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|  Immune Status / Immunodeficiency Panels using MultiTest on FACSCanto II Flow Cytometer |
| **Purpose** | The BD MultiTEST system with TruCount tubes is a lyse/no wash method of 4 color direct immunofluorescence staining of human lymphocytes. This provides a complete panel as recommended by the CDC for lymphocyte subset profile analysis. |
| **Policy Statements** | This procedure applies to all laboratory technologists performing Flow Cytometry testing, the sectionsupervisor, and section pathologist. |
| **Principle and Clinical Significance** | When samples are stained using MultiTEST reagents and TruCOUNT tubes, you can enumerate absolute counts (cells/uL) as well as lymphocyte percentages of mature T helper/inducer (CD3+/CD4+), T suppressor/cytotoxic (CD3+/CD8+) and total T (CD3+) cells within one tube. Using a second tube, you can enumerate mature T (CD3+), B (CD3-/CD19+) and NK (CD3-/CD16+56+) subsets. Lymphocyte subsets are monitored in a variety of conditions including congenital and acquired immunodeficiencies.  |
| **Test Code** | IMMP/C48P  |
| **Materials** |

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| **Reagents** | **Supplies** | **Equipment** |
| Reagents Provided in the MultiTest IMK Kit (Sufficient for 50 tests):1.) MultiTest CD3 FITC/ CD8 PE/ CD45 PerCP/ CD4 APC2.) MultiTest CD3 FITC/ CD16+56 PE/ CD45 PerCP/ CD19 APC3.) TruCOUNT Tubes (25 tubes per sealed pouch)4.) MultiTest IMK Kit Lysing Solution, 10X concentration (FACSLyse) | Reagents and Materials Required but not Provided:1.) 7 Color Setup beads2.)1X FACSLyse solution diluted from 10x concentration (See dilution instructions below)\*  3.) Reagent-grade (distilled or deionized) water 4.) K2 EDTA Vacutainer (2 mL size) or Sarstedt Microtainer tubes (500 µL size)5.) Disposable 12x75 mm polystyrene Falcon tubes with caps 5.) Sheath Fluid (BD FACSFlow) | ● Vortex Mixer● Micropipettors and tips ● BD FACSCanto II Flow Cytometer equipped with 633 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/FACSLyse:* 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. In cases where obtaining a full 2 mL, is not possible, 500µL of blood in an EDTA microtainer is acceptable.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. Store specimen at room temperature (20° to 25° C) and stain within 72 hours of draw. Analyze within 4 hours of staining.Additional Notes:* Do not use previously fixed and stored specimens.
* Whole blood samples refrigerated prior to staining may give aberrant results.
* Clotted or hemolyzed samples should be rejected.

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| **Special Safety Precautions** | BD FACSLyse contains formaldehyde. 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. |
| **Quality Control** | 1. Follow guidelines outlined in *Flow Cytometry Quality Assurance* procedure.

 [FLO 1.3 Quality Assurance in Flow Cytometry](http://khan.childrensmn.org/Manuals/Lab/SOP/Flow/Flow/204759.pdf)1. Quality Control will be accomplished as a whole test system for this procedure. BD FACS Canto software performs flow cytometric data acquisition and automated analysis. Each sample is automatically analyzed using fluorescent (CD45 vs SSC) gating of lymphocytes followed by enumeration of subset populations and TruCount beads. The system does not require an isotypic control as each tube contains its own double-negative population, which does not overlap positive populations. In addition, the software analysis algorithm uses regions, known as attractors, which define each subset. These attractors accommodate variations in subset position encountered from one specimen to the next. To help ensure quality results, BD FACS Canto software automatically provides a quality control assessment of cell cluster integrity during the analysis of each single tube.
2. 7 - Color Setup Beads are run each day of testing.
3. CD Chex Plus whole blood controls will be used to check functionality of monoclonal antibodies. Low and Normal levels are run each day of testing.
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| **Procedure** | **Specimen Processing** |
|  | **Step** | Action | **Related Document** |
|  | 1 | Remove two TruCOUNT tubes for each patient and control. NOTES: Examine the desiccant inside the foil pouch each time that tubes are removed. If the desiccant has turned blue to lavender, discard the remaining tubes. 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 stable for 30 days. Use care to protect the tubes from direct light; make sure the foil pouch is completely sealed and as much air as possible is expelled. Before use, verify that the TruCOUNT bead pellet is intact and within the metal retainer at the bottom of the tube. Bead counts vary by lot of TruCOUNT tubes. It is critical to use the correct bead count as this number is used in the calculation of absolute counts. To update lot numbers and bead counts, select Tools > Lot IDs on the menu bar. 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/CD19for each CD Chex control (Low and Normal) **Patient Samples:**Set up tubes for CD3/CD8/CD45/CD4 and CD3/CD16+56/CD45/CD19 for each patient sample |  |
|  | 3 | Pipette20µL of the appropriate antibody into the TruCOUNT tube just above the stainless steel retainer. Do not touch the bead pellet when dispensing.Reverse pipette 50µL of well-mixed, anticoagulated EDTA whole blood. Wipe the outside of the pipette tip with gauze to remove excess before dispensing into the tube (take care not to wick out any sample). Change tips for each tube when dispensing patient samples.**NOTE**: Avoid smearing blood down the sides of the tube. When using TruCOUNT tubes, volume accuracy is critical to the calculation of absolute counts. Pipette sample onto the side of the tube just above the retainer. |  |
|  | 4 | Vortex gently to mix. |  |
|  | 5 | Incubate 15 minutes, in the dark, at room temperature. (20° to 25° C) |  |
|  | 6 | Add 450 µL 1x FACSLyse solution, vortex to mix. |  |
|  | 7 | Incubate 10 minutes, in the dark, at room temperature. (20° to 25° C) |  |
|  | 8 | 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 BD FACS CANTO software, log in with username and password. Run 7 Color bead setup program at this time if you have not already done so. | [Flo-2.9-analyzing-the-performance-setup-on-bd-facscanto-ii.pdf](https://starnet.childrenshc.org/References/labsop/flow/flow/flo-2.9-analyzing-the-performance-setup-on-bd-facscanto-ii.pdf) |
|  | 2 | In the Worklist, identify control or patient samples as follows. Name: LAST\_FIRST\_TEST CODE (For controls, enter CONTROL)Sample ID: LAST\_FIRST\_TEST CODE (For controls, enter CD CHEX NORMAL or LOW)Case Number: ACCESSION\_MRN\_DOB (For controls, enter lot number)Ensure the 4 color TBNK+TruC panel is selected. |  |
|  | 3 | Save the worklist. Files are named by date.  |  |
|  | 4 | If using the auto loader, ensure tubes are loaded on the carousel in the same order that they are programmed on the worklist. Each patient test should have two tubes: 3/4/45/8 is loaded first, 3/16+56/45/19 second. Run tests by clicking PLAY.jpg. Click "Ignore" to load tubes without the carousel and follow prompts to switch tubes. |  |
|  | 5 | Visually inspect the positions of the attractors on the dot plots. If desired, you can adjust the lymphocyte gate or attractors for the tube. Any changes made to the gate, attractors or displays are applied to the current tube only. If not using the loader, plots can be adjusted as each tube is run. If using the loader, click "OK" under the "Status" column to open the lab report for each sample.* Double-click the CD45 vs SSC plot to increase (zoom) the plot size. Adjust the gate around the lymphocytes as necessary.
* Adjust the attractor’s gate to move, resize, or reorient the attractors.
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|  | 13 | Print the report. Compare with CBC printout for gross discrepancies in absolute lymphocyte count (CD45). |  |
|  | 14 | After results are entered in Sunquest, reports and CBC printouts are scanned to G: LAB/Hematology/Heme Section Procedures/Scanned Flow Results. Save files as LAST NAME\_FIRST NAME\_MRN\_ACCESSION |  |
| **Interpretation/ Results** | [Document G - Pediatric Peripheral Blood Normal Ranges T & B Cells](http://khan.childrensmn.org/Manuals/Lab/SOP/Flow/Res/204753.pdf)Logical Subset Relationships:Observations that act as internal controls used by the operator are the lymphosum which is the sum of CD3+,CD19+ and NK cells. The lymphosum should equal 95 - 105%. Also, the CD4 and CD8 values should add up to the CD3 value ± 10%. The CD3+ cells also serve as a tube to tube variability control. If the CD3 values vary by >5% the test should be repeated.If an IMMP is ordered and percentages of CD4 + CD8 do not add up to total CD3 within 10%, reflex testing for T-cell receptors is performed. See [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)  |
| **Result Reporting** | In Sunquest:Round to nearest whole numbers.Function: MEMWorksheet: ISPTest: <CR>Method: <CR>ACC. NO: Enter Sunquest accession numberSOUR: PEBL (or other specimen type code as appropriate)CD3: Averaged % CD3CD4: % CD4 (tube 1)CD8: % CD8 (tube 1)CD19: % CD19 (tube 2)CD16: % CD16+56 (tube 2)3A: Averaged absolute CD34A: Absolute CD4 (tube 1)8A: Absolute CD8 (tube 1)19A: Absolute CD19 (tube 2)16A: Absolute CD16+56 (tube 2)HSR: Helper (CD4):Suppressor (CD8) ratio (tube 1)CD4A8: Sum of % CD4 and CD8, calculated by SunquestOTRE: Reflex T-cell receptors. If 4+8 is within acceptable range as calculated by Sunquest, enter HIDE. If not, Sunquest will prompt you to type Y. TCR will be added to the accession under the ISP worksheet.<CR>A to accept results <CR> |
| **Alternate Methods** | **Pre-Washing for Non-Specific Staining (dual platform analysis):**Obtain patient CBC results. The EDTA sample must have a WBC and differential counted by Hematology in order to calculate the absolute values for each marker. Pre-wash the sample with PBS to remove plasma before the monoclonal antibodies are added. Once the patient sample has been altered by pre-washing, utilize two step analysis for IMMP and C48P using 12x75 polystyrene Falcon tubes (TruCOUNT tubes are not necessary.) Refer to [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).Immune status panels analyzed using the pre-washed sample 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) |
| **References** | BD Bioscience FACSCanto Training Manual. 23-9575-00 Rev. A. 2007, Becton, Dickinson and Company, San José, CABD FACSLyse Wash Assistant User’s Guide,Rev.23-11113-00 Rev. A ,Becton Dickenson, San José, San José, CAMultiTEST IMK Kit product Insert 23-3602-01. Becton Dickenson, San José, CABD Multitest IMK Kit product Insert 23-3602-04. Becton Dickenson, San José, CABD TruCOUNT Tubes product Insert 23-3483-05, Becton Dickenson, San José, CA |
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| **Historical Record** | **Version** | **Written/Revised by:** | **Effective Date:** | **Summary of Revisions** |
| 1 | Immune cell Phenotyping by Flow Cytometry  | 3/4/91 | Updated 1993 |
| 2 | Colleen Berglund | 08/05/97 | Supercedes procedure Immune Status Panel |
| 3 | Colleen Berglund | 9/11/1999 | Updated |
|  | 4 | Colleen Berglund | 01/13/2000 | Updated |  |  |
| 5  | Jim Berger | 02/01/2011 | Prepping Immune Status and Immunodeficieny panels using LWA |
|  | 6 | Al Quigley | 03/31/13 | Updated for CMS Web |
|  | 7 | Amanda McCaustland | 08/28/20 | Updated processing and cytometer instructions, removed references to LWA multi-test prep, added instructions for Sunquest result reporting, formatting |