Ver.250501

RESISTANT STARCH ASSAY

BC6110 (100 Assays)

FOR RESEARCH USE ONLY, DO NOT USE IT IN CLINICAL DIAGNOSIS

I. Product Description

Samples are incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase (AMG) for 16 h at 37°C, during which time non-resistant starch is solubilised and hydrolysed to D-glucose by the combined action of the two enzymes. The reaction is terminated by the addition of an equal volume of ethanol or industrial methylated spirits (IMS, denatured ethanol) and the RS is recovered as a pellet on centrifugation. This is then washed twice by suspension in aqueous IMS or ethanol (50% v/v), followed by centrifugation. Free liquid is removed by decantation. RS in the pellet is dissolved in 2 M KOH by vigorously stirring in an ice-water bath over a magnetic stirrer. This solution is neutralised with acetate buffer and the starch is quantitatively hydrolysed to glucose with AMG. D-Glucose is measured with glucose oxidase/peroxidase reagent (GOPOD) and this is a measure of the RS content of the sample. Non-resistant starch (solubilised starch) is determined by pooling the original supernatant and the washings, adjusting the volume to 100mL and measuring D-glucose content with GOPOD.

II. Applicability and Accuracy

The method is applicable to samples containing more than 2% w/w RS. With such samples, standard errors of ± 5% are achieved routinely. Higher errors are obtained for samples with RS contents < 2% w/w.

III. Reagent Composition & Preparation

SL:NO	Reagents	Storage
Bottle 1	**Amyloglucosidase** [12mL, 3,300 U/mL on soluble starch (or 200 U/mL on *p*-nitrophenyl β maltoside)] at pH 4.5 and 40°C. AMG solution should be essentially free of detectable levels of free D-glucose.	4°C
Bottle 2	**Pancreatic α amylase** (Pancreatin, 10g, 3 Ceralpha Units/mg).	-10°C
Bottle 3	**GOPOD Reagent Buffer.** Buffer (50mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.09% w/v).	4°C
Bottle 4	**GOPOD Reagent Enzymes.** Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze dried powder.	-10°C
Bottle 5	**D-Glucose standard solution** (5mL, 1.0 mg/mL) in 0.2% (w/v) benzoic acid.	RT
Bottle 6	**Resistant starch control**. Resistant starch content shown on the label.	RT

IV. Preparation of Reagent Solutions/Suspensions

1. Use the contents of **bottle 1** (3,300 U/mL AMG solution) as supplied. This solution is viscous and thus should be dispensed with a positive displacement dispenser, e.g. Eppendorf Multipette ® with 5.0mL Combitip® (to dispense 0.1mL aliquots).

   Also prepare:

   Solution 1 (dilute AMG, 300 U/mL): Dilute 2mL of bottle 1 (3,300 U/mL AMG solution) to 2mL with 0.1 M sodium maleate buffer (100mM, pH 6.0; Reagent 1; not supplied). Divide into 5mL aliquots and store frozen in polypropylene containers between uses. This is **solution 1** (dilute AMG, 300 U/mL). Stable to repeated freeze/thaw cycles and for ≥2 years below -10°C.

2. **Immediately before use**, suspend 1 g of the contents of **bottle 2** (pancreatic α-amylase) in 100mL of sodium maleate buffer (100mM, pH 6.0; Reagent 1; not supplied) and stir for 5 minute. Add 1.0mL of solution 1 (dilute AMG, 300 U/mL) and mix well. Centrifuge at >1,500 g for 10 minute and carefully decant the supernatant solution. This is **solution 2** (pancreatic α-amylase, 10 mg/mL containing 3 U/mL AMG). Use on the day of preparation.
3. Dilute the contents of the **GOPOD Reagent Buffer bottle** to 1L with distilled water (this is **solution 3**). Use immediately. Operation Procedure

   Note:

   a) On storage, salt crystals may form in the **GOPOD Reagent Buffer** these must be completely dissolved when this buffer is diluted to 1L with distilled water.

   b) This buffer contains 0.09% (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.
4. Dissolve the contents of the GOPOD Reagent Enzymes bottle in 20mL 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 ≥1 month at 4°C or ≥ 12 months below -10°C.

   If this reagent is to be stored in the frozen state, preferably it should be divided into aliquots. Do not freeze/thaw more than once.

   When the reagent is freshly prepared it may be light yellow or light pink in colour. Upon storage at 4°C the GOPOD reagent may develop a stronger pink colour. The absorbance of this solution should be less than 0.05 when read against distilled water.
5. Use the contents of bottles 5 and 6 as supplied.

V. Reagents (Not Supplied)

Reagents should be analytical purity grade (or similar).

1. **Sodium maleate buffer (Reagent 1)** (100mM, pH 6.0) plus 2mM calcium chloride dihydrate.

   Dissolve 23.2 g of maleic acid in 1600mL of distilled water and adjust the pH to 6.0 with 4 M (160 g/L) sodium hydroxide. Add 0.6 g of calcium chloride dihydrate ($CaCl_2.2H_2O$) and dissolve. Adjust the volume to 2L. Store at 4°C.
2. **Sodium acetate buffer** (1.2 M, pH 3.8).

   Add 68.6mL of glacial acetic acid (1.05 g/mL) to 800mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Adjust the volume to 1L with distilled water. Store at 4°C.
3. **Sodium acetate buffer** (100mM, pH 4.5).

   Add 5.8mL of glacial acetic acid to 900mL of distilled water and adjust to pH 4.5 using 4 M sodium hydroxide. Adjust the volume to 1L with distilled water. Store at 4°C.
4. **Potassium hydroxide solution** (2 M).

   Add 112.2 g Potassium hydroxide (KOH) to 900mL of deionised water and dissolve by stirring. Adjust volume to 1 L. Store in a sealed container. Store at room temperature.
5. **~ 50% v/v ethanol (or IMS).**

   Add 500mL of ethanol (95% v/v or 99% v/v) or industrial methylated spirits (IMS; denatured ethanol; ~ 95% v/v ethanol plus 5% v/v methanol) to 500mL of H_2O. Store in a well-sealed bottle at room temperature.
6. **99% v/v ethanol**

   99% v/v ethanol. Alternatively, 95 % v/v Ethanol or IMS (denatured ethanol; ~ 95% v/v ethanol plus 5% v/v methanol) may be used.

VI. Equipment (Recommended)

1. Grinding mill - Centrifugal, equipped with 12-tooth rotor and a 1.0 mm sieve, or similar device. Alternatively, a cyclone mill can be used for small samples.
2. Meat mincer - Hand operated or electric, fitted with a 4.5 mm screen.
3. Bench centrifuge - Capable of holding 16 x 120 mm glass test tubes, with rating of approx. 1,500 g (~ 3,000 rpm).
4. Shaking water bath set in linear motion at 100 revolutions per min on the dial (equivalent to a shake speed of 200 strokes/min), a stroke length of 35 mm and 37°C.
5. Water bath - Capable of maintaining 50 +/- 0.1°C.
6. Vortex mixer.
7. Magnetic stirrer.
8. Magnetic stirrer bars – 5 × 15 mm.
9. pH Meter.
10. Stop-watch/timer (digital).
11. Analytical balance (correct to 0.1 mg).
12. Spectrophotometer - capable of operating at 510 nm, preferably fitted with flowthrough cell (10 mm path length).
13. Pipettor - capable of delivering 100 µL; with disposable tips. Alternatively, motorised hand-held dispenser can be used.
14. Positive displacement pipettor - Equipped with 50 mL tips capable of delivering 2.0mL, 3.0mL and 4.0mL.
15. Screw cap, 16 x 125 mm.
16. Glass test tubes - 16 x 100 mm, 14mL capacity.
17. Plastic box, large enough to hold test-tube rack and serve as an ice-water bath.
18. Thermometer - Capable of reading 37 +/- 0.1°C and 50 +/- 0.1°C.
19. Volumetric flasks - 100mL, 200mL, 500mL, 1L and 2L capacity.

VII. Sample Preparation

Grind approximately 50 g of sample of grain or lyophilised plant or food product in grinding mill to pass a 1.0 mm sieve. Transfer all material to a wide-mouthed plastic jar and mix well by shaking and inversion. Industrial starch preparations are usually supplied as a fine powder, so grinding is not required. Mince fresh samples (e.g. canned beans, bananas, potatoes) in a hand operated or electric meat mincer to pass an ~ 4.5 mm screen. Determine moisture content of dry samples by AOAC Method 925.10 and of fresh samples by lyophilisation followed by oven drying according to AOAC Method 925.10.

VIII. Assay Procedure

(a) Hydrolysis and solubilisation of non-resistant starch

i. Accurately weigh a 100 ±5 mg sample directly into each screw cap tube (16 × 125 mm) and gently tap the tube to ensure that the sample falls to the bottom.
   Note: For wet samples such as minced canned beans or food products, the sample size is approx. 0.5 g (weighed accurately). With such materials, the moisture content is usually 60-80%.

ii. Add 4.0mL of solution 2 (pancreatic α-amylase (10 mg/mL) containing 3 U/mL AMG) to each tube.

iii. Tightly cap the tubes, mix them on a vortex mixer and attach them horizontally in a shaking water bath, aligned in the direction of motion.

iv. Incubate tubes at 37°C with continuous shaking (200 strokes/min) for exactly 16 h (**Note**: for linear motion, a setting of 100 on the water bath is equivalent to 200strokes/min; 100 forward and 100 reverse).

v. Remove the tubes from the water bath and remove excess surface water with a paper towel. Remove the tube caps and treat the contents with 4.0mL of ethanol (99% v/v) or IMS (99% v/v) with vigorous stirring on a vortex mixer.
vi. Centrifuge the tubes at 1,500 g (approx. 3,000 rpm) for 10 minute (non-capped).
vii. Carefully decant the supernatants and re-suspend the pellets in 2mL of 50% v/v ethanol (or 50% v/v IMS) with vigorous stirring on a vortex mixer. Add a further 6mL of 50% v/v ethanol, mix the tubes and centrifuge again at 1,500 g for 10 minute.
viii. Decant the supernatants and repeat this suspension and centrifugation step once more.
ix. Carefully decant the supernatants and invert the tubes on absorbent paper to drain excess liquid.

(b) Measurement of Resistant Starch

i. Add a magnetic stirrer bar (5 × 15 mm) and 2mL of 2 M KOH to each tube and resuspend the pellets (and dissolve the RS) by stirring for approx. 20 minute in an ice/water bath over a magnetic stirrer.
   Note: a) Do not mix on a vortex mixer as this may cause the starch to emulsify.
   b) Ensure that the tube contents are vigorously stirred as the KOH solution is added. This will avoid the formation of a lump of starch material that will then be difficult to dissolve.
ii. Add 8mL of 1.2 M sodium acetate buffer (pH 3.8) to each tube with stirring on the magnetic stirrer. Immediately 0.1mL of bottle 1 (AMG, 3,300 U/mL) mix well and place the tubes in a water bath at 50°C.
iii. Incubate the tubes for 30 minute with intermittent mixing on a vortex mixer.
iv. For samples containing > 10% RS; quantitatively transfer the contents of the tube to a 100mL volumetric flask (using a water wash bottle). Use an external magnet to retain the stirrer bar in the tube while washing the solution from the tube with the water wash bottle. Adjust to 100mL with distilled water and mix well. Centrifuge an aliquot of the solution at 1,500 g for 10 minute.
v. For samples containing < 10% RS; directly centrifuge the tubes at 1,500 g for 10 minute (no dilution). For such samples, the final volume in the tube is approx. 10.3mL (however, this volume will vary particularly if wet samples are analysed, and appropriate allowance for volume should be made in the calculations).
vi. Transfer 0.1mL aliquots (in duplicate) of either the diluted (step iv) or the undiluted (step v) supernatants into glass test tubes (16 x 100 mm), add 3.0mL of GOPOD reagent and incubate at 50°C for 20 minute.
vii. Measure the absorbance of each solution at 510 nm against the reagent blank.
   Prepare reagent blank solutions by mixing 0.1mL of 100mM sodium acetate buffer (pH 4.5) and 3.0mL of **GOPOD reagent**.
   Prepare D-glucose standards (in quadruplicate) by mixing 0.1mL of bottle 5 (D-glucose, 1 mg/mL) and 3.0mL of **GOPOD reagent**.

(c) Measurement of Non-Resistant (Solubilised) Starch

i. Combine the supernatant solutions obtained on centrifugation of the initial incubation [(a)vii] with the supernatants obtained from the subsequent two 50% ethanol washings [(a)viii and (a)ix] and adjust the volume to 100mL with 100mM sodium acetate buffer (pH 4.5) in a volumetric flask. Mix well.
ii. Incubate 0.1mL aliquots of this solution (in duplicate) with 10µL of solution 1 (dilute AMG, 300 U/mL).in 100mM sodium maleate buffer (pH 6.0) for 20 minute at 50°C. Add 3.0mL of GOPOD reagent and incubate the tubes for a further 20 minute at 50°C.
iii. Measure the absorbance at 510 nm against a reagent blank.
iv. Calculate the content of non-resistant (solubilised) starch.

Total starch content is the sum of resistant starch and non-resistant (solubilised) starch.

IX. Calculations

Calculate resistant starch, non-resistant (solubilised) starch and total starch content (%, on a dry weight basis) in test samples as follows:

Resistant Starch (g/100 g sample) (samples containing > 10% RS):

$$= \Delta A \times F \times \frac{\mathbf{100}}{\mathbf{0.1}} \times \frac{\mathbf{1}}{\mathbf{1000}} \times \frac{\mathbf{100}}{\mathbf{W}} \times \frac{\mathbf{162}}{\mathbf{180}}$$

$$= \Delta A \times \frac{\mathbf{F}}{\mathbf{W}} \times 90$$

Resistant Starch (g/100 g sample) (samples containing < 10% RS):

$$= \Delta A \times F \times \frac{\mathbf{10.3}}{\mathbf{0.1}} \times \frac{\mathbf{1}}{\mathbf{1000}} \times \frac{\mathbf{100}}{\mathbf{W}} \times \frac{\mathbf{162}}{\mathbf{180}}$$

$$= \Delta A \times \frac{\mathbf{F}}{\mathbf{W}} \times 9.27$$

Non-Resistant (Solubilised) Starch (g/100 g sample):

$$= \Delta A \times F \times \frac{\mathbf{100}}{\mathbf{0.1}} \times \frac{\mathbf{1}}{\mathbf{1000}} \times \frac{\mathbf{100}}{\mathbf{W}} \times \frac{\mathbf{162}}{\mathbf{180}}$$

$$= \Delta A \times \frac{\mathbf{F}}{\mathbf{W}} \times 90$$

Total Starch = Resistant Starch + Non-Resistant Starch

where:

ΔA = absorbance (reaction) read against the reagent blank.

F = conversion from absorbance to micrograms (the absorbance obtained for 100μg of D-glucose in the GOPOD reaction is determined and F = 100 (μg of D-glucose) divided by the GOPOD absorbance for this 100μg of D-glucose.

100/0.1 = volume correction (0.1mL taken from 100mL).

1/1000 = conversion from micrograms to milligrams.

W = dry weight of sample analysed
="as is" weight x [(100-moisture content)/100].

100/W = factor to present RS as a percentage of sample weight.

162/180 = factor to convert from free D-glucose, as determined, to anhydro-D-glucose as occurs in starch.

10.3/0.1 = volume correction (0.1mL taken from 10.3mL) for samples containing 0- 10% RS where the incubation solution is not diluted and the final volume is ~ 10.3mL. When wet samples are analysed, this volume will be larger, and this should be allowed for in the calculations.