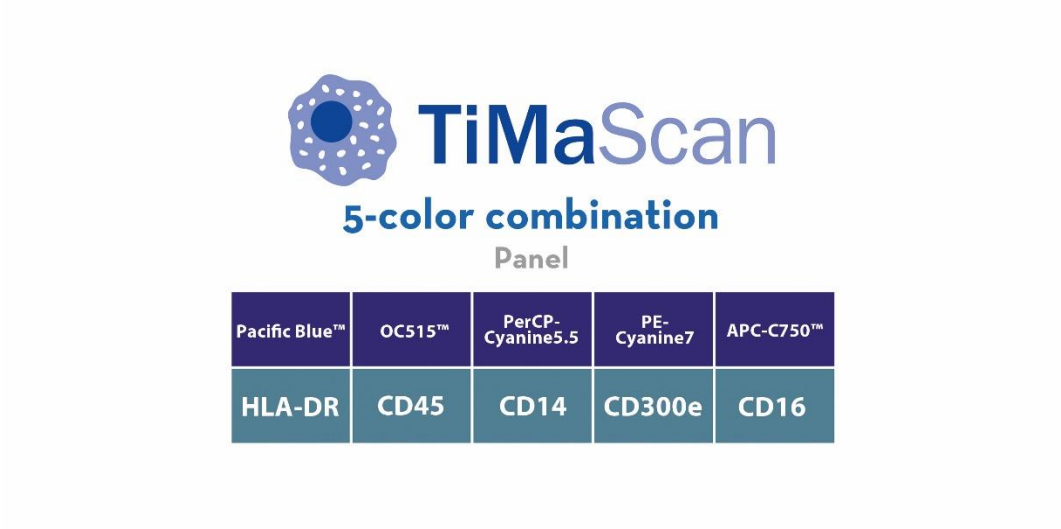


Ref: CYT-TIMASCAN-R

RUO

For Research Use Only. Not for use in diagnostic procedures.

TiMaScan™ VIALS CONTAIN LYOPHILIZED PRODUCTS. READ CAREFULLY THE FOLLOWING INSTRUCTIONS FOR RECONSTITUTION:

The lyophilized TiMaScan™ kit preserves the stability of the pre-mixed combination of antibodies. Reconstitute each lyophilized vial containing the pre-mixed combination with **150 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 each reconstituted vial before each use.
Unused volume of the reconstituted vials is stable during one month 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

TiMaScan™ kit is a combination with 5 conjugated antibodies designed for the accurate detection and enumeration of the main monocyte subsets by flow cytometry (FC), which is being studied to be relevant for monitoring different situations as tissue damage. This 5-color panel of monocyte markers has been designed leaving three fluorochrome positions free for drop-in reagents depending the context of use of this combination. This kit must be used by FC 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.
The erythrocyte population, which could hinder the detection of the target population, is lysed by using a red blood cell lysing solution.

Last revision: 2022-03-21

SUMMARY AND EXPLANATION

The monocyte-macrophage system is already abundantly present in tissues under normal homeostatic conditions. Among the monocytes three subsets can be identified: the classical (CD14+ CD16−), intermediate (CD14+ CD16+) and non-classical monocyte populations (CD14−CD16+). These subsets represent consecutive maturation stages and are all assumed to be capable of entering the tissues as macrophages.
Moreover, the different subsets have been found to differ in function and they change in both relative and absolute numbers during innate immune responses associated with infection and other disease conditions.

REAGENT COMPOSITION

TiMaScan™ kit contains sufficient volume for 25 tests distributed in lyophilized vials of 5 tests each and includes:

- **5 lyophilized vials of 5 tests for surface staining that contains**:
  - Antibody anti human CD14-PerCP-Cy5.5, clone: 47-3D6, isotype: IgG1.
  - Antibody anti human CD300e- PE-Cyanine7, clone: UP-H2, isotype: IgG1.
  - Antibody anti human CD16-APC-C750™, clone: 3G8, isotype: IgG1.
  - Antibody anti human HLA-DR-Pacific Blue™, clone: L243, isotype: IgG2a.
  - Antibody anti human CD45-OC515™, clone: GA90, isotype: IgG2a.

Fluorochrome	FITC	PE	PerCP-Cyanine5.5	PE-Cyanine7	APC	APC-C750™	Pacific Blue™	OC515™
Marker	-	-	CD14	CD300e	-	CD16	HLA-DR	CD45
Clone	-	-	47-3D6	UP-H2	-	3G8	L243	GA90
Isotype	-	-	IgG1	IgG1	-	IgG1	IgG2a	IgG2a

- **4 lyophilized tubes for compensation** of 1 test each for CD14-PerCP-Cyanine 5.5, CD300-PE-Cyanine7, CD16-APC-C750™ and CD45-OC515™ conjugates.

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

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. For information regarding identification and classification of hazards and precautionary statements on chemical substances of the product, please, refer to Material Safety Data Sheet (MSDS) available upon request at support@cytognos.com.
4. All patient specimens and materials which come into contact are considered biohazards and should be handled as if capable of transmitting infection 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.
5. 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.
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

Material required but not included

- 3 laser-equipped flow cytometer (8-color or more such as Omnicyt™) and appropriate computer hardware and software.
- 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 pipette 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. 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 7 x 10^6 cells **per tube to be stained**. 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 7 x 10^6 cells of the cell suspension per tube to be stained, by resuspending the pellet with washing buffer.

Surface Staining:

- Reconstitute the lyophilized vial using 150 µL of distilled water, mix well and place it in a roller for at least 30 min at RT before use.
- Add 25 µL of antibody mixture in a 5 mL flow cytometry tube, add other drop-in conjugated antibodies and then, add the 100 µL of cell suspension.
- Mix well. For optimal staining conditions, complete with washing buffer until a final volume of 200 µL.
- Incubate for 30 min at RT protected from light.
- Add 2 mL of an erythrocyte lysing solution containing fixatives.
- Mix well.
- Incubate for 10 min at room temperature 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 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 residual volume in each tube.
- Resuspend the cell pellet with acquisition buffer:
    - 850 µL using Omnicyt™.
    - 500 µL using other flow cytometers.
- Acquire the cells immediately after staining or store at 4°C (for 1h maximum) until measured in the flow cytometer.
- Acquire the sample at medium flow rate.

Staining steps for combined staining of TiMaScan™ surface membrane markers and cytoplasmic markers:

- Reconstitute the lyophilized vial using 150 µL of distilled water, mix well and place it in a roller for at least 30 min at RT before use.
- Add 25 µL of antibody mixture in a 5 mL flow cytometry tube and then, add the 100 µL of cell suspension.
- Mix well. For optimal staining conditions, complete with washing buffer until a final volume of 200 µL.
- 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 residual volume in each 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 each 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.
- Add cytoplasmic staining antibodies in the 5 mL tube containing the 200 µL of both Reagent B and cell suspension.
- 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:
    - 850 µL using Omnicyt™
    - 500 µL using other flow cytometers
- Acquire the cells immediately after staining or store at 4°C (for 1h maximum) until measured in the flow cytometer.
- Acquire the sample at medium flow rate.

Important Recommendations

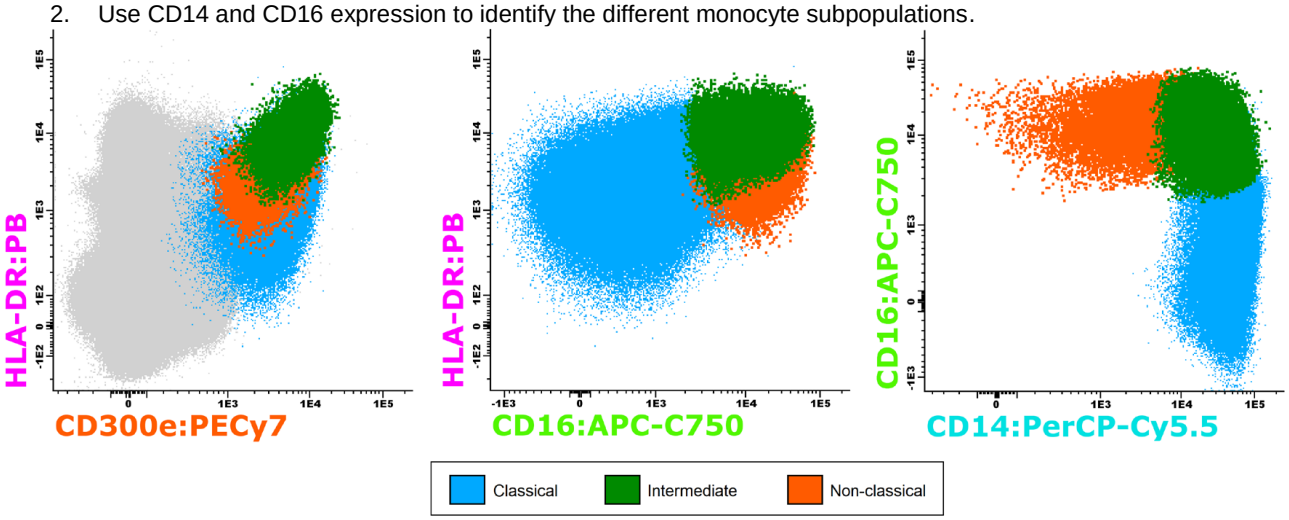
In order to achieve optimal results, EuroFlow™ Standard Operating Protocol (SOP) for Cytometer Setup should be followed. You will find a complete guide on the web site www.euroflow.org, which includes recommendations for FSC, SSC and target voltage PMT settings, compensation setup and instrument performance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

FLOW CYTOMETRY ANALYSIS

Cytognos recommends the use of the **Infinicyt™ analysis software**, which is capable to use pattern recognition and store analysis strategies to apply in batch to other samples using always the same criteria. You will find complete information about Infinicyt™ on the web site: www.infinicyt.com.

To analyze the results of the Monocyte Subsets tube we recommend follow these indications:

1. Identification the general monocyte population based on their IREM2 (CD300e) and HLA-DR expression.
2. Use CD14 and CD16 expression to identify the different monocyte subpopulations.



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.

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 13485:2016 and ISO 9001:2015 standards.

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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's 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
Σ	Contains sufficient for <n> tests

MANUFACTURER

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VERSION CONTROL

Version	Date	Modifications
1.0	2020-12-22	Document creation
2.0	2022-03-21	Reference to MSDS on information about hazard statements related to chemical substances of the product. Reference to Quality Management Systems standards ISO 13485:2016 and ISO 9001:2015 Check sodium azide percentage. Headline referring to the organization as manufacturer. Inclusion of version control chart.