Pullulanase (K-PullG6) Procedure for ChemWell®-T Auto-Analyser

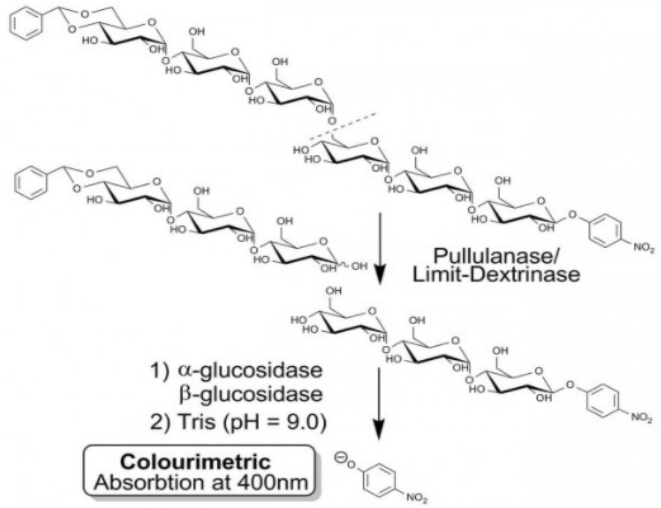
Requirements:

- Pullulanase Assay Kit **(K-PullG6)** (provides ~ 330 assays).
- **K-PullG6 (Pullulanase)** ChemWell®-T assay file.
- Use in association with the Pullulanase Assay Kit **(K-PullG6)** product data booklet.

Use:

A specific and sensitive colourimetric method for the determination of pullulanase.

Assay Principle:



Procedure:

Prepare the assay reagents and use with the **K-PullG6 (Pullulanase)** ChemWell®-T assay file.

Pullulanase Assay Kit Components:

Bottle 1: (x2) 4,6-*O*-benzylidene-4-nitrophenyl-6^3-α-D-maltotriosyl-maltotriose (BPNPG3G3) plus thermostable α and β-glucosidase Stable for > 5 years below -10ºC.

Bottle 2: Pullulanase control solution (*Bacillus licheniformis*, ~2.3 U/mL on PullG6 substrate) (exact activity stated on the vial label). in 50% v/v glycerol plus BSA (1% w/v) and sodium azide (0.02% w/v) Stable for > 4 years below -10ºC.

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: Not required for pullulansase assay.

Preparation of Kit Components:

1. Dissolve the contents of one bottle 1, in 5 mL of distilled water. This is the **PullG6 Reagent Solution**. Divide into aliquots and store in polypropylene tubes below -10ºC 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 -10ºC
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 -10ºC. Once dissolved, the standard is stable for > 1 year below -10ºC.
3. Dilute the contents of bottle 3 to 500 mL with distilled water. This is **Buffer B**. Store at 4ºC. Stable for > 2 years at 4ºC.

Preparation of Additional Reagents & Stopping Reagent

Stopping Reagent

(2% (w/v) Tris buffer, pH 9.0)

Dissolve 20 g of Tris buffer salt **(B-TRIS500)** in 900 mL of distilled water. Adjust the pH to 9.0 with 1 M NaOH and adjust the volume to 1 L. Stable for > 2 years at room temperature.

(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

(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 4ºC.

Enzyme Extraction and Dilution:

1. Add 1.0 mL of liquid enzyme preparation to 49 mL of **Buffer B** (100 mM sodium acetate, pH 5.0) using a positive displacement dispenser (these solutions can be very viscous) and mix thoroughly. **This is termed the Original Extract.**

Alternatively:

Add 1.0 g of **powder enzyme** sample to 50 mL of **Buffer B** (100 mM sodium acetate, pH 5.0) and gently stir the slurry over a period of approx. 15 min or until the sample is completely dispersed or dissolved. Clarify this solution by centrifugation (1,000 x *g*, 10 min) or by filtration through Whatman No. 1 (9 cm) filter circles. **This is termed the Original Extract**.

2. Add 1.0 mL of the **Original Extract** to 9.0 mL of **Buffer B** (100 mM sodium acetate, pH 5.0) (10-fold dilution) and mix thoroughly. This process of dilution should be repeated until a suitable concentration of pullulanase for assay is achieved.
3. Perform the assay using the **K-PullG6 (Pullulanase)** ChemWell®-T assay file.

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Pullulanase (K-PullG6)
Procedure for ChemWell®-T Auto-Analyser

Assay Parameters:

Assay volumes:

PullG6 Reagent:	0.03 mL
Sample:	0.03 mL
Stopping Reagent:	0.30 mL

Reaction time: 10 min at 37ºC
Wavelength: 405 nm
Assay type: Stopped reaction
Reaction direction: Increase

Calculation of Activity:

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable α-glucosidase and β-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)

where:

ΔE_{405}	= Absorbance (reaction) - Absorbance (blank)
Incubation Time	= 10 min
Total Volume in Cell	= 0.36 mL
Aliquot Assayed	= 0.03 mL
ε_{mM} of *p*-nitrophenol (at 405 nm) in 2% Tris buffer, pH 9	= 12.456
Extraction Volume	= 50 mL per 1 g or 1 mL
D	= Dilution of the original extract

Thus:

Pullulanase Units/mL or g:

$$= \frac{\Delta E_{405}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\varepsilon_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight}} \times D$$

$$= \frac{\Delta E_{405}}{10} \times \frac{0.36}{0.03} \times \frac{1}{12.456} \times \frac{50}{1} \times D$$

$$= \Delta E_{405} \times 4.817 \times D$$

NOTE:
The absorption coefficient (ε_{mM}) of 12.456 was experimentally determined under the conditions of the automated PullG6 Pullulanase assay using a ChemWell®-T auto-analyser.