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| C1920 Rituximab Panel | | | | | | | |
| **Purpose** | To measure the effect of B-cell depletion therapy with Rituximab 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;  215308.fig.003  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** | | ● Monoclonal Antibodies (MoAbs): Follow manufacturer's insert instructions in handling antibodies. In general, protect from light and store at 2 to 10° C. Maintain sterile technique to prevent bacterial or cross contamination of reagents.   * 1X Lysing Solution diluted   from 10x concentration  (See dilution instructions below)\*  ● PBS Buffer Solution (PBS):  A. Make PBS according to package directions. Do not add CaCl if provided.  B. Mix in a clean container and label properly including lot number, hazard information and expiration date (one month from preparation date). | |  |  | | --- | --- | | |  | | --- | | 1.) CS&T setup beads  2.) Reagent-grade  (distilled or deionized) water  3.) K2 EDTA Vacutainer  (2 mL size) BD MAPS tubes  (500 μL size).  4.)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.   * BD FACS Lyse/Wash Assistant |  |  | | --- | | *\*Dilution instruction for BD FACS 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 | 1. Peripheral blood, 2mL, freshly drawn and collected in EDTA.  2. Specimens should be stored at room temperature until processing. Specimens exposed to extreme temperatures may yield inadequate results.  3. Clotted, grossly hemolyzed specimens or under-filled tubes are not acceptable for analysis.  4. Any specimens not properly labeled should be rejected.  5. Specimen should be processed within 72 hours of draw per CDC recommendations and our validation studies. (5/13/03). | | | | | | |
| **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) | | | | | | |
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| **Daily Startup** | **Step** | | Action | | | | **Related Document** |
|  |  | | 1. Turn on the cytometer main power, log in to the computer and start BD FACS DIVA clinical software. 2. Ensure that the software is connected to the cytometer by checking the Status Bar (lower right hand corner *Connected* is displayed). 3. Any cytometer condition that needs attention will be red in the status window. If any of the fluid level(s) appears in red, service the corresponding fluid container(s). 4. Select Cytometer > Fluidics StartUp 5. Check the flow cell air bubbles.    1. Lift the flow cell access door.    2. If you see bubbles, remove them by selecting Cytometer > Cleaning Modes > Degas flow cell. 6. Check that the laser warmup has finished. When laser warmup is complete, a Ready message appears in the status bar. 7. Allow the laser to warm up for 15 to 30 minutes before running samples on the cytometer to ensure laser stability and optimal power. | | | | [FLO 1.8 CISP Comprehensive Immune Status Panel](https://starnet.childrenshc.org/References/labsop/flow/flow/flo-1.8-cisp-comprehensive-immune-status-panel.pdf) |
| **Running CS&T** |  | | 1. Pipette 350 μL of PBS into a 12X75 mm polystyrene tube. 2. Mix the CS&T dropper bottle. 3. Add one drop of CS&T beads to the tube. 4. Select Cytometer > CST. The CS&T application will open. 5. In the Setup Control window, select Check Performance from the drop-down menu. Check that the lot number of CS&T setup beads is correct. 6. Load the tube on the cytometer and click Run. 7. The Checking Cytometer Performance window will appear. 8. When the check is complete, the software will indicate set up completed successfully or unsuccessfully. Click Finish. 9. At the end of each month, print a copy of the month’s CS&T Levey-Jennings data and place in the Levey-Jennings CS&T beads section of the Flow QC binder. Date and Initial review of data. | | | | [FLO 1.8 CISP Comprehensive Immune Status Panel](https://starnet.childrenshc.org/References/labsop/flow/flow/flo-1.8-cisp-comprehensive-immune-status-panel.pdf) |
| **Procedure** | **Step** | Action | | | | | **Related Document** |
|  | 1 | Label tubes for desired antibody panel and add appropriate monoclonal antibodies to each tube:  ( FITC/ PE/ PerCP/ APC)  CD3 / CD20 / CD45 / CD19 | | | | | [FLO 1.8 CISP Comprehensive Immune Status Panel](https://starnet.childrenshc.org/References/labsop/flow/flow/flo-1.8-cisp-comprehensive-immune-status-panel.pdf) |
|  | 2 | Add 50 μL of well mixed whole blood to the tube. Vortex gently to mix. | | | | |  |
|  | 3 | Place the uncapped tube into a carousel rack. Press the carousel access button to open the carousel door of the Lyse/Wash Assistant. Place the carousel on its spindle. | | | | |  |
|  | 4 | Select the preprogrammed protocol: duo inc-lyse-wash-no fix mode and  close the carousel door to begin processing. | | | | |  |
|  | 5 | 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.* | | | | |  |
|  | 6 | Analyze prepared samples on the FACS Canto II flow cytometer. | | | | |  |
| **Data Collection** | **Step** | **Action** | | | | | **Related Documents** |
|  | 1 | In the Browser, highlight your login name. From the Main Menu, select Experiment→ New Experiment→ General→ Rituximab Panel. | | | | |  |
|  | 2 | Rename experiment with patient last name\_first name\_accession number\_hospital number\_DOB. The experiment will be archived and organized by this name. | | | | |  |
|  | 3 | Right click on Cytometer Settings (directly under the experiment name) and choose “link setup.” Choose the current 4 color compensation and click “Link”. | | | | |  |
|  | 4 | Click on plus sign to expand the specimen and view the tubes. Click on pointer next to the first tube to select it. In the Acquistion Dashboard window, click Acquire. | | | | |  |
|  | 5 | Adjust the gate around the lymphocytes as necessary. Click Record when ready to accumulate patient data. | | | | | [FLO 2.1 CD45 Gating Flow Cytometric Analysis](https://starnet.childrenshc.org/References/labsop/flow/flow/flo-2.1-cd45-gating-flow-cytometric-analysis.pdf) |
|  | 6 | Print a hard copy of the scatter worksheet. | | | | |  |
| **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. | | | | | | |
| **Calculations/**  **Result Reporting**  **References** | 1. The EDTA sample must have a WBC and Differential counted by Hematology in order to calculate the absolute values for each marker.  2. C1920s and CISPs analyzed using CD45-gating must have the absolute values manually calculated. 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)  3. All manual calculations must be checked by another tech, designated by a √ and initialed. Results should also be reviewed for correct entry into the computer.  4. Enter results (percent and absolute) for CD3, CD19, and CD20 in Sunquest. If there is a population positive for both CD19 and CD20, indicate the percentage of dual positive lymphocytes in the COM1 field with a freetext comment.  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  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** |  | | |  |  |  | |
|  | **Version** | | | **Written/Revised by:** | **Effective Date:** | **Summary of Revisions** | |
| 1 | | | Al Quigley | 11/01/17 | Initial Version | |
|  | 2 | | | Amanda McCaustland | 9/28/2018 | Updated method from single platform/MultiTEST to dual platform analysis | |