MM MRD
Multiple Myeloma Minimal Residual Disease
Panel

EuroFlow

drop-in	drop-in	FITC	PE	PerCP-Cyanine5.5	PE-Cyanine7	APC	APC-C750™
CD138	CD27	CD38	CD56	CD45	CD19	CD117	CD81
CD138	CD27	CD38	CD56	CD45	CD19	Cylgκ	Cylgλ

Ref: CYT-MM-MRD

RUO

For Research Use Only. Not for use in diagnostic procedures

THE MM MRD VIALS CONTAIN LYOPHILIZED PRODUCTS. READ CAREFULLY THE FOLLOWING INSTRUCTIONS FOR RECONSTITUTION:

The lyophilized format of the tubes to detect Multiple Myeloma Minimal Residual Disease (MM MRD) preserves the stability of the pre-mixed combination of antibodies. Reconstitute each lyophilized vial as follows:

- **Tube 1: 180 µL of distilled water.**
- **Tube 2 surface staining: 120 µL of distilled water.**
- **Tube 2 cytoplasmic staining: 70 µL of distilled water**.

Mix thoroughly each reconstituted vial in a "roller" mixer for at least 30 minutes at room temperature before use. Spin down reconstituted vials before each use.
Unused volume of the reconstituted vials is stable during four weeks from reconstitution date if stored in the dark at 2-8 ºC.

THE COMPENSATION TUBES CONTAIN LYOPHILIZED REAGENTS. READ CAREFULLY THE FOLLOWING INSTRUCTIONS FOR RECONSTITUTION:

To reconstitute the **lyophilized compensation tubes, add directly to the tube the corresponding volume of sample intended for compensation**: peripheral blood (PB) or beads**. Incubate 30 minutes at room temperature in the dark, and then proceed with conventional protocol**: EuroFlow™ standard operating protocol (SOP) for cell surface staining in the case of PB (www.euroflow.org) or manufacturer protocol for beads**.**

INTENDED USE

MM MRD kit is a pre-mixed 6-color antibody combination to be used with the drop-ins CD138 and CD27. This kit is designed for the accurate identification and discrimination of malignant and normal plasma cells (PCs) in bone marrow samples.
This kit must be used by flow cytometry qualified personnel.

PRINCIPLES OF THE PROCEDURE

Flow cytometry is a technology that allows to simultaneously evaluate different characteristics of a single cell. Flow cytometers use hydrodynamic or acoustic focusing to individually present cells to one or more laser beams. As cells are intercepted by light, a set of detectors recovers two types of signals: those generated by dispersed light (FSC/SSC), which mainly reflect cell size and internal complexity, and those related to fluorochromes light emission when cells are labelled. Recovered signals are then amplified by a series of linear and logarithmic amplifiers and converted into electrical signals to be plotted.
The fluorochrome-labeled antibodies bind specifically to the antigens they are directed against, allowing for the detection by flow cytometry of the different cell subsets.

SUMMARY AND EXPLANATION

In general, independently of 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 theEuroFlow™ MM-MRD multicolor flow cytometry panel.
This immunophenotypic characterization is not using isotype controls, basically because there are internal controls such as other leukocyte populations to define negative and positive limits (always when applying a standard operating procedure for instrument calibration. A complete guide is available on the website www.euroflow.org).
The use of standardized approaches for MM MRD detection, for both sample preparation and identification strategies, can convert flow cytometry analysis into a fast, reliable, reproducible, cost-effective and most important sensitive method among the ones currently used for this application.
The 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 [6].

REAGENT COMPOSITION

MM MRD kit contains:

Two 6-color antibody combinations containing enough reagent for 20 tests.

- **Tube 1:**
  - **4 lyophilized vials of 5 tests for surface staining that contains:**
    - 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 CD19-PE-Cyanine7 antibody, clone: SA287, isotype: IgG1.
    - Anti-human CD117-APC antibody, clone: 104D2, isotype: IgG1.
    - Anti-human CD81-APC-C750™ antibody, clone: M38, isotype: IgG1.
- **Tube 2:**
  - **4 lyophilized vials of 5 tests each for surface staining that contains:**
    - 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 CD19-PE-Cyanine7 antibody, clone: SA287, isotype: IgG1.
  - **4 lyophilized vials of 5 tests each for cytoplasmic staining that contains:**
    - Anti-human CyIgκ-APC goat antibody, clone: polyclonal.
    - Anti-human CyIgλ-APC-C750™ goat antibody, clone: polyclonal.

	Fluorochrome	Drop In	Drop In	FITC	PE	PerCP-Cy5.5	PE-Cyanine7	APC	APC-C750™
TUBE 1	Antibody	CD138*	CD27*	CD38	CD56	CD45	CD19	CD117	CD81
	Clone			-	C5.9	EO1	SA287	104D2	M38
	Isotype			-	IgG2b	IgG2a	IgG1	IgG1	IgG1
TUBE 2	Antibody	CD138*	CD27*	CD38	CD56	CD45	CD19	Cy-Igκ	Cy-Igλ
	Clone			-	C5.9	EO1	SA287	-	-
	Isotype			-	IgG2b	IgG2a	IgG1	-	-

*Anti-human CD138 and CD27 antibodies are not included in the kit. It is recommended to add these antibodies as drop-ins when staining the sample following EuroFlow™ recommendations (www.euroflow.org), CD27-BV510 (10 µL/test) and CD138-BV421 (2µL/test).

- **Compensation reagents: 4 lyophilized tubes of 1 test each to compensate** CD45-PerCP-Cyanine5.5, CD19-PE-Cyanine7, CD81-APC-C750 and Lambda APC-C750™ conjugates.

  * Consult "important recommendations" for the compensation of Lambda-APC-C750™.

- **Fixative-free ammonium chloride erythrocyte lysing solution 10X** (BulkLysis™).

- **Fixation and permeabilization solutions** (Fix&Perm®, Nordic-MUBio BV, The Netherlands).

All components contain 0,09% (m/v) sodium azide (NaN_3), except BulkLysis™ which contains 1% sodium azide.
Reagents are not considered sterile.

STORAGE CONDITIONS

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

WARNINGS AND RECOMMENDATIONS

1. For research use only. Not for use in diagnostic procedures.
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. All components contain 0,09% (m/v) sodium azide (CAS-Nr. 26628-22-8) (except BulkLysis™, which contains 1% sodium azide). Although sodium azide is used as a preservative, care should be taken to avoid microbial contamination of reagents.
4. The indications about mixture dangers are described below:
   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 contents in a container in accordance with local/regional/national/international regulation.
5. The component BulkLysis™ contains 8,3% ammonium chloride, 1% EDTA and 1% (m/v) sodium azide (CAS-No. 26628-22-8).
6. The indications about BulkLysis™ dangers are described below:
   Mixture classification according to Regulation (EC) No 1272/2008
   Acute oral toxicity, category 5; Acute dermal toxicity, category 4.
   Harmful to aquatic life with lasting effects, category 3.
   Indication(s) of danger:
   H302 Harmful if swallowed.
   H315 Causes skin irritation.
   H319 Causes serious eye irritation.
   H412 Harmful to aquatic life with long lasting effects.
   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 contents in a container in accordance with local/regional/national/international regulation.
   For more information, consult product safety data sheet.
7. All patient specimens and materials 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. It is also recommended handling 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.
8. Use of reagent with dilutions, incubation times or temperatures different from those recommended may cause erroneous results. Any such changes must be validated by the user.
9. 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

Material required but not included

- 3 laser-equipped flow cytometer (8-color or more such as Omnicyt™) and appropriate computer hardware and software.
- The markers CD27-BV510 and CD138-BV421 should be used as “drop in” according to EuroFlow™ recommendation, 10µL / test and 2µL / test, respectively.
- Tubes for sample acquisition in flow cytometer (e.g. rounded bottom, 12 x 75 mm, 5 mL tubes).
- Automatic pipette and tips.
- 50 mL tubes.
- Chronometer.
- Pasteur pipettes or vacuum system.
- Vortex mixer.
- Roller mixer or sample-shaker device.
- Centrifuge.
- Lysing solution containing a fixative agent.
- Washing buffer: filtered solution of phosphate buffered saline (PBS) containing 0,09% (m/v) NaN_3, 0,2% (m/v) bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA).

- Acquisition buffer: filtered PBS solution containing 0,2% (m/v) BSA and 2 mM EDTA (NaN_3-free).

Preparation

Sample must be collected in commercially available anticoagulant-treated tube. Use of EDTA is highly recommended [8,9].
All steps indicated below should be performed a room temperature (RT).

Recommended procedure:

1. Determine the absolute count of nucleated cells per µL of the sample.
2. Transfer the sample, containing **at least** 20 x 10^6 nucleated cells to a 50 mL tube. Notice that during bulk lysing process an unselective loss of cells occurs, and a starting amount of 10 x 10^6 cells **per each** MM-MRD tube to be stained (total of 2 tubes) is needed. Do not use more than 2 mL of sample per 50 mL of **1X** BulkLysis™ solution (dilute **10X** BulkLysis™ reagent to **1X** using distilled water at RT). If larger volumes of sample are needed (i.e. starting cell concentration is low), use several 50 mL tubes.
3. Bring the final volume to 50 mL adding 1X BulkLysis™ to each tube.
4. Mix well by manually inverting the tube and incubate for 15 min in a roller or sample-shaker device.
5. Centrifuge at 800 g for 10 min and remove supernatant using a Pasteur pipette or a vacuum system without disturbing the cell pellet (approximately 300 µL of cell suspension should remain in the tube).
6. Add 2 mL of washing buffer and resuspend the cell pellet vigorously, preferably by vortex mixing.
7. Fill up the volume of the tube containing the cell suspension up to 50 mL with washing buffer.
8. Mix well by manually inverting the tube.
9. Centrifuge at 800 g for 5 min and remove supernatant using a Pasteur pipette or a vacuum system, without disturbing the cell pellet.
10. Resuspend the cell pellet in 2 mL of washing buffer. Mix well and transfer this volume to a 5 mL flow cytometry tube.
11. Wash the 50 mL Falcon tube with 2 mL of washing buffer to recuperate any cells that might be left in the bottom.
12. Add this volume to the 5 mL tube containing the rest of the sample that was transferred in step 10.
13. Centrifuge at 540 g for 5 min and remove the supernatant by decanting or using a Pasteur pipette.
14. In case multiple 50 mL tubes have been processed, combine all the cell suspensions from each tube in one single tube before adjusting cell concentration (try to keep the sample in a low final volume, with the aim to dilute with washing buffer in case cell concentration needs to be adjusted as indicated in the next step).
15. Adjust the volume in order to obtain 100 µL containing 10 x 10^6 cells of the cell suspension **per each** MM-MRD tube to be stained (total of 2 tubes), by resuspending the pellet with washing buffer.

Staining steps for surface membrane markers only (MM-MRD Tube1):

- Reconstitute the lyophilized vial using 180 µL of distilled water, mix well and place it in a roller for at least 30 min at RT before use.
- Add 30 µL of antibody mixture in a 5 mL flow cytometry tube and add CD27 and CD138 antibodies as recommended by EuroFlow™ (10µL/test and 2µL/test, respectively). Then, add the 100 µL of cell suspension.
- Mix well. Bring the final volume to 200 µL with washing buffer for optimal staining conditions.
- 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 of residual volume in the 5 mL tube.
- Add 2 mL of washing buffer.
- 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 of residual volume in the 5 mL tube.
- Resuspend the cell pellet with acquisition buffer in a total volume of:
  - 850 µL for Omnicyt™.
  - 500 µL for other cytometers.
- Acquire the whole volume of the sample immediately after staining or store at 4°C (for 1h maximum) until measurement in flow cytometer.
- The established sample acquisition flow rate will depend on the cytometer used.

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 of distilled water, mix well and place it in a roller for at least 30 min at RT before use.
- Add 20 µL of surface staining antibody mixture in a 5 mL flow cytometry tube and CD27 and CD138 antibodies as recommended by EuroFlow™ (10µL/test and 2µL/test, respectively). Then, add the 100 µL of cell suspension.
- Mix well. Bring the final volume to 200 µL with washing buffer for optimal staining conditions.
- Incubate for 30 min at RT protected from light.
- Add 2 mL of washing buffer 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 of residual volume in the 5 mL tube.
- Resuspend the cell pellet by mixing gently.
- Add 100 µL of Reagent A (Fix&Perm®, Nordic-MUBio BV, The Netherlands) and mix thoroughly.
- Incubate for 15 min at RT protected from light.
- Add 2 mL of washing buffer.
- 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 the 5 mL tube.
- Mix vigorously by vortex mixing to resuspend the cell pellet.
- Add 100 µL of Reagent B (Fix&Perm®, Nordic-MUBio BV, The Netherlands).
- Mix well.

- Reconstitute the lyophilized vial for **cytoplasmic staining** using 70 µL of distilled water, mix well and place it in a roller for at least 30 min at RT before use.
- Add 10 µL of cytoplasmic staining antibody mixture in the 5 mL tube containing the 200 µL of both Reagent B and cell suspension.
- Mix well.
- Incubate for 15 min at RT protected from light.
- Add 2 mL of washing buffer 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 of residual volume in the 5 mL tube.
- Resuspend the cell pellet with acquisition buffer in a total volume of:
    - 850 µL using Omnicyt™.
    - 500 µL using other flow cytometers.
- Acquire the whole volume of the sample immediately after staining or store at 4°C (for 1h maximum) until measurement in flow cytometer.
- The established sample acquisition flow rate will depend on the cytometer used.

Important Recommendations

In order to achieve optimal results, EuroFlow Standard Operating Protocol (SOP) for Cytometer Setup [10] should be followed. You will find a complete guide on the web site www.euroflow.org, which includes recommendations for FSC, SSC and target voltage PMT settings, compensation setup and instrument performance monitoring.

The Lambda-APC-C750™ antibody is of goat origin and it recognizes specifically human CyIgλ. Compensation might be performed using either anti-goat beads or peripheral blood. If compensation is performed with peripheral blood, please note that three (3) washing steps are required prior to staining to remove soluble plasma proteins.

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. Infinicyt™ innovative features make the analysis and interpretation of the results easier, faster and more accurate. This analysis software was developed as part of the EuroFlow™ project "Flow cytometry for fast and sensitive diagnosis and follow-up of hematological malignancies", in which highly qualified cytometry personnel has been involved. Infinicyt™ includes a wide variety of analysis tools and exclusive diagrams. You can find complete information about Infinicyt™ on the website: www.infinicyt.com.
EuroFlow™ has created a MM MRD database using normal samples that allows for the automated identification of all populations identified using this panel [13]. MM MRD FCS files can be analyzed using the database or manually using a pre-established gating strategy. Full description of the steps required for both analyses are listed below:

Files generated in compatible 8-color flow cytometers such as Omnicyt™, can be analyzed using the EuroFlow™ database which automatically analyzes the sample and gives a standard report in different languages.

1. Go to Profile > Configure > Databases > Language and select your preferred language for MM MRD reporting.
2. Click on Databases in the main window and select the two files to be analyzed (MM MRD tube 1 and 2).
3. Select type of sample Bone Marrow.
4. Select MM MRD database.
5. Click OK.
6. Confirm all labels are correct by comparing them to the ones in the database.
7. After the automatic gating and identification process is finished, verify all events in all Check Populations to find abnormal PCs.
8. Open the automatic report.

Note: contact support@cytognos.com for any question regarding MM MRD analysis using the EuroFlow™ databases

Files generated in non-compatible 8-color flow cytometers can be analyzed manually using the EuroFlow™ MM MRD Profile.

1. Load the MM MRD Profile from the Infinicyt™ profile manager.
2. Load the Analysis Strategy
3. Modify the gates to include all plasma cells.
4. Note that samples treated with CD38 monoclonal antibody therapies might show a dimmer expression of this marker.
5. Assign events to the Plasma cell population.
6. Load the normal reference image from the profile. Classify for each of the three normal PC subpopulations (based on CD19 and CD56) the kappa and lambda populations. Verify that the ratio is within normal.
7. Classify the abnormal PC population according to its differential expression from normal plasma cells.

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 hemoglobinopathies are observed. This may cause misleading results since unlysed red blood cells are counted as leukocytes.
- Results obtained by flow cytometry may be erroneous if the cytometer lasers are 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 [1-6,9,10].

QUALITY CONTROL

- Pipettes precision and cytometer calibration should be verified to obtain optimal results.
- In multicolor 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 analyzing 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 9001:2015 standard.

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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' sole liability is limited to either replacement of the product or refund of the purchase price.

EXPLANATION OF SYMBOLS

	Use by (YYYY-MM)
REF	Catalogue number
LOT	Batch code
	Keep out of sunlight
	Storage temperature limitation
	Consult instructions for use
	Manufacturer
RUO	For research use only
	Contains sufficient for <n> tests
	Health hazard/Hazardous to the ozone layer

PRODUCED BY

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