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| C1920 Rituximab Panel | | | | | | | |
| **Purpose** | To measure the effect of B-cell depletion therapy with Riuximab on circulating B-cells in the patient. | | | | | | |
| **Policy Statements** | • This procedure applies to all laboratory technologists performing Immunology testing, the section  supervisor, and section pathologist. | | | | | | |
| **Principle and Clinical Significance** | B-cell depletion for treatment of human autoimmune diseases is often accomplished through  antibodies targeting the surface molecule CD20 (Rituximab).Treatment with these antibodies depletes  B-cells by a combination of antibody – mediated cellular toxicity (ADCC), complement – dependent  Cytotoxicity (CDC), and antibody - triggered apoptosis;    B-cell depletion with CD20 (Rituximab). Anti-CD20 mAb can direct the killing of B cells by antibody-dependent cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), or apoptosis. ADCC is triggered by the interaction between the Fc region of the antibody and the FcR on effector cells of the immune system. In CDC the Fc region is bound by the complement component C1q, which triggers a proteolytic cascade. Apoptosis occurs when CD20 molecules are cross-linked by anti-CD20 mAb in lipid rafts and activate signaling pathways leading to cell death.  Rituximab is a chimeric monoclonal antibody against the protein CD20, which is primarily found on  the surface of immune system B cells. When it binds to this protein it triggers cell death. Rituximab  destroys both normal and malignant B cells that have CD20 on their surfaces and is therefore  used to treat diseases that are characterized by having to many B cells, overactive B cells,  or dysfunctional B cells.  B-cell depletion using Rituximab treatment results in nearly undetectable circulating B-cell levels  one month after therapy and B-cell counts remain low for 6-12 months.  As bone marrow stem cells and early B-cell precursors (pro-B cells) do not express CD20, the new  B-cells repopulate the B-cell compartment once the drug has cleared the system, allowing the immune response to return to normal.  The major side effect of B-cell depletion is the risk of severe infections, which needs to be taken into consideration when evaluating the risks and benefits of B-cell depletion.  B-cell depletion offers a promising therapy for the treatment of a variety of autoimmune diseases. The treatment is usually well tolerated; however, adverse events include infusion reactions, infections and hypogammaglobulinemia. | | | | | | |
| **Test Code** | C1920 | | | | | | |
| Materials | |  |  |  | | --- | --- | --- | | **Reagents** | **Supplies** | **Equipment** | | ● Reagents Provided in the MultiTest IMK Kit: (Sufficient for 50 tests):  1.)MultiTest CD3 FITC/ CD20 PE/ CD45 PerCP/ CD19APC  2.) TruCOUNT Tubes (25 tubes per sealed pouch)  3.)MultiTest IMK Kit Lysing Solution, 10X concentration (FACSLyse)  4.) Sheath Fluid (BD FACSFlow ) | |  |  | | --- | --- | | |  | | --- | | ● Reagents and Materials  Required but not Provided:  1.) 7 Color beads  2.)1X Lysing Solution diluted  from 10x concentration  (See dilution instructions below)\*  3.) Reagent-grade  (distilled or deionized) water  4.) K2 EDTA Vacutainer  (2 mL size) BD MAPS tubes  (500 μL size).  5.)TruCount tubes or disposable  12x75 mm polystyrene Falcon  tubes with caps. | | | ● Vortex Mixer  ● Micropipettors and tips including (Eppendorf Research plus pipettor-50μL volume delivery  ● BD FACSCanto II Flow Cytometer equipped with 635 nm and 488 nm lasers  capable of detecting forward and side scatter light as well as four-color fluorescence with emission detectable in four ranges: 515-545 nm, 562-607 nm, >650 nm, and 652-668 nm. |  |  | | --- | | *\*Dilution instruction for MultiTEST IMK Lysing Solution:* Dilute to 1X with room temperature (20° |   and 25° C) deionized water (sterile water stocked by this hospital). The prepared solution is stable for  1 month.  The 10x concentrate contains a proprietary buffer buffered solution containing <15% formaldehyde  and <50% diethylene glycol. | | | | | | |
| Sample | Collect 2 mLs of blood aseptically by venipuncture into a sterile K3 EDTA or K2 EDTA (lavender top) tube.  NOTE: IMK Kit reagents and TruCOUNT tubes have been validated with both liquid and dry formulations of EDTA. Follow collection tube manufacturer's guidelines for the minimum volume of blood to be collected to ensure proper specimen dilution, especially when determining absolute count with TruCOUNT beads. In cases where obtaining a full 2 mL, is not possible, 500μL of blood in a BD MAPS tube is acceptable.  Store specimen at room temperature (20° to 25° C) and stain within 72 hours of draw and then analyze within 4 hours of staining.  Additional Notes:  1.) Do not use previously fixed and stored specimens.  2.) Whole blood samples refrigerated prior to staining may give aberrant results.  3.) Samples obtained from patients taking immunosuppressive drugs or certain antibiotics (e.g.  Keflex) could yield poor resolution.  4.) Blasts cells could interfere with test results.  5.) Hemolyzed samples should be rejected.  6.) Specimen WBC must be in the linearity range appropriate for this test system.  (WBC concentration should be between 0.2 x 10e3 to 29.7 x 10e3 WBC/μL and a  **lymphocyte concentration of 0.1 x 10e3 to 9.0 10e3 lymphocytes/μl.)** | | | | | | |
| **Special Safety Precautions**  **Quality Control** | **SAFETY PRECAUTIONS**: Formaldehyde is extremely toxic and destructive to tissue of mucous membranes, upper respiratory tract, eyes and skin. It is harmful if swallowed, inhaled or absorbed through the skin. This material is an irritant, a sensitizer, a highly toxic lachrymator and a possible mutagen Gloves and protective clothing must be worn to prevent contact with skin. See MSDS for further information regarding its irritant, corrosive and possible carcinogenic properties.  Formaldehyde Disposal: Tubes containing 1% or less of formaldehyde may be disposed of in red biohazard buckets. Stock and working dilutions of formaldehyde may be disposed of down the drain with copious amounts of water.  **1)** Follow guidelines outlined in *Flow Cytometry Quality Assurance* procedure.  [FLO-1.3-quality-assurance-in-flow-cytometry.pdf](http://intranet.childrensmn.org/References/labsop/flow/flow/flo-1.3-quality-assurance-in-flow-cytometry.pdf)  **2)** Quality Control will be accomplished as a whole test system for this procedure. Refer to pages 10-1 through 10-3 in the training manual for information on expert gating algorithm and built-in software QC checks.  **3)** 7 - Color Setup Beads FACSComp software will be utilized to optimize instrument compensation settings. Stored optimized compensation settings will be used from normal human blood for sample analysis and further adjusted for each individual specimen as necessary.  **4)** CD Chex Plus whole blood controls will be used to check functionality of monoclonal antibodies. Low and Normal levels are monitored.  **5)** BD FACS Canto software will be used to analyze samples and check tube to tube variability in conjunction with TruCOUNT tubes and TruCOUNT beads. | | | | | | |
| **Procedure** |  | | | | | | |
|  | **Step** | | Action | | | | **Related Document** |
|  | 1 | | **Remove TruCount tubes for each patient and control.**  NOTES: a.) Examine the dessicant inside the foil pouch each time that tubes are removed. If the dessicant has turned blue to lavender, discard the remaining tubes.  b.) Use tubes within 1 hour after removal from the foil pouch and do not use beyond the expiration date indicated on the package. Once the foil pouch is opened, the tubes are only good for 30 days.  c.) Use care to protect the tubes from direct light.  d.) Perform the procedure at room temperature (20° to 25° C).  e.) Before use, verify that the TruCOUNT bead pellet is intact and within the metal retainer at the bottom of the tube. If this is not the case, discard the TruCOUNT tube and replace it with another.  f.) Make sure the foil pouch is completely sealed and as much air as possible is expelled.  g.) Bead counts vary by lot of TruCOUNT tubes. It is critical to use the bead count shown on the lot of TruCOUNT tubes you are currently using. The TruCOUNT lot ID and Bead/Pellet is stored in the MultiSET software and must be updated with each new lot of tubes. Do not mix multiple lots of tubes in the same assay. | | | |  |
|  | 2 | | Label tubes for desired antibody panel:  **CD Chex Plus controls:**  Set up CD3/CD8/CD45/CD4 and CD3/CD16+56/CD45/CD19/CD20  for each CD Chex control (Low and Normal) so a complete CD Chex Plus ISP is run.  **Patient Samples:**  Set up tubes for CD3/CD20/CD45/CD19 for each patient sample. | | | |  |
|  | 3 | | Pipette 20μL of the appropriate antibody into the TruCOUNT tube just above the stainless steel retainer. Do not touch the bead pellet when dispensing.  Pipette 50μL of well-mixed, anticoagulated EDTA whole blood into the bottom of each tube.  **NOTE**: Avoid smearing blood down the sides of the tube. If whole blood remains on the side of the tube, it will not be stained with the reagents. When using TruCOUNT tubes, accuracy is critical. Pipette sample onto the side of the tube just above the retainer. | | | |  |
|  | 4 | | Vortex gently to mix. | | | |  |
|  | 5 | | Place the uncapped tubes into a carousel rack. | | | |  |
|  | 6 | | Press the carousel access button to open the carousel door of the Lyse/Wash Assistant, if it is not already open. If the instrument is in use, the door opens once the instrument pauses.  Place the carousel on its spindle. | | | |  |
|  | 7 | | Select the preprogrammed protocol: multi test prep and close the carousel door to begin processing. As the LWA scans the carousel rack, the screen changes to reflect tube replacement. Rack positions containing a tube are indicated by circles with numbers beside them. After the LWA finishes scanning, the touchscreen continues to show rack progress. The countdown clock shows an estimate of the time remaining to finish the rack. The multi test prep program will perform an incubation for 15 minutes, followed by dispensing of 450 чl FACS Lyse, performing a mixing of sample and an additional 10 minute incubation. | | | |  |
|  | 8 | | Once all tubes in a rack have been fully processed, the green status indicator light goes out, the instrument beeps, and the touchscreen displays the message *REMOVE CAROUSEL RACK* and *RACK DONE.*  Press the carousel access button to open the carousel door.  Remove the carousel rack from the LWA and close the carousel door. | | | |  |
|  | 9 | | Analyze prepared samples on the FACS Canto II flow cytometer.  If samples are not to analyzed immediately after preparation, store them in the dark at 2-8°C and analyze within 4 hours. | | | |  |
|  | **Flow Cytometry Instrument** | | | | | |  |
|  | **Step** | **Action** | | | | | **Related Document** |
|  | 1 | Launch computer, select administrator | | | | |  |
|  | 2 | Enter password (CE number, Groupwise Password) | | | | |  |
|  | 3 | Launch BD FACS CANTO software from menu | | | | |  |
|  | 4 | When the SIGN IN view appears: enter CANTO and click to accept | | | | |  |
|  | 5 | Run 7 Color bead setup program at this time if you have not  already done so. Use 7 Color beads to set up the instrument to acquire four-color Lyse/No Wash samples. Verify program using current lot of 7 Color beads.  Confirm Cytometer Setup passes specifications. Troubleshoot setup as needed, Confirm Cytometer Setup passes specifications. | | | | |  |
|  | 6 | In the Sample, enter the information as required to identify control or patient samples. For the *controls*, enter **Control** under Name, for sample ID enter Lot number of control, for Case Number enter Low or Normal control.  For *patient samples*, enter patient’s name and test under  Name: patient’s last name\_first name\_(C1920),for sample ID enter  patient’s accession number\_patient’s hospital number \_date of birth,  and then for Case Number enter patient’s accession number again. | | | | |  |
|  | 7 | Choose the appropriate test panel (Panel ► down to 4 color  TBNK +TruC ) to run for each sample in the Panel Name column. | | | | |  |
|  | 8 | Run Tests by selecting the “two tubes” icon and pressing the ► key in the main bar. | | | | |  |
|  | 9 | Select Stop Icon to view results. | | | | |  |
|  | 10 | Gently vortex the specimen to be acquired and install on the SIP.  In this pre-acquisition phase, you can preview the data before  beginning acquisition. Name worksheet file based on day of  month and year and append with number of run.  (i.e.worksheet.02.23.2011.2) | | | | |  |
|  | 11 | Click ACQUIRE and the acquisition phase will begin. Analysis is  very rapid, results are calculated and the Laboratory Report view  appears. | | | | |  |
|  | 12 | Visually inspect the positions of the attractors on the dot plots.  See procedure notes for criteria to assess attractor gates.  If desired, you can adjust the gate or attractors for the tube just  analyzed. Any changes made to the gate, attractors or  displays are applied to the current tube only.  a.) Double-click the CD45 vs SSC plot to increase (zoom) the plot size. Adjust the gate around the lymphocytes as necessary and click the CLOSE box in the4 upper left corner of the plot to un-zoom the plot.  b.) Adjust the attractor’s gate to move, resize, or reorient  the attractors. | | | | |  |
| **Interpretation**  **Procedure Notes** | [Document-G-Pediatric-Peripheral-Blood-Normal-Ranges-T-and-B-cells.pdf](http://intranet.childrensmn.org/References/labsop/flow/res/document-g-pediatric-peripheral-blood-normal-ranges-t-and-b-cells.pdf)  1. Disease relapse occurs in about 50% of patients either at the time that B-cell numbers increase to pretreatment levels or within 3 months, while in other cases clinical relapse can be delayed for years.  2. Additional Rituximab courses can induce subsequent remission. Multiple Rituximab courses are often associated with progressive decrease in circulating IgM and IgG levels.  3. Not all CD20+ B-cells are equally affected by Rituximab treatment. B-cells located in the peritoneal cavity are surprisingly resistant to depletion. While these B-cells express normal CD20 densities and are bound by CD20 monoclonal antibody, only about 50% of these cells are depleted. These location dependent sensitivities to CD20 monoclonal antibody mediated depletion could have significant consequences for therapy and may be the reason of the heterogeneity of results in human clinical trials.  4. If it is necessary to calculate the absolute counts manually, the following equations are used:      Absolute Lymphocytes = WBC x %Lymphocyte  e.g. WBC - 5.0 K/μL, %L = 35  5000 X 0.35 = 1750 (Absolute Lymph count)  Absolute CD subset = Absolute Lymphs x % CD subset  e.g. CD3 = 70%  1750 x 0.70 = 1225 (Absolute CD3 count) | | | | | | |
| **Alternate Method**  **Result Reporting**  **(Computer)**  **References** | An alternative to using the multi test prep mod on the LWA:  Manually add 450ul 1X FACS Lyse using the Eppendorf Research plus adjustable  pipette.  Vortex gently.  Incubate in dark at room temperature for 15 minutes.  The tubes are now ready to be analyzed on the FACS Canto II flow cytometer as  usual.  Enter results ( percent and absolute ) for CD3, CD19, CD20 in Sunquest.  BD Bioscience FACSCanto Training Manual. 23-9575-00 Rev. A. 2007, Becton, Dickinson and Company, San José, CA  BD FACSLyse Wash Assistant User’s Guide,Rev.23-11113-00 Rev. A ,Becton Dickenson, San José, San José, CA  MultiTEST IMK Kit product Insert 23-3602-01. Becton Dickenson, San José, CA  BD Multitest IMK Kit product Insert 23-3602-04. Becton Dickenson, San José, CA  BD TruCOUNT Tubes produce Insert 23-3483-05, Becton Dickenson, San José, CA  Review article B Cells in Autoimmune Diseases Christiane S. Hampe  Department of Medicine, University of Washington, SLU-276, 850 Republican, Seattle WA 98109, USA  Academic Editors; B.- L. Chiang and R. Klein  Copyright 2012. | | | | | | |
| **Historical Record** |  | | |  |  |  | |
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