



# Report of the European Myeloma Network on multiparametric flow cytometry in multiple myeloma and related disorders

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## ABSTRACT

The European Myeloma Network (EMN) organized two flow cytometry workshops. The first aimed to identify specific indications for flow cytometry in patients with monoclonal gammopathies, and consensus technical approaches through a questionnaire-based review of current practice in participating laboratories. The second aimed to resolve outstanding technical issues and develop a consensus approach to analysis of plasma cells. The primary clinical applications identified were: differential diagnosis of neoplastic plasma cell disorders from reactive plasmacytosis; identifying risk of progression in patients with MGUS and detecting minimal residual disease. A range of technical recommendations were identified, including: 1) CD38, CD138 and CD45 should all be included in at least one tube for plasma cell identification and enumeration. The primary gate should be based on CD38 vs. CD138 expression; 2) after treatment, clonality assessment is only likely to be informative when combined with immunophenotype to detect abnormal cells. Flow cytometry is suitable for demonstrating a stringent complete remission; 3) for detection of abnormal plasma cells, a minimal panel should include CD19 and CD56. A preferred panel would also include CD20, CD117, CD28 and CD27; 4) discrepancies between the percentage of plasma cells detected by flow cytometry and morphology are primarily related to sample quality and it is, therefore, important to determine that marrow elements are present in follow-up samples, particularly normal plasma cells in MRD negative cases.

**Key words:** flow cytometry, myeloma, monoclonal gammopathies of undetermined significance.

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Introduction

Clinical indications for the use of flow cytometry in multiple myeloma

At present, immunophenotyping is mandatory for the diagnosis and monitoring of acute leukemias and chronic lymphoproliferative disorders.<sup>1-4</sup> By contrast, in multiple myeloma, the use of multiparametric flow cytometry in many clinical diagnostic laboratories is currently restricted to clinical research studies and the differential diagnosis of unusual cases.<sup>5-7</sup> However, the generation and identification of markers that allow the unequivocal identification of plasma cells among other hematopoietic cells (such as CD138), and the identification of aberrant plasma cell phenotypes that enable us to discriminate between normal and neoplastic plasma cells,<sup>8-10</sup> means we can identify, characterize and enumerate neoplastic plasma cells even when few cells are present. This offers several advantages over other techniques and there is growing evidence in literature concerning the potential clinical benefit of immunophenotyping plasma cells by flow cytometry in patients diagnosed and/or suspected of suffering from myeloma or other plasma cell disorders. The advantages of flow cytometry in the diagnosis and monitoring of monoclonal gammopathies can be broadly categorised into three main topics (Table 1): (i) primary diagnosis of myeloma and associated disorders, based on the enumeration of plasma cells in the bone marrow and demonstration that a proportion are phenotypically abnormal, monoclonal and not reactive. The ability to assess multiple markers in combination with clonality assessment provides more specific information than can be obtained by other diagnostic techniques, such as immunohistochemistry; (ii) identification of independent prognostic markers, in particular those predicting the risk of progression for patients with MGUS and asymptomatic myeloma based on the relative proportions of abnormal and normal plasma cells; (iii) quantitative evaluation of minimal residual disease (MRD) levels for assessing efficacy of treatment and prediction of outcome, as well as the determination of stringent complete remission as defined by the International Myeloma Working Group (IMWG).<sup>11</sup> This article combines a review of the literature concerning the application of flow cytometry for the diagnosis of myeloma and other plasma cell disorders, as well as practical guidelines drawn up from an analysis of consensus views and group data analysis performed at two workshop meetings of the European Myeloma Network.

Plasma cell enumeration

Accurate quantitation of the plasma cell burden in bone marrow is essential for the diagnosis of myeloma.<sup>12,13</sup> Most laboratories assess the extent of plasma cell infiltration by morphological examination of stained bone marrow aspirate samples and trephine sections.<sup>14-17</sup> The limited use of flow cytometry in the analysis of myeloma has probably been due to the well-documented discrepancy in the plasma cell percentage observed between flow cytometry and

Table 1. Consensus medical indications of multiparametric flow cytometry immunophenotyping in the study of multiple myeloma and other monoclonal gammopathies.

Clinical application	Parameters measured by flow cytometry
Differential diagnosis between myeloma, MGUS and reactive conditions	(i) Plasma cells as a percentage of total leucocytes. (ii) Plasma cell immunophenotype (see Table 2) (iii) Plasma cell clonality (iv) Abnormal plasma cells as a percentage of total plasma cells
Prognostic markers in myeloma	Expression of specific antigens by abnormal plasma cells, e.g. CD45/CD56/CD117/CD28
Prediction of outcome for patients with MGUS and asymptomatic myeloma	Abnormal plasma cells as a percentage of total plasma cells
Detection of minimal residual disease in myeloma patients after treatment and determination of a stringent complete response	Abnormal plasma cells, identified by immunophenotype and cytoplasmic $\kappa/\lambda$ , as a percentage of either total leucocytes or as a percentage of total plasma cells; requires high sensitivity assessment

conventional microscopy in overall enumeration of plasma cells in bone marrow samples from myeloma patients.<sup>8,18-20</sup> Notably, these discrepancies affect all laboratory investigations, including cytogenetics/FISH and molecular studies.

The main reason for the discrepancy is the use of a secondary aspirate for laboratory studies, which is usually of poorer quality than the primary aspirate taken for morphological assessment. Counting errors and expression of certain adhesion molecules may also have an impact.<sup>21-23</sup> The causes of the discrepancy are described in detail in the online appendix. It was emphasized that recent studies have demonstrated that plasma cell enumeration by flow cytometry is of greater prognostic value in myeloma patients than a morphological plasma cell count.<sup>20</sup>

Plasma cell enumeration consensus

Current diagnostic criteria require morphological assessment of plasma cell percentage. This is helpful in providing a global assessment of the sample. Discrepancies between the plasma cell percentage detected by flow cytometry compared with morphological enumeration are primarily due to the sample quality and it is likely that the use of first-pull aspirate samples for immunophenotyping will largely remove the inconsistency. These discrepancies affect all laboratory studies, including cytogenetics/FISH and molecular studies: reducing sampling artefact will benefit all laboratory studies. Flow cytometric enumeration of plasma cells may be more reproducible and reliable at predicting outcome in myeloma than morphological assessment since larger number of cells are analyzed and there is less operator bias. However, further studies are required to confirm this.

### Differential diagnosis of myeloma and other monoclonal gammopathies

The primary role of flow cytometry in participating laboratories was to demonstrate abnormal and/or monoclonal plasma cells as part of the diagnosis of myeloma. A large body of evidence has been collected to demonstrate that neoplastic bone marrow plasma cells from myeloma patients and other monoclonal gammopathies display aberrant phenotypes<sup>10,24-26</sup> and restricted immunoglobulin (Ig) light chain expression at the cytoplasmic<sup>9,27</sup> and, to a lesser extent, at the surface membrane level.<sup>28</sup> Based on these features, unequivocal identification and enumeration of aberrant and normal plasma cells co-existing in a bone marrow sample can be performed.<sup>29</sup> These immunophenotypical features are described in Table 2 and below in the section *Antigen expression on normal and neoplastic plasma cells*. Therefore, the demonstration of restricted immunoglobulin coupled with an abnormal immunophenotype can be used to distinguish between reactive and neoplastic conditions. Immunophenotyping of plasma cells is recommended in the differential diagnosis between myeloma and monoclonal gammopathy of undetermined significance (MGUS), for the identification of aberrant phenotypes present in clonal plasma cells at diagnosis that could be used later during patient monitoring, and for the evaluation of minimal residual disease after therapy. Additional medical indications of multiparametric flow cytometry immunophenotypical studies at diagnosis include the differential diagnosis of unusual cases. For example, immunophenotyping can help to distinguish rare cases of IgM myeloma, where the predominant population will have the phenotype of abnormal plasma cells, from other

IgM secretory disorders which have distinct phenotype.<sup>45</sup> Similarly the demonstration of abnormal plasma cells may be useful in the diagnosis of patients with non-secretory myeloma or primary amyloidosis. Immunophenotyping at diagnosis may also be useful for the identification of potential therapeutic targets (e.g. CD52 and CD20).<sup>46,47</sup>

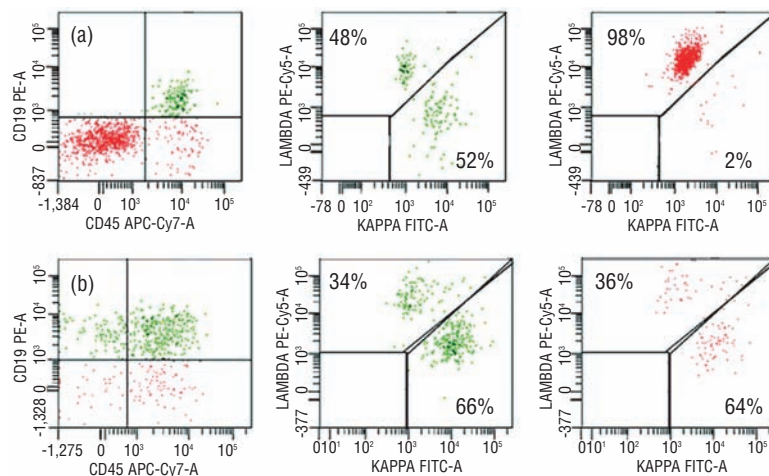
### Differential diagnosis: consensus

Demonstration of the presence of phenotypically aberrant plasma cells can be used in the differential diagnosis between MGUS, myeloma and reactive conditions.

### Identification of prognostic markers and immunophenotypical screening of cytogenetic abnormalities in myeloma and MGUS

The prognostic value of immunophenotyping has not yet been clearly established.<sup>29</sup> Several studies have demonstrated an association between antigenic profile and specific genetic abnormalities<sup>24,27,30,48,49</sup> but this is not strong enough for immunophenotyping to be used to screen for genetic abnormalities in myeloma. The detection of circulating plasma cells<sup>31-33,50,51</sup> and the CD45 expression pattern<sup>21,34</sup> are also reported to be a highly significant prognostic factor but further work is required to define the role of this assay in routine clinical use. These studies are described in more detail in the *Online appendix*.

Arguably one of the most useful prognostic factors that has been identified is the ratio of abnormal/normal plasma cells in the bone marrow of patients with MGUS and asymptomatic myeloma. This affects a large group of patients whose outcome is currently difficult to predict from presentation features. The presence of a great major-



**Figure 1.** Illustrating examples of basic immunophenotype and clonality assessment to screen for the presence of residual disease in bone marrow samples from multiple myeloma patients. The plots show bone marrow cells from two myeloma patients in morphological remission prepared using a fixation and permeabilization procedure and demonstrate typical profiles for CD19, cytoplasmic kappa and cytoplasmic lambda expression on gated plasma cells. The plots on the left show CD19 vs. CD45 expression and demonstrate typical profiles for CD19<sup>+</sup> plasma cells are colored in green and the CD19<sup>-</sup> plasma cells in red. Kappa vs. Lambda expression for CD19<sup>+</sup> normal plasma cells is shown in the middle plots and for CD19<sup>+</sup>/CD45<sup>-</sup> plasma cells in the right plots, with the percentage of gated cells noted in the relevant regions.

The upper three plots are from a patient with 0.04% total plasma cells at day 100 after high dose therapy: the CD19<sup>+</sup> plasma cells are light-chain restricted while a small population of CD19<sup>+</sup> plasma cells is polyclonal. This demonstrates that low levels of residual disease can be identified and enumerated using basic immunophenotyping and clonality assessment. The lower three plots are from a patient in continued complete remission several years after high dose therapy: the majority of plasma cells are CD19<sup>+</sup> but some CD19<sup>+</sup> plasma cells are detectable and both the CD19<sup>+</sup> and CD19<sup>-</sup> fraction appear polyclonal. CD19<sup>+</sup> plasma cells are present in normal individuals and are not necessarily neoplastic. In this case, extended analysis confirmed that both the CD19<sup>+</sup> and CD19<sup>-</sup> fractions of plasma cells were normal. Screening approaches can only exclude the presence of residual disease if all the plasma cells are CD19<sup>+</sup> in a patient known to have CD19<sup>+</sup> disease.



**Table 2.** List of most useful antigens for the detection of aberrant plasma cells in multiple myeloma.<sup>5-10,20-44</sup>

Antigen	Normal expression profile (percentage expression on normal plasma cells)	Abnormal expression profile	Percentage of myeloma cases with abnormal expression	Requirement for diagnosis and monitoring
CD19	Positive (>70%)	Negative	95%	Essential
CD56	Negative (<15%)	Strongly positive	75%	Essential
CD117	Negative (0%)	Positive	30%	Recommended
CD20	Negative (0%)	Positive	30%	Recommended
CD28	Negative/weak (<15%)	Strongly positive	15-45%	Recommended
CD27	Strongly positive (100%)	Weak or negative	40-50%	Recommended
CD81	Positive (100%)	Weak or negative	Not published	Suggested
CD200	Weakly positive	Strongly positive	Not published	Suggested

ity of abnormal plasma cells (>97% of total bone marrow plasma cells) is typically seen in myeloma while the presence of normal plasma cells (>3% of total bone marrow plasma cells) is more consistent with a diagnosis of MGUS, although the final distinction between MGUS and myeloma will be dependent on a morphological assessment of total plasma cell infiltration and on other clinical features. However, both MGUS and asymptomatic myeloma patients with a high ratio of abnormal/normal plasma cells have a greatly increased risk of progression to myeloma and this is one of the most powerful prognostic factors that can be identified at presentation.<sup>35,36</sup>

### Prognostic markers: consensus

Several marker combinations, particularly that of CD117 and CD28, show promise in predicting outcome for myeloma; (i) Immunophenotyping is of limited value for the diagnostic screening of specific genetic abnormalities in myeloma; (ii) further collaborative studies, including the exchange of original flow cytometry data files, are required to reach consensus on the relevance of CD45 expression in myeloma; (iii) the ratio between phenotypically normal and aberrant plasma cells can be used to predict the risk of disease progression in MGUS and asymptomatic myeloma.

### Detection of minimal residual disease by flow cytometry

Response assessment using serum or urine paraprotein assessment can be held back by the long half life of some immunoglobulin molecules<sup>37,52</sup> while serum free light chain is relatively insensitive.<sup>53</sup> Direct assessment of bone marrow tumor load is more predictive of outcome. Allele-specific oligonucleotide (ASO) PCR is highly sensitive but can be costly, time-consuming and can have limited applicability.<sup>38,54-61</sup> It is possible to detect neoplastic plasma cells by flow cytometry above the clinically relevant threshold of

0.01%<sup>38,57</sup> and this is more informative than conventional assessment.<sup>39,40,62</sup> Flow cytometry for residual disease detection is applicable to almost all patients, more sensitive than paraprotein or light chain assessment, and considerably cheaper than PCR analysis. A more detailed comparison of the different approaches to disease monitoring is provided in the online appendix.

### Minimal residual disease: consensus

Multiparametric flow cytometry is a feasible and adequate method for monitoring residual disease and evaluating response to therapy. This application of flow cytometry is likely to become more widespread, and will require the development of standardized approaches with defined specificity and sensitivity, along with suitable quality control schemes.

### EMN consensus approaches and techniques for flow cytometry in monoclonal gammopathies

The following sections discuss the key issues for immunophenotyping bone marrow samples at diagnosis and for detecting residual disease by flow cytometry in myeloma, with identification of consensus approaches where available. Unless otherwise stated, the methods refer to the characterization of plasma cells in bone marrow samples and most studies can be undertaken using a flow cytometer capable of detecting a minimum of three fluorochromes. Cytometers capable of detecting four or more colors are to be preferred since they can more easily identify and reproduce abnormal populations, and can reduce the time and cost involved in acquiring and analyzing data.

### Sample preparation

The majority of participants used a fixative-free erythrocyte lysis method for enumeration and phenotypical characterization while a smaller proportion of centres used whole blood/marrow methods that contain a fixative for enumeration. Fixation and permeabilization methods were frequently used in addition to the fixative-free lysis method for analysis of intracellular immunoglobulin expression. Two centres used a density gradient centrifugation approach prior to immunomagnetic separation, cell culture, and/or immunophenotyping. The percentage of plasma cells detected does not differ significantly between these approaches except for density gradient centrifugation which may result in a variable increase or decrease in the percentage of plasma cells compared with whole blood/marrow approaches. This approach is not, therefore, suitable for enumeration of plasma cells.

### Sample preparation: consensus

Any whole blood/marrow approach is suitable for plasma cell enumeration and phenotyping. Density gradient centrifugation is inappropriate for plasma cell enumeration but may be suitable for some applications.

### Primary gating antibodies

The identification of an accurate gating strategy is a critical component of a reproducible and sensitive immunophenotypical assay for the analysis of plasma cells. A variety of approaches based on CD38, CD138 and/or CD45 expression were used. The majority of centers reported a gating strategy using combined CD38, CD138 and light scatter characteristics. It was noted that there was no formally published consensus method for gating plasma cells and this was therefore addressed directly at the Leeds meeting by experimentally comparing the different gating strategies. This is described in detail in the *Online appendix*. Using CD38 vs. side scatter gives false negative results for cases with relatively weak CD38 expression on the neoplastic plasma cells. Using CD38 vs. CD138 improves the detection of small plasma cell populations but there is a high contamination rate which inhibits the ability to demonstrate an abnormal phenotype. Using CD38 vs. CD45 reduces contamination but also results in the exclusion of CD45<sup>+</sup> plasma cells, which can constitute the majority of abnormal plasma cells. The combined use of CD38, CD138 and CD45 together with light scatter characteristics provides the optimal detection rate and concordance between different operators. It is critical that the first gate is set using CD38 vs. CD138 expression rather than CD38 vs. CD45 expression to ensure that CD45<sup>+</sup> plasma cells are not excluded.

### Primary gating antibodies: consensus

It is recommended to use four or more detectors for flow cytometry analysis. Two-color immunophenotypical analyses are not feasible as at least two antigens are required to gate plasma cells accurately. CD138, CD38, CD45 and light scatter characteristics should all be assessed simultaneously in at least one tube. If using bivariate analysis, the primary gate should be set to include CD38<sup>+</sup>CD138<sup>+</sup> events. For characterization of plasma cells, further tubes should include at least two markers, preferably CD38 and CD138, with the optimal combination identified from the primary gating tube. If sufficient detectors are available, the optimal approach would include CD38, CD45 and CD138 in all tests.

### Controls for gating and immunophenotyping

The use of suitable controls is essential for any accurate analysis but there is considerable discussion within the flow cytometry community about what these should be. Isotype controls have historically been used but they do not provide a control for many of the variables that affect the level of non-specific fluorescence, including antibody concentration, fluorochrome:antibody ratio, and isoelectric point. The majority of centers did not use an isotype control for gating or analysis. It was noted that the gating antibodies should yield a discrete population of plasma cells and the expression of gating reagents was therefore controlled internally by the remaining leucocytes. For immunophenotypical characterization, centers not using an iso-

type control reported using other leucocyte populations to define positive/negative limits, or using autofluorescence alone (i.e. cells labeled with only the gating reagents). The use of non-isotype controls (such as CD3) was also reported. Guidelines for controlling protein expression analysis have been published by the Clinical and Laboratory Standards Institute<sup>63</sup> and the consensus among participants was that these guidelines are suitable for flow cytometry in the diagnosis and monitoring of plasma cell disorders.

### Controls for gating and immunophenotyping: consensus

A control for the gating reagents is not required since these are controlled internally. Controls for staining should be in accordance with standard flow cytometry procedures.<sup>63</sup>

### Number of events

The detection limit for a typical flow cytometry immunophenotyping (e.g. MRD) assay is partly determined by the minimum number of events that can reliably be used to define a population of neoplastic cells. Among participants, this varied from 10 to 100 events with the majority requiring more than 20 events. However, it has previously been shown that accurate identification of a population using up to 4-color flow cytometry immunophenotypical approaches requires at least 20 events.<sup>64,65</sup> If fewer than 100 neoplastic plasma cell events are counted, the coefficient of variation of the percent value of neoplastic plasma cells will be greater than 10%, independent of any biological or experimental variations. Therefore, it is recommended that at least 100 neoplastic plasma cell events are acquired. The number of target events need not be acquired in a single tube but can be made up of the events identified in several tubes, e.g. two tests with a minimum of 50 neoplastic plasma cell events and 500,000 total events in each test, or four tests with a minimum of 25 neoplastic plasma cell events and 250,000 total events in each test. This allows counting and biological or experimental errors to be considered simultaneously.

### Number of events: consensus

At least 100 neoplastic plasma cell events should be acquired for accurate enumeration. If an MRD assay is to have a limit of sensitivity of 0.01%, then the minimum number of total events required is 1,000,000. If the assay consists of several individual tests then the minimum requirements are the sum, not the average, of the individual tests.

### Clonality assessment

Demonstration of plasma cell clonality is important for diagnostic specimens but the relevance of clonality assessment in follow-up samples is less clear. As in other MRD approaches,<sup>66</sup> the use of  $\kappa/\lambda$  assessment alone is not suitable in an MRD setting because restricted light chain expression only becomes apparent when the monoclonal population represents more than 30% of the polyclonal

background. Assessment of intracellular heavy chain expression may also be used in this regard, but relatively few centers carry out routine analysis of both light and heavy chains at follow-up. It is possible to combine clonality with basic immunophenotype using six-color analysis to provide rapid detection of abnormal plasma cells at presentation and follow-up. In many cases, identification of a clearly defined abnormal population will rule out the need for extended immunophenotyping. Examples of 6-color screening panels at Salamanca and Leeds are: cytIgλ/cytIgk/CD19/CD56/CD38/CD45 and cytIgλ/CD19/cytIgk/CD138/CD38/CD45 respectively. Such approaches can detect neoplastic cells even when they represent as little as 0.01% of leucocytes.<sup>67</sup> An example of clonality assessment is shown in Figure 1.

It is critical that whole marrow samples are washed twice in a ten-fold excess of buffered saline solution prior to assessment of cytoplasmic immunoglobulin expression to remove cytophilic immunoglobulin. During the washing procedure, the supernatant should be removed by aspiration, not by decanting, in order to avoid excessive cell loss. Standard commercial fixation and permeabilization kits were reported to be suitable for assessment of cytoplasmic kappa/lambda detection, and participants did not report any specific advantages or disadvantages of the kits available from different companies.

#### **Clonality assessment: consensus**

Assessment of cytoplasmic κ/λ expression by flow cytometry is important to demonstrate clonality at presentation and is appropriate for the assessment of a stringent complete remission according to the IMWG criteria.<sup>11</sup>

The demonstration of phenotypically abnormal plasma cells is more sensitive and specific for the detection of residual disease than clonality assessment by immunohistochemistry and/or flow cytometry. Combined assessment of clonality with basic immunophenotype may be useful for screening at diagnosis and follow-up.

#### **Antigen expression on normal and neoplastic plasma cells**

The most commonly assessed antigens for the detection of neoplastic and normal plasma cells from published literature and workshop participants, apart from the gating reagents, include CD19, CD56, CD20, CD117, CD28, CD33, CD27, CD81, CD31, CD39, CD40, CD44, CyclinD1 and CD34. No single marker has been reported to systematically differentiate neoplastic plasma cells from their normal counterparts. There has been no formal study to identify the minimum requirements for reproducible detection of minimal residual disease and further investigation is required to identify a common panel. However, based on reported studies, a panel containing CD19 and CD56 will be applicable to at least 90% of patients, with the markers CD20, CD117, CD28 and CD27 likely to increase this to more than 95% of patients.<sup>5-10,20-29,30-44</sup> In addition, several participants had

analyzed CD81 and CD20068 and suggested that these markers should be assessed further. The proposed antigens for investigation are outlined in Table 2.

#### **Antigen expression on normal and neoplastic plasma cells: consensus**

It is not possible to define plasma cells as being phenotypically abnormal, either at diagnosis or after treatment, using only one test antigen. In addition to the plasma cell gating markers discussed above, the minimal test antigens for classifying abnormal plasma cells are CD19 and CD56. A preferred panel would incorporate CD20, CD117, CD28 and CD27.

#### **Measure of sample quality**

As discussed above, it is known that a lower percentage of plasma cells is detected by flow cytometry than by morphology. In most cases, this may be due to the provision of a blood-diluted sample. This may also be critical for MRD analysis, since the level of neoplastic plasma cells will be under-estimated in a blood-diluted sample. Normal polyclonal CD138<sup>+</sup>CD19<sup>+</sup> plasma cells are typically restricted to the bone marrow and the presence of such cells has been used to confirm that the sample is representative marrow. If only neoplastic cells are present, then the sample is MRD<sup>+</sup> but may not be representative of the marrow. B-cell regeneration is usually rapid after high-dose melphalan with autologous stem cell rescue, and if no plasma cells are present, then the sample is unlikely to be representative. However, there was anecdotal evidence from the UK Myeloma IX trial that good quality bone marrow samples containing neither normal nor neoplastic plasma cells were observed in a small proportion of patients. It may therefore be necessary to determine the levels of other cells that are predominantly restricted to the bone marrow, e.g. erythroid, myeloid and B-cell progenitors. In cases where marrow elements are not detectable, it should be stated that the sample is unsuitable for quantitative MRD analysis.

#### **Measure of sample quality: consensus**

The sample is suitable for quantitative MRD analysis if normal plasma cells (CD19<sup>+</sup>CD56<sup>-</sup> and/or polyclonal) are detectable. If normal plasma cells are not detected, the quality of the sample should be assessed by morphological assessment of a bone marrow smear made from the same sample used for flow cytometry, and/or additional flow cytometry for the presence of normal erythroid, myeloid or B-cell progenitors. If there are no marrow elements present but neoplastic plasma cells are detected, the sample should be reported as MRD-positive, but note that the sample may be unsuitable for quantitative assessment. If there are no marrow elements and no plasma cells, the sample should be reported as unsuitable for analysis.



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