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Letter to the Editors-in-Chief

Confirmed validation of an innovative PCR-assay without DNA extraction for multiplex diagnosis of factor V Leiden and prothrombin gene variants

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Sir,

Venous thromboembolic disease (VTE) is a major public health issue in developed countries with an estimated annual occurrence ranging from 1 to 2 per 1000 inhabitants and increased risk over lifetime [1]. The major occurrences of VTE are lower limbs thrombosis and pulmonary embolisms, but any vein can be affected by an inappropriate clot formation. Besides age, several risk factors have been reported, all belonging to the triad described in the 19th century by Rudolf Virchow, i.e. hypercoagulability, blood stasis and endothelial damage. Among these risk factors, some are considered transient, such as surgery, while others remain throughout life [2]. One of the permanent risk factors is thrombophilia, a hypercoagulability state which may lead to VTE. Although by itself not a disease, this predisposition increases the risk of VTE, especially in association with other risk factors. Several hypercoagulable conditions have been recognized as thrombophilia, yet their overall impact is difficult to evaluate. Recommendations have been published on the indications and content of "thrombophilia screening" [2], to which can be added search for deficiencies in antithrombin, protein S (PS) and protein C (PC), exploration of an underlying antiphospholipid syndrome (lupus anticoagulant, anti β2GP1 and anticardiolipid antibodies) and assessment of the possible presence of gene variants of factor V Leiden c.16941G > A (*F5L*) and/or prothrombin (*F2* c.*97G > A). The *F5L* gene variant, described in 1994 induces a resistance of active Factor V to active PC cleavage. *F2* c.*97G > A, described in 1996, occurs in the 3′UTR region of the prothrombin gene and leads to an accumulation of Factor II messenger ribonucleic acid (mRNA) and increased *F2* levels. Although frequent in the general population, at 5% and 1–4% for *F5L* and *F2* c.*97G > A variants respectively, both yield a low relative-risk when heterozygous ($_{htz}$), at 2–8 and 3 [3]. However, their high frequency and the high relative-risk associated to homozygous ($_{hmz}$) variants [4], double heterozygosity (both *F5L* and *F2* c.*97G > A) or association with other risk factors such as estroprogestative therapy or pregnancy, make them key variants in thrombophilia screening.

Current strategies for the detection of *F5L* and *F2* c.*97G > A mostly rely on conventional polymerase chain reaction (PCR)-based assays. However, these strategies are time-consuming and usually require to be performed on series of samples increasing the delay between blood sample collection and mutation results.

Loop-mediated isothermal amplification (LAMP) is a PCR-based assay developed during the early 2000s [5] mostly for microbial pathogens. It allows identifying nucleic sequences without requiring thermal cycling. Amplification is faster than with conventional PCR, through the use of special sets of primers and a thermostable DNA polymerase. LaCAR MDX Technologies (Liège, Belgium) applied this principle to the development of a kit applicable to thrombophilia exploration.

This is a two-stage PCR using 6 different primers for each variant allowing the loop-mediated amplification. Fluorescence-generating and quenching probes at two different wavelengths provide separate detection systems allowing for the concomitant detection of both reactions during the final melting phase. For each variant, the first phase uses 4 primers (forward, backward, then mid-loop) leading to a double-looped DNA strand where hot-spot regions are located within the loop structure. Following this double-looped DNA strand formation, a fast amplification of hot-spot regions is initiated in various locations within this strand, using 2 additional primers. The mutational status of the amplicons is then assessed using an enhancer, within the hot-spot region, complementary of the variant for the *F5L* allele and of the wild-type allele for *F2*. During the ensuing melting phase, measurement of the emitted fluorescences allows to differentiate mutated and wild-type alleles.

The objective of this study was to validate the Lamp Human FII& FVL duplex KIT (rs1799963, LaCAR MDX Technologies) by comparison with the diagnosis assays used routinely in our University Hospital laboratory. *F2* c.*97G > A detection used an Allele Specific Amplification (ASA) PCR followed by melting fusion detection on Lightcycler (LC) 96 (Thermo Fisher Scientific Waltham, USA) adapted from a published protocol [6]. Briefly, each PCR well tests 50 ng of DNA to which is added SYBR® Premix Ex Taq™ II and 10 µM each of 5 primers. The latter are a common *F2* anti-sense (5′-TCTAGAAACAGTTGCCTGGC-3′) a wild *F2* sense (5′-CACTGGGAGCATTGAGGATC-3′) and a mutated *F2* sense (5′-CACTGGGAGCATTGAGGATT-3′). Additionally, an internal control is provided by *F9*sense (5′-CTCCTGCAGCATTGAGGGAGATGGACATT-3′) and anti-sense primers (5′- CTCGAATTCGGCAAGCATACTCAATG TAT-3′). The PCR includes a pre-incubation period at 95° for 30 s, followed by 32 cycles of amplification (5 s at 95 °C then 30 s at 57 °C then 15 s at 72 °C) and a 90 s fusion phase with a gradual increase up to 75 °C. The *F5L* detection assay was carried out as reported elsewhere

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Table 1
Mutational status and nature of the 40 samples used in the validation test.

	Whole blood *N* = 17	DNA *N* = 23
F2 $_{WT}$	7	12
F2 c.*97G > A $_{hmz}$	4	6
F2 c.*97G > A $_{htz}$	6	5
F5 $_{WT}$	10	11
F5L $_{hmz}$	1	6
F5L $_{htz}$	6	6

WT: wild type; hmz: homozygous; htz: heterozygous.

[7], for all samples.

Two positive and negative controls from the Lamp Human FII&FVL duplex KIT and 40 different prospectively selected samples of blood (fresh, stored up to 2 weeks at +4 °C then frozen at −20 °C) or retrospectively selected previously extracted DNA were processed with the Lamp Human FII&FVL duplex KIT on an LC96 platform (Thermo Fisher Scientific), in 5 runs. All samples but one (amended upon repeat) yielded a correct melting curve with two peaks for the positive control and none for the negative control in each series. Samples characteristics are described in Table 1. Examples of graphical results are shown in Fig. 1.

For *F5L* tests, mean melting temperatures were 60.0 ± 0.4 °C and 67.3 ± 0.4 °C for the respective peaks. For the *F2* c.*97G > A test, they were 55.1 ± 0.6 °C and 63.4 ± 0.5 °C. Coefficients of variation of melting temperatures for the positive controls included in all runs were 0.5%, 0.2%, 0.3% and 0.5% respectively.

Repeatability of the Lamp Human FII&FVL duplex KIT was verified with a blood sample (*F2* c.*97G > A $_{htz}$/*F5L*$_{htz}$) analyzed 5 times in the same run and with two other blood samples (*F2* c.*97G > A $_{hmz}$/F5$_{Wild\ Type\ (WT)}$; *F2*$_{WT}$/*F5L*$_{hmz}$) analyzed twelve times in another run. Analysis of the positive control, one DNA sample (*F2* c.*97G > A $_{htz}$/*F5L*$_{htz}$) and two blood samples (*F2* c.*97G > A $_{hmz}$/F5$_{WT}$; *F2*$_{WT}$/*F5L*$_{hmz}$) were repeated in 5 different runs for reproducibility testing. All intermediate fidelity tests were valid. Five different operators performed the five LAMP assays and a double curve analysis was performed by 6 operators. The mean duration of the LAMP assay was 40 min (range 35–55) with 15–20 min hands-on time compared to around 80 min for a classic PCR method. There was 100% reproducibility between operators and a perfect concordance in the analysis of the different curves. Considering the 40 samples tested in the five runs, all showed similar results between the Lamp Human FII&FVL duplex KIT and routine assays for the detection of *F5L* and *F2* c.*97G > A. No amplification of the negative control was observed in any of the runs performed. In addition, two blood samples (*F2* c.*97G > A $_{hmz}$/F5$_{WT}$; *F2*$_{WT}$/*F5L*$_{hmz}$) were deposited twelve times alternatively with H_2O as blank in the wells of a plate to detect possible contamination. All these tests were correct.

This study demonstrates the good performance of the Lamp Human FII&FVL duplex KIT for *F5L* and *F2* $_{G20210A}$ mutational status assessment

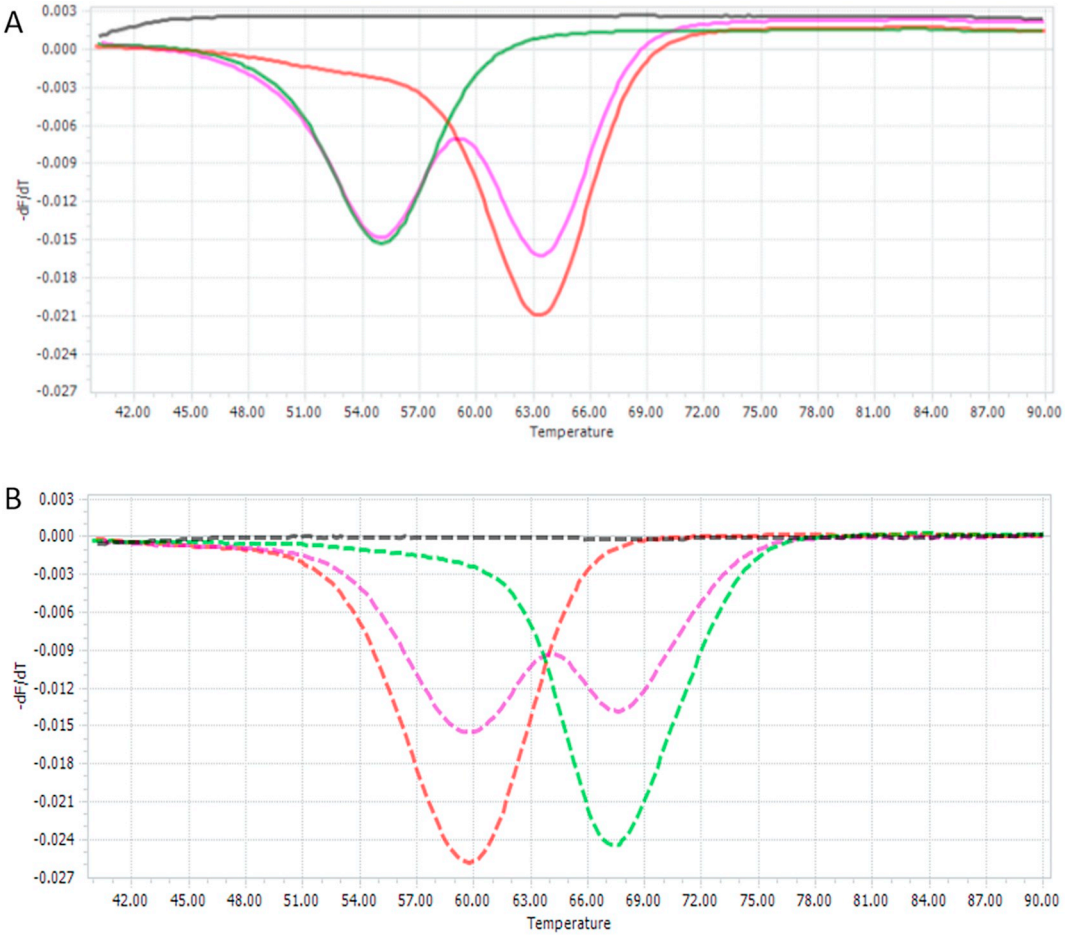

Fig. 1. Graphical representation of results obtained with the *F5L&F2* LAMP test. A. Results for the FAM (6-carboxyfluorescein) channel (*F2*). Homozygous normal samples show one peak around 55 °C (green curve), homozygous mutated samples show a peak around 63 °C (red curve). Heterozygous samples show two peaks (orange curve) and the negative control shows no peak (grey curve).
B. Results for the Texas Red channel (*F5L*). Homozygous normal samples show a peak around 67 °C (green dotted curve). Homozygous mutated samples show a peak around 60 °C (red dotted curve). Heterozygous samples show two peaks (orange dotted curve) and the negative control shows no peak (grey dotted curve). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in comparison to standard diagnostic techniques. Inter-operator reproducibility was very good (100% match) testifying of the robustness of the assay. This test allows for a fast and easy simultaneous identification of both the *F5L* and *F2* c.*97G > A mutational status in thrombophilia screening. Its main assets are this concomitant screen of both variants and possible use of whole blood, without any requirement for DNA extraction/quantification. Based on these assets, the mean overall time for reaching reportable results is of about 1 h (including 40 minute hands-off amplifications) in comparison to several hours for the two separate conventional assays. Moreover, the workload for technicians is significantly reduced without any loss in accuracy. In addition, the absence of consecutive thermal cycles also allows a time reduction of the PCR which can be achieved with classical equipment and could therefore become a routine test. The various amplification systems used for the detection of thrombophilia-associated variants have been largely compared by Emadi et al. [8] and shown to be mostly highly reliable.

Indeed, the Genoflow assay [9], reported after the meta-analysis of Emadi et al. [8] uses for each assay 10 ng of extracted DNA and involves 42 cycles of 9 min each, i.e. about 6 h. Here only 1 ng of DNA is sufficient ant the turnaround time is only 1 h. Similarly, in a very recent report [10], the Cobas F2F5 test (Roche®) was reported to provide performances improved compared to those of the LightCycler® 1.2 platform. Indeed, turnover times were 98.6 vs 420.2 min, yet still around 1.5 h, not counting the time for DNA extraction. The amount of the latter was not indicated, only mention of suspensions between 0.1 and 120 ng/μL being made, suggesting larger amounts than here.

In conclusion, the Lamp Human FII&FVL duplex KIT appears to be a valid tool for a fast and reliable detection of *F5L* and *F2* c.*97G > A mutational status in clinical practice.

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A. Desjonquères[a], A. Ménard[a], L. Detemmerman[b], C. Ternisien[a], M. Fouassier[a], B. Gillet[a], M.C. Béné[a], Y. Le Bris[a,*]

[a] *Hematology Biology, Nantes University Hospital, Nantes, France*

[b] *LaCAR MDX Technologies, Liège, Belgium*

E-mail address: yannick.lebris@chu-nantes.fr (Y. Le Bris).

* Corresponding author at: Hematology Biology, Nantes University Hospital, 9, quai Moncousu, F- 44093 Nantes Cedex 1, France.