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BRIEF REPORT

Droplet digital PCR and mile-post analysis for the detection of *F8 int1h* inversions

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Abstract

Background: *F8 int1h* inversions (Inv1) are detected in 1%–2% of severe hemophilia A (HA) patients. Long-range polymerase chain reaction (PCR) and inverse-shifting PCR have been used to diagnose these inversions.

Objectives: To design and validate a sensitive and robust assay for detection of *F8* Inv1 inversions.

Methods: Archival DNA samples were investigated using mile-post assays and droplet digital PCR.

Results: Milepost assays for Inv1 showing high specificities and sensitivities were designed and optimized. Analysis of four patients, two carrier mothers, and 40 healthy controls showed concordance with known mutation status with one exception. One patient had a duplication involving exons 2–22 of the *F8* gene instead of an Inv1 mutation. DNA mixtures with different proportions of wild-type and Inv1 DNA correlated well with the observed relative linkage for both wild type and Inv1 assays and estimated the limit of detection of these assays to 2% of the rare chromosome.

Conclusions: The milepost strategy has several inherent control systems. The absolute counting of target molecules by both assays enables determination of template quantity, detection of copy number variants, and rare variants occurring in primer and probe annealing sites and estimation of DNA integrity through the observed linkage. The presented Inv1 milepost analysis offers sensitive and robust detection and quantification of the *F8 int1h* inversions and other rearrangements involving intron 1 in patients and their mothers.

KEYWORDS
factor VIII, genetic linkage, hemophilia A, polymerase chain reaction, sequence inversion

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1 | INTRODUCTION

Inversions involving intron 1 and intron 22 of *F8* are the most common mutations in patients with severe hemophilia A (HA). Inversions in intron 1 (Inv1) result from homologous recombination between a ~1 kb repeated sequence in intron 1 (*int1h-1*) of *F8* and *int1h-2* located ~140 kb distal to *F8*. Inv1 inversions are present in 1%–2% of HA patients.[1] Inv1 genotyping has been performed using long-range polymerase chain reaction (PCR),[2] inverse-shifting PCR,[3] and an RNA-based method.[4]

Recently, droplet digital PCR (ddPCR) using a milepost strategy was described for the detection of intron 22 inversions.[5] The ddPCR produces water-in-oil emulsions that partitions the template DNA into thousands of small droplets. The presence or absence of one or several target molecules are then determined for individual droplets using flow cytometry. The number of target molecules is limited in relation to the number of droplets and a Poisson algorithm is used to estimate the true number of target molecules from the fraction of negative droplets.[6] Two target sequences can be analyzed simultaneously using TaqMan assays labelled with different fluorophores. Physical linkage between the two target sequences is then detected by a higher-than-expected fraction of droplets being positive for both targets compared with the random distribution observed for two unlinked loci. This milepost strategy can be used to investigate the existence of, for example, inversions.[7]

In the present study, we describe a milepost-based system for the detection of Inv1 in patients and carrier mothers. We also characterize the properties of the milepost systems for quantification of Inv1 chromosomes and validate the systems by analyzing a collection of archival samples with respect to Inv1 inversions.

Essentials

- Long-range- or inverse-shifting polymerase chain reaction (PCR) have been used to diagnose the common *F8* intron 1 inversions (Inv1).
- Mile-post assays were used to detect and quantitate *F8* Inv1 inversions in four patients, two carrier mothers and 40 healthy controls.
- Mile-post assays can quantitate *F8* Inv1 inversions at a level of 2% of the rare chromosome.

2 | METHODS

2.1 | DNA samples

Two HA patients with Inv1 inversions and two healthy males were used for *F8* Inv1 assay development. DNA isolation was performed using the QIAamp DNA Blood Mini Kit (Qiagen GmbH). Samples were quantified using spectrophotometry in an Eon microplate spectrophotometer (BioTek Instruments) and subsequent ddPCR analysis. Their inversion mutations had been determined using either long-range[2] or inverse-shifting PCR.[3] Four patients with Inv1 mutations, two known carrier mothers, 20 healthy females, and 20 healthy males (used as controls) were analyzed in total. The procedures were approved by The Regional Ethical Review Board in Lund and the Swedish Data Inspection Board and were in accordance with the Declaration of Helsinki.

2.2 | Droplet digital PCR and milepost analysis

Droplet digital PCR used FAM and VIC dual-labeled probe systems to detect Inv1 target sequences in a similar way to what has been previously described for Inv22 target sequences.[5] In short, probes were designed using RealTimeDesign Software, BioSearch Technology (https://www.biosearchtech.com/display.aspx?pageid=54) and ordered from DNA Technology A/S. Digital PCR was performed using a QX100 ddPCR system from Bio-Rad. The ddPCR reaction mixture contained 1xSupermix (Bio-Rad), 900 nM of each primer, 500 nM of each probe, and template DNA in the 8–50 ng range. Droplets were generated using a QX100 Droplet Generator (Bio-Rad) and PCR products amplified using Veriti 96 PCR machines (Applied Biosystems). The assays were optimized with regard to annealing temperature that defined an optimum annealing temperature of 60°C (full sequences and protocol in Table 1). Theoretically, males would be expected to show linkage to either the wild-type (WT_1) or Inv 1 assay (and the other assay would show nonlinkage). In carrier females, WT_1 and Inv 1 assays would be expected to show a highly similar degree of linkage.

TABLE 1 Description of milepost assays for detection of *F8 int1h* inversions

Primer/Probe	Sequence
Anchor, forward	AAGCGTGGCTTCCCACTGA
Anchor, reverse	GCTGGCAGGTTGGACTTTG
Anchor, probe	VIC-AAGGCTGATCTAGCTACTGTTGCCAC-BHQ1
WT locus, forward	TGCAGAGCCATCCTAGCTTG
WT locus, reverse	TGAAGGAAGCAGGATTGGGTAA
WT locus, probe	FAM-TCCCTGTGGGACTGACCTTTATTCA-BHQ1
Inv1 locus, forward	GCTCTAGCAACAGGGTTCATC
Inv1 locus, reverse	TTCGGGAAGAAAGAGCACTGTAG
Inv1 locus, probe	FAM-TGTGCCATATGCCTTCCCCAGG-BHQ1
PCR protocol: 95°C for 10 min; 40 cycles of 94°C for 30 s followed by 60°C for 1 min; 1 cycle of 98°C for 10 min; all ramp rates set at 2°C/s	

Abbreviation: PCR, polymerase chain reaction

An *RNase P* assay confirmed nonlinkage and allowed determination of target sequence copy numbers. The number of target molecules and linked fragments was estimated by counting in a QX100 droplet reader and analysis of the data was performed using QuantaSoft software (Bio-Rad). The overabundance and the proportion of linked fragments were calculated as described by Regan et al.[7]

2.3 | Multiplex ligation-dependent probe amplification and long-range PCR analysis

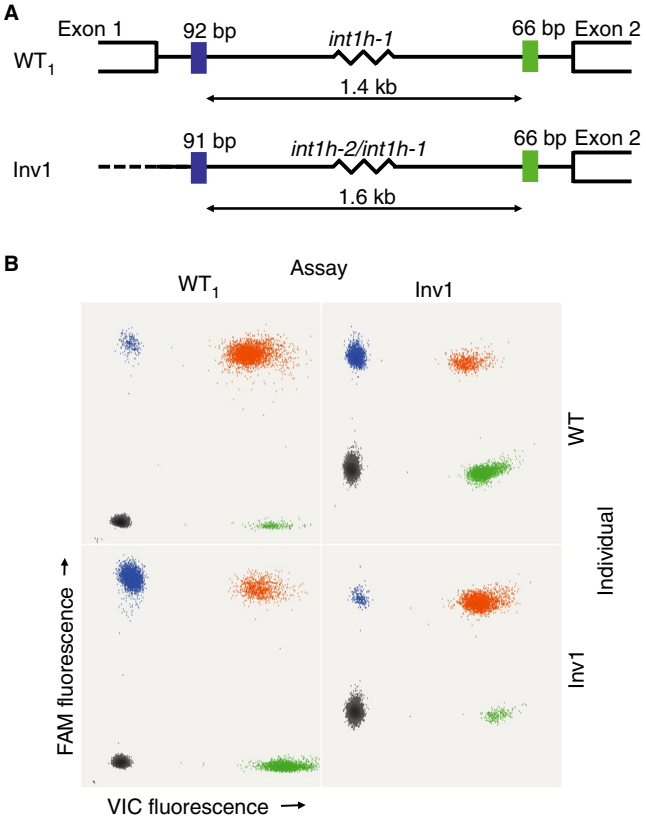
Multiplex ligation-dependent probe amplification (MLPA) analysis (P178-F8, MRC-Holland) was performed according to the manufacturer's instructions (http://www.mlpa.com). Each sample consisted of 50 ng of template DNA and the probe sets were separated on an ABI 3130xl genetic analyzer and analyzed with Coffalyzer.net (MRC-Holland). Long-range PCR analysis used the method described by Bagnall et al.[2]

3 | RESULTS AND DISCUSSION

3.1 | Detection of *F8 int1h* inversions

The flanking regions of the *int1h-1* and *int1h-2* sequences were analyzed for repeated sequences. A VIC-labeled anchor assay located in intron 1 outside of *int1h-1* and towards exon 2 and mile-post assays specific for each of the wild-type and the Inv1 chromosomes were designed (Figure 1A). These were subsequently used to analyze HA patients with the Inv1 mutations and wild-type individuals. Droplet patterns for all combinations of assays and samples are shown in Figure 1B. Equal amounts of template DNA was used to clearly demonstrate the overabundance of double-positive droplets in samples positive for each specific assay. The types of chromosomes present in the samples were identified by their higher-than-expected fractions of double-positive droplets. The number of linked fragments was calculated from the difference between the number of double-positive droplets observed for each sample and the number expected due to chance, as described by Regan et al[7] (Figure 1C). Very few droplets were observed in between the clusters in Figure 1B, indicating the high specificity of the TaqMan assays.

These assays were then used to analyze archival samples with different DNA concentrations and integrities. Four patients with Inv1 chromosomes, two known carrier mothers, and 40 healthy control samples (20 males and 20 females) with wild-type chromosomes were analyzed using Inv1 and WT_1 assays. Comparing the number of linked targets as a function of the number of FAM targets revealed considerably variation in the number of linked targets for different samples (Figure 2A,B). This variation reflects both the varying DNA concentrations and the varying integrities of the samples. The 40 healthy controls all showed the expected pattern of linkage for the WT_1 and nonlinkage for the Inv1 assays,



C

Individual	WT_1 assay				Inv1 assay			
	FAM	VIC	Linked	% Linked	FAM	VIC	Linked	% Linked
WT	6060	6080	5620	93	5580	5560	0	0
Inv 1	6400	6340	0	0	6020	6020	5480	91

FIGURE 1 Design and validation of milepost assays for Inv1 detection. (A) Positions of anchor (green) and milepost systems (blue) for detection of wild-type (WT) and Inv1 chromosomes and their positions within the *F8* gene. (B) Milepost analysis of male individuals with WT or Inv1 chromosomes using WT_1- and Inv1-specific milepost assays. (C) Calculated numbers of target molecules for the combinations of assays and individuals shown in (B)

respectively. As expected, the two carrier mothers showed equal proportions of linked molecules to both WT_1 and Inv1 assays. Three of four patients known to possess Inv1 chromosomes also showed the expected pattern of linkage for the Inv1 and nonlinkage for the WT_1 assays, respectively. One of the patients, however, failed to show linkage to the Inv1 assay but instead showed linkage to the WT_1 assay. This patient, originally analyzed using the long-range PCR assay of Bagnall et al.[2] showed twice the copy number of the FAM probe region compared with the VIC probe region indicating a duplication of the FAM probe region (Figure 2C). All four patients were subsequently reanalyzed using MLPA of all *F8* exons confirming the presence of a duplication of exons 2–22 in the patient with a FAM probe region duplication (Figure 2D). When this patient was reanalyzed using the original PCR assay,[2] an ambiguous result was obtained. The analysis showed an Inv1-positive DNA fragment for one of the reactions, but a wild-type-positive fragment for the other indicating an abnormal situation (Figure 2E). Thus, the results of all

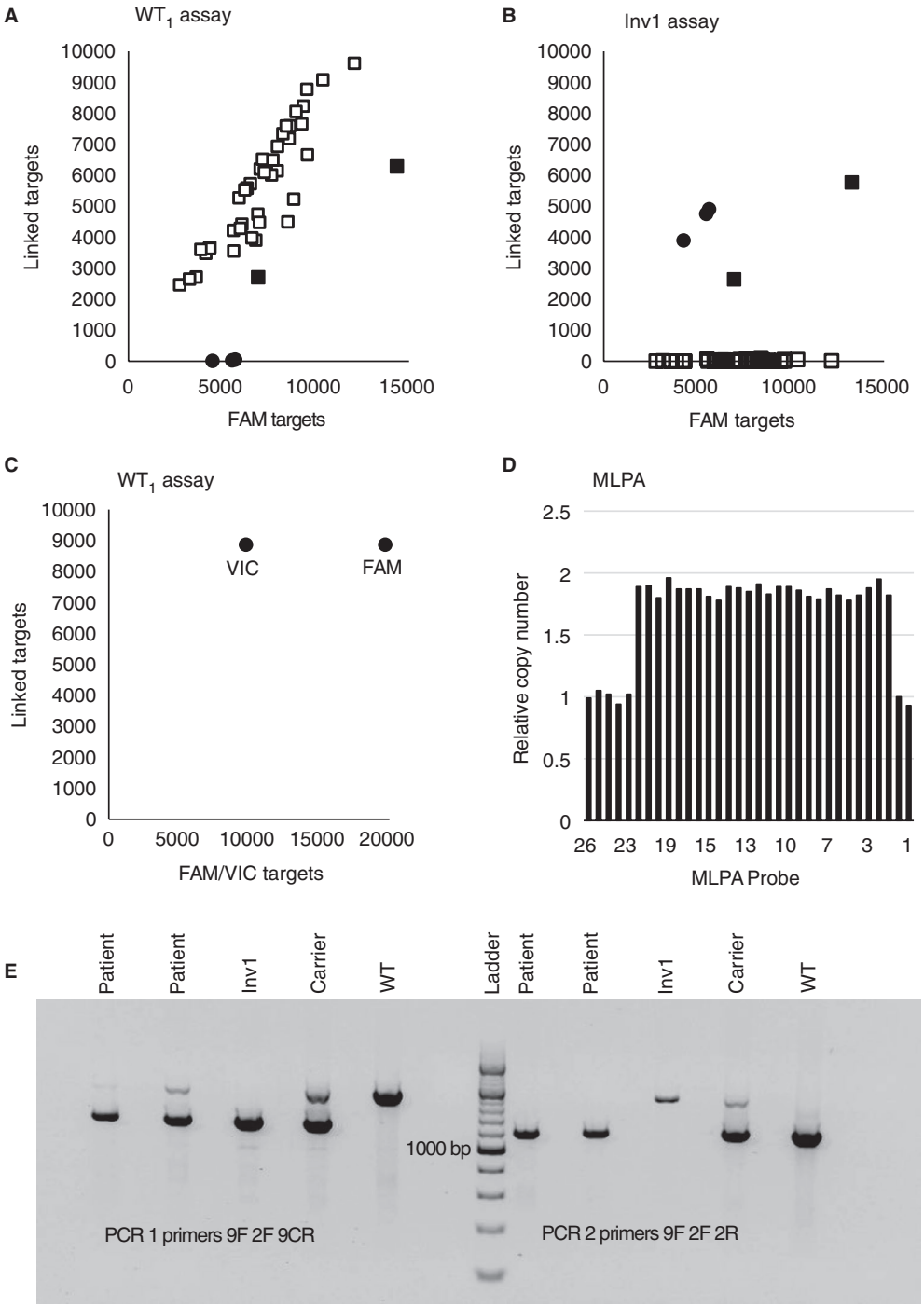


FIGURE 2 Milepost analysis for Inv1 inversions in patients, carrier mothers, and controls. The number of linked targets as a function of the number of FAM targets shown for (A) WT_1 and (B) Inv1 assays for male patients (black circles), healthy male and female controls (open squares), and carrier mothers (black squares). (C) A single patient showed instead linkage to the WT_1 assay with twice the number of FAM compared with VIC targets. (D) Multiplex ligation probe amplification (MLPA) analysis showed a duplication in this patient. (E) Ambiguous long-range polymerase chain reaction (PCR) results for the patient with duplication of exons 2–22 (duplicates of patient compared with Inv1, carrier, and wild-type individuals; primers and PCR systems as defined by Bagnall et al.[2])

three techniques are fully compatible with each other, detecting an exon 2–22 duplication.

The milepost assays have several inherent control systems. The determination of the number of target molecules tells us directly how many chromosomes are interrogated and the number of linked targets in relation to the total number of target molecules estimate the DNA integrity. If both WT_1 and Inv1 assays are used to analyze an individual, all samples are expected to show linkage to at least one of the assays and are thus capable of detecting highly fragmented samples. Because each assay is made up of two independent TaqMan assays and because they are both expected to identify very similar numbers of target molecules when a large population of

DNA molecules is analyzed, this can be used to identify rare variants occurring in any of the recognition sequences of primers and probes for the systems. This feature will also identify duplications or deletions encompassing one of the TaqMan systems of an assay, whereas a duplication or deletion encompassing both TaqMan systems of an assay can be identified by running in parallel an unlinked assay and comparing their numbers.

3.2 | Quantitative properties of milepost inversion assays

Artificial mixtures of mutant and wild-type DNA samples were then used to investigate the milepost assays for their quantitative properties. Samples with 100%, 50%, and 0% mutant DNA against a background of 0%, 50%, and 100% wild-type DNA were prepared for analysis using WT_1/Inv1 milepost assays. All mixtures were analyzed in replicates of four. Both the WT_1 and Inv1 milepost assays showed a good correspondence between the proportions of the different types of DNA in the artificial mixtures and their relative linkage (Figure 3A). The Inv1 and WT_1 assays showed relative linkages of 92% and 93%, respectively. To investigate the limit of detection of these assays, artificial mixtures were prepared with 0%, 2%, 4%, and 8% of both types of DNAs. Milepost analysis using the WT_1 and Inv1 assays showed that it was possible to discriminate between a pure DNA sample and samples containing 2% of DNA from the rare chromosome using both assays (Figure 3B).

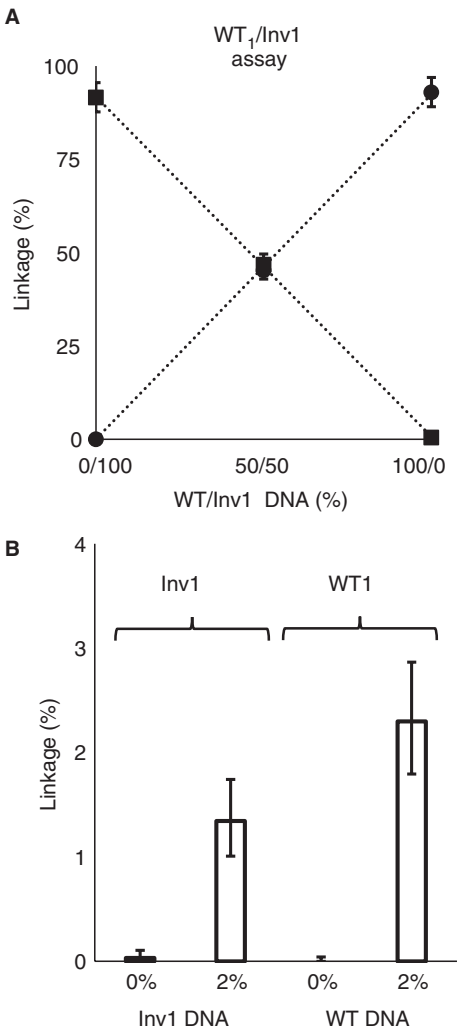


FIGURE 3 Quantitative properties of the Inv 1 inversion assays. (A) Quantitative response of WT_1 (dots) and Inv1 (squares) assays determined from artificial mixtures prepared with different proportions of mutant and wild-type DNAs. (B) Limit of detection determined by analyzing artificial mixtures where either type of DNA was present at a low proportion. The measure of uncertainty is based on the analysis of four replicates and given as 95% confidence intervals

We have previously reported a detection system for Inv22 inversions using milepost assays and ddPCR.[5] The present study reports an extension of this study including the design, optimization, and validation of technically simple milepost assays allowing for rapid and precise diagnosis of Inv1 inversions in both male patients and carrier mothers. The robustness of these assays was emphasized by the successful analysis of archival samples with widely varying DNA concentrations and integrities. Because the present strategy allows the analysis of large numbers of single molecules in individual reactions, the assay systems described here are more tolerant to DNA fragmentation than both long-range- or inverse-shifting PCR. Intron 1 and intron 22 inversions are present in almost one-half of patients with severe HA. Therefore, the development of robust quantitative methods for detection of these inversions are of major importance. We would like to argue that the use of milepost assays for both types of *F8* inversions allows for much more powerful detection of these inversions and will make standardization and cross-laboratory evaluation of these analyses easier. Furthermore, the presence of milepost systems for the detection of both types of inversions increases the clinical usefulness of these systems because both types of inversions can now be analyzed using the same platform.

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CONFLICT OF INTEREST

All declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Eric Manderstedt and Christina Lind-Halldén performed experiments. Eric Manderstedt and Christer Halldén performed data analysis and wrote the manuscript. Rolf Ljung and Jan Astermark contributed patient material. Christer Halldén supervised the study. All authors contributed to the final manuscript.

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