Online supplement

Methods for individual circulating markers

Whole blood (10cc) was drawn on arrival at the emergency department using the tube containing ethylenediaminetetraacetic acid (EDTA) or citrate. The samples were immediately delivered to the laboratory. Plasma and serum were quickly prepared from whole blood by centrifugation at 3000 g for 15 min at room temperature. The plasma and serum were carefully transferred into appropriately labeled micro-centrifuge tubes by using a sterile transfer pipette tip. The samples were apportioned into 1 ml aliquots from 5 to 7 vials and stored at –80 ˚C for later analysis. Detailed methods for individual blood marker are described below:

1) Interleukin-6 (IL-6, pg/ml)

Whole blood was drawn using the EDTA tube, and plasma concentration of IL-6 was measured using a quantitative sandwich ELISA kit (IL-6 Quantikine, R&D Systems, MN). In brief, 100μl of assay diluent was added and 100μl of standards, control and samples was incubated in microplate wells pre-coated with monoclonal anti-human IL-6 antibody for 2hours at room temperature. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6, IL-6 conjugate, was added to the each wells and the wells were incubated 2hours at room temperature. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-6 bound in the initial step. The color development is stopped and the intensity of the color is measured at 450nm. The concentration of IL-6 in the samples was then determined by comparing the O.D. of the samples to the standard curve. Intra- and inter-assay coefficients of variance (CV) were 7.4 % and 8.9 %, respectively. The minimum detectable dose (MDD) of the

kit is typically 0.70 pg/ml (manufacturer's information).

2) Matrix-metalloproteinase-9 (MMP-9, ng/ml)

Whole blood was drawn using the citrate tube, and plasma concentration of MMP-9 was measured using a commercially available ELISA kit (e-bioscience, Abingdon, UK). In brief, 100μl of diluted standards and samples was added and 50μl of biotin-conjugate was added in anti human MMP-9 monoclonal antibody pre-coated microplate. After incubation for 2hours shaking 100rpm on orbital microplate shaker at room temperature, the wells were washed 4 times. 100μl of diluted streptavidin-HRP was added in the wells and the wells were incubated for 1hour shaking 100rpm on orbital microplate shaker at room temperature. Following a wash to removes any unbound antibody-enzyme reagent, 100μl of TMB substrate solution was added in the wells for 10minutes. The remaining conjugated was allowed to react with the substrate solution (TMB). The reaction was stopped by addition of 100μl stop solution and absorbance of the resulting yellow product is measured at 450nm. The concentration of MMP-9 in the samples was then determined by comparing the O.D. of the samples to the standard curve. The measurement range of human MMP-9 starts at 0.23ng with an upper limit of quantification of 15ng/ml. After pre-dilution 1:10 with the assay buffer (1x), plasma samples were diluted 1: 20 for MMP-9. The measured concentration of sample from standard curve was calculated by dilution factor, because samples had been diluted prior to the assay. Intra-assay and inter-assay CV were 9.2 % and 12.6 %, respectively. The MDD of the kit is typically 0.05 ng/ml (manufacturer's information).

3) Tumor necrosis factor-alpha (TNF-α, pg/ml)

Whole blood was drawn using the EDTA tube, and plasma concentration of TNF-α was measured using a quantitative sandwich ELISA kit (R&D Systems, Minneapolis,

USA). In brief, after adding 50μl of buffered protein base with preservative, 200μl of standards and samples were incubated in TNF-α monoclonal antibody pre-coated microplate. After 2hours, the wells were washed 4-times with wash solution. Horseradish peroxidase conjugated polyclonal anti-human TNF-α antibody was added in the wells and the wells were incubated for 2hours at room temperature. Following a wash to removes any unbound antibody-enzyme reagent, 200μl of substrate solution was added to the wells and color development was stopped by stop solution. The intensity of the color was measured at 450nm within 30minutes. The concentration of TNF-α in the samples was then determined by comparing the O.D. of the samples to the standard curve. Intra-assay and inter-assay CV were 6.7 % and 9.3 %, respectively. The MDD of the kit is typically 1.6 pg/ml (manufacturer's information).

4) Plasminogen activator inhibitor-1 (PAI-1, ng/ml)

Whole blood was drawn using the citrate tube, and plasma concentration of PAI-1 was measured using a commercially available active PAI-1 functional assay kit (Innovative Research, Novi, MI, USA). In brief, adding 100 μl of diluted standards and samples, the urokinase coated microtiter wells were incubated for 30minutes shaking 300rpm on orbital microplate shaker at room temperature. After washing 3 times, 100μl of BSA mixed PAI-1 primary antibody was added in the wells and the wells were incubated for 30minutes, shaking at ca.300rpm on orbital microplate shaker at room temperature. Following a washing, 100μl of BSA mixed PAI-1 conjugated secondary antibody was added the wells were incubated for 30minutes, shaking at ca.300rpm on orbital microplate shaker at room temperature. After washed 3 times, 100μl of tetramethylbenzidine (TMB) substrate was added to each well and the wells were incubated for 10minutes avoiding direct sunlight. The enzyme reaction was stopped by

the addition of 50µl stop solution and absorbance of the resulting yellow product is measured at 450nm with a spectrophotometer. The concentration of PAI-1 in the samples was then determined by comparing the O.D. of the samples to the standard curve. Intra-assay and inter-assay CV were 5.7 % and 7.9 %, respectively. The MDD of the kit is typically 0.181ng/ml (manufacturer’s information).