University of Washington
Department of Laboratory Medicine
Harborview Medical Center
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DRIED BLOOD SPOT HIGH-SENSITIVITY C-REACTIVE PROTEIN (hsCRP) ASSAY

The CRP Assay is a sandwich ELISA used to measure the CRP concentration in dried blood spot (DBS) samples. It is an adaptation of a previously published method (McDade TW, J Burhop and J Dohnal (2004) High sensitivity enzyme immunoassay for C reactive protein in dried blood spots. Clinical Chemistry 50:652-654). A punch from a DBS card containing either a CRP assay calibrator, a quality control (QC) sample or a patient sample is eluted in a buffer solution. The elution solution from each sample is transferred to a well on an ELISA microtiter plate. The bottom face of each well of the plate is pre-coated with a mouse monoclonal antibody (mAb) that recognizes a distinct antigenic determinant on the CRP molecule. CRP in the elution solution is bound by the anti-CRP mAb (solid phase immobilization). A conjugate solution containing goat anti-CRP Ab coupled to peroxidase (enzyme-linked antibody) is then added to each well, resulting in CRP molecules being sandwiched between the solid phase and enzyme-linked antibodies. After incubation, the wells are washed to remove unbound material. A tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) solution is added; H_2O_2, cleaved by the peroxidase, reacts with TMB and causes the solution to develop color**.** The CRP concentration is directly proportional to the absorbance of the solution; absorbance is measured spectrophotometrically. A standard curve is constructed by plotting the absorbance values of the calibrators against their known CRP concentrations. Using the standard curve, the absorbance values of the QC samples and patient samples are read as CRP concentrations. Acceptability of the assay is determined by comparing the CRP concentrations of the QC samples with their established values.

DBS CRP assay calibrators were constructed from pooled human plasma with a negligible CRP concentration (negligible CRP plasma; University of Washington Department of Laboratory Medicine, Seattle, WA; UW Lab Med) spiked with CRP concentrate (Cell Sciences, Canton, MA) and serially diluted with negligible CRP plasma to the desired final CRP concentration. Three DBS QC samples were constructed from a separate pool of human plasma, either undiluted (high CRP concentration QC sample) or diluted with negligible CRP plasma (medium CRP concentration QC sample and low CRP concentration QC sample). Each calibrator and QC sample solution was mixed with a constant volume of washed human erythrocytes (UW Lab Med), pipetted in 75µl aliquots onto No. 903 filter paper (Whatman, Piscataway, NJ) and dried for 4hr at RT (23°c). The final CRP

concentration of each calibrator and QC sample solution was determined from analyses of plasma from the sample solution-erythrocytes mixture on a UniCel DxC 800 Synchron Clinical System (Beckman Coulter, Miami, FL). DBS calibrators, QC samples and study samples were sealed in Ziploc bags with desiccant packs and stored at -70°c until processing. Immediately prior to processing, DBS were warmed to RT and then a single 3.2mm (1/8in) diameter punch was punched from each DBS into a microtiter plate well (Greiner Bio-One, Monroe, North Carolina). Microtiter plates were either immediately assayed or were firmly sealed and stored at -70°c pending assaying.

Immediately prior to assaying, microtiter plates were warmed to RT. 200µl CRP elution buffer (hsCRP Sample Diluent; Percipio, Inc, Manhattan Beach, CA) was added to each microtiter plate well. The plate was sealed and gently shaken for 1hr on a Delfia Plateshake microplate shaker (PerkinElmer, Waltham, MA) to elute CRP. 20µl of eluent was transferred from each well of the elution plate to an ELISA microtiter plate. 100µl of CRP Enzyme Conjugate Reagent (Percipio) was added to each ELISA microtiter plate well, the plate was gently shaken for 30sec, incubated at RT for 45min in the dark and then washed 5X with di/ddH_2O. 100µl of TMB Reagent (Percipio) was added, the plate gently shaken for 30sec and then placed on a microtiter plate reader (Synergy HT, BioTek, Winooski, VT). The absorbance of each well (OD) was read at 370nm excitation after the OD of the high calibrator on the plate reached 1.5 (approximately 20min). A 5-parameter weighted calibration curve, constructed by plotting the assigned concentrations of the calibrators against the recorded absorbance values, was used to convert the blank-subtracted OD value of each sample into a DBS CRP concentration (Gen 5 Software, BioTek). Acceptability of the assay was determined by comparing the CRP concentrations of the QC samples with their established values.

The CRP assay lower limit of detection was 0.035mg/L, within-assay imprecision (CV) was 8.1% and between-assay imprecision was 11.0%. The CRP concentrations of 452 DBS samples analyzed by the DBS assay correlated with the CRP concentrations of paired plasma samples (Pearson R = 0.95) and were linearly related (DBS assay CRP value = 0 + plasma CRP value X 0.843).

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DRIED BLOOD SPOT EBV ASSAY

The EBV Assay is an indirect ELISA used to measure the titer of EBV IgG antibodies in dried blood spot (DBS) samples. It is an adaptation of a previously published method (McDade, T W, Stallings, JF, et al. (2000) Epstein-Barr virus antibodies in whole blood spots: A minimally invasive method for assessing an aspect of cell-mediated immunity. Psychosomatic Medicine 62(4): 560-568). A punch from a DBS card containing a quality control (QC) sample or a patient sample is eluted in a buffer solution. The elution solution from each sample, assay calibrator, and assay control sample is transferred to an ELISA microtiter plate. The bottom face of each well of the plate is pre-coated with Epstein-Barr Viral Capsid p18 peptide that is recognized by IgG antibody to Epstein-Barr Viral Capsid Antigen (EBV VCA IgG). EBV VCA IgG in the transferred solutions complexes with the bound p18 peptide (solid phase immobilization). After incubation, the wells are washed to remove unbound material. A conjugate solution containing an anti-IgG Ab coupled to peroxidase (enzyme-linked antibody) is then added to each well, resulting in EBV VCA IgG being simultaneously bound by the immobilized solid phase and the enzyme-linked antibodies. After incubation, the wells are washed to remove unbound material. A tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) solution is added; H_2O_2, cleaved by the peroxidase, reacts with TMB and causes the solution to develop color. The reaction is stopped after a fixed period of time. The EBV VCA IgG concentration is directly proportional to the absorbance of the solution; absorbance is measured spectrophotometrically. A standard curve is constructed by plotting the absorbance values of the calibrators against their assigned EBV VCA IgG concentrations. Using the standard curve, the EBV VCA IgG concentrations of the QC samples, patient samples and assay control samples are determined from the absorbance values.

DBS EBV assay calibrators and assay control samples were supplied by the ELISA manufacturer (DiaSorin, Stillwater, MN). Three DBS QC samples were constructed from separate pools of human plasma with either low, medium or high concentrations of EBV VCA IgG (University of Washington Department of Laboratory Medicine, Seattle, WA; UW Lab Med). QC sample plasma was mixed with a constant volume of washed human erythrocytes (UW Lab Med), pipetted in 75µl aliquots onto No. 903 filter paper (Whatman, Piscataway, NJ) and dried for 2hr at RT (23°c). The final EBV VCA IgG concentration of each DBS QC sample was determined from

repeated analysis of the sample on an EBV VCA IgG ELISA (Diasorin). DBS QC samples and study samples were sealed in Ziploc bags with desiccant packs and stored at -70°c until processing. Immediately prior to processing, DBS were warmed to RT and then a single 3.2mm (1/8in) diameter punch was punched from each DBS into a microtiter plate well (Greiner Bio-One, Monroe, North Carolina). Microtiter plates were either immediately assayed or were firmly sealed and stored at -70°c pending assaying.

Immediately prior to assaying, microtiter plates were warmed to RT. 200µl Sample Diluent elution buffer (DiaSorin) was added to each microtiter plate well and the plate was sealed and gently shaken for 1hr on a Delfia Plateshake microplate shaker (PerkinElmer, Waltham, MA). The elution solution was then mixed with an additional 200µl Sample Diluent (DiaSorin). 100µl was transferred from each well of the elution plate, and from each calibrator and assay control sample solution, to an ELISA microtiter plate (DiaSorin). All calibrators and controls were assayed in duplicate. The plate was gently shaken for 30sec, incubated at 37° for 60min in the dark and then washed 5X with Wash Buffer (DiaSorin). 100µl of Diluted Enzyme Tracer (DiaSorin) was added to each ELISA microtiter plate well, the plate incubated at 37° for 60min in the dark and then washed 5X with Wash Buffer (DiaSorin). 100µl of Chromagen/Substrate (DiaSorin) was added, the plate gently shaken for 30sec, incubated at RT for 15min in the dark, and then 100µl of Stop Solution (DiaSorin) was added. The plate was placed on a microtiter plate reader (Synergy HT, BioTek, Winooski, VT) and the absorbance of each well (OD) was read at 450nm. A standard curve, constructed by plotting the assigned concentrations of the calibrators against the recorded absorbance values, was used to convert the OD value of each sample into a DBS EBV VCA IgG concentration (Gen 5 Software, BioTek). Acceptability of the assay was determined by comparing the EBV VCA IgG concentrations of the QC samples with their established values.

The EBV VCA IgG assay lower limit of detection was 9 AU/mL, within-assay imprecision (CV%) was 8.1% and between-assay imprecision was 11.0%. The EBV VCA IgG concentrations of 162 DBS samples correlated with the EBV VCA IgG concentrations of paired plasma samples (Pearson R = 0.95) and were linearly related (DBS assay EBV VCA IgG value = 1.145 + plasma EBV VCA IgG value X 0.575).

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DRIED BLOOD SPOT HDL CHOLESTEROL FLUORIMETRIC ASSAY

The dried blood spot (DBS) HDL cholesterol fluorimetric assay involves a series of coupled enzymatic reactions. A punch from a DBS card containing an HDL cholesterol (HDL-C) assay calibrator, quality control (QC) sample or patient sample is eluted with deionized water. The elution solution is mixed with a reagent containing anti-human β-lipoprotein antibody (to bind non-HDL lipoproteins into nonreactive complexes) and a flurogen. A second reagent, containing cholesterol ester hydrolase, cholesterol oxidase and peroxidase, is then added. The cholesterol ester hydrolase catalyzes the conversion of HDL-C into cholesterol, which is in turn oxidized by cholesterol oxidase and produces hydrogen peroxide (H_2O_2) as a byproduct. In the presence of peroxidase, H_2O_2 reacts with the non-fluorescent fluorogen to produce a fluorescent fluorophore. The HDL-C concentration is directly proportional to the fluorescent intensity of the solution; fluorescence is measured spectrophotometrically. A standard curve is constructed by plotting the fluorescence values of the calibrators against the known HDL-C concentrations. Using the standard curve, the fluorescence values of the QC samples and patient samples are read as HDL-C concentrations. Acceptability of the assay is determined by comparing the HDL-C concentrations of the QC samples with their established values.

DBS HDL-C assay calibrators were constructed from high HDL-C concentration pooled human plasma (University of Washington Department of Laboratory Medicine, Seattle, WA; UW Lab Med) serially diluted with 7% bovine serum albumin in phosphate buffered saline (BSA/PBS; Sigma Aldrich, St. Louis, MO) to the desired final HDL-C concentration. Two DBS QC samples were constructed from a separate pool of human plasma, either undiluted (high HDL-C concentration QC sample) or diluted with BSA/PBS (low HDL-C concentration QC sample). Each calibrator and QC sample solution was mixed with a constant volume of washed human erythrocytes (UW Lab Med), pipetted in 75µl aliquots onto No. 903 filter paper (Whatman, Piscataway, NJ) and dried for 4hr at RT (23°c). The final HDL-C concentration of each calibrator and QC sample solution was determined by analysis on a UniCel DxC 800 Synchron Clinical System (Beckman Coulter, Miami, FL). DBS calibrators, QC samples and study samples were sealed in Ziploc bags with desiccant packs and stored at -70°c until processing. Immediately prior to processing, DBS were warmed to RT and then a single 3.2mm (1/8in) diameter punch was punched from each DBS into a microtiter plate well

(Greiner Bio-One, Monroe, North Carolina). Microtiter plates were either immediately assayed or were firmly sealed and stored at -70°c pending assaying.

Immediately prior to assaying, microtiter plates were warmed to RT. 400µl diH_2O HDL-C elution buffer was added to each microtiter plate well. The plate was sealed and gently shaken for 1hr on a microplate shaker (Delfia Plateshake, PerkinElmer, Waltham, MA). 40µl of eluent was transferred from each well of the elution plate to an assay microtiter plate. This was followed by addition of 75µl of HDL-C assay reagent 1 comprised of 99% EZ HDL Cholesterol Reagent 1 (Trinity Biotech, St Louis, MO) and 1% Cayman ADHP (10-acetyl-3,7-dihydroxyphenoxazine; Cayman Chemical, Ann Arbor, MI). The plate was gently shaken for 30sec and then incubated at 37°c for 25min. At the conclusion of this incubation, 25µl of HDL-C assay reagent 2 (EZ HDL Cholesterol Reagent 2; Trinity Biotech) was added, the assay plate shaken for 30sec and then incubated at 37°c for 30min. The fluorescence intensity (RFU) of each well was read at 530/25nm excitation and 590/35nm emission by a Synergy HT Microtiter Plate Reader (BioTek, Winooski, VT). A linear regression calibration curve, constructed by plotting the assigned concentrations of the calibrators against the recorded fluorescence values, was used to convert the RFU value of each sample into a DBS HDL-C concentration (Gen5 Software, BioTek).

The HDL-C assay lower limit of detection was 15mg/dl, within-assay imprecision (CV) was 5.3% and between-assay imprecision was 9.9%. The HDL-C concentrations of 91 DBS samples analyzed by the DBS assay correlated with the HDL-C concentrations of paired plasma samples (Pearson R = 0.83) and were linearly related (DBS assay HDL-C value = 39.55 + plasma HDL-C value X 1.097). Correction for hemoglobin absorbance did not improve the agreement between the plasma and DBS HDL-C values.

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Harborview Medical Center
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DRIED BLOOD SPOT TOTAL CHOLESTEROL FLUORIMETRIC ASSAY

The dried blood spot (DBS) total cholesterol fluorimetric assay involves a series of coupled enzymatic reactions. A punch from a DBS card containing a total cholesterol (TC) assay calibrator, quality control (QC) sample or patient sample is eluted with a buffer solution. The elution solution is incubated with assay reagent containing cholesterol ester hydrolase, cholesterol oxidase, peroxidase, and a fluorogen. The cholesterol ester hydrolase catalyzes the conversion of cholesterol esters to cholesterol, and this and de novo cholesterol is oxidized by cholesterol oxidase, producing hydrogen peroxide (H_2O_2) as a byproduct. In the presence of peroxidase, the H_2O_2 reacts with the non-fluorescent fluorogen to produce a fluorescent fluorophore. The TC concentration is directly proportional to the fluorescent intensity of the solution; fluorescence is measured spectrophotometrically. A standard curve is constructed by plotting the fluorescence values of the calibrators against the known TC concentrations. Using the standard curve, the fluorescence values of the QC samples and patient samples are read as TC concentrations. Acceptability of the assay is determined by comparing the TC concentrations of the QC samples with their established values.

DBS TC assay calibrators were constructed from high TC concentration pooled human plasma (University of Washington Department of Laboratory Medicine, Seattle, WA; UW Lab Med) serially diluted with 7% bovine serum albumin in phosphate buffered saline (BSA/PBS; Sigma Aldrich, St. Louis, MO) to the desired final TC concentration. Two DBS QC samples were constructed from a separate pool of human plasma, either undiluted (high TC concentration QC sample) or diluted with BSA/PBS (low TC concentration QC sample). Each calibrator and QC sample solution was mixed with a constant volume of washed human erythrocytes (UW Lab Med), pipetted in 75µl aliquots onto No. 903 filter paper (Whatman, Piscataway, NJ) and dried for 4hr at RT (23°c). The final TC concentration of each calibrator and QC sample solution was determined by analysis on a UniCel DxC 800 Synchron Clinical System (Beckman Coulter, Miami, FL). DBS calibrators, QC samples and study samples were sealed in Ziploc bags with desiccant packs and stored at -70°c until processing. Immediately prior to processing, DBS were warmed to RT and then a single 3.2mm (1/8in) diameter punch was punched from each DBS into a microtiter plate well (Greiner Bio-One, Monroe, North Carolina). Microtiter plates were either immediately assayed or were firmly sealed and stored at -70°c pending assaying.

Immediately prior to assaying, microtiter plates were warmed to RT. A TC elution buffer comprised of 400µl ddH_2O with 0.1% Triton X-100 (Sigma) was added to each microtiter plate well. The plate was sealed and gently shaken for 1hr on a microplate shaker (Delfia Plateshake, PerkinElmer, Waltham, MA). 20µl of eluent was transferred from each well of the elution plate to an assay microtiter plate. This was followed by addition of 100µl of TC assay reagent comprised of 70% Cholesterol Chromogen (Synermed, Westfield, IN), 29% Cholesterol Enzyme (Synermed) and 1% Cayman ADHP (10-acetyl-3,7-dihydroxyphenoxazine; Cayman Chemical, Ann Arbor, MI). The plate was gently shaken for 30sec and then incubated at 37°c for 30min. The fluorescence intensity (RFU) of each well was read at 530/25nm excitation and 590/35nm emission by a microtiter plate reader (Synergy HT, BioTek, Winooski, VT). A linear regression calibration curve, constructed by plotting the assigned concentrations of the calibrators against the recorded fluorescence values, was used to convert the RFU value of each sample into a DBS TC concentration (Gen5 Software, BioTek).

The TC assay lower limit of detection was 27mg/dl, within-assay imprecision (CV) was 4.0% and between-assay imprecision was 4.7%. The TC concentrations of 89 DBS samples analyzed by the DBS assay correlated with the TC concentrations of paired plasma samples (Pearson R = 0.91) and were linearly related (DBS assay TC value = 59.35 + plasma TC value X 1.12). Correction for hemoglobin absorbance did not improve the agreement between the plasma and DBS TC values.