WHITE PAPER: Freezing Blood Samples for Deferred Immunophenotyping

D. Coupar, J. Thompson, N.Kaenzig. March 2019
Cytomark Limited. Whiteleaf Business Centre, 11 Little Balmer, Buckingham, MK18 1TF, UK

Abstract

A study conducted by the Canadian Clinical Research Organisation (CRO), Caprion found that whole blood samples stored in Cytomark's TransFix/EDTA Vacuum Blood Collection tubes (TVT) are suitable for immunophenotyping after being frozen at -80°C. Caprion also observed that a competing product, Streck's Cyto-Chex Blood Collection Tubes (BCT), were not useable after storage at -80°C.

Cytomark performed a short study to determine whether Caprion's findings could be replicated. This study used the TVTs and BCTs to compare the immunophenotype of fixed peripheral whole blood samples at time zero, and after freezing at -80°C for 7 days. The structural integrity of the frozen TVTs and BCTs were also examined. Optimisation of storage conditions was further investigated to improve immunophenotyping after freezing.

TVTs showed the best cell percentage differences from the control with average lymphocyte cell recovery of 77% compared to 26% for the BCTs. All three BCTs cracked during the freezing process while all of the TVTs remained intact. Storage optimisation experiments showed tubes frozen at an angle and thawed at 37°C performed best overall.

These results raise the possibility that whole blood samples for immunophenotyping may be stored at -80°C for extended periods beyond 7 days. Further studies will aim to demonstrate the upper limit of time the sample will remain stable and suitable for immunophenotyping. Experiments will also be conducted to examine the suitability for use in phosphoflow assays.

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 TVTs and BCTs are both designed to stabilise whole blood for up to 14 days[(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)].

A study conducted by the Canadian CRO, Caprion indicated that samples stored in TVTs are suitable for immunophenotyping after being frozen at -80°C. Caprion also observed that blood samples collected in the BCTs were not useable after storage at -80°C [(4)].

Cytomark designed a study to determine whether Caprion's findings could be replicated. The ability to store blood collected in TVTs at -80°C offers 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.

Methods

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

Day 0: Blood from three healthy donors was collected in triplicate into a BD EDTA Vacutainer (Control), TVTs and BCTs, and 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 and BCTs were also examined for structural integrity after freezing. Collection tubes that were cracked were put in 50ml centrifuge tubes to prior to thawing.

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

Results

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. Blood stored in the BCTs (Figure 1iii) shows that the forward and side scatter characteristics for granulocytes (black) and monocytes (yellow) have decreased to a greater extent than the TVTs and the monocyte population has shifted into the lymphocytes. 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).

(i) Control day 0 *(ii) TVTs day 7* *(iii) BCTs day 7*

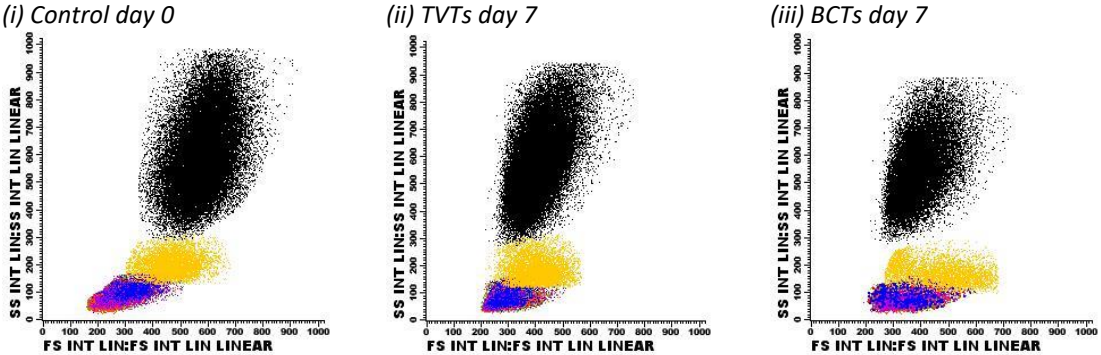

Figure 1i-iii. Dot plots depicting leukocyte forward scatter (FSC) vs side scatter (SSC) characteristics of blood collected in the Control at day 0 (i), TVTs day 7 (ii) and BCTs day 7(iii)

(i) Control day 0 (ii) TVTs day 7 (iii) BCTs day 7

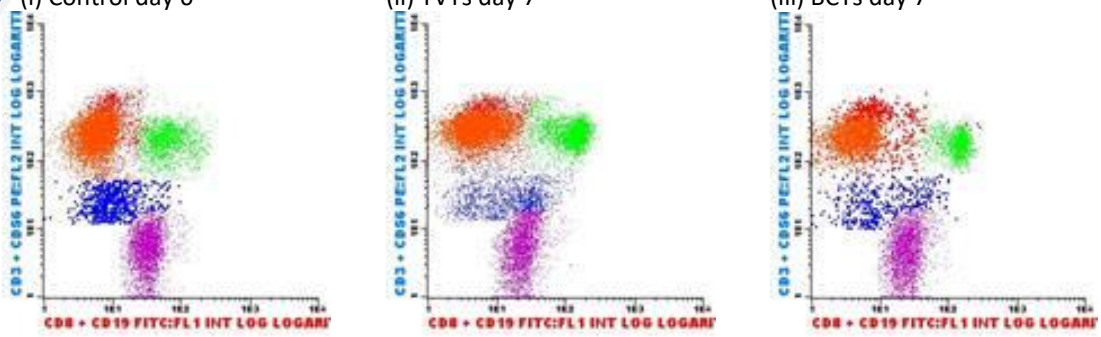

Figure 2i-iii. Dot plots depicting lymphocyte CD8 + CD19 vs CD3 + CD56 expression characteristics for the Control day 0 (i), TVTs day 7 (ii) and BCTs day 7 (iii) samples.

The effects of freezing on immunophenotyping and tube integrity

Table 2 summarises the flow cytometry functional testing conducted on the TVTs and BCTs 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 whereas all the BCTs tubes cracked.

The average lymphocyte recovery (CD45 Count) for the TVTs is 77% and lymphocyte subset recovery ranged from 51-80% across all donors. The average lymphocyte recovery for the BCTs is 26% and lymphocyte subset recovery ranged from 16-45% across all donors.

DAY 7 % CELL RECOVERY		% CD3	% CD4	CD3 Count	CD4 Count	CD8 Count	CD45 Count	B Cell Count	NK Cell Count	Granulocyte Count	Monocyte Count
TVT	Donor 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
BCT	Donor A	81.5	103.1	26.5	27.3	26.6	32.5	45.4	24.5	38.6	48.8
	B	90.5	108.6	21.1	22.9	19.9	23.3	34.7	18.1	34.8	53.0
	C	90.2	112.3	18.9	21.2	16.7	20.9	29.2	16.1	28.1	29.6

Table 2. Tests are summarised above in terms of % total cell count recovery from the same tube and donor on Day 0.

The effects of freeze thaw methods on immunophenotyping and tube integrity

Table 3 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 3 shows that tubes stored under condition 3 (TVTs stored at a 45° angle and thawed at 37°C) performed best overall.

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 3. 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.

Discussion

Immunophenotyping

The TVTs showed the best cell percentage differences from the Control with average lymphocyte recovery of 77% compared to 26% for the BCTs. The TVTs leukocyte cell recovery is 51-80% across all parameters and donors compared to 16-45% for the BCTs. The TVTs gave the best results in terms of recovery of cells after preservation.

The study to optimise the freeze, thaw and storage conditions showed that the yield of cells after thawing at room temperature was markedly less than when thawed at 37°C. The T cell lymphocyte recoveries from condition 1 were 79.8 – 83.3% lower than those in the Control. In comparison, the T cell lymphocyte recoveries from conditions 2-4 were no higher than 31.8% different. Condition 3 gave the best results.

Tube integrity

The TVTs are the only tube able to withstand being frozen at -80°C without compromising tube integrity. When optimising storage conditions, hairline fissures were seen in the lower sidewalls of tubes that were stored under conditions 1 and 2 (fast freeze, stored upright). These were not seen for tubes stored under conditions 3 (fast freeze, stored at 45° angle) and 4 (freeze at -1°C per minute). Storing tubes at an angle prevents a 'plug' of ice from forming and reduces the chance of swelling at the base of the tubes.

Conclusion

The TVTs are suitable for freezing whole blood at -80°C and the effects of long-term storage of TransFix stabilised whole blood samples need to be established. Experiments are underway to determine the upper limit of time in storage for frozen whole blood samples to still be viable. We recommend that all cellular markers and antibodies should first be validated for compatibility with TransFix and freezing. Finally, we suggest that the ability to freeze samples in TVTs prompts future investigation as to whether this product is suitable for phospho-specific flow cytometry. 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.