

Rapid Detection of Coagulation Factor V Using PlexTaq® for Direct Blood PCR Applications

Introduction

Blood contains an array of coagulation factors poised to protect blood vessels from bleeding and injury but, a delicate balance in the coagulation pathway must be maintained to prevent potentially life-threatening conditions. Coagulation Factor V (FV) serves an essential role in this pathway by acting as a cofactor for conversion of prothrombin to thrombin. Insufficiency of Factor V leads to a blood disorder known as parahemophilia or Owren's disease which causes a range of symptoms from mild bleeding episodes to potentially fatal hemorrhages¹. Alternatively, the presence of mutations that prevent the degradation of Factor V can cause a state of hypercoagulability.² The cleavage resistant allele, called Factor V Leiden, can be a risk factor for serious complications such as deep venous thrombosis or pulmonary embolism and has been associated with a higher risk of myocardial infarction, stroke, and miscarriage.³ For these reasons, Factor V genetic testing is recommended in certain clinical settings. The allele has been detected in 20% of idiopathic first venous thrombosis cases, and 60% of venous thrombosis cases in pregnant women. Knowledge of the presence of Factor V Leiden in patients and relatives can influence management and prevent serious complications. The most reliable test for identifying Factor V Leiden relies on PCR amplification.^{3,4}

Blood contains a host of factors that can be inhibitory for PCR, so most genetic analysis depends on the extraction and purification of DNA from samples. Extraction methods can be time consuming as well as cost and reagent intensive.³ Extraction and purification steps result in substantial DNA loss, with recovery rates that vary from 10% to 80% depending on the starting sample type.⁵ Amplification of DNA directly from blood simplifies molecular assay workflows and makes them more accessible by reducing the amount of sample handling, speeding up the processing time, and preventing sample loss. In this study we demonstrate how PlexTaq® 5x qPCR Multiplex Master Mix (myPOLS Biotech GmbH, part of Medix Biochemica) can be used directly in whole blood samples up to 20% of the reaction volume and retain multiplexing consistency.

The targets used in this study include Coagulation Factor V (Factor V), Actin-B1 (ACTB1) and an internal amplification control (IAC). PlexTaq® was compared with 3 products from leading competitors that have been marketed for multiplex performance.

Methods

PlexTaq® 5x qPCR Multiplex Master Mix and three competitors were used to amplify three targets in multiplex: 85 bp-long fragment of Coagulation Factor V gene (NG_011806.1), 185 bp-long fragment of Actin-B1 gene (NG_007992.1), and IAC a 120 bp internal amplification control which was spiked into reactions at a concentration of 10,000 copies per reaction. A dilution series of whole human blood preserved in K3 EDTA was performed in PCR grade water and added to reactions at the indicated concentrations in Figures 1 - 4. As a positive control gDNA purified from HeLa cells was added in lieu of whole blood at a concentration of 1 ng/μl. Samples were amplified following the qPCR program shown in Table 1 followed by a melting curve analysis from 45 - 85°C. Samples were detected in real time using TaqMan (ACTB1 and IAC) or hybridization (Factor V) probes directed against the above targets. Reaction setup and final primer concentrations were applied according to the competitor manufacturer recommendations. PlexTaq® 5x qPCR Multiplex Master Mix was also used in a 2-step qPCR protocol to compare the results. Program for 2-step protocol is shown in Table 2. PCR products were also subjected to visualization on a 2.5% agarose gel at the endpoint.

Table 1

Step	Temperature	Time	Cycles
1.	95°C	2 minutes	1 cycle
2.	95°C	30 seconds	50 cycles
3.	60°C	30 seconds	
4.	72°C	30 seconds	

Table 2

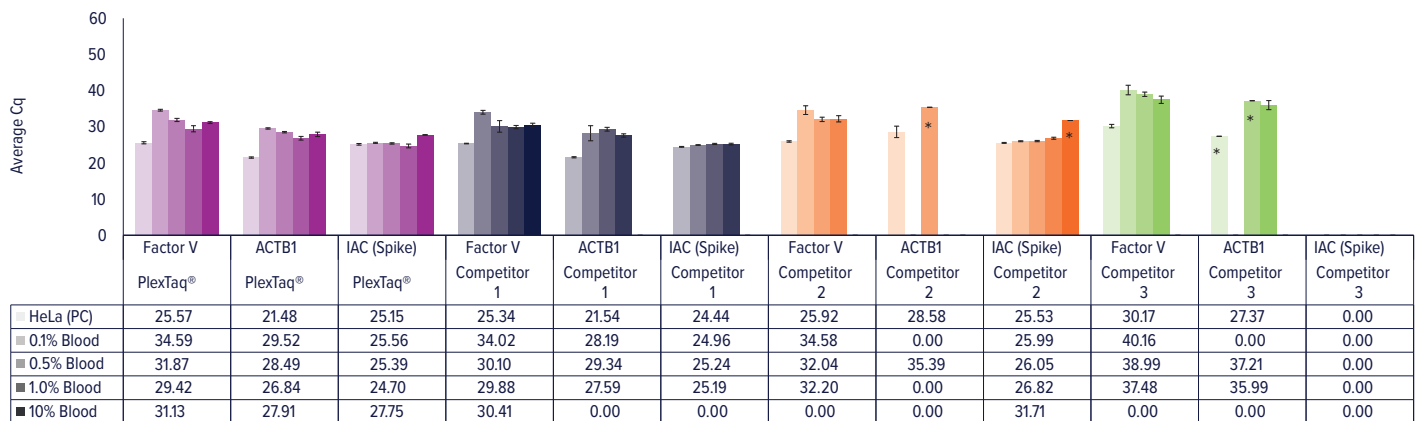
Step	Temperature	Time	Cycles
1.	95°C	2 minutes	1 cycle
2.	95°C	10 seconds	50 cycles
3.	60°C	40 seconds	

Results

The average C_q results for three targets in 4 different concentrations of whole blood are shown for 4 different master mixes marketed for multiplex performance in Figure 1. PlexTaq® 5x qPCR Multiplex Master Mix detects all three targets in blood up to 10% compared to leading suppliers. Competitor 1 detected all three targets in concentrations up to 1% blood but failed to amplify 2 out of 3 targets at the 10% blood concentration. Competitor 2 and 3 failed to amplify ACTB1, a commonly used housekeeping gene, at low blood concentrations. Competitor 3 failed to amplify the spiked target even without the presence of blood and failed to detect Coagulation Factor V at 10% blood. Sample amplification and melting curves for the Coagulation Factor V target are shown in Figure 2. PlexTaq amplifies consistently and specifically while displaying a higher fluorescence signal than competitors. Blood has

a quenching effect on the fluorescence signal which can be seen in the amplification curves, to demonstrate the actual yield of the targets, products from PlexTaq® and Competitor 1 assays were analyzed by 2.5% agarose gel at the endpoint. This gel visualization demonstrates that at 10% blood, PlexTaq® is able to amplify all three targets while Competitor 1 only amplifies 1 of 3 targets and shows a lower yield (Figure 3A). PlexTaq® was also tested at the endpoint with 0.5% and 20% Blood with several common anti-coagulation treatments (K3 EDTA, Na Heparin and Na Citrate). In all cases, PlexTaq® was able to amplify all three targets even in 20% Blood (Figure 3B). The data for Figures 1-3 used the competitor recommended 3-step PCR program (Table 1), but PlexTaq® can also be used in a 2-step protocol without sacrificing performance (Table 2). Figure 4 compares average C_q values across the two protocols.

PlexTaq® 5x qPCR Multiplex Master Mix vs. leading competitors



* 2 of 3 replicates failed to amplify.

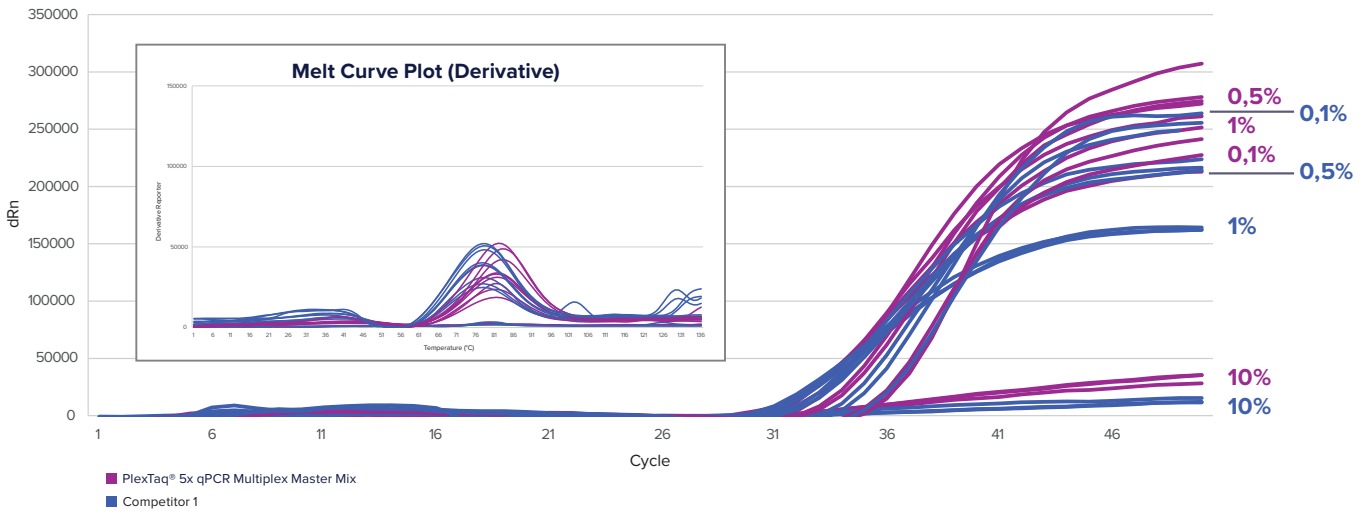
All samples tested with appropriate negative controls.

Figure 1. PlexTaq® 5x qPCR Multiplex Master Mix performs consistently in blood compared to leading suppliers

PlexTaq® 5x qPCR Multiplex Master Mix vs. leading competitors

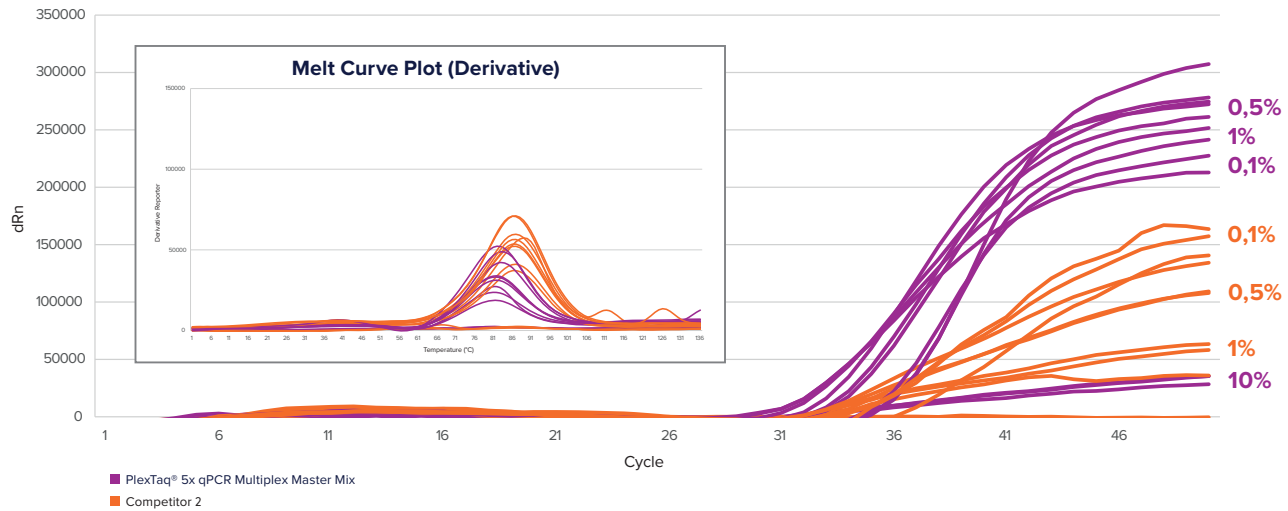
Amplification plot

PlexTaq® vs Competitor 1



Amplification plot

PlexTaq® vs Competitor 2



Amplification plot

PlexTaq® vs Competitor 3

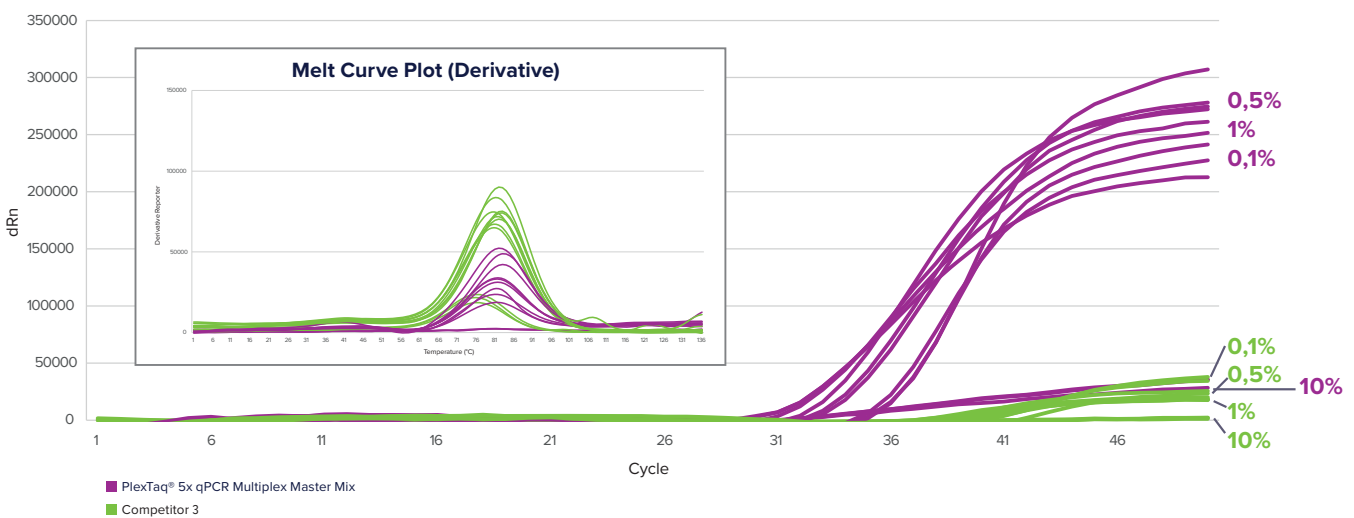


Figure 2. Examples of Reaction Curves and Melting curve data for Coagulation Factor V

Agarose gel visualization of PCR products at endpoint

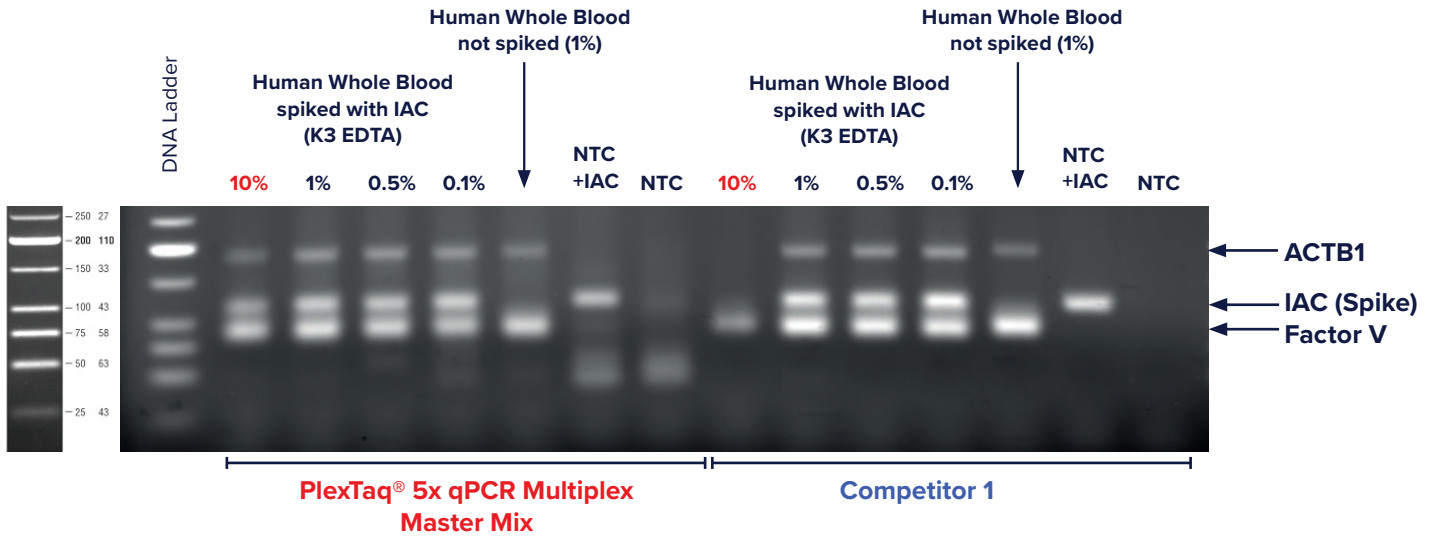


Figure 3A. Agarose gel visualization of PCR products at endpoint

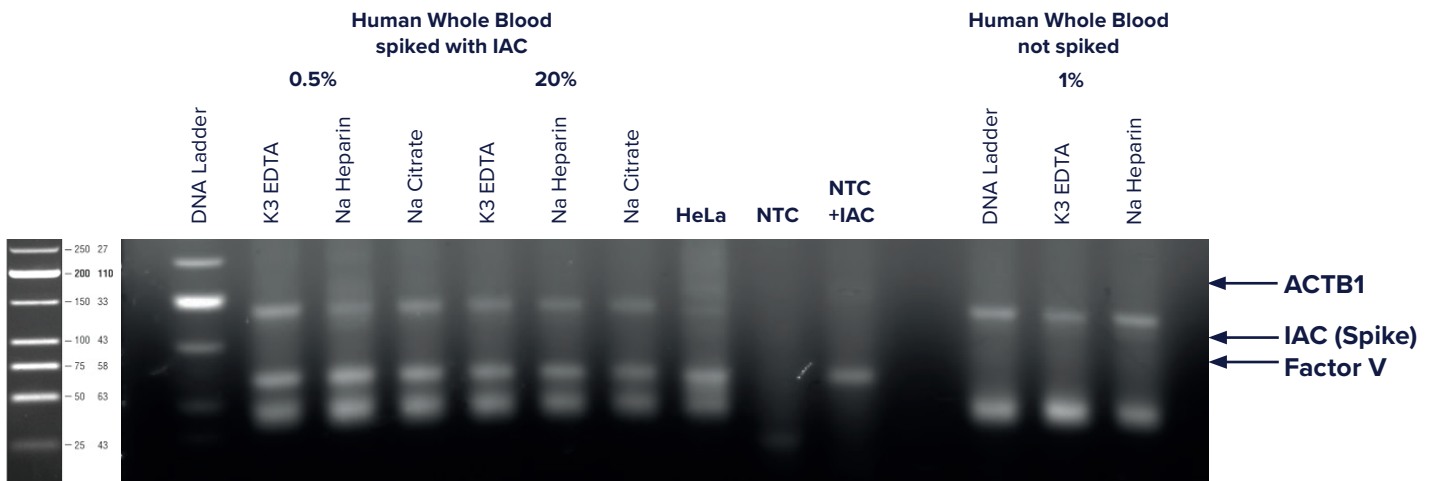


Figure 3B. Agarose gel visualization of PCR products at endpoint

PlexTaq® 5x qPCR Multiplex Master Mix can be used with a 2-step protocol

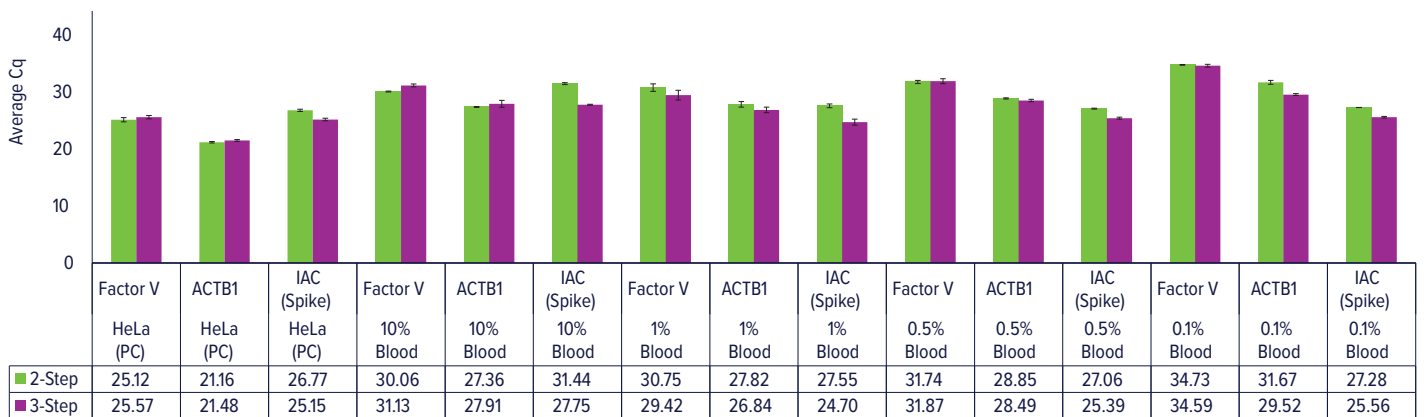


Figure 4. PlexTaq® 5x qPCR Multiplex Master Mix can be used with a 2-step protocol

Table 3

Characteristic	PlexTaq® 5x qPCR Multiplex Master Mix	Competitor 1	Competitor 2	Competitor 3
Multiplex performance up to 1% whole blood	✓✓✓	✓✓✓	✓✓	✗
Multiplex performance in 10% whole blood	✓✓✓	✓	✓	✗
Time to result (manufacturer recommended protocol)	⌚	⌚⌚	⌚	⌚
Price per reaction	\$	\$\$\$\$	\$	\$\$
Standard deviation between replicates	σ	σ σ	σ σ	σ σ σ
Concentration	5x	2x	2x	2x
Reversible hot-start	✓	✗	✗	✗
Hot-start type	Aptamer	Antibody	Antibody	Antibody
Maximum multiplex targets	30	20	30	20
Lyo-ready	✓	✗	✗	✗
Quality	ISO13485	RUO	RUO	RUO

Conclusions

This study demonstrated that PlexTaq® 5x qPCR Multiplex Master Mix performs consistently in whole blood compared to leading suppliers without compromising on other features or price point. Although PlexTaq is a mix designed for multiplexing it is inhibitor tolerant when compared to other mixes designed for multiplexing. PlexTaq® not only performs in whole blood but also has other desirable features including a 5x concentration (allowing for more primers, probes, and sample), an aptamer hot-start that can bind both before and after amplification, a lyo-ready formulation which enables room temperature storage upon freeze drying, and was designed and manufactured under the ISO13485 quality standard. All these features are available while still maintaining a competitive price point. These characteristics are summarized in Table 3. These features allow for development of direct blood amplification assays for clinically relevant targets such as Coagulation Factor V.

References:

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