ZAP-70 assessment by flow Cytometry: improving assay sensitivity, robustness and workflow using an isoclonic control and PerFix-nc.

For distribution Outside US only.
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INTRODUCTION

ZAP-70 testing by flow cytometry is a fast and cost-friendly alternative method to molecular assays (Crespo et al., N Engl J Med 2003). However, since the method was established in 2003 the analysis strategy for assessment of ZAP-70 expression has been a matter of debate. In order to overcome the operator sensitivity seen with manual gating methods (Crespo et al., N Engl J Med 2003) a mean fluorescence intensity-based method was proposed (Shankey et al. Cytometry B Clin Cytom. 2006). By defining a background-corrected T/B ZAP-70 ratio a more objective approach was achieved. A recent publication questions this approach, demonstrating that the level of ZAP-70 expression in T cells is donor dependent which impacts the sensitivity of the T/B method. The use of an isoclonic control as internal negative control and normalization factor is shown to be a more accurate approach (Rizzo et al., Cytometry B Clin Cytom. 2013).

Instead of time-consuming classical or Triton-based intracellular staining methods a PerFix-nc-based protocol is applied that supersedes the majority of washing steps, thus improving reproducibility and lab efficiency. PerFix-nc kit also allows for simultaneous detection of surface & intracellular antigens simplifying the workflow.

The use of ZAP-70-PE and the utility of unlabeled ZAP-70 as an isoclonic control, combined with a PerFix-nc-based fixation and permeabilization protocol, provides a robust and fast procedure overcoming the so far unmet challenges of ZAP-70 flow cytometry assays.
ZAP-70 Antibodies  

<table>
<thead>
<tr>
<th>Clone: SBZAP (IgG1 Mouse)</th>
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<tbody>
<tr>
<td>Anti-ZAP-70-Purified*</td>
</tr>
<tr>
<td>Anti-ZAP-70-PE</td>
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* Please note that the concentration of ZAP-70 purified is a 100x excess of ZAP-70-PE concentration.

PerFix-nc Kits

<table>
<thead>
<tr>
<th>RUO</th>
<th>CE-IVD &amp; IVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 tests</td>
<td>B10825</td>
</tr>
<tr>
<td>150 tests</td>
<td>B10826</td>
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</tbody>
</table>

For more information regarding the PerFix-nc kit, please refer to the regulatory status available in your country.

The use of Isoclonic negative controls has shown to be very valuable in flow cytometry. The negative signal obtained with the incubation of a fluorochrome-conjugated antibody and a large excess of its unlabeled counterpart represents the true non-specific background of the tested antibody itself. To validate ZAP-70 assay and increase confidence in results obtained assessing expression of ZAP-70 on abnormal populations, ZAP-70-PE can be used together with the unlabeled ZAP-70 antibody. The isoclonic ratio can be calculated as the ratio between ZAP-70 MFI on abnormal B-cells from the test tube (ZAP-70-PE only) and the isoclonic control tube (ZAP-70-PE in presence of ZAP-70 purified).

CE-IVD & IVD: This reagent complies with the IVD and CE classifications.
CE-marked products are sold in European Union countries.
IVD products are sold in countries where they comply with applicable regulation.

ZAP-70 expression in Abnormal B-cells and T-cells from abnormal Peripheral Blood Mononuclear Cells (PBMC).

Staining of different lymphocytes subsets. Lymphocytes were first gated from a FSC/SSC biparametric histogram (not shown). The biparametric histogram (CD19-FITC/CD5-APC) shows NK cells (light blue), T-cells (green), residual normal B-cells (red) and abnormal B-cells (dark blue).

Monoparametric histogram overlay of ZAP-70 labeling with ZAP-70-PE and ZAP-70-PE in the presence of ZAP-70 purified for abnormal B-cells.

Monoparametric histogram overlay of ZAP-70 labeling with ZAP-70-PE and ZAP-70-PE in the presence of ZAP-70 purified for T-cells.
STAINING PROTOCOLS

Whole Blood

1. Dispense 50 µL of blood into empty tube
2. Add **5 µL of Fixative Reagent R1**, mix tube
3. Incubate 15 min. at Room Temperature. Then, mix tube.
4. Add **300 µL of Permeabilizing Reagent R2**. Mix tube
5. Add 20 µL ZAP-70 purified**, mix tube immediately and incubate 10 min at Room Temperature
6. Add 20 µL ZAP-70-PE and appropriate volumes of surface markers, mix tube immediately
7. Incubate 45 min. at Room Temperature
8. Add **3 mL of Final Reagent 1X R3**, mix tube
9. Centrifuge 5 min. at 500 g, aspirate supernatant into waste
10. Re-suspend cells in 500 µL of Final Reagent 1X R3. Hold at 4°C, protect from light, until analysis

PBMC

1. Resuspend the PBMC in 100% serum (FCS or FBS), at a concentration ranging from “normal” (5.10^6/mL) to “high” (4.10^7/mL).
2. Pipet 50 µL of these cells, into the bottom of each appropriately labeled tube and add **2,5 µL of Fixative Reagent R1**, mix tube
3. Incubate 15 min. at Room Temperature
4. Add **150 µL of Permeabilizing Reagent R2**.
5. Add 20 µL ZAP-70 Purified*, mix tube immediately and incubate 10 min at Room Temperature
6. Add 20 µL ZAP-70-PE and appropriate volumes of surface markers, mix tube immediately
7. Incubate 45 min. at Room Temperature
8. Add **3 mL of Final Reagent 1X R3**, mix tube
9. Centrifuge 5 min. at 500 g, aspirate supernatant into waste
10. Re-suspend cells in 500 µL of Final Reagent 1X R3. Hold at 4°C, protect from light, until analysis

* Please note that the concentration of ZAP-70 purified is a 100x excess of ZAP-70-PE concentration.

! For a staining without the isoclonic control, please skip step 5 !