Freezing Blood Samples for Deferred Immunophenotyping

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Introduction

Blood degrades over time affecting the immunophenotype and cell numbers [(1)]. This is a challenge for clinical trials assessing the immune profile of patients where samples may not be immediately tested. To protect against sample degradation, TransFix/EDTA Vacuum Blood Collection Tubes (TVTs) are designed to stabilise whole blood for up to 14 days at 2-8°C[(2)], however clinical studies may require samples to be preserved for many months whilst comparing long term effects of drug exposure. Freezing samples is therefore a potential solution for long-term storage, but cryopreservation techniques can require hands-on time which is undesirable at collection sites.

The methodology of fixation and freezing can be used to prepare samples for phosphoflow to detect the phosphorylation of intracellular signalling molecules. This technique is used to determine pharmacodynamic effects of small molecular inhibitors targeting signalling kinases and is therefore of interest for drug discovery [(3)].

Objectives

Previous studies have indicated that samples stored in TVTs are suitable for immunophenotyping after being frozen at -80°C [(4)]. We designed a study to confirm these previous finding. The ability to store blood collected in TVTs at -80°C may then offer a simple, cost effective way to preserve samples for future analysis of immunophenotype, and opens the possibility that the TVTs could be suitable for phosphoflow applications or for use as internal controls.

Methods

Immunophenotyping of blood using TVTs after storage at -80°C

Day 0: Blood from three healthy donors was collected in triplicate into a BD EDTA Vacutainer (Control) and TVTs, and then immediately immunophenotyped using a standard TBNK panel on a Beckman Coulter Navios flow cytometer. All blood tubes to be frozen were inserted in a polystyrene rack and placed in the -80°C freezer.

Day 7: After storage for 7 days at -80°C, all samples were thawed at 37°C for 30 minutes and tested in triplicate as per Day 0. The TVTs were also visually examined for structural integrity after freezing.

Optimisation of storage method for immunophenotyping and tube integrity

It was hypothesised that altering the freezing method could improve the immunophenotype and would reduce the effect of freezing on tube integrity. On Day 0, blood from one healthy donor was collected into the Control, and 4x TVTs at the same venepuncture. Each tube was then subject to one of the conditions in Table 1 for 7 days, then immunophenotyped as previously described.

	Freezing condition	Storage Condition	Thawing Condition
Condition 1	Fast freeze (placed directly at -80°C)	Tube stored upright	Thaw at room temperature
Condition 2	Fast freeze (placed directly at -80°C)	Tube stored upright	Thaw at 37°C
Condition 3	Fast freeze (placed directly at -80°C)	Tube stored at a 45° angle	Thaw at 37°C
Condition 4	Slow freeze (Δ-1°C per minute)	Tube stored upright	Thaw at 37°C

Table 1. *Summary of conditions for freezing, storage and thawing of TVTs*

The effects of freeze thaw methods on immunophenotyping and tube integrity

Table 2 summarises the flow cytometry functional testing conducted on the stabilised TVTs (conditions 1-4). All values relate to the percentage difference between the parameter result and the equivalent result for the Control on Day 0. Table 2 shows that tubes stored under condition 3 (TVTs stored at a 45° angle and thawed at 37°C) performed best overall. All tubes storage under condition 3 remained intact.

DAY 7 % DIFF	% CD3	% CD4	CD3 Count	CD4 Count	CD8 Count	CD45 Count	B Cell Count	NK Cell Count	Granulocyte Count	Monocyte Count
Condition 1	0.4	17.2	79.8	83.3	78.0	79.8	86.1	78.2	30.9	67.8
Condition 2	0.3	7.8	20.6	26.8	18.1	20.4	40.5	23.7	0.1	11.0
Condition 3	0.1	6.9	22.0	22	13.6	16.3	42.8	12.0	0.6	13.5
Condition 4	1.7	10.5	31.8	31.8	22.3	22.5	44.7	10.4	2.7	26.8

Table 2. *Tests are summarised above in terms of parameter percentage difference from the EDTA result from Day 0. Best results are highlighted in green and worst in red.*

Forward scatter (FSC) vs side scatter (SSC) characteristics after freezing

Blood stored in the TVTs (Figure 1ii) shows that the forward and side scatter characteristics for granulocytes (black) and monocytes (yellow) are decreased from the Control day 0 (Figure 1i), but all populations including the lymphocytes are still clearly identifiable. Dot plots CD8 + CD19 vs CD3 + CD56 show clear populations on day 7 for the TVTs (Figure 2ii) similar to the Control at day 0 (Figure 2i).

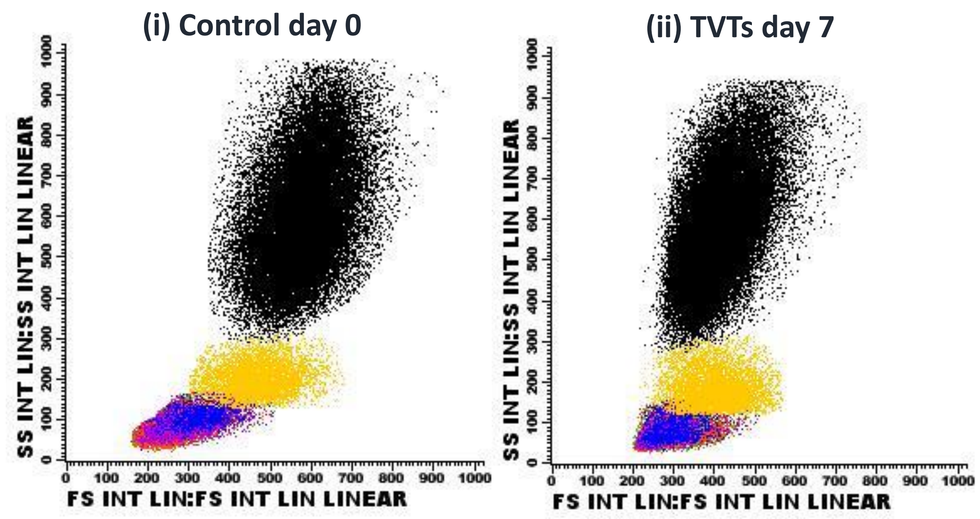

Figure 1i-ii*. Dot plots depicting leukocyte forward scatter (FSC) vs side scatter (SSC) characteristics of blood collected in the fresh Control at day 0 (i) and thawed TVTs day 7 (ii)*

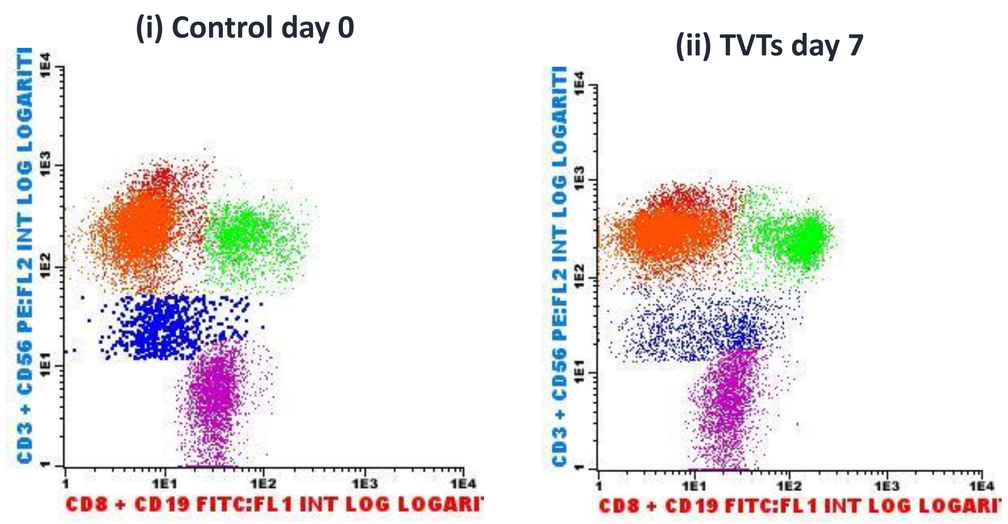

Figure 2i-ii. *Dot plots depicting lymphocyte CD8 + CD19 vs CD3 + CD56 expression characteristics for the fresh Control at day 0 (i) and thawed TVTs at day 7 (ii)*

The effects of freezing on immunophenotyping and tube integrity

Table 3 summarises the flow cytometry functional testing conducted on the TVT stabilised blood. All values relate to the percentage of total cell count recovery between the parameter result and the equivalent result for the same tube on Day 0. All TVTs remained intact after storage at -80°C for 7 days.

The average lymphocyte recovery (CD45 Count) for the TVTs is 77% and lymphocyte subset recovery ranged from 51-80% across all donors.

	Donor	% CD3	% CD4	CD3 Count	CD4 Count	CD8 Count	CD45 Count	B Cell Count	NK Cell Count	Granulocyte Count	Monocyte Count
% Cell Recovery	A	93.7	94.9	70.4	66.8	72.8	75.1	55.4	124.7	93.8	80.2
	B	92.7	93.5	74.0	69.2	73.3	79.8	50.8	76.5	90.9	66.6
	C	96.9	89.9	72.2	65.0	71.1	74.5	65.1	81.8	102.5	86.4

Table 3. *Day 7, % cell recovery. Tests are summarised above in terms of % total cell count recovery from the same tube and donor on Day 0.*

Conclusions and further work

The samples stored in TVTs showed average lymphocyte recovery of 77% after storage at -80°C and thawed after 7 days. The TVTs were also able to withstand being frozen at -80°C without compromising tube integrity. This suggests the TVTs may be suitable for freezing whole blood at -80°C. We foresee an application of stabilising and freezing samples to create internal quality controls.

Further work is required to determine the upper limit of time in storage for frozen whole blood samples to still be viable for immunophenotyping. Future investigation into whether this product is suitable for phospho-specific flow cytometry would also be of interest as we foresee a tube that is suitable for both immunophenotyping and phosphoflow assays could be highly advantageous for clinical research.

References

1. Ekong T, Kupek E, Hill A, Clark C, Davies A, Pinching A. Technical influences on immunophenotyping by flow cytometry. The effect of time and temperature of storage on the viability of lymphocyte subsets. J Immunol Methods. 1993; 164: 263–273. **2**. Harrison D, Ward R, Bastow S, Parr A, Macro S, and Wallace P K. Interlaboratory Comparison of the TransFix®/EDTA Vacuum Blood Collection Tube with the 5 mL Cyto-Chex® BCT. Cytometry Part B (Clinical Cytometry). 2018; 9999: 1–12. **3.** Sommer U, Morales J, Groenewegen A, Muller A, Naab J, Woerly G, Kamphausen E, Marsot H, Bennett P, Kakkanaiah V, et al. Implementation of highly sophisticated flow cytometry assays in multicenter clinical studies: considerations and guidance. Bioanalysis. 2015; 7:1299–1311. **4.** Pouliot P., Sugden S., Chagnon-Choquet J., Lelaidier M., Young K., Belanger B., Gagnon D., Poulin J-F. Extension of whole blood sample stability for flow cytometry analysis through the use of specialized blood collection tubes. POSTER presented at the European Bioanalysis Forum 2017 by Caprion Biosciences Inc.