Instructions for use

ROTI®Quant universal

Colorimetric protein concentration analysis

0120

A. The kit contains

ROTI®Quant universal Reagenz 1 (0118): 500 ml (0120.1) / 200 ml (0120.2)

 Danger H318-H315 P280- P305+P351+P338-P310

ROTI®Quant universal Reagenz 2 (0119): 40 ml (0120.1) / 16 ml (0120.2)

 - H411 P273

One kit is sufficient for 500 (200) assays in cuvettes or 5000 (2000) assays in microtitre plates.
Contents of this Kit may not be bought separately.

B. Storage at room temperature. Do not cool or freeze the product. A deposit may precipitate in reagents 1 or 2 in exceptional cases, e.g. when shipped at low temperatures. The crystals should then be dissolved by carefully heating and canting them. Do not use reagents which have changed colour or which seem to be microbially contaminated.

C. Mechanism

ROTI®Quant universal is a colorimetric reagent for detecting and quantifying soluble proteins, ***highly similar to the test system known as BCA Protein Assay***. Like this quantitation assay, ROTI®Quant universal is based on a biuret reaction in combination with a highly specific colorimetric enhancer reaction.
During the so called ***Biuret reaction*** (complex formation of tripeptides and larger polypeptides with copper ions) the Cu^{++} ions (cupric form) of reagent 2 are reduced to Cu^{+} ions (curpous form) in the alkaline environment of the test. The terminus stems from the chemical similarity of this process to the reaction of the molecule Biuret with copper ions. The complex is coloured light lilac, while the intensity of the colour is proportional to the number of peptide bonds (and, therefore, amino acids) participating in the reaction. The colour of solubilised Cu^{+} ions can only be seen in high concentrations, thus, protein quantitation would only be possible after very long incubation time.
Enhancement of the resulting faint colour (by approx. 100fold) is then achieved by chelation of the cuprous Cu^{+} ions with ***PCA***, a molecule highly similar to bicinchoninic acid (BCA). The resulting chelate complex is strongly coloured in a dark, slightly brownish purple (while BCA results in violet colouring).
The colour development of the copper-specific enhancer reaction is also directly proportional to the concentration of the protein/peptide present. The resulting purple copper complex exhibits a strong light absorption with a maximum of 503 nm, although the wide maximum of the absorption curve also permits highly sensitive and no-loss measurements at 492 nm. The reaction does not form a true end point measurement: the colour develops slowly at room temperature, this, however, can be accelerated in heat. High sensitivity, therefore, can be achieved even after a short time.

Standardisation

The amount of protein in the measured sample (and subsequently the concentration of protein in the quantified solution) is to be analyzed by comparing the test measurement with the series of dilution of a protein standard of known concentration (Diagram A). Standard and sample must always be treated in the same way. The linear measuring range of the assay stretches from 5 up to 2000 µg/ml (0.5 to 200 µg total protein).

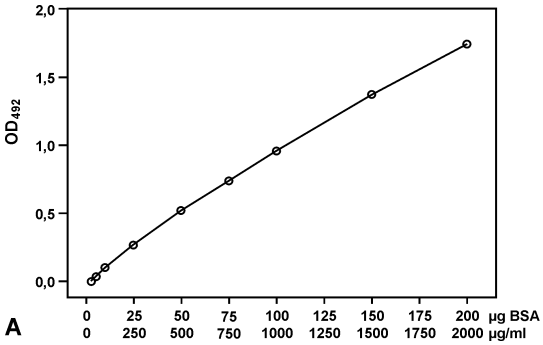


Figure A
Roti®-Quant universal typical standard plot of BSA concentrations (Bovine Serum Albumin). Adjusted to zero value. Measurement of 100 µl each of protein solution.

Tolerance towards interfering substances
ROTI®Quant universal is extremely tolerant towards detergents (see fig. D), alcohols or buffers (fig. B), which interfere severely with other measuring methods, e.g. testing according to Bradford. The table listed below indicates the tolerance of the kit towards a selection of the most commonly used reagents.

Figure B
Tolerance of Roti®-Quant universal tests towards detergents and other additives, examples. Adjusted to zero value. Measurement of 100 µl each of protein solution in a macro preparation. (With courtesy of Dr. Angelika Böttger, Zoological Institute, Ludwig-Maximilians-University of Munich).

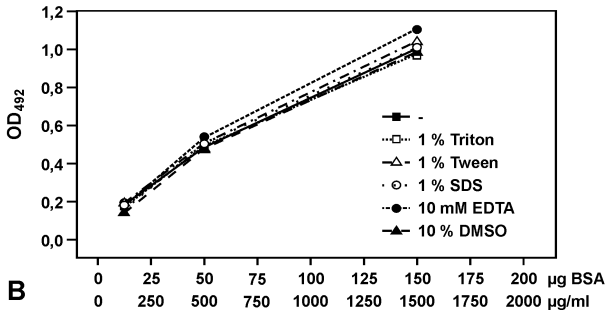


Table 1: Tolerance towards interfering factors

Substance	Concentration
Ammonium sulphate	1.5 M
DMF	10 %
DMSO	10 %
DTE	1 mM
DTT	1 mM
EDTA	10 mM
EGTA	not compatible
Ethanole	10 %
Glucose	10 mM
Glycerol	10 %
Glycin	0.1 M
Guanidine-HCl	4 M
HCl	0.1 M
HEPES	0.1 M
Imidazole	50 mM
MES pH 6.1	0.1 M
Methanole	10 %
MOPS	0.1 M

Substance	Concentration
NaOH	0.1 M
NP-40	5 %
PIPES	0.1 M
RIPA buffer	undiluted
SDS	5 %
Sodium acetate pH 4.8	0.2 M
Sodium azide	0.2 %
Sodium carbonate	0.1 M
Sodium chloride	1 M
Sodium deoxycholate	5 %
Sodium phosphate	0.1 M
Sucrose	40 %
Thimerosal	0.01 %
Tris	250 mM
Triton X-100	5 %
Triton X-114	1 %
Tween 20	5 %
Urea	3 M

A very small number of substances, however, still interfere with ROTI®Quant universal reagents. They are substances with highly reducing potential (also reducing sugars), strong acids or bases, or chelating substances. Following table (Table 2) lists some interfering agents.

Table 2: Inhibiting Factors

Substance	max. tolerated concentration
Ammonium sulphate	1.5 M
Bicine	20 mM
Bis-Tris	33 mM
DTT / DTE	1 mM
EDTA	10 mM
EGTA	none
Glucose	10 mM
Guanidin-HCl	4 M
Imidazole	50 mM
β-Mercaptoethanol	0.01 %
Sodium azide	0.2 %
Tricine	25 mM

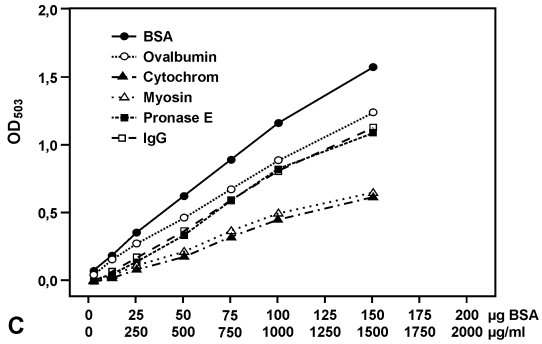
In order to measure the protein concentration in spite of interfering factors we recommend using the following strategy:

- Dilute the sample until it falls below the critical value of the interfering substance
- Dialysis or gel filtration of the sample to remove the interfering substance
- Precipitating the proteins through TCA
- If a chelating substance is involved, then the quantity of the applied Reagent 2 can be increased: to 7.5: 1 or 5: 1 (ratio reagent 1: reagent 2, standard value: 15: 1)

Please ensure that the standard proteins are also treated here in the same way as the samples.

Dependence on the protein structure

Although quantification with ROTI®Quant universal is less dependent on the exact protein structure, as is the case with Bradford assay, the exact amino acid compound and the primary structure of the proteins can, however, influence the result (fig. C). Therefore, when measuring proteins which deviate greatly in their conformation to globular proteins, we recommend using similar proteins as a standard (e.g. bovine gammaglobuline with immunoglobuline).



C

Figure C

Measurement deviation of different standard proteins with Roti®-Quant universal. Adjusted to zero value.

D. Measurement

Preparation volume

Measuring can be carried out in centrifuge tubes/cuvettes (macro preparation) or in microtitre plates (micro preparation) (see table 3).

Table 3	Macro preparation	Micro preparation
Sample volume	50-100 µl	≤50 µl
Ratio sample: working solution	1:10 oder 1:20	1:2 or 1:4
Recommended measurement at	503 nm (Photometer)	492 nm ((Microplate-Reader)

Protein standard

Pipette the dilution series of the protein standard into clean tubes (Table 4). We recommend using a freshly diluted protein standard (e.g. Albumin Fraktion V, Prod. No. T844.1). If possible use the same dilution buffer for standard proteins and samples. Albumin stock solution (2 mg/ml) can be frozen in aliquots and stored at -20 °C.

Table 4: Dilution series of standards			
Solution	End conc. BSA (µg/ml)	Vol. dilut. buffer	Vol. and origin BSA
A	2000	0	400 µl Stock solution (2.0 mg/ml)
B	1500	125	375 µl Stock solution (2.0 mg/ml)
C	1000	325	325 µl Stock solution (2.0 mg/ml)
D	750	325	325 µl Solution B
E	500	325	325 µl Solution C
F	250	325	325 µl Solution E
G	125	325	325 µl Solution F
H	50	450	300 µl Solution G
I	25	400	100 µl Solution G
K	5	400	100 µl Solution I
Blank value	0	400	0

Samples
Prepare a 1:10 dilution from each of your samples, or preferably a 1:20, 1:50 or 1:100 dilution.
This will enable you at any rate, to gain a measurement in a linear quantifiable range.

Working solution
Mix 15 parts of Reagent 1 and 1 part of Reagent 2 in a clean vessel. The staining solution will remain stable for 24 h at room temperature and protected from light.
Following formula can be used to determine the working solution required in total:
(Number of protein standard dilutions + blank + number of samples to be measured)
multiplied by the number of replications equals:
the working solution in ml in the macro preparation
the working solution in ml in microplate preparation divided by 10

Example:	10 standard dilutions + 1 blank + 6 samples	= 17
	All measurements x 3, i.e. 17 x 3	= 51
	Macro preparation	51 ml working solution
	Microplate preparation	5.1 ml working solution

Pipetting protocol
Macro preparation: Volume ratio sample: working solution should be 1:10 to 1:20
(Microplate preparation is always indicated in brackets: 1:2 to 1:4). This means that for a macro preparation 10 to 20 times as much working solution as sample must be used (for a micro preparation 2 to 4 times as much):
1. Pipette 100 µl or 50 µl (50 µl) each of the standard, the blank and your samples into a clean test tube.
2. Add 1 ml (100 µl) working solution.
3. Mix by inverting or pipetting repeatedly.
4. Incubate the closed tubes at
   2 hours at room temperature
   or 30 minutes at 37 °C
   or 15 minutes at 60 °C
5. Stop the reaction by cooling the tubes at room temperature.
   Please note: Although the reaction has no true end point and the colour continues to develop at room temperature, no significant deviation arises in the different tubes during measurement. However, we recommend measuring all tubes in a period of
   15 minutes.
6. Reset the photometer to 0 at wave length 503 nm or 492 nm by measuring pure water in a clean cuvette (microtitre plate).
7. Transfer the blank control, the protein standards and your samples into the cuvettes (microtitre plates) and measure all solutions at the same wave length.
8. Adjust the measured value by subtracting the blank value from the measured value.
9. Plot the protein concentration of the standard on a graph – in µg/ml on the x-axis – against the corrected measurements – on the y-axis. The protein concentration of your sample can be determined on the calibration curve.

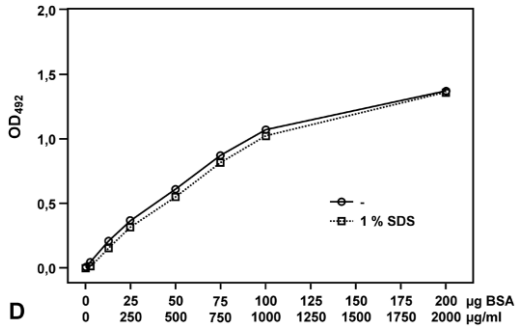


Figure D
Typical standard plot of a microassay. Comparison of BSA without SDS and BSA under presence of 1 % SDS.
Assay: 50 µl protein solution + 100 µl working solution, measured after 60 mins at 37 °C.

E. Trouble shooting

Problem	Possible reasons	**Solution**
No colour in all tubes	Sample buffer contains chelating component	- Dialysis, desalting or Probe precipitation of sample - Increase amount of reagent 2 in the working solution (e.g. to 2:15 or 4:15)
Colour of samples lighter lower measured values than expected	Protein concentration too low	- Concentrate proteins by precipitation or Spectra/Gel Absorbent - Increase incubation temperature
	pH-value of sample strongly alkaline or acidic	- Dialysis, desalting or precipitation of sample
	Measuring at false wavelength	- Use wavelength between 480 and 515 nm
	Incubation temperature too low	- Increase incubation temperature
Colour of samples darker / higher measured values than expected	Protein concentration too high	- Dilute sample - Lower incubation temperature
	Sample contains lipids or lipoproteins	- Reextract proteins
	Sample contains a reducing reagent or thiol	- Dialysis, desalting or precipitation of sample
Photometer has no 503 or 492 nm filter		- Measurement between 480 and 505 nm possible, sensitivity can however decrease below 492 or 503 nm

F. Additionally required equipment and reagents

BSA (Prod. No. T844.1)
SpectraGel Absorbent (Prod. No. 0115.1)
Cuvettes (Prod. No. XK20.1)
Microtitre plates (Prod. No. CNP7.1 + XT79.1 (lid))

ROTI®Quant universal

1 Mini-Kit (approx. 200 assays in cuvettes)	**0120.2**
1 Kit (approx. 500 assays in cuvettes)	**0120.1**

Carl Roth GmbH + Co. KG
Schoemperlenstraße 3-5 • 76185 Karlsruhe
P.O. Box 100121 • 76231 Karlsruhe
Phone: +49 (0) 721/ 5606-0
Fax: +49 (0) 721/ 5606-149
info@carlroth.com • www.carlroth.com
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