Comparison of minimal residual disease detection in multiple myeloma between the DuraClone and EuroFlow methods

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In this study, the minimal residual disease (MRD) levels in patients with multiple myeloma (MM) were assessed by comparing the new 8-color single-tube multiparameter flow cytometry method (DuraClone), which reduces the cost of antibodies and labor burden of laboratories, with the EuroFlow next-generation flow (NGF) method. A total of 96 samples derived from 69 patients with MM were assessed to determine the total cell acquisition number (tCAN), percentages of total and normal plasma cells (PCs), and MRD levels using two methods. We found that the tCAN was significantly higher with EuroFlow-NGF than with DuraClone (median 8.6 × 10^6 vs. 5.7 × 10^6; p < 0.0001). In addition, a significant correlation in the MRD levels between the two methods was noted (r = 0.92, p < 0.0001). However, in the qualitative analysis, 5.2% (5/96) of the samples showed discrepancies in the MRD levels. In conclusion, the DuraClone is a good option to evaluate MRD in multiple myeloma but it should be used with caution.

Recently, an increasing number of patients with multiple myeloma (MM) achieve complete response (CR) because of novel agents, and this is well associated with good progression-free survival and overall survival1–4. The minimal residual disease (MRD) levels are used to stratify patients who achieve CR and predict their outcomes5–9. In 2016, the International Myeloma Working Group (IMWG) approved the definition of the MRD criteria, such as Flow-MRD negative and Sequencing MRD negative10. A Flow-MRD-negative case is assessed by multiparameter flow cytometry (MFC) with a minimum sensitivity of 1/10^5 nucleated cells and equivalent methods, such as EuroFlow next-generation flow (NGF). EuroFlow-NGF, which uses two 8-color tubes, is a highly sensitive (cutoff value, 2 × 10^-6) and standardized way of detecting MRD11, and the IMWG advocates the use of EuroFlow or equivalent methods to assess MRD. Although it is more rapid and cost-effective in detecting MRD compared with next-generation sequencing5,6,9,12, the EuroFlow method is too expensive (< 350 USD/sample) to perform under public medical insurances in some countries, including Japan12,14. The DuraClone RE PC kit (DuraClone panel) is a recently developed 8-color single-tube dry antibody panel that uses the Kaluza software (Beckman Coulter, Brea, USA) for the identification of abnormal plasma cells (aPCs) to detect MRD in MM with a sensitivity of 1.0 × 10^-515. Although the DuraClone panel is widely available, whether it can reduce the cost of antibodies and labor burden of laboratories remains to be validated, and the relationships in the PCs and MRD detection between EuroFlow-NGF and DuraClone are unclear. Therefore, we hypothesized that the DuraClone method could offer the same performance as that offered by EuroFlow-NGF for the detection of MRD in patients with MM. In this study, we performed a comparison between the two methods.

Methods

Patients and samples. The MRD test was prospectively performed in patients with IMWG-defined symptomatic MM who were treated at Kameda Medical Center and Kanazawa University Hospital in Japan from January 2017 to November 2018. As part of routine clinical care, 4 mL of bone marrow aspirate (first bone marrow aspiration pull) was collected in tubes containing ethylenediaminetetraacetic acid. The samples were then
mixed, measured, and split evenly (2 mL each). One sample was analyzed with the DuraClone 8-color single-tube panel at Kameda Medical Center, and the other was analyzed with the EuroFlow-NGF 8-color 2-tube panel at Kanazawa University Hospital. Within 48 h after collection, up to 20 million cells or the entire volume of the sample was used. All study methods were carried out in accordance with the Declaration of Helsinki.

Eight-color MFC analysis using the DuraClone and EuroFlow-NGF methods. The technical details and validation of the EuroFlow-NGF have been reported previously11. In brief, the EuroFlow method uses ammonium chloride-based bulk lysis, followed by surface staining using antibodies against CD138-BV421, CD27-BV510, CD38 multi-epitope (ME)-FITC, CD56-PE, CD45-PerCP Cy5.5, CD19-PECy7, CD117-APC, and CD81-APC-C750 in Tube 1 and surface/intracellular staining using antibodies against CD138-BV421, CD27-BV510, CD38 ME-FITC, CD56-PE, CD45-PerCP Cy5.5, CD19-PECy7, cytoplasmic (cy) Igκ-APC, and cyIgλ-APC-C750 after permeabilization in Tube 2, as previously described11,14. Anti-CD38 ME antibody was used to prevent the interference of anti-CD38 monoclonal antibody such as daratumumab. The FACSCanto II (BD Biosciences) flow cytometer was used to acquire all samples, and the gating and identification of clonal aPCs were manually performed by experts using the Infinicyt software (Cytognos, Salamanca, Spain) for the EuroFlow-NGF method.

On the other hand, the DuraClone method15 was performed with fixative-free erythrocyte lysis (VersaLyse Lysing Solution, Beckman Coulter), followed by surface staining with the prefixed dry reagent of antibodies against CD138-APC, CD38-PB (non-ME antibody), CD56-APC-A750, CD19-PC5.5, CD45-KrO, CD200-PC7, CD81-FITC, and CD27-PE in a single tube to identify aPCs. The Navios flow cytometer (Beckman Coulter, USA) was used to acquire the samples. Cell doublets were excluded by omitting the events with high time of flight (TOF)15 (Fig. 1A) and debris were excluded by omitting the events with lower forward scatter peak signals than lymphocytes (Fig. 1B). Furthermore, dye aggregates were eliminated with excluding the high fluorescent events with CD81-FITC and CD56-APC-A750 (Fig. 1C). Following these procedures, primary identification of plasma cells was performed by gating the events with CD38-PB high and CD138-APC high (Fig. 1D). Additionally, plasma cells were selected with the gating of CD38 and CD45 (Fig. 1E). The samples following anti-CD38 monoclonal antibody treatment, removal of doublets (Fig. 2A), debris (Fig. 2B), and aggregates (Fig. 2C) was...
performed as well as the samples without using anti-CD38 monoclonal antibody, but primary identification of plasma cells was performed CD38-PB negative and CD138-APC high (Fig. 2D). Additionally, plasma cells are selected with the gating of CD138 and CD45 (Fig. 2E), and identification of aPCs was manually performed using a radar plot, which is a projection of the intensity of the released light energy from each eight fluorochrome on a 2-dimensional series of spokes projecting from a central point analyzed by the Kaluza v1.5a software (Beckman Coulter) as previously described in both samples with or without using anti-CD38 monoclonal antibody15 (Figs. 1F, 2F). Using DuraClone, the analysis strategy bases on the different-from-normal approach as there is no specific marker of abnormality known yet but only deviations from the normal expression pattern which are CD45med, CD38high, CD138med/high, CD19positive, CD56dim/neg, CD27high, CD81high, CD200dim/neg. Abnormal plasma cells can deviate by a diversity of abnormal patterns, e.g. CD45low/neg, CD38med, CD138 all high, CD19neg, CD56dim/neg, CD27dim, CD81high, CD200high, or as another example, CD45med, CD38high, CD138med/high, CD19neg, CD56high, CD27med, CD81dim/neg, CD200dim/neg16. In addition to original method of surface staining (Tube 1), we additionally performed intracellular staining of cyIgκ-FITC and cyIgλ-PE (DAKO, product code: FR481) concomitant with CD38-PB (non-ME antibody, Beckman Coulter, product code: B09683), CD138-APC (Beckman Coulter, product code: A87787), CD19-PC5.5 (Beckman Coulter, product code: A66328), CD56-APC-A750 (Beckman Coulter, product code: B46024), CD45-KrO (Beckman Coulter, product code: B36294), CD200-PC7 (Beckman Coulter, product code: B43299) after permeabilization with PerFix (Beckman Coulter) in Tube 2 to confirm the clonality of the abnormal cells as our original method. In Tube 2, elimination of cell doublets and debris were performed by omitting the events with high TOF (Fig. 3A) and by omitting the events with lower forward scatter peak signals than lymphocytes (Fig. 3B) as performed in Tube 1. Furthermore, dye aggregates were eliminated with excluding the high fluorescent events with using cylgx-FITC and CD56-APC-A750 instead of CD81-FITC and CD56-APC-A750 in Tube 1 (Fig. 3C). Primary identification of plasma cells was performed by gating the events with CD38-PB high and CD138-APC high in samples without the treatment of anti-CD38 antibody (Fig. 3D) and with CD38-PB negative and CD138-APC high in those with the treatment of anti-CD38 antibody. Plasma cells were selected with the gating of CD45-KrO and CD38-PB (Fig. 3E) in samples without the treatment of anti-CD38 antibody or CD45-KrO and CD138-APC in those with the treatment of anti-CD38 antibody, and clonality of plasma cells were confirmed by cylgx-FITC and cylgA-PE (Fig. 3F). We splitted evenly (1 mL each) for surface staining and intracellular staining in the DuraClone analysis. These two tubes were not merged in DuraClone analysis. The correlation of the total cell acquisition number, percentages of the total and normal PCs, and MRD levels in both methods was then analyzed in the DuraClone

Figure 2. Overview of the gating strategy of plasma cells and discrimination of normal and abnormal plasma cells with radar plot by DuraClone method in patients who received anti-CD38 antibody. Light scatter characteristics are used to exclude doublets with high time of flight (TOF) (A), debris with lower scatter peak than lymphocytes (B) and aggregates with high fluorescent events with CD81-FITC and CD56-APC-A750 (C). PCs are captured by gating CD138 + CD38 − events (D). PCs are selected with CD138 high events using CD45 (E) Abnormal PCs are detected with radar plot (F). Red dots: myeloma cells, Blue dots: normal plasma cells. FS TOF forward scatter time of flight, FS INT forward scatter intensity, SS INT side scatter intensity.
Statistical analysis. Wilcoxon signed-rank test was used for the comparison of paired variables. Spearman’s correlation coefficient was applied for evaluating the correlation of paired data. All statistical analyses were performed using the EZR software package (Saitama Medical Center/Jichi Medical University, Saitama, Japan)\(^{17}\), which is a graphical user interface for R version 3.0.4.0 (R Foundation for Statistical Computing, Vienna, Austria). \(P\) values < 0.05 were considered statistically significant (two-sided).

Ethics. The local ethic committees of Kameda Medical Center and Kanazawa University approved this study.

Consent to participate. All patients provided written consent in accordance with the Declaration of Helsinki.

Consent for publication. All patients provided written consent in accordance with the Declaration of Helsinki.

Results
A total of 96 samples derived from 69 patients were analyzed, and 21 patients were assessed repeatedly in different states of disease. The patient characteristics (diagnosis and clinical status by the IMWG response criteria for MRD evaluation) are summarized in Table 1. The number of cells obtained using EuroFlow-NGF (median 8.6 × 10^6; range 1.1 × 10^6–11.7 × 10^6) was significantly higher than that obtained by the DuraClone panel (median 5.7 × 10^6; range 0.5 × 10^6–18.3 × 10^6; \(p < 0.0001\)), and the correlation was not so high (\(r = 0.40; p < 0.0001\)) (Fig. 4A). In the
91 (94.8%) and 75 (78.1%) of 96 samples assessed by the EuroFlow-NGF and DuraClone, respectively, >3 million cells were acquired as recommended by the NCI myeloma working group panel18. In addition, 81 (84.3%) and 56 (58.3%) of the 96 samples assessed by the EuroFlow-NGF and DuraClone methods, respectively, achieved an acquisition of 5 million cells as recommended by the IMWG10.

Next, we compared the percentages of the total and normal PCs between the EuroFlow-NGF and DuraClone methods. Relatively a high-correlation was noted in the percentages of the total (\( r = 0.79; p < 0.0001 \)) and normal (\( r = 0.75; p < 0.0001 \)) PCs (Fig. 4B–C).

Finally, we compared the efficiency of MRD detection between the two methods. In the quantification of the MRD levels, a high-correlation in MRD levels was observed between the two methods (\( r = 0.92; p < 0.0001 \)).

### Table 1. Characteristics of the patients’ diagnosis and clinical status when each sample was analyzed.

| Diagnosis (69 patients) | Clinical status (96 samples) |
|------------------------|------------------------------|
| IgGκ 31 (44.9%) PR 5 (5.2%) | IgGλ 8 (11.5%) VGPR 28 (29.1%) |
| IgGκ 9 (13.0%) CR 14 (14.5%) | IgGλ 8 (11.5%) sCR 41 (42.7%) |
| IgAκ 8 (11.5%) CLR 8 (8.3%) | BJκ 5 (7.2%) |
| BJλ 8 (11.5%) | |

Figure 4. Comparison between the DuraClone and EuroFlow-NGF methods. Total cell acquisition (A), percentages of total plasma cells (PCs) (B) and normal PCs (C), quantitative study of minimal residual disease (MRD) detection (D), and qualitative study of MRD detection (E).
### Table 2. Overview of the samples showing discrepancy of MRD negativity between DuraClone and EuroFlow-NGF methods.

| Sample no | Clinical status | Diagnosis | Total cell of tube1 | Abnormal PC | Abnormal PC ratio (MRD) | Abberant expression | MRD negativity* | Abnormal PC | Abnormal PC ratio (MRD) | MRD negativity* | PFS, post-MRD assessment (months) |
|-----------|----------------|-----------|---------------------|-------------|-------------------------|---------------------|----------------|-------------|-------------------------|----------------|-------------------------------|
| 1         | CR             | IgGκ      | 28,53,031           | 13,91,147   | 100                     | Positive            | CD56 +         | 91,70,213   | 0                       | <2 × 10⁻⁶      | Negative                      |
| 2         | sCR            | Igκκ      | 1,42,04,76         | 70,82,588   | 268                     | Positive            | CD56 +, CD200 + | 92,29,865   | 0                       | <2 × 10⁻⁶      | Negative                      |
| 3         | CR             | BJκ       | 1,09,44,839        | 56,55,587   | 32                      | Negative            | CD56 +         | 84,12,160   | 94                      | 1.1 × 10⁻⁵     | Positive                      |
| 4         | CR             | BJκ       | 87,01,500          | 44,10,891   | 6                       | Negative            | CD56 +         | 87,41,417   | 127                     | 1.5 × 10⁻⁵     | No progression                |
| 5         | VGPR           | Igκλ      | 22,42,709          | 10,08,320   | 6                       | Negative            | CD56 +, CD19 + | 73,41,906   | 78                      | 1.1 × 10⁻⁵     | No progression                |

Note: *MRD negativity was 1 × 10⁻⁵ in both DuraClone and EuroFlow-NGF.

Discussion

This study demonstrated that the DuraClone method was not comparable to EuroFlow-NGF in total cell acquisition number, PC plasma cells, MRD minimal residual disease, NGF next-generation flow, CR complete response, sCR stringent CR, VGPR very good partial response, PFS progression-free survival, NA not assessed due to be transferred to another hospital. The thresholds of MRD negativity was 1 × 10⁻⁵ in both DuraClone and EuroFlow-NGF.
addition to surface staining by the fusion of both surface and cytoplasmic staining tubes, however, DuraClone could not fuse surface and cytoplasmic staining cells together, thus counting of aPCs was performed only by surface staining tube using the radar plot. For these reasons, DuraClone has the potential to overestimate aPCs. To date, three methods for monitoring MRD have been developed, namely, PCR, allele-specific quantitative PCR, and next-generation sequencing, and emerged as attractive and sensitive approaches. Among these methods, EuroFlow-NGF is currently the most cost-effective and widely available method. Compared with the EuroFlow 2-tube method (< 350 USD/sample), the DuraClone single-tube method (< 100 USD/sample) is expected to be more cost-effective and can be used under public medical insurance in countries such as Japan.

In summary, we found Duraclone is a good option to evaluate MRD as the EuroFlow 8-color 2-tube method in MM but it should be used with caution and some discrepancies between them may have resulted because of the difference in the number of cells analyzed and antibody panels. Therefore, a sufficient cell acquisition number (> 5 × 10⁶) is essential to achieve high sensitivity in MRD detection in MM.

Data availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
T.Y., K.N., H.T. and K.M. designed the research study, performed the research, analyzed the data and wrote the manuscript; T.Y., K.N., H.T., M.F. and S.N. and K.M. performed the research.
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Competing interests
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Additional information
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