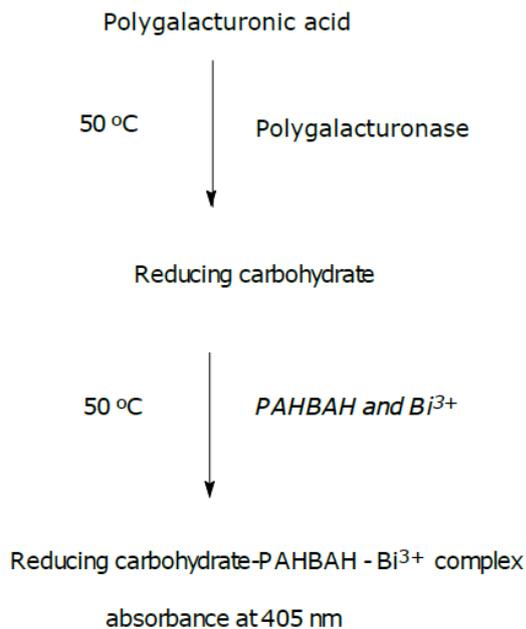


# PGNU(PL), pectinase activity determination

## Principle

Polygalacturonase hydrolyzes polygalacturonic acid and releases reducing carbohydrate. This reaction is stopped by an alkaline reagent including p-hydroxybenzoic acid hydrazide (PAHBAH) and  $\text{Bi}^{3+}$ , which complexes with reducing sugar, producing color, detected at 405 nm. The produced color is proportional to the polygalacturonase activity. Enzymatic reaction and absorption measurement proceed automatically in the Konelab analyzer.

Enzyme reaction:



## Reaction 1: Enzyme reaction

Parameter	Reaction conditions
Temperature(°C)	50
pH	4.5 at room temperature
Substrate conc.	1.36 g/L
Enzyme conc.	[0.0074; 0.0296] PGNU(PL)/ml
Reaction time [sec.]	300

## Reaction 2: Stop and detection reaction

Parameter	Reaction conditions
Temperature(°C)	50
Reaction time before measurement [sec.]	400
Wave length [nm]	405
PAHBAH conc.	47 mM
Bi <sup>3+</sup> conc.	5 mM
Tartrate conc.	62 mM
NaOH conc.	176 mM

## Definition of unit

The polygalacturonase activity is determined relative to an enzyme standard. The result is given in the unit PGNU(PL).

## Parameters

The following parameters were determined by validation:

Parameters	Facts
Specificity	The method is specific for enzymes which release reducing sugars from polygalacturonic acid
Calibration range and normal range	[0.010; 0.041] PGNU(PL)/ml. The analytical range of the standard curve

## Equipment

Equipment	Manufacturer
Konelab 30 Analyzer or Konelab Prime	Thermo Fisher Scientific
Diluter	E.g. Hamilton Microlab
Analytical balance	E.g. Sartorius, Mettler
Balance	E.g. Sartorius, Mettler
pH meter	E.g. Radiometer, Mettler
Ultrapure water	E.g. Milli-Q, Direct 8, Ad-vantage A10 or reference A+
Magnetic stirrer plates	-

## Chemicals

Name	CAS no.	Brand
Polygalacturonic acid sodium salt	9049-37-0	Information regarding critical chemicals is available upon request
p-Hydroxybenzoic acid hydrazide (PAHBAH) C <sub>7</sub> H <sub>4</sub> O <sub>2</sub> N <sub>2</sub> H <sub>4</sub>	5351-23-5	Information regarding critical chemicals is available upon request
Brij® L23 30 % (w/v) in H <sub>2</sub> O C <sub>12</sub> E <sub>23</sub>	9002-92-0	E.g. Sigma Art. no.: B 4184
Sodium acetate trihydrate CH <sub>3</sub> COONa·3H <sub>2</sub> O	6131-90-4	E.g. Sigma Art. no.: 32318
Glacial acetic acid CH <sub>3</sub> CO <sub>2</sub> H	64-19-7	E.g. Sigma Art. no.: P33209
Sodium hydroxide 500 mM NaOH	1310-73-2	E.g. Sigma Art. no.: 38217
Potassium sodium tartrate KNaC <sub>4</sub> H <sub>4</sub> O <sub>6</sub> ·4H <sub>2</sub> O	6381-59-5	E.g. Sigma Art. no.: 25508
Bismuth (III) acetate (CH <sub>3</sub> CO <sub>2</sub> ) <sub>3</sub> Bi Purity ≥ 99%	22306-37-2	E.g. Alfa Art. no.: 17574

IMPORTANT: Read the Safety Data Sheets (SDS) for the chemicals

## Reagents

### 0.67M CH<sub>3</sub>COOH, 0.33M CH<sub>3</sub>COONa, 3H<sub>2</sub>O

Example: Preparation of 5 L

Step	Action
1	Weigh out 222.0 ± 0.4 g of CH <sub>3</sub> COONa, 3H <sub>2</sub> O in a beaker
2	While stirring, transfer quantitative to a 5 L volumetric flask containing approx. 4500 ml ultrapure water
3	Stir until completely dissolved
4	Add 192 ml of CH <sub>3</sub> COOH to the solution using a measuring cylinder. IMPORTANT: Use the smallest possible cylinder to measure out the whole amount filling to the mark only in one operation step
5	Fill to the mark with ultrapure water and stir
6	Storability: 1 month, room temperature

### 0.67M CH<sub>3</sub>COOH, 0.33M CH<sub>3</sub>COONa, 3H<sub>2</sub>O, pH 4.5

Example: Preparation of 30mL

Step	Action
1	Add 30 ml <b>0.67M CH<sub>3</sub>COOH, 0.33M CH<sub>3</sub>COONa, 3H<sub>2</sub>O</b> into a beaker
2	Adjust pH to 4.50 ± 0.01 with NaOH or HCl as appropriate
3	Storability: 1 day at room temperature

**15% (w/v) Brij® L23**

Example: Preparation of 1 L

Step	Action
1	Weigh out 508.0±0.4g of Brij® L23 into a beaker. NOTE: Heat Brij® L23 in the manufacturer's container using a water bath which temperature is 35 - 45°C when Brij® L23 turns very stiff or does not come out easily
2	Add approx. 300 ml of ultrapure water and stir
3	Transfer the Brij® L23 quantitatively to a 1 L volumetric flask
4	Fill to the mark with ultrapure water
5	Stir until homogenous
6	Storability: 2 months in refrigerator

**50g/L potassium sodium tartrate, 20g/L PAHBAH, 5.52g/L Bismuth (III)-acetate**

Example: Preparation of 500 ml

Step	Action
1	Before the weighing of Bismuth (III)-acetate, grind down big particles of the chemical. TIP: Weigh out a bit more than needed to a weighing boat and crush/grind the chemical using a metal spoon. Weigh from this material in step 2 and discard the rest
2	Weigh out 2.760 ± 0.002 g of Bismuth (III)-acetate powder using a weighing boat
3	Transfer quantitative to a 500 ml beaker using 0.5M NaOH
4	Weigh out 25.00 ± 0.02 g potassium sodium tartrate, tetrahydrate into a weighing boat and transfer quantitative to the beaker using 0.5M NaOH
5	Weigh out 10.00 ± 0.02 g of PAHBAH into a weighing boat and transfer quantitative to the beaker using 0.5M NaOH
6	Add up to approx. 450 ml 0.5M NaOH. IMPORTANT: Wrap immediately in aluminium foil to protect from light
7	Stir for 15 ± 5 minutes. NOTE: If there is still particle undissolved after 10 minutes, grind it down using a metal spoon which is rinsed into the solution by 0.5M NaOH after the grinding, and then stir for additional 5 minutes
8	Check if all the powder is dissolved. If not, discard it and prepare a new portion
9	Transfer the solution quantitative to a 500 ml volumetric flask and rinse the beaker several times to be sure all the solution is transferred to the flask. TIP: Use a dark volumetric flask or wrap in tin foil, to protect from light
10	Fill to the mark by 0.5M NaOH. IMPORTANT: Use a dark volumetric flask or wrap in tin foil, to protect from light
11	Stir for 5 minutes
12	Storability: 4 days at room temperature. IMPORTANT: The storability is only obtained if the NaOH has been opened for less than 14 days at the preparation. IMPORTANT: The reagent should retain the original colourless to light yellow color, and a noticeable change in the color to visibly darker yellow should lead to discarding. IMPORTANT: Keep the reagent in a light resistant flask or wrapped in tin foil

**0.03M CH<sub>3</sub>COOH, 0.02M CH<sub>3</sub>COONa,3H<sub>2</sub>O, 0.015% (w/v) Brij® L23, pH 4.5**

Example: Preparation of 1 L

Step	Action
1	Add 50 ml of <b>0.67M CH<sub>3</sub>COOH, 0.33M CH<sub>3</sub>COONa,3H<sub>2</sub>O</b> into a 1000 ml volumetric flask
2	Add 1.0 ml <b>15% (w/v) Brij® L23</b> into the same flask
3	Add approx. 90% of the total volume of ultrapure water
4	Adjust pH to 4.50 ± 0.01 using NaOH or HCl as appropriate
5	Fill to the mark with ultrapure water and stir
6	Storability: 1 day at room temperature

**5 g/L polygalacturonic acid, 0.03M CH<sub>3</sub>COOH, 0.02M CH<sub>3</sub>COONa, 3H<sub>2</sub>O**

Example: Preparation of 100 ml

Step	Action
1	Weigh out 0.5000 ± 0.0005 g of polygalacturonic acid
2	Take 80 ml of ultrapure water in a beaker and place on a magnetic stirrer
3	Add the polygalacturonic acid slowly along the sides of the vortex. Cover with glass plate.
4	Dissolve with constant stirring without heating for precisely 5 minutes. Make sure that no lump is formed
5	Remove glass plate and place the beaker on a heater with stirring. IMPORTANT: Stirring while heating can make the temperature even in the whole beaker
6	When the temperature reaches 80°C, start the stop watch and turn off the heater
7	Allow the solution to boil gently and make sure that the temperature remains between 80 and 85°C. The solution can be removed from the hot plate for this purpose. IMPORTANT: Check if substrate solution is clear. If not, discard it and prepare a new portion
8	After precisely 2 minutes, immediately transfer the beaker to an ice-water bath (0°C) and cover it with a glass plate. Cool to 25°C (this takes approx. 5-6 min) and remove from ice-water bath. IMPORTANT: Keep the solution at this stage until ready to run the analysis. Proceed to next steps at the last moment
9	Transfer to a 100 ml volumetric flask
10	Add 5 ml of <b>0.67M CH<sub>3</sub>COOH, 0.33M CH<sub>3</sub>COONa,3H<sub>2</sub>O, pH 4.5</b> to the flask
11	Fill to the mark with ultrapure water and stir gently. IMPORTANT: Use for analysis as soon as possible (within approx.10 minutes)
12	Storability: 1 hour after step 8, room temperature. IMPORTANT: Keep the solution in a light resistant bottle or wrapped in tin foil

**0.03M CH<sub>3</sub>COOH, 0.02M CH<sub>3</sub>COONa, 3H<sub>2</sub>O, pH 4.5**

Example: Preparation of 100 ml

Step	Action
1	Add 5 ml <b>0.67M CH<sub>3</sub>COOH, 0.33M CH<sub>3</sub>COONa,3H<sub>2</sub>O, pH 4.5</b> into a 100 ml volumetric flask
2	Fill to the mark with ultrapure water and stir for one minute
3	Storability:1 day at room temperature

## Blank

No blank.

## Standard

The standard is available upon request.

Preparation:

Step	Action																																								
1	<b>Stock solution I:</b> Weigh out an amount of enzyme standard corresponding to 4068 units																																								
2	Transfer the standard enzyme sample to a 200 ml measuring flask and fill to the mark with sample diluent ( <b>0.03M CH<sub>3</sub>COOH, 0.02M CH<sub>3</sub>COONa,3H<sub>2</sub>O, 0.015% (w/v) Brij® L23, pH 4.5</b> ) and stir for 15 minutes. Stock solution I stability: 6 hours at room temperature																																								
3	<b>Stock solution II:</b> Futher dilute 50x with sample diluent ( <b>0.03M CH<sub>3</sub>COOH, 0.02M CH<sub>3</sub>COONa,3H<sub>2</sub>O, 0.015% (w/v) Brij® L23, pH 4.5</b> ) on Hamilton dilutor. Stock solution II stability: Use immediately. <b>IMPORTANT: All dilutions are prepared on a diluter with plastic tube, dilute as stated. The dilutor with steel tube CANNOT be used</b>																																								
4	<b>Working solutions:</b> The standard curve is a 7-point standard curve with a factor of 4 between the highest and the lowest standard points. The recommended total volume of Hamilton dilution is 1200 µl																																								
5	The standard solutions are prepared by diluting the stock solution with dilution buffer ( <b>0.03M CH<sub>3</sub>COOH, 0.02M CH<sub>3</sub>COONa,3H<sub>2</sub>O, 0.015% (w/v) Brij® L23, pH 4.5</b> ) on a dilutor directly into the sample cups according to the table below: <table border="1"><thead><tr><th>Standard no.</th><th>Dilution ratio</th><th>Std solution [µL]</th><th>Dilution buffer [µL]</th><th>Conc. [PGNU(PL)/ml]</th></tr></thead><tbody><tr><td>1</td><td>40</td><td>35</td><td>1365</td><td>0.010</td></tr><tr><td>2</td><td>30</td><td>50</td><td>1450</td><td>0.014</td></tr><tr><td>3</td><td>24</td><td>60</td><td>1380</td><td>0.017</td></tr><tr><td>4</td><td>20</td><td>75</td><td>1425</td><td>0.020</td></tr><tr><td>5</td><td>15</td><td>100</td><td>1400</td><td>0.027</td></tr><tr><td>6</td><td>12</td><td>125</td><td>1375</td><td>0.034</td></tr><tr><td>7</td><td>10</td><td>150</td><td>1350</td><td>0.041</td></tr></tbody></table> Storability in Konelab cups: Use immediately	Standard no.	Dilution ratio	Std solution [µL]	Dilution buffer [µL]	Conc. [PGNU(PL)/ml]	1	40	35	1365	0.010	2	30	50	1450	0.014	3	24	60	1380	0.017	4	20	75	1425	0.020	5	15	100	1400	0.027	6	12	125	1375	0.034	7	10	150	1350	0.041
Standard no.	Dilution ratio	Std solution [µL]	Dilution buffer [µL]	Conc. [PGNU(PL)/ml]																																					
1	40	35	1365	0.010																																					
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5	15	100	1400	0.027																																					
6	12	125	1375	0.034																																					
7	10	150	1350	0.041																																					

## Control sample

It is advisable to include a control sample in each run. Prepare the control sample in the same way as described for the samples below always using the same preparation procedure.

## Samples

Prepare samples according to the following procedure:

Step	Action
1	Weighing out between 0.5-1.79g of sample and note the precise weight. Transfer the sample quantitatively to a measuring flask and dissolve in dissolution buffer ( <b>0.03M CH<sub>3</sub>COOH, 0.02M CH<sub>3</sub>COONa, 3H<sub>2</sub>O, 0.015% (w/v) Brij® L23, pH 4.5</b> )
2	Stir on a magnetic stirrer for 15 minutes. Storability: 6 hours at room temperature
3	The samples are further diluted using dilution buffer ( <b>0.03M CH<sub>3</sub>COOH, 0.02M CH<sub>3</sub>COONa, 3H<sub>2</sub>O, 0.015% (w/v) Brij® L23, pH 4.5</b> ) on a Hamilton dilutor. The activity/concentration in the final dilution should if possible be approx. 0.025 PGNU(PL)/ml. Storability in Konelab cups: Use immediately. <b>IMPORTANT: All dilutions are prepared on a diluter with plastic tube, dilute as stated. The dilutor with steel tube CANNOT be used</b>

## Procedure

Step	Action																
1	Prepare the reagents, dilutions of standard sample, control sample and samples so valid for use																
2	Start up the Konelab																
3	Place the reagents in the Konelab: <table border="1" data-bbox="256 987 1437 1350"> <thead> <tr> <th>Reagents</th> <th>Reagent konelab name</th> <th>Container volume [ml]</th> <th>Storability in konelab</th> </tr> </thead> <tbody> <tr> <td>50g/L potassium sodium tartrate, 20g/L PAHBAH, 5.52g/L Bismuth (III)-acetate</td> <td>PAHBAH/S</td> <td>20</td> <td>1 day</td> </tr> <tr> <td>5 g/L polygalacturonic acid, 0.03M CH<sub>3</sub>COOH, 0.02M CH<sub>3</sub>COONa, 3H<sub>2</sub>O</td> <td>PGNUPLSUB/S</td> <td>20</td> <td>-</td> </tr> <tr> <td>0.03M CH<sub>3</sub>COOH, 0.02M CH<sub>3</sub>COONa, 3H<sub>2</sub>O, pH 4.5</td> <td>PGNUPLBUF/S</td> <td>20</td> <td>1 day</td> </tr> </tbody> </table> <p>IMPORTANT: PGNUPLSUB/S must be prepared freshly before use</p>	Reagents	Reagent konelab name	Container volume [ml]	Storability in konelab	50g/L potassium sodium tartrate, 20g/L PAHBAH, 5.52g/L Bismuth (III)-acetate	PAHBAH/S	20	1 day	5 g/L polygalacturonic acid, 0.03M CH <sub>3</sub> COOH, 0.02M CH <sub>3</sub> COONa, 3H <sub>2</sub> O	PGNUPLSUB/S	20	-	0.03M CH <sub>3</sub> COOH, 0.02M CH <sub>3</sub> COONa, 3H <sub>2</sub> O, pH 4.5	PGNUPLBUF/S	20	1 day
Reagents	Reagent konelab name	Container volume [ml]	Storability in konelab														
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0.03M CH <sub>3</sub> COOH, 0.02M CH <sub>3</sub> COONa, 3H <sub>2</sub> O, pH 4.5	PGNUPLBUF/S	20	1 day														
4	Place the standards, control sample, and samples in the Konelab in the stated order. NOTE: 40 samples can be analyzed in one run																
5	Start analyzing at the Konelab																

## Calculation

Procedure for calculation:

Step	Action
1	The activity of the enzyme samples is determined relative to the standard curve
2	Based on the results in Abs for the standard dilutions, a standard curve is drawn with the activities of the standards in PGNU(PL)/ml as the x-values and the Abs of the standards as the y-values
3	<p>The enzyme activity of the diluted samples is read from the standard curve. Calculation of activity of a sample in PGNU(PL)/g is calculated in the formula:</p> $PGNU(PL)/g = \frac{S \cdot V \cdot F}{W}$ <p>S = Reading from the standard curve in PGNU(PL)/ml            V = Volume of the measuring flask used in ml            F = Dilution factor for second dilution            W = Weight of sample in g</p>
4	<p>EXAMPLE: 0.7629 g sample is dissolved in a 100 ml measuring flask and further diluted 1250 times using a diluter on the konelab an Abs of 2.036 is measured from the standard curve an activity of 0.03107 PGNU(PL)/ml is calculated.</p> $Activity = \frac{0.03107 \cdot 100 \cdot 1250}{0.7629} = 5091 \text{ PGNU(PL)/g}$

## Approval

### Standard curve

Parameter	Accept criteria
<b>Standard</b>	
Quality of fit (Lower r <sup>2</sup> limit)	r <sup>2</sup> ≥ 0.9945
Curve appearance	Logitlog4

## Control sample

It is advised only to approve runs where the control sample is within ± 2 standard deviations of the declared value.

## Samples:

The analytical result (= average of 2 weighings on three different standard curves) must be CV ≤ 9.72%.

## Statement of analysis results

The result must be reported with three significant digits.

# Configurations

Konelab test definition:

Test definition		Arena 7.2 ARIN		Page: 1	
PGNU(PL)		Novozymes S/N ***** k**			
Date: 29/11/2017		***** Laboratorium			
Time: 09: 30					
Last change date 29/11/2017 09: 30					
Tick length (sec) 4.5					
Full name					
Online Name	PGNU(PL)	Test In Use	YES		
Test type	Photometric		LOW	HIGH	
		Test limit	*	*	Abs
Result unit	Abs	Initial absorbance	*	*	A
Number of Decim.	5	Dilution limit	*	*	Abs
		Secondary dil 1+	0.0	0.0	
		Critical limit	*	*	Abs
		Reflex test limit	*	*	Abs
		Reflex test			
Acceptance	Automatic	Reference class	LOW	HIGH	In Use
Dilution 1+	0.0				
Sample type	Sample type 5	Correction factor	1.00		
		Correction bias	0.00	Abs	
		Temperature	50.0	°C	
Calibration type	None				
Factor	1.00	Bias		0.00	
Bias correction in use	NO				
Manual QC in Use	NO	Routine QC in Use		NO	
Blank	Sample	Normal cuvette			
Reagent	PGNUPLSUB/S	Volume(ul)		30	
Disp. With	Extra	Add. Volume(ul)		60	
Wash reagent	PGNUPLBUF/S	Repl. Reagent		PGNUPLBUF/S	
Reagent wash	Before dispense				
Syringe speed	Slow				
Incubation		Time (sec)		240	
Sample		Volume(ul)		80	
Disp. With	Extra	Add. Volume(ul)		50	
Dilution with	Water	Wash reagent		Water	
Incubation		Time (sec)		300	
Reagent	PAHBAH/S	Volume(ul)		60	
Disp. With	Extra	Add. Volume(ul)		50	
Wash reagent	PAHBAH/S				
Reagent wash	Before dispense				
Syringe speed	Slow				
Incubation		Time (sec)		400	
Measurement	End point				
Wavelength (nm)	405 nm	Side wavel. (nm)		None	
Meas.type	Fixed timing				

Reagent definition:

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=====
Reagent definition      Arena 7.2 ARIN                      Page: 1
                       Novozymes S/N ***** k**
                       ***** Laboratorium

14/01/2016 08: 43
=====
```

Reagent	PAHBAH/S	Lot	Expiry date (dd/mm/yyyy)
Stable on board (days)	1		
Alarm limit (ml)	1.0		
Information			
Vial volume	20 ml		
Barcode id			
Syringe speed	Slow		

Reagent definition:

```
=====
Reagent definition      Arena 7.2 ARIN                      Page: 1
                       Novozymes S/N ***** k**
                       ***** Laboratorium

29/11/2017 09: 30
=====
```

Reagent	PGNUPLSUB/S	Lot	Expiry date (dd/mm/yyyy)
Stable on board (days)	0		
Alarm limit (ml)	1.0		
Information			
Vial volume	20 ml		
Barcode id			
Syringe speed	Slow		

Reagent definition:

```
=====
Reagent definition      Arena 7.2 ARIN                      Page: 1
                       Novozymes S/N ***** k**
                       ***** Laboratorium

29/11/2017 09: 30
=====
```

Reagent	PGNUPLBUF/S	Lot	Expiry date (dd/mm/yyyy)
Stable on board (days)	1		
Alarm limit (ml)	1.0		
Information			
Vial volume	20 ml		
Barcode id			
Syringe speed	Slow		

## Handling of enzymes and chemicals

Enzymes and enzyme solutions should be handled in a fume hood or in closed containers. Avoid inappropriate handling of enzymes and enzyme solutions, which may result in aerosol/dust generation. Avoid inhalation of dust aerosols and contact with skin and eyes. Handling of chemicals and disposal of waste must be performed according to valid procedures.

# Validity

Valid from August 2018.

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## About Novozymes

Novozymes is the world leader in biological solutions. Together with customers, partners and the global community, we improve industrial performance while preserving the planet's resources and helping build better lives. As the world's largest provider of enzyme and microbial technologies, our bioinnovation enables higher agricultural yields, low-temperature washing, energy-efficient production, renewable fuel and many other benefits that we rely on today and in the future. We call it Rethink Tomorrow.

August, 2018 · Luna No. 2018-13525-01

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