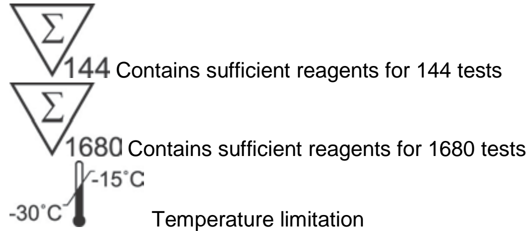
HOLOGIC®

Invader® Factor II
REF 95-452: 144 tests or REF 95-456: 1680 tests

IVD *In vitro* diagnostic medical device

Rx only



Contains sufficient reagents for 144 tests

Contains sufficient reagents for 1680 tests

Temperature limitation

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I. INDICATIONS AND USE

A. Intended Use

The Invader® Factor II test is an *in vitro* diagnostic test intended for the detection and genotyping of a single point mutation (G to A at position 20210) of the human Factor II gene in isolated genomic DNA obtained from whole blood potassium EDTA samples from patients with suspected thrombophilia.

B. Clinical Significance

The prothrombin (Factor II) mutation refers to a base change from guanine (G) to adenine (A) at nucleotide position 20210 in the prothrombin gene.[1] The G20210A mutation is associated with an elevation of prothrombin levels to about 30 percent above normal in heterozygotes and to 70 percent above normal in homozygotes.[1,2] The mutation is the second most common inherited risk factor for thrombosis. In the United States, the prevalence of the mutant allele is 1.1 percent in Caucasians and Hispanics and 0.3 percent in African Americans.[3] This mutation is present in about 2 percent of the population and in 6 percent or more of patients who present with a first venous thromboembolism (VTE). Six to 12 percent of individuals who are heterozygous for Factor V Leiden (FVL) and have VTE also have the prothrombin G20210A mutation (double heterozygotes).[4,5,6] Heterozygotes are at a two- to four-fold increased risk of an initial thrombosis.[5,7] Individuals who are doubly heterozygous for FVL and prothrombin G20210A (about 1 in 1,000 Americans) have an estimated 20-fold increased risk when compared to individuals without either mutation, suggesting a multiplicative elevation in risk.[3,4,5]

C. Principles and Procedures

The Invader® Factor II test utilizes the Invader Plus® chemistry with DNA isolated from human whole blood for the detection of the targeted sequence polymorphism. Specifically, the Invader Plus® chemistry utilizes a single-tube, two phase reaction, including target amplification and signal generation (mediated by Invader® chemistry). Invader Plus® reaction mixes are assembled by combining the Factor II Oligo Mix, Universal Enzyme Mix, and Universal Buffer. In a 96-well plate, reaction mix is combined with purified genomic DNA samples, as well as four (4) controls included with the test. The No DNA Control is used by the interpretive software to set the "noise" component of the run for "signal-to-noise" calculations. The genotype-specific controls (WT, HET, MUT) ensure reagents were assembled correctly and perform according to the specifications. The 96-well plate is transferred to an appropriately programmed thermal cycler for target amplification and signal generation. In the target amplification phase of the reaction, amplification is carried out using "two-step" cycling conditions (i.e., denaturation & annealing/extension). Following amplification, Taq polymerase is inactivated by a 10 minute incubation at 99°C, after which the thermal cycler proceeds to 63°C to initiate the signal generation (Invader®) phase of the reaction (see Figure 1).

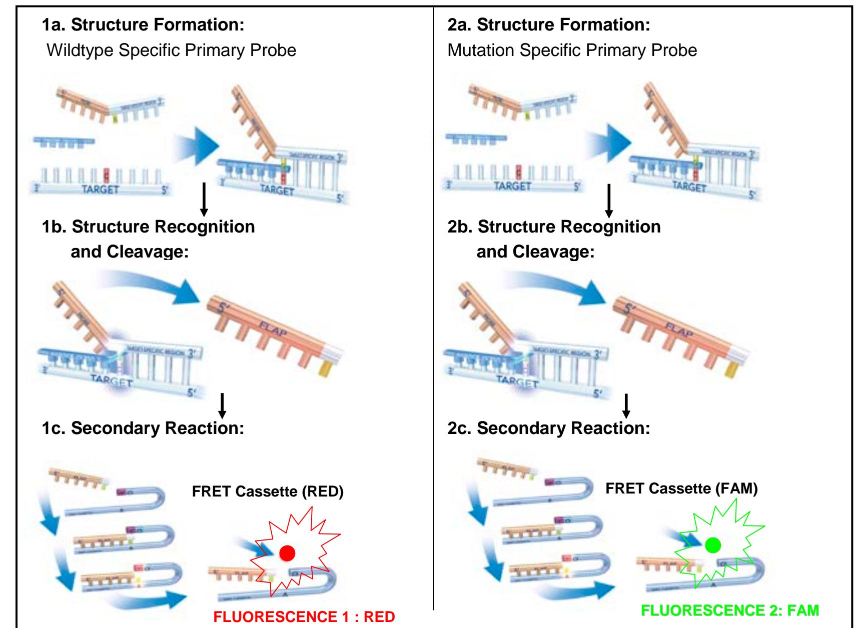


Figure 1: Invader® Signal Generation Phase

During the signal generation phase, a discriminatory Primary Probe transiently hybridizes to the amplified target sequence along with an Invader® oligonucleotide, to form an overlapping structure. The 5'-end of the Primary Probe includes a 5'-flap that does not hybridize to the target DNA. The 3'-nucleotide of the bound Invader® oligonucleotide overlaps the Primary Probe, and does not hybridize to the target DNA. The Cleavase® enzyme recognizes this overlapping structure and cleaves off the unpaired 5'-flap of the Primary Probe, releasing it as a target-specific product. The Primary Probe is designed to have a melting temperature aligned with the Invader® reaction temperature so that under the isothermal reaction conditions (~63°C) the Primary Probes cycle on and off the target DNA. This allows for multiple rounds of Primary Probe cleavage for each DNA target resulting in an accumulation of the number of released 5'-flaps. The released 5'-flap transiently hybridizes with a corresponding FRET cassette forming an overlapping structure that is recognized and the fluorophore is cleaved from the FRET cassette by the Cleavase® enzyme. The 5'-flap is designed to have a melting temperature aligned with the Invader® reaction temperature, so that the 5'-flaps cycle on and off of the corresponding FRET cassettes. This allows for multiple rounds of FRET cassette cleavage for each 5'-flap, and an accumulation of released fluorophore. When the FRET cassette is cleaved, a fluorophore and quencher are separated, generating detectable fluorescence signal. The format uses two different discriminatory Primary Probes, one for the mutant allele and one for the wild type allele (Figure 1). Each Primary Probe is assigned a unique 5'-flap, and distinct FRET cassette, with a spectrally distinct fluorophore. By design, the released 5'-flaps will bind only to their respective FRET cassettes to generate a target-specific signal, linking the wild type allele with one fluorophore (Fluorescence 1: RED) and the mutant allele with the second fluorophore (Fluorescence 2: FAM).

II. MATERIALS AND METHODS

A. Reagents Provided

Note: For information on any hazard and precautionary statements that may be associated with reagents, refer to the Safety Data Sheet Library at www.hologicsds.com.

Table 1: Reagents Provided

Reagent	Vial Label Abbreviation
Factor II Oligo Mix	None
Universal Buffer	B
Universal Enzyme Mix	E
Invader® Factor II WT	None
Invader® Factor II HET	None
Invader® Factor II MUT	None
No DNA Control	C4

B. Reaction Mix

All of the Invader® Factor II reagents are supplied in concentrations ready for use. The amount of reagents required for each reaction is summarized in Table 2. Make sure to mix reagents well prior to use.

C. Other Materials Provided

Invader Call Reporter® software and Invader® Factor II software

Software User Manual for Invader® Factor II (MAN-01687)

Both software programs and the software user manual are provided along with the first order/shipment of the Invader® Factor II test. Contact Hologic Technical Support (1 888 484 4747) if an additional copy is needed.

D. Materials and Reagents Needed But Not Provided

- Thermal cycler with heated lid capable of holding set temperatures within +/- 1°C.
- Multi-well Fluorometer (see Software User Manual for Invader® Factor II, MAN-01687, for fluorometer software specifications)
- Computer (See Software User Manual for Invader® Factor II, MAN-01687, for computer specifications)
- Pipette tips, filter barrier
- 96-well plates
- Optically Clear Adhesive Plate sealers
- Nuclease-free water
- Mineral oil
- Microcentrifuge tubes
- Commercially Available DNA Extraction kit (or validated in-house laboratory method)
- General laboratory equipment as needed (tube racks, micropipettors, multichannel pipettor, microcentrifuge, vortex mixer).

E. Storage and Handling

NOTE: Product requires multiple storage temperatures for reagents.

Immediately upon receipt, genotype-specific controls are to be stored at +2°C to +8°C. All other components of the kit should be stored between -30°C to -15°C in a non-frost-free freezer.

Prior to use, allow reagents to equilibrate to room temperature, excluding the Universal Enzyme Mix which should remain between -30°C to -15°C until just prior to use. Minimize reagent exposure to light. Do not subject the reagents to more than 15 freeze-thaw cycles.

F. Indications of Instability

When properly stored, the reagents are stable through the dating indicated on the label. There are no obvious signs to indicate instability of this product. However, genotype-specific controls should be included on each run, as an increase in non-specific fluorescence signal may indicate reagent instability. If this is observed, contact Hologic Technical Support (1 888 484 4747).

G. Specimen Collection and Preparation for Analysis

Clinical Specimens: Human whole blood samples should be anti-coagulated with potassium EDTA. DNA extraction may be accomplished using commercially available DNA extraction chemistries, capable of obtaining DNA concentrations greater than 5 ng/μL for use in the Invader® Factor II test.

Genotype-Specific Control Samples: Genotype-specific (i.e., WT, HET, MUT) controls are provided with the test. Genotype-specific controls consist of synthetic DNA in a blood-like matrix, and are not infectious. Genotype-specific controls must be extracted prior to use, and can serve as a DNA extraction control as well if prepared using the same method as the blood samples. Prior to extraction, genotype-specific controls should be vortexed (30-60 seconds) to re-suspend the contents.

DNA Storage: The purified DNA from samples and genotype-specific controls can be used immediately or safely stored in elution buffer as per the DNA extraction kit manufacturer's recommendation.

DNA Preparation: Extracted clinical specimen and genotype-specific control DNA must be diluted 1:20 in nuclease-free water just prior to running the Invader® Factor II test (see Section IV A. 3). The level of DNA present in the extracted genotype-specific controls may not be detectable with certain quantitation methods and is not quantifiable by spectrophotometer measurements.

III. SAFETY ISSUES

A. Safety and Handling Precautions

1. Universal safety precautions should be used when handling any human whole blood samples. Specimens should be disposed of according to local requirements.
2. Product components (product residuals and packaging) can be considered laboratory waste. Dispose of unused reagents and waste in accordance with applicable federal, state, and local regulations.

B. Precautions

1. The Invader® Factor II test is intended for *in vitro* diagnostic use.
2. These components have been tested as a unit. Do not interchange components from their sources or from different lots. Do not pool reagents from different lots or from different vials of the same lot.
3. Take reasonable precautions when handling reagents. Use disposable gloves when handling suspected carcinogens or toxic materials.
4. Do not smoke, eat or drink in areas where specimens or reagents are being handled.
5. Avoid contact of eyes and mucous membranes with reagents. If reagents come in contact with sensitive areas, wash with copious amounts of water.
6. Patient specimens and all materials coming into contact with them should be handled as if capable of transmitting infection and disposed of with proper precautions. Never pipette by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
7. Avoid microbial contamination of reagents as this could produce incorrect results.
8. Incubation times and temperatures other than those specified may give erroneous results.
9. The reagents have been optimally formulated and further dilution may result in loss of performance.
10. Do not use reagents after their expiration date.
11. Use fresh mineral oil for each reaction set-up (do not transfer these reagents back to the original container once they have been dispensed).
12. The provided genotype-specific controls are in a blood-like matrix and are not infectious. Material can be used in a Bio-Safety Level 1 laboratory.
13. **IMPORTANT CONTAMINATION PRECAUTIONS: This product generates amplified DNA targets. When performing the test, caution must be taken to prevent amplicon contamination of work areas. Always use barrier pipette tips for pipetting procedures. Perform the amplification set up in an isolated area with dedicated pipettes. Use tips and tubes that are DNase/RNase free.**

C. Toxicity of Invader® Reagents

The Invader® Factor II test reagents are not controlled as dangerous substances and no toxicity has been determined.

IV. INSTRUCTIONS FOR USE

A. Invader® Test Step by Step Procedure

Software Set up

1. Open the Invader Call Reporter® software and complete the testing information. Details for using the software can be found in the software user manual (Software User Manual for Invader® Factor II, MAN-01687).
   a. Enter the name of the operator.
   b. In the dropdown "Menu Selection" select the Factor II test.
   c. Enter the number of samples to be tested in the space provided.
   d. Click the "Proceed to Mix Preparation" button located in the lower right corner of the window.
   e. On the Mix Preparation tab, fill in the green shaded boxes for Lot Numbers and Expiration Dates for the reagents used during the testing.
   f. If desired, click the "View/Save PDF" button located in the upper right corner of the window. Print the PDF and then close the PDF window.
   g. On the Sample Placement tab, enter the Sample IDs into the list on the left side of window. The Sample ID list runs down columns (i.e., wells E1 through H1, followed by A2 through H2, and then A3 through H3…).
   h. Verify all samples are entered in the list and are in correct position of the sample grid.
   i. If desired, click the "View/Save PDF" button located in the upper right corner of the window. Print the PDF and then close the PDF window.
   j. Close the Invader Call Reporter® software.
2. Confirm the thermal cycler is programmed as stated in Figure 3.

Sample Preparation (1:20 Dilution)

3. Dilute extracted genotype-specific controls and all extracted sample DNAs (1:20) using 5 µL of genotype-specific control/sample and 95 µL nuclease-free water in a 0.5 mL tube or similar consumable. Mix the diluted genotype-specific controls/samples thoroughly. Do not dilute the No DNA Control (Control 4) prior to use.

Mix Preparation

4. Remove the reagents (Oligo Mix, Universal Buffer, No DNA Control) from their respective storage locations and allow them to equilibrate to room temperature for approximately 30 minutes. Do not remove the Universal Enzyme Mix from the -30°C to -15°C freezer until just prior to use.
5. Vortex the components of the reaction mix thoroughly and spin down the contents in a microcentrifuge for 3 – 5 seconds.
6. Combine the components of the reaction mix as shown in Table 2, or print out from Mix Preparation tab of software, in a microcentrifuge tube.

Note: The prepared reaction mixture must be used within 30 minutes.

Note: No DNA Control and genotype-specific controls must be placed in the correct wells for proper data interpretation. Refer to Figure 2.

Table 2: Invader® Factor II Reaction Mix

Component	µL/well	Number of Rxns	25% Overage	Total Volume (µL)
Factor II Oligo Mix	7.5 µL	*k*	1.25	=7.5*k*(1.25)
Universal Buffer	2.0 µL	*k*	1.25	=2.0*k*(1.25)
Universal Enzyme Mix	0.5 µL	*k*	1.25	=0.5*k*(1.25)
Total Mix Volume	**10 µL**	***k***	**1.25**	**=10*k*(1.25)**

7. Vortex the reaction mix thoroughly and spin down the contents in a microcentrifuge for 3-5 seconds.

Plate Set up

8. Reaction mix may be aliquoted into a 96-well plate to facilitate the use of a multi-channel pipettor.
9. Add 10 µL of reaction mix to the bottom of each well designated for each control and sample of the reaction plate.
10. Dispense 10 µL of the appropriate control or sample (genotype-specific controls and all samples are diluted (See step 3), Control 4 (NDC) is undiluted) to bottom of the appropriate well of the reaction plate (See Figure 2). Mix by pipetting up and down 3 – 5 times upon addition to ensure reaction homogeneity. Change pipette tips between every addition.

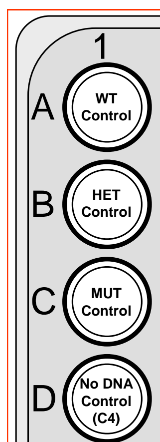
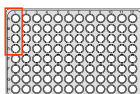


Figure 2: Plate Position of Controls in a 96-well plate.



11. Overlay all control and sample wells with 20 µL of fresh mineral oil by dispensing along the side of the wells. Change pipette tips between every addition.
12. Cover the reaction plate with optically clear adhesive film. Thoroughly secure the film to the surface of the plate.
13. Visually confirm no bubbles exist in the reaction wells. If bubbles are visible, remove bubbles (e.g., centrifuge plate briefly).
14. Place the reaction plate in the thermal cycler.

Step	Description	Temperature (°C)	Time	Cycles
1	Denature	95	2 Minutes	2
2	Anneal / Extend	72	2 Minutes	
3	Denature	94	0.5 Minutes	28
4	Anneal / Extend	72	1.5 Minutes	
5	Heat Inactivate Polymerase	99	10 Minutes	1
6	Invader® Reaction	63	1 Minute	
7	Hold	10	Hold	

Figure 3: Invader Reaction Program

15. Start the Invader reaction program.
16. When the Invader reaction program is complete, the reaction plate can be held in the thermal cycler at +10°C or stored in a refrigerator (+2°C to +8°C), protected from light, overnight.

Data Collection

17. Allow the reaction plate to equilibrate to room temperature on the bench top at least 1 minute prior to reading the plate.
18. Visually confirm no bubbles exist in the reaction wells. If bubbles are visible, remove bubbles (e.g., centrifuge plate briefly).
19. Read the reaction plate on a multi-well fluorometer according to manufacturer's instructions. Verify parameters match Table 3.

Table 3: Recommended Multi-well Fluorometer Settings

Setting Category	Measurement 1 (FAM)	Measurement 2 (Red)
Mode:	Fluorescence Top Reading	Fluorescence Top Reading
Do not use "Plate with cover" option		
Excitation Wavelength (Bandwidth):	485 (20) nm	560 (20) nm
Emission Wavelength (Bandwidth):	535 (25) nm	612 (10) nm
Number of Reads (or flashes):	10	10
Integration Time:	20 µs	20 µs
Lag Time:	0 µs	0 µs
Settle Time:	0 ms	0 ms
Multiple Reads per Well:	Not selected	Not selected
Label Name:	Label 1	Label 2

20. Place the 96-well plate to be analyzed onto the plate carrier with the A1-well oriented to the upper left corner of the plate carrier. Do not remove the optically clear adhesive film from the surface of the plate. Read the entire plate according to manufacturer's instructions.

NOTE: If the No DNA Control (NDC) signal is not greater than 600 counts for FAM or Red fluorescence, re-read the plate adjusting the gain setting(s) accordingly so that each value is greater than 600 counts and the reader is in the linear dynamic range according to the manufacturer's instructions.

Data Analysis

21. Open the Invader Call Reporter® software.
22. Select the plate(s) to be analyzed by highlighting the appropriate row in the blue "Active Assay" field.
23. Click the "Load Selected" button in lower left area of the window. This should allow the "Results" tab to be selected.
24. Click on the "Results" tab.
25. Select the Raw Data File by clicking on the "Select File" button and select the appropriate file in the browser.
26. Select the appropriate Worksheet in the raw data file from the available choices in the dropdown menu.
27. Click the "Import Raw Data" button to populate data fields.
28. If desired, click the "View/Save PDF" button located in the upper right corner of window. Print the PDF and then close the PDF window.
29. Click on the "Summary" tab to view sample validity and genotype results.
30. If desired, click the "View/Save PDF" button located in the upper right corner of window. Print the PDF and then close the PDF window.
31. If desired, click the "Finish Active Assay" button to delete run information when testing and analysis is completed.

B. Quality Control Procedures

Differences in blood processing and technical procedures in the user's laboratory may produce variability in results, necessitating regular evaluation of laboratory designated controls in addition to the following procedures.

Prior to initial use of this test in the user's laboratory, the performance of the test may be verified by testing a number of positive and negative samples with known characteristics. These quality control tests should be repeated for each new lot or a change in test parameters.

Test verification on a daily basis may be accomplished through the proper use of the above-mentioned laboratory designated controls, as described in this section.

The No DNA Control (C4) is used to establish the amount of signal generated in the absence of target.

Test runs are valid when the genotype-specific controls yield the appropriate genotype results (Table 4). If any of the genotype-specific controls are called incorrectly or EQ (equivocal), the run is invalid and must be repeated. A test run with invalid control results will fail to provide sample results. In the event of a control failure, all samples in the run should be re-tested.

Unexplained discrepancies in control results should be referred to Hologic Technical Support (1 888 484 4747). See the Troubleshooting section of this package insert for additional information.

All quality control requirements should be performed in conformance with local, state, and/or federal regulations or accreditation requirements.

C. Interpretation of the Results

Results from the Invader® Factor II test are reported to the user as a genotype "call", indicating which genotype was detected in the sample (WT, HET, MUT). The results also report sample validity and run validity. Genotype calls and corresponding nucleotides are shown in Table 4.

Table 4: Interpretation of Results

Genotype	Invader® Factor II Genotype Call	Nucleotides at Position 20210
Homozygous Wild Type	WT	GG
Heterozygous	HET	GA
Homozygous Mutant	MUT	AA

The Results in the Invader Call Reporter® software display sample and control data. If results are invalid or not displayed, refer to the Troubleshooting section of this package insert and the Software User Manual for Invader® Factor II (MAN-01687).

The "Summary" tab in the Invader Call Reporter® software displays results for all samples and controls in a condensed format. If results are invalid or not displayed, refer to the Troubleshooting section of this package insert or in the Software User Manual for Invader® Factor II (MAN-01687).

If any of the controls are invalid, sample results will not be displayed.

D. Limitations

General Limitations:
Reagents may demonstrate unexpected performance in previously untested samples. The possibility of unexpected performance even in tested blood samples cannot be completely eliminated due to the biological variability of sample matrices. Contact Hologic Technical Support (1 888 484 4747) with any documented unexpected result(s).

Specific Limitations:
Accurate results were obtained when mutations A20207C, C20209T, A20218G and C20221T were tested using Invader® Factor II. It is recommended that the laboratory assess the possibility of any additional rare mutations that may generate false Factor II results and report this as a limitation, if applicable.

E. Summary of Expected Results

1. Accuracy compared to bi-directional DNA Sequencing

Human whole blood samples (n = 336) underwent DNA extraction and subsequent bi-directional DNA sequence analysis. The same DNA samples were then analyzed using the Invader® Factor II test. The observed agreement between the Invader® Factor II test and bi-directional DNA sequencing was 100% (336/336). The overall agreement with bi-directional sequencing was 100% (336/336), with 99.11%, one-sided lower 95% confidence limit (see Table 5).

Table 5: Agreement between the Invader® Factor II Test and Bi-directional DNA Sequencing				
Factor II Genotype*	**Number Tested**	**Number of Valid Results on 1st Run**	**Number of Correct Genotype Calls on 1st Run**	**First Run Agreement**
Homozygous Wild Type (GG)	305	305	305	100%
Heterozygous (GA)	24	24	24	100%
Homozygous Mutant (AA)	7	7	7	100%
Total	336	336	336	100%

* Genotype determined through bi-directional DNA sequencing

2. Reproducibility

a. Inter-laboratory Reproducibility: A multi-center external study was conducted to determine the reproducibility of the Invader® Factor II test. A single lot of the Invader® Factor II test was used to compare the test performance at three different study sites. Blood samples for each genotype were extracted at each site. DNA from the samples underwent subsequent Invader® analysis at each site on each of five (5) non-consecutive days. Results were obtained using the Factor II Invader Call Reporter® software (see Tables 6 and 7).

Table 6: Inter-laboratory Reproducibility of Invader® Factor II Test

Site	Operator	Samples tested	First Pass			Final (Following Single Retest)			Final % Agreement
			Correct Calls	No Calls (Invalid, EQ)	Miscalls	Correct Calls	No Calls (Invalid, EQ)	Miscalls	Final Correct Calls / Samples Tested
Site 001	1	90	90	0	0	90	0	0	100%
	2	90	90	0	0	90	0	0	100%
Site 002	1	90	90	0	0	90	0	0	100%
	2	90	90	0	0	90	0	0	100%
Site 003	1	90	90	0	0	90	0	0	100%
	2	90	54	36*	0	88	2†	0	97.78%†
All	All	540	504	36*	0	538	2†	0	99.63%†

*These "No Call" results were due to an "Invalid Control" result on 2 independent runs. Upon an "Invalid Control" result, the call reporting software automatically prevents the display of all sample genotypes, which resulted in 36 "No Call" samples. Upon retraining of the Operator, and retesting of the 2 runs (see Figure 4), all controls reported "Valid". †From the 36 "No Call" samples, only 2 samples did not provide any genotype results, however upon re-testing both samples were found to be in agreement with sequencing.

Table 7: Factor II Summary of Agreement Data for all Three Sites

Analyses	Number of Comparisons	Number of Agreements	Percent Agreement	One-Sided Lower 95% Confidence Limit
Within Operator (Within-day)	9 (samples) x 2 (operators) x 5 (days) x 3 (sites) = 270	270	100%	99.0%
Between-days (Within-Operator)	9 (samples) x 10 (day pairs) x 4 (2 reps per day) x 2 (operators) x 3 (sites) = 2160	2160	100%	99.6%
Between-operator (within-site)	9 (samples) x 4 (2 reps per operator) x 25 (day pairs between 2 operators) x 3 (sites) = 2700	2700	100%	99.6%
Between-Sites	9 (samples) x 20 (tests at site a) x 20 (tests at site b) x 3 (site pairs) = 10800	10800	100%	99.6%

Table 7: Factor II Summary of Agreement Data for all Three Sites

b. Lot-to-Lot Reproducibility: Whole blood samples were extracted and subjected to bi-directional DNA sequencing. The same DNA samples were then analyzed using the Invader® Factor II test with three different lots of the reagents. The observed agreement between all three lots of the Invader® Factor II test and bi-directional DNA sequencing was 100% (48/48) (see Table 8). Across all genotypes tested, across all three (3) lots, the overall agreement with bi-directional sequencing was 100% (48/48), with 93.95%, one sided lower 95% confidence limit.

Table 8: Lot-to-Lot Agreement between Invader® Factor II and Bi-directional DNA Sequencing

Factor II Genotype (bi-directional sequencing)	Number of Genotypes Tested	Replicates per Sample	Correct Genotype Calls			Invalid Calls			Percent Agreement
			Lot 1	Lot 2	Lot 3	Lot 1	Lot 2	Lot 3	
Homozygous Wild Type (GG)	3	4	12	12	12	0	0	0	100%
Heterozygous (GA)	1	4	4	4	4	0	0	0	100%
Total	4	4	16	16	16	0	0	0	100%

3. Upper and Lower Limits of Detection

Forty (40) replicates of genomic DNA samples representing the wildtype and heterozygous Factor II genotypes were tested at concentrations of both 5 ng/µL and 80 ng/µL, prior to 1:20 dilution for the Invader reaction, and the Invader results compared to bi-directional sequencing. For each concentration, there was 100% (80/80) agreement with bi-directional sequencing. Across all genotypes tested, for a given DNA concentration, the one sided lower 95% confidence limit was 96.32%. Samples were also tested beyond the recommended concentrations of DNA, at 10-fold extremes of the recommended range (e.g., 0.5 ng/µL and 800 ng/µL). At these extreme concentrations, there was 44.70% (45/80) agreement at 0.5 ng/µL and 100% (80/80) agreement at 800 ng/µL concentrations.

4. Interfering Substances

Heparin (1500 U/dL human whole blood), bilirubin (10 mg/dL human whole blood), cholesterol (300 mg/dL human whole blood), potassium EDTA (1.8 mg/mL human whole blood), hemoglobin (up to 0.2% in human whole blood), and ethanol-based wash buffer (5% in DNA sample) had no impact on Invader® Factor II performance.

F. Troubleshooting

Table 9: Troubleshooting Guide

Observation	Probable Cause	Solution
Errors occur during data import: “Check FAM & Red gain settings and read the whole plate again. (Partial plate reads are not allowed.)” “Check FAM gain setting and read the whole plate again. (Partial plate reads are not allowed.)” “Check Red gain setting and read the whole plate again. (Partial plate reads are not allowed.)”	Fluorometer issues.	See Invader Call Reporter® Invader® Factor II Software User Manual troubleshooting guide.
	Incubation period was longer than specified length of time recommended.	Verify that the Invader Reaction Program is as specified (Section IV.A. Figure 3).
High No DNA Control FAM or Red Signal	Contamination	Re-run test taking care not to contaminate samples or reagents.
	Bubbles in reaction well.	Remove bubbles (e.g., centrifuge plate briefly) and re-read.
Bubbles in reaction well.	Over-mixing	Remove bubbles (e.g., centrifuge plate briefly).
No DNA Control is Invalid-Result for one or more Genotype-specific Control is Invalid	Incorrect control volume or no control added to well.	Vortex each reagent before adding to reaction mix.
		Verify correct reagent volumes were added to the reaction mix.
		Verify all reagents were added to the reaction mix.
		Vortex reaction mix before adding to the 96-well plate.
		Visually confirm that no volume discrepancies exist in the 96-well plate by viewing the bottom side of the plate.
	Evaporation of reaction mix/sample during run.	Verify reactions are covered by 20 µL of mineral oil.
	Evidence of contamination during control preparation or reaction mix preparation.	Use DNase/RNase-free aerosol barrier tips at all times.
		Do not allow pipette tips to touch any surface except the solution being pipetted.
No DNA Control is Invalid-Result for one or more Genotype-specific Control is Invalid	Evidence of contamination during genotype-specific control preparation or reaction mix preparation.	Use sterile tubes for preparing reaction mixes.
		Wear gloves at all times
	Controls in wrong location on plate	Verify control well location (Section IV.A. Figure 2)
	Gain setting too low (NDC value <600 counts).	Adjust gain setting so NDC is above 600 counts.
No DNA Control is Invalid-Result for one or more Genotype-specific Control is Invalid	Genotype-specific controls not extracted	Extract genotype-specific controls using standard laboratory method.
	Improper preparation of reaction mix	Vortex each reagent before adding to reaction mix.
		Verify correct reagent volumes were added to the reaction mix.
		Verify all reagents were added to the reaction mix.
		Vortex reaction mix before adding to the 96-well plate.
		Visually confirm that no volume discrepancies exist in the 96-well plate by viewing the bottom side of the plate.
Result for sample is “Low Signal”, “EQ” or “Invalid”	Insufficient sample DNA used in the reaction.	Verify concentration of at least 5 ng/µL prior to dilution and reaction set up.
		Verify 1:20 dilution made correctly (Sample Preparation section IV.A).
		If the DNA concentration is <5 ng/µL pre-dilution, repeat the DNA extraction and purification protocol to obtain purified DNA at a higher concentration.
		Repeat sample with Invader® Factor II test.
	Bubbles in reaction well.	Remove bubbles (e.g., centrifuge plate briefly) and re-read.
	DNA sample inhibition	Repeat DNA extraction from specimen.
		Refer to package insert performance characteristics (Interfering substances Section IV.E.4).
		Verify 1:20 dilution made correctly (Sample Preparation section IV.A).

Table 9: Troubleshooting Guide		
Observation	**Probable Cause**	**Solution**
Result for sample is “Low Signal”, “EQ” or “Invalid”	Incorrect sample volume or no sample added to well.	Verify that no volume discrepancies exist in the 96-well plate by viewing the bottom side of the plate.
	Improper preparation of reaction mix	Vortex each reagent before adding to reaction mix.
		Verify correct reagent volumes were added to the reaction mix.
		Verify all reagents were added to the reaction mix.
		Vortex reaction mix before adding to the 96-well plate.
		Visually confirm that no volume discrepancies exist in the 96-well plate by viewing the bottom side of the plate.
	Evaporation of reaction mix/sample during run.	Verify reactions are covered by 20 µL of mineral oil.
		Verify the 96-well plate is firmly sealed with optical clear adhesive cover before incubating.
		Verify thermal cycler heated lid is firmly closed.
		If the thermal cycler requires a compression pad, verify that the compression pad is seated properly on top of the 96-well plate.
	Insufficient DNA amplification.	Verify the correct Invader Reaction Program was used (Section IV.A. Figure 3). Repeat sample test.
		Verify all reagents have been added to reaction.
	96-well plate incompatible with thermal cycler or positioned incorrectly within thermal cycler.	Verify the 96-well plate is compatible with the thermal cycler, is firmly seated in the thermal cycler and secured properly.
	Sample DNA degradation (DNA may degrade if stored at room temperature).	Store extracted DNA as indicated in the DNA extraction and purification protocol prior to the Invader® test.

Troubleshooting Re-test Procedure

If the established criteria for an acceptable genotype call (i.e., WT, HET, or MUT) are not met by a given sample, it is identified as either “Low Signal” or “EQ” and the sample(s) must be re-tested. A given extraction of a sample that has two EQ (equivocal) results in a row cannot be called by the Invader® Factor II test. If a sample fails to produce the minimum fold-over-zero then the Invader test gives a “Low Signal” result, and the sample must be re-tested. Figure 4 illustrates the recommended re-test process for samples with “Low Signal”, “EQ” or “Invalid” results.

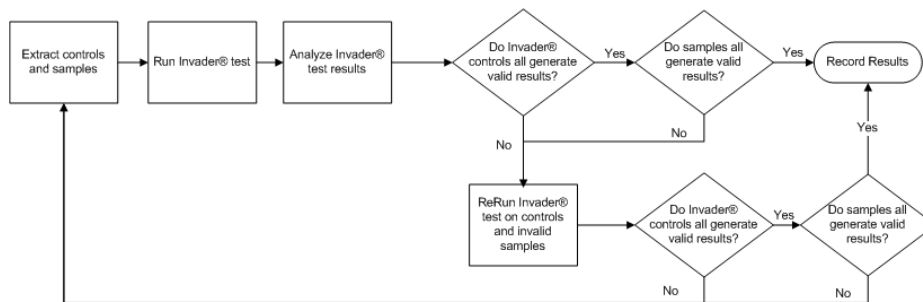


Figure 4: Recommended testing process for samples producing “Low Signal”, “EQ” or “Invalid” results with the Invader® Factor II test.

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VI. CONTACT INFORMATION

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Part Number 15-3216, Revision 102
2017-07