β-GLUCANASE
(Malt and Microbial)

ASSAY PROTOCOL

K-MBGL

07/23

(AZO-BARLEY GLUCAN METHOD)

(100 Assays per Kit)

INTRODUCTION:

β-glucanases are endogenous enzymes which can be used in the preparation of food, feed and beverage products to degrade potentially detrimental β-glucans. β-glucans are a major structural component of cereal cell walls (e.g. barley and oat). Due to their intrinsic water-soluble nature these β-glucans, in their high molecular weight undegraded form, can cause issues in brewing processes such as increased viscosity, delayed filtration as well forming precipitate in the final product.

This **K-MBGL** assay kit allows for an accurate and reliable measurement of both malt and microbial β-glucanase.

PRINCIPLE:

Malt extract is incubated with Azo-Barley glucan substrate under defined conditions. The dyed substrate is depolymerised by malt or microbial 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 12).

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 Neogen and consist of:

Bottle 1: (x2)	Concentrated Buffer Solution. Store at 4°C. See individual label for expiry date.
Bottle 2: (x2)	Azo-Barley Glucan Substrate. Store at 4°C. See individual label for expiry date.
Bottle 3:	Malt Control Flour. Store at room temperature. See individual label for expiry date.

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) Concentrated Buffer:

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**) contains 40 mM sodium acetate buffer plus 40 mM sodium phosphate buffer, pH 4.6.

Store at 4°C.

(B) Azo-Barley glucan substrate:

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

Store at 4°C between use and heat to 30°C before dispensing.

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.

REQUIRED REAGENTS (NOT SUPPLIED):

1. **Precipitant Solution A:**

Dissolve 40.0 g sodium acetate ($CH_3COONa.3H_2O$) and 4.0 g zinc acetate in 150 mL distilled water. Adjust pH to 5.0 with concentrated HCl, then adjust volume to 200 mL. To this solution add 800 mL of methyl cellosolve (methoxyethanol) and mix well. This is the **Precipitant Solution A** (EGME/Zn acetate Precipitant Solution). Store at room temperature. An alternative precipitant (**Precipitant B**; IMS/Zn acetate) is given in Appendix A.

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

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

If the volume of **Concentrated Buffer Solution** (**bottle 1**) is insufficient, more extractant buffer can be prepared as follows: Dissolve 5.44 g of sodium acetate ($CH_3.COONa.3H_2O$) and 6.24 g of sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) 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 L. Store at 4°C.

EQUIPMENT (RECOMMENDED):

1. Centrifuge tubes (16 x 120 mm; 17 mL capacity).
2. Bench Centrifuge.
3. Mill with 0.5 mm screen: Tecator Cyclotec® mill (or equivalent) for milling malt sample.
4. Positive displacement pipettor (e.g. Eppendorf Multipette® with 12.5 mL Combitip®) to dispense the viscous substrate solution.
5. Pipettor, 0 to 1.0 mL (e.g. Gilson Pipetman®) to dispense malt extract.
6. Adjustable-volume dispensers: 0-5.0 mL (for precipitant solution)
   0-10.0 mL (for acetate buffer)
7. Top-pan balance.
8. Spectrophotometer set at 590 nm.
9. Vortex mixer.
10. Thermostated water bath set at 30°C or 40°C (dependent on assay protocol used).
11. Stop clock.

CONTROLS AND PRECAUTIONS:

1. Assay the malt β-glucanase within one 1 h 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 0.5 mL of the Azo-Barley glucan substrate and mixing thoroughly; then add 0.5 mL of malt extract, mix thoroughly, and centrifuge as in step 5 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 **Extraction Buffer** and re-assayed. 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 x 120 mm; 17 mL capacity).
3. Add 8.0 mL of **Extraction Buffer** (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 min period at room temperature (less than 30°C), with occasional mixing.
5. Centrifuge the tubes and contents at 1,000 *g* for 10 min.
6. Use the supernatant (malt extract) from this step in the assay

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 min.
2. Pre-incubate malt extracts (from step 6 above) at **30°C** for 5 min.
3. To each tube of 0.5 mL Azo-Barley glucan substrate add a **0.5 mL** aliquot of malt extract, mix vigorously and then incubate at **30°C** for exactly **10 min** (from time of addition).
4. At the end of the 10 min 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 min and then stir them again.
6. Centrifuge the tubes and contents at 1,000 *g* for 10 min.
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 the equation given in **Figure 1**: Malt β-Glucanase standard curve.

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

AZO-BARLEY GLUCAN
LOT 90301
Y = M X + C
M= 574; C = 2.5; X = Absorbance.
Thus: Y = 574 x Abs. + 2.5

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.

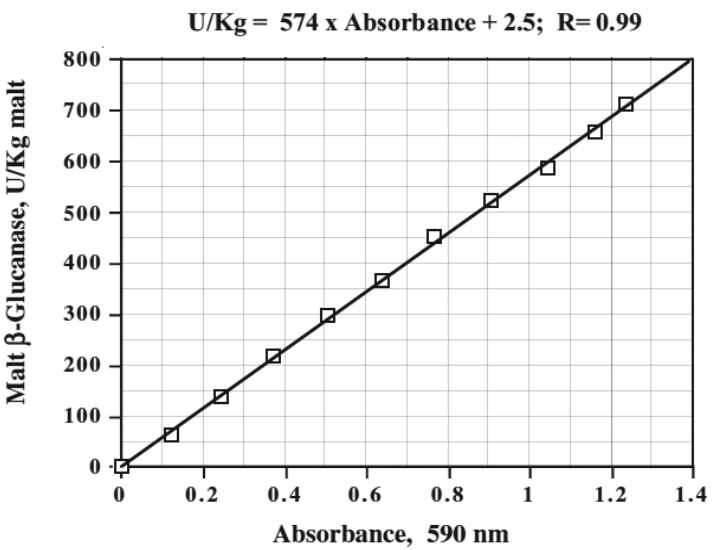

Figure 1. **Malt β-Glucanase** standard curve on Azo Barley Glucan (Lot 90301) at pH 4.6 and 30°C for 10 min, using 3 mL of **Precipitant Solution A** (EGME/Zn acetate Precipitant Solution).

Preparation of Calibration Curve for Malt β-Glucanase

The calibration curve and the resultant equation relating absorbance (590 nm) 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:

U/kg of malt	=	U/0.5 mL of extract x 16 x 2,000
	=	U/0.5 mL x 32,000

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:

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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 A:

Alternative Precipitant Solution (B)

As 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: ~ 95% ethanol + 5% methanol).

Precipitant Solution B:

Dissolve 30.0 g sodium acetate ($CH_3COONa.3H_2O$) 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 min.
2. Pre-incubate malt extracts (from step 6, page 4, "Enzyme Extraction") at **30°C** for 5 min.
3. To each tube of 0.5 mL Azo-Barley glucan substrate add a **0.5 mL** aliquot of malt extract, mix vigorously and then incubate at **30°C** for exactly **30 min** (from time of addition).
4. At the end of the 30 min incubation period add 3.0 mL of **Precipitant Solution B** and stir the tube contents vigorously.
5. Allow the tubes to stand for 5 min at room temperature and stir them again.
6. Centrifuge the tubes and contents at 1,000 *g* for 10 min.
7. Read the absorbance (at 590 nm) of the supernatant solutions of each sample and the reaction blank against distilled water.
8. Calculate activity by reference to the equation given in **Figure 2**: Malt β-Glucanase standard curve (using **Precipitant Solution B**).

CALCULATION OF ACTIVITY:

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

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

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.

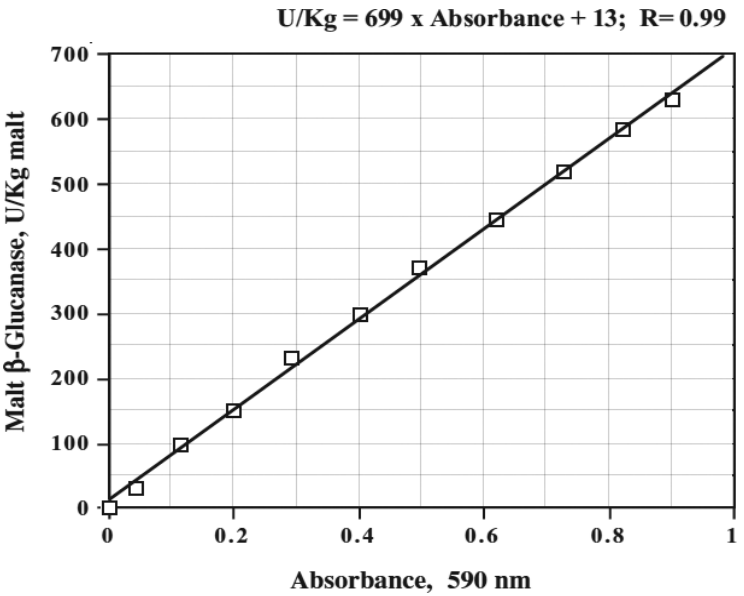


Figure 2. **Malt β-Glucanase** standard curve on Azo Barley Glucan (Lot 90301) at pH 4.6 and 30°C for 30 min, using 3 mL of **Precipitant Solution B** (IMS/Zn acetate).

APPENDIX B:

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 L. Add BSA (0.5 g) plus sodium azide (0.2 g). 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 min.

2. Pre-incubate suitably diluted cellulase in assay buffer [0.1 M sodium acetate buffer (pH 4.5)] at **40°C** for 5 min.

3. To each tube of 0.5 mL Azo-Barley glucan substrate add a **0.5 mL** aliquot of cellulase solution, mix vigorously and then incubate 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 either **Precipitant Solution A** or **B** and stir the tube contents vigorously.

5. Allow the tubes to stand for 5 min at room temperature and stir them again.

6. Centrifuge the tubes and contents at 1,000 g for 10 min.

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 **Figure 3**: Cellulase (EG II) standard curve (when using **Precipitant Solution A**; EGME/Zn acetate) or **Figure 4**: Cellulase (EG II) standard curve (when using **Precipitant B**; IMS/Zn acetate. See Appendix A for details.).

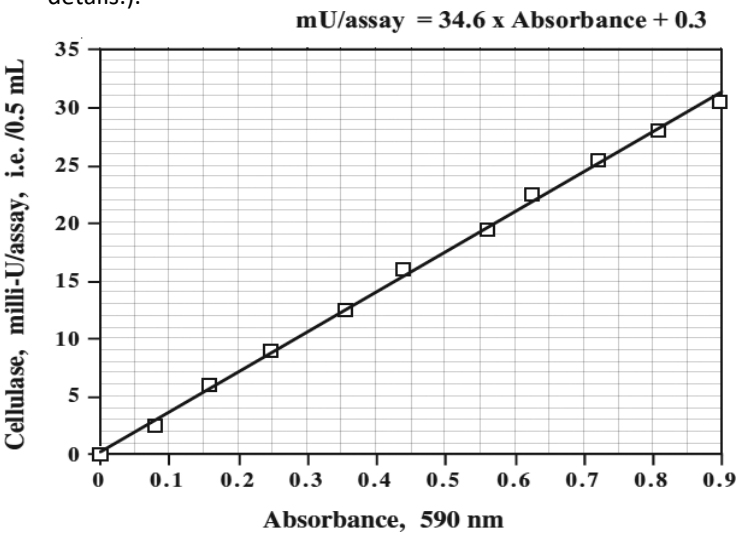


Figure 3. **Cellulase** (EG II) standard curve on Azo Barley Glucan (Lot 90301) at pH 4.5 and 40°C for 10 min, using 3 mL of **Precipitant Solution A** (EGME/Zn acetate).

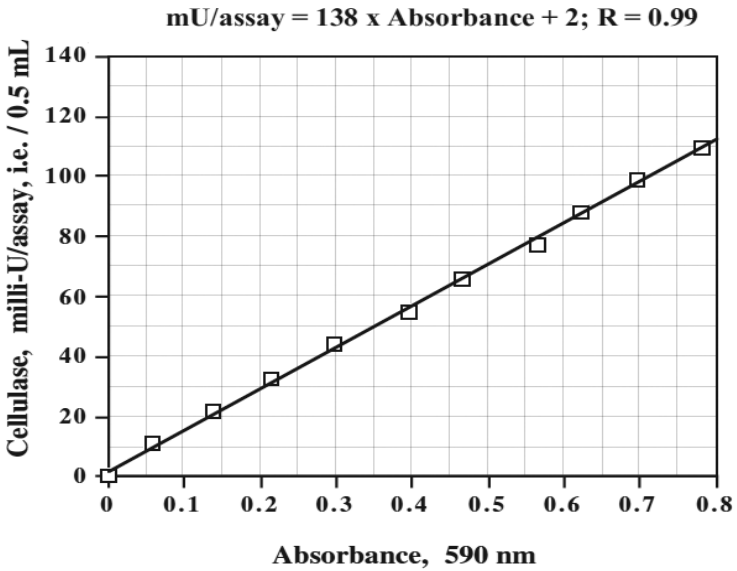


Figure 4. **Cellulase** (EG II) standard curve on Azo Barley Glucan (Lot 90301) at pH 4.5 and 40°C for 10 min, using 3 mL of **Precipitant Solution B** (IMS/Zn acetate).

APPENDIX C:

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 ($NaH_2PO_4.2H_2O$) 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 1 L. 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 min.
2. Pre-incubate suitably diluted lichenase in Assay Buffer [0.1 M sodium phosphate buffer (pH 6.5)] at **40°C** for 5 min.
3. To each tube containing 0.5 mL Azo-Barley glucan substrate add a **0.5 mL** aliquot of lichenase solution, mix vigorously and then incubate 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 either **Precipitant Solution A** or **B** (See Appendix A for details.) and stir the tube contents vigorously.

5. Allow the tubes to stand for 5 min at room temperature and then stir again.
6. Centrifuge the tubes and contents at 1,000 *g* for 10 min.
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 Figure 5: Lichenase standard curve (**Precipitant A**; EGME/Zn acetate) or Figure 6: Lichenase standard curve (**Precipitant B**; IMS/Zn acetate. See Appendix A for details.).

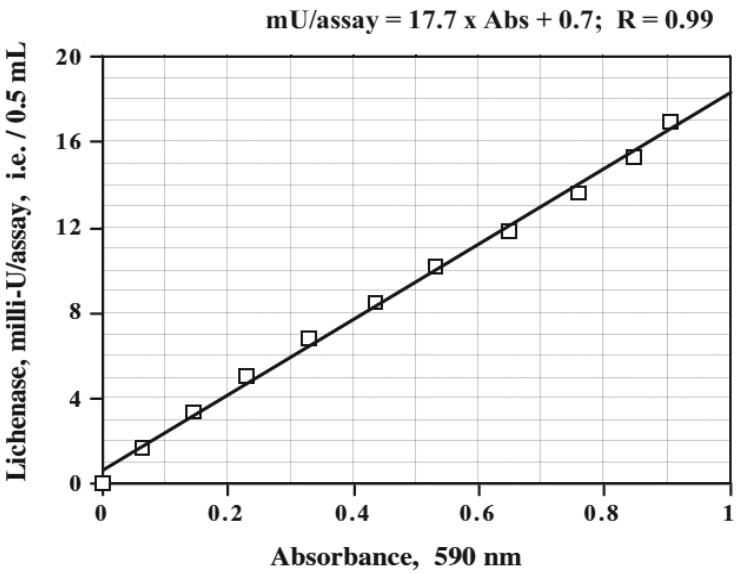


Figure 5. **Lichenase** standard curve on Azo Barley Glucan (Lot 90301) at pH 6.5 and 40°C for 10 min, using 3 mL of **Precipitant Solution A** (EGME/ Zn acetate).

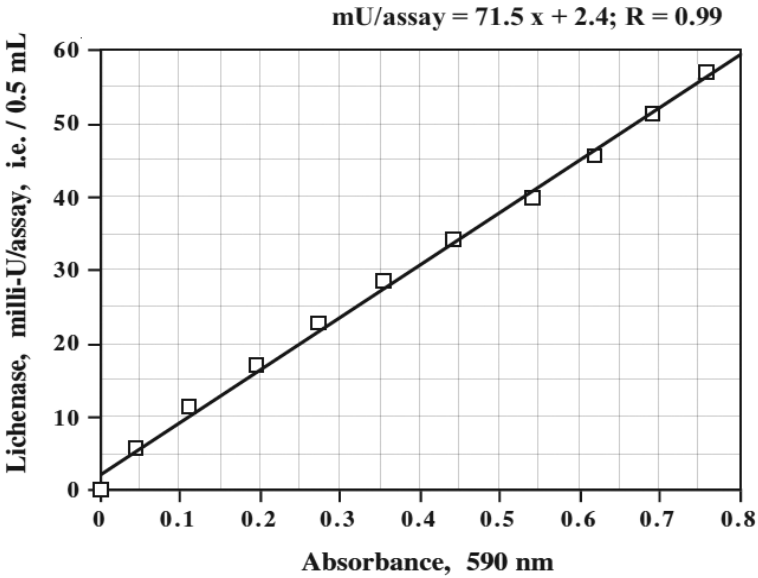
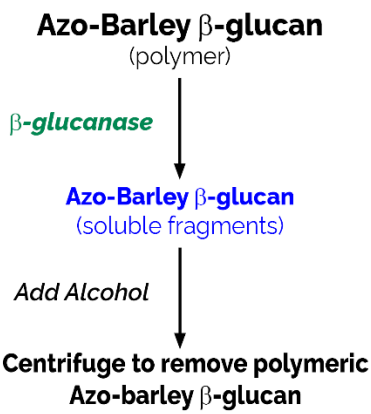


Figure 6. **Lichenase** standard curve on Azo Barley Glucan (Lot 90301) at pH 6.5 and 40°C for 10 min, using 3 mL of **Precipitant Solution B** (IMS/Zn acetate).



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

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Without guarantee

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