HEIRS Laboratory Test Descriptions & References

All testing was performed at Fairview-University Medical Center Clinical Laboratory unless noted otherwise.

Transferrin Saturation

Iron analysis and unbound iron binding capacity (UIBC) are required for the determination of transferrin saturation. Total iron binding capacity (TIBC) is the sum of the iron and UIBC valus; transferrin saturation is the quotient of iron divided by the TIBC expressed as percent. For HEIRS screening visits, the participant samples from the London Ontario Field Center were analyzed at the MDS Laboratory by a ferrozine based colorimetric iron[1] and UIBC[2] method on a Hitachi 917 analyzer (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). All other samples were analyzed at Fairview-University Medical Center Clinical Laboratory using the same methods on a Hitachi 911 analyzer. In order to determine comparability between testing sites, quality control comparisons were analyzed at the Fairview-University Laboratory on approximately 2% of the samples tested at MDS Laboratory.

Serum Ferritin

Ferritin was analyzed on the Hitachi 911 analyzer using a turbidimetric antibody method[3]. (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, ferritin antibody bound to latex forms an antigen-antibody complex with ferritin in the sample. Turbidity measured at 700 nm is directly proportional to the concentration of ferritin.

C282Y, H63D, and Other Polymorphism Detection

Invader Assay: For the initial screen (IS) samples, C282Y and H63D genotyping was performed using a modification of the Invader assay (Third Wave Technologies, Inc. Madison, WI.). In this assay[4], two oligonucleotides (wildtype or mutant probe and Invader oligo) hybridize in tandem to a specific region of DNA generating a structure that is recognized and cleaved by the Cleavase VIII enzyme. This structure includes an unpaired "flap" on the 5' end of the wildtype or mutant probe. Cleavage releases the 5' flap, which serves as the Invader oligo in the second cleavage reaction on a FRET oligonucleotide probe, which is tagged with a fluorophore quenched by an internal dye. Upon cleavage, the 5'-fluoresein labeled product is detectable using a fluorescence plate reader. The assay was modified to include 12 cycles of allele-specific PCR reaction in order to increase the amount of DNA available for the reaction.

PCR-RFLP Method: All IS samples that were homozygous for the mutant allele, 2% of randomly selected IS samples, and CCE participant samples were confirmed by genotyping using a PCR-RFLP based method in which the amplified product is cut with a restriction enzyme specific for each mutation[5]. In addition, Family Study participant samples were genotyped using the PCR-RFLP method. The primers used for C282Y genotyping were described by Debra Leonard in a personal communication and are not located over the 5569G/A single nucleotide polymorphism described by Jeffreys et al. [6]

Serum Glucose

Glucose was analyzed on the Hitachi 911 analyzer using a hexokinase method[7] (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, hexokinase catalyzes the phosphorylation of glucose by ATP. G-6-P is oxidized to 6-phosphogluconatein the presence of NADP

by G-6-PDH. Formation of NADPH measured at 304nm is directly proportional to the concentration of glucose.

Serum Insulin

Analysis of insulin will be performed on the DPC Immulite Analyzer (Diagnostic Products Corporation, Los Angeles CA) using solid phase immunometric assay[8]. In this assay, insulin binds to antibody on a solid phase and anti-insulin alkaline phosphatase conjugate reacts with a different antigenic site on the insulin molecule to form a sandwich complex. Light generated by the reaction of a chemiluminescent substrate added to the bound antigen-antibody-conjugate complex is proportional to the concentration of insulin.

Alanine Aminotransferase (ALT)

ALT was analyzed on the Hitachi 911 analyzer using a colorimetric method[9] (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, alpha-ketoglutarate reacts with L-alanine in the presence of ALT to form L-glutamate and pyruvate. An indicator reaction utilizes the pyruvate for a kinetic determination of NADH consumption, which is directly proportional to the enzyme activity.

Aspartate Aminotransferase (AST)

AST was analyzed on the Hitachi 911 analyzer using a colorimetric method[9] (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, alpha-ketoglutarate reacts with L-aspartate in the presence of AST to form L-glutamate and oxaloacetate. An indicator reaction utilizes the oxaloacetate for a kinetic determination of NADH consumption, which is directly proportional to the enzyme activity.

Serum Gamma Glutamyltransferase (GGT)

GGT was analyzed on the Hitachi 911 analyzer using a colorimetric method[10] (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, L-gamma-glutamyl-3-carboxyy-4-nitroanailide reacts with glycylglycine in the presence of GGT to form 5-amino-2-nitrobenzoate and L-gamma-glutamyl-glycylglycine. The photometric determination of the rate of 5-amino-2-nitrobenzoate production is directly proportional to the GGT activity.

C-Reactive Protein (CRP)

CRP was analyzed on the Hitachi 911 analyzer using a turbidimetric immunoprecipitation method[11] (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, antibody to human CRP forms insoluble antigen-antibody complexes with CRP in the sample. Turbidity measured at 340nm is directly proportional to the concentration of CRP.

Hemogram, differential, platelet and reticulocyte count

An automated hemogram (complete blood count), differential, and platelet count was performed using the Beckman Coulter GenS (Beckman/Coulter, Fullerton, CA). This analysis included WBC, RBC, hemoglobin, hematocrit, MCV, MCH, MCHC, RDW, differential, and platelet.

Reticulocyte count was analyzed in samples when the MCV or hemoglobin values were below the lower limit of the gender-specific reference range. Reticulocyte count was analyzed at ARUP Laboratory using flow cytometry.

Haptoglobin

Haptoglobin was analyzed in samples when the MCV or hemoglobin values were below the lower limit of the gender-specific reference range. Haptoglobin was analyzed on the Hitachi 911 analyzer using a turbidimetric antibody method[11] (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, antibody to human haptoglobin forms insoluble antigen-antibody complexes with haptoglobin in the sample. Turbidity measured at 340nm is directly proportional to the concentration of haptoglobin.

Lactate dehydrogenase (LD)

LD was analyzed in samples when the MCV or hemoglobin values were below the lower limit of the gender-specific reference range. LD was analyzed on the Hitachi 911 analyzer using a colorimetric method[13] (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In this assay, NAD and lactate are converted to pyruvate and NADH in the presence of LDH. The rate of NADH formation is directly proportional to LDH activity.

Bilirubin, total, direct and indirect

Bilirubin was analyzed in samples when the MCV or hemoglobin values were below the lower limit of the gender-specific reference range. Total and direct bilirubin was analyzed on the Hitachi 911 analyzer using a colorimetric method[14] (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). In the total bilirubin assay, bilirubin is coupled with a diazonium ion in the presence of a solubilizing agent and a strongly acid medium. The production of azobilirubin is measured photometrically and is directly proportional to the concentration of total bilirubin. In the direct bilirubin assay, diazotized sulfanilic acid without an accelerator converts primarily conjugated bilirubin, but little unconjugated bilirubin, to an azobilirubin isomer, which is measured photometrically. Indirect bilirubin is calculated by subtracting the direct bilirubin from the total bilirubin.

Hemoglobin Identification and Hemoglobin A2 Quantitation

Participants with MCV or hemoglobin values below the lower limit of the gender-specific reference range were evaluated for presence of genetic variants of hemoglobin and for hemoglobin A2 quantitation. These tests were performed at ARUP Laboratory using high performance liquid chromatography (HPLC) with reflex to electrophoresis or extended HPLC when abnormal hemoglobin is detected.

Hepatitis B Surface Antigen (HBsAg)

HBsAg was analyzed using an immunometric assay[15] on the Vitros ECi (Ortho-Clinical Diagnostics, Inc., Raritan, NJ). In this method, sample is added to wells that are coated with mouse monoclonal antibody to hepatitis B surface antigen. Antigen present in the sample binds to the antibody. Then, mouse monoclonal antibody to hepatitis B surface antigen conjugated with horseradish peroxidase is added. This reacts with the antigen-antibody complex. When luminogenic substrate and electron transfer agent are added to the reaction mixture, chemiluminescence develops proportional to the amount of HbsAg present. Results are reported as positive or negative.

Hepatitis C Antibody (Anti-HCV)

Anti-HCV was analyzed using an immunometric assay[16] on the Vitros ECi (Ortho-Clinical Diagnostics, Inc., Raritan, NJ). In this method, sample is incubated in wells that are coated with recombinant HCV antigens C22-3, C200, and NS-5. If antibody is present in the sample, human immunoglobulins in the sample bind to the coated well forming an antigen-antibody complex. This complex is incubated with a solution containing horseradish peroxidase labeled mouse antibodies directed against human immunoglobulins. When luminogenic substrate and electron transfer agent are added to the reaction mixture, chemiluminescence develops proportional to the amount of anti-HCV present. Results are reported as positive or negative.

Lymphocyte cryopreservation

Cyopreservation of lymphocytes involves isolation of peripheral blood mononuclear cells (PBMC) from anticoagulated whole blood using density gradient centrifugation[17, 18] A Vacutainer-CPT™ tube (Bectin Dickenson, NJ), which contains a density adjusted solution for separating the mononuclear cells from other blood sample elemnets, anticoagulant, and a gel barrier was used for blood sample collection. After separation, the PBMC are washed in Hanks Balanced Salt Solution (HBSS). Then, the PBMC are suspended in a protein-rich media consisting of 20% fetal calf serum in RPMI-1640 + hepes. A cell count is made using a hemocytometer. Cells were diluted 1:1 with DMSO and frozen in liquid nitrogen.

DNA Isolation

For the Initial Screening (IS) samples, buffy coat was collected from EDTA anticoagulated whole blood and spotted on to FTA®-treated paper (Whatman® Clifton, NJ). 1.5 mm punches from the buffy coat spot was washed to remove hemoglobin and other interfering substances and dried overnight. These washed punches were used in the Invader or PCR-RFLP assays C282Y and H63D genotyping.

For CCE and Family Study participant samples, DNA was isolated using commercial Puregene® reagents[19] (Gentra System, Inc., Minneapolis, MN). This method utilizes sodium dodecylsulfate for cell lysis and a salt precipitation method for protein removal. DNA is isolated using isolated in isopropanol and stored in DNA Hydration Solution (Tris-EDTA buffer).

References:

1. Siedel J, Wahlefeld AW, Ziegenhorn J. Improved FerroZine-based reagent for the determination of serum iron (transferring iron) without deproteinization. Clin Chem 1984;30(6):975. Abstract.

2. Goodwin JF, Bramlett M, Guillemette M. Dierct measurement of serum iron and binding capacity. Clin Chem 1966;12:47.

3. Morikawa K, Oseko F, Morikawa S. A role for ferritin in hematopoiesis and the immune system. Leuk-Lymphoma 1995;18(5-6):429-433.

4. Lyamichev V, Mast AL, Hall JG, Prudent JR, Kaiser MW, Takova T, Kwiatkowski RW, Sander TJ, de Arruda M, Arco DA, Neri BP, Brow MA. Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. Nat Biotechnol. 1999 Mar;17(3):292-6.

5. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, Dormishian F, Domingo Jr. R, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, Kronmal GS, Lauer P, Lee VK, Loeb

DB, Mapa FA, McClellend E, Meyer NC, Mintier GA, Moelleer N, Moore T, Morikang E, Prass CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nature Genetics 1996;13:399-408.

6. Jeffrey GP, Chakrabarti S, Hegele RA, Adams PC. Polymorphism in intron 4 of HFE may cause overestimation of C282Y homozygote prevalence in haemochromatosis. Nature Genetics 1999;22:325-326.

7. Peterson JI, Young DS. Evaluation of the hexokinase-glucose-6-phosphate dehydrogenase method of determination of glucose in urine. Anal Biochemistry 1958;23:301.

8. AL Babson, DR Olson, T Palmieri, AF Ross, DM Becker, and PJ Mulqueen The IMMULITE assay tube: a new approach to heterogeneous ligand assay Clin Chem 1991;37:1521-1522

9. Bergmeyer HU. Scheibe P. Wahlefeld AW. Optimization of methods for aspartate aminotransferase and alanine aminotransferase. Clin Chem 1978;24(1):58-73.

10. Szasz G. A kinetic photometric method for serum gamma-glutamyl transpeptidase. Clin Chem 1969;15:124.

11. Burtis CA, Ashwood ER. Tietz Textbook of Clinical Chemistry. 3rd ed. Philadelphia PA: WB Saunders Co;1999:215.

12. Grimaldi E, Scopacasa F. Evaluation of Beckman-Coulter® GenS™ hematology analyzer. Lab Hematology 1998;4:264-268.

13. Bergmeyer HU. Methods of Enzymatic Analysis III. 3rd ed. Weinheim: Verlag Chemie; 1983:126-133.

14. BW Perry, BT Doumas, DD Bayse, T Butler, A Cohen, W Fellows, CC Garber, B Howell, T Koch, S Krishnamurthy, A Louderback, RB McComb, D Miller, RR Miller, RN Rand, and R Schaffer. A candidate reference method for determination of bilirubin in serum. Test for transferability Clin Chem 1983;29:297-301.

15. Wei R, Knight GJ, Zimmerman DH, Bond HE. Solid-phase enzyme immunoassay for hepatitis B surface antigen. Clin Chem 1977;23:813-815.

16. Chien D, Choo Q-L, et al. Diagnosis of hepatitis C virus (HCV) infection using an immunodominant chimeric polyprotein to capture circulating antibodies: reevaluation of the role of HCV in liver disease. Proc Natl Acad Sci 1992;89:10,011-10,015.

17. Mittal KK. Isolation and purification of lymphocytes from peripheral blood. Fotino Laboratory AACHT Manual I-2-1.

18. Ting A and Morris PT. A technique for lymphocyte preparation from stored heparinized blood. Vox Sang 1971;20:561-563.

19. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acid research 1988;16:1215.