

Comment 3: References are needed for the standard methods for identification of hemolysis and the specific equipment and protocols followed.

#### Protocol for identification of hemolysis

Hemolysis events are determined using several criteria and measuring instruments as follows:

##### 1. LDH Measurement

Required equipment: Syringe, 70% alcohol swab, tourniquet, plaster, and 3 cc plain tube.

Procedure:

- a. Draw 3 cc of blood from the median cubital vein.
- b. Place the sample in a plain tube.
- c. Centrifuge at 3000 rpm for 10 minutes.
- d. Use the resulting serum to test LDH with a Cobas C311 chemistry analyzer.

##### 2. Bilirubin Measurement

Required equipment: Syringe, 70% alcohol swab, tourniquet, plaster, and 3 cc plain tube.

Procedure:

- a. Draw 3 cc of blood from the median cubital vein.
- b. Place the sample in a plain tube.
- c. Centrifuge at 3000 rpm for 10 minutes.
- d. Test the resulting serum for indirect bilirubin using a Cobas C311 chemistry analyzer.

##### 3. Haptoglobin Measurement

Required equipment: Syringe, 70% alcohol swab, tourniquet, plaster, and 3 cc EDTA tube.

Procedure using ELISA method:

- a. Prepare reagents, samples, and standards, designating wells in the plate for standard, blank, and sample.
- b. Add 100  $\mu$ L of standard and sample to designated wells, cover with a sealer, and incubate at 37°C for 90 minutes.
- c. After incubation, discard the liquid from each well without washing.

- d. Add 100  $\mu\text{L}$  of Biotinylated Detection Ab solution to each well and incubate at  $37^{\circ}\text{C}$  for 60 minutes.
- e. Discard the liquid and add 350  $\mu\text{L}$  of wash buffer to each well, let it sit for 1 minute, aspirate, and dry. Repeat this washing step three times.
- f. Add 100  $\mu\text{L}$  of HRP conjugate solution to each well, cover with a new sealer, and incubate at  $37^{\circ}\text{C}$  for 30 minutes.
- g. Discard the liquid again, and repeat the wash procedure five times.
- h. Add 90  $\mu\text{L}$  of substrate reagent to each well, cover, and incubate at  $37^{\circ}\text{C}$  for 15 minutes.
- i. Add 50  $\mu\text{L}$  of stop solution to each well.
- j. Read the Optical Density (OD) at 450 nm using a microplate reader.

#### 4. Free Hemoglobin Measurement

Required equipment: Syringe, 70% alcohol swab, tourniquet, plaster, and 3 cc EDTA tube.

Procedure (similar ELISA method as for Haptoglobin):

Follow a similar ELISA procedure as outlined for haptoglobin, including incubations, washing steps, and Optical Density (OD) reading at 450 nm.

#### 5. Hemoglobinuria Measurement

Required equipment: Urine collection tube.

Procedure using ELISA method:

Follow similar ELISA steps as outlined for haptoglobin, including incubation, washing, and Optical Density (OD) reading at 450 nm.