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STANDARD OPERATION PROCEDURE FOR
Von Willebrand Factor (vWF) antigen ELISA

PRINCIPLE OF ASSAY
This assay employs a double antibody sandwich ELISA.

REAGENTS

Coating antibody:
96 well microtitre plates are coated with DAKO rabbit anti-human von Willebrand factor (code No. A 0082) diluted to a protein concentration of 10 mg/ml (1/500). Plasma sample or standard containing von Willebrand factor is added to a microtest well. After an incubation period allowing 95% of vWF to bind to the coating antibody the wells are emptied and washed to remove unbound material.

Detection antibody:
DAKO peroxidase-conjugated rabbit anti-human von Willebrand Factor (code No. A 0226) diluted 1:4000 is added to each well and incubated to allow reaction with the bound vWF. The wells are emptied and washed to remove unbound conjugate. 1,2 phenylenediamine dihydrochloride substrate (OPD) and hydrogen peroxide (as activator) are then added, and the released yellow product is measured photometrically on a microplate reader. The colour development is proportional to the concentration of vWF in the sample. After stopping the reaction with H_2SO_4, the concentration is read from the standard curve on the same plate.

Other Reagents and materials:
96 well microtitre plates:Ultra-high binding polystyrene 4HBX from Immulon.
Standard: 9th British Standard for blood coagulation factors from NIBSC.
Stabiliser: Immunoassay stabiliser from Advanced Biotechnologies Inc.

Controls:

An in-house control of pooled plasma is used as a control on each plate
The vWF mean concentration of this plasma is assigned after at least 20 separate runs. A Levey-Jennings curve is generated, and QC results are plotted on this for each run. Westgard rules are used to monitor internal quality control.

Coating Buffer(Buffer A)
NaCl: 42.37g
NaH_2PO_4: 1.96g
Na_2HPO_4: 5.32g
Make up to 5L in water and adjust to pH 7.2 with concentrated NaOH.
Remove 150ml of solution for coating buffer

Washing Buffer (Buffer B)
Add to the remaining 4.85L of Buffer A
NaCl 100.62g
Tween 20 4.85 ml

REAGENT PREPARATION

Coating the 96 well plate

For 5 plates: 110µl coat antibody (AO82) in 55ml of Buffer A.
(Antibody coat concentration = 1/500)
Using 12 channel pipette, add 100µl of diluted coating antibody to each well.
Cover the plate with parafilm and aluminium foil and leave overnight at +4°C.

Stabilising
Empty the wells and wash once with Buffer B as described in the wash procedure.
Add 100µl of stabiliser to each well and leave on the bench, uncovered for ½ hour.
Empty the wells, but do not wash. Leave the plates to dry on the bench for 1 hour.
Cover the plate with parafilm and include a desiccant pouch. Wrap in aluminium foil and store at 4°C. Under these storage conditions, plates are stable for up to 1 month.

Conjugate (HRP)
Add 3 µl HRP to 12 mls washing buffer and mix carefully by inversion; make up 5 minutes before use.

Substrate
GLOVES MUST BE WORN WHEN HANDLING OPD (carcinogenic) HYDROGEN PEROXIDE AND ACID (corrosive).
Add 2 tablets OPD to 12 mls distilled H_2O and mix carefully by inversion; make up approx. 10 minutes before use.
Add 5µl H_2O_2 (30%) and mix immediately before adding substrate to plate.

Acid (Stop) Solution
Slowly add 50ml of concentrated sulphuric acid to 540ml water in fume cupboard.
This solution will be quite hot. Cover, label and allow to cool.

Dilutions for Assay

Standard Curve (9th British Standard Assigned value 73 IU/dl)

Final conc.	Dilution
220 IU/dl	60 µl pool + 740 µl washing buffer
146 IU/dl	40 µl pool + 760 µl washing buffer
110 IU/dl	30 µl pool + 770 µl washing buffer
73 IU/dl	20 µl pool + 780 µl washing buffer
37 IU/dl	10 µl pool + 790 µl washing buffer

Samples(citrated plasma)
20 µl sample + 780 µl washing buffer (including normal pool as an internal QC).
Ensure all standard, control and sample dilutions are well mixed using a whirlimixer.
Make up standards immediately prior to addition to plate.

Wash Procedure
The plate should be washed between incubations as follows:
Fill each well with washing buffer.
Soak for 20 seconds.
Invert the plate and decant the buffer into sink.
Remove excess buffer by grasping the plate firmly and smartly rapping the inverted.
plate onto a clean paper towel.
This procedure should be performed 3 times.
Alternatively the MRW plate washer may be use using a suitable programme.

Assay procedure

Make up worksheet using a 96 well template format and enter standard and sample details.
Add 100µl of diluted standard, sample and control to the appropriate wells.
Incubate at RT for 30mins on plate shaker at a setting of 5.

Wash X3

Add 100 µl conjugate (HRP) prepared as above.
Incubate at RT for 30mins on plate shaker as before.

Wash X3

Add 100 µl substrate prepared as above. Substrate should be added to each row at 5 or 10 second intervals.

Incubate at RT for 3mins on bench.

Add 100 µl 1M H_2SO_4 (stop). The acid should be added to the rows at the same intervals (5 or 10 seconds) as the addition of the substrate to ensure that each well has the same incubation time.

Read immediately on plate reader MR5000 using the relevant programme (492nm). This will plot a log/log curve and read the sample ODs from it. Plates should be read as soon as possible because colour development may continue after the addition of the acid.

Write results onto the work sheet and then key into the relevant data-base.
Samples with values greater then 300 IU/dl or less than 50 IU/dl should be repeated.
File worksheets in correct study folder. The control result is plotted onto the Levey-Jennings chart. Westgard quality assurance rules must be observed.
Discard the contents of the plate down the sink and flush through with plenty of cold running water.