

High-Throughput Genotyping Using the Invader® Assay

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Abstract

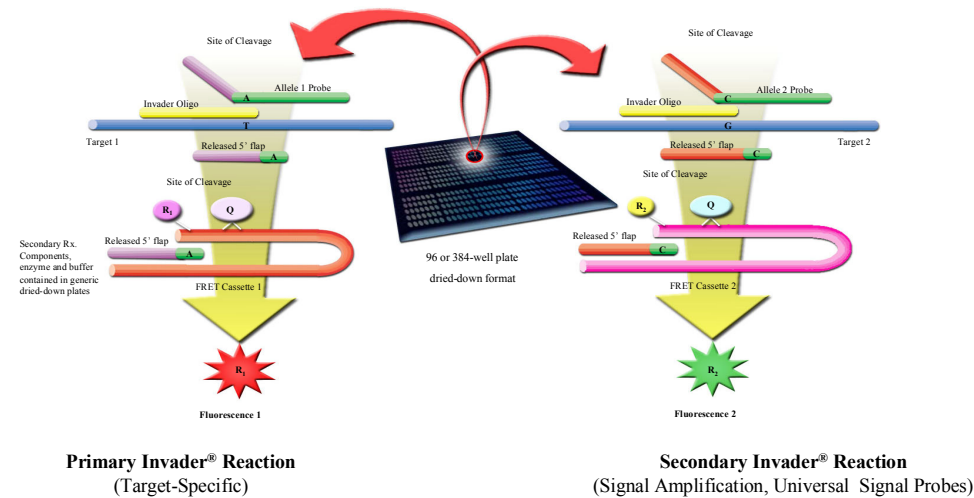
The opportunity for understanding disease and improving patient care is accelerating with the rapid growth of sequence-specific knowledge of the human genome. SNP discovery and disease association studies are important steps in achieving the goal of personalized medicine. Combining the Invader® Assay with high-throughput robotic platforms brings this frontier of medicine closer to reality.

The Invader® Assay is based on the specificity of the Cleavase® enzyme, which recognizes and cleaves the structure formed when two allele-specific oligonucleotides hybridize to a target sequence with a region of overlap. This reaction is biphased and signal detection is based on Fluorescence Resonance Energy Transfer (FRET). The Invader® Assay can be run directly on genomic DNA samples, or the Invader® Assay can be combined with multiplex PCR, allowing for the determination of 100+ genotypes from a limited amount of sample.

The simplicity of the assay lends itself to automation. Results will be presented from mid to high-throughput systems. Miniaturization efforts have led to the development of an ultra high-throughput platform, which has the added benefit of a direct genomic assay requiring only nanogram to sub-nanogram amounts of DNA.

Invader® FRET Assay - Biplex SNP Detection

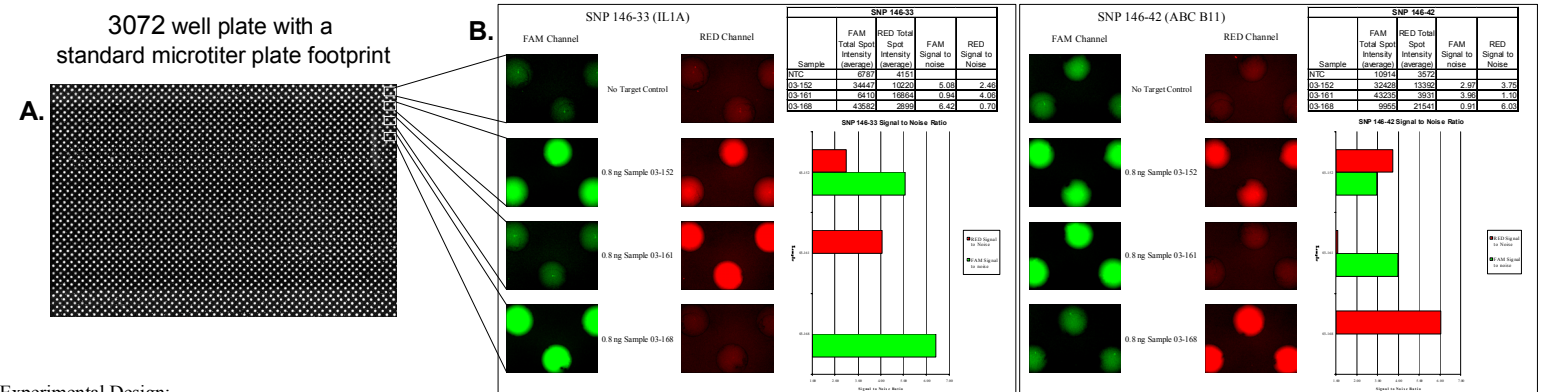
The Invader® Assay is a homogenous single tube assay where two specific Invader® reactions occur simultaneously. Target detection and allele discrimination takes place in the primary Invader® reaction, while signal amplification takes place in the secondary Invader® reaction using a set of universal signal probes. The entire assay is isothermal and simple to set up.



The primary Invader® reaction detects the presence of a specific target sequence. The two target specific oligonucleotides, the Invader® oligo and the allele specific probe, hybridize directly to genomic DNA and form an overlapping complex. The Cleavase® enzyme recognizes this structure and cleaves off the 5' non-complementary sequence, or "flap", of the allele specific probe. The reaction temperature is near the melting temperature of the allele specific probe, which is present in excess. After cleavage, the allele specific probe dissociates from the target and is replaced by another uncleaved allele specific probe. The generation of cleaved flap sequences is linear with respect to time and target level, with a rate of ~1000 cleaved flaps generated per hour per target molecule. In the case of a multiplex SNP assay, the two allele specific probes have different 5' flap sequences.

The secondary Invader® reaction amplifies signal via fluorescence resonance energy transfer (FRET). Each signal probe has a hairpin structure with a 3' overhanging end. A fluorescent dye is attached to the 5' end of the signal probe, and a quenching dye is located nearby. The cleaved 5' flap sequences from the primary reaction act as an Invader® oligo in the secondary reaction. The Cleavase® enzyme cleaves off the 5' reporter dye, releasing it from the nearby quencher. The reaction temperature is near the melting temperature of each flap sequence. After the cleavage event, the 5' flap sequence dissociates from the cleaved signal probe and hybridizes to another signal probe. The generation of fluorescent signal is linear with time, with a rate of ~1000 reporter dyes released per hour per 5' flap sequence.

Direct Detection of Less than 1 ng of Genomic DNA in a High Density Array

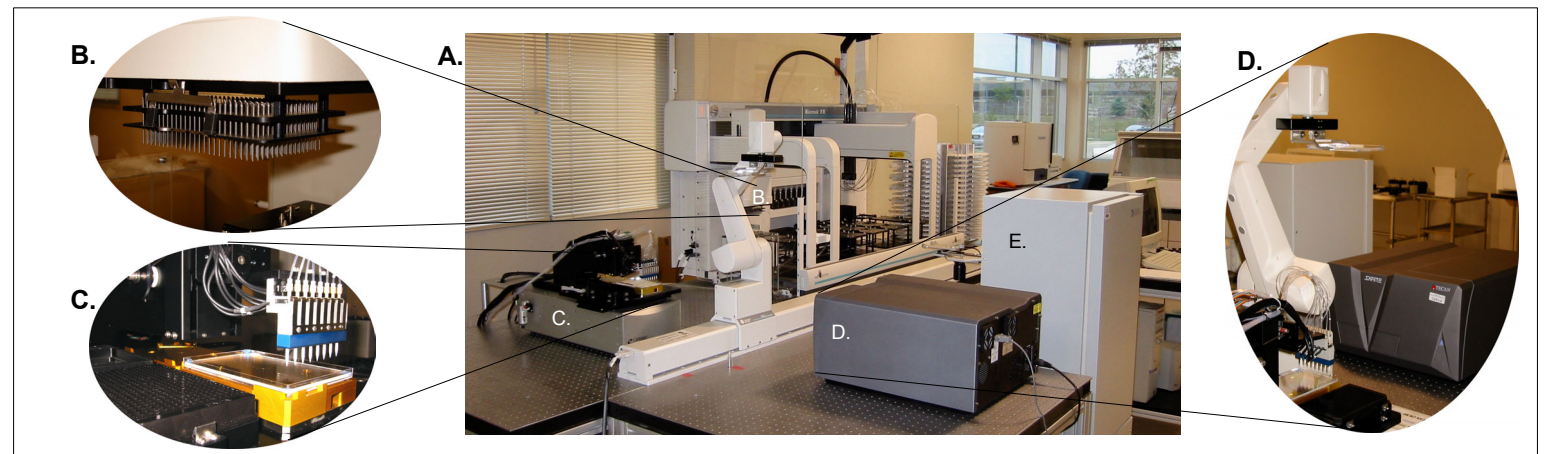


Experimental Design:

A. 3072 plate (48 columns by 64 rows) created using the Cartesian Pipetting platform. In order to illustrate a 3072 plate, 40 nl of 1 uM fluorescein was pipetted into each well and visualized using an AlphaArray™ 7000 from Alpha Innotech, Inc.. Average well diameter is 1 mm.

B. Two different SNP assays were set up on a 3072 plate. The plate was set up with four rows of a no target control interspersed with four rows of the above indicated sample. 40 nl of each were added per well. Samples were at a concentration of 20 ng/ul and were pre-denatured before addition. This material was allowed to come to dryness, taking approximately 30 seconds. Plates were overlaid with mineral oil as an evaporative barrier and 40 nl of probe/invader/FRET/enzyme mix was dispensed through the oil layer to the well below using the Cartesian platform. Reactions were incubated at 63°C for three hours. Clusters of wells were visualized on a fluorescence microscope (Nikon, Inc., Labphot 2®, 4X objective, with a Q Imaging™ Retiga EX™ CCD camera. The grayscale images were colorized using ImageQuant™ software from Molecular Dynamics, Inc.). Numerical values were obtained using a conventional fluorescence plate reader (Tecan SAFIRE) and were averaged across all related wells.

Automation of Nanoliter Volume, High Density Invader® Assays



Automation: This photo shows the nanoliter volume, high density platform as it is currently being configured at Third Wave Technologies. Throughput: 400,000 genotypes per day.

- A. Beckman SAGIAN™ CORE system: Biomek® FX dual bridge unit with Span-8 and multi-channel heads using an ORCA on a 3 meter rail.
- B. 384-well pin tool from V&P Scientific, Inc. with floating, slotted pins. Used to create 3072 pattern and to spot target samples.
- C. Cartesian Technologies, Inc., PreSys™4040C with 8 channel dispense head. Capable of rapid, nanoliter pipetting. Dispenses Invader® reagents directly through mineral oil onto 3072 plate.
- D. Tecan SAFIRE fluorescence plate reader with tunable monochromator. Capable of reading a 3072 plate, bi-plexed, in approximately 10 minutes.
- E. LiCONic StoreX40, high temperature, automated incubator.

Summary

Third Wave has developed a miniaturized platform for the Invader® Assay that requires less than 1 ng of human genomic DNA per bi-plexed reaction. This platform has 3072 wells in a microtiter plate footprint. The reactions can be detected using a standard fluorescence plate reader. Reactions can be set-up, incubated and read using readily available automation systems.

This system will be particularly useful for situations with limiting amounts of DNA and for cases where screening of large numbers of samples is desired.