**Supplementary materials**

***Standard operation procedures (essential)***

Daily cleaning and calibration procedures are recommended. Compensation process must be done every month or when necessary, according to the stability of the flow cytometer parameters.

Flow cytometer FACSCalibur: to ensure the cytometer stability, BD Calibrite beads (BD) ™ are used daily with the BD FACSComp software (BD) ™ , in order to adjust the detectors voltages, between the target channel values and perform a sensitivity test, comparing the signal separation of each parameter with the minimum expected value.1

FACS Canto II settings must be performed daily using Setup & Tracking (CS&T) beads (BD Biosciences) ™ and Eight-peak Rainbow bead calibration (Spherotech, Lake Forest, IL, USA) ™ as previously described.2

Routine preventive maintenance should be performed periodically, that is, at least every six months

***Staining of membrane surface antibodies (recommended):***

* 100μL of sample per tube are incubated for 30 minutes at RT and in the dark with pre-titrated saturated amounts of 4-color combinations of fluorochrome-conjugated MoAb - FITC, PE, PerCPCy5, APC (Tables 1 and 2).
* Non-nucleated erythrocytes are lysed using FACS Lysing solution – BD BiosciencesTM, San Jose, CA, USA - cat. Reference 349202, according to the manufacturer's instructions.
* Sequentially, the remaining cells are centrifuged, washed twice in phosphate buffered saline (PBS; pH 7.4) and resuspended in 500 mL of PBS.
* Intracellular staining is performed using a permeabilizing reagent (Fix&Perm - InvitrogenTM, Camarillo, CA, USA reagent kit) as recommended by the manufacturer, after staining the cell surface membrane markers.
* Finally, the cell pellet is resuspended in 500 mL of acquisition buffer.

***Combined staining of intracellular and membrane surface markers (recommended):*** after performing the protocol described above:

* Add 2 mL of wash buffer to the cell pellet. Mix well in vortex.
* Centrifuge for 5 min at 540 g.
* Discard the supernatant using a Pasteur pipette or vacuum system, without disturbing the cell pellet, leaving approximately 100 mL of residual volume in each tube.
* Resuspend the cell pellet by mixing gently. Add 100 μL of Reagent A (fixative; Fix&Perm, Nordic-MUbio, Catalog number: GAS-002-1, Susteren, Netherlands) and mix using a vortex for 1-2 seconds.
* Incubate for 15 min at RT protected from light.
* Add 2 mL of wash buffer to the cell pellet. Mix well in vortex.
* Centrifuge for 5 min at 540 g. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 100 μL of residual volume in each tube.
* Mix vigorously by vortexing to fully resuspend the cell pellet.
* Add 100 μL of Reagent B (permeabilizing solution; Fix&Perm). Mix well by vortexing.
* Add the intracellular antibodies in the appropriate volume. Mix well by vortexing.
* Incubate for 15 min at RT protected from light.
* Add 2 mL of wash buffer to the cell pellet. Mix well by vortexing.
* Centrifuge for 5 min at 540 g. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 100 μL residual volume in each tube.
* Resuspend the cell pellet in 500 μL of acquisition buffer.

***Sample acquisition (essential)***

Data acquisition is recommended to be performed immediately after sample staining preparation. When it's not possible, the sample should be stored at 4 ° C for a maximum of 1 hour. The acquisition of the total sample in each tube must be done with "medium" flow rate set.

***Recommendations for reporting results (recommended)***

ALL residual cells should be reported as a percentage of all bone marrow nucleated cells (CD45 + and CD45 negative cells) and not as a percentage of the total sample (without excluding debris), for a better comparison with molecular techniques, because DNA is isolated only from nucleated cells3. Although the lysis process can affect the total number of nucleated cells because ammonium chloride also lyses some nucleated erythroid cells, the impact of different lysis approaches appears to have a negligible effect on the detected MRD level. However, the method used must be clearly reported.3

It is essential that the report indicates the sensitivity level of the test, which is determined by the limit of detection (LOD) and the lower limit of quantification (LLOQ), especially in cases where no disease has been detected.4

Conventionally, 20 homogeneous events were considered as a minimum value to compose a population of cells in a flow cytometric data file. This implies that the LOD can be estimated by dividing 20 (cells) by the total number of cells analyzed and multiplied by 100% (20 ÷ total cells analyzed x 100%). Likewise, it is also widely accepted that more than 50 events are a standard limit for the reproducible enumeration of a population of cells in a flow cytometer data file. Consequently, LLOQ can be estimated as 50 (cells) divided by the total number of cells analyzed and multiplied by 100% (50 ÷ total cells analyzed x 100%).3,5-7

Additional recommendation to be reported is the pattern of intensity of antigen expression in residual leukemic cells compared to their normal counterparts. It is necessary to include the percentage of positive cells for each marker. This is useful for monitoring MRD detection.3

Information about the inadequate quality of the sample, such as clots, hemodilution, hypocellularity, etc., must be reported, to be considered a collection of a new sample.8

Finally, the report must be clear, whether the MRD was detected or not and whether it was not possible to conclude at that time for any reason.

**References:**

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**Table S-1.** Clones, fluorochromes and titration of MoAb

|  |  |  |  |
| --- | --- | --- | --- |
| **Monoclonal Ab** | **Fluorochrome** | **Clones** | **Amount (μL)** |
| **CD20** | FITC | L27 20 / 2H7 | 20 |
| **CD38** | FITC | HB7 | 10 |
| **CD45** | FITC | 2D1 | 10 |
| **CD15** | FITC | MMA | 10 |
| **CD65** | FITC | 88H7 | 3 |
| **TdT** | FITC | HT6 / E17-1519 | 3 |
| **CD7** | FITC | 4H9/ M-T701 | 10 |
| **CD44** | FITC | L178 | 20 |
| **CD10** | PE | HI10A | 5 |
| **CD66c** | PE | KOR-SA3544 / B6.2 | 5 |
| **CD123** | PE | 9f5 / 7G31 | 5 |
| **CD81** | PE | JS-81 | 20 |
| **CD73** | PE | AD-2 | 20 |
| **CD304** | PE | 12C2 | 5 |
| **NG2** | PE | 7.1 | 5 |
| **CD3** | PE | SK7 / UCHT1 | 20 |
| **CD7** | PE | M-T701 | 10 |
| **CD99** | PE | TÜ12 | 5 |
| **CD117** | PE | 104D2 | 20 |
| **CD34** | PerCPcy5.5 | 8G12 | 5 |
| **CD3** | PerCPcy5.5 | SK7 | 5 |
| **CD5** | PerCPcy5.5 | L17F12 | 10 |
| **CD19** | APC | SJ25C1 / HIB19 | 20 |
| **CD7** | APC | M-T701 | 2 |
| **CD1a** | APC | HI149 | 5 |
| **CD13** | APC | WM15 | 20 |
| **CD33** | APC | P67.6 | 3 |
| **CD56** | APC | NCAM16.2 | 5 |
| **CD45RA** | APC | HI 100 | 5 |

**Table S-2.** Median Fluorescence Intensity values of MoAb used for 4-color MFC assays in 17 samples (10 BCP-ALL, 4 T-ALL, 1 T-ALL MRD, 1 BCP-ALL MRD, 1 normal)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **MoAb** | **BCP-ALL**  (min-max)  median | **Normal mature**  **B cells**  (min-max)  median | **MoAb** | **T-ALL**  (min-max)  median | **Normal mature**  **T cells**  (min-max)  median |
| **FITC** | | | | | |
| **CD20** | (164,7-5001,1)  282,9 | (3304,3-24029,3)  6683,4 | **NuTdT** | (319,0-2074)  2031,0 | (158-556,6)  390,3 |
| **CD45** | (622,4-3190,8)  1451,8 | (12956,9-28686,8)  18275,1 | **CD45** | NA | (24456,3-28750,5)  26603,4 |
| **CD38** | (145,8-2832,7)  694,6 | (114,9-337)  209,4 | **CD7** | (1836-7546,2)  2631,6 | (1714,4-10260)  2580,7 |
| **CD15+CD65** | (70,7-5231,0)  183,0 | (11,2-140,4)  100,6 | **CD44** | 357,4 | 3774,2 |
| **PE** | | | | | |
| **CD10** | (31,3- 37581,4)  3789, 5 | (-203-71,9)  13,2 | **CD7** | (5921,8-15024,1)  7981,5 | (235,6-5608)  3989,5 |
| **CD19** | (201,7-11433)  1481,9 | (349,1-4161,8)  1806 | **CD99** | (632,3-4747,3)  3945,3 | (204,1-3495)  652,8 |
| **CD66c+CD123** | (57-4459)  502,5 | (-106-149,8)  63,6 | **CD10+CD117** | (65,6-150,6)  108,1 | (25,7-35,3)  30,5 |
| **CD73+CD304** | (50,7-727)  524,2 | (557,5-962)  759,7 |  |  |  |
| **NG2** | (26,9-1939)  49,4 | (16,4-53)  31,3 |  |  |  |
| **PerCPCy5.5** | | | | | |
| **CD34** | (108,5-5766)  1195,7 | (51,8-177,5)  86,7 | **cyCD3** | (349,2-5983,1)  3403,7 | (167,9-12748)  4874,7 |
|  |  |  | **CD5** | (1052,1-3824,7)  3815,5 | (6330,3-20360,0)  14525,6 |
|  |  |  | **CD34** | NA | NA |
| **APC** | | | | | |
| **CD19** | (478,3-11057,2)  4777,5 | (3800,3-14905,8)  4678,6 | **CD7** | NA | (8968,5-11058)  10013,2 |
| **CD81** | (2608-48062,8)  13439,4 | (4304-10566,5)  7270,5 | **CD1a** | (34-273)  68,5 | (27,1-2228)  80,8 |
| **---** | **---** | **---** | **CD45RA** | (55,73-202,87)  81,7 | (78-2710)  1367,7 |

NA: not available for the analyzed samples