

DIRECTED EVOLUTION FOR THE DEVELOPMENT OF AN INHIBITOR-RESISTANT DNA POLYMERASE FOR IMPROVED TARGET AMPLIFICATION AND QUANTIFICATION OF CRUDE SAMPLES WITH REAL-TIME PCR

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INTRODUCTION

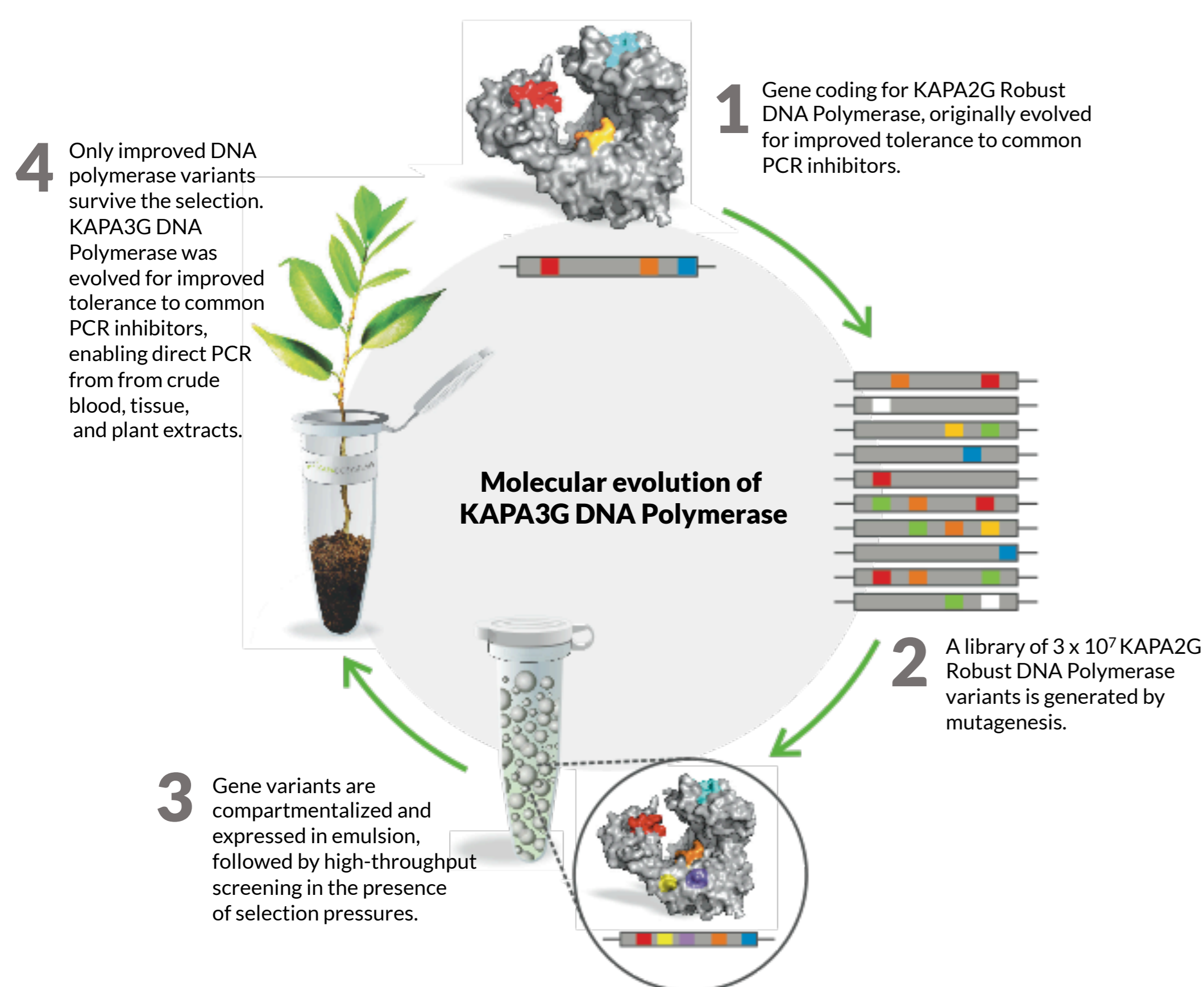
As sample quantities continue to grow for high-throughput gene expression and genotyping, real-time PCR applications such as pathogen detection, mouse genotyping, and genetically modified organism (GMO) screening, so does the need for greater levels of detection and process efficiency.

Enzymatic inhibition is an inherent challenge of PCR amplification when working with complex biological samples, thus requiring DNA extraction and purification methods prior to sample analysis. Robust purification methods are available, but impose additional time, cost, and DNA loss with even the most efficient processes that can potentially lead to false negatives of low copy targets. While crude extraction methods alleviate process burdens, higher levels of inhibitors lead to inefficient amplification and variable results. Wild-type enzymes such as *Taq* and *Tth* are not suitable for high-throughput amplification directly from crude samples.

Utilizing high-throughput directed evolution and buffer optimization, we have developed a novel, highly processive third-generation DNA polymerase, KAPA PROBE (KP) FORCE, not only capable of crude sample amplification directly from plant, tissue and blood samples, but also with 5'-3' exonuclease activity required for probe-based quantitative PCR (qPCR).

DIRECTED EVOLUTION

KP FORCE contains a novel, third-generation (3G) DNA polymerase engineered via directed evolution for improved tolerance to common plant-derived inhibitors. Improved buffer optimization broadened the enzyme's inhibitor tolerance to tissue and blood samples.



OBJECTIVES

We assessed the ability of KP FORCE to amplify a variety of blood, tissue, and plant DNA templates after crude sample extraction and in the presence of relevant inhibitors using probe-based qPCR assays.

We compared KP FORCE with KP FAST and current equivalent industry-leading PCR master mix, focusing on PCR efficiency and threshold cycle (C_q) metrics.

METHODS

Targets. The human β -actin, mouse (*Mus musculus*) β -2-microglobulin, grapevine (*Vitis vinifera*) *adh2* and *E. coli* 16S genes were amplified with 200 nM gene-specific primers and 200 nM (FAM/Iowa Black[®]) hydrolysis probes. A 5- or 10-fold dilution series of purified human, mouse, plant or bacterial genomic DNA was included in all assays.

Crude samples. A single donor specimen was used to produce fresh, haemolyzed and frozen EDTA samples, as well as plasma, buffy coat and dried blood spots. Mouse tail material was prepared by extraction using KAPA Express Extract, and blood was prepared as a dried blood spot. Crude preparations of grapevine were prepared by both DTT and β ME crude extraction, and direct punches were also used.

Real-time PCR. Probe-based qPCR was performed using KP FORCE, KP FAST and equivalent reagents on a Qiagen RotorGene Q Real-time PCR Cycler. Each enzyme was supplied as a master mix, and we performed the qPCR using the recommended cycling conditions for each master mix. For our in-house enzymes, we used a fast cycling protocol (95°C for 3–5 min / 45–50 cycles of 95°C for 10 sec; 60°C for 20 sec). No template controls (NTCs) were run under the same conditions for each assay.

Multiplex assay. Mouse gDNA was amplified with 4 primer/probe sets (Biosearch Technologies) targeting the *Isg20* (FAM/BHQ-1), *F13a1* (CAL Fluor Orange 560), *Camtal* (Quasar 670), *Ube20* (Quasar 705) genes. 500 nM primers and 110 nM probes were used with the following cycling conditions: 95°C for 30 sec/50 cycles of 95°C for 3 sec, 60°C for 30 sec.

Inhibitor assays. A dilution series of human, mouse and grapevine genomic DNA was spiked with inhibitors commonly encountered in crude lysates of blood, tissue or plant samples. The concentration of each inhibitor used was set in range-finding experiments as that which caused a $<1 C_q$ delay with KP FORCE.

RESULTS

We determined the efficiency, performance and sensitivity of KP FORCE vs KP FAST and other industry master mix in a 4-plex assay (Fig. 1). Our novel enzyme demonstrates consistent performance and reliably detected multiple targets.

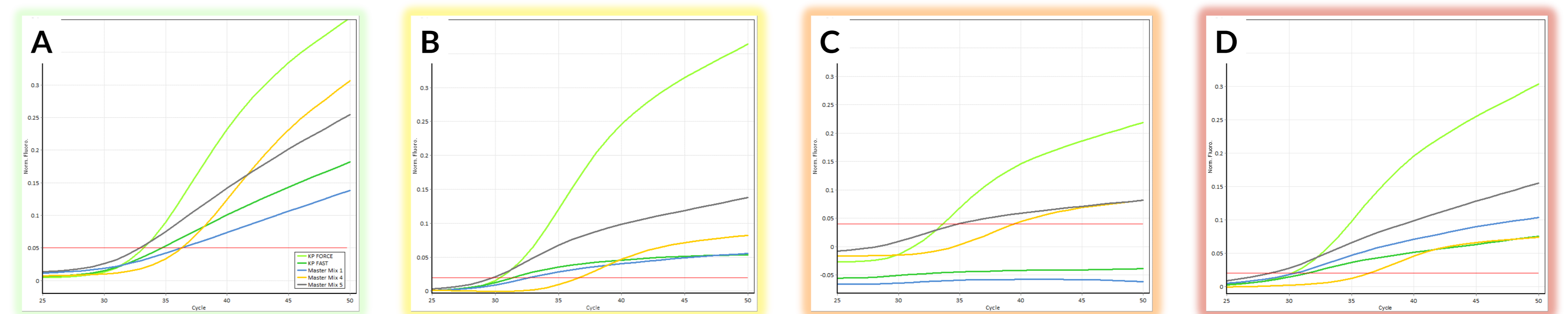


Fig. 1. Four targets amplified with KP FORCE and three equivalent PCR master mix in a multiplex assay. Mouse gDNA was amplified targeting the (A) *Isg20* (FAM/BHQ-1), (B) *F13a1* (CAL Fluor Orange 560), (C) *Camtal* (Quasar 670) and (D) *Ube20* (Quasar 705) genes. 500 nM primers and 110 nM probes were used with the following cycling conditions: 95°C for 30 sec/50 cycles of 95°C for 3 sec, 60°C for 30 sec.

We then determined the ability of KP FORCE to amplify target DNA in direct PCR or from crude lysate compared with KP FAST (Fig. 2). KP FORCE consistently amplified target DNA from crude samples using a variety of extraction methods without an observable delay in C_q and in most cases out-performs KP FAST, demonstrated by earlier C_q values. In addition, KP FORCE amplified low input targets (down to <10 copies/reaction).

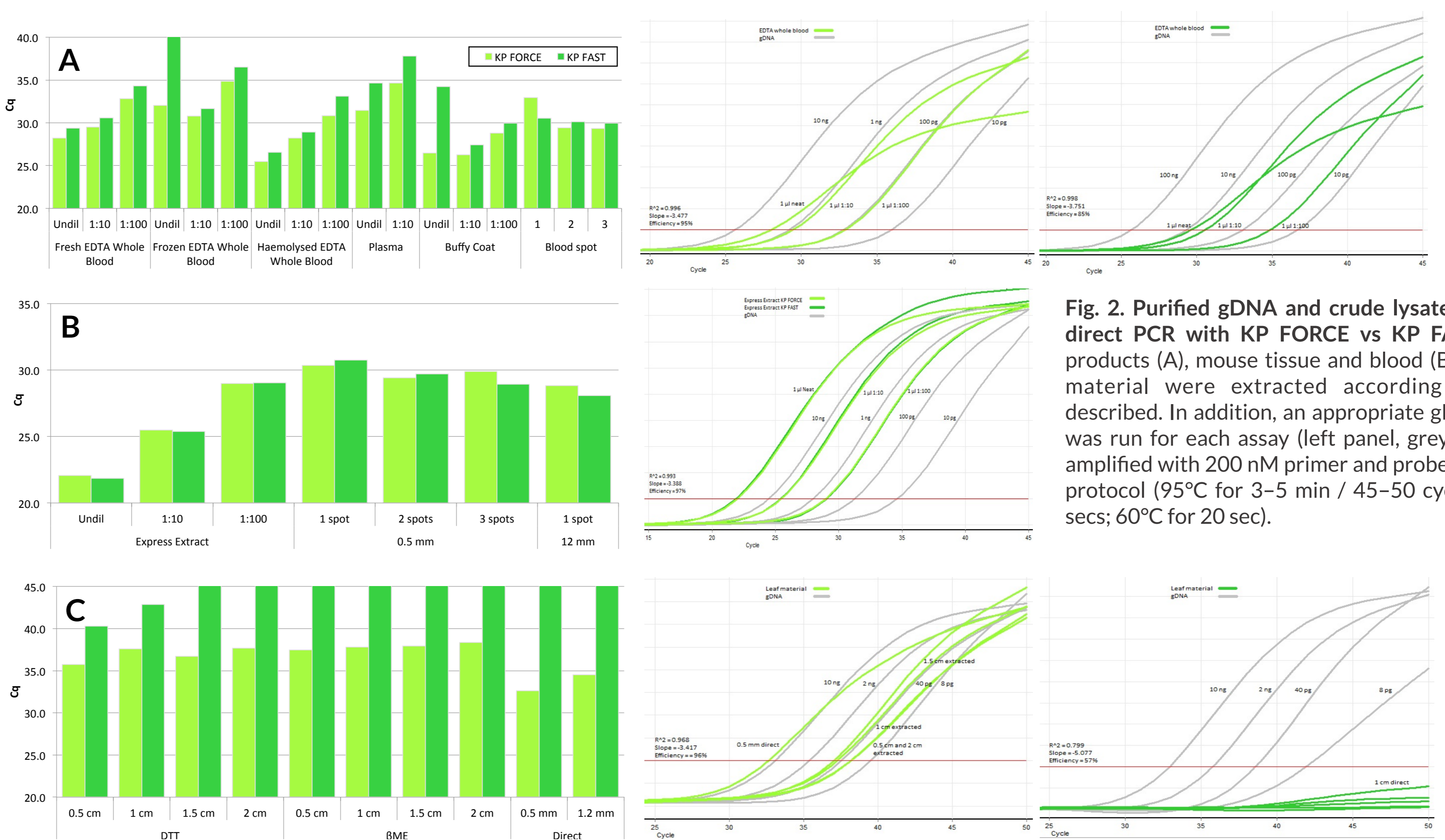


Fig. 2. Purified gDNA and crude lysate amplification and direct PCR with KP FORCE vs KP FAST. Human blood products (A), mouse tissue and blood (B) and plant leaf (C) material were extracted according to the methods described. In addition, an appropriate gDNA dilution series was run for each assay (left panel, grey curves). DNA was amplified with 200 nM primer and probe using a fast cycling protocol (95°C for 3–5 min / 45–50 cycles of 95°C for 10 sec; 60°C for 20 sec).

Finally, we determined the performance of KP FORCE in the presence of common PCR inhibitors encountered in blood, tissue and plant samples (Fig. 3). KP FORCE matched or outperformed the majority of master mix tested with $<1 C_q$ delay compared with controls for each inhibitor.

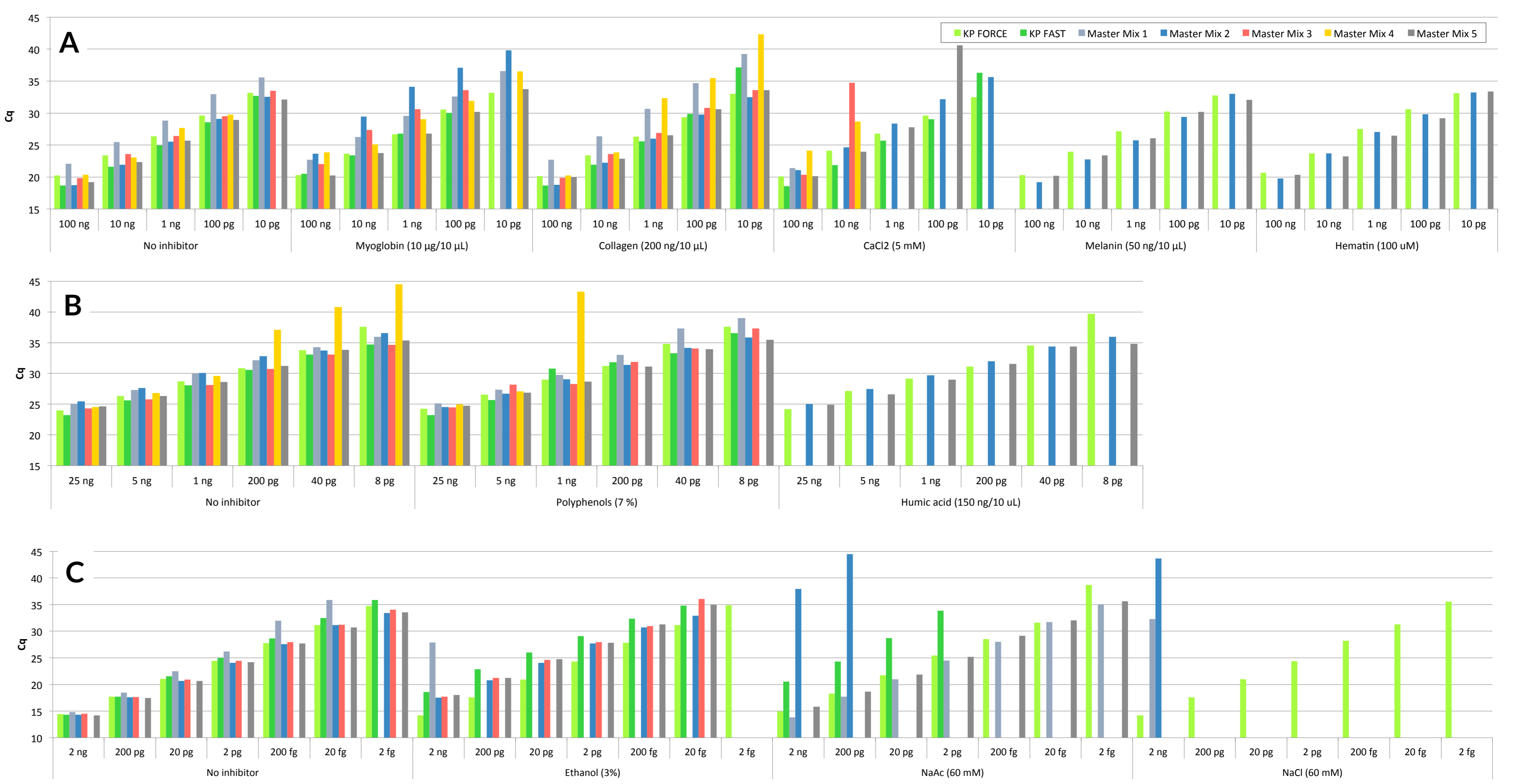


Fig. 3. DNA amplified in the presence of PCR inhibitors using KP FORCE, KP FAST or equivalent polymerase master mix. A dilution series of (A) human, (B) grapevine or (C) *E. coli* gDNA was amplified with 200 nM primer and probe at the recommended cycling conditions for each master mix. The concentration of each inhibitor used was set in range-finding experiments as that which caused a $<1 C_q$ delay with KP FORCE.

CONCLUSION

KP FORCE is capable of amplifying DNA from samples after crude extraction without an observable delay in C_q . At inhibitor concentrations that did not result in a $>1 C_q$ delay for the novel enzyme, competing reagents typically displayed much greater delays in their corresponding C_q values. Our novel enzyme and optimized buffer in the form of KP FORCE offers improved sensitivity with low input targets and direct amplification from crude lysates from blood, tissue and plant.