

Homocysteine Microplate STE Assay Instruction Manual 1

CALDON BIOTECH, INC.

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Microplate STE-Based Assay of Homocysteine

Instruction Manual

Catalog Number

96 Tests Per Kit

INTENDED USE

The Homocysteine Microplate STE Assay 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.

INTRODUCTION

Homocysteine (Hcy) is a thiol containing amino acid produced by the intracellular demethylation of the essential amino acid methionine. Intracellular Hcy either enters the transsulfuration pathway or the remethylation cycle as shown in Fig. 1. Approximately 50% Hcy enters the transsulfuration pathway, where it is irreversibly combined with serine by the B6-dependent enzyme cystathionine beta-synthase to form cystathionine. This is then metabolized to cysteine and

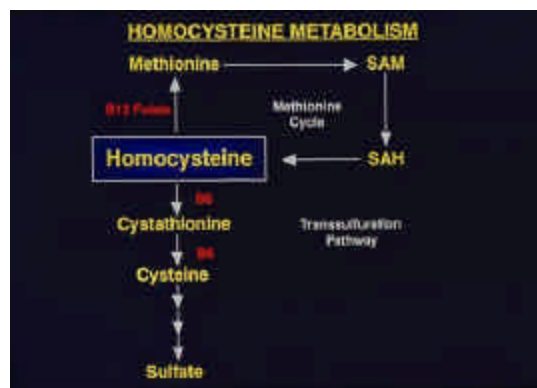


Figure 1.

ultimately to sulfate which is excreted in the urine. In the remethylation pathway, Hcy is recycled to methionine by two different reactions catalyzed by betaine-Hcy methyltransferase and methionine synthase, which requires 5-methyltetrahydrofolate as methyl donor and vitamin B12 as a cofactor. When the enzymatic reactions involved in the two metabolic pathways of intracellular Hcy are impaired either due to genetic defects of enzymes for Hcy metabolism or the nutritional deficiency of vitamins such as folate, B12 and B6, Hcy accumulates in the cells and is exported to the circulation (1-4). Approximately 80% of circulating Hcy in the blood is protein bound by disulfide linkage (5). The remaining unbound Hcy combines by oxidation either with itself to form the dimer homocystine or with cysteine to form the mixed disulfide cysteine-Hcy. Only a small amount circulates as free Hcy. Total Hcy (tHcy) represents the sum of all forms of Hcy including forms of oxidized, protein bound and free. Severely elevated concentrations of tHcy are found in subjects with **homocystinuria**, a rare genetic disorder most commonly caused by a deficiency of cystathionine beta-synthase. Typical clinical manifestations of patients with homocystinuria include mental retardation, early arteriosclerosis and arterial and venous thromboembolism (4,6). Other less severe genetic defects including polymorphisms in genes encoding for enzymes that participate in the Hcy metabolism such as methylenetetra-hydrofolate reductase (MTHFR) and methionine synthase are also found in patients who have moderately elevated plasma levels of Hcy (7-8). Several pathogenic mechanisms have been suggested to explain how increased Hcy concentrations may cause atherosclerosis and vascular diseases (9). These include

- (1) direct toxicity to endothelial cells in the vessel wall with a subsequent increase in platelet adhesiveness;
- (2) promotion of vascular smooth muscle cell growth and an inhibitory effect on endothelial cell growth;
- (3) modifying of bloodclotting factors with a consequent increase in tendency to thrombosis; and
- (4) adverse effects on lipid metabolism.

Epidemiological studies have demonstrated strong correlation 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 tHcy was associated with an odds ratio for coronary artery disease (CAD) of 1.6 (95% confidence interval (CI), 1.4 to 1.7) for men and 1.8 (95% CI, 1.3 to 1.9) for women. The odds ratio for cerebrovascular disease was 1.5 (95% CI, 1.3 to 1.9) or the same increase in risk as for 0.5 mmol/L increase in cholesterol (10). Peripheral arterial disease also showed a strong association. Patients with **chronic renal disease** have a high morbidity and mortality due to arteriosclerotic CVD. Elevated concentration of tHcy is a frequently observed finding in the blood of these patients. Initial investigation suggested that the markedly elevated plasma tHcy found in end-stage renal disease patients contributes independently to their excess incidence of fatal and non-fatal CVD outcomes (11). Although they may lack some of the vitamins involved in the metabolism of Hcy, the increased levels of tHcy are mainly due to impaired removal of Hcy from the blood by the kidney (12). Factors that affect Hcy concentrations also include drug

interactions besides the above mentioned genetic defects, nutritional (vitamin) deficiency, and renal disease. Drugs that interfere with Hcy metabolism such as nitric oxide (anesthesia), methotrexate (folate antagonist for treatments of malignancies, psoriasis, RA and some autoimmune diseases), phenytoin (anticonvulsant) and biguanides (oral antidiabetic drugs) may increase tHcy concentrations (13).

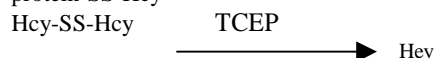
ASSAY PRINCIPLE

Homocysteine Microplate STE Assay is a Substrate-Trapping- Enzyme (STE) based EIA-like assay for the determination of tHcy in blood. STE Technology™ employs genetically engineered enzymes that lack catalytic activity but specifically and tightly trap their substrate or product molecules as the basis for diagnostic tests (14). Plasma samples are pretreated in vials with a reducing agent, TCEP, to reduce the protein bound Hcy to free Hcy that is subsequently converted to S-adenosyl-L-homocysteine (SAH) by SAH hydrolase and quantitated by the STE based assay.

Reduction

Hcy, mixed disulfide and protein bound forms of Hcy in the plasma sample are reduced to free Hcy by use of tris(2-carboxyethyl) phosphine hydrochloride (TCEP).

protein-SS-Hcy



*R1-SS-Hcy

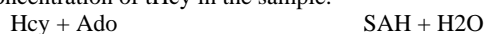
*R1 is any thiol-residue.

Enzymatic conversion

The reduced Hcy in the sample is enzymatically converted to S-adenosyl-L-homocysteine by SAH hydrolase and excess adenosine (Ado) (15).

STE assay

After the above pre-treatment, samples are transferred into wells of a microtiter plate for STE assay. The solid-phase STE assay is based on the competition between SAH in the sample and SAH conjugated on horseradish peroxidase (HRP-SAH) for binding sites on a SAH specific trapping enzyme, 123 which is biotinylated, and binds to the wells of avidin coated microtiter plate. After removal of the unbound HRP-SAH, The bound HRP activity is measured by the absorbance at 450 nm in the presence of the enzyme substrates (TMB/H₂O₂) and is inversely correlated to the concentration of tHcy in the sample.



Homocysteine Microplate STE Assay				
Reagents	Color code	Contents	Format	Volumes
Reagent A Assay buffer	white	50 mM Phosphate buffer	Ready to use	20 mL
Reagent B Adenosine/TCEP	yellow	Adenosine, Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), Tris buffer	Ready to use	1.2 mL
Reagent C SAH-hydrolase	white	Recombinant S-adenosyl-L-homo-cysteine hydrolase, phosphate buffer and glycerol	Ready to use	1.2 mL
Reagent D Enzyme inhibitor	green	Adenosine analog, phosphate buffer	Ready to use	12 mL
Reagent E Adenosine deaminase	red	Adenosine deaminase, phosphate buffer, and glycerol, 0.05% NaN ₃	Ready to use	6 mL
Reagent F DEAE-Sephadex	blue	DEAE-Sephadex in phosphate buffer, 0.05% NaN ₃	Ready to use	12 mL
Reagent G SAH capturing enzyme	blue	123-biotin conjugate, Tris buffer, glycerol	Concentrated Dilute 51x before use	0.1 mL
Reagent H HRP conjugate	brown	HRP-SAH, glycerol, Phosphate buffer, geneticin	Ready to use	3.5 mL
Reagent I HRP substrate	brown	TMB+ (Tetramethylbenzidine)	Ready to use	15 mL
Reagent J Stop solution	white	1 M phosphoric acid	Ready to use	15 mL
Wash buffer	white	Phosphate buffer, Tween 20	Concentrated, Dilute 5x before use	50 mL
Calibrators	pink	S-adenosyl-L-homocysteine of 2.5 to 60 μmol/L in human plasma, 0.05% NaN ₃	Ready to use	6x 100 μL
Microtiter strips		Coated with avidin	Ready to use	12x8 wells

MATERIALS NEEDED BUT NOT PROVIDED IN THE ASSAY KIT

- .1.5 mL Microcentrifuge tubes (e.g. Eppendorf tubes) for pre-treatment of samples.
- .Pipettes/multipipettes of 20 μ L, 200 μ L, and 1000 μ L.
- .Pipetting reservoirs (55 mL).
- .Incubator or water bath of 37C.
- .Microtiter plate reader (450nm).
- .Hcy control (see Quality Control section for more information).

PRECAUTIONS

- The Homocysteine Microplate STE Assay is for *in vitro* diagnostic use (research use only in the U.S.).
- The Homocysteine Microplate STE Assay only measures L-homocysteine and not D-homocysteine.
- Controls and calibrators contain diluted plasma originating from human blood samples. The source materials have been tested and found to be negative for HIV-1, HIV-2, HTLV-I, and Hepatitis B and C. However, the handling of blood derivatives should be done according to recommended procedures for handling infectious material. HAS publication no.(CDC) 84-8395 or local/national guidelines on laboratory safety procedures should be referred.
- Some of the reagents contain <0.1% sodium azide, NaN₃, as preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metal azide. On disposal, flush with a large volume of water to prevent azide build up.
- Reagents with different lot numbers must not be interchanged.
- Equilibrate all reagents and plate to room temperature before use is required for obtaining consistent results.
- Do not use the kit after the expiration date on the outer box.

PREPARATION AND STORAGE OF ASSAY COMPONENTS

- All reagents should be refrigerated (2-8C). Reagents are stable until expiration date when stored and handled as directed. An opened reagent kit is stable for 12 weeks when stored at 2-8C and handled as directed in this package insert.
- The sample pre-treatment solution (*ABC Mixture*) has to be freshly made just before use by mixing **Reagent A**, **B**, and **C**.
- The **wash buffer** solution should be 5-fold diluted (to 50 mL of the concentrated wash buffer is added 200 mL water) with distilled water before use. The prepared **wash buffer** is stable for 4 weeks when stored at room temperature (18- 25C).
- The **Reagent G** is 51-fold concentrated. Use the 5-fold diluted wash buffer as diluent to make **Reagent G** working solution. Only the necessary volume of **Reagent G** working solution should be made each time.
- It is important that the microtiter strips are kept dry, i.e.in the sealed bag with drying capsules, and refrigerated. Leave the microtiter strips in the sealed bag at room temperature (18-25C) for minimum of 1 hour before use.
- Only the necessary number of microtiter strips should be kept in the frame during the run. Unused strips should be kept in the sealed bag with drying capsules. .Avoid exposure of the kit to temperatures exceeding 37C as this may denature the enzymes.

SPECIMEN COLLECTION AND PREPARATION

- Synthesis of Hcy will take place in red blood cells after sampling, and release Hcy into the plasma, resulting in an artificial increase of Hcy in plasma at a rate of about 10% per hour at room temperature (16- 17). Therefore, it is very important to centrifuge blood samples immediately after collection to separate the plasma from the blood cells. If immediate centrifugation of EDTA stabilized blood is not possible, the artificial increase can be reduced by keeping the blood on ice and separating the plasma from the cells within 1 hour (17). Addition of sodium fluoride to the blood has been shown to prevent significant increase of Hcy for 2 hours after sampling (18). Addition of 3-deazaadenosine to inhibit SAH synthesis in red cells has been suggested (19), however, this method **can not be used** for Hcy assay with the Homocysteine Microplate STE Assay since 3-deazaadenosine interferes the enzymatic conversion step of the assay.
- EDTA-plasma from anti-coagulated whole blood is recommended in the scientific literature (20-21). EDTA-plasma can be used with the Homocysteine Microplate STE Assay. Serum samples have been found to have 5-10% higher normal range than that of plasma samples (22).
- .Food consumption can affect circulating Hcy levels. Protein rich meals give higher tHcy values and should be avoided late in the day before sampling (21, 23).
- Standardized sampling procedures are crucial due to the above mentioned influencing factors.
- Repeated freezing and thawing does not affect the plasma Hcy concentration (24). However, complete

- mixing of thawed samples is required before use.
- .After separation of plasma from cells, Hcy is stable for at least 4 days at room temperature (22). It is stable for several weeks at 0-2C, and for several months, possibly for years, when kept frozen at -20C(16, 24).

ASSAY PROCEDURE

Take out the whole kit from 4C storage, and equilibrate all reagents to room temperature before use. Leaving the kit at room temperature for minimum of 1 hour before use is recommended and is important for obtaining good results. We recommend running the calibrators in duplicates and performing a new calibration curve for each run to avoid run-to-run variations using coated microtiter plates.

Reagent Preparation

a. Reagent ABC Mixture:

Prepare **Reagent ABC Mixture** fresh prior to the start of the assay. Volumes needed per 20 samples (dead volume not included) are:

2.6 mL **Reagent A** (130 μ L per sample)

0.2 mL **Reagent B** (10 μ L per sample)

0.2 mL **Reagent C** (10 μ L per sample)

Mix well using vortex or mixer.

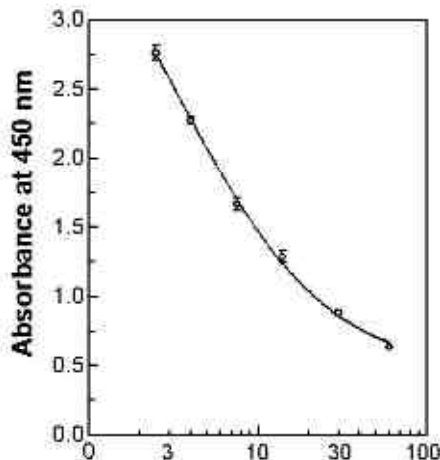
Assay

1. To microcentrifuge tubes (*e.g.* 1.5-ml Eppendorf tube) add 150 μ L of **Reagent ABC Mixture** and 10 μ L of plasma sample, or calibrator or control. Cap tubes and Vortex well. Incubate 30 min at 37C.
2. Pipette 100 μ L of **Reagent D** into each tube. Vortex well. Incubate 10 min at room temperature (18-25C).
3. Pipette 50 μ L of **Reagent E** into each tube. Vortex well. Incubate 15 min at room temperature (18-25C).
4. Pipette 100 μ L of **Reagent F** into each tube. Incubate 10 min at room temperature (18-25C).
Note: Vigorously shake Reagent F bottle to mix the bead solution before pipetting each time.
5. Pipette 25 μ L of the above pre-treated sample or calibrator or control solutions (supernatant) from step 4 into the wells of the provided microtiter plate strips. *Note: Do not disturb the bead pellet (red color) at the bottom of tubes while pipetting.*
6. Preparation of **Reagent G**: The provided **Reagent G** is 51x concentrated solution. Before use, dilute the concentrated **Reagent G** with the wash buffer (1x). Each test will need 25 μ L of the diluted **Reagent G**. For example, for 20 samples, pipette 10 μ L of the concentrated **Reagent G** into 0.5 mL of the wash buffer (1x) in a test tube or in a micro centrifuge tube, mix well to make 0.51 mL of **Reagent G** working solution (dead volume is not included). The **Reagent G** working solution should be made just before use.
7. Pipette 25 μ L of **Reagent G** working solution into each well containing sample, or calibrator or control. Incubate for 5 min at room temperature, and then add 25 μ L of **Reagent H** Incubate 30 min at room temperature (18-25C). Use the enclosed film to cover the wells and shield the microtiter plate from lights during all the incubations.
8. Decant the plate and blot on paper towels. Wash the wells 3 times with 300 μ L/well of diluted **Wash Buffer** (1x). Blot the plate on paper towels after each wash.
9. Pipette 100 μ L of **Reagent I** (room temperature) into each well. Incubate 10 min at room temperature (18-25C) (shield from lights).
10. Pipette 100 μ L of **Reagent J (Stop Solution)** into each well.
11. Shake and read at 450 nm within 15 min. Automatic plate shaker is preferred to ensure proper mixing.

CALIBRATION CURVE

Six calibrators of SAH concentrations ranging from 2.5 to 60 μ mol/L are provided for construction of a calibration curve for each run of the assay. We recommend that a four parameter logistic curve fit be used for preparing the calibration curve and calculation of unknown samples. Below is an example of a calibration curve using the four-parameter logistic curve fit.

**Homocysteine Microplate STE Assay
Calibration Curve (2.5-60 μ M)**



- Example only. Not to be used to determine values.

REFERENCE RANGE

The reference range should be determined by each laboratory to confirm with the characteristics of the population being tested. The tHcy concentration in plasma of healthy individuals can vary with age, gender, geographical area, and genetic factors.

The “normal” range for fasting tHcy concentrations is approximately 5 to 15 $\mu\text{mol/L}$. The upper limit of this range, however, should be revised downward because the increased risk of atherosclerosis associated with tHcy levels in this range has been well documented (24-27). A plasma tHcy concentration of 12 $\mu\text{mol/L}$ was used as the upper limit of the “normal” range in the European Concerted Action Project (28).

Scientific literature reports that men have higher tHcy levels than women, and post menopausal women have higher tHcy values than pre-menopausal women (29). The plasma tHcy values will normally increase with age, giving a reference range among elderly (>60 years) of 5-20 $\mu\text{mol/L}$ (30-31).

The U.S. National Health and Nutrition Examination Survey (NHANES) reported homocysteine levels in a representative sample of the U.S. population as follows (32):

	12-19 years age	=60 years age	Cut-off for “high” levels
Male	4.3-9.9 μM	5.9-15.3 μM	= 11.4 μM
female	3.3-7.2 μM	4.7-11.6 μM	= 10.4 μM

Certain with abnormal Hcy metabolism may have normal fasting plasma tHcy levels and may require provocative testing (i.e. methionine loading) to expose this abnormality (33).

The tHcy test results should be interpreted considering all other test results and the clinical status of the patients.

PERFORMANCE DATA

Assay Precision

Precision was tested by running 3 levels of controls for 8 days, 4 replicates per run of each level.

Precision data are summarized in the table below.

	Average Hcy $\mu\text{mol/L}$	Intra assay precision	Total precision
Low	6.8	9.1%	11.6%
Med	11.3	8.8%	9.8%
High	22.0	4.5%	7.6%

Analytical Sensitivity and Dynamic Range

The quantification limit of the Homocysteine Microplate STE Assay was tested to be 1 $\mu\text{mol/L}$ with a CV value < 20%.

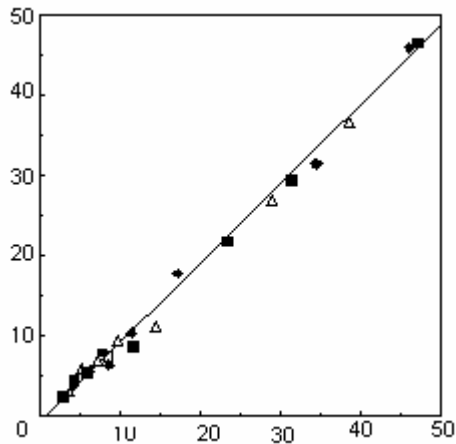
The dynamic range of the assay is 1-60 $\mu\text{mol/L}$.

Hcy ($\mu\text{mol/L}$)	Average result ($\mu\text{mol/L}$)	SD ($\mu\text{mol/L}$)	CV%
4	3.83	0.22	5.71
3	3.25	0.44	13.62
2	1.94	0.09	4.55
1	0.86	0.14	16.01
0.5	0.60	0.22	37.36

Linearity of Diluted Plasma Samples

If the Hcy concentration of a sample exceeds the upper limit of the calibration curve (60 $\mu\text{mol/L}$), the sample should be diluted with **Reagent A** (assay buffer), and re-analyzed. The linearity of the Homocysteine Microplate STE Assay was evaluated by diluting three high patient samples with varying amounts of **Reagent A** as a diluent.

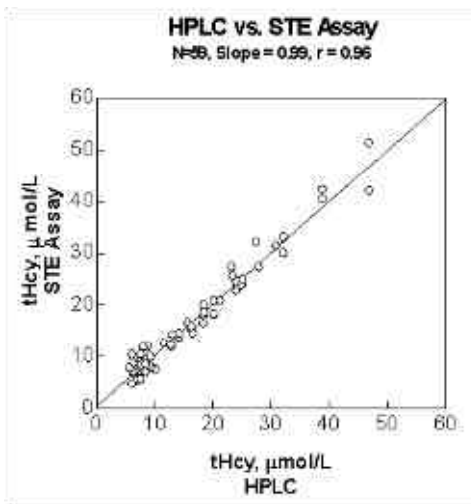
The results below show that the average recovery in % for each dilution step is within 90-110% of the calculated theoretical value.

Dilution recovery of 3 samples

Theoretical tHcy \propto mol/L
 Slope = 0.99
 Intercept = -0.78 mol/L
 Correlation coefficient (r^2) = 0.99

Method Comparison

The Homocysteine Microplate STE Assay was compared to in-house HPLC method described by Ubbink J. B. et al. (34). A comparison of 59 patient samples ranging from 4-52 μ mol/L tHcy is shown in the figure below:

**Interfering Substances**

Bilirubin, hemoglobin, lipids, red blood cells protein and sodium fluoride were spiked into plasma samples and tested for interference by Homocysteine Microplate STE Assay.

The following interferences were found.

Interfering substances	Interfering substance concentration	% Interference
Bilirubin	0.4 mg/mL	0.2
Hemoglobin	4 mg/mL	-0.2
Triglycerides	4 mg/mL	-0.1
Protein	5 mg/mL	-0.1
Na-Fluoride	10 mg/mL	0.0

Since there is no antibody used and no immunochemical reactions involved in this STE-based tHcy assay, there is no HAMA effect for this assay. Specimens from patients carrying human anti-mouse antibody (HAMA) can also be tested for tHcy with the Homocysteine Microplate STE Assay.

Cross Reactivity

The cross reactivity was examined by spiking solutions of Adenosine, S-adenosyl-L-methionine (SAM), cystathionine, methionine, L-cysteine, glutathionine, and thiolactone in concentrations more than 10-fold higher than physiological concentrations. The following results were found when tested with Homocysteine Microplate STE Assay.

MEASURING RANGE

The calibrator range is from 2.5 to 60 μ mol/L.

LIMITATIONS

.Specimens from patients who are on drug therapy involving S-adenosyl-methionine may show falsely

Compound	Concentration	% Cross reactivity
Adenosine	0.1 mmol/L	- 0.9
SAM	0.5 mmol/L	10.0
Cystathionine	0.5 mmol/L	0.4
L-cysteine	40 mmol/L	0.0
Gluthathione	4 mmol/L	0.0
Thiolactone	40 mmol/L	0.0

elevated levels of tHcy.

- .Clinical limitations related to metabolic interference from patients who are taking methotrexate, carbamazepine, pheytain, nitrous oxide, anticonvulsants or 6-azauridine triacetate, may have higher levels of tHcy due to metabolic interference with Hcy metabolism.

QUALITY CONTROL

We recommend each laboratory to use a Hcy control with known concentration.

The controls contain L-Hcy in diluted human plasma (non-reactive for HIV-1/2 antibody, HCV antibody, Hepatitis B core antibody, HTLV-1 antibody, HIV-1 antigen and Hepatitis B surface antigen) in the following concentrations:

Control level	Mean Value Hey(μ mol/L)	Range Hey(μ mol/L)
Normal	7.0	5.6-8.4
High	23.0	18.4-27.6

Example only, for actual values see Control Package Insert.

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Product Safety Information:

Calibrators/Low, Medium, High Controls/Reagent
A/Reagent D/Reagent F



R22-32
S36

Harmful
Reagent K



Corrosive

R22 Harmful if swallowed.
R23 Toxic by inhalation.
R24 Toxic in contact with skin.
R25 Toxic if swallowed.
R26 Very toxic by inhalation.
R27 Very toxic in contact with skin.
R28 Very toxic if swallowed.
R29 Contact with water liberates toxic gas.
R30 Can become highly flammable in use.
R31 Contact with acids liberates toxic gas.
R32 Contact with acid liberates very toxic gas.
S36 Wear suitable protective clothing.