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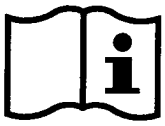

Useŕs Manual

Homocysteine ELISA

Enzyme immunoassay for the quantitative determination of total L-homocysteine in human serum or plasma

IVD

CE

REF DE2925

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1. INTENDED USE

The Homocysteine Enzyme Immunoassay (EIA) is intended for the quantitative determination of total L-homocysteine in human serum or plasma. The device can assist in the diagnosis and treatment of patients suspected of having hyperhomocysteinemia and homocystinuria.

2. SUMMARY AND EXPLANATION OF THE TEST

Homocysteine (Hcy) is a thiol-containing amino acid produced by the intracellular demethylation of methionine. Hcy is exported into plasma where it circulates mostly in its oxidized forms bound to plasma proteins.[1, 2 ,3, 4] Smaller amounts of reduced homocysteine and disulfide homocystin (Hcy-SS-Hcy) are present. Total homocysteine represents the sum of all Hcy species found in plasma and serum (free plus protein-bound). Hcy is either metabolised to cysteine or to methionine. In the vitamin B_6 dependent transsulphuration pathway Hcy is irreversibly catabolised to cysteine. A major part of Hcy is remethylated to methionine, mainly by the folate and cobalamin- dependent enzyme methionine synthase. Hcy accumulates and is excreted into the blood when these reactions are impaired.[2,4] Severely elevated concentrations of Hcy are found in subjects with homocystinuria, a rare genetic disorder of the enzymes involved in the metabolism of Hcy. Patients with homocystinuria exhibit mental retardation, early arteriosclerosis and arterial and venous thromboembolism.[1, 5] Hcy reducing therapy improves the prognosis for this disease.[5] Other less severe genetic defects which lead to moderately elevated levels of Hcy are also found.[6, 7,8] Epidemiological studies have investigated the relationship between Hcy levels in blood and cardiovascular disease (CVD). A meta analysis of 27 epidemiological studies, including more than 4000 patients, estimated that a 5 µmol/L increase in Hcy was associated with an odds ratio for coronary artery disease (CAD) of 1.6 for men and 1.8 for women, or the same that is associated with 0.5 mmol/L (20 mg/dL) increase in cholesterol. Peripheral arterial disease also showed a strong association.[9] Certain patient groups with anemia and/or asthenia also demonstrate increased levels of plasma- or serum Hcy. [10,11] Patients with chronic renal disease experience an excess morbidity and mortality due to arteriosclerotic CVD. Elevated concentration of Hcy is a frequently observed finding in the blood of these patients. Although such patients may lack some of the vitamins involved in the metabolism of Hcy, the increased levels of Hcy are mainly due to impaired removal of Hcy from the blood by the kidney.[12, 13] Drugs such as methotrexate, carbamazepine, phenytoin, nitrous oxide and penicillamine interfere with the Hcy metabolism and may give elevated levels of Hcy.[14, 15]

3. ASSAY PRINCIPLE

Homocysteine Enzyme Immunoassay (EIA) is an enzyme immunoassay for the determination of Hcy in blood.[16] Protein-bound Hcy is reduced to free Hcy and enzymatically converted to S-adenosyl-L-homocysteine (SAH) in a separate procedure prior to the immunoassay.[17] The enzyme is specific for the L-form of homocysteine, which is the only form present in the blood.

4. REDUCTION

Hcy, mixed disulfide and protein-bound forms of Hcy in the sample are reduced to free Hcy by use of dithiothreitol (DTT).

*R1 is any thiol-residue.

5. ENZYMATIC CONVERSION

Hcy in the test sample is converted to S-adenosyl-L-homocysteine by the use of SAH hydrolase and excess adenosine (Ad).

$$\text{Hcy} + \text{Ad} \xrightarrow{\text{SAH-hydrolase}} \text{SAH} + H_2O$$

The following solid-phase enzyme immunoassay is based on competition between SAH in the sample and immobilised SAH bound to the walls of the microtitre plate for binding sites on a monoclonal anti-SAH antibody. After removal of unbound anti-SAH antibody, a secondary rabbit anti-mouse antibody labelled with the enzyme horse radish peroxidase (HRP) is added. The peroxidase activity is measured spectrophotometrically after addition of substrate, and the absorbance is inversely related to the concentration of Hcy in the sample.

6. WARNINGS AND PRECAUTIONS

1. IVD For in-vitro Diagnostic Use Only.
2. Reagent D contains 0.15% merthiolate (≦ 0.074% mercury). Please handle and dispose of properly.
3. 0.01% merthiolate is used as preservative in some reagents. Each kit contains less than 0.028% mercury. Please handle and dispose of appropriately.
4. Reagent F contains mouse antibodies and Reagent G contains rabbit antibodies.
5. Reagent S contains 1.6 N acidic solution, and is classified as “Danger”. Please handle and dispose of properly (See section “Product Safety Information”).
6. Calibrators, Controls, Reagent A and Reagent E (N.B. WARNING - See section "Product Safety Information") contain less than 0.10% sodium azide as preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.
7. ⚠ Controls contain sera originating from human blood samples. The source materials havebeen tested and found to be negative for Hepatitis B Surface Antigen (HBsAG), HIV-1 Antigen (HIVAg), HCV antibody, HIV-1/2 antibody, HTLV-1/2 antibody and Hepatitis B core Antibody (HBc). However, blood derivatives should be handled according to recommended procedures for handling infectious material. HHS publication no. (CDC) 93-8395[18] or local/national guidelines on laboratory safety procedures should be consulted.
8. Reagents with different lot numbers must not be interchanged.
9. Do not use the kit after the expiration date on the outer box.

Caution: Federal law restricts this device to sale by or on the order of a physician.

7. KIT COMPONENTS

REF DE2925 Homocysteine EIA Kit, 96 wells

Kit Components	Solution	Component Description	Volume
[REAG A] / **BUF**	Assay buffer	Phosphate buffer, sodium azide	54 ml
[REAG B] / **ADENO DTT**	Adenosine/ DTT	Adenosine / dithiothreitol, citric acid	3.5 ml
[REAG C] / **SAH HYDROL**	SAH-hydrolase	Recombinant S-adenosyl-L-homocysteine hydrolase, trisbuffer, glycerol,methylparaben	3.5 ml
[REAG D] / **ENZ INH**	Enzyme inhibitor	Merthiolate, phosphate buffer	55 ml
[REAG E] / **ADENO DEAM**	Adenosine deaminase	Adenosine deaminase, phosphate buffer, sodium azide, BSA, phenol-red dye	55 ml
[REAG F] / **Ab**	a-SAH antibody	Monoclonal mouse-anti-S-adenosyl-L-homocysteine antibody, BSA, merthiolate	25 ml
[REAG G] / **ENZ CONJ**	Enzyme conjugate	Rabbit anti-mouse-antibody enzyme conjugate, BSA, horse radish peroxidase, blue dye	15 ml
[REAG H] / **SUB NMP**	Substrate solution	N-methyl-2-pyrrolidon, propyleneglycol	15 ml
[REAG S] / **STOP SOLN**	Stop solution	1.6 N acidic solution	20 ml
WASH SOLN 10x	Wash buffer	Phosphate buffer, merthiolate, Tween 20, BSA	60 ml
CAL 1 – 6	Calibrators	S-adenosyl-L-homocysteine (2, 4, 8, 15, 30, 50 µmol/L) in buffer with preservative	6 x 1.5 ml
SORB MT	Microtitre strips	Coated with S-adenosyl-L-homocysteine	12 x 8 wells

WASH SOLN 10x is concentrated and must be diluted (1+9) with purified water before use.
All other components are ready to use.

REF DE3329 Homocysteine EIA Control Kit

Kit Components	Component Description	Volume
CONTROL L	7.0 µmol/L homocysteine in diluted serum samples of human origin, phosphate buffer and preservative	1.5 ml
CONTROL M	12.5 µmol/L homocysteine in diluted serum samples of human origin, phosphate buffer and preservative	1.5 ml
CONTROL H	25.0 µmol/L homocysteine in diluted serum samples of human origin, phosphate buffer and preservative	1.5 ml

All controls are ready to use.

Homocysteine EIA Wash buffer

Kit Components	Component Description	Volume
WASH SOLN 10x	Phosphate buffer, merthiolate, Tween 20, BSA	1000 ml

WASH SOLN 10x is concentrated and must be diluted (1+9) with purified water before use.

8. MATERIALS REQUIRED BUT NOT PROVIDED IN THE KIT:

- Homocysteine controls (see section “Quality Controls” for more information)
- Plastic or glass tubes for pre-treatment of samples
- Pipettes / multipipettes 25 µL, 100 µL, 200 µL and 500 µL or 8 channel multipipette for 100 µL and 200 µL
- Volumetric flask 50 ml and 600 ml
- Incubator, 37 °C
- Washer and reader (450 nm) for microtitre plates

9. PREPARATION AND STORAGE OF KIT COMPONENTS

1. Components should be stored refrigerated (2 - 8 °C). Store all bottles upright and tightly capped. The components are stable until the stated expiration date when stored and handled as directed. Once the components in the Homocysteine Enzyme Immunoassay (EIA) Kit are opened they are stable for 12 weeks when stored at 2-8 °C.
2. The sample pre-treatment solution has to be made by mixing Reagent A, B and C (see Section “Procedure”). The solution is stable for one hour and has to be freshly made for each run.
3. The Wash buffer must be diluted (1+9) with distilled water before use. The prepared Wash buffer is stable for 4 weeks when stored at room temperature (18-25 °C).
4. Reagent D and H are stored in dark bottles to avoid exposure to light.
5. It is important that the microtitre strips are kept dry, i.e. in the sealed bag with drying capsules, and stored refrigerated. Equilibration for a minimum of two hours is required to reach room temperature (18 - 25 °C). Leave the strips in the bag during equilibration.
6. Only the necessary number of microtitre strips should be kept in the frame during the run. Unused strips should be kept in the sealed bag with drying capsules.
7. Avoid exposure of the kit to temperatures exceeding 37 °C as this may denature the enzymes.

10. SPECIMEN COLLECTION AND PREPARATION

EDTA-plasma or serum may be used with the Homocysteine Enzyme Immunoassay (EIA) assay.

As synthesis of Hcy continues in red blood cells after drawing, it is very important to prepare specimens as follows:

- Serum samples should be allowed to clot for no more than 30 minutes before centrifugation and separation of serum. Serum samples should be kept on ice prior to separation.
- EDTA-plasma samples must be centrifuged or put on ice immediately after drawing. EDTA-plasma samples may be kept on ice for up to 6 hours prior to separation by centrifugation.

Food consumption can affect circulating homocysteine levels. Protein rich meals give higher homocysteine values and should be avoided late in the day before sampling.[19, 20]
Standardised sampling procedures are crucial due to the above mentioned influencing factors. Complete mixing of thawed samples is required before use.

Plasma or serum samples may be stored for 12 weeks at 2-8°C, for up to 3 weeks at room-temperature (18-25°C) and have been shown to be stable for at least 8 months if frozen at minus 20°C.

11. LIMITATIONS

- If an automatic pipetting station is used, thorough washing of the tubing after addition of the blue coloured Reagent G may be needed - preferably with diluted acid followed by water. Any remaining solution in the tubing will interfere with the next assay step; i.e. addition of Reagent H.
- The washing procedure is critical for obtaining good precision. If manual washing is required, use 4 times 350 µL instead of 3 times 400 µL. After washing, empty the wells on paper towels.
- Avoid exposure of the kit to temperatures exceeding 37 °C as this may denature the enzymes.
- Specimens from patients who are on drug therapy involving S-adenosyl-methionine may show falsely elevated levels of homocysteine.
- Specimens from patients who have received preparations of mouse monoclonal antibodies for diagnosis or therapy may contain human anti-mouse antibody (HAMA). HAMA, present in serum or plasma specimens, may interfere in immunoassays which utilise mouse monoclonal antibodies. These specimens should not be assayed with the Homocysteine Enzyme Immunoassay (EIA) assay.
- Specimens from patients taking methotrexate, carbamazepine, phenytoin, nitrous oxide, anti-convulsants or 6-azauridine triacetate, may have elevated levels of homocysteine due to metabolic interference with the homocysteine metabolism.

12. PROCEDURE

Make sure all solutions and microtitre strips are equilibrated to room temperature before use. Leaving the kit at room temperature over night is recommended. We recommend running the calibrators in duplicate and to performing a new calibration curve for each run to avoid run-to-run variations using coated microtitre plates.

Sample pre-treatment procedure

1. Sample pre-treatment solution must be made up no more than 1 hour prior to the start of the assay. Volume needed per 10 samples (no dead volume calculated):
   4.5 ml **REAG A**
   0.25 ml **REAG B**
   0.25 ml Mix **REAG C**
2. Dilute calibrators and samples/controls in plastic or glass tubes as follows:
   25 µL calibrator/sample/control
   + 500 µL sample pre-treatment solution
   Mix well.
   Incubate for 30 minutes at 37 °C (Cap the tubes or cover with parafilm during incubation).

Note: Proceed with step 3 before the samples have cooled.

3. Add 500 µL **REAG D**
   Mix well.
   Incubate for 15 minutes at 18-25 °C.
4. Add 500 µL **REAG E**
   Mix well.
   Incubate for 5 minutes at 18-25 °C.

Microtitre plate procedure

5. Pipette 25 µL diluted calibrator / sample / control from step 4 into the wells of the SAH-coated microtitre strips.
6. Add 200 µL **REAG F** to each well.
   Incubate for 30 min at 18-25 °C.
   Use the enclosed lid during all incubations.
7. Wash with diluted Wash buffer (WASH SOLN 10x + purified water).
   Use 3 x 400 µL. If manual washing is required, use 4 times 350 µL instead of 3 times 400 µL. After washing, empty the wells on paper towels.
8. Add 100 µL **REAG G** to each well.
   Incubate for 20 min at 18-25 °C.
9. Wash with diluted Wash buffer (WASH SOLN 10x + purified water).
   Use 3 x 400 µL. If manual washing is required, use 4 times 350 µL instead of 3 times 400 µL. After washing, empty the wells on paper towels.
10. Add 100 µL **REAG H** to each well.
    Incubate for 10 min at 18-25 °C.
11. Add 100 µL **REAG S** to each well.
12. Shake and read at 450 nm within 15 minutes (Automatic plate shaker is preferred to ensure proper mixing).

13. INTERPRETATION OF RESULTS

Results should be interpreted considering all other test results and the clinical status of the patient.
We recommend that a four parameter logistic curve fit is used for preparing the calibration curve and calculation of unknown samples.

14. QUALITY CONTROL

We recommend that each laboratory use a homocysteine control with known value.
Demeditec provides a set of low, medium and high controls, REF DE3329.
The controls contain L-homocysteine in processed human serum at the following concentrations:

Control	Mean Value Hcy (µmol/L)	Range HCY (µmol/L)
CONTROL L	7.0	5.6 – 8.4
CONTROL M	12.5	10.0 – 15.0
CONTROL H	25.0	20.0 – 30.0

15. REFERENCE RANGE

The reference range should be determined by each laboratory to confirm the characteristics of the population being tested. As a point of reference, the following data may be used until the laboratory has analysed a sufficient number of samples to determine its own reference range.
The Hcy concentration in plasma or serum of healthy individuals varies with age, gender, geographical area and genetic factors. Scientific literature reports reference values for adult male and females between 5 and 15 µmol/L, men having higher values than women, and post menopausal women having higher homocysteine values than pre menopausal women.[21, 22, 23] Hcy values will normally increase with age, giving a reference range among an elderly population (> 60 years) of 5 - 20 µmol/L.[24] In countries with folic acid fortification programmes, reduced levels of Hcy may be observed.[25, 26]

Samples from 382 males and females (100 Scandinavians; 54 males aged 30 - 60, 46 females aged 29- 70. 185 Hispanics; predominantly males aged 20 - 65. 97 Americans; 54 males aged 16 - 74, 43 females aged 15 - 79), apparently healthy, without information on current medications, disease states or known risk conditions for elevated homocysteine, were tested using the Homocysteine Enzyme Immunoassay (EIA). The median value of the homocysteine concentration among Scandinavians was 8.4 µmol/L, among Hispanics 8.9 µmol/L and among Americans 9.3 µmol/L.
The homocysteine reference range was established based on 95% confidence limits as 5 - 15 µmol/L for the Scandinavian population, 3.6 - 15.0 µmol/L for the American population and 2.9 - 16.0 µmol/L for the Hispanic population.

16. MEASURING RANGE

The calibrator range is from 2 to 50 µmol/L.

17. PERFORMANCE DATA

Assay Precision

Precision of the Homocysteine EIA assay was evaluated according to NCCLS Protocol EP5-T2. Three levels of controls were assayed for 20 days with 4 replicates per run at each level. Precision data are summarised in Table 1.

Table 1: Precision			
Samples	Average HCY µmol/L	Within run precision	Total Precision
Control Low	6.1	8 %	10 %
Control Medium	10.5	7 %	9 %
Control High	20.6	8 %	10 %

Limit of quantification

The quantification limit (CV < 20%) is 1.0 µmol/L.

Linearity of diluted plasma samples

If the homocysteine concentration of a sample exceeds the range of the calibration curve, the sample should be diluted with Reagent A and reanalysed. The linearity was evaluated by diluting four high patient samples with varying amounts of Reagent A as diluent.

Linear regression analysis gave:
Slope: 0.98
Intercept: -0.4 µmol/L
Correlation coefficient r^2: 0.99

Method Comparison

The Homocysteine Enzyme Immunoassay (EIA) was compared to the University of Bergen HPLC method.[27] A comparison of 164 patient samples ranging from 3 - 37 µmol/L homocysteine gave the linear regression shown in Figure 1.

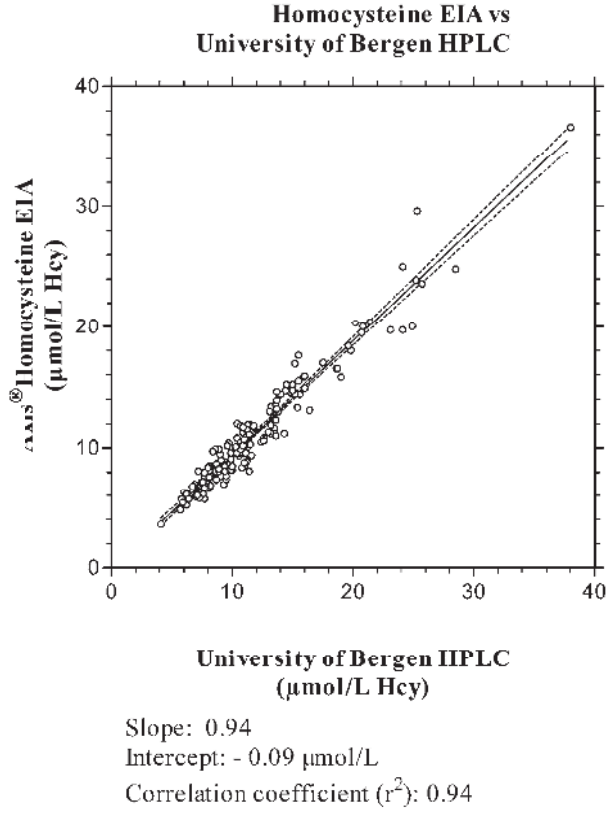

Figure 1: Method Comparison

Interfering Substances
Bilirubin, haemoglobin, lipids, red blood cells, protein and sodium fluoride were spiked into plasma samples and tested for interference by Homocysteine Enzyme Immunoassay (EIA). The assay demonstrated less than 10% interference in the presence of:
bilirubin (0.5 g/L), haemoglobin (10 g/L), triglycerides (10 g/L), red blood cells (5.0% v/v), protein (80 g/L) and sodium-fluoride (10 g/L).

Cross-reactivity
Cross-reactivity was tested for compounds that may interfere with the Homocysteine Enzyme Immunoassay (EIA) assay. The assay demonstrated 16% cross-reactivity in the presence of S-adenosyl-L-methionine (0.5 mmol/L) and less than 1% cross-reactivity in the presence of: Adenosine (5.0 mmol/L), cystathionine (0.5 mmol/L), L-cysteine (100 mmol/L), glutathione (100 mmol/L) and thiolactone (0.5 mmol/L).

18. PRODUCT SAFETY INFORMATION

 **DANGER** **REAG S**	**WARNING** H314 – **PREVENTION** P264 – P280 – **RESPONSE** P303+361+353 - P305+351+338 – P405 -	 Causes severe skin burns and eye damage. Wash hands thoroughly after handling. Wear protective gloves / protective clothing / eye protection / face protection. IF ON SKIN (or hair): Remove immediately all contaminated clothing. Rinse skin with water/shower. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Store locked up.