CLONALPATH-PLUS™
Differentiation between MGUS and MM

Ref: CYT-CP45

For research use only

INTENDED USE
Clonalpath-Plus™ is a four-color direct immunofluorescence reagent (CD38-FITC/CD56-PE/CD19-PECy5/CD45-APC) for use in flow cytometry designed for the identification and characterization of normal and tumoral plasma cells (PC). This product has been optimized in order to differentiate between monoclonal gammophathy of undetermined significance (MGUS) and multiple myeloma (MM) on the basis of the number of immunophenotypically normal PC.

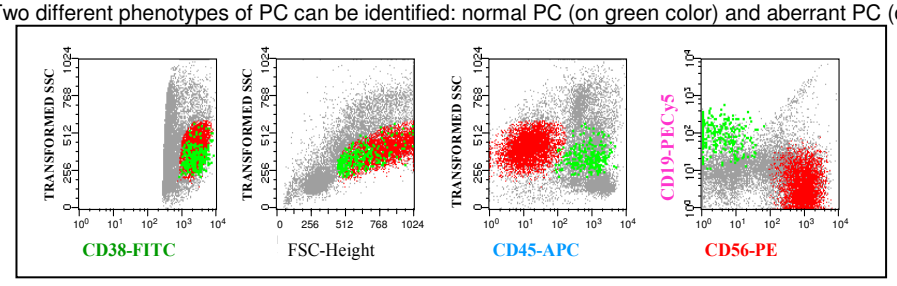
SUMMARY AND EXPLANATION
Monoclonal gammopathies are a heterogeneous group of diseases characterized by the expansion of monoclonal plasma cells (PC) that produce a monoclonal immunoglobulin (M-component), which is detectable in serum and/or urine. Although multiple myeloma (MM) represents the prototype of monoclonal gammopathy, the most common plasma cell disorder is the monoclonal gammophathy of undetermined significance (MGUS) [1]. The importance of accurate differentiation between MM and MGUS is clear. Unsuitable delay of treatment for multiple myeloma permits the development of clinical symptoms and complications, including bone fracture and renal failure. On the other hand, chemotherapy for MGUS exposes patients to an unnecessary treatment.
Clonalpath-Plus™ is a reagent focused on contributing to establish the differentiation between MGUS and MM based on the characterization of tumoral/clonal and normal/polyclonal plasma cells, identified by immunophenotype [2]. Identification of clonal plasma cells can be of clinical interest for the differential diagnosis between the different entities (MGUS and MM), but also for monitoring residual disease after treatment and for the detection of contaminating plasma cells in peripheral blood derived products to be used in autologous transplant [3-6]. It has been also described the ratio between normal and clonal plasma cells in bone marrow provides prognostic information [7].

PRINCIPLES OF THE PROCEDURE
Currently, overall consensus exists on the use of the CD38/CD56/CD19/CD45 four-color combination of monoclonal antibody reagents for the identification and enumeration of normal and pathological plasma cells from a sample in case of MM, MGUS and plasmacytoma[2, 8]. CD38 is used for the identification of PC as strong positive cells for this marker and CD56, CD19 and CD45 are used for the characterization and differentiation of normal and pathologic PC. The following table shows the percentage of analyzed cases and the intensity of expression of these different markers [2]:

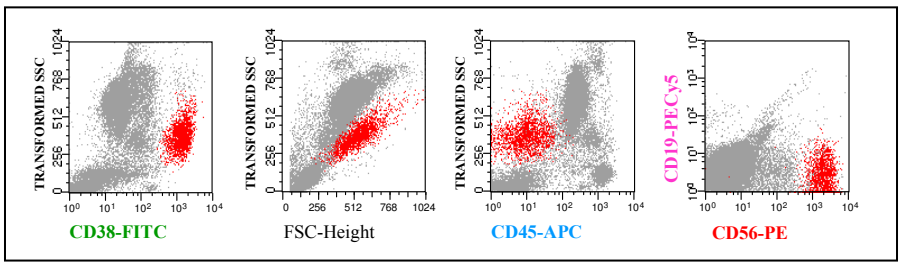
Antigen	Expression in normal PC	Expression in pathological PC	
		MGUS	MM
CD38	100% (+++)	100% (++/+++)	100% (++)
CD56	0%	69% (+/++)	67% (+/++)
CD19	100% (+)	5,5% (+)	3,2% (+)
CD45	100% (++)	45% (+/++)	44% (+/++)

Normal PC show intermediate SSC and FSC values, $CD38^{+++}$, $CD56^{-/low}$ and most of them co-express CD19 and CD45. Clonal PC from MM and MGUS frequently show higher FSC and SSC characteristics together with a slightly lower intensity of expression of CD38, they commonly are $CD56^{+}$ and $CD19^{-}$, and typically CD45 is absent in a major fraction of clonal PC.
There are two different PC subpopulations in the bone marrow from MGUS patients [8, 9]. One of these PC subpopulations shows identically phenotypic characteristics to those of normal PC while the second and predominant PC subpopulation shows these markers with an aberrant pattern in a high percentage of cases. The presence of immunophenotypically normal PC, which is a constant finding in MGUS patients, is a rare event in MM and when it is present, its frequency is significantly lower than that observed in MGUS [5, 8-10]. MGUS cases display a percentage of inmunophenotypically normal PC ($CD38^{+++}CD56^{-}CD19^{+}CD45^{+}$) from the total $CD38^{+++}$ PC population higher than 3% while MM patients present a percentage of inmunophenotypically normal plasma cells from total PC lower or equal than 3%.

- The following figure shows representative CD38+ gate data on bone marrow sample from a MGUS patient stained with the reagent. Two different phenotypes of PC can be identified: normal PC (on green color) and aberrant PC (on red color).



- The following figure shows representative data on bone marrow sample from a MM patient stained with the reagent. All PC show an aberrant phenotype (on red color)



REAGENT COMPOSITION

Clonalpath-Plus™ is provided in phosphate buffered saline with 0.1% sodium azide. It contains the following mixture of monoclonal antibodies (MAb):

- Fluorescein isothiocyanate (FITC)-labeled CD38, clone LD38; isotype IgG1
- Phycoerythrin (PE)-labeled CD56, clone C5.9, isotype IgG2b.
- Phycoerythrin-cyanine (PECy5)-labeled CD19, clone J4.119, isotype IgG1
- Allophycocyanine (APC)-labeled CD45, clone BHPT-1, isotype IgG1

Purification: Affinity chromatography
Amount per 1 ml vial: 50 tests (20 µl reagent to 10^6 cells)
Reagents are not considered sterile.

STORAGE CONDITIONS

The reagent is stable until the expiration date shown on the label, when stored at 2-8ºC. The reagent should not be frozen or exposed to direct light during storage or during incubation with cells. Keep the reagent vial dry. 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. This product is supplied ready to use.
3. The reagent is stable until the expiration date shown on the label if it is properly stored. Do not use after the expiration date shown on the label. If the reagents are stored in conditions different from those recommended, such conditions must be validated by the user.
4. 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.
5. It contains 0.1% 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.
    - Sodium azide (NaN_3) is harmful if swallowed (R22), if swallowed, seek medical advice immediately and show this container or label (S46).
    - Wear suitable protecting clothing (S36).
    - Contact with acids liberates very toxic gas (R32).
    - Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in metal drains where explosive conditions may develop.
6. All patient specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection [11], 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.
7. Use of the reagent with incubation times or temperatures different from those recommended may cause erroneous results. Any such changes must be validated by the user.

PROCEDURE

Material included

Clonalpath-Plus™ sufficient for 50 determinations (20 µl reagent to 10^6 cells).

Material required but not included

- 488 nm ion argon laser-equipped flow cytometer and appropriate computer hardware and software.
- 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 (100µL) and tips.
- Micropipette with tips.
- Chronometer
- Vortex Mixer
- Isotypic control reagent
- Quicklysis™ lysing solution
- Wash buffer as phosphate buffered saline (PBS) containing 0,1% sodium azide.

Preparation

Freshly obtained peripheral blood, bone marrow and other body fluid specimens should be stored at 18-22ºC for periods no longer than 24 hours.

- Whole blood sample must be taken aseptically by means of a venipuncture[12] in a sterilized tube for blood collection containing an appropriate anticoagulant (use of EDTA is recommended). The analysis requires one hundred (100) µl of the whole blood sample per tube, assuming a normal range of approximately 4 to 10 x 10^3 leucocytes per µl. For samples with a high white blood cell count, dilute samples with PBS to obtain a concentration of cells approximately equal to 1 x 10^4 cells/µL. Hemolyzed samples or samples with suspended cell aggregates should be rejected.
- Bone marrow sample should be pass 3 or 4 times through a syringe in order to disaggregate cell clumps. Perform a white blood cell count of the sample and dilute samples with PBS to obtain a concentration of cells approximately equal to 1 x 10^4 cells/µl.

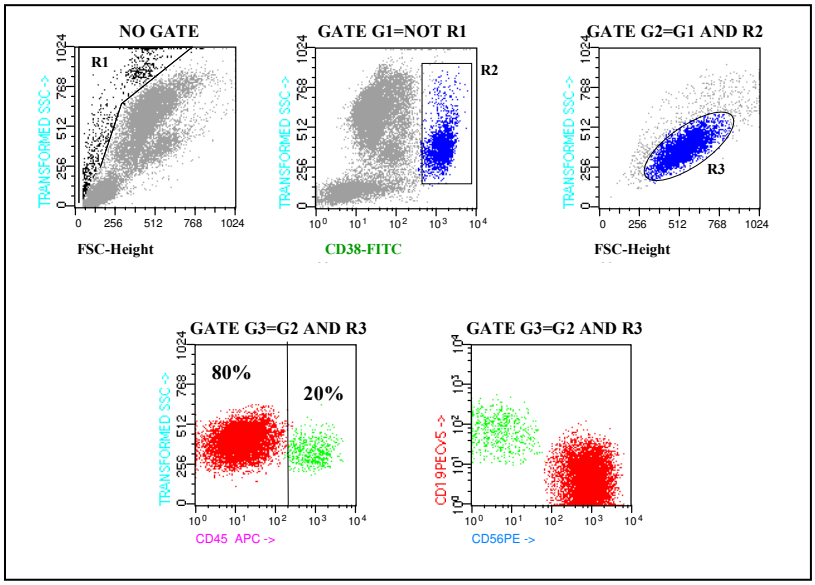
1. Mix 100µl of the sample with 20µl of Clonalpath-Plus™. To evaluate the non-specific binding of the reagent, an appropriated isotype control tube can be prepared.
2. Incubate for 10-15 minutes at room temperature in the dark.
3. Add 2 ml of Quicklysis™* erythrocyte lysing solution and incubate the sample for 10 minutes at room temperature in the dark.
4. For the phenotypic characterization of PC a minimum of 1000 cells of interest should be acquired. For minimal residual disease studies, at least 100 events corresponding to pathological PC should be collected to allow unequivocal identification and accurate enumeration of tumoral PC.
   Acquire directly on the flow cytometer within the first four hours of finishing the sample preparation. If the samples are not acquired immediately after preparation, they should be stored at 2-8ºC in the dark. Calibration of the instrument must be done according to the manufacturer's advice. Before acquiring samples, adjust the threshold or discriminator to minimize debris and ensure populations of interest are included. Before acquiring the sample on the flow cytometer, mix the cells on the vortex at low speed to reduce aggregation.

*Note: The use of other lysing solutions may require the elimination of the lysed red blood cells. Follow the manufacturer's recommended protocol of the lysing solution used.

Flow cytometry analysis

Check that the cytometer is correctly aligned and standardized for light dispersion (FSC/SSC on linear scale) and fluorescent intensity (FL1, FL2, FL3 and FL4 on logarithmic scale) and that the right color compensation has been set following the instructions of the cytometer manufacturer.

1. Use the FCS vs SSC dot plot to exclude dead cells, platelets and debris as exemplified in the following figure.
2. Select PC (shown on blue color) according to their highest fluorescence intensity for CD38-FITC and their FSC/SSC distribution.
3. Discriminate immunophenotypically normal PC and aberrant PC present in the sample:
   - **Normal plasma cells**, shown on green color, **display positivity for CD19 and CD45, and they are generally negative for the CD56 antigen.**
   - Aberrant plasma cells, shown on red color, frequently display a different pattern: they commonly are CD56 positive, CD19 negative, and typically CD45 is absent in a major fraction of tumoral PC.



4. Report on the percentage of:
   - The total plasma cell population from the events acquired after excluding those corresponding to dead cells, debris and platelets.
   - The immunophenotypically normal PC from the total PC. When the percentage of normal PC is higher than 3%, it is compatible with a MGUS while MM patients usually present a number of normal PC lower or equal than 3% from the total PC.
   - The immunophenotypically pathological PC from the total PC. Percentage of pathological PC provides information about tumor load which can be of help during the following up of the patient.

LIMITATIONS

- Blood and bone marrow samples should be stored at 18-22ºC and be tested within the 24 hours after they are obtained.
- It is advisable to acquire stained samples on the cytometer as soon as possible to optimize the results. Nonviable cells may stain nonspecifically. Prolonged exposure of blood and bone marrow samples to lytic reagents may cause white cell destruction and loss of cells from the target population.
- When using whole blood and bone marrow procedures, all red blood cells may not lyse under following conditions: nucleated red blood cells, abnormal protein concentration or hemoglobinopathies. This may cause falsely decreased results due to unlysed red blood cells being counted as leukocytes.
- Results obtained by flow cytometry may be erroneous if the cytometer laser is misaligned or the gates are improperly set

QUALITY CONTROL

- To obtain optimum results it is advisable to verify the precision of pipettes and that the cytometer is correctly calibrated.
- The fluorochromes fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), R-phycoerythrin-cyanine 5 (PECy5) and allophycyanin (APC) emit in different wavelengths but show a certain spectral overlapping which must be corrected by means of electronic compensation if combinations of different monoclonal antibodies are used conjugated with these fluorochromes. The optimum levels of compensation can be established by analysis in a dot-plot diagram of cells from healthy individuals stained with mutually exclusive monoclonal antibodies conjugated with the fluorochromes to be used in the test.

- To evaluate the non-specific binding of the reagent, an appropriated isotype control tube can be prepared.

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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 (use by YYYY-MM)**
	**Storage temperature limitation**
	**Consult instruction for use**
RUO	**For research use only**
	**Batch code**
	**Code number**
	**Manufacturer**

PRODUCED BY

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103472ENG **Last revision**: ORAN 26/07/2010