AGE-CML ELISA

Photometric Enzyme-Linked ImmunoSorbentAssay (ELISA) for the quantitative determination of Carboxymethyl Lysine (CML) respectively Advanced Glycation Endproducts (AGE)

3x 96 tests (48 duplicate determinations including six standards)

Store kit components as recommended (2-8°C and -20°C)

For Research Use Only, Not for use in diagnostic procedures

Instruction Manual
Version 6, April 2011

MICROCOAT

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1 Preface

CAUTION The following reagents, used in the assay described in this instruction manual, are toxic and should be handled with care:

- ABTS solution

Kit contents

Label	Content including function	Storage
Bi-BSA-AGE	• 250µl	at -20°C for up to 12 months
AGE standards (six different concentrations and a positive control)	• 500µl, ready-to-use • Standard a: 0ng/ml • Standard b: 7.15 ng/ml • Standard c: 14.3 ng/ml • Standard d: 27.85 ng/ml • Standard e: 50.63 ng/ml • Standard f: 101.04 ng/ml • Positive control: 24ng/ml (∓ 4ng/ml)	at -20°C for up to 12 months
Assay buffer	• 100ml, ready-to-use • Slightly opaque solution	at 2-8°C for up to 12 months
MAB <CML> HRP conjugate	• 40µl • AGE specific antibody conjugated to horse radish peroxidase	at 2-8°C for up to 12 months
ABTS solution	• 40ml, ready-to-use • Clear solution, slightly yellowish	at 2-8°C for up to 12 month
Washing buffer 10x concentrate	• 100ml • Clear solution, foaming possible • Has to be diluted 1 + 9 with bi-distilled water	at 2-8°C for up to 12 months
3x SA-MTP	• Microtiterplates coated with streptavidin	at 15-25°C for up to 36 months
9x Adhesive foil	• Adhesive Acetate foil for coverage of MTPs	

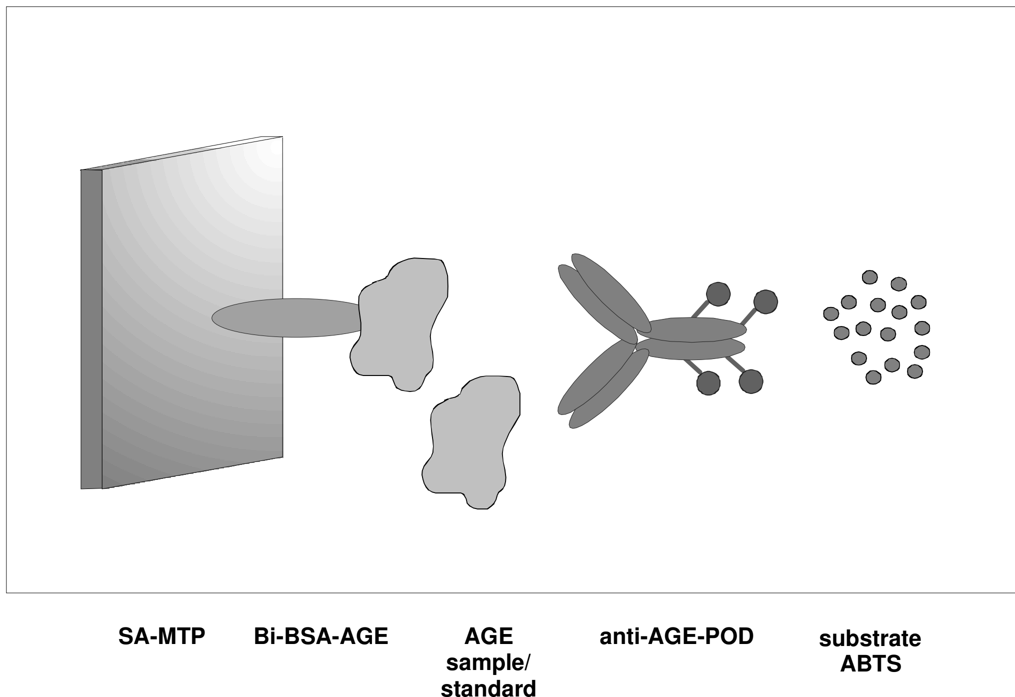
Additional equipment required To perform assays with this ELISA kit, you will need the following equipment:

- calibrated pipettes, multi-channel pipettes, single channel Multipipettor (with stepping mechanism for precise repeat dispensing)
- MTP shaker
- MTP reader
- Proteinase K (for digestion of serum and plasma samples)
  e.g. Proteinase K : Roche Applied Science Order No. 161 519

2 Introduction

2.1 Product overview

The AGE-ELISA is designed for the quantitative in vitro determination of Carboxymethyl Lysine (CML) in sample material within streptavidin microtiter plates (SA-MTP). The level of CML corresponds to the level of Advanced Glycation Endproducts (AGE). In this manual CML is used as a synonym for AGE and vice versa.
The test principle is shown in the following figure:



SA-MTP **Bi-BSA-AGE** **AGE sample/ standard** **anti-AGE-POD** **substrate ABTS**

Test principle - basic Steps

Step	Description
1	In the first step the biotinylated BSA- AGE is bound to the streptavidin coated surface of the microtiter plate. Unbound Bi-BSA-AGE is washed away.
2	The sample / standard is added. The AGE specific antibody conjugated to horse radish peroxidase is added. Bound biotinylated AGE and AGE of sample / standard compete for antibody. Unbound antibody is washed away.
3	The bound peroxidase (POD) is developed by the substrate 2.2-anzio-bis(3-ethylbenzthiazolin-6-sulfonicacid))ABTS/Perborate and determined photometrically. The color intensity is inversely proportional to the concentration of AGE.

Sample material Serum, heparin- / citrate-plasma (no EDTA-plasma!), urine

Assay time The assay time is 2.5 hours.

3 Procedures and required materials

3.1 Preparation of working solutions

The working solutions below are calculated for one microtiterplate (assay volume: 100µl). The provided amounts of reagents are exactly calculated - DO NOT prepare higher amounts of working solutions!

Solution	Reconstitution/Preparation of working solution	Stability of solution	For use in step
Bi-BSA-AGE	To prepare 14ml solution, add 70µl Bi-BSA-AGE solution to 14ml Assay buffer Result: Slightly opaque solution	Prepare shortly before use!	1
MAB<CML>HRP conjugate	To prepare 14,5ml reagent, add 10µl MAB<CML>HRP conjugate to 14.5ml Assay buffer Result: slightly opaque solution	Prepare shortly before use!	3
Washing buffer	Dilute 30ml Washing buffer 10x with 270ml double distilled water	1 day at 2-8°C	1 - 4

3.2 Before you begin

Reagents

- All reagents necessary to perform the assay are supplied with this kit.
- Reagents and MTP modules of different lots MUST NOT be used in one test series
- For dilution purposes, only use double distilled water.
- Bring all working solutions to 15-25°C before use.
- DO NOT aliquot standard, antibodies or conjugate.
- Centrifuge standard and conjugate briefly before use and mix carefully; DO NOT vortex!
- A precipitate may form in the standard. It has no influence on the standard. It can be removed by centrifugation.

Additional Equipment Required

- Use only pipettes that are carefully calibrated to their target volume
- Use plastic disposables, avoid glass ware
- The test is designed for use in combination with a MTP shaker, a MTP washer and a MTP reader.
  Note: If no shaker is used, signal levels will be considerably lower and may vary, thereby negatively influencing the test precision.
- Calculation software: Rodbart equation (4 parameter fit) is suggested to evaluate the samples or the “data analysis program” for data evaluation (when provided with the kit)..
- When Proteinase K digestion is used for sample preparation a waterbath or thermoblock is recommended

Assay

- Pipet thoroughly to ensure accurate transfer of the small volumes.
- Perform a separate calibration curve with each test.
- Perform all measurements in duplicate.
- Strictly perform the assay at the recommended incubation times and temperature.
- Use always the same procedure to minimize inter-assay variances.

MTP layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	ABTS-Blank	ABTS-Blank	Sample 1	Sample 1								
B	0 ng/ml	0 ng/ml	Sample 2	Sample 2								
C	7.15 ng/ml	7.15 ng/ml										
D	14.3 ng/ml	14.3 ng/ml										
E	27,85 ng/ml	27,85 ng/ml										
F	50.63 ng/ml	50.63 ng/ml										
G	101.04 ng/ml	101.04 ng/ml									Sample 39	Sample 39
H	positive control	positive control									Sample 40	Sample 40

3.3 Sample preparation

- Samples have to be diluted in Assay buffer. Typical dilutions vary between 1:10 and 1:200 and have to be identified for each sample.
- It is recommended that serum, plasma and eventually other samples should be enzymatical treated before measurement (e.g. with Proteinase K).

3.3.1 Pre-treatment of serum / plasma samples with Proteinase K

Reagents and Solutions

- Washing buffer from the AGE-CML assay, (after dilution 1+9 with distilled water)
- Proteinase K : Roche Applied Science Order No. 03 115 887 001 (1.25 ml)

Sample Pretreatment:

Incubate in a closed vial:
100 µl washing buffer
5 µl sample (serum, plasma)
5 µl Proteinase K

Incubate 2 h at 37°C (waterbath or thermoblock)
Afterwards Proteinase K is inactivated at 80°C for 10 minutes (time starts only when 80°C are reached !)
Cool sample to room temperature.
(a PCR thermocycler is useful for this procedure but not obligatory)

The digested sample is now ready for the AGE-CML ELISA.
Sample dilution by this procedure is 22x.

3.4 Procedure (AGE-CML determination)

ELISA

Follow these steps to determine the amount of AGE-CML in different samples. The amounts given below are calculated for one microtiterplate. To obtain reproducible results please follow strictly the specifications. We strongly recommend to measure samples and standards in duplicate.

Note: All incubation steps have to be carried out at **15-25°C**!
The antibody and standard dilutions have to be prepared shortly before use!

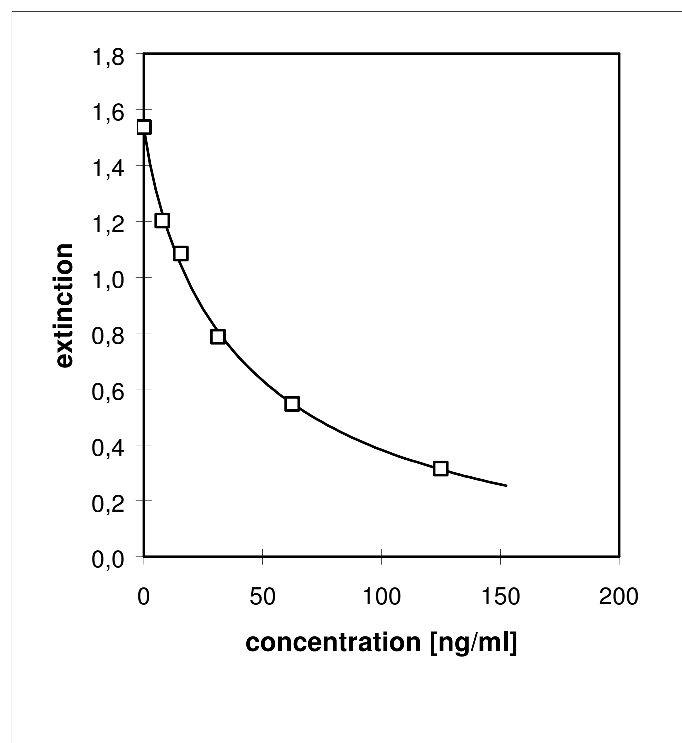
Step	Action	Volume / well	Time
1	• Pipet Bi-BSA-AGE solution in all wells (except A1 and A2). • Cover the MTP tightly with an adhesive cover foil. • Incubate under constant shaking at 600 rpm.	100 µl	1h
	• Wash 3 times with washing buffer. • Remove the washing fluid by aspirating or tapping (especially after the last washing step, tap thoroughyl upside down onto blotting paper to remove all remaining liquid)	3x 300 µl	3x 1 min
2	• Pipet the standards and positive control thoroughly in wells • Pipet the diluted samples thoroughly in wells • Pipet MAB<CML>HRP conjugate solution immediately afterwards in wells (except A1 and A2) • Cover the MTP tightly with an adhesive cover foil. • Incubate under constant shaking at 600 rpm.	50 µl 50 µl 50 µl	1h
	• Wash 3 times with washing buffer • Remove the washing fluid by aspirating or tapping	3x 300 µl	3x 1 min
3	• Pipet ABTS solution in all wells • Cover the MTP tightly with an adhesive cover foil. • Incubate under constant shaking at 600 rpm	100 µl	30 min
	Photometric measurement: • Measure the optical density of each well at 405nm (reference wavelength 492nm) on a microplate reader (you can subtract absorbance at reference wavelength 492 nm). • if reading for standard a (0 ng/ml) is <1.000 E, prolong incubation to 45 or 60 min		

4 Results

4.1 Calculation

Plotting the Standard curve Use the standards provided in the kit to prepare a six point calibration curve. Use Rodbart equation (4 parameter fit) or the "data analysis program" for data evaluation (when provided with the kit).
For drawing choose a linear/linear plot.

Standard curve The standard curve has to be determined individually for each experiment!
The following standard curve is shown as an example.



Calculating samples

Calculate mean of duplicate determinations.
Read the sample concentrations off the curve and multiply by the dilution factor (e.g. 22x for Proteinase K pretreatment).

Results

The results refer to monomeric epitopes, irrespective of the protein to which the CML is attached

4.2 Measuring range

approx. 7.15 -101.04 ng/ml AGE-Carboxymethyl Lysine

4.3 Quality control

This product has been function tested using blood serum, heparin- / citrate –plasma and urine.

5 Appendix

5.1 Trouble shooting

Trouble shooting table This table describes various trouble shooting parameters.

Problem	Possible cause	Recommendation
Unexpected color development	Inadequate incubation time and temperature	Ensure that incubation-intervals are correct and that all reagents achieve 15-25℃ before using in the test
	Uncontrolled water ingredients influence the test negatively	Always use double distilled water for reconstitution and preparation the working solutions; take care that the water is not microbially contaminated!
	Substrate or vial used to aliquot substrate contaminated with oxidative active substances	• DO NOT directly pipet from the substrate bottle • Check the vial for contamination
Questionable readings	Non-suitable filters in the MTP reader have been used	Check the filters in the MTP reader for the correct wavelenght
Weak or no signal	Sodium azide, ß-mercapthoethanol and DTT interfere with the peroxidase activity	Only use samples and solutions without Sodium azide, ß-mercapthoethanol and DTT
Drift	Unequal distribution of temperature in wells	Ensure that all reagents achieve 15-25℃ prior to use and use the recommended incubation times and temperatures
	Evaporation of fluids	Check the adequate fixation of the adhesive cover foils during the incubation steps
Poor precision	Non-homogenous sample after freezing	Mix sample before pipetting
	Turbidity or particles in the sample	• Centrifuge sample to pellet particles • Mix sample well before pipetting
	Carry over between samples/standard	Change pipet tips between each pipetting step
	Unequal volumes added to the wells	Check pipette function and recalibrate if necessary
	Inadequate aspiration of fluids	There should be remained no fluid in the wells after aspiration
	Washing was incomplete	Ensure that the automatic washer is working properly
	Unequal mixing of reagents during incubation	Use a plate shaker to ensure adequate mixing

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