

Dissolve 30.0 g sodium acetate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) and 3.0 g zinc acetate in 250 mL distilled water. Adjust the pH to 5.0 with conc. HCl, adjust the volume to 300.0 mL. To this solution add 700.0 mL of industrial methylated spirits (IMS, 95%) and mix well.

Store in a well sealed glass bottle at room temperature.

Assay of Malt β -glucanase Activity using Precipitant B:

1. Dispense (with a positive displacement dispenser) **0.5 mL** aliquots of Azo-Barley glucan substrate solution (pre-warmed to **30°C**) into centrifuge tubes and pre-incubate the tubes and contents at **30°C** for 5 minutes.
2. Pre-incubate malt extracts (from step 5, page 4, "Enzyme Extraction") at **30°C** for 5 minutes.
3. To each tube of 0.5 mL Azo-Barley glucan substrate add a **0.5mL** aliquot of malt extract, mix vigorously and then incubate at **30°C** for exactly **30 minutes** (from time of addition).

- At the end of the 30 minute incubation period add 3.0 mL of **Precipitant Solution B** and stir the tube contents vigorously.
- Allow the tubes to stand for 5 minutes at room temperature and stir them again.
- Centrifuge the tubes and contents at 1,000g for 10 minutes.
- Read the absorbance (at 590 nm) of the supernatant solutions of each sample and the reaction blank against distilled water.

CALCULATION OF ACTIVITY:

1. Malt β -glucanase is calculated using the equation:

$$Y = MX + C \quad \text{[EQUATION 1]}$$

where: Y = malt β -glucanase activity (in U/kg of malt)
 M = slope of calibration graph
 X = absorbance of reaction at 590 nm (minus blank)
 C = intercept on Y-axis

Values for M and C vary slightly between batches of substrate.

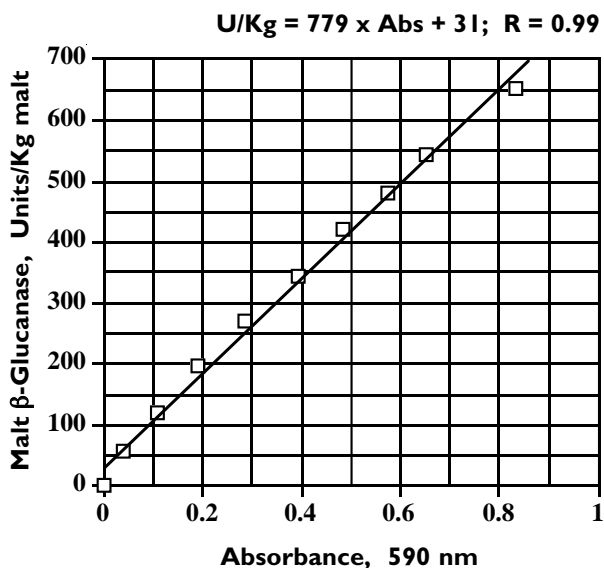


Figure 2. Malt β -Glucanase standard curve on Azo Barley Glucan (Lot 60602) at pH 4.6 and 30°C for 30 min, using 3 ml of **Precipitant Solution B** (IMS/Zn acetate).

APPENDIX C:

Assay of Cellulase (EG II) Using Azo-Barley Glucan.

Assay Buffer: Sodium acetate buffer (100 mM, pH 4.5) + BSA.

Add 5.7 ml of glacial acetic acid (1.05 g/ml) to 900 ml of distilled water. Adjust the pH of the solution to pH 4.5 by the addition of 2 M (8 g/100 ml) sodium hydroxide solution. Adjust the volume to 1 litre. Add BSA (0.5g) plus sodium azide (0.2g). Store 4°C.

Assay Procedure:

1. Dispense (with a positive displacement dispenser) **0.5 mL** aliquots of Azo-Barley glucan substrate solution (pre-warmed to **40°C**) into centrifuge tubes and pre-incubate the tubes and contents at **40°C** for 5 minutes.
2. Pre-incubate suitably diluted cellulase in assay buffer [0.1 M sodium acetate buffer (pH 4.5)] at **40°C** for 5 minutes.
3. To each tube of 0.5 mL Azo-Barley glucan substrate add a **0.5mL** aliquot of cellulase solution, mix vigorously and then incubate at **40°C** for exactly **10 minutes** (from time of addition).
4. At the end of the 10 minute incubation period add 3.0 mL of either **Precipitant Solution A** or **B** and stir the tube contents vigorously.
5. Allow the tubes to stand for 5 minutes at room temperature and stir them again.
6. Centrifuge the tubes and contents at 1,000g for 10 minutes.
7. Read the absorbance (at 590 nm) of the supernatant of each sample and the reaction blank against distilled water.
8. Calculate activity by reference to Standard Curve 3 (**Precipitant A**; EGME/Zn acetate) or Standard Curve 4 (**Precipitant B**; IMS/Zn acetate).

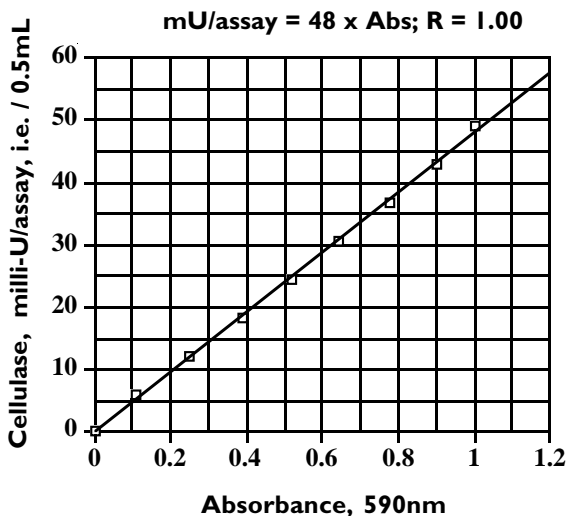


Figure 3. Cellulase (EG II) standard curve on Azo Barley Glucan (Lot 60602) at pH 4.5 and 40°C for 10 min, using 3 ml of **Precipitant Solution A** (EGME/Zn acetate).

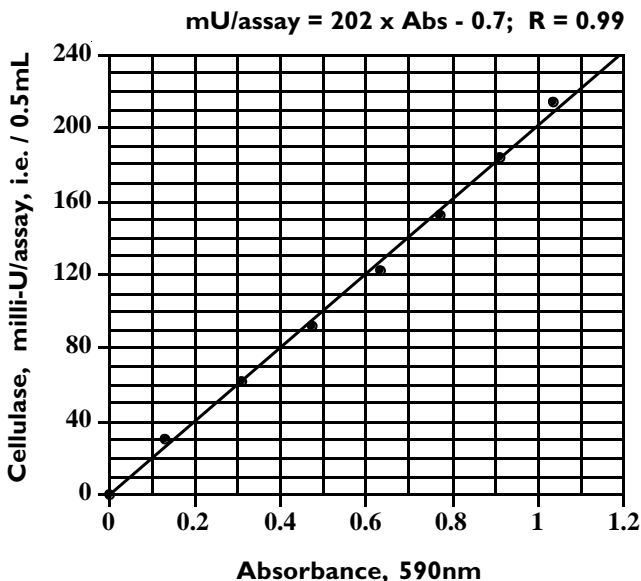


Figure 4. Cellulase (EG II) standard curve on Azo Barley Glucan (Lot 60602) at pH 4.5 and 40°C for 10 min, using 3ml of **Precipitant Solution B** (IMS/Zn acetate).

APPENDIX D:

Assay of endo-1,3:1,4- β -Glucanase (Lichenase) Using Azo-Barley Glucan.

Assay Buffer: Sodium phosphate buffer (100 mM, pH 6.5)

Dissolve 15.6 g sodium dihydrogen orthophosphate dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) in 900 mL of distilled water and adjust the pH to 6.5 by the addition of 1 M sodium hydroxide (40 g/L). [Approximately 25 mL is required]. Add BSA (0.5 g) plus sodium azide (0.2 g). Adjust the volume to one litre. Store at 4°C.

Assay Procedure:

1. Dispense (with a positive displacement dispenser) **0.5 mL** aliquots of Azo-Barley glucan substrate solution (pre-warmed to **40°C**) into centrifuge tubes and pre-incubate the tubes and contents at **40°C** for 5 minutes.
2. Pre-incubate suitably diluted lichenase in Assay Buffer [0.1 M sodium phosphate buffer (pH 6.5)] at **40°C** for 5 minutes.
3. To each tube containing 0.5 mL Azo-Barley glucan substrate add a **0.5mL** aliquot of lichenase solution, mix vigorously and then incubate at **40°C** for exactly **10 minutes** (from time of addition).
4. At the end of the 10 minute incubation period, add 3.0 mL of either **Precipitant Solution A** or **B** and stir the tube contents vigorously.
5. Allow the tubes to stand for 5 minutes at room temperature and then stir again.
6. Centrifuge the tubes and contents at 1,000g for 10 minutes.
7. Read the absorbance (at 590 nm) of the supernatant of each sample and the reaction blank against distilled water.
8. Calculate activity by reference to Standard Curve 5 (**Precipitant A**; EGME/Zn acetate) or Standard Curve 6 (**Precipitant B**; IMS/Zn acetate).

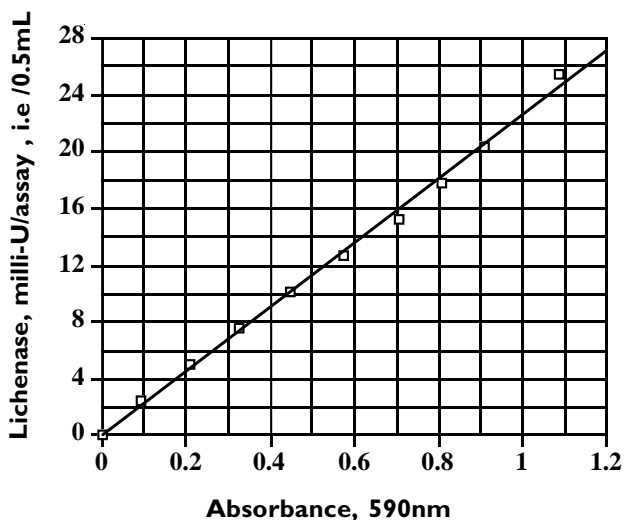


Figure 5. Lichenase standard curve on Azo Barley Glucan (Lot 60602) at pH 6.5 and 40°C for 10 min, using 3 ml of **Precipitant Solution A** (EGME/Zn acetate).

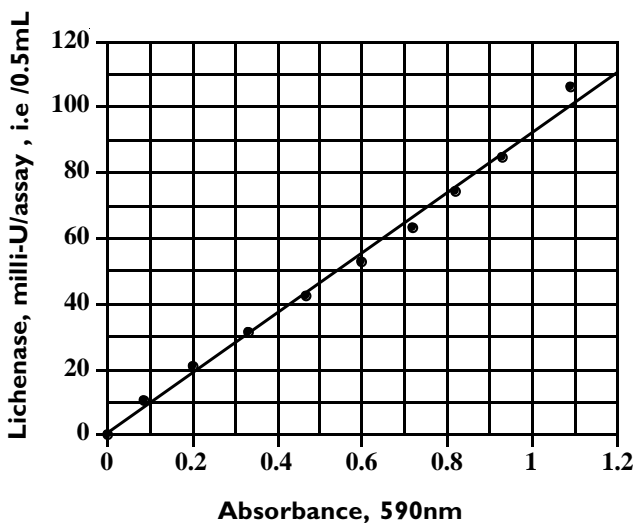
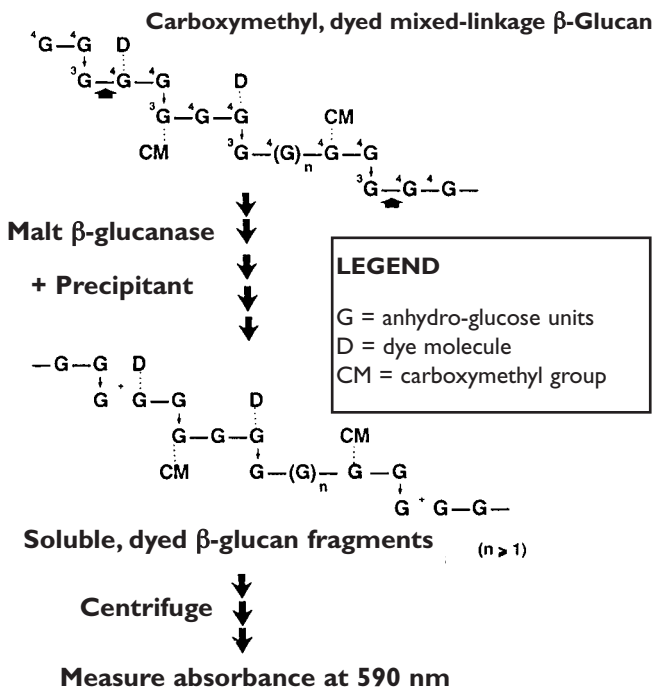


Figure 6. Lichenase standard curve on Azo Barley Glucan (Lot 60602) at pH 6.5 and 40°C for 10 min, using 3 ml of **Precipitant Solution B** (IMS/Zn acetate).



Scheme 1. Theoretical basis of the malt β -glucanase assay employing Azo-Barley Glucan.



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