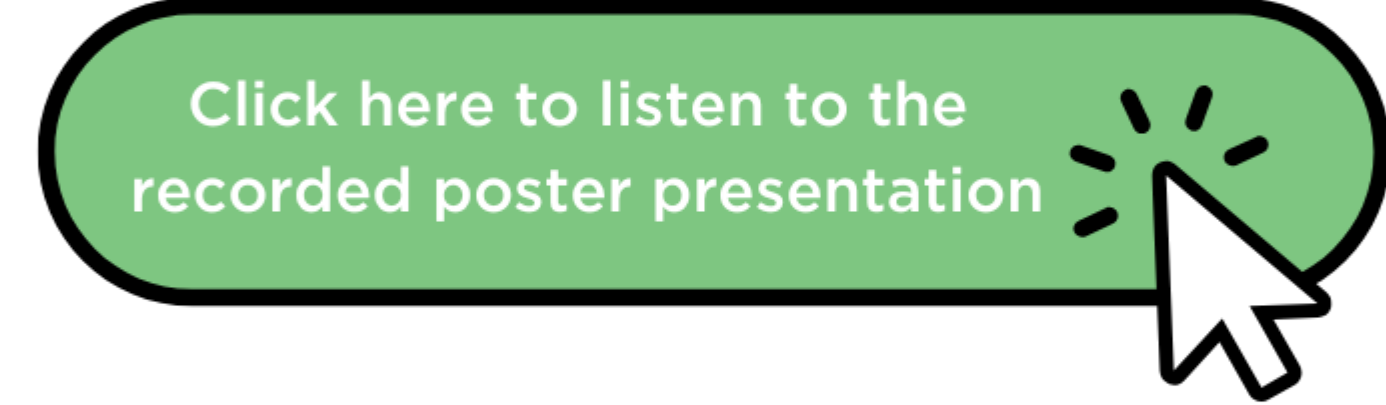


Development of a Novel Surrogate Blister Fluid Matrix for Flow Cytometry Assay Validation to Support Bioanalysis

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INTRODUCTION

Blister fluid is a matrix rich in immune infiltration but rarely analyzed by flow cytometry. The cantharidin-induced blister model generates a local inflammation in the form of a blister on the skin that can be used for assessing leukocyte trafficking during the evaluation of novel anti-inflammatory drug candidates in healthy volunteers.

Developing a flow cytometry assay for blister fluid is challenging due to the limited availability of fresh blister fluid material. To overcome this limitation, we developed an innovative surrogate matrix enabling a fit-for-purpose validation of a flow cytometry assay to support clinical trials requiring blister fluid bioanalysis.

METHODS

Blister fluids are generated with the application of Cantharidin on the skin surface (Figure 1). Different strategies were evaluated to engineer a surrogate blister fluid matrix suitable for the assessment of a flow cytometry assay performance (Figure 2). To assess the performance of those surrogate matrices, a flow cytometry assay has been used (Figure 3) (Table 1).

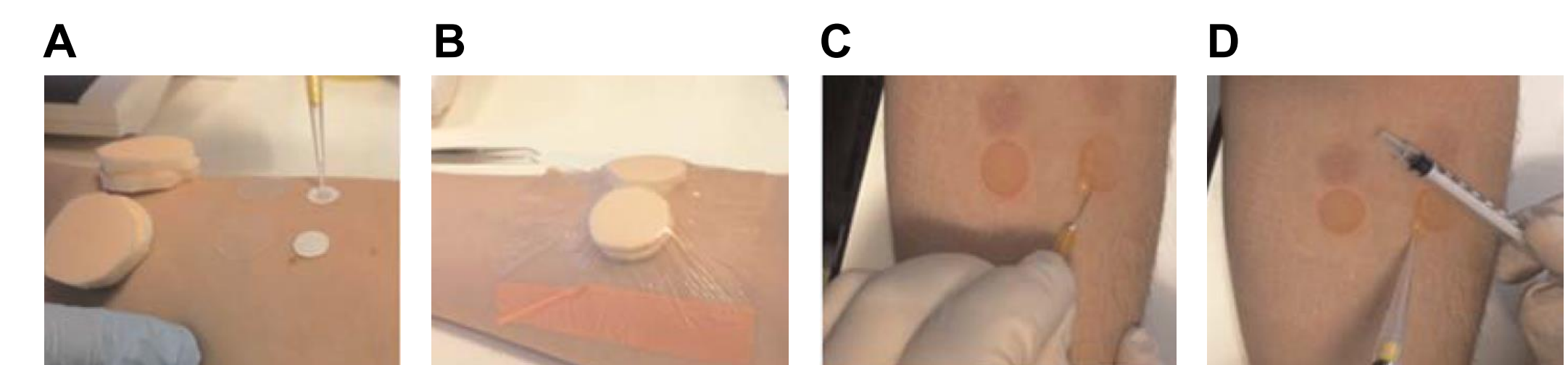


Figure 1. Cantharidin-Induced Blister Fluid
 (A) 25 µL of Cantharidin (0.1% solution in acetone) is pipetted onto paper discs. (B) Paper discs are covered with a 16-mm film disc and then with foam pads covered with an adhesive dressing. (C) The fluid is extracted by piercing the epidermis at the lateral border using a 26-gauge sterile needle. (D) The edema is allowed to drain, and a 1 mL syringe is very softly (only light pressure can be applied on the surface of the blister to avoid rupture) rolled across the blister to guide fluid aspiration. The fluid is carefully aspirated using a siliconized sterile tip and immediately transferred to a sterile siliconized 1.5 mL Eppendorf tube containing 50 µL sodium citrate.

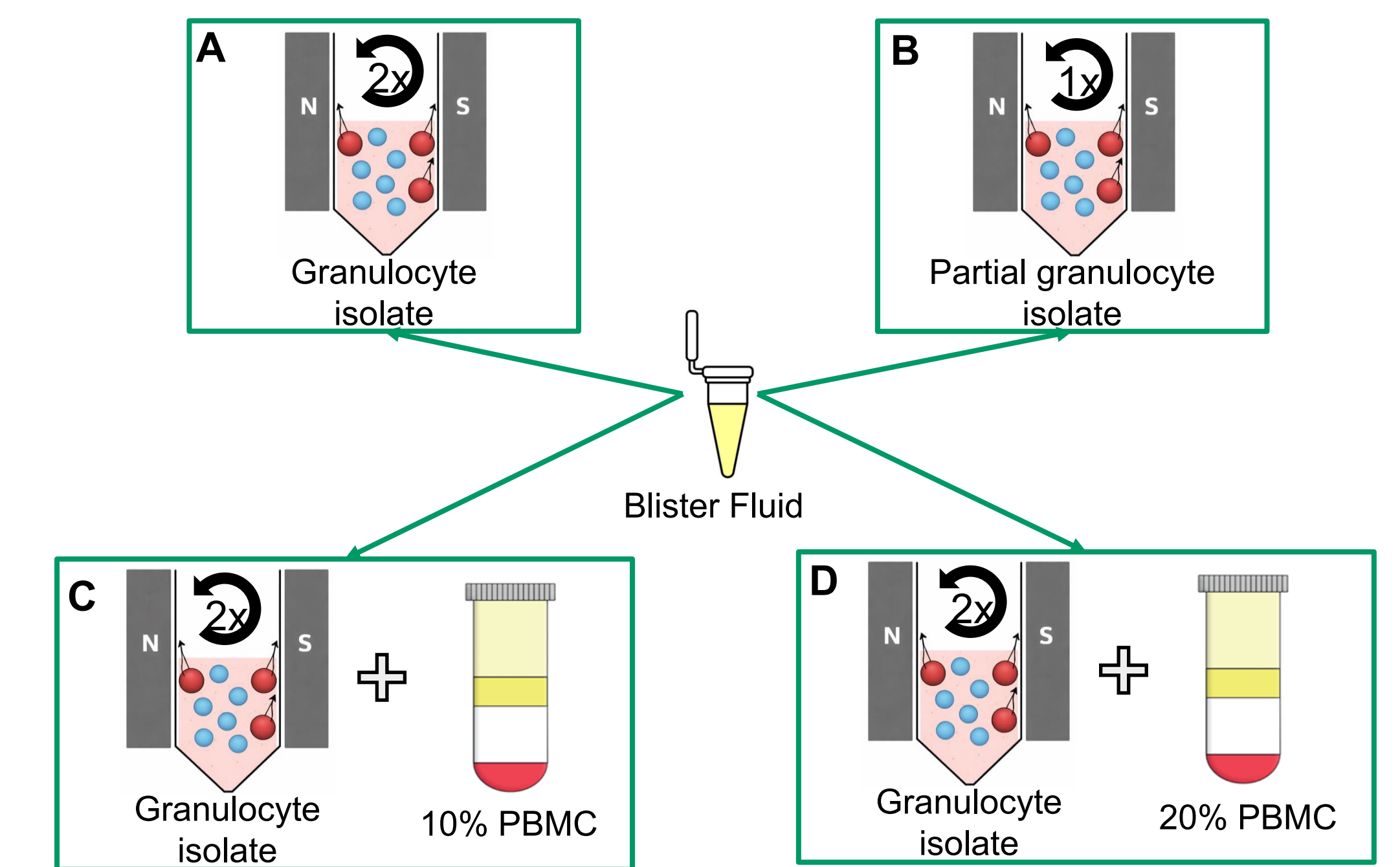


Figure 2. Different Surrogate Matrices of Blister Fluid
 Multiple surrogate matrices have been tested and compared to cantharidin-induced blister fluid. Whole blood collected in sodium heparin vacutainer have been used to perform granulocyte isolation (Stemcell cat# 19659) and/or isolate PBMC with Ficoll/Sepramate. (A) A granulocyte isolate has been achieved by following the manufacturer's recommended protocol, which consists of 2 rounds of magnetic separation. (B) Partial granulocyte isolation has also been tried by performing only one round of magnetic isolation. (C-D) Autologous PBMC were added to granulocyte isolates at 10% (C) or 20% (D).

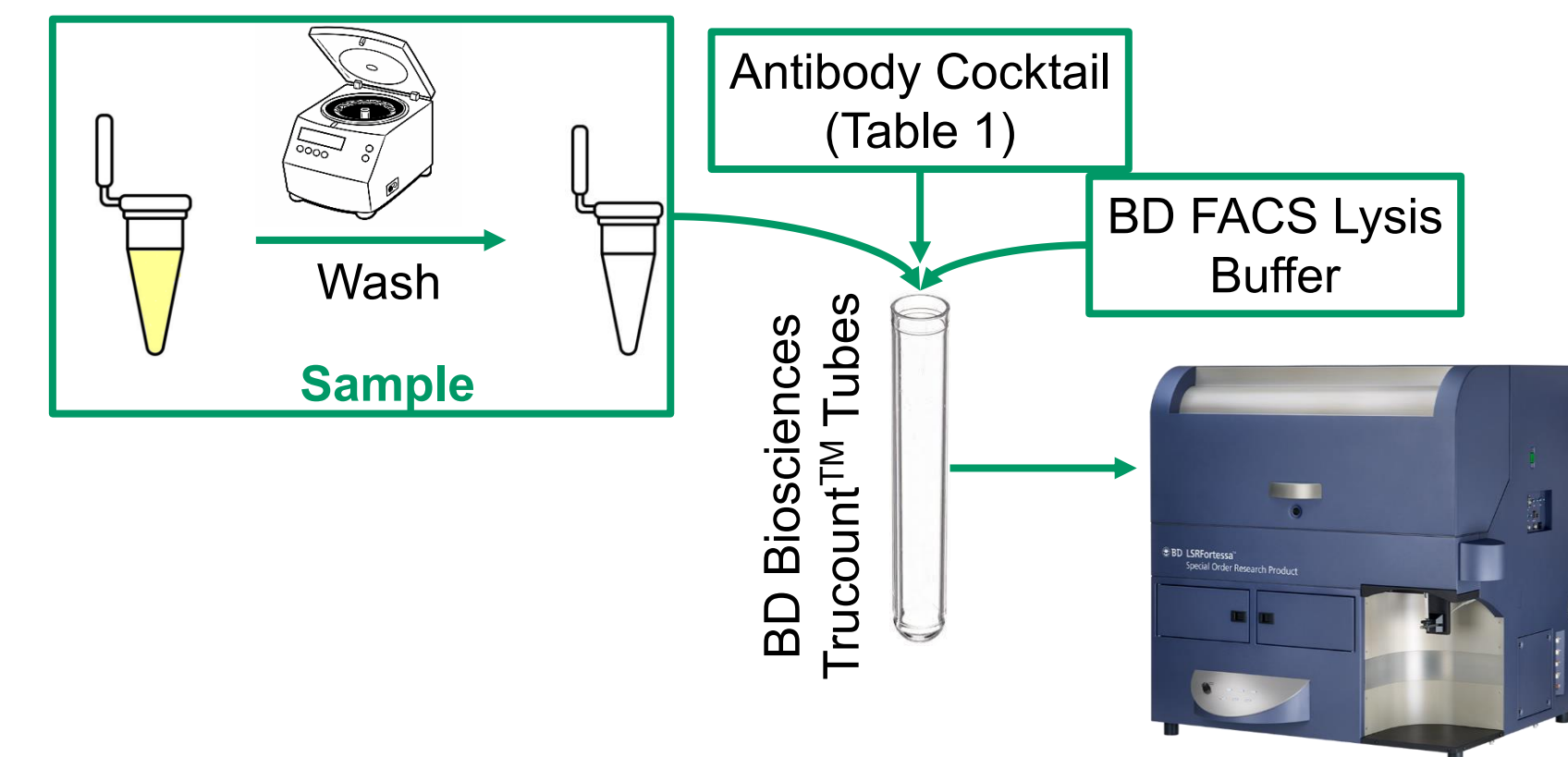


Figure 3. Sample Staining Method
 Samples (i.e., blister fluid, isolated cell fractions, PBMC) are used whole or mixed accordingly. Then, a single wash is performed to remove the supernatant and resuspended in staining buffer. The whole sample is then added to a BD Biosciences Trucount™ tube (BD Biosciences cat# 663028). A Lyse-no wash staining procedure is performed with the addition of the staining cocktail (Table 1) and FACS™ Lysing Solution (BD Biosciences cat# 349202). Stained samples are acquired on a BD FACS Fortessa.

Table 1. Flow Cytometry Antibody Cocktail and Readout List

Marker	Readout	Expression Profile
Viability FVS510	Living Cells	Viability
CD45 APC-H7	CD45+ Leukocytes	ViabilityCD45+
CD3 FITC	CD3+ T cells	ViabilityCD45+SSC-A ^{low} CD3+CD19/CD20-
CD19 R718	CD19/CD20+ B cells	ViabilityCD45+SSC-A ^{low} CD3-CD19/CD20+
CD20 R718	Monocytes	ViabilityCD45+SSC-A ^{Mid} CD14 ⁺
CD14 BV605	Neutrophils	ViabilityCD45+SSC-A ^{high} CD15 ⁺ CD16 ⁺
CD15 BV786	Eosinophils	ViabilityCD45+SSC-A ^{high} CD15 ⁺ CD16 ^{low} CD193 ⁺
CD16 BV711		
CD193 BV421		

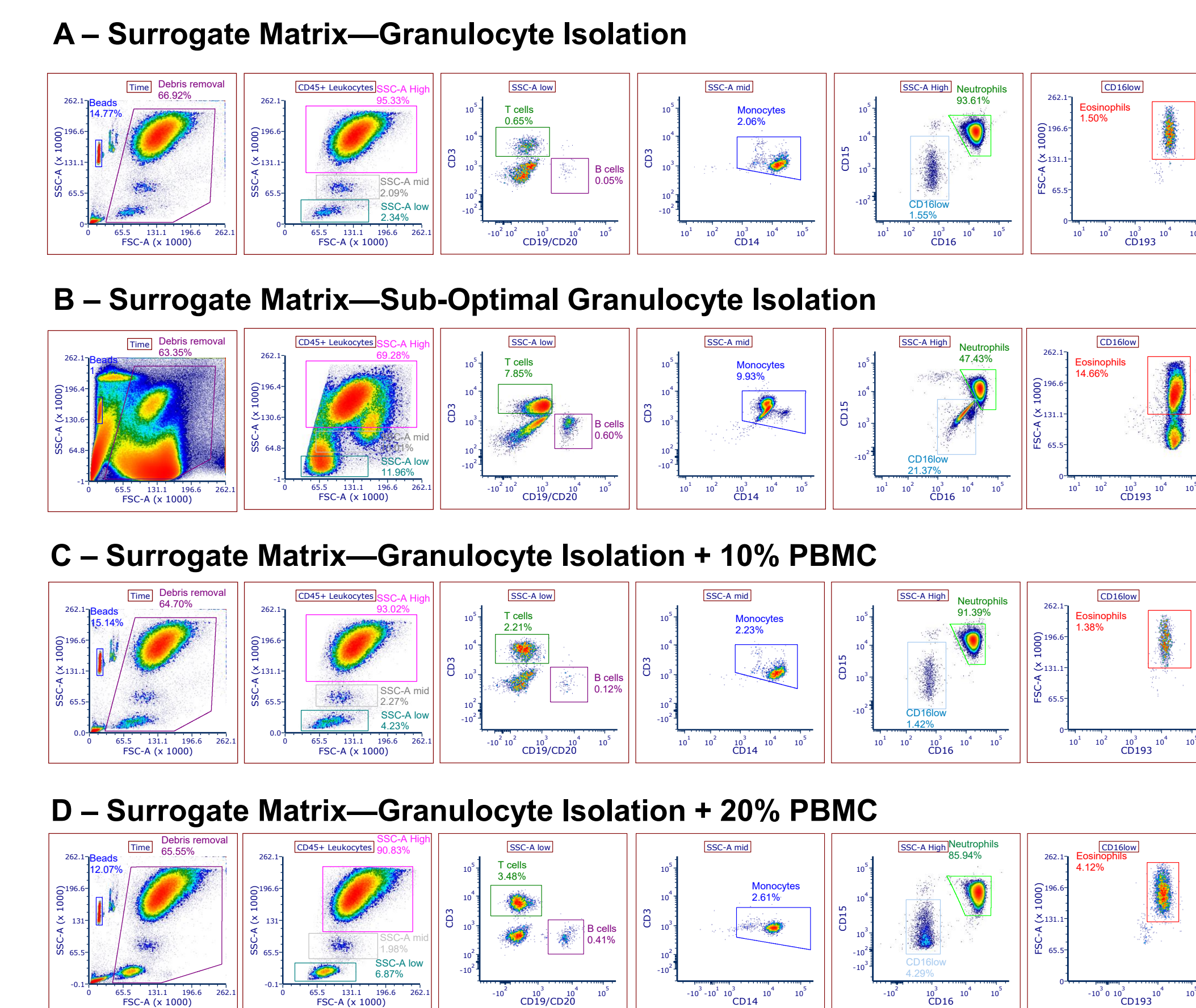


Figure 4. Flow Cytometry Analysis of Blister Fluid Surrogate Matrices
 (A) Whole blood underwent a granulocyte isolation as per the manufacturer's recommended protocol. (B) Whole blood underwent a sub-optimal granulocyte isolation (i.e., only one round of magnetic isolation performed). (C-D) Whole blood underwent a granulocyte isolation protocol and autologous addition of freshly isolated at a ratio of 10% (panel C) and 20% (panel D).

RESULTS

The different surrogate matrix strategies were tested with the objective of comparing them to theoretical values in terms of granulocyte and lymphocyte content (Figure 4).

A pan-granulocyte isolation kit did not have enough lymphocyte content to be comparable to blister fluid. A sub-optimal granulocyte isolation (i.e., only performing 1 round of magnetic separation compared to the standard protocol of two rounds) was tested to increase the lymphocyte content. However, too much debris led to flow analysis issues. To increase lymphocyte/monocyte content, different ratios of freshly isolated autologous PBMC were added to the granulocyte isolates. Frequencies of leukocytes for each immune cell subset are summarized in Table 2. The granulocyte isolated with 20% autologous fresh PBMC was identified as the best suitable surrogate matrix for cantharidin-induced blister fluid.

Table 2. Frequency (relative to leukocytes) of Populations for Different Surrogate Matrices

Cell Population	Granulocytes Isolation	Sub-optimal granulocytes Isolation	Granulocytes Isolation + 10 %PBMC	Granulocytes Isolation + 20 %PBMC	Blister Fluid (theoretical values) ¹
T cells	[0.62 – 0.65] %	[2.0 – 2.2] %	[2.0 – 2.2] %	4.9 (± 2.5) %	2 – 5 %
B cells	[0.05 – 0.06] %	[0.15 – 0.17] %	[0.15 – 0.25] %	0.6 (± 0.3) %	0.02 – 0.05 %
Monocytes	[2.0 – 2.1] %	[2.5 – 4.6] %	[2.3 – 2.8] %	1.3 (± 0.4) %	12 – 22 %
Neutrophils	[93.2 – 93.4] %	[11.9 – 23.4] %	[88.1 – 91.3] %	85.9 (± 4.5) %	50 – 70 %
Eosinophils	[1.5] %	[10.8 – 14.7] %	[1.4 – 2.2] %	3.6 (± 1.1) %	0.2 – 2.7 %

1. Dinh et al. BJCP 2011

Two healthy volunteers underwent the Cantharidin procedure (presented in Figure 1), and blister fluids were analysed by flow cytometry (Figure 5). Data was compared to 12 independent surrogate matrices isolated using the procedure outlined in Figure 4 panel D (i.e., granulocyte isolates + 20% autologous PBMC) as part of a fit-for-purpose validation to assess both the robustness of the flow cytometry method and the comparison between the two matrices. Given the interest for the clinical study was T cells, neutrophils, and eosinophils, the comparison was focused on those sub-populations specifically. (Tables 3 and 4)

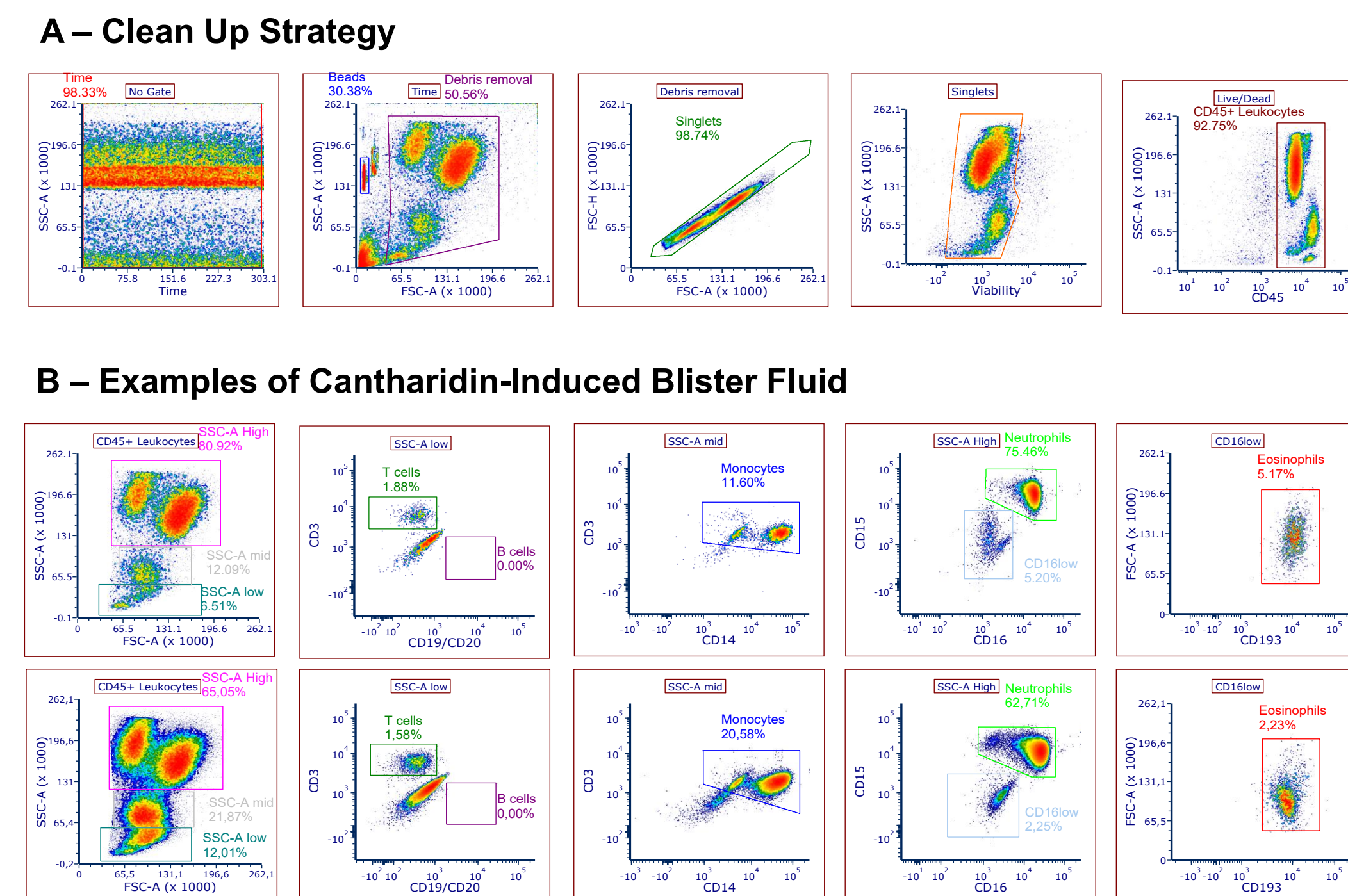


Figure 5. Flow Cytometry Analysis of Cantharidin-Induced Blister Fluids
 (A) Example of the clean-up strategy applied to all samples analyzed. First dot plot is a time gate allowing the removal of all fluidic instabilities, then, followed by an FSC-A / SSC-A dot plot to gate on beads and remove debris. FSC-A / FSC-H allows the removal of doublets. Finally, the last 2 dot plots are gating on viable cells and CD45 to have a final cleaned-up sample representing all leukocytes. (B) Example of cantharidin-induced blister fluid showing all readouts (i.e., from left to right – T cells, B cells, Monocytes, Neutrophils, and Eosinophils).

Table 3. Data of 12 Independent Surrogate Matrix Samples (Using Figure 4 – Panel D) Compared to Cantharidin-Induced Blister Fluid

Sample	Surrogate Matrix			Blister Fluid		
	T cells	Neutrophils	Eosinophils	T cells	Neutrophils	Eosinophils
	% of Leukocytes					
1	1.0	90.9	5.0	1.6	64.2	2.8
2	9.3	76.4	4.4	1.8	73.8	6.3
3	4.1	89.5	2.8			
4	7.4	84.7	3.0			
5	6.8	82.8	3.2			
6	4.0	87.5	2.7			
7	3.5	89.9	3.6			
8	3.3	87.7	4.1			
9	3.0	86.3	5.6			
10	3.6	89.2	2.0			
11	7.6	80.4	3.6			
12	10.3	60.7	19.1			
Range:	[1.0 – 10.3]	[60.7 – 90.9]	[2.0 – 19.1]	[1.6 – 1.8]	[64.2 – 73.8]	[2.8 – 6.3]

Table 4. Fit-for-Purpose Validation Summary – Frequencies and Absolute Counts

Intra-Assay Precision (24 independent triplicates evaluated)						
For all readouts, at least 23 out of 24 independent triplicates had %CV ≤30%.						
Inter-Assay Precision (12 independent samples evaluated (in triplicate))						
For all readouts, at least 10 out of 12 independent samples, the %Difference were within ±30%.						
Sensitivity (LLOQ) (3 independent samples serial diluted 1 in 2 for a total of 5 times)						
Lowest absolute counts reported where the %CV of the triplicate is lower or equal to the Precision evaluation.						
	Leukocytes CD45+	T cells CD3+	B cells CD19+CD20+	Monocytes	Neutrophils	Eosinophils
Absolute Counts	694	37	110	107	524	35
Processed Sample Stability (3 independent processed samples acquired at baseline (within 2 hours of the experimental end time) versus the same processed samples stored at 4 °C for 22.6 hours)						
For all readouts, at least 2 out of 3 samples, the % difference were within ±20%.						

CONCLUSION

The surrogate matrix that most closely resembled cantharidin-induced blister fluid leukocyte compositions consisted of a granulocyte concentrate using a granulocyte magnetic negative selection kit with the autologous addition of 20% freshly isolated PBMC, both derived from the same donor.

This innovative surrogate matrix approach facilitated the development and fit-for-purpose validation of a flow cytometry assay for bioanalysis of blister fluid samples. The resulting immunophenotyping assay proved to be a reliable and reproducible method for analyzing non-cryopreserved blister fluid specimens, expanding analytical capabilities to study local inflammation in clinical trials.

This fit-for-purpose validated flow cytometry assay supported clinical trial #NCT06799416.