



***Gc-globulin  
(Actin-free)  
ELISA Kit***



ANTIBODYSHOP

## Gc-globulin (Actin-free) ELISA Kit (KIT 034)

For the *in vitro* determination of actin-free Gc-globulin in human serum and plasma

February 2005

Batch: GG-0401CE

**Please read these instructions carefully**

### INTENDED USE

To aid the diagnosis of organ damage and the prognosis of critically ill patients, especially those with hepatic necrosis including hepatic necrosis after acetaminophen (paracetamol) overdose, and patients with multiple trauma.

### CLINICAL SIGNIFICANCE

Gc-globulin (group-specific component globulin), also known as vitamin D binding protein (DBP), is a multifunctional plasma glycoprotein of molecular mass 51-58 kDa.<sup>1,2</sup> It is structurally related to serum albumin and acts as a carrier protein for vitamin D, but it can also be converted by partial deglycosylation into a macrophage activating factor known as DBP-MAF or Gc-MAF.<sup>3</sup> One of its most important functions, however, is to act as an actin scavenger.<sup>4</sup> Actin is the most abundant protein in eukaryotic cells and is released into the circulation by dead or dying cells. There it can form long filaments which may trigger intravascular coagulation if not rapidly removed. This can lead to a condition resembling multiple organ dysfunction syndrome (MODS).<sup>5</sup> In the actin scavenging system, actin is depolymerized by gelsolin and strongly bound by Gc-globulin, allowing for the rapid clearance of actin-Gc-globulin complexes. This consumes Gc-globulin. Low Gc-globulin levels, both the total level and the actin-free level, which is an index of residual actin-scavenging capacity, can act as prognostic markers in situations of organ damage such as fulminant hepatic failure,<sup>6,7</sup> acetaminophen (paracetamol) overdose<sup>8,9</sup> and multiple trauma.<sup>10,11</sup> Other conditions such as septic shock may also be associated with reduced Gc-globulin levels and complex formation with actin,<sup>12</sup> but this has been less extensively studied.

Gc-globulin is an acute phase protein, whose synthesis by the liver is increased by inflammation. Circulating Gc-globulin may therefore show various responses in critically ill patients, depending on the balance between the rate and duration of Gc-globulin depletion, due to complex formation with actin, and the rate of new Gc-globulin synthesis.<sup>13</sup> After acetaminophen (paracetamol) overdose, the mean fall in the actin-free Gc-globulin level is more marked than the mean fall in the total Gc-globulin level.<sup>9,14</sup>

In fulminant hepatic failure, using a cutoff value of  $\leq 100$   $\mu\text{g/mL}$  for serum total Gc-globulin on admission (determined by rocket immunoelectrophoresis) gave positive predictive values for patient non-survival of 100% for acetaminophen-related cases and 79% for other cases, with corresponding negative predictive values (patient survival with levels  $> 100$   $\mu\text{g/mL}$ ) of

53% and 60%. These predictive values are slightly better than those obtained with the King's College Hospital (KCH) criteria.<sup>6</sup> In acute liver failure after acetaminophen overdose, using a cutoff value of  $\leq 120$   $\text{g/mL}$  for serum total Gc-globulin on day 2 gave a positive predictive value for the development of hepatic encephalopathy (coma grade II) of 75%, while the negative predictive value was 91%.<sup>9</sup> Lee et al.<sup>14</sup> used a cutoff value of  $\geq 34$   $\mu\text{g/mL}$  for serum unbound (i.e. actin-free) Gc-globulin to predict survival in fulminant hepatic failure, obtaining positive predictive values of 68% for early sera and 89% for later sera.

In cases of multiple trauma, using a cutoff value of  $\leq 200$   $\mu\text{g/mL}$  for serum total Gc-globulin on admission (determined by rocket immunoelectrophoresis) gave a positive predictive value for patient non-survival of 69% and a negative predictive value (patient survival with levels  $> 200$   $\mu\text{g/mL}$ ) of 84%. Sensitivity and specificity were 56% and 90%, respectively. These values are closely comparable to those obtained from the trauma injury and severity score (TRISS).<sup>10</sup>

### PRINCIPLE OF THE ASSAY PROCEDURE

The assay is an ELISA performed in microwells coated with a monoclonal antibody capable of binding human Gc-globulin whether or not it is complexed with actin. Coat-bound actin-free Gc-globulin is detected with a horseradish peroxidase (HRP)-conjugated monoclonal antibody whose binding to Gc-globulin is blocked by the presence of bound actin. This is followed by color development via incubation with a chromogenic substrate. The assay is a rapid two-step procedure:

Step 1. Aliquots of calibrators, diluted serum samples and any controls are incubated with HRP-conjugated detection antibody in the coated microwells. Only actin-free Gc-globulin will bind to both coat and detection antibody, while unbound materials are removed by washing.

Step 2. A chromogenic peroxidase substrate containing tetramethylbenzidine (TMB) is added to each test well. The HRP linked to the bound detection antibody reacts with the substrate to generate a colored product. The enzymatic reaction is stopped chemically, and the color intensity is read at 450 nm in an ELISA reader. The color intensity (optical density) is a function of the concentration of actin-free Gc-globulin originally added to each well. The results for the calibrators are used to construct a calibration curve from which the concentrations of actin-free Gc-globulin in the samples are read.

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### PRINCIPLE OF THE ASSAY PROCEDURE



### KIT COMPONENTS

Item	Contents	Quantity
1	12 strips of 8 Microwells each coated with Gc-globulin antibody (96 wells) + frame (ready to use)	1 plate
2	3X Sample Diluent Concentrate	60 mL
3	Gc-globulin Calibrators (ready to use). 0, 1, 2, 5, 10, 20, 50, 100 ng/mL	8 x 1 mL
4	25X Wash Solution Concentrate	1 x 30 mL
5	HRP-conjugated Gc-globulin (Actin-free) Antibody (ready to use)	1 x 7 mL
6	TMB Substrate (ready to use)	1 x 12 mL
7	Stop Solution (ready to use)	1 x 16 mL
8	Polypropylene U-microwell plate (96 wells)	1 plate

**Note:** Liquid reagents contain the preservatives thimerosal or Bronidox L.

### MATERIALS REQUIRED BUT NOT PROVIDED

- Adjustable micropipettes covering the range 1-1000  $\mu$ L and corresponding disposable pipette tips
- Polypropylene tubes to contain up to 1000  $\mu$ L
- Tube racks
- Adjustable 8- or 12-channel micropipette (50-250  $\mu$ L range) or repeating micropipette (optional)
- Clean 250-mL and 1000-mL graduated cylinders
- Deionized or distilled water
- Cover for microplate
- Clean container for diluted Wash Solution
- Apparatus for filling wells during washing procedure (optional)
- Lint-free paper towels or absorbent paper
- Disposable pipetting reservoirs
- Timer (60-minute range)
- Calibrated ELISA plate reader capable of reading at 450 nm (preferably subtracting reference values at 650 or 620 nm)
- Sodium hypochlorite (household bleach 1:10 dilution) for decontamination of specimens, reagents, and materials

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### PRECAUTIONS

#### For *in vitro* diagnostic use only

1. The Gc-globulin calibrators were prepared from Gc-globulin purified from human plasma. Each blood unit used for its preparation was tested by approved methods and found to be nonreactive for hepatitis B surface antigen (HBsAg) and antibodies against human immunodeficiency virus (HIV), and hepatitis C virus (HCV). However, as no test method can offer complete security that infectious agents are absent, the calibrators and patients' specimens should be handled at Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the CDC/NIH manual "Biosafety In Microbiology and Biomedical Laboratories", 1999. Solutions containing human serum should be treated as potentially infectious and handled accordingly.
2. Use separate pipette tips for each sample, calibrator and reagent to avoid cross-contamination.
3. Use separate reservoirs for each reagent. This applies especially to the TMB Substrate.
4. After use, decontaminate all specimens, reagents and materials by soaking for at least 30 minutes in sodium hypochlorite solution (household bleach diluted 1:10).
5. To avoid droplet formation during washing, aspirate the wash solution into a bottle containing bleach.
6. Reagents in this kit are preserved with up to 0.375% thimerosal, also called thiomersal or merthiolate, corresponding to 0.015% in the final diluted solution, or 0.06% Bronidox L corresponding to 0.02% in the final diluted solution. These may be toxic if ingested.
7. The Stop Solution contains 0.5 M sulfuric acid and can cause irritation or burns to the skin and eyes. If contact occurs, flush immediately with water.
8. Do not interchange components from kits with different batch numbers. The components have been standardized as a unit for a given batch.
9. Hemolyzed, hyperlipemic, heat-treated or contaminated specimens may give erroneous results.
10. Do not dilute serum specimens directly in the antibody-coated microwells.
11. Do not touch or scrape the bottom of the microwells when pipetting or aspirating fluid.
12. Incubation times and temperatures other than those specified may give erroneous results.
13. Do not allow the wells to dry once the assay has begun.
14. Keep the TMB Substrate away from bright light.
15. Do not reuse microwells or pour reagents back into their bottles once dispensed.

### STABILITY AND STORAGE

1. Store the kit with all reagents at 2-8°C. Do not freeze.
2. Use all reagents before the expiry date on the vial labels.
3. Diluted Wash Solution remains stable for 3 months at 2-8°C.
4. Diluted Sample Diluent remains stable for 4 weeks at 2-8°C.
5. For subsequent use, store unused well strips in the foil pouch with the desiccant provided and reseal.

### COLLECTION OF SPECIMENS

**Handle and dispose of all blood, serum or plasma specimens as if they were potentially infectious. See Precautions, sections 1, 2, 4 and 5.**

Determination of Gc-globulin in a single specimen requires 10 µL of serum or plasma. Blood specimens should be collected aseptically into a plain or heparinized tube by qualified staff using approved venepuncture techniques. Serum or plasma should be prepared by standard techniques for clinical laboratory testing. Cap the specimens and store them at 2-8°C for assay within 24 hours. If the assay cannot be performed within 24 hours or if the specimen is to be shipped, cap the specimen and keep it frozen at -20°C or below. Avoid repeated freezing and thawing. Do not use hemolyzed, hyperlipemic, heat-treated or contaminated specimens.

### PREPARATION OF REAGENTS AND SAMPLES

1. Bring all specimens and reagents to room temperature (20-25°C). Mix specimens thoroughly by gentle inversion and if necessary clear visible particulate matter by low-speed centrifugation.
2. Determine the number of specimens to be tested (in duplicate) plus any internal laboratory control specimens (in duplicate) plus any reagent blank wells. Add 16 wells for the 8 calibrators (in duplicate). Remove the number of microwell strips required and replace the remainder in the foil pouch with desiccant at 2-8°C.
3. Wash Solution: Dilute the 25X Wash Solution Concentrate by pouring the total contents of the bottle (30 mL) into a 1000-mL graduated cylinder and add distilled or deionized water to a final volume of 750 mL. Mix thoroughly and store at 2-8°C.

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4. Sample Diluent: Dilute the 3X Sample Diluent Concentrate by pouring the total contents of the bottle (60 mL) into a 250-mL graduated cylinder and add distilled or deionized water to a final volume of 180 mL. Mix thoroughly and store at 2-8°C. If not using all 96 wells a proportionally smaller amount of sample diluent should be prepared.
5. Gc-globulin Calibrators: ready to use. The assigned concentrations are indicated on their labels. Do not dilute further.
6. HRP-conjugated Gc-globulin (Actin-free) Antibody: ready to use, do not dilute further.
7. TMB Substrate: ready to use, do not dilute further.
8. Stop Solution: ready to use, do not dilute further.
9. Patient specimens: Dilute each specimen in a recorded proportion with Sample Diluent to obtain at least 150 µL of diluted solution that can be set up in duplicate wells at 50 µL per well. An initial screening at a dilution of 1/5000 is suggested for most samples, followed by reassay of out-of-range samples at lower or higher dilution, as appropriate.  
The 1/5000 dilution requires a pre-dilution of e.g. 10 µL of sample + 490 µL of Sample Diluent (giving a 1/50 dilution) followed by a further 1/100 dilution, e.g. 10 µL of sample diluted 1/50 + 990 µL Sample Diluent (giving a final dilution of 1/5000).

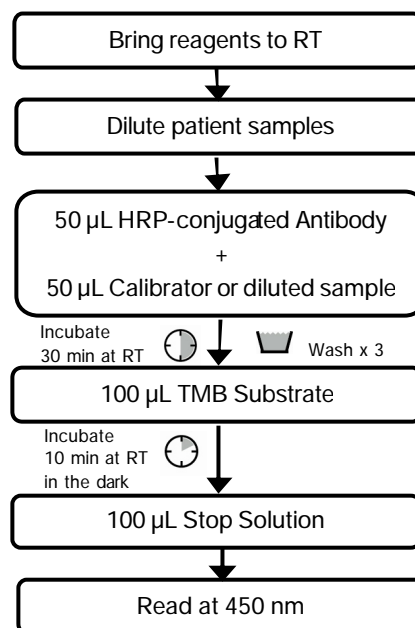
### ASSAY PROCEDURE

1. Prepare the assay protocol, assigning the appropriate wells for setting up calibrators, diluted patient specimens and any internal laboratory controls in duplicate. If a reference wavelength of 650 or 620 nm is not available on the ELISA reader, a reagent blank well can be assigned. This is set up with 50 µL of Sample Diluent instead of diluted serum or plasma and processed like the other wells.
2. Dilute samples according to the expected Gc-globulin concentrations (1/5000 will be suitable for most samples).
3. Transfer a sufficient volume of calibrators, diluted samples and any internal laboratory controls to the polypropylene U-microwell plate for the required numbers of 50 µL-transfers.
4. Pipette 50 µL volumes of HRP-conjugated Antibody into the corresponding positions in the coated microwell strips. Then with a multichannel pipette rapidly transfer 50 µL volumes of the calibrator solutions, diluted samples and internal controls from the U-wells into the corresponding duplicate wells already containing the detection

antibody. This method of sample addition is recommended to reduce the difference in incubation time between the first and last samples added to the assay wells.

5. Cover the wells and incubate for **30 minutes** at room temperature on a shaking platform set at 200/minute.

### SCHEMATIC OVERVIEW OF THE PROCEDURE



6. Aspirate the contents of the microwells and wash the microwells three times with at least 300 µL of diluted Wash Solution. If washing is performed manually, empty the microwells by inversion and gentle shaking into a suitable container, followed by blotting in the inverted position on a paper towel. A dwell time of 1 minute before emptying is recommended for at least the last wash of the cycle. The vigor with which diluted Wash Solution is filled into or emptied from the wells influences final color development. Manual pipetting, which may be very gentle and lead to high color development, is only recommended in the absence of alternatives such as filling the wells by immersion, using a multi-channel manual washing dispenser, or using an automatic washing apparatus.
7. Dispense 100 µL of TMB Substrate (ready to use) into each microwell. The use of a multichannel micropipette is recommended to reduce pipetting time. Cover the wells and incubate for **exactly 10**

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**minutes** at room temperature in the dark. Start the clock when filling the first well.

8. Add 100  $\mu$ L Stop Solution (ready to use) to each well, maintaining the same pipetting sequence and rate as in Step 7. Mix by gentle shaking for 20 seconds, avoiding splashing. Read the wells within 30 minutes.
9. Read the optical densities (absorbances) of the wells at 450 nm in an appropriate microplate reader (reference wavelength 650 or 620 nm). If no reference wavelength is available, the value of the reagent blank well is subtracted from each of the other values before other calculations are performed.

### CALCULATION OF RESULTS

The basic principle is to construct a calibration curve by plotting the mean of duplicate optical density values for each Gc-globulin Calibrator on the y-axis against the corresponding Gc-globulin concentrations in ng/mL on the x-axis. The calibration curve must meet the validation requirements. The Gc-globulin concentration of each diluted sample is then found by locating the point on the curve corresponding to the mean of duplicate optical density values for the diluted sample and reading its corresponding concentration in ng/mL from the x axis. The concentration of Gc-globulin in the undiluted sample is calculated by multiplying this result by the sample dilution factor.

This procedure can be performed manually using graph paper with linear x and y scales. A smooth curve can be drawn through the points or adjacent points can be joined by straight lines. The latter procedure may slightly over- or underestimate concentration values between points when the curve is slightly convex to the left or right, respectively. Although the curve may approximate to a straight line, it is both practically and theoretically incorrect to calculate and draw the straight line of best fit to all the points and then read the results from this.

The procedure can also be performed by an ELISA reader software program incorporating curve-fitting procedures. The procedure of choice is to use linear x and y axes with 4-parameter logistic curve fitting.

Diluted samples that give a mean optical density value above that for the 100 ng/mL Gc-globulin Calibrator or below that for the 1 ng/mL Gc-globulin Calibrator are out of the range of the assay and their concentrations should be noted as  $>100$  ng/mL and  $<1$  ng/mL respectively. The corresponding concentrations in the undiluted sera are calculated  $>(100 \times \text{dilution factor})$  ng/mL and  $<(1 \times \text{dilution factor})$  ng/mL, respectively. These samples should be reassayed at higher and lower dilutions for high- and low-reading

samples, respectively. The new dilution factors should be those estimated to give optical density values that fall well within the range of the calibration curve. Dilutions as low as 1/5 are unlikely to be required and should not be used.

### VALIDATION OF CALIBRATION CURVES

The mean optical density values for the 100 ng/mL and 0 ng/mL Gc-globulin Calibrators should be  $>1.5$  and  $<0.05$ , respectively (reagent blank subtracted when applicable). Any curvature of the calibration curve should be slight when the results are plotted on linear axes.

**Out-of-line points for individual calibrators:** One or more individual calibrators may give anomalous OD readings. One or both of the duplicate values may be out of line, and the mean of the duplicates may be out of line. This error is significant if it impairs satisfactory curve fitting by the 4-parameter logistic method, which, as a result of the anomalous value, is shifted away from other calibrator points that are in fact correct. The calibrator points and fitted curve should always be examined for correct fit before any calculations of concentration from it are accepted. A poorly fitting curve will also be revealed by a high value for the sum of residual squares. If only one calibrator is affected, which is not the highest calibrator, two courses of action are possible:

i) An erroneous singlet or duplicate result should be eliminated from the curve, and the remaining results refitted by the 4-parameter logistic procedure. If a satisfactory fit is obtained, provisional concentration results can be calculated from it.

ii) If no satisfactory fit can be obtained in this way, but the curve is otherwise consistent, provisional results can be obtained from straight lines or simple cubic spline fitting between the means of duplicates, omitting the erroneous point.

If two or more calibrators are affected, the assay should be repeated.

A deviant result for an individual calibrator can be due to operator error or to calibrator deterioration. If both duplicate values are consistently out of line in successive assays, the calibrator is faulty and should be omitted.

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### INTERPRETATION OF RESULTS

The full range of Gc-globulin concentrations in serum or plasma from healthy human donors (n=108) as measured by this assay is 92 to 332 µg/mL. These figures are lower than corresponding values for total Gc-globulin (approximately 200 to 600 µg/mL). Release of actin into the blood and removal of actin-Gc-globulin complexes commonly results in a consumption of Gc-globulin so that both the total and Actin-free Gc-globulin levels are reduced.

Clinical studies have used cutoff values of ≤100 µg/mL, ≤120 µg/mL and ≤200 µg/mL for the total Gc-globulin concentration in serum to define abnormally low values that may have prognostic significance in different clinical contexts (see Clinical Significance). Further studies are necessary to establish similar cutoff values for actin-free Gc-globulin. Pending more detailed data, values <100 µg/mL (the 2.5 centile value) should be regarded as potentially indicative of a reduced reserve of Actin-free Gc-globulin.

### QUALITY CONTROL

Laboratories intending to perform repeated assays should establish their own high-reading and low-reading control sera, stored in small (e.g. 50 µL) aliquots at -20°C or below. An aliquot of each should be thawed and tested in each assay and a record kept of successive results. This serves as a control of test performance, test integrity and operator reliability. The results should be examined for drift (tendency for successive results to rise or fall) or significant deviation from the mean of previous results. Values not deviating by more than 20% from the mean of previous values can be taken to indicate acceptability of the assay. Aliquots of control serum should not be refrozen for repeated assay once thawed, and if a further assay is performed, fresh control aliquots and fresh dilutions of patient specimens should be used.

### LIMITATIONS

Altered concentrations of Gc-globulin in serum do not necessarily imply the existence of a particular disease. Physicians must interpret the significance of the Gc-globulin values in the light of the clinical features.

### PERFORMANCE CHARACTERISTICS

#### Limit of detection

The lowest concentration giving an OD reading greater than 2 SD above the mean zero calibrator reading (n = 6) was 0.4 ng/mL, corresponding to a serum concentration of 2.0 µg/mL in a specimen diluted 1/5000.

**Intraassay (within-run) reproducibility:** Two sera were run in 6 replicates to determine within-run reproducibility. The following results were obtained (CV = coefficient of variation):

Intraassay	Serum 1	Serum 2
Mean Gc-globulin conc.	197 µg/mL	287 µg/mL
SD	7 µg/mL	10 µg/mL
CV	3.5%	3.6%

**Interassay (between-run) reproducibility (different days/operators):** The same two sera were run in duplicate in 4 different assays carried out by at least 2 operators on different days to determine between-run reproducibility. The following results were obtained:

Interassay	Serum 1	Serum 2
Mean Gc-globulin conc.	196 ng/mL	288 ng/mL
SD	6 µg/mL	23 µg/mL
CV	3.1%	7.9%

**Analytical recovery:** A serum sample was spiked with various amounts of purified human Gc-globulin and analyzed in the assay. Recovery was calculated as "Measured"/"Calculated"\*100%

Recovery	Measured	Calculated	Recovery
Sample 1	51.0 ng/mL	50.4 ng/mL	102%
Sample 2	32.4 ng/mL	32.9 ng/mL	99%
Sample 3	23.0 ng/mL	24.5 ng/mL	94%

**Sample material:** The Gc-globulin concentration in 3 sets of serum, citrate, heparin and EDTA plasma was measured and compared to the serum values.

Sample	Set 1	Set 2	Set 3
Serum	326 µg/mL	249 µg/mL	199 µg/mL
Citrate	72%	80%	84%
Heparin	90%	97%	97%
EDTA	86%	90%	98%

### Correlation

A method comparison between the Gc-globulin (Actin-free) ELISA kit and rocket immunoelectrophoresis was performed on 49 patient samples. The fitted Deming regression line was  $y = 1.00x + 0.73$ . The measured Gc-globulin values ranged between 9.7 and 319 µg/mL.

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### REFERENCES

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### LIABILITY

This kit is only intended for the *in vitro* determination of Gc-globulin in human serum and plasma.

The kit is only intended for use by qualified personnel carrying out research or diagnostic activities.

If the recipient of this kit passes it on in any way to a third party, this instruction must be enclosed, and said recipient shall at own risk secure in favor of AntibodyShop A/S all limitations of liability herein.

AntibodyShop A/S shall not be responsible for any damages or losses due to using the kit in any way other than as expressly stated in these Instructions.

The liability of AntibodyShop A/S shall in no event exceed the commercial value of the kit.

AntibodyShop A/S shall under no circumstances be liable for indirect, special or consequential damages, including but not limited to loss of profit.



### For *in vitro* diagnostic use only

The Gc-globulin (Actin-free) ELISA kit (KIT 034) conforms to Directive 98/79/EU of the European Parliament and Council of 27 October 1998 on *in vitro* diagnostic medical devices. FDA approval for diagnostic use in the USA has not been sought.

