

HOLOGIC[®]

INVADER[®] DNA ASSAY PROCEDURE

Cleavase[®] X Invader[®] Reagent Core Kit for Genomic DNA

Fluorescence Resonance Energy Transfer (FRET) Detection Format

Arm Dye Code 1=FAM, 3=RED

Catalogue No. 91-291

PRINCIPLES OF THE INVADER ASSAY FOR SNP DETECTION:

In the Invader assay, a specific "invading" oligonucleotide, upstream from the cleavage site, and a partially overlapping downstream probe form a specific structure when bound to a complementary DNA template (Figure 1). This structure is recognized and cut at a specific site by Cleavase enzymes, resulting in release of the 5' arm of the probe oligonucleotide. This fragment then serves as the "Invader" oligonucleotide in a secondary reaction, resulting in specific cleavage of the signal probe by Cleavase enzymes. Fluorescence signal is generated when the signal probe, labeled with dye molecules capable of fluorescence resonance energy transfer, is cleaved. When the targeted nucleotide is not present, cleavage does not occur, the probe arm is not released, and a fluorescent signal is not generated. The signal generated by the wild-type specific signal probe is related to the signal generated by the mutant specific signal probe, thus allowing a genotype call to be made.

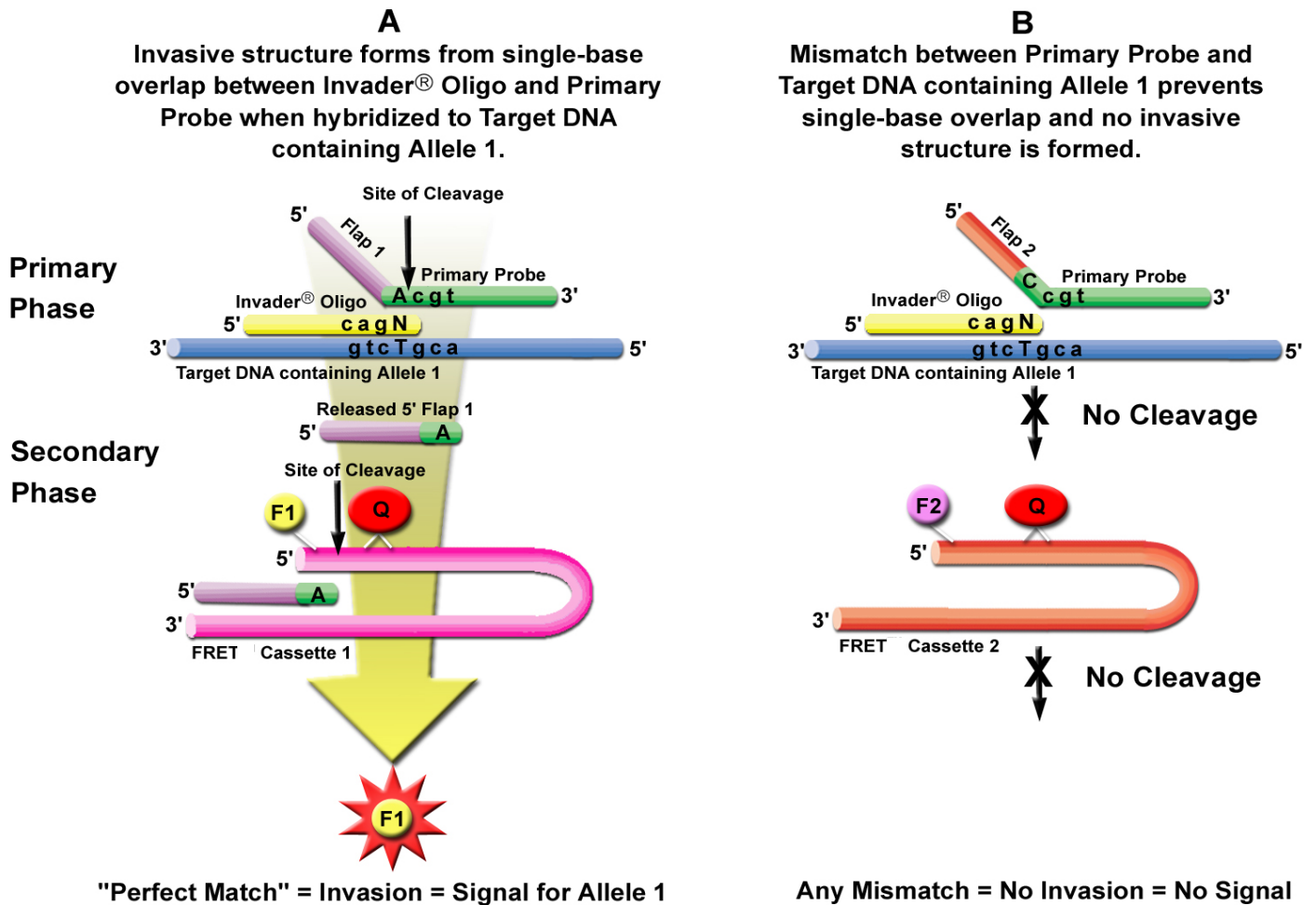


Figure 1.

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 (1 bottle, 21.0 ml).

⌘ = Arm-Dye Code Arm 1=FAM, Arm 3=RED

Note: The ⌘ symbol indicates the Arm-Dye Code. This kit can only be used with Probe Mix tubes having the corresponding symbol.

Cleavase X Enzyme/MgCl₂ Solution (1 bottle, 6.0 ml)

No Target Blank (NTB) (1 bottle, 2.8 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.

Reagent Storage:

Store all liquid reagents at -20°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)

Genomic DNA Sample preparation:

There are a number of commercially available kits for DNA extraction from plants; the following kits have been used successfully:

Nucleon™ Phytopure™ Kit (Amersham Life Science, Buckinghamshire, England, Cat. No. RPN 8510)

Gentra Puregene™ Kit (Gentra Systems, Minneapolis, MN, Cat. No. D-7000A)

QIAGEN Dneasy™ Plant mini kit (QIAGEN, Inc., Valencia, CA, Cat. No. 69103)

INSTRUMENTS REQUIRED, BUT NOT PROVIDED:

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).

DNA QUANTITATION:

The quantitation of DNA is recommended to determine the optimum sample type and DNA extraction method to ensure an appropriate quantity of DNA is used for testing. Once an acceptable sample preparation method has been established, it is no longer necessary to routinely perform DNA quantitation. Various commercially available or in-house methods can be employed for DNA quantitation. The following is a recommended quantitation method using the PicoGreen® DNA Quantitation Kit (Molecular Probes, Eugene, OR, Cat. No.: P-11495).

1. Prepare 1X TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7) from 20X stock which is supplied in the kit. To make 50 ml, add 2.5 ml of 20X TE to 47.5 ml sterile, distilled, DNase free water. 50 ml is sufficient for 250 assays.
2. Dilute the DNA standard from 100 µg/ml to 2 µg/ml with 1X TE. For each standard curve, prepare 175 µl of a 2 µg/ml stock by adding 3.5 µl of the 100 µg/ml stock to 171.5 µl 1X TE.
3. Prepare the standard curves in the microtiter plate as shown in this table. Add the DNA solution to the 1X TE buffer in the microplate well.

Final [DNA] (ng/well)	Vol. (µl) 2 µg/ml DNA Standard	Vol. (µl) 1X TE Buffer
0	0	100
5	2.5	97.5
10	5	95
20	10	90
40	20	80
60	30	70
80	40	60
100	50	50

4. For each unknown, add 2 µl of sample to 98 µl of 1X TE in the microplate well. Mix by pipetting up and down.
5. Prepare a 1:200 dilution of the PicoGreen reagent in 1X TE. For each standard and each unknown sample, a volume of 100 µl will be needed. For example, 2 standard curves with 8 points each will require 1.6 ml. To calculate the total volume of diluted PicoGreen reagent needed, determine the total number of samples and unknowns you will be testing and multiply this number by 100 µl (if using a multichannel pipet, make extra reagent). The PicoGreen reagent is light sensitive and should be kept wrapped in foil while thawing and in the diluted state. Vortex well.
6. Add 100 µl of diluted PicoGreen to every standard and sample. Mix by pipetting up and down.
7. Cover the microplate with foil and allow to incubate at room temperature for 2–5 minutes. While the plate is incubating, power up the plate reader and establish the following settings:

	<u>Wavelength/Bandwidth</u>
Excitation:	~485nm/20nm
Emission:	~530nm/25nm

To ensure that the sample readings remain in the detection range of the fluorometer, the instrument's gain should be set so that the sample containing the highest DNA concentration yields a fluorescence intensity near the fluorometer's maximum.

8. Read the plate.
9. Generate a standard curve and determine the concentration of DNA in the unknown samples.

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 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 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																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
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P																								

3. Denature the genomic DNA samples 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 as formatted in the experimental layout grid.

7. Following the actual experimental plate layout grid (see Step 1), pipette 3 μ l of Master Mix into the appropriate wells of the 384-well plate.
8. Overlay each reaction with 6 μ l 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 following parameters:

		<u>Wavelength/Bandwidth</u>
RED dye:	Excitation:	~560nm/20nm
	Emission:	~620nm/40nm
FAM dye:	Excitation:	~485nm/20nm
	Emission:	~530nm/25nm

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

TROUBLESHOOTING GUIDE:

Problem	Possible Solutions
No Signal or Low Signal for Standards and Samples	<p>Assay:</p> <ul style="list-style-type: none"> Mixing inconsistencies. Be sure the samples and probes are mixed thoroughly and consistently. This can be done by pipetting up and down several times as you add reagents, samples, and probe mixes. Verify that all reagents were added in the correct sequence. Verify that all reagents were stored at the proper temperature, indicated in the package insert. Check that the components match the list in the package insert. <p>Instrument:</p> <ul style="list-style-type: none"> Adjust the gain of the fluorescence plate reader. The gain or sensitivity should be set to maximize signal and minimize the variation. Make sure the proper "96-well plate type" has been selected in the fluorescence reader. Verify that the coordinates of the plate are programmed correctly in the fluorescence instrument. Signal should be read in the middle of the well and at an optimal distance from the plate for best results. Incubations should be conducted in properly calibrated heating units. Use Mineral oil to prevent evaporation of sample.
No signal or Low Signal for Samples only	<p>Sample Prep:</p> <ul style="list-style-type: none"> Not enough DNA was used in the assay. The DNA sample should be quantitated using the PicoGreen Assay. PicoGreen can underestimate the amount of DNA if proteins are present. Contact Molecular Probes for further assistance. Quantitating by A_{260}/A_{280} can lead to an overestimation of the amount of DNA in the sample due to RNA contamination. A low A_{260}/A_{280} reading (<1.5) indicates there is an overabundance of protein in the sample. <p>Assay:</p> <ul style="list-style-type: none"> Mixing inconsistencies. Be sure the samples and probes are mixed thoroughly and consistently. This can be done by pipetting up and down several times as you add the sample or the probe mix. <p>Instrument:</p> <ul style="list-style-type: none"> Verify that the reader is set to the correct excitation and emission. If possible, run a diagnostic test on the machine to ensure that the instrument and light source are working properly.
High Signal in No Target Controls	<p>Assay:</p> <ul style="list-style-type: none"> Make sure that pipet tips are changed after each use. 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.
Fluorescent Signal is Offscale	<p>Assay/Instrument:</p> <ul style="list-style-type: none"> Lower the gain of the fluorescence plate reader. Confirm that the incubations were done for the correct amount of time.
Variable Readings	<p>Assay:</p> <ul style="list-style-type: none"> Mix well after adding each sample, and when adding Signal Probe Mix by pipetting up and down several times. Careful pipetting is essential for reducing variation in the assay. Use only <u>calibrated pipettes</u>. It is important that drops of liquid are not present on the outside of the tip, especially when dispensing small volumes. Multi-channel pipetors will reduce time, but should be used with special care. Be sure that all tips are on securely. Carefully inspect each tip when pipetting to ensure all tips draw and dispense the proper volume. Verify that tips are changed between samples and reagents. <p>Instrument:</p> <ul style="list-style-type: none"> Verify that the reader is set to the correct excitation and emission. If possible, run a diagnostic test on the machine to ensure that the instrument and light source are working properly.
Controls Yield Incorrect Genotype	<p>Assay:</p> <ul style="list-style-type: none"> Controls mixed up or contaminated.
Low Signal for One Sample - Significantly Below Background	<p>Assay:</p> <ul style="list-style-type: none"> Signal Probe Mix was not added to the sample.

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