Megazyme

MIXED-LINKAGE BETA-GLUCAN

ASSAY PROCEDURE (McCLEARY METHOD)

K-BGLU 07/11

(100 Assays per kit)

AACC Method 32-23
AOAC Method 995.16
EBC Methods 3.11.1, 4.16.1 and 8.11.1
ICC Standard Method No. 166

INTRODUCTION:

For some time the brewing, food and ingredient industries have identified the need to develop an accurate, convenient and reliable method for assaying mixed-linkage β-glucan in barley, malt, wort and beer. The Megazyme method meets all of these requirements. It is simple to use and fifty to one hundred (50-100) samples can be assayed in a day. The method has now been adapted for the measurement of β-glucan in oats and oat fibre products (see page 10).

PRINCIPLE:

Samples are suspended and hydrated in a buffer solution of pH 6.5 and then incubated with purified lichenase enzyme and filtered. An aliquot of the filtrate is then hydrolysed to completion with purified β-glucosidase. The D-glucose produced is assayed using a glucose oxidase/peroxidase reagent (Scheme 1, page 14).

ACCURACY:

In barley containing 4.0 % (w/w) total β-glucan, the method is accurate to 4.0 ± 0.1 % (w/w).

EVALUATION:

This assay method has been successfully evaluated by Analytical Committees of the Royal Australian Chemical Institute and the European Brewing Convention. A modified version (Streamlined Method, page 10) is AOAC Official Method 995.16, and an AACC (Method 32-23) and ICC (Method No. 168) recommended/standard method.

SPECIFICITY:

The assay is specific for mixed-linkage [(1-3)(1-4)]-β-D-glucan.

KITS:

Kits suitable for performing 100 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Lichenase [specific, *endo*-(1-3)(1-4)-β-D-glucan 4-glucanohydrolase] suspension (1 mL).
Stable for > 3 years at 4°C.

Bottle 2: β-Glucosidase (1 mL) suspension.
Stable for > 3 years at 4°C.

Bottle 3: **GOPOD Reagent Buffer.** Buffer (48 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.4 % w/v).
Stable for > 4 years at 4°C.

Bottle 4: **GOPOD Reagent Enzymes.** Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze-dried powder.
Stable for > 5 years at -20°C.

Bottle 5: D-Glucose standard solution (5 mL, 1.0 mg/mL) in 0.2 % (w/v) benzoic acid.
Stable for > 5 years at room temperature.

Bottle 6: Standardised barley flour control. β-Glucan content shown on vial label.
Stable for > 5 years at room temperature.

Bottle 7: Standardised oat flour control. β-Glucan content shown on vial label.
Stable for > 5 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Dilute the contents of bottle 1 (lichenase) to 20.0 mL with 20 mM sodium phosphate buffer (pH 6.5). Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and keep cool during use if possible.
Stable for > 2 years at -20°C.

> **NOTE:** It is imperative that the lichenase is not cross-contaminated with β-glucosidase.

2. Dilute the entire contents of bottle 2 (β-glucosidase) to 20.0 mL with 50 mM sodium acetate buffer (pH 4.0). Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and keep cool during use if possible. Stable for > 2 years at -20°C.

3. Dilute the contents of bottle 3 (GOPOD Reagent Buffer) to 1 L with distilled water (this is solution 3). Use immediately.

> **NOTE:**
> 1. If the concentrated buffer is stored at -20°C, it will form salt crystals that must be completely dissolved when this buffer is diluted to 1 L with distilled water.
> 2. This buffer contains 0.4 % (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.

4. Dissolve the contents of bottle 4 in 20 mL of solution 3 and quantitatively transfer this to the bottle containing the remainder of solution 3. Cover this bottle with aluminium

foil to protect the enclosed reagent from light. This is Glucose Determination Reagent **(GOPOD Reagent)**. Stable for ~ 3 months at 2-5°C or > 12 months at -20°C.

If this reagent is to be stored in the frozen state, preferably it should be divided into aliquots that should be freeze/thawed only once during use.

When the reagent is freshly prepared it may be light yellow or light pink in colour. It will develop a stronger pink colour over 2-3 months at 4°C. The absorbance of this solution should be less than 0.05 when read against distilled water.

BUFFERS (NOT SUPPLIED):

1. **Sodium phosphate buffer** (20 mM, pH 6.5)
   Dissolve 3.12 g of 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 100 mM sodium hydroxide (4 g/L) (approx. 50 mL is required). Adjust the volume to 1 L. Add 0.2 g of sodium azide. Stable for 2 months at 4°C.

2. **Sodium acetate buffer** (50 mM, pH 4.0)
   Add 2.9 mL of glacial acetic acid to 900 mL of distilled water. Adjust to pH 4.0 by the addition of 1 M sodium hydroxide solution. Adjust the volume to 1 L. Add 0.2 g sodium azide. Stable for 2 months at 4°C.

3. **Sodium acetate buffer** (200 mM, pH 4.0)
   Add 11.6 mL of glacial acetic acid to 900 mL of distilled water. Adjust to pH 4.0 by the addition of 1 M sodium hydroxide solution. Adjust the volume to 1 L. Add 0.2 g sodium azide. Stable for 2 months at 4°C.

EQUIPMENT (RECOMMENDED):

1. Polypropylene tubes/containers with caps (35.0 mL capacity).
2. Glass test-tubes (12 mL capacity).
3. Micro-pipettors, e.g. Gilson Pipetman® (100 µL and 200 µL).
4. Positive displacement pipettor e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.1 mL aliquots of buffer and buffered β-glucosidase solution).

5. Adjustable-volume dispensers
   0-5.0 mL (for phosphate buffer).
   3.0 mL (for glucose oxidase/ peroxidase reagent).
   0-25.0 mL (for distilled water).
6. Laboratory oven.
7. Analytical and top-pan balances.
8. Spectrophotometer set at 510 nm (see point 1 under Useful Hints).
9. Vortex mixer.
10. Thermostated water bath set at 40°C (or 50°C for the **Streamlined Method** as detailed on pages 10-13).
11. Stop watch.
12. Whatman No. 41 filter circles.
13. Centrifuge (in conjunction with preparation of malt, wort and beer).
14. Laboratory mill with 0.5 mm screen (e.g. Frisch pulverisette 14®).
15. Boiling water bath.

CONTROLS AND PRECAUTIONS:

1. With each set of determinations, reagent blanks and D-glucose standards of 50 µg and/or 100 µg are included, in duplicate.

   The **reagent blank** comprises 0.1 mL distilled water + 0.1 mL sodium acetate buffer + 3.0 mL of **GOPOD Reagent.**

   The **glucose standard** comprises 0.1 mL sodium acetate buffer + 0.1 mL D-glucose standard (50 µg/0.1 mL or 100 µg/0.1 mL) + 3.0 mL **GOPOD Reagent.**
2. With each set of determinations at least one standard barley flour is also included.
3. With each new batch of **GOPOD Reagent** the time for maximum colour formation with 100 µg of D-glucose standard should be checked. This is usually approx. 15 min.
4. It is imperative that the lichenase enzyme preparation is **not** cross-contaminated with the β-glucosidase preparation (the reverse is not a problem).

USEFUL HINTS:

1. To reduce handling time during the final (absorbance reading) stage of the assay it is suggested that a spectrophotometer with a flow-through cell be used.

2. After reaction of samples with lichenase it is suggested that the volume of the reaction mixture* is adjusted to 30.0 mL by the addition of 24.0 mL of distilled water via a dispenser.

* Assume the volume to be 6.0 mL; approx. 0.2 mL is lost during the heating step.

3. In step 5 of the assay procedure, if the solution becomes very viscous after the 5 min boiling step, add 5.0 mL of distilled water and stir well on a vortex mixer. After reaction with lichenase adjust the volume to 30.0 mL by the addition of 19.0 mL of distilled water.

NOTE: If the solution is very viscous there may be some problem with the diffusion of lichenase. Adding 5.0 mL of distilled water will alleviate this problem.

4. If glass, rather than polypropylene, tubes are used in step 5 of the assay procedure, reduce the time of incubation in the boiling water bath to 45 sec initially, vortex the contents and incubate for a further 45 sec in the boiling water bath (i.e. total of 1.5 min).

ASSAY PROCEDURE FOR BARLEY (EBC Method 3.11.1):

1. Mill barley to pass a 0.5 mm screen using a Tecator Cyclotec® mill (uniform, fine milling is essential).
2. Accurately weigh barley flour samples (approx. 0.5 g) of known moisture content* into polypropylene tubes (refer to Equipment, point 1).

* See footnote under Example Results Sheet on page 6.

3. Add an aliquot (1.0 mL) of aqueous ethanol (50 % v/v) to each tube to aid in the subsequent dispersion of samples.
4. Add 5.0 mL of sodium phosphate buffer (20 mM, pH 6.5) and stir the tubes on a vortex mixer.
5. Incubate the tubes in a boiling water bath for approx. **2** min (see point 3 and 4 under Useful Hints). Remove the tubes and vigorously stir them on a vortex mixer. Heat the tubes for a further **3** min in the boiling water bath (mixing after 2 min prevents formation of a lump of gel material).

6. Cool the tubes to 40°C and add 0.2 mL of **lichenase** (10 U) to each tube. Cap the tubes, stir, and incubate at 40°C for 1 h.
7. Adjust the volume in each tube to 30.0 mL by the addition of distilled water (see point 2 under Useful Hints).
8. Thoroughly mix the contents of the tubes and filter an aliquot from each tube through a Whatman No. 41 filter circle (or centrifuge an aliquot at approx. 1,000 *g* for 10 min).
9. **Carefully and accurately** transfer aliquots (0.1 mL) from each filtrate to the bottom of **three** test tubes.
10. Add an aliquot (0.1 mL) of sodium acetate buffer (50 mM, pH 4.0) to one of these (the reaction blank), while to the other two (the reaction) add 0.1 mL of **β-glucosidase** (0.2 U) in 50 mM acetate buffer (pH 4.0). Incubate the tubes at 40°C for 15 min.
11. Add **GOPOD Reagent** (3.0 mL) to each tube and incubate at 40°C for 20 min (see point 3 under Controls and Precautions).
12. Measure the absorbance at 510 nm for each reaction (E_A) and reaction blank (E_{Bl}).

NOTE: With the **GOPOD Reagent** employed in this kit, the colour complex which is formed is stable for at least 2 h at room temperature.

EXAMPLE RESULTS SHEET:

Sample	Sample weight (mg)		Absorbances (510 nm)			β-Glucan
	Fresh	Dry (corrected)	E_{Bl}	E_A	ΔA	% (w/w)
e.g. Clipper	495	420	0.012	0.460 0.455	0.448 0.443	2.86 2.83

$$\text{Dry weight} = \text{fresh weight} \times \frac{100 - \text{moisture content (\%)}}{100}*$$

* In general this is determined by NIR reflectance. Alternatively this can be determined by observing weight loss on storage of flour samples (0.5 g) at 80°C for 20 h. The moisture content of cereal flour samples is consistently in the range of 10-14 %.

ASSAY PROCEDURE for MALT, SPENT GRAIN, BEER and WORT.

A. Malt (EBC Method 4.16.1):

1. To 1.0 g of malt flour (milled to pass a 0.5 mm screen) or lyophilised barley samples removed during the malting process, add 5.0 mL of aqueous ethanol (50 % v/v).
2. Incubate in a boiling water bath for 5 min. Mix the contents on a vortex stirrer and add a further 5.0 mL of 50 % (v/v) aqueous ethanol. Mix.
3. Centrifuge for 10 min at 1,000 *g*. Discard the supernatant.
4. Resuspend the pellet in 10.0 mL of 50 % (v/v) aqueous ethanol, centrifuge and discard the supernatant (as in step 3. above).
5. Suspend the pellet in 5.0 mL of sodium phosphate buffer (20 mM, pH 6.5).
6. Assay for β-glucan as per the Assay Procedure for barley from step 5.

B. Spent Grain:

Either wash spent grain with hot water (approx. 75°C), and then lyophilise, or lyophilise without washing. Mill this material to pass a 0.5 mm screen and analyse for β-glucan content and perform calculations by the same procedure as employed for the malt samples.

C. Beer or Wort (EBC Method 8.11.1):

1. De-gas beer by heating an aliquot to approx. 80°C in a boiling water bath. Allow to cool.
2. To 5.0 mL of wort or degassed beer in a pre-weighed centrifuge tube (12 mL capacity) add 2.5 g of finely milled ammonium sulphate crystals.
3. Seal the tube with Parafilm® and dissolve the ammonium sulphate by careful inversion (to avoid frothing).
4. Allow the tube to stand for approx. 20 h at 4°C.
5. Centrifuge at 1,000 *g* for 10 min on a bench centrifuge.
6. Discard the supernatant.

7. Resuspend the pellet by thoroughly vortexing with 1.0 mL of 50 % (v/v) aqueous ethanol. Add a further 10.0 mL of 50 % (v/v) aqueous ethanol and mix well by inversion of the tube.
8. Centrifuge at 1,000 *g* for 5 min. Discard the supernatant.
9. Repeat the ethanol-washing procedure by resuspending the pellet, etc. as in steps 7. and 8. above.
10. Discard the supernatant.
11. Resuspend the pellet in sodium phosphate buffer (20 mM, pH 6.5): for wort, adjust the volume to 4.8 mL (by weight) for beer, adjust the volume to 1.8 mL (by weight).
12. Add 0.2 mL lichenase (10 U) and incubate at 40°C for 5 min. Centrifuge at 1,000 *g* for 10 min then proceed as per the assay procedure for barley starting from step 9.

NOTE: For wort samples containing low levels of β-glucan, incubate a larger aliquot of sample solution (up to 0.5 mL) with β-glucosidase. Use this larger aliquot size also for the blank. The D-glucose standard must also be adjusted accordingly with distilled water. Modify calculations accordingly.

CALCULATIONS:

A. For barley, malt and spent grain

$$\beta\text{-glucan (\% w/w)} = \Delta A \times F \times 300 \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

$$= \Delta A \times \frac{F}{W} \times 27$$

B. For wort

$$\beta\text{-glucan (mg/L)} = \Delta A \times F \times 10{,}000 \times \frac{1}{1000} \times \frac{5}{5} \times \frac{162}{180}$$

$$= \Delta A \times F \times 9$$

C. For beer

$$\beta\text{-glucan (mg/L)} = \Delta A \times F \times 10{,}000 \times \frac{1}{1000} \times \frac{2}{5} \times \frac{162}{180}$$

$$= \Delta A \times F \times 3.6$$

where:

ΔA = Absorbance after β-glucosidase treatment (reaction) minus reaction blank absorbance.

F = A factor for the conversion of absorbance values to µg of glucose.

$$= \frac{100\ (\mu g\ \text{of D-glucose})}{\text{absorbance of } 100\ \mu g\ \text{of D-glucose}}$$

300 = Volume correction (i.e. 0.1 mL taken from 30.0 mL).

10,000 = Volume adjustment factor (0.1 mL was analysed but results are presented per litre of sample).

$\frac{1}{1000}$ = Conversion from µg to mg.

$\frac{100}{W}$ = Factor to express β-glucan content as a percentage of dry flour weight.

W = The calculated dry weight of the sample analysed, in mg (refer to example results sheet on page 6).

$\frac{5}{5}$ = Volume correction factor. For wort samples, 5.0 mL aliquots were treated with precipitant (ammonium sulphate) and the volume was readjusted to 5.0 mL (i.e. 4.8 mL + 0.2 mL lichenase).

$\frac{2}{5}$ = Volume correction factor. For beer samples, 5.0 mL aliquots were treated with precipitant (ammonium sulphate) and the volume was readjusted to 2.0 mL (i.e. 1.8 mL + 0.2 mL lichenase).

$\frac{162}{180}$ = A factor to convert from free D-glucose, as determined, to anhydro-D-glucose, as occurs in β-glucan

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

ASSAY OF MIXED-LINKAGE BETA-GLUCAN IN OAT AND BARLEY FLOUR AND FIBRE SAMPLES
- STREAMLINED METHOD -

AOAC Method 995.16
AACC Method 32-23
ICC Standard Method No. 168

INTRODUCTION:

This procedure is ideal for all dry samples particularly those containing high levels of β-glucan (e.g. processed oat bran products).

METHOD:

1. Mill barley, oats or fibre sample (approximately 50 g) to pass a 0.5 mm screen using a Fritsch pulverisette 14® (Fritsch GmbH Idar-Oberstein, Germany) or alternative centrifugal mill.
2. Add flour sample (80-120 mg; weighed accurately) to a glass centrifuge tube (16 x 120 mm; 17 mL capacity). Tap the tube to ensure that all sample falls to the bottom of the tube.
3. Wet the sample with 0.2 mL of aqueous ethanol (50 % v/v) to aid dispersion. Add sodium phosphate buffer (4.0 mL, 20 mM, pH 6.5) and stir the contents on a vortex mixer.
4. On mixing, immediately place the tube in a boiling water bath and incubate for **60 sec**. Vigorously stir the mixture on a vortex mixer, incubate at 100°C for a further **2 min**, and stir again.
5. Incubate the tube plus contents at **50°C** and allow to equilibrate for 5 min.
6. Add **lichenase** (0.2 mL, 10 U) and stir the tube contents. Seal the tube with parafilm and incubate for **1 h** at 50°C, with regular vigorous stirring (i.e. 3-4 times) on a vortex mixer. In fact, continuous stirring using a device such as the Megazyme Multistir Incubation Bath (cat. no. G-IBMKIII) is recommended.
7. Add sodium acetate buffer (5.0 mL, 200 mM, pH 4.0) and vigorously mix the tube contents on a vortex mixer.

8. Allow the tube to equilibrate to room temperature (5 min), and centrifuge (1,000 *g*, 10 min). Carefully and accurately dispense aliquots (0.1 mL) into the bottom of three test tubes (12 mL capacity) using a Gilson Pipetman® or a Rainin EDP-2® motorised dispenser.
9. Add **β-glucosidase** (0.1 mL, 0.2 U) in 50 mM sodium acetate buffer (pH 4.0) to two of these tubes (the reaction). To the third (the reaction blank), add 50 mM acetate buffer (0.1 mL, pH 4.0). Incubate all tubes at **50°C** for **10 min**.
10. Add **GOPOD Reagent** (3.0 mL) to each tube, and incubate at 50°C for a further **20 min**.
11. Remove the tubes from the water bath and measure the absorbance (510 nm) within 1 h.

STANDARDS AND CONTROLS:

With each set of determinations (usually 20-30 samples), include D-glucose standards of 50 and 100 µg (in 0.1 mL), as well as two control flours of known β-glucan content.

Calculate the β-glucan content (dry weight basis) by allowing for moisture content (refer to page 6).

REAGENTS:

Mixed linkage β-Glucan Test Kit from Megazyme International Ireland Ltd (see page 1).

NOTE:

1. In the analysis of β-glucan in cooked, toasted or extruded cereal products, the sample should be pre-extracted with aqueous ethanol to remove free sugars and to reduce the levels of fats and oils. This is simply achieved using steps 1. to 4. as for malt flour samples (page 7), except that in this case, 0.2 g sample size is used, and after the lichenase treatment step (Step 6, page 10), the amount of sodium acetate buffer added is reduced to 2.0 mL. Calculations are adjusted appropriately to allow for the different final volume (i.e. 6.4 mL instead of 9.4 mL).
2. Reaction blank determinations should be performed for all processed products to account for free D-glucose in the sample.
3. The addition of sodium acetate buffer and the cooling of the tube after the lichenase treatment step assists in producing a clearer supernatant solution on centrifugation.

CALCULATIONS:

$$\beta\text{-glucan (\% w/w)} = \Delta A \times F \times 94 \text{ (or 64)} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

$$= \Delta A \times \frac{F}{W} \times 8.46 \text{ (or 5.76)}$$

where:

ΔA = Absorbance after β-glucosidase treatment (reaction) minus reaction blank absorbance.

F = A factor for the conversion of absorbance values to µg of glucose.

$$= \frac{100 \text{ (µg of D-glucose)}}{\text{absorbance of 100 µg of D-glucose}}$$

94 = Volume correction factor (0.1 mL out of 9.4 mL was analysed for cereal samples).

64 = Volume correction factor (0.1 mL out of 6.4 mL was analysed for cooked, toasted and extruded cereal products).

$\frac{1}{1000}$ = Conversion from µg to mg.

$\frac{100}{W}$ = Factor to express β-glucan content as a percentage of dry flour weight.

W = The calculated dry weight of the sample analysed in mg (refer to example results sheet on page 6).

$\frac{162}{180}$ = A factor to convert from free D-glucose, as determined, to anhydro-D-glucose, as occurs in β-glucan.

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

COMPARISON OF METHODS:

The Megazyme streamlined β-glucan method has been compared to AACC Method 32-22 (the AACC modification of the original Megazyme method[1]) in an interlaboratory evaluation, and the results obtained with both methods were very similar. Results with the Megazyme "Streamlined Method" are shown in Table 1. With this method, more than 100 samples can be analysed by a single analyst in one day. This compares to about 20 samples with AACC Method 32-22.

Table 1: Method Performance for Determination of β-D-Glucan in Oats by Streamlined Enzymatic Method.[a]

Sample	Mean, % dry basis	S_r	S_R	RSD_r %	RSD_R %	r^b	R^c
Oat flour	2.73	0.083	0.241	3.1	8.8	0.232	0.675
Oat bran	6.39	0.296	0.456	4.6	7.1	0.829	1.277
Rolled oats	4.27	0.283	0.315	6.6	7.4	0.792	0.882
Oat bran (breakfast cereal)	3.93	0.484	0.484	12.3	12.3	1.355	1.355
Instant oat bran	8.00	0.480	0.524	6.0	6.6	1.344	1.467

[a] Based on results from 8 laboratories; no outliers identified
[b] $r = 2.8 \times s_r$
[c] $R = 2.8 \times s_R$

STANDARDISATION OF ENZYME ACTIVITY:

β-Glucosidase was standardised using *p*-nitrophenyl β-glucoside as substrate. One unit is defined as the amount of enzyme required to release one µmole of *p*-nitrophenol from *p*-nitrophenyl β-glucoside (10 mM) per min at pH 4.0 and 40°C. **Lichenase** activity was determined on barley β-glucan (10 mg/mL) in sodium phosphate buffer (pH 6.5) at 40°C using the Nelson/Somogyi reducing sugar procedure (refer to reference 1, page 14 of this booklet). One Unit of activity is defined as the amount of enzyme required to release one µmole of D-glucose reducing-sugar equivalents per min under the defined assay conditions.

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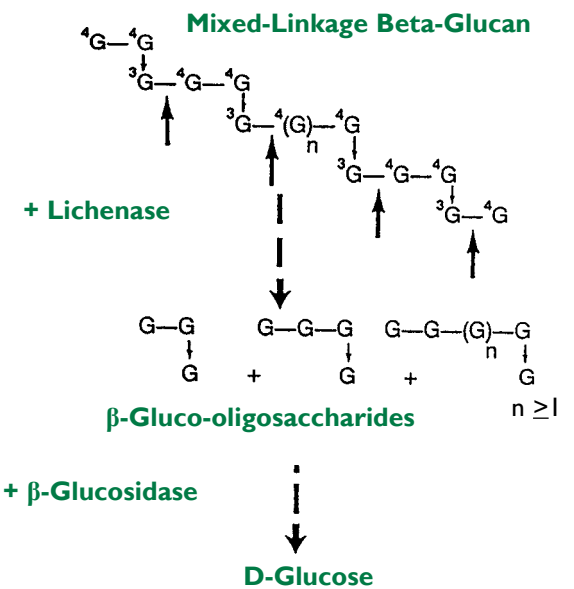
4. McCleary, B. V. and Mugford, D. C. (1992). Interlaboratory evaluation of β-glucan analysis methods. In "The changing role of oats in human and animal nutrition". Proceedings of the Fourth International Oat Conference, Adelaide, Australia. Oct 19-23.



Scheme 1. Principle of the mixed-linkage beta-glucan assay procedure.

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