

# Megazyme

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## **PULLULANASE/ LIMIT-DEXTRINASE**

### **ASSAY PROCEDURE (PullG6 METHOD)**

K-PullG6 04/20

FOR THE MEASUREMENT OF  
MICROBIAL PULLULANASE AND  
MALT LIMIT-DEXTRINASE

(100/200 Assays per Kit)



## INTRODUCTION:

Pullulanase (EC 3.2.1.4) and limit dextrinase (EC 3.2.1.142) cleave the  $\alpha$ -1,6-linkages in pullulan, amylopectin and  $\alpha$ - and  $\beta$ -limit dextrans of starch. Pullulanase is obtained from microbial sources and finds widespread application in the starch processing industry. In combination with amyloglucosidase (AMG; EC 3.2.1.3) it gives more efficient conversion of starch to glucose, reducing the formation of isomaltose and giving up to 1% greater yield of glucose.<sup>1,2</sup> High maltose syrups are produced from starch using combinations of pullulanase and  $\alpha$ -amylase.<sup>1,3</sup> Limit dextrinase is found in cereals, notably malted barley, where it plays a key role during the mashing stage of the brewing process. Together with  $\alpha$ -amylase (EC 3.2.1.1),  $\beta$ -amylase (EC 3.2.1.2) and  $\alpha$ -glucosidase (EC. 3.2.1.20), it catalyses the degradation of starch to fermentable carbohydrates (mostly maltose and D-glucose).

Pullulanase activity in industrial enzyme preparations can conveniently be assayed with borohydride reduced pullulan (cat. no. **P-PULLBH**) using an appropriate reducing sugar method (Nelson and Somogyi). In crude enzyme preparations, the activity can be more specifically detected using soluble dyed substrates (Red Pullulan – cat. no. **S-RPUL**) or dyed and cross-linked pullulan in powder form (AZCL-Pullulan – cat. no. **I-AZPUL**). The activity can be accurately quantified using AZCL-Pullulan in tablet form (Limit-Dextrizyme Tablets – cat. no. **T-LDZ**).<sup>4</sup> The same substrates can be used for the measurement of limit-dextrinase although the assay of this enzyme in crude malt and mash samples is complicated by a number of factors, most notably the low levels of enzyme activity present and the ability of limit-dextrinase to carry out transglycosylation as well as hydrolytic cleavage. The transglycosylation problem can be circumvented provided that the sample is devoid of malt dextrans. This is accomplished by the addition of amyloglucosidase to the buffer employed during the extraction of limit-dextrinase and is discussed in detail in the [Limit-Dextrizyme data booklet](#). While these dyed polysaccharide substrates are very useful and widely used, they do not readily lend themselves to automated analysis procedures.

This kit (**K-PullG6**) provides a simple robust method for the measurement of pullulanase and limit dextrinase. The kit assay employs a novel reagent, PullG6 that is highly specific for pullulanase and limit dextrinase and is not hydrolysed by  $\alpha$ -amylase (EC 3.2.1.1),  $\beta$ -amylase (EC 3.2.1.2), amyloglucosidase (EC 3.2.1.3) or  $\alpha$ -glucosidase (EC. 3.2.1.20). It has also been shown to be unaffected by the transglycosylation activity of limit-dextrinase. Most importantly, this reagent is suitable for use with high throughput auto-analyser and microplate assay formats.

## PRINCIPLE:

The principle of the assay is shown in Appendix C (page 12). The PullG6 reagent is composed of a defined substrate, namely 4,6-*O*-benzylidene-4-nitrophenyl-6<sup>3</sup>- $\alpha$ -D-maltotriosyl-maltotriose (BPNPG3G3), coupled with the ancillary enzymes  $\alpha$ -glucosidase and  $\beta$ -glucosidase. Upon hydrolysis of the substrate at the 1,6- $\alpha$ -linkage by pullulanase or limit-dextrinase, the released 4-nitrophenyl- $\beta$ -maltotriose is immediately hydrolysed to glucose and 4-nitrophenol by the concerted action of the  $\alpha$ -glucosidase and  $\beta$ -glucosidase enzymes in the reagent mixture. The reaction is terminated and phenolate ions are developed by addition of dilute alkali. The absorbance is read at 400 nm and the value obtained correlates directly with pullulanase or limit dextrinase activity.

## ACCURACY:

Standard errors of less than 5% are achieved routinely.

## SPECIFICITY:

The assay is absolutely specific for pullulanase/limit-dextrinase.

## KITS:

Kits suitable for performing 100/200 assays are available from Megazyme. The kits contain the full assay method plus:

### Bottle 1: (x2)

Each vial contains 4,6-*O*-Benzylidene-4-nitrophenyl-6<sup>3</sup>- $\alpha$ -D-maltotriosyl-maltotriose (BPNPG3G3) plus thermostable  $\alpha$  and  $\beta$ -glucosidase.  
Stable for > 5 years below -10°C.

### Bottle 2:

Control pullulanase solution (*Bacillus licheniformis*, ~ 2.3 U/mL on PullG6 substrate) (exact activity stated on vial label) in 50% v/v glycerol plus BSA (1% w/v) and sodium azide (0.02% w/v).  
Stable for > 4 years below -10°C.

### Bottle 3:

Concentrated sodium acetate buffer (25 mL, 2 M, pH 5.0) plus BSA (10 mg/mL) and sodium azide (0.09% w/v).  
Stable for > 4 years at room temperature.

### Bottle 4:

Control malt flour of standardised limit-dextrinase activity, ~ 0.28 U/g on PullG6 substrate) (exact activity stated on vial label). Stable for > 4 years; store sealed below -10°C.

## PREPARATION OF REAGENT SOLUTIONS:

1. Dissolve the contents of one of bottle 1 in 5 mL of distilled water. This is the **PullG6 Reagent Solution**. Divide into appropriately sized aliquots and store in polypropylene tubes below  $-10^{\circ}\text{C}$  between use and on ice during use. Do not dissolve the contents of the other bottle until required. Once dissolved, the reagent is stable for  $> 2$  years below  $-10^{\circ}\text{C}$ .
2. With a positive displacement pipette, dispense 1 mL of the contents of bottle 2 into a 25 mL volumetric flask and dilute to volume with **Buffer B**. Divide into appropriately sized aliquots and store below  $-10^{\circ}\text{C}$ . Once dissolved, the standard is stable for  $> 1$  year below  $-10^{\circ}\text{C}$ .
3. Dilute the contents of bottle 3 to 500 mL with distilled water. This is **Buffer B**. Store at  $4^{\circ}\text{C}$ . Stable for  $> 2$  years at  $4^{\circ}\text{C}$ .
4. The limit-dextrinase extraction/activation procedure for the pre-milled standard is exactly the same as that described for barley samples on page 7. Simply continue from point 2 - "Accurately weigh 0.5g...".

## PREPARATION OF ADDITIONAL BUFFERS (not supplied):

### For the assay of pullulanase:

#### (A) Concentrated Acetate Buffer (Sodium acetate, 1 M, pH 5.0)

Add 60.0 g of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water. Adjust the pH of this solution to 5.0 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) Acetate Extraction/Dilution Buffer for Pullulanase Assays (Sodium acetate, 100 mM, pH 5.0 containing sodium azide, 0.02% w/v and BSA, 0.05% w/v)

Add 100 mL of concentrated acetate buffer A to 850 mL of distilled water. Adjust the pH to 5.0 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, 0.5 g BSA and dissolve. Stable for  $> 2$  years at  $4^{\circ}\text{C}$ .

### CAUTION

Dissolve the reagents and adjust the pH to 5.0 before adding the sodium azide. Adding sodium azide to an acidic solution results in the release of a poisonous gas.

Powdered maleic acid is an irritant and thus should be handled with due care.

## **For the assay of limit dextrinase in barley malt:**

### **(C) Dilution Buffer**

**[Sodium maleate (100 mM, pH 5.5) plus sodium azide, 0.02% w/v]**

Dissolve maleic acid (5.8 g, Sigma cat. no. M0375) in 400 mL of distilled water and adjust the pH to 5.5 with sodium hydroxide solution (2 M) (requires about 35 mL). Add sodium azide (0.1 g) and readjust the pH to 5.5. Adjust the volume to 500 mL. Store at room temperature. Stable for > 2 years at room temperature.

### **(D) Extraction/Activation Buffer for limit-dextrinase assays**

**[Sodium maleate (100 mM, pH 5.5) plus sodium azide, 0.02% w/v plus 25 mM dithiothreitol]**

Prepare immediately before use, add dithiothreitol (0.1 g) to 25 mL of Buffer C.

## **For all assays:**

### **(E) Stopping Reagent [2% w/v Tris buffer solution (pH 9.0)]**

Dissolve 20 g of Tris buffer (Megazyme cat. no. **B-TRIS500**) in 900 mL of distilled water. Adjust the pH to 9.0 with 1 M NaOH. Adjust the volume to 1 L. Stable for > 2 years at room temperature.

## **EQUIPMENT (RECOMMENDED):**

1. Glass test tubes (12 mL and 20 mL capacity).
2. Disposable 13 mL polypropylene tubes, e.g. Sarstedt cat. no. 60.541.685 PP ([www.sarstedt.com](http://www.sarstedt.com)).
3. Pipettors, 0.1 and/or 0.2 mL (e.g. Gilson Pipetman<sup>®</sup>) to dispense enzyme extract and substrate.
4. Adjustable-volume dispenser:
  - 0-10 mL (for Extraction Buffer).
  - 0-5 mL (for Stopping Reagent).
5. Positive displacement pipettor, e.g. Eppendorf Multipipette<sup>®</sup>
  - with 5.0 mL Combitip<sup>®</sup> (to dispense 0.5 mL aliquots of concentrated enzyme solutions).
  - with 25 mL Combitip<sup>®</sup> (to dispense various aliquots of dilution buffers).
6. Top-pan balance.
7. Spectrophotometer set at 400 nm.
8. Vortex mixer (optional).
9. Thermostated water bath set at 40°C.
10. Stop Clock.

1. Bench centrifuge or Whatman GF/A glass fibre filter paper circles (9 cm diameter).

## CONTROLS AND PRECAUTIONS:

1. The freeze-dried substrate is extremely stable at room temperature, however, when dissolved it should be stored at 0-5°C during use and below -10°C between use. If the number of assays performed at any one time is limited, it is recommended that the substrate be divided into 2-3 mL aliquots and stored in the frozen state.
2. On storage at 0-5°C, the blank absorbance values will increase from 0.03 to about 0.05 in 5 days. This does not affect the performance of the substrate but obviously these values must be determined at the same time as the assay is performed. Blank absorbance values as high as 0.2 do not affect the reliability or accuracy of the assay.
3. The assay format should be standardised with the enclosed control pullulanase preparation. The activity of this solution is shown on the enclosed vial.
4. The time of incubation of the assay must be carefully controlled (i.e. exactly 10 min).
5. Incubation temperatures must be accurately controlled (i.e. 40°C).
6. After addition of Tris buffer solution to the reaction tube, the tubes must be stirred vigorously to ensure thorough mixing.

### NOTE:

A single Reaction Blank is normally sufficient for each batch of samples being analysed. To obtain this blank value for pullulanase samples, 3 mL of stopping reagent should be added to 0.1 mL of substrate solution. Then add 0.1 mL of enzyme extract. For limit dextrinase samples use 1.5 mL in place of 3 mL stopping reagent. The spectrophotometer employed should be standardised with a 4-nitrophenol standard in 2% Tris buffer solution ( $\epsilon_{mM} = 18.1$ ). 4-Nitrophenol solution (10 mM) can be obtained from Sigma (cat no. N7660).

## USEFUL HINTS:

1. If the absorbance values for a particular assay are greater than 1.20, the enzyme extract should be diluted with the appropriate buffer and re-assayed.

2. The number of pullulanase assays which can be performed per kit can be increased to 200 by halving the volumes of all reagents used and employing semi-micro spectrophotometer cuvettes.

## PULLULANASE ASSAY

### ENZYME EXTRACTION:

#### Liquid Preparations

1. Add 1.0 mL of liquid enzyme preparation (using a positive displacement dispenser) to **Buffer B** (49 mL) and mix thoroughly. This is termed the **Original Extract**.
2. Dilute 1.0 mL of original extract 10-fold by addition to 9.0 mL of **Buffer B** and mix thoroughly. Repeat this step until a dilution suitable for assay is obtained.

#### Powder Preparations

1. Add 1 g of enzyme powder preparation to 50 mL of **Buffer B** and gently stir the slurry over a period of about 15 min or until the sample is completely dispersed or dissolved.
2. Clarify this solution (the **Original Extract**) by centrifugation (1,000 g, 10 min) or filtration through Whatman No. 1 (9 cm) filter circles.
3. Dilute 1.0 mL of this solution 10-fold by addition to 9.0 mL of **Buffer B** and mix thoroughly. Repeat this step until a dilution suitable for assay is obtained.

### ASSAY PROCEDURE:

1. Dispense 0.10 mL aliquots of PullG6 Reagent Solution (unbuffered) into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate pullulanase extract at 40°C for 5 min.
3. To each tube containing PullG6 Reagent Solution (0.1 mL), add 0.1 mL of pre-equilibrated pullulanase extract directly to the bottom of the tube. Incubate at 40°C for exactly 10 min (from time of addition).
4. At the end of the 10 min incubation period, add exactly 3 mL of Stopping Reagent and stir the tube contents vigorously.
5. Read the absorbance of the solutions and the reaction blank at 400 nm against distilled water.

## CALCULATION OF ACTIVITY:

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable  $\alpha$ -glucosidase and  $\beta$ -glucosidase, required to release one micromole of 4-nitrophenol from BPNPG3G3 in one minute under the defined assay conditions, and is termed a **PullG6 Unit (PU)**. The conversion of PullG6 Units to International Units is discussed in Appendix B (page 9).

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

### where:

$\Delta A_{400}$  = Absorbance (reaction) - Absorbance (blank)

Incubation Time = 10 min

Total Volume in Cell = 3.2 mL

Aliquot Assayed = 0.1 mL

$\epsilon_{\text{mM}}$  of *p*-nitrophenol (at 400 nm) in 2% Tris buffer solution = 18.1

Extraction Volume = 50 mL per 1 g or 1 mL

Dilution = Dilution of the original extract

### Thus:

PullG6 Units /g or /mL of original preparation

$$= \frac{\Delta A_{400}}{10} \times \frac{3.2}{0.1} \times \frac{1}{18.1} \times \frac{50}{1.0} \times \text{Dilution}$$
$$= \Delta A_{400} \times 8.8 \times \text{Dilution}$$

## LIMIT-DEXTRINASE ASSAY

### ENZYME EXTRACTION:

1. Mill barley (10-50 g sample) to pass a 0.5 mm screen (e.g. with a Fritsch centrifugal mill).
2. Accurately weigh 0.5 g of flour into a 13 mL polypropylene tube.
3. To each tube add 8.0 mL of **Buffer D**, mix the contents well and close the screw-cap tightly.
4. Allow the enzyme to extract over 5 h at 40°C, vortexing for ~ 10 sec once per h.
5. Filter an aliquot of the solution through a Whatman GF/A glass fibre filter paper, or centrifuge an aliquot at 1,000 g for 10 min. **Assay enzyme activity** within 2 h.

## ASSAY PROCEDURE:

1. Dispense 0.10 mL aliquots of PullG6 Reagent Solution (unbuffered) into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate limit-dextrinase extract at 40°C for 5 min.
3. To each tube containing PullG6 Reagent Solution (0.1 mL), add 0.1 mL of pre-equilibrated limit-dextrinase extract directly to the bottom of the tube. Incubate at 40°C for exactly 30 min (from time of addition).
4. At the end of the 30 min incubation period, add exactly 1.5 mL of Stopping Reagent and stir the tube contents vigorously.
5. Read the absorbance of the solutions and the reaction blank at 400 nm against distilled water.

## CALCULATION OF ACTIVITY:

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable  $\alpha$ -glucosidase and  $\beta$ -glucosidase, required to release one micromole of 4-nitrophenol from BPNPG3G3 in one minute under the defined assay conditions, and is termed a **PullG6 Unit (PU)**. The conversion of PullG6 Units to International Units is discussed in Appendix B (page 9).

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

### where:

$\Delta A_{400}$  = Absorbance (reaction) - Absorbance (blank)

Incubation Time = 30 min

Total Volume in Cell = 1.7 mL (malt limit-dextrinase extracts)

Aliquot Assayed = 0.1 mL

$\epsilon_{\text{mM}}$  of *p*-nitrophenol (at 400 nm) in 2% Tris buffer solution = 18.1

Extraction Volume = 8 mL per 0.5 g

Dilution = Dilution of the original extract

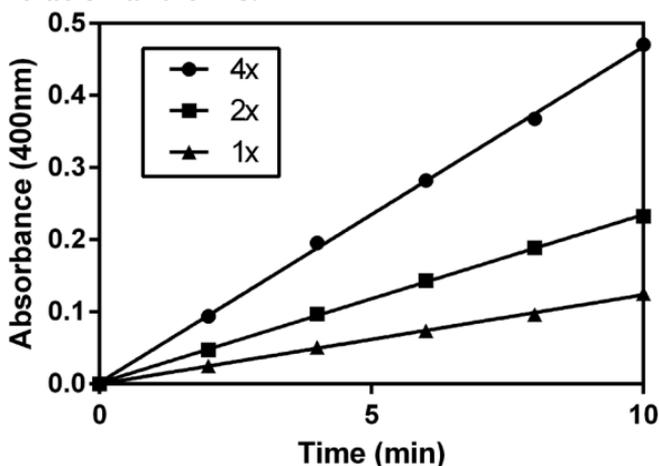
### Thus:

PullG6 Units/gram of milled malt:

$$\begin{aligned} &= \frac{\Delta A_{400}}{30} \times \frac{1.7}{0.1} \times \frac{1}{18.1} \times \frac{8}{0.5} \times \text{Dilution} \\ &= \Delta A_{400} \times 0.5 \times \text{Dilution} \end{aligned}$$

## APPENDIX:

### A. Linearity of the assay procedure with enzyme concentration and time.



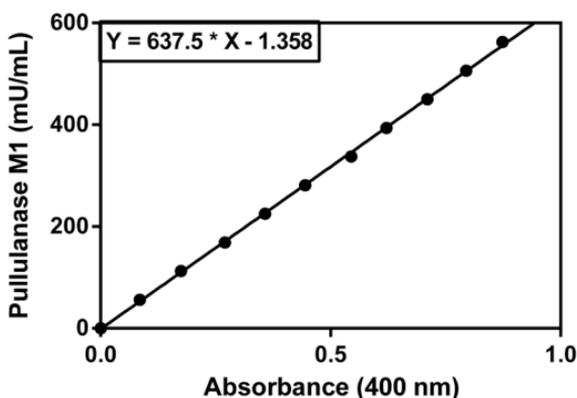
**Figure 1.** Linearity of PullG6 assay with pullulanase M2 (*B. licheniformis*) (cat. no. **E-PULBL**) in sodium maleate buffer (pH 5), containing BSA (0.05%) and sodium azide (0.02%). Reaction was terminated at intervals by adding Tris buffer solution (3.0 mL, 2%).

### B. Conversion of PullG6 Units to International Units.

**One International Unit (IU)** of activity is defined as the amount of enzyme required to release one micromole of glucose reducing-sugar equivalents per minute under defined conditions of temperature and pH.

Figure 2 shows the standard curve relating the activity of Pullulanase M1 (*K. planticola*) (cat. no. **E-PULKP**) on borohydride reduced pullulan (cat. no. **P-PULLBH**) (Nelson-Somogyi) to absorbance increase on hydrolysis of PullG6. This allows the conversion of PullG6 Units to International Units if desired.

$$\begin{aligned} \text{Pullulanase M1 (K. planticola) U/mL (borohydride reduced pullulan)} \\ &= 0.638 \times (\text{Absorbance}) - 0.001 \\ &\quad \text{or} \\ &= 3.60 \times \text{PullG6 Units/mL} \end{aligned}$$



**Figure 2.** Standard curve relating the activity of Pullulanase M1 (*K. planticola*) (cat. no. **E-PULKP**) on borohydride reduced pullulan (cat. no. **P-PULLBH**) (Nelson-Somogyi) to absorbance increase on hydrolysis of PullG6.

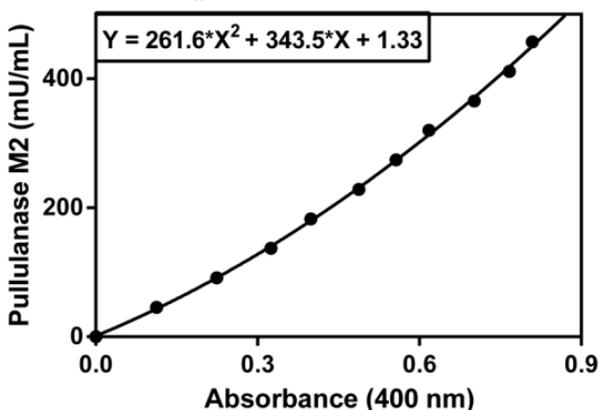
Figure 3 shows the standard curve relating the activity of Pullulanase M2 (*B. licheniformis*) (cat. no. **E-PULBL**) on borohydride reduced pullulan (cat. no. **P-PULLBH**) (Nelson-Somogyi) to absorbance increase on hydrolysis of PullG6. As the relationship is slightly non-linear, the conversion of PullG6 Units to International Units is derived from the linear portion of this curve (absorbance < 0.3).

**Pullulanase M2 (*B. licheniformis*) U/mL (borohydride reduced pullulan)**

$$= 0.262 \times (\text{Absorbance})^2 + 0.344 \times (\text{Absorbance}) + 0.001$$

or

$$= 2.88 \times \text{PullG6 Units/mL (provided the absorbance is } < 0.3)$$



**Figure 3.** Standard curve relating the activity of Pullulanase M2 (*B. licheniformis*) (cat. no. **E-PULBL**) on borohydride reduced pullulan (cat. no. **P-PULLBH**) (Nelson-Somogyi) to absorbance increase on hydrolysis of PullG6.

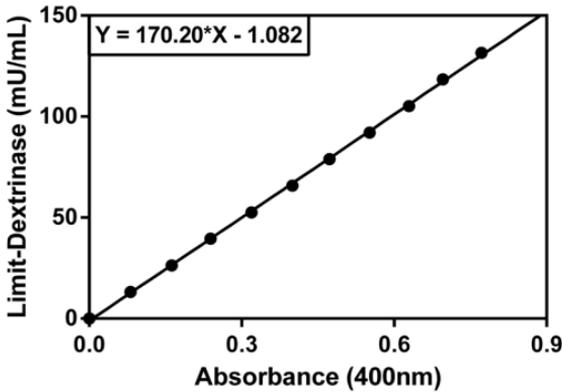
Figure 4 shows the standard curve relating the activity of a limit-dextrinase sample extracted from rice on borohydride reduced pullulan (cat. no. **P-PULLBH**) (Nelson-Somogyi) to absorbance increase on hydrolysis of PullG6. This allows the conversion of PullG6 Units to International Units if desired.

**Rice Limit-Dextrinase U/mL (borohydride reduced pullulan)**

$$= 0.170 \times (\text{Absorbance}) - 0.001$$

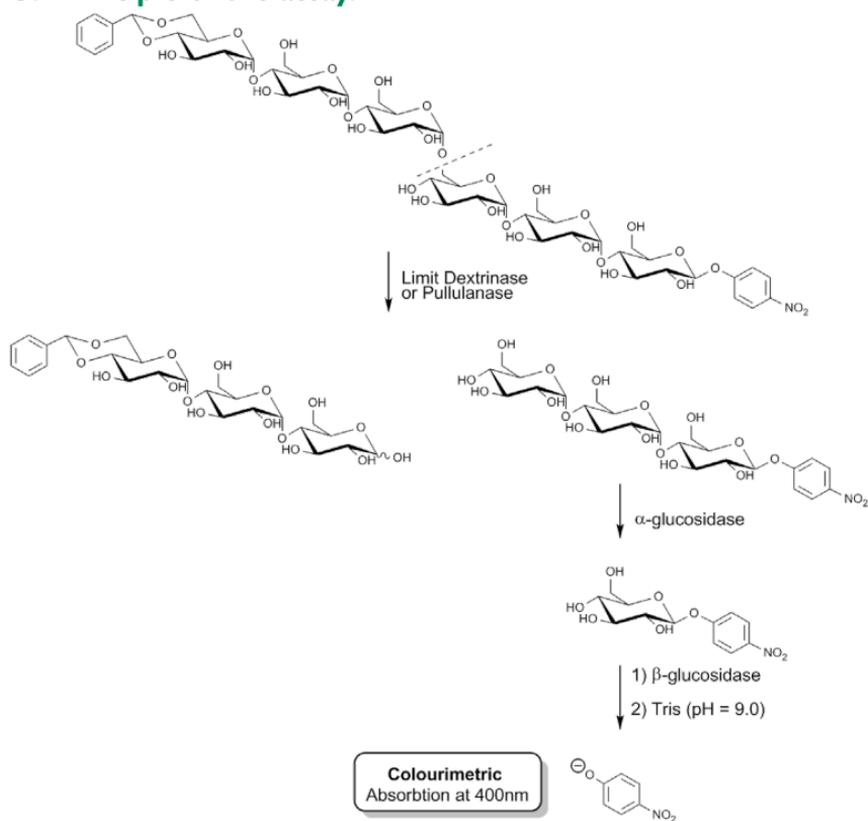
or

$$= 5.31 \times \text{PullG6 Units/mL}$$



**Figure 4.** Standard curve relating the activity of a limit-dextrinase sample extracted from rice on borohydride reduced pullulan (cat. no. **P-PULLBH**) (Nelson-Somogyi) to absorbance increase on hydrolysis of PullG6.

### C. Principle of the assay.



### Scheme 1. Theoretical basis of the PullG6 assay procedure.

Upon hydrolysis of the substrate at the 1,6- $\alpha$ -linkage by pullulanase or limit-dextrinase, the released 4-nitrophenyl- $\beta$ -maltotriose is immediately hydrolysed to glucose and 4-nitrophenol by the concerted action of the  $\alpha$ -glucosidase and  $\beta$ -glucosidase enzymes in the reagent mixture. The reaction is terminated and phenolate ions are developed by addition of dilute alkali.

### REFERENCES:

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4. McCleary, B. V. (1992). Measurement of the Content of Limit-Dextrinase in Cereal Flours. *Carbohydr. Res.*, **227**, 257-268.







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