

# Immunophenotypic approach to the identification and characterization of clonal plasma cells from patients with monoclonal gammopathies

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## PURPOSE

The procedure described here provides general instructions for the immunophenotypical identification of tumoral/clonal plasma cells (PC) in bone marrow, peripheral blood, malignant effusions and other tissues (plasmacytomas, tonsillar tissues, lymph nodes) and for distinguishing them from normal/polyclonal plasma cells.

## SAMPLE STORAGE

Freshly obtained peripheral blood, bone marrow and other body fluid/tissue specimens should be stored at stable room temperature (18-22°C) for periods no longer than 24 hours. If required, storage for longer periods should be at lower temperatures (2-8 °C) (1, 2).

## EQUIPMENT AND MATERIALS

1. Multiparameter 4- or 5- color flow cytometer equipped with a 488-nm laser or a 488-nm and 635-

nm lasers and filters for the detection of at least green, orange, red and deep-red fluorescence.

2. Computer workstation equipped with appropriate software for calibration, quality control, data acquisition, transfer and analysis.
3. Additional computer support (PC's, CD's, ZIP).
4. Benchtop centrifuge at 540 x g adapted for 5 mL tubes and/or 96-well plates.
5. Refrigerator (at ≈ 4°C) for reagent storage.
6. 12 x 75 mm polystyrene tubes.

**Note: Alternatively 96-well culture plates can be used.**

7. Set of calibrated micropipettes capable of dispensing in the range of 5-10 µL and in the range of 100-1000 µL volumes.
8. Appropriate pipette tips.
9. Vortex mixer and tube roller.

## REAGENTS AND SOLUTIONS

1. Monoclonal antibodies directly conjugated to fluorochromes optimized for detection of cell surface

and intracellular epitopes resistant to conditions of fixation and permeabilization. Two different groups of monoclonal antibodies should be used in multicolor combinations: 1) CD38 and CD138 for the identification of plasma cells (3, 4) and 2) CD56, CD19, CD45 and CD38 for the characterization of clonal vs normal PC (6, 7, 9).

**Note:** Currently, overall consensus exists on the use of the CD38/CD56/CD19/CD45 or CD38/CD56/CD19/CD45/CD138 (3, 6, 7, 22) four- or five-color combinations of monoclonal antibody reagents for the identification and enumeration of normal and pathological plasma cells from a sample in case of multiple myeloma (MM), monoclonal gammopathy of undetermined significance (MGUS), and plasmacytoma. In turn, in Waldenström's macroglobulinemia other combinations may be more effective for the distinction between normal and clonal PC (25) such as surface immunoglobulin light chains (e.g. s $\lambda$ s $\kappa$ /CD38 (or CD138)/CD20). Additional combinations may be required and used according to the aims of the immunophenotypic study.

2. Cell wash buffer (filtered -0.40  $\mu$ m pore-) phosphate buffered saline (-PBS-) with 0.1-0.2% sodium azide and 0.1-2% protein solution -e.g. bovine serum albumin, BSA-, pH=7.6.
3. Non-nucleated red cell lysing solution.

**Note:** Non-nucleated red cell lysing solutions with or without a fixative can be used. The most frequently used red cell lysing solutions include: 1x ammonium chloride, Optilyse and Versalyse (both from Immunotech, Marseille, France), FACSLysing (Becton/Dickinson Biosciences, San José, CA), Quicklysis (Cytognos, Salamanca, Spain), Whole Blood Lysing Solution (Caltag Laboratories, San Francisco, CA) or Uti-Lyse™ (DAKOCytomation, Glostrup, Denmark).

4. A combination of fixation and permeabilization solutions for staining of antigens inside the cell.

**Note:** At present several reagent kits which contain ready to use permeabilization and fixation solutions and that can be applied as reference reagents (19) are commercially available: Fix & Perm (Caltag Laboratories), Intraprep (Immunotech), Intrastain (DAKO Cytomation).

5. PBS with 0.5% w/vol paraformaldehyde
6. Reagents and cells for instrument set-up

**Note:** For more detailed information please see the European Working Group in Clinical Cell Analysis (EWGCCA) guidelines (23).

## SPECIMEN

One of the following types of specimens containing clonal/pathological plasma cells may be studied:

1. EDTA anticoagulated bone marrow.

**Note:** Since in a bone marrow specimen cell aggregates exist, adequate mechanical disaggregation should be used (e.g. passing the sample through a 25G gauge syringe).

2. EDTA anticoagulated peripheral blood.
3. Malignant effusions (e.g. ascitic or pleural effusions).
4. Solid tissues (e.g. plasmacytomas, tonsils, lymph nodes).

**Note:** Solid tissues should be disaggregated by cutting them into small pieces (1 mm<sup>3</sup>) either manually or with semi-automated instruments - e.g. Medimachine (DAKOCytomation) or BD Medimachine (Becton/Dickinson Biosciences). After this, the sample should be passed through a 25G gauge syringe.

5. Immortalized plasma cells (e.g. myelomatous plasma cell lines).

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## QUALITY CONTROL PROCEDURES

1. Careful selection of fluorochrome-conjugates for each monoclonal antibody reagent. Measurement of basal autofluorescence levels of plasma cells (negative control; Fig. 1) or fluorescence from cells exposed to isotype-matched irrelevant immunoglobulins.
2. Alignment of Optics and Fluidics.
3. Procedure for multicolor compensation.
4. Procedure for checking instrument linearity.

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## PROCEDURE

a) *Protocol for staining of plasma cell surface antigens in samples containing non-nucleated red cells*

1. Label the tubes according to the monoclonal antibody combinations.
2. Add the appropriate amounts of each of the selected monoclonal antibodies.

**Note:** Typically between 0.1 and 1.5  $\mu$ g of antibody in a volume of 5-10  $\mu$ L are added to a final incubation volume of  $\leq$  200  $\mu$ L.

3. In each of the tubes, place between 50  $\mu$ L and 100  $\mu$ L of the sample containing up to 10<sup>6</sup> nucleated cells; gently mix the tubes for 10 seconds.

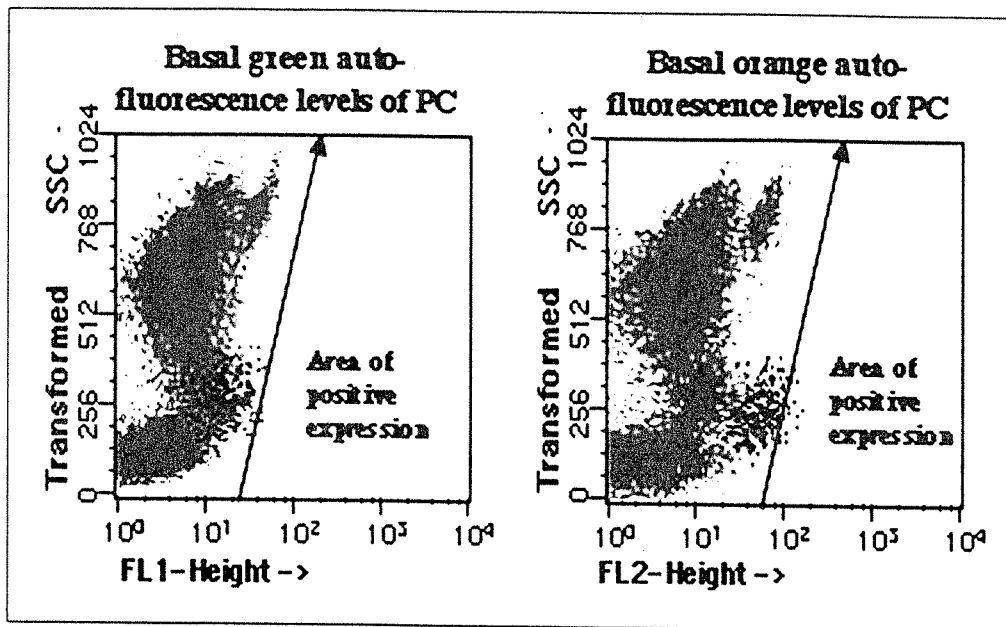


Fig. 1

4. Incubate for 15 minutes at room temperature in the darkness.
5. Add 2 mL/tube of the non-nucleated red cell lysing solution and gently mix.
6. Incubate for 10 to 15 minutes at room temperature in the darkness.
7. Centrifuge the sample for 5 minutes at 500 g.
8. Discard the supernatant with a Pasteur pipette and resuspend the cell pellet.
9. Add 2 mL/tube of filtered PBS.
10. Centrifuge for 5 minutes at 500 g.
11. Discard the supernatant with a Pasteur pipette, resuspend the cell pellet and add 0.5 mL/tube of filtered PBS containing 0.5% paraformaldehyde.
12. Read in the flow cytometer or store at 4°C protected from light for a maximum of 24 hours.

**b) Protocol for staining of plasma cell surface antigens in non-nucleated red cell-free samples (e.g. cell lines, isolated/purified plasma cells)**

Proceed with steps 1 through 4 and 9 through 12 as in section A.

**c) Protocol for staining of surface immunoglobulin (slg) kappa and lambda light-chains (or Ig heavy chains) in combination with other membrane antigens in samples containing free soluble Igs (e.g.: blood, bone marrow)**

1. Add 2 mL of filtered PBS in a tube.
2. Place between 50 and 100 µL of the sample containing up to 10<sup>6</sup> nucleated cells in the same tube and vortex the sample for a few seconds.
3. Centrifuge for 5 minutes at 500 g.
4. Discard the supernatant with a Pasteur pipette and resuspend the cell pellet in 2 mL/tube of filtered PBS; gently mix for a few seconds.

5. Centrifuge for 5 minutes at 500 g.
6. Discard the supernatant with a Pasteur pipette and resuspend the cell pellet.
7. Label the tubes according to the monoclonal antibody combinations containing the anti-human Ig reagents in combination with other plasma cell markers.

**Note: Steps 1 to 7 are used to eliminate soluble Ig molecules, Ig light-chains and Ig heavy chains present in the sample; soluble Igs could decrease the intensity of staining for the Ig present at the surface of plasma cells and other mature B-cells to near undetectable levels.**

8. Proceed with steps 2 to 12 of the protocol described above in section A.

**d) Protocol for staining of slg kappa and lambda light-chains (or Ig heavy chains) in combination with other membrane antigens in non-nucleated red cell-free samples containing free soluble Ig molecules (e.g. spinal fluid)**

1. Proceed with steps 1 to 7 of the protocol described in section C.
2. Proceed with steps 2 to 8 of the protocol described above in section B.

**e) Protocol for the simultaneous staining of intracellular and surface plasma cell antigens**

**Note: This protocol combines staining for antigens inside plasma cells with detection of antigens expressed on the surface of the plasma cells, which are useful for the identification and characterization of plasma cells (e.g. combined staining for CD38 and**

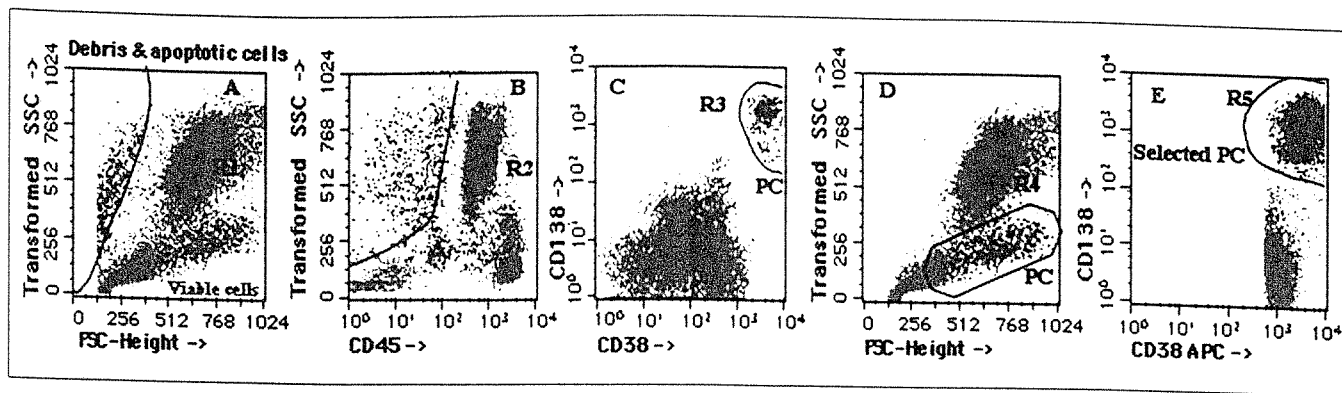


Fig. 2

### CD138 on the cell surface and cytoplasmic kappa and lambda Ig light-chains to assess clonality among plasma cells).

1. Label the tubes according to the monoclonal antibody combinations to be applied.
2. Add the appropriate amounts of each of the monoclonal antibodies directed against the surface antigens.
3. To each tube add between 50  $\mu$ L and 100  $\mu$ L of sample containing up to  $10^6$  nucleated cells; gently mix the tubes for a few seconds.
4. Incubate for 15 minutes at room temperature in the darkness.
5. Add 2 mL/tube of filtered PBS.
6. Centrifuge at 500 g for 5 minutes.
7. Discard the supernatant with a Pasteur pipette and resuspend the cell pellet in a total volume of <math><50 \mu\text{L}</math>.
8. Add 100  $\mu$ L/tube of a fixative reagent (see above section on reagents and solutions); gently mix.

**Note: Membrane fixation avoids extensive uncontrolled cell loss after permeabilization.**

9. Incubate for 15 minutes at room temperature in the darkness.
10. Add 2 mL/tube of filtered PBS.
11. Centrifuge at 500 g for 5 minutes.
12. Discard the supernatant with a Pasteur pipette and resuspend the cell pellet in a volume of <math><50 \text{ mL}</math>.
13. Add 100  $\mu$ L/tube of a permeabilizing solution and the appropriate amounts of those monoclonal antibodies directed against the intracellular antigens to be detected; gently mix.

**Note: Membrane permeabilization allows intra-cellular staining.**

14. Incubate for 15 minutes at room temperature in the darkness.
15. Add 2 mL/tube of filtered PBS.
16. Centrifuge at 500 g for 5 minutes.

17. Discard the supernatant with a Pasteur pipette and resuspend the cell pellet.
18. Add 0.5 mL/tube of filtered PBS.
19. Read in the flow cytometer or store at 4°C protected from light for a maximum of 24 hours.

**Note: Samples should be preferably acquired immediately after stained, although they can be stored overnight at 4 °C.**

### DATA ACQUISITION AND ANALYSIS

#### Creation of gating regions

1. Two-dimensional dot-plots should be generated in which the *forward scatter* (FSC) and *side scatter* (SSC) light scatter parameters are correlated between them and with each fluorescence emission. Additional bivariate dot-plots of all possible combinations of two-different fluorescence emissions should be established.
2. Broad electronic regions are set in a FSC vs. SSC dot-plot (R1 in Fig. 2) and a SSC vs. CD45 (R2 in Fig. 2) bivariate plot to exclude cell debris/ platelets.

**Note: Cell debris are excluded by gating (G1) events as followed: G1=R1 and R2.**

3. Set another broad electronic region in a CD38 vs CD138 dot plot (R3 in Fig. 2); in G1 all viable cells present in the sample will be included; G1 plus R3 will be enriched in plasma cells and should include all plasma cells.
4. Draw another region (R4 in Fig. 2) on FSC vs SSC dot-plot to better define (G1 and R3 and R4) the population of plasma cells.

#### Data acquisition

1. Place appropriate instrument settings.

**Note: Appropriate instrument settings should be preferably established according to the**

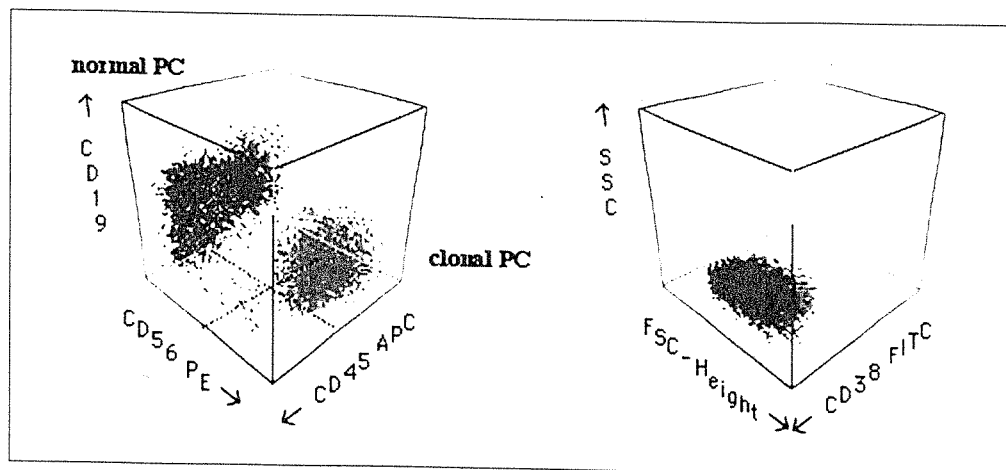


Fig. 3

protocol proposed by the EWGCCA (23); alternatively, the instrument set-up protocol specifically recommended by the manufacturer may be used.

2. Place a threshold on FSC to exclude debris as much as possible without excluding lymphocytes or non-nucleated red cells (Fig. 2A).
3. Acquire between 20 and 30x10<sup>3</sup> nucleated cells corresponding to the whole sample cellularity in a list mode file without any selection of regions or gates, apart from the threshold set in FSC.
4. If information on <10<sup>4</sup> plasma cells was acquired in the first step (as frequently occurs during investigation of minimal residual disease) activate

an electronic live gate (R5 from Fig. 2: R5= G1 plus R3 plus R4) and perform a second acquisition step in which information is stored exclusively on those events contained in this electronic live gate.

**Note:** For the phenotypic characterization of plasma cells a minimum of 10<sup>3</sup> cells of interest should be acquired using either a single or double-step acquisition. For minimal residual disease studies, at least 100 events corresponding to pathological plasma cells should be collected to allow unequivocal identification and accurate enumeration (coefficient of variation of <10%) of neoplastic, phenotypically aberrant, plasma cells.

TABLE I - SUMMARY OF THE MOST RELEVANT MOLECULES DESCRIBED IN PLASMA CELLS

	Expression in normal PC	Expression in pathological PC		
		MGUS	MM	WM
CD19	100% (+)	5.5% (+)	3.2% (+)	100% (+)
CD20	0%	24%(+)	23% (+)	100% (+/++)
CD38	100% (+++)	100% (++/+++)	100% (++)	100% (++)
CD45	100% (++)	45% (+/+++)	44%(+/+++)	100% (+/+++)
CD56 (NCAM)	0%	69% (+/+++)	67% (+/+++)	0%
CD138 (syndecan-1)	100% (+)	100% (+)	100% (+)	56% (+ <sup>d</sup> )
CD28	10% (+ <sup>d</sup> )	47% (+/+++)	41% (+/+++)	unknown
CD13	17% (+ <sup>d</sup> )	22% (+ <sup>d</sup> )	28% (+ <sup>d</sup> )	unknown
CD33	7% (+ <sup>d</sup> )	6% (+/+++)	24% (+/+++)	unknown
CD117 (c-kit ligand)	0%	23% (+)	27% (+)	unknown
HLA-Iα	100% (++)	100%(+++)	100% (++/+++)	100% (unknown)
β2-microglobulin	100% (++)	100% (+++)	100% (++)	100% (unknown)
CD40	100% (+++)	100% (+++)	100% (++)	unknown
CD126	0% (+)	60% (+)	100% (+)	unknown
Cytoplasmic light chain Ig*	Polyclonal	Monoclonal	Monoclonal	Monoclonal (75% of cases)

Results expressed as percentage of cases with more than 15% PCs positive for the antigens (intensity of expression) except for \*. +<sup>d</sup>: dim positivity; +: positive; ++: strong positive; +++: very strong positive

### Data analysis

If a combination of CD19, CD38, CD56 and CD45 has been employed to stain the sample, the following protocol can be used for data analysis:

1. Use the FSC vs. SSC and SSC vs CD45 dot-plots to exclude dead cells, platelets and debris as exemplified in Figure 2 (e.g.: G1).

**Note: "Transformed SSC" is preferred to SSC to better distinguish plasma cells with low SSC values and granular lymphocytes.**

2. Select the CD38 strong-positive events (e.g.: G1 plus R3 in Fig. 2).
3. Redefine the gated CD38 strong-positive plasma cells in a FSC/SSC bivariate dot-plot as being a homogenous population of cells in SSC with a relatively heterogeneous FSC distribution (e.g.: R4 in Fig. 2).
4. Report on the percentage of plasma cells identified from the whole sample cellularity and from the CD45<sup>+</sup> leukocytes (G1 events identified as defined in steps 1 to 3 as R5=G1 plus R3 and R4).
5. Identify the presence of pathological versus normal plasma cells in the sample among the CD38 strong-positive plasma cells (Fig. 3) based on their different immunophenotypic and light scatter characteristics:
  - a) define the presence of normal plasma cells as those plasma cells showing intermediate SSC and FSC values, CD38<sup>high</sup> and CD56<sup>-/low</sup>; most of normal PC co-express CD19 and CD45.
  - b) define the presence of pathological/clonal phenotypically aberrant plasma cells and report on its percentage from the total CD38<sup>high</sup> plasma cell population; as compared to normal PC, clonal PC from MM, MGUS and plasmacytomas frequently show higher FSC and SSC characteristics together with a slightly lower intensity of expression of CD38; in addition, they commonly are CD56<sup>+</sup> and CD19<sup>-</sup>; typically, CD45 is absent in a major fraction of clonal plasma cells in these disease conditions.

**Note: If this combination of markers does not allow the unequivocal identification of myelomatous plasma cells, additional markers (e.g. CD28, CD117, CD33, CD13, CD86, sIg) may be of help in this regard (Tab. I). In case of Waldenström's macroglobulinemia, clonal plasma cells can not be distinguished from normal plasma cells based on CD38, CD19, CD45, and CD56 expression but they frequently show abnormal sIgM/ $\kappa^+$  and CD20 expression.**

6. Report on the percentage of: 1) the total plasma cell population, 2) the normal plasma cells and 3) the pathological cells identified. In addition, record the total number of events acquired, after excluding those

corresponding to dead cells, debris and platelets, as well as the total number of CD45<sup>+</sup> leukocytes plus CD138<sup>+</sup>/CD38<sup>high</sup> plasma cells analyzed.

7. Calculate the percentage of pathological plasma cells from: 1) the total number of plasma cells, 2) the total number of white blood cells, and 3) the total number of cells in the sample.

**Note: Percentage of pathological plasma cell provides information about tumor load and helps to discriminate between MGUS and MM (6, 7, 11).**

8. Calculate the mean fluorescence intensity (MFI) and CV obtained for each marker specifically for each population of plasma cells as well as their FSC and SSC characteristics.

**Note: Comparison of the MFI for individual markers between pathological PC and normal PC helps in detecting aberrant expression of these proteins.**

**For other antibody combinations the staining could be measured following the same steps.**

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### CLINICAL SIGNIFICANCE AND INTERPRETATION

The study of plasma cells by immunophenotypic techniques is complex due, to a large extent, to their low representation in bone marrow aspirates and other types of specimens (4, 6, 8, 9, 11, 26). Identification of clonal plasma cells can be of clinical interest for the differential diagnosis between distinct plasma cell dyscrasias (6, 7), for staging purposes (3, 5), for monitoring residual disease after treatment (9, 11, 26) and for the investigation of the presence of contaminating plasma cells in peripheral blood (13-15) derived-products to be used in an autologous transplant. It has been also described that the ratio between normal and clonal plasma cells in bone marrow provides prognostic information (27).

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### LIMITATIONS AND TROUBLESHOOTING

If an appropriate number of events has been acquired, the lower limit of sensitivity is of  $<10^{-4}$  (one plasma cell in  $10^4$  total cells) or of 3 cells/ $\mu$ L if  $>50 \mu$ L of sample are available to be studied (6, 9, 27).

In rare occasions ( $<5\%$  of the cases) pathological plasma cells may show immunophenotypic characteristics identical to those of normal plasma cells (6, 7, 9).

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