HOLOGIC®

INSTRUCTIONS FOR USE
Cleavase® X Enzyme Invader® Reagent Core Kit for Genomic DNA
Arm-Dye Code 1=FAM, 3= RED
Catalog No. 91-219

PRINCIPLES OF THE INVADER DNA ASSAY:
The Invader DNA Assays use Cleavase enzymes to recognize and cleave specific structures formed by the addition of two oligonucleotides to a nucleic acid target (Figure 1).

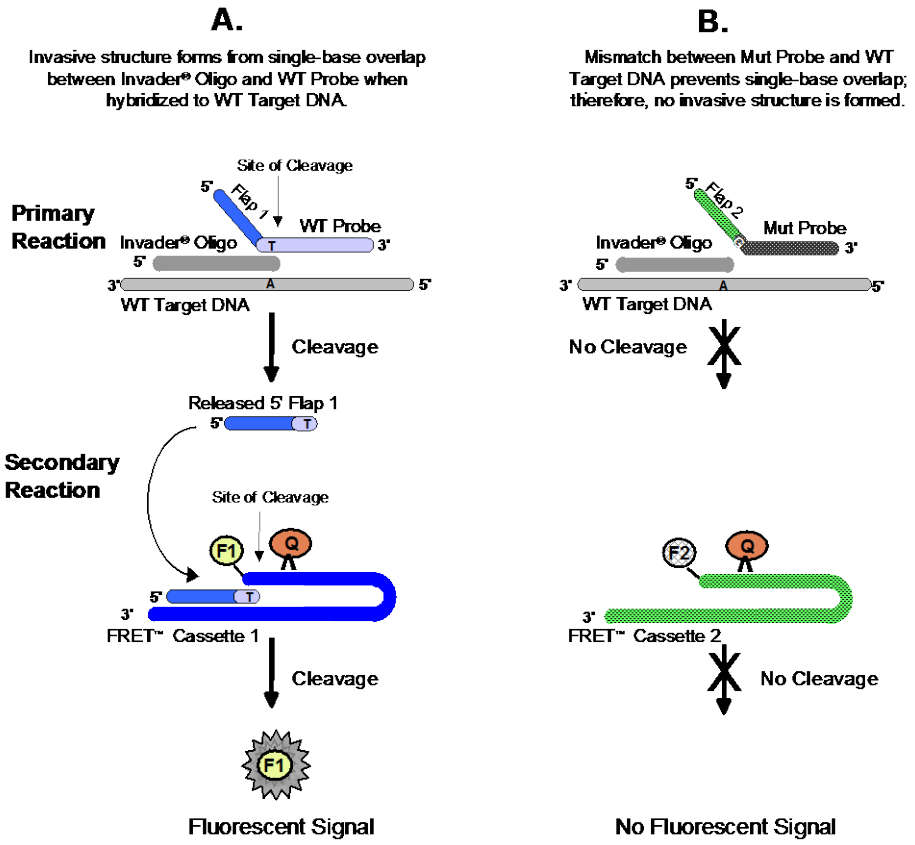

Figure 1.

Invader Reaction:
In the Invader DNA Assay, two oligonucleotides (a discriminatory Primary Probe and an Invader Oligo) hybridize in tandem to the target DNA to form an overlapping structure. The 5'-end of the Primary Probe includes a 5'-flap that does not hybridize to the target DNA (Figure 1). The 3'-nucleotide of the bound Invader Oligo overlaps the Primary Probe, but need 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 close to the reaction temperature. Thus, under the isothermal assay conditions, Primary Probes, which are provided in excess, cycle on the target DNA. This allows for multiple rounds of Primary Probe cleavage for each target DNA, and amplification of the number of released 5'-flaps.

In the secondary reaction, each released 5'-flap can serve as an Invader Oligo on a fluorescence resonance energy transfer FRET Cassette to create another overlapping structure that is recognized and cleaved by the Cleavase enzyme (Figure 1). When the FRET Cassette is cleaved, the fluorophore (F) and quencher (Q) are separated, generating detectable fluorescence signal. Similar to the initial reaction, the released 5'-flap and the FRET Cassette cycle, resulting in amplified fluorescence signal. The initial and secondary reactions run concurrently in the same well.

The biplex format of the Invader DNA Assay enables simultaneous detection of two DNA sequences in a single well. Most often, this involves detection of two variants of a particular polymorphism. The biplex format uses two different discriminatory Primary Probes, each with a unique 5'-flap, and two different FRET Cassettes, each 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.

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.hologic.com/sds.

FRET Mix (2 tubes, 1.1 ml each).
Dye Code Arm 1=FAM, Arm 3=RED
Cleavase X Enzyme - $MgCl_2$ Solution (1 tube, 500 µl)
No Target Blank (1 tube, 180 µl)

REAGENTS REQUIRED, BUT ORDERED SEPARATELY

Probe Mix (1.75 ml)
Assay-specific probes and Invader oligonucleotide to detect the two alleles of the polymorphism of interest.

STORAGE REQUIREMENTS

Component	Storage Requirement
Probe Mix	–15°C to –30°C
FRET Mix	–15°C to –30°C
Cleavase X Enzyme - $MgCl_2$ solution	–15°C to –30°C
No Target Blank	–15°C to –30°C

SUPPLIES AND EQUIPMENT REQUIRED, BUT NOT PROVIDED

Reagents:

Mineral oil for reaction overlay to prevent evaporation. Mineral oil produced by Sigma is recommended (catalog number M-3516 or M-5904). It is imperative that a high grade of mineral oil be used, because lower grades may interfere with fluorescence readings.

Consumable Supplies:

Sterile, DNase/RNase-free disposable aerosol barrier pipet tips
96-well or 384-well microplates
Sterile 0.6 ml or 1.7 ml microcentrifuge tubes
Sterile 8-tube strip or microplate (optional)
Disposable plastic trough (optional)
Plate sealing tape (optional)

Sample Preparation Kits:

The following commercially available DNA extraction kits are recommended for purifying DNA from whole blood, buffy coat, or cell lines:

QIAGEN QIAamp® Blood Kit
Gentra Systems PUREGENE® Kit
Gentra Systems GENERATION® Products

If using a DNA isolation/preparation method other than those recommended (or if modifying one of the recommended methods), verify that the yield of DNA is sufficient (**at least** 25 ng/µl) and consistent by using the PicoGreen® assay. If using Gentra Systems GENERATION products for genomic DNA preparation, the OliGreen® assay should be used for DNA quantitation as needed.

Equipment:

Thermal cycler **OR** oven capable of holding 63° ± 1°C.

Fluorescence plate reader (top reading; set for the appropriate excitation and emission wavelengths).

Miscellaneous laboratory equipment (tube racks, micropipetors, multichannel pipet, microcentrifuge, vortex mixer, plate centrifuge-for manual setup only).

PRECAUTIONS

1. Use only calibrated pipets, thermal cyclers, or ovens. Do not use heat blocks, because the microtiter plates will warp, producing unreliable results.
2. Use sterile disposable aerosol barrier pipet tips for each addition and transfer to avoid cross-contamination.
3. To help avoid contamination do not let the tips touch previously dispensed reagents.
4. Be sure the samples and reagents are mixed by vortexing thoroughly and consistently before use.
5. Follow the order of addition of reagents given in the **Invader DNA Assay Procedure** below.
6. Visually check the level (volume) of reagents in each plate well to verify that reagents and/or sample have been added as required.
7. Do not pool reagents from different lots or from different vials of the same lot.
8. Do not use a reagent after its expiration date.
9. Use freshly dispensed mineral oil for reaction overlay.
10. Do not transfer any reagents back to the original container once they have been dispensed.
11. Dispose of unused reagents and waste in accordance with applicable country, federal, state, and local regulations.
12. Before reading a plate, allow the lamp in the fluorescence plate reader to warm up for at least 10 minutes and verify that the plate is aligned properly in the fluorescence plate reader.

THE INVADER DNA ASSAY PROCEDURE – 96-WELL REACTION FORMAT

The following procedure applies to assays setup manually or with automated liquid handlers. For a specific automated liquid handler system, follow the manufacturer's recommendations.

1. Plan the microtiter plate assay layout. It is recommended to have duplicate No Target Blanks (see example below).
2. Experimental Plate Layout: (*fill in as appropriate and required*)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

An Example Layout Follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Allele 1 Control											
B	Allele 2 Control											
C	NTB											
D	NTB											
E												
F												
G												
H												

3. Denature the genomic DNA samples (**at least** 25 ng/µl) by incubating them at 95°C for 10 minutes. Allow the DNA to cool to room temperature before use. The cooling process can be accelerated by placing the samples on ice. Volumes greater than 150 µl may require longer incubation for complete denaturation.
4. **CAUTION**: If using a 96-well plate or 0.2-ml microtubes for denaturation, do not fill with more than 150 µl of the DNA sample, to help ensure that samples heat evenly. Cap each microtube or each filled well of the 96-well plate before heating. **Do not use adhesive sealing film on the 96-well plate.** Do not remove the caps until the DNA sample has reached room temperature.
5. Calculate and prepare the appropriate amount of Master Mix, as shown in the table below. We recommend the preparation of a master mix sufficient for testing of the complete set of samples.
   Note: It is recommended to prepare a fresh mix each time.

Master Mix Component	(A) Volume per reaction	(B) Number of reactions	(C) Multiplication factor *	(A) x (B) x (C) = Volume of reagent required
Probe Mix	3 µl		1.2x	
FRET Mix	3.5 µl		1.2x	
Cleavase X Enzyme - $MgCl_2$ Solution	1 µl		1.2x	
Total volume	7.5 µl		1.2x	

*** This multiplication factor allows for 20% overage. For automated liquid handlers, the amount of overage may vary, depending on the dead volume and overdraws. The actual number of reactions obtained from a tube will vary, based on the liquid handler used and the number of times each tube is used.**

6. Add 7.5 µl of the appropriate control(s) or sample DNA target at 25 ng/µl (approximately 180 ng of genomic DNA) as formatted in the experimental layout grid.
7. Following the actual experimental plate layout grid (see Step 1), pipette 7.5 µl of Master Mix into the appropriate wells of the 96-well plate.
8. Overlay each reaction with 15 µl of mineral oil.
9. At this point, it is optional to cover the plates with an adhesive cover and spin at 1000 rpm in a Beckman GS-15R centrifuge (or equivalent) for 10 seconds to force the probe and target into the bottom of the wells.
10. Incubate the plate(s) at 63°C for 4 hours in a thermal cycler or incubator such as the BioOven III. After 4 hours incubation at 63°C lower the temperature to 4°C if a thermal cycler is being used or to room temperature if an incubator is being used.
11. Analyze the microtiter plate(s) on a fluorescence plate reader using the appropriate parameters.

THE INVADER DNA ASSAY PROCEDURE – 384-WELL REACTION FORMAT

The following procedure applies to assays setup manually or with automated liquid handlers. For a specific automated liquid handler system, follow the manufacturer's recommendations.

1. Plan the microtiter plate assay layout. It is recommended to have duplicate No Target Blanks.
2. Experimental Plate Layout: (*fill in as appropriate and required*)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
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3. Denature the genomic DNA samples (**at least** 25 ng/µl) by incubating them at 95°C for 10 minutes. Allow the DNA to cool to room temperature before use. The cooling process can be accelerated by placing the samples on ice. Volumes greater than 150 µl may require longer incubation for complete denaturation.
4. **CAUTION**: If using a 96-well plate or 0.2-ml microtubes for denaturation, do not fill with more than 150 µl of the DNA sample, so that samples heat evenly. Cap each microtube or each filled well of the 96-well plate before heating. **Do not use adhesive sealing film on the 96-well plate.** Do not remove the caps until the DNA sample has reached room temperature.
5. Calculate and prepare the appropriate amount of Master Mix, as shown in the table below. We recommend the preparation of a master mix sufficient for testing of the complete set of samples.
   Note: It is recommended to prepare a fresh mix each time.

Master Mix Component	(A) Volume per reaction	(B) Number of reactions	(C) Multiplication factor *	(A) x (B) x (C) = Volume of reagent required
Probe Mix	1.2 µl		1.2x	
FRET Mix	1.4 µl		1.2x	
Cleavase X Enzyme - $MgCl_2$ Solution	0.4 µl		1.2x	
Total volume	3 µl		1.2x	

*** This multiplication factor allows for 20% overage. For automated liquid handlers, the amount of overage may vary, depending on the dead volume and overdraws. The actual number of reactions obtained from a tube will vary, based on the liquid handler used and the number of times each tube is used.**

6. Add 3 µl of the appropriate control(s) or sample DNA target at 25 ng/µl (approximately 75 ng of genomic DNA) as formatted in the experimental layout grid.
7. Following the actual experimental plate layout grid (see Step 1), pipette 3 µl of Master Mix into the appropriate wells of the 384-well plate.
8. Overlay each reaction with 6 µl of mineral oil.
9. At this point, it is optional to cover the plates with an adhesive cover and spin at 1000 rpm in a Beckman GS-15R centrifuge (or equivalent) for 10 seconds to force the probe and target into the bottom of the wells.
10. Incubate the plate(s) at 63°C for 4 hours in a thermal cycler or incubator such as the BioOven III. After 4 hours incubation at 63°C lower the temperature to 4°C if a thermal cycler is being used or to room temperature if an incubator is being used.
11. Analyze the microtiter plate(s) on a fluorescence plate reader using the appropriate parameters.

DETERMINE WHICH ALLELE IS REPORTED BY FAM OR RED

Referring to the arm-allele combination listed on the probe mix vial for each assay, and the arm-dye code of the Invader Reagent Core Kit, determine which allele is reported by FAM or RED dyes:

For example:

If, the probe mix vial label lists 1=C 3=T, and the arm-dye code is 1=FAM, 3=RED
Then, the FAM dye will report the C allele, and the RED dye will report the T allele.

CALCULATION OF FOLD OVER ZERO AND ALLELIC RATIOS

1. Determine Fold Over Zero (FOZ) values as follows:

   F Probe FOZ = (raw counts from F Probe for the sample) / (average raw counts from F Probe for the No Target Blank)

   R Probe FOZ = (raw counts from R Probe for the sample) / (average raw counts from R Probe for the No Target Blank)

2. Calculate the Net FOZ for each sample by subtracting 1 from the FOZ values calculated in step 1 above.

   Net FOZ for F Probe = F Probe FOZ -1

   Net FOZ for R Probe = R Probe FOZ -1

3. Calculate the Allelic Ratio for each sample using the two Net FOZ values.

$$\text{Allelic Ratio} = \frac{\text{Net FOZ for F Probe}}{\text{Net FOZ for R Probe}}$$

NOTE: In cases where the Net FOZ value is ≤ 0, set the value to 0.01 for the calculation of the Allelic Ratio.

RECOMMENDED INTERPRETATION CRITERIA FOR DETERMINATION OF SAMPLE GENOTYPES

Using the FOZ and the Allelic Ratio calculations, the results for each sample can be classified as:

Homozygous for F Allele;
Homozygous for R Allele;
Heterozygous;
Equivocal; or
Invalid.

Sample determinations are valid under the following two criteria. Samples not meeting both criteria are invalid and must be repeated.

1. The Allelic Ratio, (as calculated above), falls into the ranges in the chart below and
2. The appropriate FOZ values, for a given sample, meet the criteria identified in the chart below:

Allelic Ratio	FAM FOZ (F Allele)	Redmond Red FOZ (R Allele)	Interpretation of Results
≥4.0	≥1.6	≥0.01	Homozygous for F Allele
≤0.25	≥0.01	≥1.6	Homozygous for R Allele
≥0.4 to ≤2.5	≥1.3	≥1.3	Heterozygous
>2.5 to <4.0	(Invalid due to Allelic Ratio)	(Invalid due to Allelic Ratio)	Equivocal (EQ1)
>0.25 to <0.4	(Invalid due to Allelic Ratio)	(Invalid due to Allelic Ratio)	Equivocal (EQ2)

Each laboratory may wish to establish its own ranges for Allelic Ratios for interpreting results. When equivocal results are obtained, the sample should be re-tested in duplicate. If the sample still generates an equivocal result, the concentration of the DNA sample should be verified.

TROUBLESHOOTING GUIDE

Problem	Possible Solution
No Signal or Low FOZ Signal	**Sample Preparation:** • Insufficient DNA was used in the assay. The DNA sample should be quantitated using the PicoGreen or OliGreen assay. Only samples with a concentration of **at least** 25 ng/µl should be used in the Invader DNA Assay. • Quantitating by A_{260}/A_{280} may lead to an overestimation of the amount of DNA in the sample due to the presence of RNA contamination. • The DNA sample(s) may not have been completely denatured. Verify that the sample was denatured at the correct temperature and for an appropriate time (the amount of time required for denaturation will vary with sample volume). • The DNA sample preparation method used caused inhibition. A low A_{260}/A_{280} ratio (< 1.5) indicates that the sample contains excess protein. **Assay:** • The plate may not be oriented properly. Orient the plate so that well A-1 is in the upper left-hand corner. • The wrong Invader Reagent core kit is being used with the Probe Mix. Check to see that the proper Invader Reagent core kit is being used with the Probe Mix. • Mixing inconsistencies. Be sure the samples, reagents and Master Mix are mixed thoroughly and consistently. Make sure that all liquid is expelled from the tip during additions. • Dispensing accuracy. For the manual format, be sure the plates have been spun down after the addition of Master Mix. For the automated format, be sure that the tip height is properly adjusted, so that solutions are delivered close to the bottom of the wells. To avoid contamination do not let the tips touch previously dispensed reagents. Verify that the correct volume of each reagent was added and that the reagents were added in the correct sequence. • DNA and/or the Master Mix may not have been added. Verify that your pipet is functioning correctly. For the automated format, verify that you have sufficient volume in the reservoir for the automated liquid handler. • The correct volume of mineral oil may not have been added. Verify that the correct volume of mineral oil was added. Lower volumes may result in sample evaporation. • High variation in the No Target Blank duplicates may lead to low FOZ signal. Repeat run. • The reagents were not stored properly. Verify that all reagents are stored at the proper temperature, as indicated in this package insert. Avoid subjecting reagents to repeated freeze/thaw cycles. • No Target Blank well(s) was/were contaminated with sample or exogenous nucleases. Take appropriate steps to avoid cross-contamination. The pipet tips may have been contaminated. Make sure that pipet tips do not touch any surfaces other than the solution being pipetted, because nucleases may be present. Do not touch pipet tips with hands. • The plate may not have been incubated at 63°C for 4 hours. Confirm that the plate was incubated properly. **Instrument:** • The plate was not oriented properly during assay setup and/or plate reading. Orient the plate so that well A-1 is in the upper left-hand corner. • The gain settings were not properly adjusted. Adjust the gain of the fluorescence plate reader so that you are in the linear dynamic range of the respective reader. • The lamp was not turned on long enough before reading the plate. Allow the lamp in the fluorescence plate reader to warm up for at least 10 minutes before reading the plate. • The incorrect plate coordinates were used when the plate was read. Make sure the correct plate coordinates have been used, for the particular plate type being used. The probe should be set to read in the middle of the well and at an optimal distance from the plate. Consult the instrument user manual for detailed instructions. • The temperature was not maintained at 63° ± 1°C. Incubations should be conducted in properly calibrated heating units. Checking these units on a regular basis using a thermocouple thermometer equipped with a probe traceable to NIST standards is recommended. • Make sure that the plate is firmly seated in the thermal cycler. • The plate was read at the incorrect excitation/emission wavelengths. Verify that the correct filters are installed or settings programmed properly for the respective fluorescence plate reader being used. If possible, run a diagnostic test on the fluorescence plate reader to ensure that the instrument and light source are working properly. Verify that two scans were performed at the recommended wavelengths.
High Signal in the No Target Blank	**Assay:** • **Use DNase/RNase free aerosol barrier tips and sterile tubes for making the Master Mix.** • Wear gloves when setting up the assay. • Make sure that pipet tips do not touch any other surfaces except the solution being pipetted, since nucleases may be present. • Do not touch pipet tips with hands.

TROUBLESHOOTING GUIDE - CONTINUED

Problem	Possible Solution
Fluorescent Signal Is Off-scale	**Assay:** • **Use DNase/RNase free aerosol barrier tips and sterile tubes for making the Master Mix.** • Confirm that the incubations were done for the correct amount of time and at the correct temperature. **Instrument:** • Adjust the gain of the fluorescence plate reader so that you are in the linear dynamic range of the respective reader. • Allow the lamp in the fluorescence plate reader to warm up for at least 10 minutes before reading the results.
The No Target Blank Values for the F dye and R dye Are Not the Same.	**Instrument:** • The No Target Blank values for the F and R dye scans will vary based on the gain setting and from assay to assay. It is not uncommon for the No Target Blank values to be different for the F and R dye scans.
The Net FOZ Value from Genomic Samples Is Less Than 0.	**Assay:** • Genomic samples can produce a Net FOZ value less than 0 for the non-reactive probe. This may be due to differences in the matrix of the sample compared to the matrix of the No Target Blank. **NOTE: In cases where the Net FOZ value is ≤ 0, set the value to 0.01 for the calculation of the Allelic Ratio.**
The Allelic Ratio of Genomic Sample Is Close to the Limit of the Recommended Value or Equivocal.	**Assay:** • The Allelic Ratios provided should only be used as a guideline. • Check the concentration of DNA using the PicoGreen or OliGreen assay since low FOZ can lead to ratios near the limits or to Equivocal results.

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Part Number 15-1515, revision 101