SpecWorks™ Protease Assay Kit

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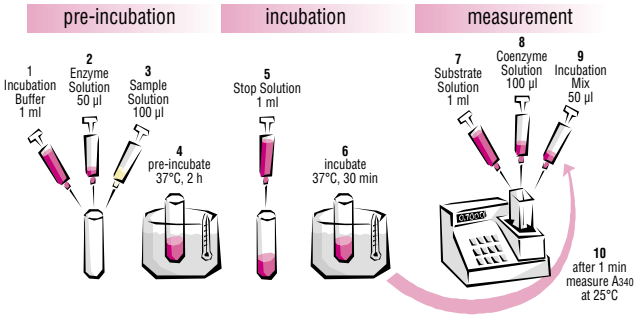
Description

The SpecWorks Protease Assay Kit is designed for the quantitative determination of the endoproteinase content in microbial, animal or plant cell extracts, purified fractions, or other biological samples. All live cells and tissues contain proteases, which are liberated when processing animal or plant cells or cells of micro-organisms. Protease activity can affect the separation and storage of proteins, and must often be removed by separation or inactivation.

Endoproteinases catalyse the hydrolytic cleavage of proteins within the polypeptide chain and may thus be analytically detected. In the SpecWorks Protease Assay, an enzyme (dehydrogenase), which is completely inactivated by the first proteolytic cleavage within the polypeptide chain, serves as substrate. After exposure of the dehydrogenase to the sample, the remaining dehydrogenase activity of the enzyme is measured by a simple spectrophotometric assay. The content of endoproteinases in the sample is inversely proportional to the residual activity of the dehydrogenase.

The assay detects proteases with an optimal pH in the neutral to alkaline range. Inactive protease-inhibitor complexes are not detected.



Measuring range

Measuring ranges: 20–320 ng/ml (calculated on Alcalase® 0.6 Anson-E/g (compare Alcalase standard included with the test kit)

5–100 ng/ml (calculated on trypsin from bovine pancreas; activity 40 U/mg (BAEE, 25°C, pH 8.0)

The measuring range is based upon a preincubation period of 2 hours at 37°C. The sensitivity of the measurement can be doubled by prolonging the preincubation period to 4 hours. The sensitivity of measurement depends on the protein content of the sample solution. With lower protein content, sensitivity is higher and the lower concentrations of protease can be detected.

Components

- 5.5 ml **Bottle ①: Incubation-buffer concentrate** (10-fold concentrated solution, black screw-cap)
- 1 vial **Bottle ②: Enzyme** (lyophilisate, contains dehydrogenase, red screw cap, volume is 3 ml when reconstituted)
- 60 ml **Bottle ③: Stop solution** (ready-to-use)
- 60 ml **Bottle ④: Substrate solution** (ready-to-use)
- 5 vials **Bottle ⑤: Coenzyme** (lyophilisate; contains NAD, volume is 1.1 ml each when reconstituted)
- 1 ml **Bottle ⑥: Alcalase® Standard** (solution with 0.6 Anson-E/g, 1.28 g enzyme/ml, white screw cap) (The main component of Alcalase is subtilisin A from *Bacillus licheniformis*)

Storage

All reagents are stored at 4°C and are usable at least up to the expiration date stated on the kit.

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Preparation of Reagent Solutions

Important: *Use protease-free deionized water for the preparation of the reagent and sample solutions. This can be accomplished by boiling deionized water.*

Incubation buffer ❶

In a separate vessel, dilute the required amount of incubation-buffer concentrate: 1 part buffer concentrate plus 9 parts deionized water (1 ml of concentrate is sufficient for 10 macro-assays). This solution is usable for 3 months if stored at 2–8°C and if contamination is prevented. Any turbidity developing during storage does not cause any interference.

Note: *When measuring Ca^{2+}-dependent proteases such as trypsin and proteinase K, use the incubation/$CaCl_2$ buffer ❾ (for preparation see below)*

Enzyme solution ❷

Dissolve the contents of one bottle ② (dehydrogenase) with 3 ml of deionized water (sufficient for 50 macro-assays). The solution is usable for 3 months if stored at 2–8°C and if contamination is prevented.

Stop solution ❸

The stop solution is ready-to-use. Should crystals appear in the solution, these can be dissolved by warming carefully to 37°C and swirling gently. The solution is usable up to the expiry date stated on the pack if stored at 2–8°C and if contamination is prevented.

Substrate solution ❹

The substrate solution is ready-to-use. It is usable up to the expiry date stated on the pack if stored at 2–8°C and if contamination is prevented.

Coenzyme solution ❺

Dissolve the contents of one bottle ⑤ (coenzyme) with 1.1 ml of deionized water (sufficient for 10 macro-assays). The solution is usable for 2 weeks if stored at 2–8°C and if contamination is prevented.

Standard solution ❻

The Alcalase Standard is ready-to-use. It is usable up to the expiry date stated on the pack if stored at 2–8°C and if contamination is prevented.

Tris/HCl/albumin buffer solution ❼ for sample dilution

This buffer solution is not included with the kit.

Dissolve 6.06 g of tris(hydroxymethyl) aminomethane in 800 ml of deionized water, adjust the pH to 8.8 with 1 N hydrochloric acid, then add 0.2 g of bovine serum albumin fraction V and dissolve. Adjust again to pH 8.5 with 1 N hydrochloric acid and make up to 1 liter with deionized water.

$CaCl_2$/dilution buffer ❽ for sample dilution

This buffer solution is not included with the kit.

Dissolve 29.4 mg of $CaCl_2$ dihydrate in 80 ml of Tris/HCl/albumin buffer solution ❼, then make up to 100 ml with buffer solution ❼.

$CaCl_2$/incubation buffer ❾

This buffer solution is not included with the kit.

Dissolve 2.94 mg of $CaCl_2$ dihydrate in 8 ml of incubation buffer ❶, then make up to 10 ml with buffer ❶.

Preparation of the Sample

Cells, tissues, other semisolid or solid samples

Homogenize cells, tissues, and other semisolid or solid samples using standard procedures. Dilute the homogenate with Tris/HCl/albumin buffer solution ❼ or $CaCl_2$/dilution buffer ❽ to place the protease content within the measuring range. Several dilutions may be necessary to achieve the correct concentration. Stir or shake the diluted homogenate for at least 15 min; thereafter use directly as sample solution. Any slight turbidity of the sample solution does not interfere with the measurement. Filter or centrifuge very turbid sample solutions.

Liquid samples

Dilute liquid samples with Tris/HCl/albumin buffer solution ❼ or $CaCl_2$/dilution buffer ❽ to place the protease content within the measuring range. Several dilutions may be necessary to achieve the correct concentration. Any slight turbidity of the sample solution does not interfere with the measurement. Filter or centrifuge very turbid sample solutions.

Note: *Use $CaCl_2$/dilution buffer ❽ for sample dilution whenever the protease to be measured (e.g. trypsin, proteinase K) requires Ca^{++}.*

Samples with high protease content

Samples diluted at least 100-fold with ❼ or ❽ can be used directly as described in "Assay Procedure" below.

Samples with low protease content

Samples diluted less than 100-fold with ❼ or ❽ may produce interference from components in the buffer (see "Interferences", page 9). Prepare and measure samples for which interferences are to be expected as described in "Treatment of unknown samples", page 7.

Preparation of the Standards

In order to produce the most accurate results, the standards must be carefully prepared. Two standards are recommended for use with the kit; the Alcalase® protease, which is supplied as a 1.28 g/ml stock solution, or trypsin (not supplied), which is available in highly purified dry form that can be accurately weighed to prepare stock solutions.

In each case, standards 1–5 are prepared as dilutions in a relevant buffer, which is buffer solution ❼ for Alcalase, and $CaCl_2$/dilution buffer ❽ for trypsin. If your sample is expected to contain low levels of protease and a high concentration of other proteins (> 1 mg/ml, e.g., milk) and the sample is to be measured without dilution, a set of standards 1–5 should be prepared using the sample as diluent. In this case the standards will not be free of protease activity, but the residual dehydrogenase activity after incubation should not be less than 70% (see also "Plotting the standard curve" under "Treatment of unknown samples"). Also note that some samples with high protein content may depress the sensitivity of the test (e.g., the detection limit for Alcalase in milk is about 2 µg/ml); in this case, standards with higher protease concentrations may be required.

Alcalase® standards

Important: *Prepare the standards **immediately** before the measurement for the plotting of the standard curve.*

In the following dilution steps, the standard solution ❻ included with the kit (0.6 Anson-E/g Alcalase®, 1.28 g/ml) is diluted to concentrations of 20, 40, 80, 160, and 320 ng/ml in Tris/HCl/albumin buffer solution ❼, for analysis in duplicate as described in "Assay procedure". Allow Alcalase to warm to room temperature prior to dilution to ease pipetting.

Dilution steps:

First dilution (1.28 mg Alcalase®/ml buffer solution ❼): Pipet 25 µl of Alcalase® standard solution ❻ into a sterile 50 ml tube containing 25 ml buffer solution ❼ for a 1:1,000 dilution. Pipetting error can be minimized by warming the stock solution to room temperature.

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Standard 1 (320 ng Alcalase®/ml): Pipet 25 µl of the first dilution (containing 1.28 mg of Alcalase®/ml) into a sterile tube or bottle containing 100 ml buffer solution ❼.

Standard 2 (160 ng Alcalase®/ml): Mix 1 ml of standard 1 (containing 320 ng Alcalase®/ml) + 1 ml of buffer solution ❼.

Standard 3 (80 ng Alcalase®/ml): Mix 1 ml of standard 2 (containing 160 ng Alcalase®/ml) + 1 ml of buffer solution ❼.

Standard 4 (40 ng Alcalase®/ml): Mix 1 ml of standard 3 (containing 80 ng Alcalase®/ml) + 1 ml of buffer solution ❼.

Standard 5 (20 ng Alcalase®/ml): Mix 1 ml of standard 4 (containing 40 ng Alcalase®/ml) + 1 ml of buffer solution ❼.

Trypsin standards

*Important: Prepare the standards **immediately** before the measurement for the plotting of the standard curve.*

Prepare the trypsin standards containing 100, 50, 25, 12.5, and 5 ng/ml as described below and analyse in duplicate as described in "Assay procedure".

Dilution steps:

First dilution (400 µg trypsin/ml $CaCl_2$/dilution buffer ❽): Weigh out exactly 20 mg of trypsin and transfer into a sterile 50 ml tube. Add 50 ml 1 mM hydrochloric acid and mix gently but thoroughly until dissolved (avoid foaming). Perform all subsequent dilutions immediately.

Standard 1 (100 ng trypsin/ml): Pipet 25 µl of the first dilution (containing 400 µg trypsin/ml) into a sterile tube or bottle containing 100 ml dilution buffer ❽.

Standard 2 (50 ng trypsin/ml): Mix 1 ml of standard 1 (containing 100 ng trypsin/ml) + 1 ml of dilution buffer ❽.

Standard 3 (25 ng trypsin/ml): Mix 1 ml of standard 2 (containing 50 ng trypsin/ml) + 1 ml of dilution buffer ❽.

Standard 4 (12.5 ng trypsin/ml): Mix 1 ml of standard 3 (containing 25 ng trypsin/ml) + 1 ml of dilution buffer ❽.

Standard 5 (5 ng trypsin/ml): Mix 1 ml of standard 4 (containing 12.5 ng trypsin/ml) + 1.5 ml of dilution buffer ❽.

Assay Procedure

Single determination, macro-assay

Two sets of incubations are performed for each assay: first, Incubation Mixes allow exposure of the sample to the dehydrogenase, and, second, Measurement Mixes assay the residual dehydrogenase enzyme activity. Prepare enough standards 1–5 and sample blank to allow for duplicate readings.

1. Assemble the following components in sealable tubes with a minimum capacity of 3 ml. All components must be combined within 5 min.

Incubation mixes

	Blank (BL)	Standard	Sample
Incubation buffer ❶	1.0 ml	1.0 ml	1.0 ml
Enzyme solution ❷	0.05 ml	0.05 ml	0.05 ml
Sample solution			0.1 ml
Standards 1–5		0.1 ml	
Tris/HCl/albumin buffer solution ❼ (or sample solution; see under "Treatment of unknown samples")	0.1 ml		

2. Close the tubes, mix, and preincubate for exactly 2 h at 37°C.

3. Add 1.0 ml Stop solution ❸ to each tube. Close the tubes once more, mix, and incubate for exactly 30 min at 37°C. Take the tubes out of the water bath and perform all subsequent steps within about 5 min at 25°C.

Note: *For accurate timing of multiple samples, stagger the assembly of the various reactions at appropriate intervals. Please see "Measurement of multiple samples" (page 8) for recommendations on performing multiple determinations.*

4. Into semi-micro cuvettes having a filling volume of about 2 ml, pipet the following components.

Measurement mixes

	Blank (BL)	Standard	Sample
Substrate solution ❹	1.0 ml	1.0 ml	1.0 ml
Coenzyme solution ❺	0.1 ml	0.1 ml	0.1 ml
Incubation mix "sample"			0.05 ml
Incubation mix "standard"		0.05 ml	
Incubation mix "blank"	0.05 ml		

5. Mix cuvette contents and measure absorbance at 340 nm (A_1) after about 1 min. After exactly another 2 min measure absorbance A_2. Calculate $A_2 - A_1 = \Delta A$ per 2 min.

Micro-assays

For a micro-assay all volumes stated can be halved. Measure in a micro-cuvette having a minimum measuring volume of 0.5 ml.

Measuring conditions

Wavelength: 340 nm

Optical path length: 1 cm

Measuring temperature: 25°C

Adjust the photometer to absorbance 0 against deionized water.

Prevent foaming before carrying out the measurement. Do not measure in the meniscus.

With a suitable photometer the Measurement mixes can be read kinetically: Mix the cuvette contents and, after 1 min, measure the increase in absorbance each min over a period of 2 min. For the evaluation use ΔA/min.

Calculation

Calculation of the residual activities of the substrate enzyme of the standards and the samples = sample values

$$\text{sample value (residual activity in \%)} = \frac{\Delta A_{sample}}{\Delta A_{BL}} \times 100 \quad [\%]$$

or

$$\text{sample value (residual activity in \%)} = \frac{\Delta A/min_{sample}}{\Delta A/min_{BL}} \times 100 \quad [\%]$$

Plotting the standard curve

For each kit used plot a new standard curve, as required, with Alcalase® or trypsin as the standard enzyme. If the kit is not yet used up, plot the standard once again after 6 weeks.

From the $\Delta A_{standard}$ of standards 1 to 5 and ΔA_{BL} of the sample blank calculate the residual activities of the substrate enzyme using the formula above. From the duplicate determinations calculate the

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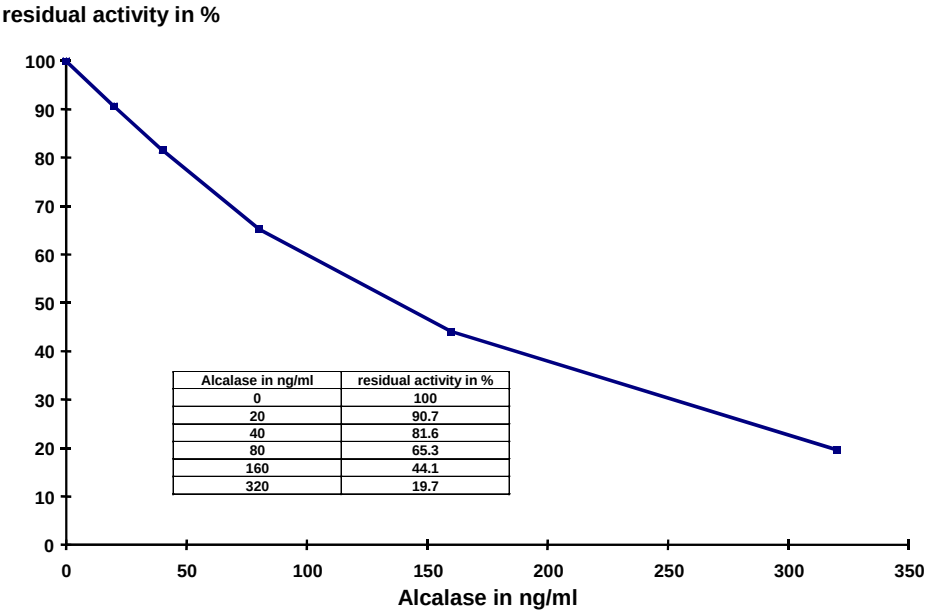
mean values and plot them against the concentrations of standards 1 to 5 (see Fig. 1). The sample blank is entered as the 100% value of the residual activity.

Fig. 1 Example standard curve for the determination of protease with Alcalase® as standard enzyme.



Alcalase in ng/ml	residual activity in %
0	100
20	90.7
40	81.6
80	65.3
160	44.1
320	19.7

Checking the standard curve

For Tris/HCl/albumin buffer solution ❼ or the $CaCl_2$/dilution buffer ❽ ΔA_{BL} per 2 min should be between about 0.6 and 0.9. If the value is < 0.4, it is advisable to repeat the determination of the blank with a fresh solution ❼ or ❽.

Calculation of the protease content of the sample

It is recommended to regularly check the sample value of the freshly prepared standard 4 (40 ng Alcalase®/ml or 12.5 ng trypsin/ml, respectively). If the residual activity expressed in % is outside of the ± 8% range of the original value, it is advisable to plot a new standard curve.

From ΔA_{sample} and ΔA_{BL} calculate the sample value (residual activity of the sample in %) using the formula above. The protease content of the sample can then be read off from the standard curve (Fig. 1).

In prediluted samples (compare dilute homogenates of solid samples) multiply the value read off from the standard curve with the dilution factor.

If the sample value is outside the standard curve (e.g. < 10–20%), dilute the sample solution, e.g. 1 + 2 with Tris/HCl/albumin buffer solution ❼ or $CaCl_2$/dilution buffer ❽ (dilution factor 3) and repeat the determination.

Treatment of unknown samples

Pretesting of the unknown sample

Prepare the sample solution as described in “Preparation of the sample solution” and preincubate for a period of 2 h as described in “Assay procedure”. For the measurement of the sample blank add the sample solution to the preincubation mix after 1 h 55 min and preincubate for another 5 min. Incubate and measure the sample and the sample blank as described above. From ΔA_{sample} and ΔA_{SB} calculate the residual activity of the sample solution (= sample value). A residual activity below 95 % indicates the presence of protease.

Plotting the standard curve

If the protease content in the unknown sample is to be determined exactly, a standard curve must be constructed as follows. If the residual value in the pretest is below 70%, dilute the sample

solution with Tris/HCl/albumin buffer solution ❼ or $CaCl_2$/dilution buffer ❽ (e. g. 1 + 3, dilution factor 4) such that the sample value (residual activity) in the pretest is above 70%. Add Alcalase® standard solution ❻ to the (diluted, if necessary) sample solution as described under "Preparation of the standard solutions". Preincubate for 2 h, incubate and measure the standards 1 to 5 and the sample solution (diluted, if necessary) as described under "Determination procedure". Enter the sample value of the sample solution without Alcalase® for the 100% value of the residual activity. Calculate the sample values of the standards and plot the standard curve. Trypsin may be used instead of Alcalase®; see "Preparation of the standard solutions".

Determination of unknown samples

Dilute unknown sample solutions with TRIS/HCl/albumin buffer solution ❼ or $CaCl_2$/dilution buffer ❽, if necessary, and process them as described in "Pretesting of the unknown sample". Read off the protease concentration of the samples from the standard curve above and multiply the result with the dilution factor, if necessary.

Measurement of multiple samples

Photometer with 6-position cuvette holder

Fig. 2 shows an example schedule of staggered measurements with a photometer with 6-position cuvette holder. Up to six assays can be measured per series of measurements.

With each series of samples of the same kind measure a sample blank. Thus, for example, a series of 34 cell homogenates and 2 sample blanks can be processed within 250 min.

Fig. 2: Schedule for processing 6 sample groups containing up to 6 samples each

Time	Series of measurements: sample groups					
(min)	1	2	3	4	5	6
0	start preincub.					
20		start preincub.				
40			start preincub.			
60				start preincub.		
80					start preincub.	
100						start preincub.
120	start incubation					
140		start incubation				
150	measuring					
160			start incubation			
170		measuring				
180				start incubation		
190			measuring			
200					start incubation	
210				measuring		
220						start incubation
230					measuring	
250						measuring
No. of samples	6	12	18	24	30	36

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Photometer with single cuvette holder

If the photometric measurement of the samples is carried out with a single cuvette holder, **four** samples per series of measurements can be processed as follows.

Example for one measuring series:

Start of preincubation of samples 1 to 4 at	0 min
Incubation of samples 1 to 4 at	120 min
Start of pipetting of the measurement mixes of samples 1 to 4 at	150, 150½, 151, 151½ min
Measuring of A_1 and A_2 of sample 1 at	152 and 154 min
Measuring of A_1 and A_2 of sample 2 at	152½ and 154½ min
Measuring of A_1 and A_2 of sample 3 at	153 and 155 min
Measuring of A_1 and A_2 of sample 4 at	153½ and 155½ min

Additional series of measurements (maximum of 6) can be started at intervals of 20 min.

Interferences

Buffer solutions were checked for possible interferences of the protease determination with 16.7 ng/ml of trypsin. No interference was observed with the following concentrations of various components in the sample solution:

Component	Concentration	Component	Concentration
Bovine serum albumin	500 µg/ml	Ammonium sulfate	25 mM
Glycine	50 mM	NaCl	50 mM
Glycerol	5%	$CaCl_2$	5 mM
EDTA	450 mM	$MgCl_2$	5 mM
HEPES	40 mM	Sucrose	10 mM
Tris/HCl pH 8.0	100 mM	Tween 20	0.5%
Tris/HCl pH 7.0	50 mM	Triton X-100	0.05%
Phosphate buffer pH 6.0	10 mM	β-Mercaptoethanol*	0.5 mM
Phosphate buffer pH 7.0	10 mM	Dithiothreitol DTT*	0.5 mM

* if necessary, neutralise with iodine acetamide or remove by dialysis

Other factors

Several other factors may cause interference in the assay, including:

- Decrease in the sensitivity of the test due to high salt content or extreme pH of the sample. Predilute such samples (neutralize, if necessary), or dialyse them against water or change the buffer by gel filtration.
- Decrease in the sensitivity of the test by high protein concentrations in the sample.
- Proteases may be inhibited by protease inhibitors such as those added to cell homogenates. Inhibition may be tested by adding a known amount of protease standard to the sample and measuring vs. the standard by itself.

References

G Sarath, et al. (1989) Protease Assay Methods. *in* "Proteolytic Enzymes–A Practical Approach", R J Beynon & J S Bond (editors)., pp. 25–55. IRL Press at Oxford University Press