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Product Information

Homocysteine Assay Kit

Catalog Number **MAK354**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

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 trans-sulfuration 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.

The 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 (λ_{ex} = 658 nm/ λ_{em} = 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.

The kit is suitable for the determination of total homocysteine concentration in plasma or serum.

Components

The kit is sufficient for 100 fluorometric assays in 96 well plates.

Homocysteine Assay Buffer Catalog Number MAK354A	25 mL
Disulfide Reducing Agent (DTT) Catalog Number MAK354B	300 μL
Homocysteine Enzyme Mix Catalog Number MAK354C	1 vial
Fluorogenic Probe Solution Catalog Number MAK354D	5 mL
Developer Solution Catalog Number MAK354E	5 mL
Homocysteine Disulfide Standard Catalog Number MAK354F	1 vial

Reagents and Equipment Required but Not Provided.

- Pipetting devices and accessories (e.g., multichannel pipettor)
- White flatbottom 96 well plates
- Fluorescence multiwell plate reader

Precautions and Disclaimer

For R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions.

Homocysteine Assay Buffer, Fluorogenic Probe Solution, and Developer Solution – Warm to room temperature, protected from light prior to use.

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 10× stock solution. Divide into aliquots and store at –20 °C, protected from light. Avoid repeated freeze/thaw cycles.

Homocysteine Disulfide Standard – Reconstitute in 220 µL of ultrapure water for a 1 mM solution. Store at –20 °C when not in use, stable for 5 freeze/thaw cycles.

Homocysteine Assay Buffer with DTT (used for assay reactions) – 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. Once prepared, keep buffer with DTT on ice and use within 4 hours.

Storage/Stability

The kit is shipped on wet ice. Store components at –20 °C, protected from light. Briefly centrifuge small vials prior to opening.

Procedure

Sample Preparation

Notes:

- 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, it is suggested to perform a pilot experiment to ensure readings are within the range of the standard curve (50–500 pmol/well or 5–50 µM). Samples with higher levels of homocysteine may be diluted with Homocysteine Assay Buffer or PBS.
- 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, it is suggested to spike samples with a known amount of Homocysteine Disulfide Standard (50 pmol).

1. Collect plasma or serum samples by standard methods (keep on ice for immediate use or store at –80 °C for future experiments).
2. Add 10 µL of undiluted serum/plasma to desired well(s) in a white, flat bottom 96 well plate.
3. For each test sample, prepare a parallel sample well to serve as the sample background control.
4. Add 160 µL of Homocysteine Assay Buffer **with DTT** to all sample reaction wells (bringing the volume to 170 µL/well).

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 (with DTT). Prepare Homocysteine Standards in desired wells of white 96 well plate according to Table 1. Adjust the volume to 170 µL/well with Homocysteine Assay Buffer (with DTT), mix well. Each mole of Homocysteine Disulfide Standard generates 2 moles of free L-homocysteine upon reduction.

Table 1.
Preparation of Homocysteine Standards

Well	25 µM Premix	Homocysteine Assay Buffer (with DTT)	Free Homocysteine (pmol/well)
1	0 µL	170 µL	0
2	1 µL	169 µL	50
3	2 µL	168 µL	100
4	4 µL	166 µL	200
5	6 µL	164 µL	300
6	8 µL	162 µL	400
7	10 µL	160 µL	500

Assay Reaction

1. Incubate the plate at 37 °C for 30 minutes with gentle shaking to liberate free homocysteine in sample and standard curve wells.
2. Remove the plate from the incubator and allow it to cool to room temperature for 5 minutes.
3. Prepare a 1× solution Homocysteine Enzyme Mix by diluting the reconstituted 10× stock with Homocysteine Assay Buffer **(without DTT)** 10-fold. For each test sample and standard curve reaction, prepare 30 µL of 1× Homocysteine Enzyme Mix (containing 3 µL reconstituted 10× stock and 27 µL Homocysteine Assay Buffer) per well.
4. For each sample background control well, prepare 30 µL of Homocysteine Assay Buffer **(without DTT).**

5. Using a multichannel pipette and reagent reservoir, add 30 µL of 1× Homocysteine Enzyme Mix to each test sample and standard curve well.
6. Add 30 µL of Homocysteine Assay Buffer **(without DTT)** to each sample background well.
7. Mix well and incubate the plate at room temperature for 5 minutes, protected from light. Note: **The 5 minute enzymatic reaction incubation time must be consistent for both the standard curve and sample wells.**
8. During the 5 minute incubation period, prepare enough Fluorogenic Developer Mix for the number of reactions being performed. For each reaction well, mix 30 µL of Fluorogenic Probe Solution and 20 µL of Developer Solution. Use immediately, do not store.
9. Using a multichannel pipette and reagent reservoir, add 50 µL of Fluorogenic Developer Mix to all sample, background control, and standard curve wells and mix well (bringing the final volume to 250 µL/well).

Measurement

Following addition of Fluorogenic Developer Mix, incubate the plate for 15 minutes at room temperature with continuous shaking (to ensure adequate mixing). Measure the fluorescence of all sample, background, and standard curve wells at λ_{ex} = 658 nm/λ_{em} = 708 nm in endpoint mode.

Results

1. For the Homocysteine Standard Curve, subtract the zero standard (0 pmol/well) reading from the remaining standard readings.
2. Plot the background-subtracted values and calculate the slope of the standard curve.
3. For test samples, calculate the corrected sample fluorescence (F_s) by subtracting the background control RFU reading from the sample reading: $F_s = RFU_S - RFU_{BC}$.
4. For unspiked samples, apply the F_s values to the standard curve to get B pmol of homocysteine in the well.

Total Homocysteine (pmol/µL or µM) =

$$(B/V) \times D$$

where:

B = the amount of homocysteine, calculated from the standard curve (in pmol)

V = the volume of sample added to the well (10 µL)

D = 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.

Amount of Homocysteine in Spiked Sample Well (B) =

$$\frac{F_s}{(F_{s + spike}) - F_s} \times (2 \times \text{Homocysteine Disulfide Spike (pmol)}$$

Figure 1.
Typical Homocysteine Standard Curve

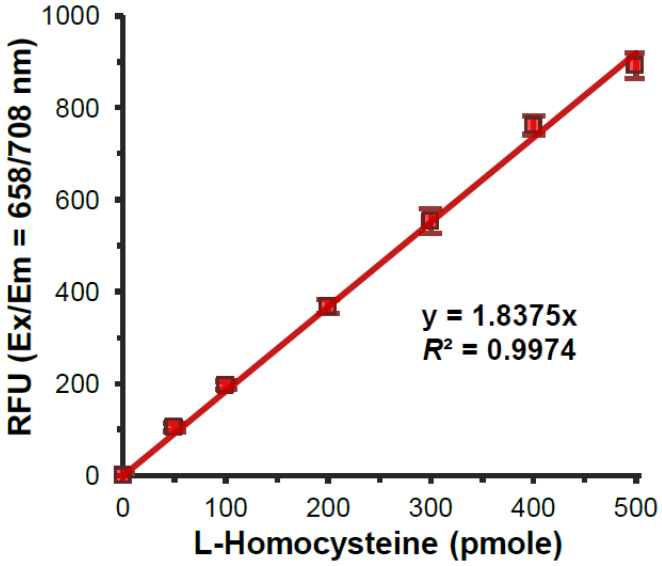
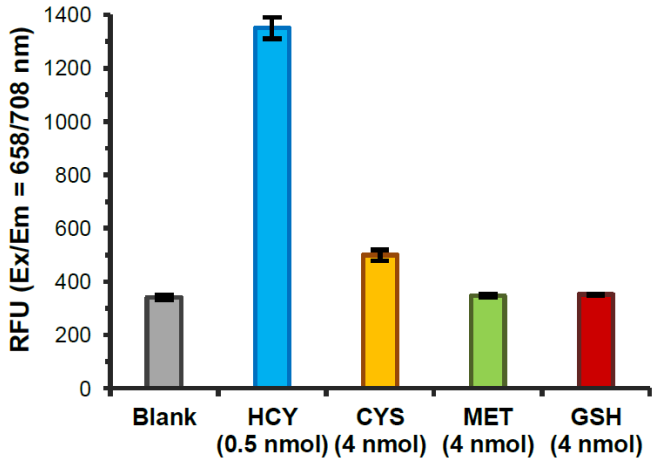
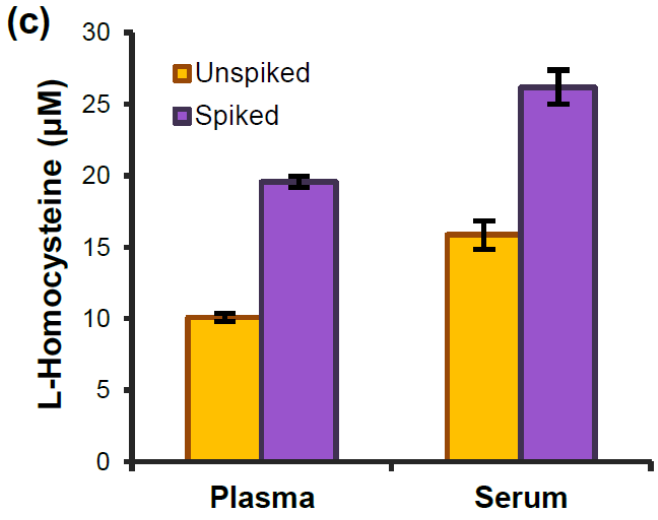


Figure 2.
Specificity for Detection of Homocysteine



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%.

Figure 3.
Estimation of Total HCY



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 procedure.

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