METHOD OF ANALYSIS		No: 61914 Version 2
Department: DFS/R&D/Analysis	Title: Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (COBAS-Analyzer)	
Date of issue: 20030516	Product: Not applicable	Product code: Not applicable
Compiled by: E.S. Edink Date:		Validated method Yes
Checked by Expert: W. Bijleveld Date:	Approved by team manager L.A.M. Jansen Date:	Approved by QA/QC-officer: M.M. Immerzeel Date:

1 SAFETY AND ENVIRONMENT

Restrictions for working with chemicals and VMT-samples are mentioned in the work instructions concerning management, storage and use of chemicals, the handling of dangerous substances and standard rules for VMT laboratories. These restrictions are also applicable for material that have been in contact with VMT samples

When working with strong acids, bases, carcinogenic matters and toxic matters etc. take all necessary precautions

When working with highly concentrated enzyme preparations take all necessary precautions. Avoid inhalation of dust add/or prolonged contact with unprotected skin

2 PRINCIPLE

2.1 Application

This method is applicable for the determination of proline specific endoprotease from *Aspergillus niger*

2.2 Description of the method

EndoPro catalyses the hydrolysis of N-carbobenzoxy-glycine-proline-*p*-nitroanilide (Z-Gly-Pro-pNA). The amount of liberated *p*-nitroaniline (pNA) formed in time is a measure for the EndoPro activity and is determined spectrophotometrically by measurement of the absorption at 405 nm.

METHOD OF ANALYSIS	No. 61914 Version 1
Title: Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (COBAS-Analyzer)	Date of issue: 20030516

The enzyme is incubated in the presence of Z-Gly-Pro-pNA in a citric acid-phosphate buffer pH 4.6 at and 37 °C. The liberated pNA in time is measured spectrophotometrically at 405 nm. The increase in absorbance at 405 nm in time is a measure for the EndoPro activity.

This is an absolute method. The results are related to the molar extinction coefficient of p-nitroaniline at 405 nm and pH 4.6.

2.3 Unit definition

The activity is expressed in PPU units (Proline Protease Units)
One PPU is defined as the amount of enzyme required to release one micromole of pNA from Z-Gly-Pro-pNA in one minute under the defined assay conditions (pH 4.6, T=37°C and in 0.37 mM Z-Gly-Pro-pNA)

2.4 Measuring-range

The measuring-range is 0.02 – 0.12 PPU/ml

2.5 Summary validation report

validation item	sample type	criterium	determined	acceptation
system precision	control	record	0,3%	yes
repeatabilty	UF	record	0,6%	yes
	Ferm. Broth	record	0,6%	yes
	Ferm Filtr.	record	0,7%	yes
intermediate precision	UF	<5%	5,5%	no
	Ferm. Broth	<5%	1,6%	yes
	Ferm Filtr.	<5%	1,9%	yes
accuracy	UF	>90% and<110%	103,9%-99,5%-97.5%	yes
	Ferm. Broth	>90% and<110%	100,8%-96,7%-91.0%	yes
	Ferm Filtr.	>90% and<110%	101,1%-99,9%-93,3%	yes
linearity	control	linear in the 0.02-0.12 PPU/ml range, p>0.1	linear in the 0.02-0.12 PPU/ml range, p=0.122	yes
robustness	control	record	99,1%-105,9%	yes

Table 1: Results from validation of method 61914, UF is UF concentrate, Ferm. Broth is Fermentation broth sample and Ferm. Filtr is Fermentation filtrate sample

METHOD OF ANALYSIS	No. 61914 Version 1
Title: Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (COBAS-Analyzer)	Date of issue: 20030516

3 APPARATUS AND CONDITIONS

3.1 Apparatus

Clinical analyser	: COBAS Mira Plus
Balance, accuracy at 0.01 g	: Sartorius 1405MP or 1213MP
Balance, accuracy at 0.001 g	: Mettler AE200 or AJ100
Balance, accuracy at 0.01 mg	: Mettler AT201
Diluter:	: Hamilton model ML 500
pH meter:	: Radiometer PHM 82

COBAS sample racks 1,2 and 3 for 30 sample cups
COBAS reagent racks 5S number 2 provided with a 4, 10 and 35 ml container
COBAS sample cup, contents 0.7 ml
COBAS reagent containers, contents of 4,10 and 35 ml
COBAS cuvette segments, 15 pieces in a rack, 0.6 cm light path, ABX

Or equivalent equipment

3.2 Conditions

Not applicable.

4 MATERIALS

4.1 Chemicals

Citric acid monohydrate	: Merck 1.00244
Disodium hydrogenphosphate dihydrate ($Na_2HPO_4.2H_2O$)	: Merck 1.06580
N-carbobenzoxy-glycyl-L-proline-4-nitroanilide =99.0% (Z-Gly-Pro-pNA)	: Fluka 96286
1,4-dioxane	: Merck 1.09671

Or equivalent chemicals

4.2 References, standards and controls

p-Nitroaniline standard preparation from Sigma, cat no N- 2128, with an officially assigned content.

EndoPro control preparation with an officially assigned activity. The activity is expressed in PPU. Store the control preparation in the freezer. Store amounts for daily use in the freezer as well.

METHOD OF ANALYSIS	No. 61914 Version 1
Title: Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (COBAS-Analyzer)	Date of issue: 20030516

4.3 Reagents

Water:
Ultra High Quality (UHQ) water, conductivity = 0.10 µS.cm

Citric Acid solution, 1 M:
Weigh 210 g citric acid monohydrate in a 1000 ml volumetric flask. Dissolve in water, make up to volume with water and mix. This solution is stable for 1 year at room temperature.

Citric acid - phosphate buffer pH 4.6:
Dissolve 10.0 g citric acid monohydrate and 15.1 g disodium hydrogenphosphate dihydrate in approx. 800 ml water. Adjust the pH to 4.60 +/- 0.03 at approx. 20°C with citric acid solution 1M. Quantitatively transfer the solution to a 1000 ml volumetric flask with water. Make up to volume with water and mix. This solution is table for 1 month in the refrigerator.

Dioxane / Citric acid-phosphate buffer pH 4.6 mixture (40 / 60):
Mix 100 ml dioxane with 150 ml of citric acid-phosphate buffer pH 4.6

Substrate solution, 20 mM:
Dissolve 21.33 mg +/- 0.05 mg Z-Gly-Pro-pNA in 10.0 ml 1.4-dioxane in a 25 ml volumetric flask. Make up to volume with citric acid-phosphate buffer pH 4.6 and mix. Always use a freshly prepared solution.

5 PROCEDURE

5.1 Preparation

Not applicable.

5.2 Pretreatment reference

Not applicable.

5.3 Pretreatment standard

Weigh, approx. 138 mg pNA standard accurately to within 0.1mg, in a 100 ml volumetric flask. Dissolve in approx. 80 ml of dioxane / citrate-phosphate buffer pH 4.6, make up to volume with the same mixture and mix. Make the following dilutions in labelled test tubes (S1-S5):

encoding	Dilution factor	PNA (mM) , approx.	
		dilution	assay
S1	12	0.83	0.15
S2	15	0.67	0.12
S3	20	0.50	0.09
S4	30	0.33	0.06
S5	60	0.17	0.03

METHOD OF ANALYSIS	No. 61914 Version 1
Title: Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (COBAS-Analyzer)	Date of issue: 20030516

5.4 Pretreatment control

Allow the control sample to attain room temperature before use. Weigh accurately to within 1 mg and in duplicate amounts of control sample corresponding to approx. 3.5 PPU in 50 ml volumetric flasks. Add approx. 40 ml citric acid-phosphate buffer pH 4.6 and dissolve by stirring on a magnetic stirrer. Make up to volume with the same buffer and mix. Store these solutions in ice until starting the incubation. Carry out the incubation within 1 hour of preparing the enzyme solutions.

5.5 Pretreatment samples

Prepare the samples with citric acid - phosphate buffer pH 4.6 to a final activity of 0.07 PPU/ml according to the application methods concerned. Store these solutions in ice until starting the incubation. Carry out the incubation within 1 hour of preparing the enzyme solutions.

5.6 Preparation measurement

Not applicable

5.7 Measurement

Analyse the standard solutions, controls and samples as follows:
Start with the standard solutions (S1 –S5) followed by the samples and controls in a random order. Place the controls not at the beginning or at the end of the series. Place every 10 samples a S3 standard solution and finish the series with the standard solution S3.

1. Priming

- Press the “INFO” button.
- Enter “6” (system checks).
- Enter “1” (prime).
- Enter “2” (“up-samp” appears on screen next to Z-position).
- Press “F1” (start) and flush 10 times. The sample needle will be positioned above the central wash position in order to allow checking of diluent stream continuity at daily start up.
- Check the syringe for the absence of air bubbles.
- Press “F1” (stop).
- Enter “3” (“up-reagent” appears on screen next to Z-position).
- Press “F1” (start) and flush 10 times. The reagent needle will be positioned above the central wash position in order to allow checking of diluent stream continuity at daily start up.
- Check the syringe for the absence of air bubbles.
- Press “F1” (stop).

METHOD OF ANALYSIS	No. 61914 Version 1
Title: Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (COBAS-Analyzer)	Date of issue: 20030516

2. Racks

- On the appropriate position on the Cobas place one “reagent rack 5s, number 2” provided with:
  at position 1, one 35 ml container filled with citric acid buffer pH 4.6
  at position 2-1, one 4 ml container filled with substrate solution
  at position 2-B, one 10 ml container filled with citric acid buffer pH 4.6
- Place one to three “sample 30 racks”, dependent on the number of runs, coded 1, 2 and 3 on the Cobas.
- Place the 0.7 ml sample cups filled with control and sample solutions in the sample racks, starting at rack 1 position 1.

3. Entering work list

- Press “ROUTINE”.
- Enter “1” (sample position).
- Press “F2” (to).
- Enter the number of runs to be determined and press “ENTER”.
- Press “X” (Endo Protease program) and press “ENTER”
  For the content of the programs see annex 1.
  The work list has been completed now.
- Note: The work list should be empty before starting a new test. If the worklist is not empty it should be cleared as follows:
  When previous series were completed:
- Press “INFO”.
- Enter “2” (patient file).
- Press “F2” (interim report).
- Press “F4” (delete) followed by “ENTER”.
- Press “SPACE”.
  When previous series were not completed:
- Press “ROUTINE”.
- Press “F1” (display).
- Press “F3” (delete) followed by “ENTER”.
- Repeat this until all tests have been deleted.
- Press “START” to begin the analysis.

4. Switching off the Cobas Mira analyser

- Remove the cuvette segments used.
- Press “INFO” button.
- Enter “6” (system checks)
- Enter “1” (prime)
- Enter “2” (“up-samp” appears on screen next to Z-position)
- Enter “F1” (start) and flush 10 times.
- Press ”INFO”.
- Enter “2” (patient file).

METHOD OF ANALYSIS	No. 61914 Version 1
Title: Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (COBAS-Analyzer)	Date of issue: 20030516

- Press “F2” (interim report).
- Press “F4” (delete) followed by “ENTER”.
- Press “SPACE”.
- Empty external waste collecting vessel and wash container.
- Remove sample cups from sample racks.
- Remove reagent containers from rack 5s.
- Switch off the Cobas Mira.

6 CALCULATION

Carry out the calculation with the aid of the computer program available for this analysis
If this is impossible carry out the calculations as follows:

6.1 Molar extinction coefficient of p-nitroaniline

Prepare a calibration curve by plotting the absorbance at 405 nm versus the known amount of pNA of the standards (S1 – S5). This calibration curve must be fitted according linear regression ($y = ax + b$, in which y = the absorbance, x = pNA (mM), a = slope of the calibration curve and b = intercept of the calibration curve). Calculate the molar extinction coefficient of pNA as follows:

Mol. Extinction coefficient of pNA ($mM^{-1}cm^{-1}$) : slope of the calibration curve (a) / 0.6

in which:
0.6 = cuvette length (cm)

6.2 Activity

Activity is calculated as follows:.

(ΔOD per minute / E*b) x V_t/V_s * 1000 = PPU per ml

in which:
E = mol. extinction coefficient of pNA at 405 nm and pH 4.6 [$cm^{-1}.mol^{-1}$]
b = 0,6 [cm]
V_t = total volume in cuvette, 0.27 [ml]
V_s = sample volume, 0.02 [ml]
1000 = correction factor: mol/l → µmol/ml

Calculate the activity in the samples according to the application methods concerned.

METHOD OF ANALYSIS	No. 61914 Version 1
Title: Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (COBAS-Analyzer)	Date of issue: 20030516

7 ASSESSMENT

7.1 Requirements

- The sample solution should have an activity within the measuring-range
- The level of the controls has to be between –3s and + 3s (see intermediate precision)
- The difference between the controls may not deviate more than 2.8s (see
- The difference between samples may not deviate more than 2.8s (see repeatability)

The results of the control samples have to be imported into the control data sheet with the aid of the computer program available for this analysis. After the processing the results have to be evaluated.

7.2 Actions

- Repeat the analysis with an adjusted dilution when the outcome is outside the measuring range.
- Repeat the analysis whenever the controls do not comply with the requirements.
- Repeat the sample analysis whenever the difference between samples do not comply with the requirements

7.3 Authorisation

After a training period by a for this method authorized laboratory technician, a technician will be authorized for this method when she/he succeeds on performing the test single-handed, whereby the control and selected samples meet the criteria mentioned above.

8 REFERENCES

- Method of analysis 62186 “Proline specific endoprotease activity determination, ELAN method”
- Memo 20030103 “Optimization of endoprotease activity determination, ELAN method (62186) for application on COBAS”
- Validation report VR ANA 61914

9 REMARKS

Due to the low solubility of the substrate in aqueous solutions, proline specific endoprotease activity in this assay is not determined under Vmax conditions. Slight variations in substrate concentration will influence the measured activity. Thus it’s very important to weigh the amount of substrate within the prescribed range.

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ANNEX 1

Programming diluent name

- Press "PROG". The program screen lights up.
- Enter "6" (system parameters). The system parameters screen light up.
- Enter "2" (diluent names). The diluent names screen lights up.
- Press "F1" (modify).
- Enter "CITR" and press "ENTER".

The diluent name has now been programmed.

Programming racks

- Press "PROG". The program screen lights up.
- Enter "5" (racks). The racks screen light up.
- Enter "1" (reagent 5s). The rack reagent 5s screen lights up.
- Enter "2" and press "ENTER". The rack reagent 5s nr 1 lights up.
- Press "F1" (modify).
- Go to rack position 1.
- Enter "1" (SR on) and press "ENTER".
- Enter "CITR" and press "ENTER".
- Go to rack position 2.
- Enter "1" (SR on).
- Enter "EPRO" and press "ENTER".

The rack has now been programmed.

Programming the test

- Press "PROG". The program screen lights up.
- Enter "2". The test screen lights up.
- Press "X" for endoprotease determination. The test routine screen lights up.
- Enter "EPRO" for endoprotease determination followed by "ENTER".
- Press "ENTER". The test routine EPRO screen lights up.
- This new screen must be filled out as follows:

GENERAL

- Measurement mode: "ABSORB".
- Reaction mode: enter "3" (R-S-SR1), "ENTER".
- Calibration mode: enter "1"(FACTOR), "ENTER".
- Reagent Blank: enter "1"(NOBLANK), "ENTER".
- Cleaner: enter "1" (NO), "ENTER".
- Wavelength: enter "2" (405), "ENTER".
- Decimal position: enter "4", "ENTER".
- Unit: enter"32" (ΔA/min), "ENTER".

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Title: Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (COBAS-Analyzer)	Date of issue: 20030516

ANALYSIS

- Post dil. factor: press “SPACE” (NO).
- Conc. factor: press “SPACE” (NO).
- Sample cycle: enter “1”, “ENTER”.
- Volume: enter”20”, “ENTER”.
- Diluent name: enter “11” (citr), “ENTER”.
- Volume: enter “10”, “ENTER”.
- Reagent cycle: enter “1”, “ENTER”.

- Volume: enter ”180”, “ENTER”.
- Start R1 cycle: enter “2”, “ENTER”.
- Volume: enter ”50”, “ENTER”.
- Diluent name: enter “11” (citr), “ENTER”.
- Volume: enter “10”, “ENTER”.

CALCULATION

- Sample limit: enter “3.500” (NO), “ENTER”.
- Point: enter “CB”, “ENTER”.
- Reac. direction: enter “1” (increase), “ENTER”.
- Check: enter “1”(on), “ENTER”.
- Convers. factor: enter “1.0000, “ENTER”.
- Offset: enter “0.0000”, “ENTER”.
- Test range low: press “SPACE” (NO).
- Test range high: press “SPACE” (NO).
- Norm. range low: press “SPACE” (NO).
- Norm. range high: press “SPACE” (NO).
- Number of steps: enter “1”, “ENTER”.
- Calc. step A: enter “3” (kinsearch).
- Readings first: enter “6”, “ENTER”.
- Readings last: enter “20”, “ENTER”.
- Reaction limit: press “SPACE” (NO)

CALIBRATION

- Calib. factor: enter “1.0000”, “ENTER”.

CONTROL

- CS1 pos: press “SPACE” (NO).
- CS2 pos: press “SPACE” (NO).
- CS3 pos: press “SPACE” (NO).

METHOD OF ANALYSIS	No. 61914 Version 1
Title: Spectrophotometric determination of Proline specific endoprotease activity with N-carbobenzoxy-glycine-proline p-nitroanilide as substrate: absolute method (COBAS-Analyzer)	Date of issue: 20030516

METHOD OF ANALYSIS	No: 61914 Version 2
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HISTORY	
Version	Description of the modification
1	First version
2	Validated method: No→Yes, New format