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Abstract

The Invader® DNA Assay is a homogeneous, isothermal reaction based on signal, not target, amplification, using a FRET-based detection method. The Invader® product platform is evaluated for qualitative and quantitative, high-throughput genomic analysis in plants. As model systems for this study, a single nucleotide polymorphism (SNP) detection, as well as a transgene copy number determination assay was developed. The SNP assay detects a genetic marker linked to an agronomic trait in tomato, and the copy number determination assay targets a transgenic sequence in lettuce. Both assays are configured in biplex format, which allows simultaneous detection of two target sequences, two SNP alleles or a transgene and an internal reference sequence, in a single well of a 96 or 384-well microtiter plate, using standard fluorescence plate readers. Our results indicate that, while both formats give identical genotype results, the 384-well format is more sensitive, requiring either a shorter incubation time, or the addition of less sample DNA per reaction. We will also describe the analytical validity of the Invader® DNA Assay for SNP genotyping and transgene copy number determination, comparing the Invader® product platform to conventional methods (e.g. Cleaved Amplified Polymorphic Sequence (CAPS) analysis versus SNP genotyping using the Invader® DNA Assay). These results indicate that the Invader® product platform increases sample throughput in a cost effective manner, enabling the researcher to screen plants for research and breeding effectively and accurately.

Introduction

As the interest in SNP-genotyping and transgene detection and copy number determination in plant and animal genetics grows, there is an increased need for accurate and cost-effective detection platforms. Available PCR-based methods can be labor-intensive and may be prone to target contamination, leading to false positive or equivocal results. The Invader product platform described here provides a highly accurate alternative to PCR-based methods. It relies on signal, not target, amplification, virtually eliminating the risk of cross-contamination. It was successfully employed for SNP detection in tomato and transgene copy number determination in lettuce.

In the Invader® DNA Assay an oligonucleotide hybridizes upstream of the cleavage site to the target sequence. The 3'-most base of the Invader® oligonucleotide overlaps a downstream hybridizing primary probe. This structure is recognized by the Cleavase® enzyme and the primary probe is cleaved, releasing a 5' flap sequence. This 5' flap then acts as an invading oligonucleotide in the secondary reaction, hybridizing to the FRET™ cassette, hybridizing to the FRET™ cassette. When this hybridization occurs the Cleavase® enzyme recognizes the structure and cleaves the FRET™ cassette, releasing the fluorophore from the quencher (see Fig. 1). In the biplex format the two FRET™ cassettes in the reaction release spectrally distinct reporter fluorophores. The resulting increase in fluorescence is directly proportional to the amount of target in the reaction and can be measured on a fluorescence plate reader. For SNP detection, signal from only one fluorophore indicates a homozygote for a specific allele while signal from both fluorophores indicates a heterozygous genotype. For copy number determination, one probe set is designed to detect an endogenous sequence serving as an internal control, while the other probe set is designed to detect the transgene. Calculating the ratio between the transgene and the internal control probe signals allows for a quantification of the transgenic sequence to be made.

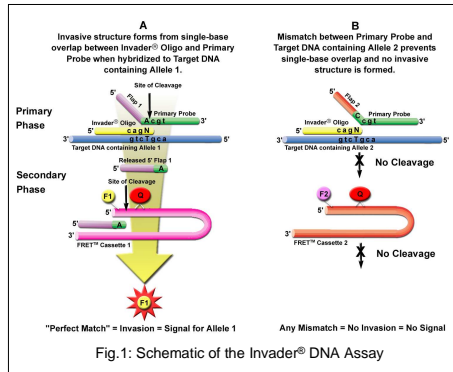


Fig. 1: Schematic of the Invader® DNA Assay

Material and Methods

Invader® DNA Assay reaction conditions:

The Invader® SNP Assay reactions were carried out on 70–150 ng of tomato genomic DNA in 96-well format or 30–65 ng in 384-well format. The Invader® copy number assay used 80 ng of lettuce genomic DNA. All reactions were carried out in biplex format at 63° C.

Sample preparation:

Genomic DNA was extracted from tomato and lettuce seedlings using a CTAB isolation method adapted from Murray and Thompson (1980). DNA samples were quantitated by PicoGreen® (Molecular Probes, Eugene, Oregon) according to manufacturer's protocol.

PCR based methods:

A tomato trait linked marker was amplified by PCR. After restriction enzyme digestion, fragments were resolved on an agarose gel. The *npfl* marker gene was amplified from lettuce genomic DNA using Taq polymerase (Roche Diagnostics, Indianapolis, Indiana).

References

Murray, H.G., and Thompson W.F., 1980. Rapid isolation of high molecular weight DNA. *Nucleic Acids Res.* 8: 4321-4325.

Results

1. SNP detection in tomato DNA

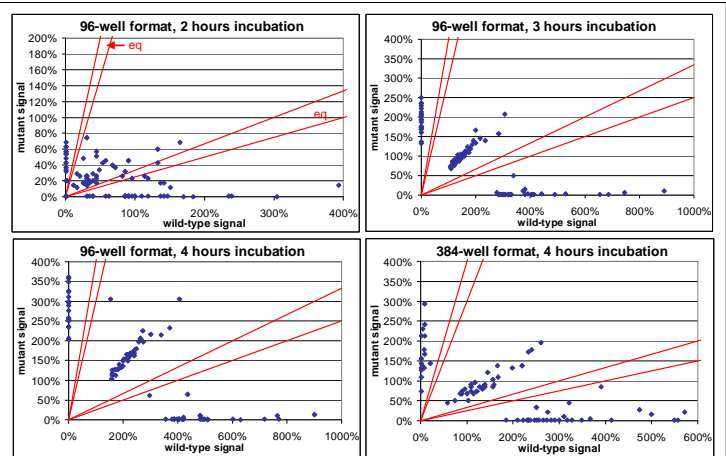


Fig. 2: SNP detection in tomato DNA. 96-well data from 2, 3, and 4-hour incubation times, and 384-well data from a 4-hour incubation time. Wild-type and mutant signals are graphed. Equivocal zones are indicated by "eq" in upper left graph. Data in equivocal zones give no clear genotype call. In the 96-well format the amount of DNA varied from 70–150 ng per reaction; in the 384-well format the amount of DNA varied from 30–65 ng per reaction. From 3 hours onward, clear genotype calls can be made.

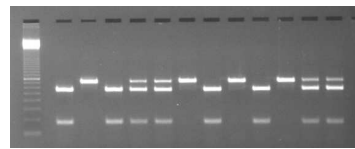


Fig. 3: CAPS analysis of tomato plants showing all three genotypes.

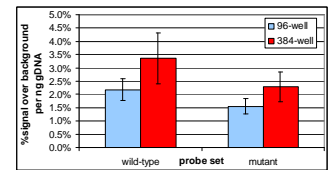


Fig. 4: Comparison of signal generation per ng of DNA between 96 and 384-well formats

2. *npfl* copy number determination and GUS histochemical analysis in transgenic lettuce:

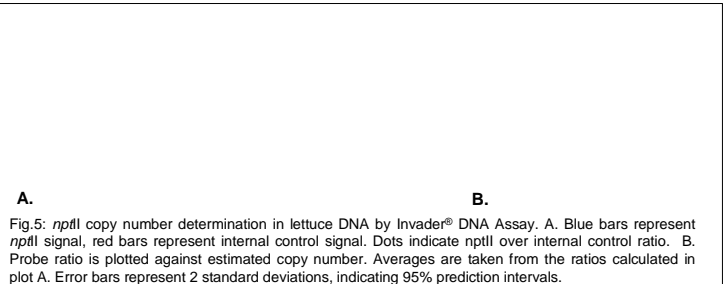


Fig. 5: *npfl* copy number determination in lettuce DNA by Invader® DNA Assay. A. Blue bars represent *npfl* signal, red bars represent internal control signal. Dots indicate npfl over internal control ratio. B. Probe ratio is plotted against estimated copy number. Averages are taken from the ratios calculated in plot A. Error bars represent 2 standard deviations, indicating 95% prediction intervals.

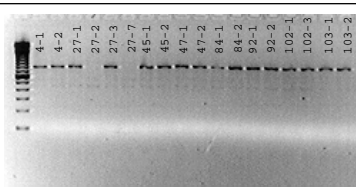


Fig. 6: PCR amplicons of *npfl* transgene resolved by agarose gel electrophoresis

Table 1: Summary of Invader™ *npfl* transgene copy number results of T1 plants and GUS histochemical analysis of T2 populations of a subset of T1 plants

T1 code	Copy # as determined by Invader	GUS activity (leaf assay)	# GUS (+) plants in T2
4-1	>10	-	0/30
4-2	>10	-	n.d.
27-1	1	+	n.d.
27-2	0	-	n.d.
27-3	1	+	n.d.
27-7	0	-	n.d.
45-1	1	+	22/30
45-2	1	+	n.d.
47-1	1	-	0/30
47-2	1	-	n.d.
84-1	1	+	n.d.
84-2	2	+	30/30
92-1	2	-	0/30
92-2	1	-	n.d.
102-1	1	+	21/30
102-3	1	+	n.d.
103-1	1	+	23/30
103-2	1	+	n.d.

Conclusions

The Invader® DNA Assay was successfully used for SNP genotyping purposes in tomato as well as copy number determination of transgenic sequences in lettuce. The Invader® DNA Assay results were consistent between 96- and 384-well formats and 100% concordant with PCR-based methods. The 384-well format proved to be more sensitive than the 96-well format, either reducing the required incubation time or the amount of sample DNA, resulting in an increased sample testing throughput. Calculated for the tomato SNP Assay the signal produced per nanogram of genomic DNA increased by as much as 100% with an average of 53% using the 384-well format. Both the 96- and the 384-well format gave consistent results over at least a 2–3 fold target concentration range, eliminating the need for precise target quantification. The Invader® DNA Assay proved to be a valuable alternative to PCR-based methods, increasing accurate sample testing throughput in a cost-effective manner.