



Total Plasma Homocysteine Kit

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MF-9080

INSTRUCTION MANUAL

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Section 1. Introduction

Homocysteine is a naturally occurring amino acid, high levels of which have been associated with coronary artery disease [1,2]. It occurs as the free thiol (homocysteine), its disulfide (homocystine), and conjugated with other thiols and proteins through disulfide linkages. The bulk of plasma homocysteine (> 85%) occurs in conjugated form, rendering it inaccessible to common analytical techniques. Samples must therefore be treated with a reducing agent before analysis, to liberate homocysteine as the free thiol.

The commonly used reducing agents present problems, however. Sodium borohydride, for example, is most effective when the reduction process is performed at 50 °C. And it tends to foam, so an antifoaming agent such as *n*-amyl alcohol [1] or methanol [3] must be added. Another reducing agent, tri-*n*-butylphosphine [4,5], is effective under a variety of conditions, but presents the danger of explosion in its concentrated form. In contrast, the BAS procedure presented here uses a safe reducing agent that does not cause foaming and is effective at room temperature (18 °C and above). Moreover, homocysteine is stable when the samples emerge from the reduction step of the BAS procedure.

Previous assays of plasma homocysteine have used either an electrochemical detector [1,3] or a fluorescence detector [4,5]. Fluorescence procedures have the disadvantage of requiring a derivatization step in addition to the required reduction step. And the derivatives are marginally stable. An electrochemical detector oxidizes homocysteine directly, so no derivatization is needed.

Two kinds of working electrodes have been used successfully for the detection of homocysteine: mercury/gold amalgams [1] and gold [3]. The gold electrode requires less preparation and maintenance, exhibits good selectivity for thiols, and does not require the use of toxic mercury. BAS has found, however, that a platinum electrode provides all these advantages and is more rugged than a gold electrode. We have therefore chosen a platinum electrode for this kit.

Finally, many determination methods for homocysteine either use no internal standard, or use a thiol rather than a disulfide. In contrast, our internal standard is a disulfide, which must be reduced before it can be detected. Included with every sample and calibrator, the internal standard provides a check on the reduction step and all subsequent steps in the assay.

Section 2. Components and Required Materials

Total Plasma Homocysteine Kit, 100 Samples (MF-9026)	Quantity	Description
	1 bottle	Homocysteine standard, 1 g (CR-2020)
	1 bottle	Homocysteine standard, 1 g (CF-1505)
	2 bottles	MP-4 Plasma Homocysteine Mobile Phase, 750 mL each (CF-1301)
	1 bottle	Reagent A, 15 mL
	1 bottle	Reagent B, solid
	1 bottle	Reagent C, 35 mL
	1 each	BAS UniJet [®] Column (MF-8957)
	1 each	Plasma Homocysteine Manual (MF-9080)
Total Plasma Homocysteine Replacement Kit, 100 Samples (MF-9027)	Quantity	Description
	1 bottle	MP-4 Plasma Homocysteine Mobile Phase, 750 mL (CF-1301)
	1 bottle	Reagent A, 15 mL
	1 bottle	Reagent B, solid
	1 bottle	Reagent C, 35 mL
Internal Standard	1 bottle	Internal Standard, 0.1 g (CF-1550)

**Additional Materials
Required**

Mechanical pipettors

Disposable gloves

Class A volumetric flasks, 100, 50, 25 and 5 mL

Class A volumetric pipets, 5 and 10 mL

250-mL class A graduated cylinder

22-mL glass vials with inert-lined caps

Vortex mixer

Analytical balance

Polypropylene microcentrifuge tubes (at least 1.7 mL — VWR 20170-620 or equiv.)

Perchloric acid

Microcentrifuge (Eppendorf 5416 with 16T60-11 rotor, or equiv.)

Vials for autosampler (if required)

Microfilter apparatus (BAS MF-5500) and Microfilters, 0.45 μm (BAS MF-5655), or alternatively, precolumn filter, 0.5 μm (BAS MR-4135).

**Additional Reagents
Required**

Deionized water (DI H ₂ O)	Type I reagent-grade water (ASTM) or HPLC-grade deionized water
Perchloric acid	AR-grade, 70 %

Instrumentation

Liquid Chromatograph

Single-channel, pulse-capable electrochemical detector with platinum working electrode *

Autosampler (optional)

Data-reduction software, integrator, or chart recorder

* The BAS Homocysteine Kit has been tested and validated on the BAS-200 Liquid Chromatograph and the LC-4C electrochemical detector with ChromGraph™ Control. While the kit should work with any pulse-capable electrochemical detector, BAS does not warrant that the kit will perform to specifications with any but the two tested models. The user should determine the feasibility of using a different detector prior to running any unknown samples.

Section 3. Specimen Collection, Preservation, and Storage

Patients should fast, as some studies indicate that homocysteine concentration in plasma declines for several hours after eating [10]. Blood samples should be collected in EDTA tubes and maintained at 0-4 °C for consistency. Separate the plasma from the blood cells by centrifugation at 2000 x g for 5 min at 0-2 °C [6]. This should be done within one hour of collection, because erythrocytes continue to export homocysteine in collected whole blood [7]. Store plasma at -80 °C if it will not be analyzed immediately.

Section 4. Experimental Assay Procedure

Preparation of Assay Reagents

0.1 M perchloric acid: Add 2.2 mL 70% perchloric acid to about 200 mL DI H₂O in a 250-mL graduated cylinder, then add water to the 250-mL mark. Mix. Store at room temperature and use within three months.

Reagent B: Reagent B is shipped dry to improve its stability. Dilute to the working concentration by adding 8.0 mL DI H₂O directly to the bottle. Mix. Store at 4 °C and use within three months.

Reagent C: Reagent C is shipped double-strength to improve its stability. Dilute to the working concentration by adding 35 mL DI H₂O directly to the bottle. Mix. Write "diluted" on the label. Store at room temperature and use within three months.

Preparation of Stock Standards

Homocysteine stock: Dissolve 27.0 mg DL-homocysteine in 0.1 M HClO₄ using a 50-mL volumetric flask. Refrigerate. Make fresh monthly.

Homocystine stock: Dissolve 26.8 mg DL-homocystine in 0.1 M HClO₄ using a 50-mL volumetric flask (may require sonication). Refrigerate. Make fresh monthly.

Internal Standard stock: Dissolve 12.5 mg Internal Standard in 0.1 M HClO₄ using a 25-mL volumetric flask. Refrigerate. Make fresh monthly.

Preparation of Test and Calibration Standards

Homocysteine Test Solution: Combine 20 µL homocysteine stock and 20 µL Reagent B with 20 mL DI H₂O. Store at 4 °C. The test solution is used to ensure that the column and detector are equilibrated and performing properly (Section 6).

Internal Standard Solution: Put 200 µL Internal Standard stock into a 5-mL volumetric flask and fill to the mark with DI H₂O. Refrigerate. Make fresh weekly.

Homocystine Calibration Standards: Homocystine in the calibrators is reduced to homocysteine in the assay. "Level" refers to the concentration of homocysteine in plasma, after reduction. Dilute the highest level into a 100-mL volumetric flask using a micropipet. Dilute the others into 22-mL vials using volumetric pipets. Refrigerate. Make fresh weekly.

<i>Level</i>	<i>Amount</i>	<i>DI H₂O</i>
20 µmol/L	1.0 mL stock	to 100 mL
15 µmol/L	15.0 mL of 20 µmol/L	5.0 mL
10 µmol/L	10.0 mL of 20 µmol/L	10.0 mL
5 µmol/L	10.0 mL of 10 µmol/L	10.0 mL
0 µmol/L		20 mL

If patients with unusually high levels of homocysteine are expected, add higher level calibrators as appropriate.

Some laboratories prefer to dilute each calibration standard individually from stock, rather than serially. In this case, add 1.0, 0.75, 0.50 and 0.25 mL stock to a final volume of 100 mL for the 20, 15, 10 and 5 $\mu\text{mol/L}$ calibration standards.

Preparation of QC Pools for Homocysteine Determinations

Add the indicated amount of homocysteine stock to 25 mL EDTA plasma. Additional pools can be created, if desired, by modifying the spike volume.

Remember that the final concentration of homocysteine will be the sum of the spike plus the endogenous level in the plasma. For this reason, the plasma used for the QC pools should have a low endogenous level of homocysteine ($\leq 10 \mu\text{mol/L}$).

<i>Pool</i>	<i>Approx. Added Conc. ($\mu\text{mol/L}$)</i>	<i>Spike Volume (μL)</i>
LOW	0	0
HIGH	20	125

Mix the plasma thoroughly after adding the homocysteine spike and before subdividing it. Subdivide the pools into small plastic tubes and freeze at $-80 \text{ }^\circ\text{C}$.

Calibration Scheme

We recommend the endogenous (standard addition) method, in which pooled plasma is spiked with known amounts of standard homocysteine.* Linear regression of the ratio (homocysteine area \div internal standard area) versus concentration results in an equation of the form:

$$\{1\} \text{ Ratio} = \text{Intercept} + \text{Concentration} \times \text{Slope}$$

for the calibrators. To calculate homocysteine concentration for a patient sample, Intercept is set to zero, so the equation above reduces to

$$\{2\} \text{ Concentration} = \text{Ratio} \div \text{Slope}$$

If you prefer not to use the internal standard, substitute the area of the homocysteine peak for "ratio" in equations {1} and {2}.

* It is possible to use external aqueous (no plasma) calibrators with this kit, but these are somewhat less stable than plasma-containing calibrators. If you wish to use this approach you should use a $4 \text{ }^\circ\text{C}$ autosampler and process small batches of samples and calibrators every 8 hours (see Section 7). Substitute $200 \mu\text{L DI H}_2\text{O}$ for the pooled plasma in the calibrators. In this case, use equation {1}.

Assay Procedure

1. Keep all experimental and QC samples frozen until ready to use.
2. Thaw samples and pooled plasma at room temperature. Plasma should be vortexed immediately before adding it to the reaction.
3. Combine the following in microcentrifuge tubes, in the order given (see Table 4.1):
 - 100 μL water (for unknowns) or 100 μL calibration standards
 - 100 μL Internal Standard solution
 - 100 μL Reagent A
 - 200 μL patient plasma (unknowns) or pooled plasma (calibrators)
 - 50 μL Reagent B
4. Cap the tubes and vortex for 5 seconds. Allow to react at room temperature (18 °C or above) for 10 minutes.
5. Add 500 μL Reagent C to each tube. Vortex for 10 seconds.

IMPORTANT: Reagent C must be diluted before use.
6. Centrifuge the tubes for 5 minutes at 8,400 x g or higher (10,000 rpm for the Eppendorf 5416 centrifuge).
7. Remove an aliquot of the supernatant and discard the pellet.
8. Filter the samples using BAS MF-1 Microfilters and 0.45 mm regenerated cellulose membranes. This step may be omitted if a 0.5 mm precolumn filter is installed in the chromatograph.
9. Inject 10 μL into the chromatograph. For greatest precision, overfill a 10- μL loop with 30 μL of sample.

Table 4.1 Assay Protocol

ID	Water	Calibration Standard	Internal Standard Solution	Reagent A	Pooled Plasma	Patient or QC Plasma	Reagent B
20 $\mu\text{mol/L}$ Calibrator		100	100	100	200		50
15 $\mu\text{mol/L}$ Calibrator		100	100	100	200		50
10 $\mu\text{mol/L}$ Calibrator		100	100	100	200		50
5 $\mu\text{mol/L}$ Calibrator		100	100	100	200		50
0 $\mu\text{mol/L}$ Calibrator		100	100	100	200		50
Patient Samples or QC Samples	100		100	100		200	50

Section 5. Instrument Conditions

Chromatographic Conditions	Column	BAS UniJet 3 μ m C-18, 100 x 2 mm, MF-8957
	Mobile Phase	BAS MP-4 homocysteine mobile phase, CF-1301
	Detector	Amperometric with dual platinum electrodes in series. Only the upstream electrode is used.
	Chromatograph	BAS-200B or BAS-480 with ChromGraph [®] Control software
	Flow Rate	0.4 mL/min
	Autosampler	BAS Sample Sentinel or equivalent
	Loop Volume	10 μ L
	Sample Volume	30 μ L
	Run Time	10 minutes
	Column Temperature	35 $^{\circ}$ C
	Approximate Retention Time	3-4 minutes for homocysteine

ChromGraph[®] Control Conditions

Method Name: HOMOCYS

Data Acquisition Options: Trigger Type = Auto, Start Data Time = 0, End Data Time = 10, Data Rate = 150 ppm, Detectors Enabled = EC1 (BAS-200) or LC-4C on DA-1 (LC-4C).

Temperature Options: Oven = 35 $^{\circ}$ C, Bottle A = 35 $^{\circ}$ C

Pump Gradient Schedule: Flow Rate = 0.4 mL/min. Low Pressure Limit = 200 psi, High Pressure Limit = 4000 psi, Synchronize Start = off (not checked)

<i>Time</i>	<i>Bottle A</i>	<i>Bottle B</i>	<i>Bottle C</i>
0.0	100%	0	0
1.0	100%	0	0

BAS-200 Detector Schedule:

<i>Time</i>	<i>Potential mV</i>	<i>Range μAfs</i>	<i>Filter Hz</i>	<i>Offset %</i>
0.0	+600	0.2	0.1	10.0
7.0	+1100	10	0.1	0.0
7.1	0	10	0.1	0.0
7.2	+600	10	0.1	0.0
8.5	+600	1	0.1	0.0
9.5	+600	0.2	0.1	0.0
10.0	+600	0.2	0.1	0.0

LC-4C Detector Schedule:

<i>Time</i>	<i>Potential mV</i>	<i>Range μAfs</i>	<i>Filter Hz</i>	<i>Offset %</i>
0.0	+600	0.5	0.1	*
7.0	+1100	10	0.1	
7.1	0	10	0.1	
7.2	+600	10	0.1	
8.5	+600	1	0.1	
9.5	+600	0.5	0.1	
10.0	+600	0.5	0.1	

* The LC-4C does not have an automatic offset. Use the offset-adjusting knob under the autozero toggle switch (which must be in the ON position) to adjust the baseline as follows. Once the detector is equilibrated, perform two blank runs (no injections). During the first two minutes of the second run, turn the adjusting knob so the baseline you see on the screen is offset about 10% upwards. This will ensure that the baseline remains visible even if there is negative drift during subsequent runs. (This is purely for esthetic reasons - ChromGraph software can process the data even if the baseline drops below zero.)

DA-5 Filter Options (LC-4C only): None.

Autosampler Conditions

Model	BAS Sample Sentinel
Injection Type	Pull
Injection Volume	30 µL
Loop Size	10 µL
Options	Input Polarity/Inject Hold Active = Hi
Communications Cable	BAS-200B: EW-4456 DA-5: EW-4454
Flush Fluid	Filtered, degassed DI H ₂ O
Flush volume	400 µL
Cycle Time	9 minutes
Temperature	4 °C

Section 6. Liquid Chromatography

The system requires overnight equilibration for consistent performance. Both the column and the electrochemical detector must be equilibrated.

Column

Connect the column to the chromatograph using plastic high-pressure fingertight fittings only. Metal fittings will damage the column connectors.

Flush a new column for 10 minutes with 40:60 (v:v) acetonitrile:water at 0.4 mL/min. Then, start the mobile phase at a flow rate of 0.4 mL/min.

The mobile phase should flow to waste for at least two hours. After this initial period it can be recycled into the solvent reservoir. The mobile phase should be recycled except when plasma samples are being injected.

Retention time of homocysteine may shorten over the life of the column. This is normal, and will not affect the quality of the separation.

The column should produce a pressure of 2000 ± 500 psi. The system pressure should be stable. (Warning: while you can obtain useful data at pressures above 3000 psi, you should not allow the system to exceed 4000 psi. Should the pressure exceed 4000 psi, shut the system down and seek the source of the problem [Section 8]).

Electrochemical Detector

Wipe the platinum electrode with methanol before installing it on the system. Once mobile phase is flowing, turn on the detector by pressing the WARMUP or ON button in the detector schedule. This will apply a potential of +600 mV to the platinum electrode at an insensitive gain range of 1 μ Afs. The electrode should be allowed to equilibrate for 2-5 hours at this potential. Then press EQUIL to set the proper gain range. Then press ZERO. Begin cycling the detector as described below.

Detector Care and Maintenance

When set at a constant potential of +600 mV, the platinum electrode slowly passivates – peak heights decline in a regular progression from run to run. The detector schedule includes built-in cleaning pulses that prevent this passivation. It is important that these pulses, which occur at 7 minutes after injection, come after peak 3 (Figure 7.1) has eluted. If for some reason peak 3 is eluting late, you must change the detector schedule so the pulses occur later.

It may take several hours or overnight until the detector stabilizes and provides consistent peak heights. Homocysteine test solution (Section 4) may be injected during this equilibration period.

To maintain its sensitivity, the detector should be cycled through its schedule even when the system is idle. To do this, set the instrument for 255 runs and allow it to cycle on its own. When using ChromGraph software, go to the Data Acquisition Options section of the Method. Check trigger type Auto, and enter 255 as the number of runs. Use a Data Name that won't be confused with samples, such as NULL. Press EQUIL, then RUN.

Samples should always be injected immediately after the previous run, so the detector has equilibrated for the same amount of time before each injection. Before starting a batch of samples, inject 5 test runs of homocysteine test solution (Section 4). Do not begin injecting the samples until the peak areas from these test runs are consistent.

If the working electrode is ever replaced, polished, or electrochemically cleaned, it must be equilibrated as above.

Idle Periods

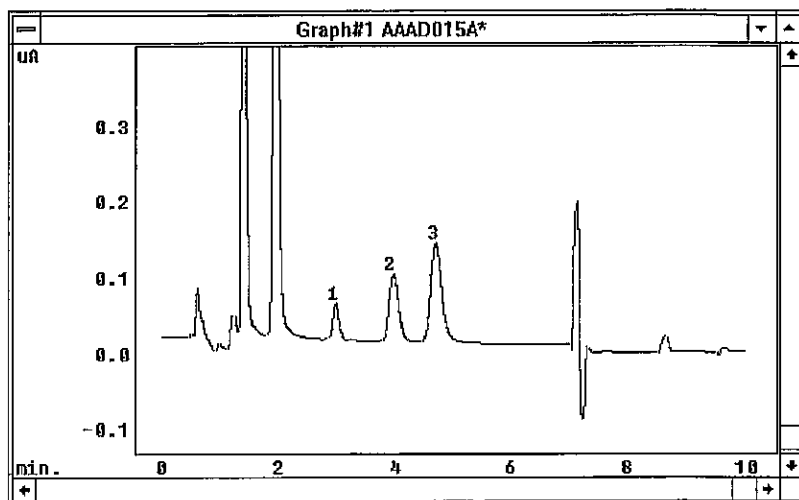
Should the system not be used in a continuous mode, place the waste line into the MP-4 mobile phase reservoir (BAS-480 system) or into the recycle connector (BAS-200B) and recirculate the mobile phase until you are ready to process new samples. Continue to pulse the electrode by running the ChromGraph Control method. These steps will ensure that the system is ready to accept samples at any time. The system may be left in this idle mode for an indefinite period. To remove the system from operation entirely, consult the operating manual for shutdown procedures.

Section 7. Results and Discussion

Typical Run

Figure 7.1 shows a separation of a human plasma sample. This is the typical quality of chromatograms obtained with the BAS plasma homocysteine kit.

Figure 7.1. Separation of a typical plasma sample. Peak 1 = homocysteine. Peak 2 = cys-gly. Peak 3 = Internal Standard. The cleaning pulses are apparent at 7 minutes.



Sample Stability

Plasma sample and plasma calibrator extracts are stable at 4 °C for up to 24 hours. We strongly recommend a refrigerated autosampler to ensure this level of stability.

Aqueous calibrators degrade faster than plasma samples. You can achieve accurate results by this method only if you use a 4 °C autosampler and process your samples and calibrators in small batches every 8 hours.

Accuracy

The following results were obtained when pooled plasma was spiked with known amounts of homocystine, reduced with the kit reagents, and quantified against external standards on two LCEC systems. Results are in $\mu\text{mol/L}$. Expected value is the sum of the average unspiked plasma value, plus the spike.

<i>Sample Type</i>	<i>Expected Value</i>	<i>Instrument 1 (BAS LC-4C)</i>	<i>Instrument 2 (BAS-200B)</i>
Plasma	-	15.1	15.4
Plasma	-	15.4	15.5
Plasma	-	14.8	15.1
Plasma + 5	20.2	20.3	20.3
Plasma + 5	20.2	20.4	20.4
Plasma + 5	20.2	20.0	19.9
Plasma + 15	30.2	30.6	30.3
Plasma + 15	30.2	30.2	30.4
Plasma + 15	30.2	29.9	29.6
Plasma + 25	40.2	40.6	38.9
Plasma + 25	40.2	40.0	39.6
Plasma + 25	40.2	36.6	39.7

Linearity

Both external and endogenous calibrators show excellent linearity, with coefficients of determination (r^2) typically in the 0.999 range. A regression line for external calibrators is shown in Figure 7.2., and one for endogenous calibration is shown in Figure 7.3.

Figure 7.2. Linear regression of external calibrators, from 10-80 $\mu\text{mol/L}$.

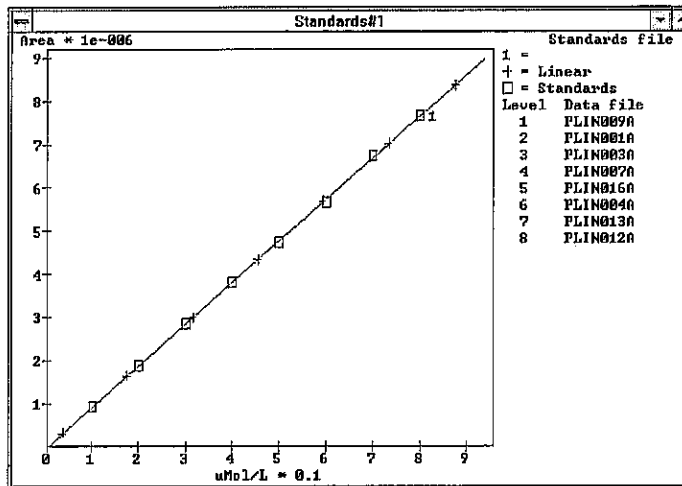
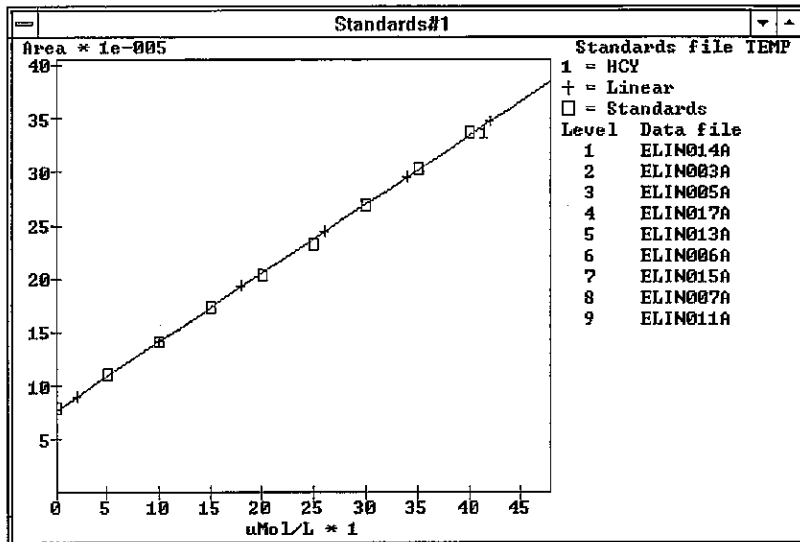


Figure 7.3. Linear regression of endogenous calibrators, from 0-40 $\mu\text{mol/L}$ added to pooled plasma.



Precision

Homocysteine content of eight replicates of pooled plasma was determined over several days by the sample addition (endogenous) method, using the internal standard. Calibrators were made fresh daily. Results are in $\mu\text{mol/L}$.

	<i>DAY 1</i>	<i>DAY 2</i>	<i>DAY 3</i>
	8.7	9.0	8.7
	8.8	8.8	8.8
	8.8	9.0	8.8
	8.7	8.9	8.7
	8.8	8.8	8.8
	8.8	8.9	8.8
	8.7	8.8	8.7
	8.8	8.8	8.7
<i>Means</i>	8.8	8.9	8.7

Within-day RSD = 0.6 %

Among-day RSD = 2.3 %

Limit of Detection

Cannot be determined. This is an endogenous compound, and homocysteine-free plasma is not available for spiking at low levels. Normal values of homocysteine in human plasma are thousands of times higher than typical limits of detection for LCEC.

Interfering Compounds

No interfering compounds have been encountered. The following related compounds may elute during a run, but do not interfere (homocysteine elutes at about 4 minutes):

<i>Compound</i>	<i>Approximate Retention Time (min)</i>
n-acetyl cysteine	0.6
glutathione	1.0
cysteine	1.5
captopril	1.6
cys-gly	5-6
penicillamine	6-7*
cysteamine	22.5
thioguanosine	23.5

** may coelute with Internal Standard*

Expected Values

There is a fine line between normal homocysteine values and those that might signal increased cardiovascular risk. Interpretation is thus best left to the physician. Published literature does contain some guidelines, however.

Selhub [8] reported homocysteine values from 1401 men and women. He found a mean concentration of 11.9 $\mu\text{mol/L}$, with a range of 3.5 to 66.9. Values above 14.0 $\mu\text{mol/L}$ were considered high.

Wu et al. [3] measured homocysteine levels in 434 people. Subjects with cardiovascular disease had a mean homocysteine concentration of 13.4 $\mu\text{mol/L}$, while control subjects had a mean of 10.1. Homocysteine levels ranged from 6-19 $\mu\text{mol/L}$.

Lussier-Cacan et al. [9] compared homocysteine levels in 584 healthy subjects. Males averaged 9.7 $\mu\text{mol/L}$, while females averaged 7.6.

Section 8. Troubleshooting

No Peak for Internal Standard

1. Was the internal standard diluted properly?
2. Were the assay ingredients added in the proper amounts, and in the proper order?
3. Was the reduction allowed to proceed for a full 10 minutes?
4. Is Reagent B out of date, or was it stored improperly?
5. Is the peak eluting at the same time as the cleaning pulse?

Non-Linear Calibration Curve

1. Were the calibrator solutions diluted properly?
2. Were the assay ingredients added in the proper amounts, and in the proper order?
3. Was the reduction allowed to proceed for a full 10 minutes?
4. Is Reagent B out of date, or was it stored improperly?
5. Is the Internal Standard peak area the same for all the calibrators?
6. Have the peak areas been measured correctly and entered into the regression correctly?

Poor Reproducibility, Within Day

1. Are you using good pipetting technique and validated pipettes?
2. Is the calibration curve linear?
3. Are you carefully pipetting the reagents down into the tube?

Poor Reproducibility, Between Day

1. Are you using the same calibrators each day and storing them at 4° C?
2. Are you using good pipetting technique and calibrated pipettes?
3. Are you repeatedly freezing and thawing the same plasma sample?
Homocysteine levels may increase after each freeze/thaw cycle.
4. Is Reagent B out of date, or was it stored improperly?

**High Pressure
(> 4000 psi)**

1. Is the flow rate correct (0.4 mL/min)?
2. Is an in-line filter clogged?
3. Is there a clog in the tubing between the injector and the column?
4. Is the column itself clogged? This can happen if unfiltered samples are injected. Try reversing the column. If the pressure decreases you have washed off particles that were clogging the column's frit. You can continue to operate the column in reverse, but should begin filtering the samples before injection, or install an in-line filter before the column.

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