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Homocysteine Assay Kit (Fluorometric)

(Catalog # K531-100; 100 Reactions; Store at -20°C)

I. Introduction:

Homocysteine is a non-proteogenic amino acid synthesized intracellularly by removal of the *N*-methyl group from the essential amino acid methionine. Homocysteine is exported from cells into the blood, where it exists mainly as an oxidized disulfide species, either as a dimer or bound to cysteine residues of serum proteins. The reduced form of homocysteine ('free' homocysteine) can be metabolized into cysteine via the transsulfuration pathway; however, it can also undergo intramolecular cyclization, forming the highly reactive pro-oxidant homocysteine thiolactone. Subsequent *N*-homocysteinylation of protein lysine residues by the reactive thiolactone disrupts protein conformation, leading to formation of cytotoxic protein aggregates. Homocysteinylated proteins may also act as autoantigens, triggering arterial inflammation and atherosclerosis. Elevated plasma homocysteine concentration is a clinical biomarker for increased risk of cardiovascular disease, ischemic stroke and myocardial infarction. Severely elevated homocysteine levels (hyperhomocysteinemia) are correlated with a 4-fold increase in mortality due to heart attack and a 16-fold increase in the likelihood of recurrent stroke. BioVision's Homocysteine Assay Kit allows for quantification of total homocysteine in biological fluids such as plasma and serum. The assay is based on the reduction of homocysteine disulfides to free homocysteine, which is cleaved by a homocysteine-selective enzyme, generating an intermediate product. The intermediate reacts with a probe solution to form a stable fluorophore that emits in the far-red spectrum (Ex/Em = 658/708 nm). The assay is not affected by physiological concentrations of other biological thiols (such as cysteine, methionine and glutathione), is high-throughput adaptable and can detect as low as 5 µM homocysteine.

II. Applications:

Estimation of total homocysteine concentration in biological fluids (serum/plasma)

III. Sample Type

· Human or animal plasma or serum

IV. Kit Contents:

Components	K531-100	Cap Code	Part Number
Homocysteine Assay Buffer	25 ml	WM	K531-100-1
Disulfide Reducing Agent (DTT)	300 µl	Blue	K531-100-2
Homocysteine Enzyme Mix	1 vial	Green	K531-100-3
Fluorogenic Probe Solution	5 ml	NM	K531-100-4
Developer Solution	5 ml	NM	K531-100-5
Homocysteine Disulfide Standard	1 vial	Yellow	K531-100-6

V. User Supplied Reagents and Equipment:

- Multiwell fluorescence microplate reader
- · Precision multi-channel pipette and reagent reservoir
- · White 96-well plates with flat bottom

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C and protect from light. Briefly centrifuge all small vials prior to opening. Allow the Homocysteine Assay Buffer to warm to room temperature prior to use. Read entire protocol before performing the assay procedure.

- Disulfide Reducing Agent (DTT): Provided as a 100 mM stock solution. Aliquot and store at -20°C, avoid repeated freeze/thaw cycles.
- Homocysteine Enzyme Mix: Reconstitute in 330 µl of Homocysteine Assay Buffer to generate a 10X stock solution. Divide into aliquots and store at -20°C, protected from light. Avoid repeated freeze/thaw cycles.
- Fluorogenic Probe Solution: Warm to RT prior to use. Store at 4°C, protected from light.
- Developer Solution: Warm to RT prior to use. Store at 4°C, protected from light.
- Homocysteine Disulfide Standard: Reconstitute in 220 µl of dH₂O for a 1 mM solution. Store at -20°C, stable for 5 freeze/thaw cycles.

VII. Homocysteine Assay Protocol:

1. Sample Preparation: Aliquot enough Homocysteine Assay Buffer for the number of reactions to be performed. Add Disulfide Reducing Agent (DTT) to Homocysteine Assay Buffer at a 1:100 ratio (10 µl of 100 mM DTT stock per 1 ml of Homocysteine Assay Buffer) immediately prior to use. Collect plasma or serum samples by standard methods (keep on ice for immediate use or store at -80°C for future experiments). Add 10 µl of undiluted serum/plasma to desired well(s) in a white, flat bottom 96-well plate. For each test sample, prepare a parallel sample well to serve as the sample background control. Add 160 µl of Homocysteine Assay Buffer (with DTT) to all sample reaction wells (bringing the volume to 170 µl/well).

Notes

- · Always prepare fresh Homocysteine Assay Buffer with DTT. Once prepared, keep buffer with DTT on ice and use within 4 hrs.
- The normal physiological range for homocysteine in human plasma is 6-12 μM; however levels can be dramatically higher in certain
 cases. For unknown samples, we suggest doing a pilot experiment to ensure readings are within the range of the standard curve (50500 pmol/well or 5-50 μM). Samples with higher levels of homocysteine may be diluted with Homocysteine Assay Buffer or PBS.

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- Plasma or serum samples exhibiting lipemia or excessive turbidity should be clarified by centrifugation prior to use. Hemolytic samples should not be used, due to release of homocysteine from lysed red blood cells.
- To ensure accurate determination of homocysteine in test samples or for samples with a low concentration of homocysteine, we recommend spiking samples with a known amount of Homocysteine Disulfide Standard (50 pmol).
- 2. Standard Curve Preparation: Prepare a 25 μM solution of Homocysteine Disulfide Standard by adding 10 μl of the 1 mM Homocysteine Disulfide stock to 390 μl of Homocysteine Assay Buffer. Add 0, 1, 2, 4, 6, 8, and 10 μl of the 25 μM Standard into a series of wells, generating 0, 50, 100, 200, 300, 400 and 500 pmol of free homocysteine/well upon disulfide reduction (each mole of Homocysteine Disulfide Standard generates 2 moles of free L-homocysteine upon reduction). Adjust the volume to 170 μl/well with Homocysteine Assay Buffer (with DTT).

3. Homocysteine Enzyme Mix Reaction:

- a. Incubate the plate at 37°C for 30 mins with gentle shaking to liberate free homocysteine in sample and standard curve wells. Remove the plate from the incubator and allow it to cool to room temperature for 5 mins.
- b. Prepare a 1X solution Homocysteine Enzyme Mix by diluting the reconstituted 10X stock with Homocysteine Assay Buffer (without DTT) at a 1:10 ratio. For test sample and standard curve reactions, prepare 30 μl of 1X Homocysteine Enzyme Mix (containing 3 μl reconstituted 10X stock and 27 μl Homocysteine Assay Buffer) per well. For sample background control wells, prepare 30 μl of Homocysteine Assay Buffer (without DTT).
- c. Add 30 µl of 1X Homocysteine Enzyme Mix to each test sample and standard curve well and 30 µl of Homocysteine Assay Buffer (without DTT) to each sample background well. Mix well and incubate the plate at room temperature for 5 mins, protected from light.
- d. During the 5 min incubation period, prepare enough Fluorogenic Developer Mix for the number of reactions being performed. For each reaction well, mix 30 µl Fluorogenic Probe Solution and 20 µl Developer Solution. Add 50 µl Fluorogenic Developer Mix to all sample, background control and standard curve wells and mix well (bringing the final volume to 250 µl/well).

Notes:

- The 5 min enzymatic reaction incubation time *must be consistent for both the standard curve and sample wells*. We recommend using a multi-channel pipette and reagent reservoir for addition of Homocysteine Enzyme Mix and Fluorogenic Developer Mix.
- Once prepared, the Fluorogenic Developer Mix should be used immediately. Do not store Fluorogenic Developer Mix.
- 4. Measurement: Following addition of Fluorogenic Developer Mix, incubate plate for 15 min at RT with continuous shaking (to ensure adequate mixing). Measure the fluorescence of all sample, background and standard curve wells at Ex/Em = 658/708 nm in endpoint mode.
- **5. Calculations:** For the Homocysteine Standard Curve, subtract the zero standard (0 pmol/well) reading from all of the standard readings, plot the background-subtracted values and calculate the slope of the standard curve. For test samples, calculate the corrected sample fluorescence (F_s) by subtracting the background control RFU reading from the sample reading: $F_s = RFU_S RFU_{BC}$. For unspiked samples, apply the F_s values to the standard curve to get B pmol of homocysteine in the well.

Sample Total Homocysteine Concentration = $\frac{B}{V} \times D$ = pmol/µl = µM

Where: **B** is the amount of homocysteine, calculated from the standard curve (in pmol)

 ${f V}$ is the volume of sample added to the well (10 μ l)

D is the sample dilution factor (if applicable, D=1 for undiluted samples)

Note: For spiked samples, calculate B by subtracting the corrected sample reading (F_s) from the corrected spiked sample reading ($F_{s+spike}$). Each mole of spiked Homocysteine Disulfide Standard is equivalent to 2 moles of free Homocysteine upon reduction.

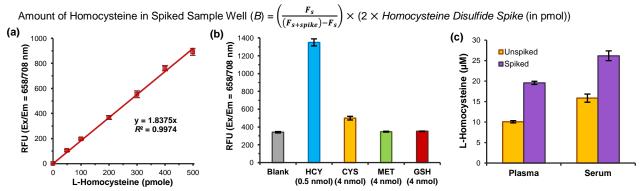


Figure: (a) Homocysteine Standard curve. (b) Specificity for detection of homocysteine (HCY) over other thiols. At an 8-fold molar excess versus HCY, cysteine (CYS) contributes ≤15% interference, while methionine (MET) and glutathione (GSH) contribute ≤2%. (c) Estimation of total HCY in single-donor human plasma and serum (10 μl), spiked with 50 pmol Homocysteine Disulfide Standard (equivalent to 100 pmol or 10 μM free HCY). Total HCY concentrations for plasma and serum samples were 10.1 ± 0.28 μM and 15.9 ± 0.99 μM, with respective spike recoveries of 95.1% and 103.4%. Data are mean ± SEM of 3 replicates, assayed according to the kit protocol.

VIII. RELATED PRODUCTS:

Cysteine Assay Kit (K558) AHCY Inhibitor Screening Kit (K326) Homocysteine α,γ-Lyase, Recombinant (P1117) Adenosylhomocysteinase (AHCY) Activity Fluorometric Assay Kit (K613) Cystathionine β -Synthase Activity Assay Kit (K998) Cystathionine β -Synthase Inhibitor Screening Kit (K695)



Gentaur Europe BVBA Voortstraat 49, 1910 Kampenhout BELGIUM Tel 0032 16 58 90 45 info@gentaur.com



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