Ref: CYT-MM-MRD

For research use only

**MM-MRD VIALS ARE A LYOPHILIZED PRODUCT. READ CAREFULLY THE FOLLOWING INSTRUCTIONS FOR RECONSTITUTION:**

The lyophilized Multiple Myeloma Minimal Residual Disease (MM-MRD) panel preserves the stability of the pre-mixed combination of antibodies. Reconstitute each lyophilized vial containing the pre-mixed combinations with distilled water as follows:

- Tube 1: 180 µl distilled water.
- Tube 2 surface staining: 120 µl distilled water.
- Tube 2 cytoplasmic staining: 70 µl distilled water.

Mix well and let the solution at least 30 minutes. Unused volume of reconstituted vial is stable during four weeks from date of reconstitution if it is stored in the dark at 2-8º C.

**INTENDED USE**

MM-MRD panel is a pre-mixed 6 colour antibody combinations designed for the accurate identification and discrimination of malignant and normal plasma cells (PCs) in bone marrow samples from treated MM patients. This reagent must be used by flow cytometry qualified personal.

**PRINCIPLES OF THE PROCEDURE**

Flow cytometry is an innovative technology by means of which different cell characteristics are simultaneously analyzed on a single cell basis. This is achieved through hydrodynamic focusing of cells that pass aligned one by one and they are illuminated by a laser beam. The interaction of the cells with the laser beam generates signals of two different types: those generated by dispersed light (Forward Scatter (FSC) / Side Scatter (SSC)), which mainly reflects the size of the cell and its internal complexity, and those related to the emission of light by the fluorochromes present in/on the cell. These signals become electric impulses which are amplified and registered as digital signals to be processed by a computer. When the reagents are added to the sample, the mixture of fluorochrome-labeled antibodies present in the reagents bind specifically to the antigens they are directed against, allowing the detection by flow cytometry of the different cell subsets. The erythrocyte population, which could hinder the detection of the target population, is eliminated by the use of a red blood cell lysis solution previous to acquire the sample on the cytometer.

**SUMMARY AND EXPLANATION**

In general, independently the pathology, MRD is defined by the presence, at low frequencies, of residual malignant cells post therapeutic intervention (1). In the particular case of MM, studies have demonstrated that the presence of a 0,01% aberrant plasma cells (considered, at present, MRD positive cases) is associated with a poor outcome (1). Recent surveys regarding the current practices in MM-MRD detection by flow cytometry revealed great heterogeneity in methodology: antibody panel, identification strategy and numbers of events acquired (2). The 2008 European Myeloma Network report (3) together with recent publications (4, 5) establish CD38, CD138 and CD45 analysis in combination with CD19, CD56, CD27, CD81 and CD117 as consensus on the best suited method for MRD detection in MM. It was also established that cytoplasmic kappa(κ)/lambda(λ) light chain staining may be helpful to clarify atypical findings in rare disease subtypes. This consensus is translated and improved in the two EuroFlow MM-MRD multicolor flow cytometry combinations.

The immunophenotypic characterization is not using isotype controls, basically because there are internal controls as other leukocyte populations to define negative and positive limits (always when applying a standard operating procedure for instrument calibration you will find a complete guide on the web site www.EuroFlow.org).
Using standardized approaches for MM-MRD detection, for both sample preparation and identification strategy, can convert flow cytometry analysis into a fast, reliable, reproducible, cost effective and most importantly sensitive method among currently used for this application.

MM-MRD kit is carefully designed to specifically recognize by flow cytometry the following antigens CD38, CD45, CD56, CD19, CD81, CD117, cytoplasmic IgKappa and IgLambda. The light chains used for clonality assessment are polyclonal. The use of polyclonal instead over monoclonal antibodies has demonstrated advantages for the detection of intracellular immunoglobulin light chains in the clonality assessment of PCs.

**REAGENT COMPOSITION**

**Material included**

MM-MRD kit is supplied as 2 different 6-color combinations containing enough volume for 20 tests containing:

- Fixative free ammonium chloride erythrocyte lysing solution (BulkLysis™).
- Fixation and permeabilization solutions (Fix&Perm®, Nordic-MUBio BV, The Netherlands) for the cytoplasmic detection of immunoglobulin light chains Kappa/Lambda sufficient for 20 tests.
- Following reagents for specific tandem compensation ready to use in liquid format sufficient for 5 tests (5µl/test):
  - CD45-PerCP-Cyanine 5.5
  - CD19-PE-Cyanine7
  - CD81-APC-C750
  - Lambda APC-C750

Tube 1 contains the following lyophilized antibodies mixture for surface staining (4 lyophilized vials of 5 tests each one):

- Anti-human CD38-FITC antibody, multi-epitope.
- Anti-human CD56-PE antibody, clone: C5.9, isotype: IgG2b.
- Anti-human CD45-PerCP-Cyanine 5.5 antibody, clone: EO1, isotype: IgG2b.
- Anti-human CD81-APC-C750 antibody, clone: M38, isotype: IgG1.

Tube 2 is composed by:

- Lyophilized antibodies mixture for surface staining (4 lyophilized vials of 5 tests each one):
  - Anti-human CD38-FITC antibody, multi-epitope.
  - Anti-human CD56-PE antibody, clone: C5.9, isotype: IgG2b.
  - Anti-human CD45-PerCP-Cyanine 5.5 antibody, clone: EO1, isotype: IgG2b.

- Lyophilized antibodies mixture for cytoplasmic staining (4 lyophilized vials of 5 tests each one):
  - Anti-human CyIg-APC goat antibody, clone: polyclonal.
  - Anti-human CyIgk-APC-C750 goat antibody, clone: polyclonal.

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<th>PerCP-Cyanine5.5</th>
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*CD27 and CD138 are not included in the kit. It is recommended to add these antibodies to the cocktail before use.

All components contains sodium azide (NaN₃) ≤0.09% (m/v). Reagents are not considered sterile.

**Material required but not included**

- 3 laser-equipped flow cytometer (8 colors) and appropriate computer hardware and software.
- Anti-human CD27 and CD138 antibodies must be added as a drop in (EuroFlow™ recommendation-CD27 BV510 and CD138 BV421).
- Test tubes suitable for obtaining samples in the flow cytometer used. Usually tubes with a rounded bottom for 6 ml, 12x 75 mm are used.
- Automatic pipette and tips
- 50 ml tubes
- Chronometer
- Vortex Mixer
- Centrifuge
• Lysing solution containing a fixative agent
• Washing buffer: phosphate buffered saline (PBS) containing ≤0.09% (m/v) sodium azide and bovine serum albumin (BSA) 0.5% (m/v).

**STORAGE CONDITIONS**
The reagent is stable until the expiration date shown on the label, when stored at 2-8º C. The reagents should not be frozen or exposed to direct light during storage or during incubation with sample. Keep all reagent vials in a dry place. Once opened, the vial must be stored in a vertical position to avoid any possible spillage.

**WARNINGS AND RECOMMENDATIONS**
1. For research use only.
2. Alteration in the appearance of the reagent, such as the precipitation or discoloration indicates instability or deterioration. In such cases, the reagent should not be used.
3. It contains ≤0.09% (m/v) sodium azide (CAS-Nr. 26628-22-8) as a preservative, but even so care should be taken to avoid microbial contamination of reagent or incorrect results may occur.

**Indication(s) of danger:**
- H302 Harmful if swallowed

**Safety advice:**
- P264 Wash thoroughly after handling.
- P270 Do not eat, drink or smoke when using this product.
- P301+P312 If swallowed, call a poison center or doctor/physician if you feel unwell.
- P301+P330 If swallowed, rinse mouth.
- P501 Dispose of contents/container in accordance with local/regional/national/international regulation.

4. All patient specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection (7), and disposed according to the legal precautions established for this type of product. Also recommended is handling of the product with appropriate protective gloves and clothing, and its use by personnel sufficiently qualified for the procedures described. Avoid contact of samples with skin and mucous membranes. After contact with skin, wash immediately with plenty of water.

5. Use of the reagent with dilutions, incubation times or temperatures different from those recommended may cause erroneous results. Any such changes must be validated by the user.
6. Any serious incident relating to the product must be reported to Cytognos S.L. as well as the competent professional authority of the Member State in which the user is established.

**PROCEDURE**
**Preparation**
Sample must be collected in commercially available anticoagulant-treated tube (use of EDTA is recommended) (8, 9).

1. Determine the absolute count of leukocytes per µl for the sample to be processed.
2. Transfer the sample containing at least 10 x 10^6 nucleated cells for each MRD tube to be stained to a 50 ml tube (consider that during BulkLysis process some cells are unselectively lost). Do not use more than 2 ml of sample per 50 ml of lysing solution. If larger volumes of sample need to be processed (i.e. starting cells concentration is low), use several 50 ml tubes.
3. Fill the tube up to reach 50 ml volume with BulkLysis™ (CYT-BL) (diluted to 1X in distilled water and at room temperature - RT).
4. Mix well and incubate for 15 min in a roller or sample-shaker device.
5. Centrifuge at 800 g for 10 min and remove the supernatant using a Pasteur pipette or a vacuum system without disturbing the cell pellet. Typically, 300 µl of cell suspension should remain in the tube.
6. Add 2 ml of PBS + 0.09% (m/v) of NaN₃ + 0.5 % (m/v) of BSA and resuspend the cell pellet vigorously.
7. Complete the volume of the tube containing the cell suspension up to 50 ml final volume with PBS + 0.09% (m/v) of NaN₃ + 0.5 % (m/v) of BSA.
8. Mix well.
9. Centrifuge at 800 g for 5 min and remove the supernatant using a Pasteur pipette or a vacuum system, without disturbing the cell pellet.
10. Resuspend the cell pellet in 2 ml of PBS + 0.09% (m/v) of NaN₃ + 0.5 % (m/v) of BSA. Mix well and transfer this volume to a 5 ml “FACS tube”.
11. Wash the 50 ml Falcon tube with 2 mL of PBS + 0.09% (m/v) of NaN₃ + 0.5 % (m/v) of BSA more to recover cells that might have left in the original tube. Add this volume to the 5 ml tube containing the rest of the sample transferred in step 10.
12. Centrifuge at 540 g for 5 min and remove the supernatant by decanting or using a Pasteur pipette. If the remaining cell volume is lower than 300 µl, PBS + 0.09% (m/v) of NaN₃ + 0.5 % (m/v) of BSA will be added to reach a volume of at least 300 µl.
13. In case multiple 50 ml tubes were used (because it was needed to lyse large sample volumes) the cell suspensions from the same sample should be combined at this moment, before adjusting cell concentration. Try to keep the final volume low, so that, in case that cell concentration needs to be adjusted as indicated in the next step, it can be easily done by diluting with the recommended buffer.
14. Adjust the final cells concentration to $1 \times 10^5$ cells/µl, by resuspending the pellet with PBS + 0,09% (m/v) of NaN$_3$ + 0,5% (m/v) of BSA.

15. Adjust the volume in order to obtain 100 µl containing $10 \times 10^6$ cells of the cell suspension per each tube to be stained/acquired.

**Staining steps for surface membrane markers only (MM-MRD Tube1):**
Reconstitute the lyophilized vial using 180 µl distilled water, mix well and let the solution at least 30 minutes.
Add 30 µl of antibody mixture and proper volume of CD27 and CD138 (not included in the mixture). Then add the cell suspension ($10 \times 10^6$ cells per tube).
Mix well. For optimal staining conditions, if needed, complete with PBS until a final volume of 200 µl.
Incubate for 30 min at RT protected from light.
Add 2 ml of fixative lysing solution.
Mix well.
Incubate for 10 min at RT protected from light.
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.
Add 2 ml of PBS+0,5% (m/v) BSA + 0,09% (m/v) NaN$_3$ to the cell pellet.
Mix well.
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 PBS + 0,5% (m/v) BSA (without NaN$_3$).
Acquire the cells immediately after staining or (if not immediately acquired) store at 4°C (for 1h maximum) until measured in the flow cytometer.
Acquire the sample in medium flow rate.

**Staining steps for combined staining of surface membrane and cytoplasmic markers (MM-MRD Tube 2):**
Reconstitute the lyophilized vial for surface staining using 120 µl distilled water, mix well and let the solution at least 30 minutes.
Add 20 µl of antibody mixture in a tube and proper volume of CD27 and CD138 (not included in the mixture). Then add the cell suspension ($10 \times 10^6$ cells per tube).
Mix well.
If needed, add PBS in order to obtain a final staining volume of 200 µl.
Incubate for 30 min at RT protected from light.
Add 2 ml of PBS + 0,5% (m/v) BSA + 0,09% (m/v) NaN$_3$ to the cell pellet.
Mix well.
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 by mixing gently.
Add 100 µl of Reagent A (fixative; Fix&Perm®, Nordic-MUBio BV, The Netherlands) and mix thoroughly.
Incubate for 15 min at RT protected from light.
Add 2 ml of PBS + 0,5% (m/v) BSA + 0,09% (m/v) NaN$_3$ to the cell pellet.
Mix well.
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 by mixing thoroughly.
Add 100 µl of Reagent B (permeabilizing solution; Fix&Perm®, Nordic-MUBio BV, The Netherlands).
Mix well.
Reconstitute the lyophilized vial for cytoplasmic staining using 70 µl distilled water.
Add 10 µl of antibody mixture in the tube containing the 200 µl Reagent B+cell suspension.
Mix well.
Incubate for 15 min at RT protected from light.
Add 2 ml of PBS + 0,5% (m/v) BSA + 0,09% (m/v) NaN$_3$ to the cell pellet.
Mix well.
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 PBS + 0,5% (m/v) BSA (without NaN$_3$).
Acquire the cells immediately after staining or (if not immediately acquired) store at 4°C (for 1h maximum) until measured in the flow cytometer.
Acquire tube completely at medium flow rate.
Important Recommendations
In order to achieve optimal results, it is needed to follow the EuroFlow Standard Operating Protocol for Cytometer Setup (10). You will find a complete guide (Cytometer Setup SOP) on the web site www.EuroFlow.org, which includes recommendations for FSC, SSC and target voltage PMT settings, compensation setup and instrument performance monitoring.

Flow cytometry analysis
Cytognos recommends the use of the analysis software Infinicyt™ (11), which provides a revolutionary approach for data integration and multidimensional analysis of flow cytometry data. Its innovative features make the analysis and interpretation of the results easier, faster and more accurate. Infinicyt™ comprises exclusive tools that allow for a better identification and description of the different cell populations. It is being developed as part of the EuroFlow™ project “Flow cytometry for fast and sensitive diagnosis and follow-up of haematological malignancies”, in which highly qualified cytometry professionals are involved. Infinicyt™ includes a wide variety of analysis tools and exclusive graphics which allow analysis with a reference picture, or calculate new virtual parameters. You will find complete information about Infinicyt™ on the web site: www.infinicyt.com.

1. Load the Plasma Cell MM-MRD Profile.
2. Load the Analysis Strategy.
3. Check the PC population/s and assure their positive and high expression for CD38, CD138 and the expression of CD45 from dim to negative. Whenever needed, move the analysis gates according to the image of each individual case.
4. Assign the PC population.
5. Load the normal PC reference image from profile.
6. Classify the PC population according to CD19 and CD56 expression.
7. Check for each of the four PC subpopulations the kappa/lamba ratio.
8. Check for abnormal expression on malignant PC of CD27 (aberrant low to negative expression), CD117 (aberrant positive expression) and CD81 (abnormal completely negative expression).

LIMITATIONS
It is advisable to acquire stained samples as soon as possible to optimize results. Non-viable cells may show unspecific staining. Prolonged exposure of samples to lytic reagents may cause white cell destruction and targeted population cell loss. When using whole blood lysing procedures some red blood cells may not lyse, for instance if there are nucleated red blood cells or if abnormal protein concentration and haemoglobinopathies are observed. This may cause misleading results since unlysed red blood cells are counted as leucocytes.

Results obtained by flow cytometry may be erroneous if cytometer laser is misaligned or if gates are incorrectly set. Knowledge of antigen normal expression pattern and its relation to other relevant antigens is paramount to carry out an adequate analysis.

QUALITY CONTROL
- Pipettes precision and cytometer calibration should be verified to obtain optimal results.
- In multicolour panels, fluorochromes emit in wavelengths that can show certain spectral overlap which must be corrected by electronic compensation. Optimal compensation levels can be established by analysing cells from healthy individuals stained with mutually exclusive monoclonal antibodies conjugated with appropriate fluorochromes.
- This product has been manufactured in accordance with standards of production and quality system of the ISO 13485:2012 standard.

REFERENCES

WARRANTY
This product is warranted only to conform to the quantity and contents stated on the label. There are no warranties that extend beyond the description on the label of the product. Cytognos’s sole liability is limited to either replacement of the product or refund of the purchase price.

EXPLANATION OF SYMBOLS

PRODUCED BY
CYTOGNOS SL
Polígono La Serna, Nave 9
37900 Santa Marta de Tormes
Salamanca (España)
Phone: + 34-923-125067
Fax: + 34-923-125128
Ordering information: admin@cytognos.com
Technical information: support@cytognos.com
www.cytognos.com