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Letter to the Editors

# Additional Stability Testing of Cryopreserved Intestinal Biopsies for Downstream Flow Cytometric Analysis

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*Dear Editors*

The use of flow cytometry (FC) in therapeutic clinical trials involving patients with gastrointestinal (GI) diseases may accelerate drug development by providing insights into pharmacokinetic/pharmacodynamic relationships and target engagement, as well as by identifying responder populations. The application of FC in these trials, however, has been constrained by the practical limitations of storing and shipping intestinal biopsy samples prior to cell extraction and FC analysis, and the potential impact of handling and storage conditions on viable cell yield. Furthermore, there has been a lack of standardization for these processes. We have undertaken research to attempt to address these constraints to the inclusion of translational science in multi-center clinical trials.

Our previous work, Wildenberg et al. (2018, 459:50-54), published in this journal, demonstrated that it was feasible to store intestinal biopsies from patients with Crohn's disease under conditions that allow for subsequent processing with preservation of adequate numbers of viable cells for valid FC analyses. This study demonstrated that storage of intestinal tissue biopsies at −20 °C in DMSO/citrate buffer for up to 48 h resulted in sufficient viable cell yield for FC analysis without affecting subsequent marker-positive cell proportions. Although these preliminary findings provide support for the shipping and storage of intestinal biopsies for centralized FC analysis in multicenter clinical trials, some practical limitations remain. For example, in the clinical trial setting, where patient enrolment and study procedures occur in an ongoing fashion and frequently over an extended period of time, it is impracticable from a resource perspective for a central laboratory to process study samples as they are collected from individual patients. Furthermore, contemporary clinical trials in the inflammatory bowel diseases are most often conducted on a global scale. Shipping and processing of biopsy samples by a central laboratory within a 48 h timeframe in this context would compound costs and add logistical hurdles that might discourage industry support for the inclusion of translational science in the multi-center clinical trial setting. In this regard, longer-term storage, and batch processing of study biopsy samples would be a preferred method. This approach would reduce both the variability and costs associated with more frequent assays and expansive resource utilization.

To that end, we developed an addendum to our original study protocol to examine the effect of longer-term storage condition prior to cell isolation and FC on viable cell yield and the proportions of immune cell phenotypes from intestinal biopsies. Consistent with the original protocol, the study was performed at Tytgat Institute (Academic Medical Center, Amsterdam, the Netherlands) under the original ethics approval, and with informed consent. Biopsies (N = 180) were procured from surgically resected inflamed or non-inflamed ileal and/or colonic tissue from four patients undergoing Crohn's disease-related surgeries (colectomy [n = 2], subtotal colectomy, ileo-cecal resection). Two biopsies were pooled and processed as a single sample, resulting in 90 samples per surgical specimen and 18 samples per condition tested. The effects of 5 different storage conditions on cell viability and subsequent FC analyses were compared in this study addendum; immediate (< 1 h from sample receipt) processing, storage at −20 °C and −80 °C for 24 h with subsequent overnight storage on dry ice (to mimic real life shipping conditions) followed by long-term (2–3 months) storage at −20 °C and − 80 °C, and immediate long-term storage at −20 °C and − 80 °C. Subsequent methods for cell isolation, staining, and FACS analysis were as previously described (Wildenberg et al. 2018, 459:50–54).

Analysis of freshly processed biopsy samples resulted in a mean (standard deviation, SD) yield of 36916.7 (59194.7) live immune cells as identified by CD45+ staining. Storage of biopsies for greater than or equal to two months at either −20° or − 80 °C significantly reduced the mean [standard deviation] number of live CD45+ cells compared to immediate processing (372.0 [607.1] and 4065.3 [4489.7], respectively, $p < 0.001$ for both comparisons with immediate processing). Sufficient cell yield (≥ 300 live CD45+ cells) for subsequent FC analysis was obtained from 77.8% (14/18) of samples processed immediately compared to 22.2% (4/18) and 27.8% (5/18) of samples stored at −20 °C (with and without simulated shipment; $p < 0.001$ chi square test) and 77.8% (14/18) and 94.4% (17/18) of samples stored at −80 °C (with and without simulated shipping; $p = 0.439$).

We further evaluated the effect of storage on the composition of specific cell types in the samples and observed a significant decrease in the proportion of CD3+ and CD4+ cells after storage at −20 °C

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**Table 1**
Marker positive cells according to storage condition and time to processing (subset of samples with ≥300 live CD45+ cells).

| | | Storage condition | | | | |
|---|---|---|---|---|---|---|
| s | Within | Immediate processing | −20 °C dry ice | −20 °C | −80 °C dry ice | −80 °C |
| CD3+ | Live CD45+ leukocytes | 40.4 (15.6) | 22.6 (12.7)[1] | 23.7 (9.2)[1] | 38.5 (11.1) | 41.1 (9.4) |
| CD4+ | Live CD3+ αβT cells | 57.3 (18.1) | 17.7 (4.3)[2] | 20.7 (15.5)[2] | 49.0 (15.0) | 54.9 (13.6) |
| CD8+ | Live CD3+ αβT cells | 25.5 (9.3) | 51.2 (27.1) | 49.0 (38.3) | 22.2 (12.1) | 16.7 (10.2) |
| CD14+ | Live CD45+ leukocytes | 3.45 (3.61) | 5.99 (5.24) | 5.03 (5.02) | 2.69 (1.65) | 1.93 (1.44) |

[1] $p < 0.05$.
[2] $< 0.001$ compared to immediate processing.

compared to samples that were immediately processed (Table 1 and Fig. 1, panels A and B). The proportion of CD8+ and CD14+ cells was unaffected by storage temperature, although the standard deviations for the proportions of these cells in samples stored at −20 °C were larger than for those observed for samples stored at −80 °C (Table 1 and Fig. 1, panels C and D).

In conclusion, our previous work demonstrated that it is feasible to store mucosal biopsies at −20 °C for 48 h in DMSO/citrate buffer before further processing with preservation of adequate numbers of viable cells for valid FC analyses suggesting that centralized FC is possible. These data provided a basis for performing additional stability testing (including shipping and longer storage). Results from this protocol addendum suggest that lower temperatures (−80 °C) may be required for longer-term storage of mucosal biopsies, and that long-term storage at −20 °C may both reduce viable cell yield, and alter the proportion of certain immune cells detected on subsequent FC analysis. We cannot, however, exclude the possibility that our results were confounded by selection bias and/or that the effects that we observed on cell proportion were the result of significantly fewer samples with ≥300 live CD45+ cells for analysis after storage at −20 °C (albeit a result of decreased viability). Overnight storage of biopsies on dry ice to mimic real life shipping conditions to a central laboratory did not appear to affect live cell yield or immune cell proportions for the markers studied (CD3+, CD4+, CD8+, CD14+).

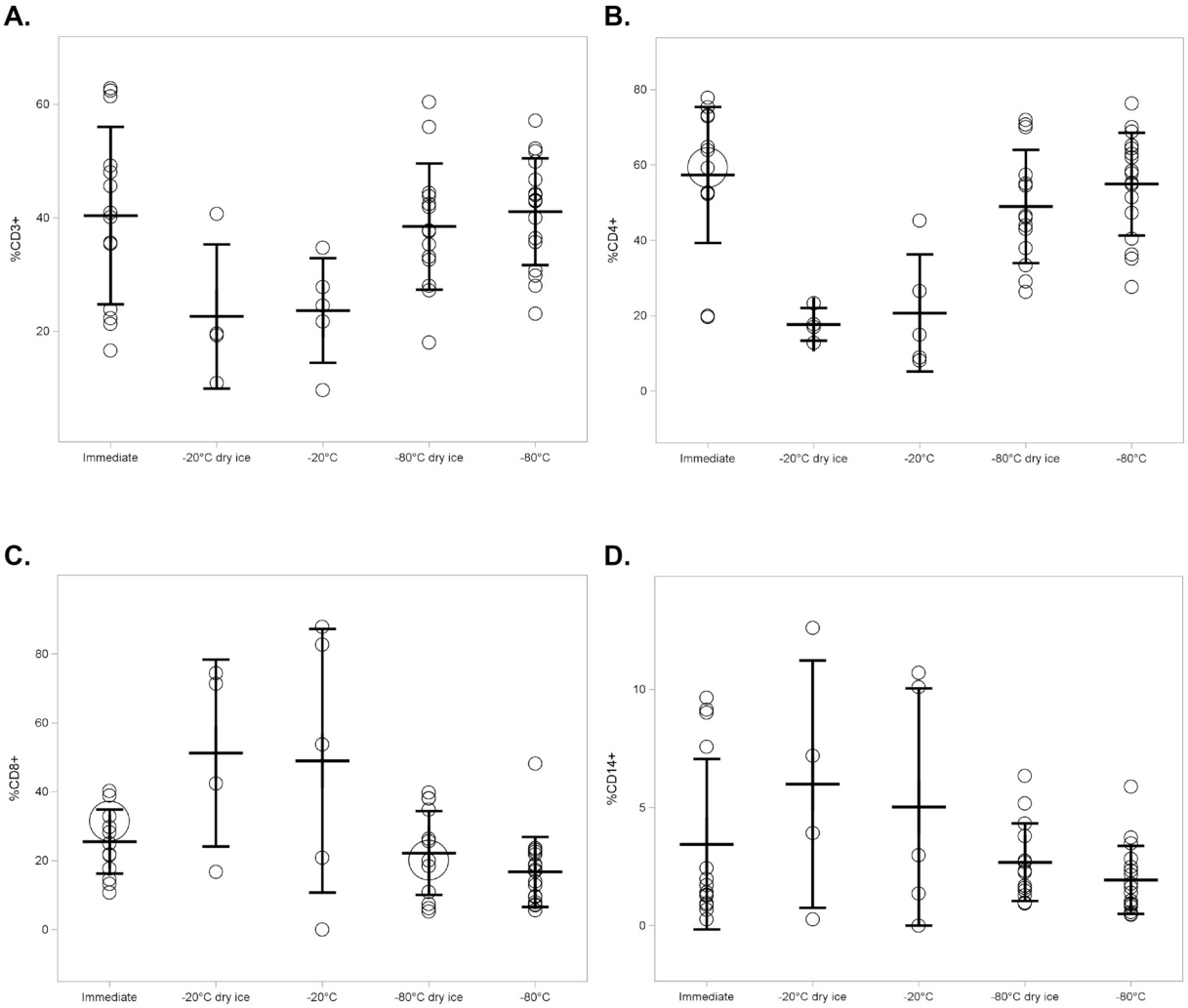


**Fig. 1.** Mean proportion of immune cell phenotypes according to storage condition. Bars represent standard deviation, and circle size is proportional to sample size. (A) %CD3+, (B) %CD4+, (C) %CD8+, (D) %CD14+.

## Potential competing interests

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