FastQ B*27 direct - Technical Report

Performance data of the FastQ B*27 direct kit and effect of the Blood Booster reagent on results with fresh blood samples in real-time PCR

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Introduction

Whole blood or buffy coat is the most commonly used sample material in diagnostic procedures including a lot of PCR applications. As DNA isolation is a time-consuming procedure and a cost factor in diagnostics labs it is desirable to be able to perform PCR amplification directly from blood samples. However, blood is difficult to use in PCR for some reasons like the presence of potentially inhibitory substances like haemoglobin or immunoglobulin G (Nishimura et al. 2000, Sidstedt et al. 2018) and limited accessibility of the DNA in the lymphocytes (Burckhardt 1994). Some of these obstacles are overcome by diluting the samples in high PCR volumes or using highly processive Taq polymerases that are commercially available from different vendors. Nevertheless, amplification – especially from fresh blood samples – often remains less reliable than from purified DNA (Burckhardt 1994, https://www.researchgate.net/post/Any_advice_in_blood_direct_PCR_Fresh_vs_frozen_samples 2015).

In this study we present data on the real-time PCR amplification of the HLA-B*27 alleles with the FastQ B*27 direct kit. This kit consists of an amplification reaction mix with sequence specific PCR primers and likewise sequence specific TaqMan® probes for the real-time detection of the HLA-B*27 alleles in combination with a PCR buffer and enzyme mix optimised for the amplification from whole blood. The diagnostic sensitivity and specificity of the FastQ B*27 direct kit was determined with whole blood samples and buffy coats that were collected more than 24 hrs before the test. The amplification with fresh blood samples turned out to be less reliable and we show the stabilizing effect of a reagent called Blood Booster. Fresh blood samples (< 12 h) were amplified with and without and the Blood Booster reagent and the reactions were compared.

Materials and Methods

120 blood samples pre-typed presence or absencefor HLA-B*27 were tested to determine the diagnostic sensitivity and specificity of the FastQ B*27 direct Primermix without the blood booster reagent. The blood samples were a mixture of whole blood and buffy coat. These samples were collected between 1 day and > 24 months (stored at ≤ -20°C) before the testing date. They have been generally been stored at 2-8°C or ≤ -20°C with short term storage periods at ambient temperature. These samples were tested according to the instructions for use from the manufacturer with a 1:50 dilution on the CFX 96 real-time PCR Cycler (Biorad).

Additionally, samples have been tested in different dilutions to test the robustness of the test (225 samples in total at dilution 1:25, 1:50, 1:100 see results below)

To determine the effect of the blood booster reagent six fresh blood samples that were not older than 12h were collected in EDTA sample collection vessels (S-Monovette® 4,9 ml, 90 x 13 mm, Kalium-EDTA, Sarstedt) from healthy voluntary donors. Informed consent was obtained from all participants. Three of these donors were positive for HLA*B27 (VB1, VB2, VB3) and the other three were negative (VB4, VB5, VB6). Due to some complications during sampling for VB1 only around 500 µl blood could be drawn into the EDTA sampling vials, resulting in an unusually high EDTA concentration.

All samples were amplified in 8 replicates according to the instructions for use from the manufacturer on a CFX 96 real-time PCR Cycler (Biorad). Cq values were determined and the coefficient of variance (CV) calculated for the 8 replicates. For the B*27 specific amplification quantification cycle (Cq) values ≤ 30 were rated as positive and for the internal control (human growth hormone) Cq values ≤ 20.

Results and Discussion

Testing of 120 pre-typed samples resulted in a diagnostic sensitivity and specificity of 100% (no discrepancies observed, see Table 1).

		Fast Q B*27	
		Positive	Negative
Pre-defined result	Positive	19 (Tp)	0 (Fn)
	Negative	0 (Fp)	101 (Tn)

Table 1: Summary of the results used to calculate the diagnostic sensitivity and specificity of the FastQ B*27 direct kit (without blood booster, samples older than 24 hrs) Tp = true positive, Tn = true negative, Fp= false positive, Fn = false negative

The results for different dilutions show that a reliable tying is possible at dilutions of the blood sample between 1:25 and 1:100. No discrepancies were observed and quantification cycle values (Cq) were similar for the different dilutions. Using a 1:50 dilution should, therefore, give a reliable result even when using blood samples with unusually high or low lymphocyte counts.

Dilution	No. of samples	B*27 (Cq)	Internal amplification control (Cq)
1:100	32 (11 positive)	13,88 – 23,11	6,74 – 17,9
1:50	161 (36 positive)	7,19 – 22,53	4,36 – 19,59
1:25	32 (11 positive)	14,94 – 24,26	6,17 – 16,7

Table 2: Summary of results with different dilutions with the FastQ B*27 direct kit (without blood booster, samples older than 24 hrs). Cq = quantification cycle.

Though the results with older blood samples and buffy coat did not show any discrepancies for the pre-typed HLA-B*27 feature, fresh blood samples collected less than 12 hrs before the testing gave randomly false negative results. The blood booster reagent enabled a reliable typing with those samples as well.

Figure 1 shows the amplification curves for the eight replicates of the six blood samples with and without the Blood Booster reagent. Without the Blood Booster the Cq values vary and there were between 2 and 7 false-negative reactions with the B*27 positive blood samples whereas the curves are closely together with the Blood Booster and there were no false negative reactions. Notably for sample VB1 – which had a small blood volume and a resulting high EDTA concentration - only one of the eight replicates showed a correct B*27 amplification without the Blood Booster. It is known from the literature that EDTA concentrations > 2 mM show a strong inhibitory effect in real-time PCR (Sidstedt et al. 2018) which in this test is consistently overcome by the Blood Booster. A similar effect, but less pronounced is observed for the amplification of the internal amplification control.

Figures 2 and 3 show the average Cq value and the coefficient of variation (CV) of the Cq values for the six blood samples; the addition of the Blood Booster improved both values. The average CV for the specific B*27 amplification could be improved from 24% to 6% (n=3) and for the internal amplification control from 7% to 4% by adding the Blood Booster.

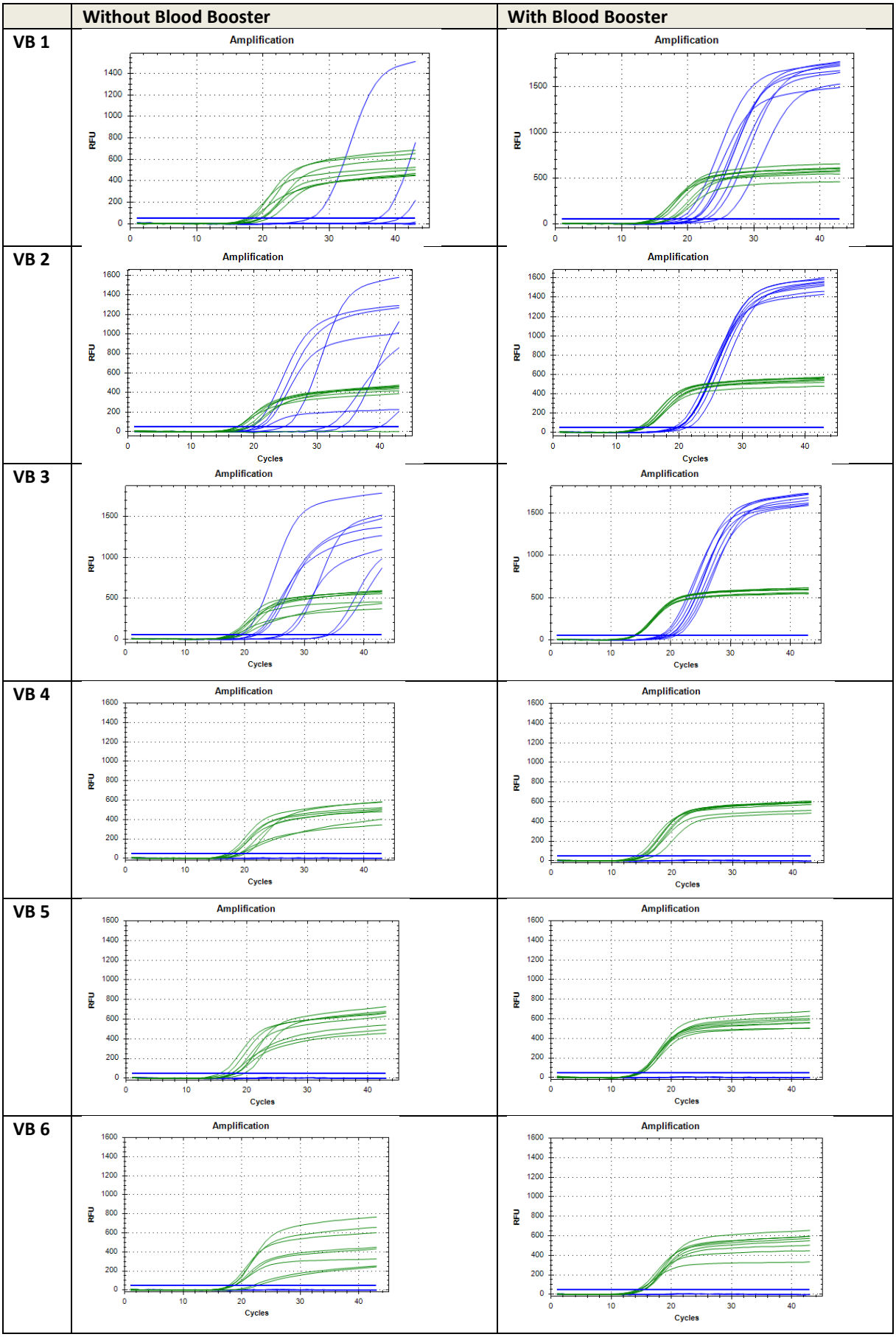

Figure 1: Amplification curves of the 8 replicates for six blood samples with the FastQ B*27 direct kit
Green curves = Internal amplification control / blue curves = specific amplification of HLA-B*27

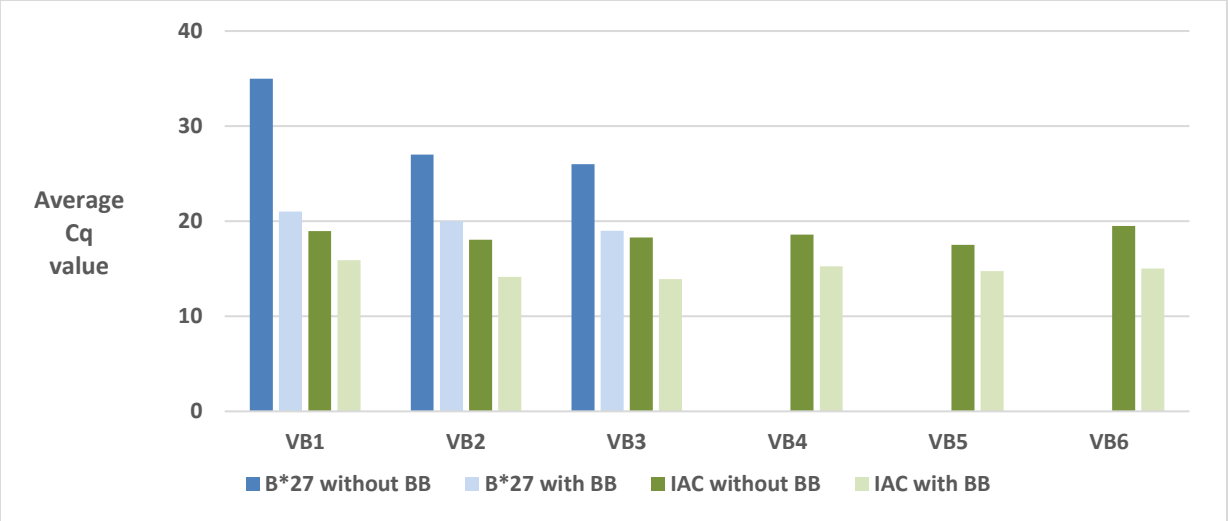

Figure 2: Average Cq value for the specific amplification of the B*27 alleles and the internal amplification control for the six blood samples (VB1 – VB6) (BB= Blood Booster, IAC = Internal amplification control

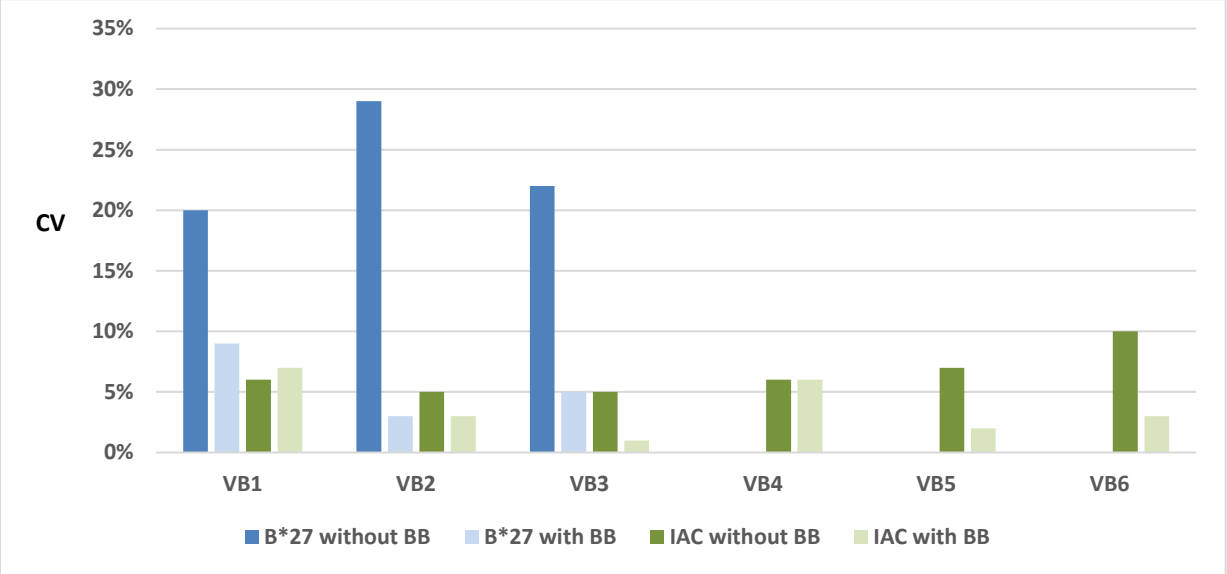

Figure 3: Average coefficient of variation (CV) for the specific amplification of the B*27 alleles and the internal amplification control for the six blood samples (VB1 – VB6) (BB= Blood Booster, IAC = Internal amplification control

Conclusion

The Blood Booster has a significant stabilizing effect on the direct real-time amplification from fresh blood samples and enables a reliable diagnostic directly from blood for the specific amplification of the B*27 alleles. The Blood Booster will be evaluated for other tests in the FastQ product line in the near future.

Literature

Burckhardt J, Amplification of DNA from whole blood, Genome Research 1994 (3):239-143

Nishimura N, Nakayama T, Tonoike H, Kojima K, Kato S, Direct polymerase chain reaction from whole blood without DNA islolation, Ann Clin Biochem 2000 (37): 674-680

Sidstedt M, Hedman J, Romsos EL, Wadsö L, Steffen CR, Vallone PM, Radström P, Inhibition mechanisms of haemoglobin, immunoglobulin G, and whole blood in digital and real-time PCR, Analytical and Bioanalytical Chemistry 2018 (410): 2569-2583