Application Note

High Throughput SNP Detection in Soybeans

High Throughput SNP Detection in Soybean Employing Melting Curve Analysis on a LightCycler 480 and a Nanodrop Express Low-Volume Dispenser

Jim Johnson, Mary Cornett and Dave Hunter
Innovadyne

Introduction

The Innovadyne Nanodrop platform has been used extensively for applications in Drug Discovery including High Throughput Screening and Assay Development. There are an increasing number of other applications requiring similar capabilities and throughput as those in Drug Discovery. This report focuses on a genomic application in which the Nanodrop technology has proven to be an efficient, cost effective tool in the preparation of PCR reactions for SNP detection in soybeans employing Melting Curve Analysis.

Melting Curve Analysis is used in the detection of single nucleotide polymorphisms (SNP). Melting curve analysis, a technology patented by Roche Applied Science, relies on the addition of fluorescently labeled, sequence-specific oligonucleotide probes during PCR setup. After PCR, a "melting curve" is generated by slowly heating the amplicon/probe heteroduplex and measuring the dramatic changes in fluorescence that result when the probe denatures, or "melts," away from the amplicon. Melting curve analysis exploits the fact that even a single mismatch between the labeled probe and the amplicon will significantly reduce the melting temperature. Thus, probe/amplicon heteroduplexes containing destabilizing mismatches, such as the SNP, melt off at lower temperatures than probes bound to a perfectly matched target DNA. Through the use of a real time PCR system, such as the Roche LightCycler 480, the resulting fluorescence due to the melting point of an amplified reaction can be continuously measured as the temperature of the thermocycler is increased. Melting curves of a population are then compared to known melting points of a target SNP via software manipulation, and a call is made for each reaction.

Figure 1. Innovadyne Nanodrop Express

Methods and Materials

Eight sets of 94 F2 soybean plants were tested for resistance or susceptibility to Mi (Meloidogyne incognita or southern root-knot nematode). These plants were from the cross of G03-4332 and one of the several resistant soybean strains. Genomic DNA was extracted from individual parental genotypes and individual field-grown F2 plants from seed tissue. PCR primers and probes were designed with the LightCycler Probe Design Software (Roche Diagnostics, Indianapolis, IN, USA). Tests were performed using a SimpleProbe for SNP199 labeled with fluorescein at the 5′ end and blocked with phosphate at the 3′-end. For actual probe and primer sequences please refer to reference 1. Each set was done in one quadrant of a 384-well plate for a total of two plates (four sets per plate) with a total reaction volume of 3 μL per well. Extracted template, primers and probes were dispensed with the Innovadyne Nanodrop Express 16-channel dispenser (see Figure 1).

SNP genotyping, using fluorescent melting curve analysis of these hybrid species, was performed in the LightCycler 480 system (see Figure 2).

Figure 2. Roche LightCycler® 480

The PCR reaction mix for the SimpleProbe assay consisted of 20-30 ng of genomic DNA, 1 µM of SNP199 forward primer, 0.5 µM of SNP199 reverse primer, 0.2 µM of SimpleProbe, 1.0 mM MgCl2, and 0.5x of LightCycler 480 Genotyping Master mix. After initial denaturation of 5 min at 95 °C, 45 PCR cycles were performed with 10 s of denaturation at 95 °C, 15 s of annealing at 55 °C, and 20 s extension at 72 °C. A final melting cycle was performed by raising the temperature to 95 °C for 2 min, lowering the temperature to 40 °C for 2 min and increasing the temperature to 85 °C with continuous fluorescent acquisition followed by a cool down to 40 °C.

The fluorescent signal (F) was plotted in real time against temperature (T) to produce melting curves for each sample. Melting curves were then converted into negative derivative curves of fluorescence with respect to temperature (-dF/dT) by the LightCycler Data Analysis software (Roche Diagnostics, Indianapolis, IN, USA). See Figure 3 for an example of melting curves. The software then groups similar melting curves and automatically calls genotypes based on melting standards for known genotypes in the experiment or software-defined melting standards.

Results and Discussion

Because the 'melting point' of the PCR is related to the degree to which the DNA sequences are matched, plants having a higher Tm (65.24 °C) are complementary with respect to the susceptible DNA sequence while plants with the lower Tm (60.94 °C) are determined to be resistant. Plants exhibiting both peaks are labeled heterozygous. Up to 288 plants from each resistant parent plant were tested to see if the crossbred plants contained the SNP199 sequence known to confer resistance to the root-knot nematode. Results from the melting curve analysis can be seen in Figure 4.

SNP Marker

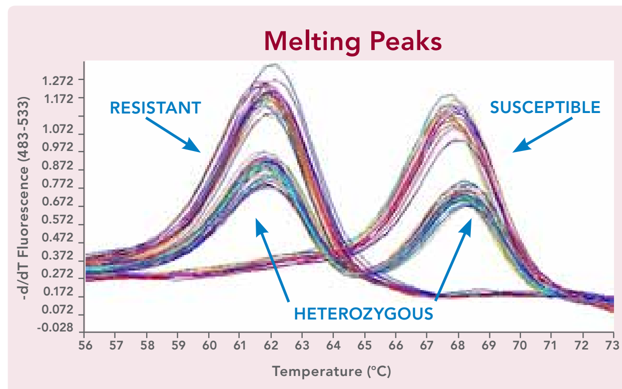

Figure 3. Typical melting curve peak fluorescence data for genotyping experiments on a Roche LightCycler Real Time PCR System.

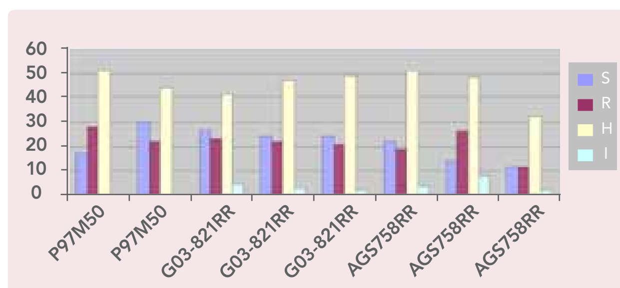

Figure 4. Melting curve results for each set of experiments. The X-axis line names the resistant parent plant in use. The designations S, R, H, and I refer to the number of test plants determined to be susceptible, resistant, heterozygous, or indeterminate for southern root-knot nematode.

SNP Maker data courtesy of Dr. H. Roger Boerma, David Hulbert, University of Georgia.

Conclusions

Due to the nature of marker-assisted selection for agricultural crops, i.e. few markers assayed against many individuals, Melting Curve Analysis is a cost effective and efficient means for SNP detection. Costs typically run around $.09 per 2 SNP with 3 uL reactions. Throughput is greatly enhanced with Melting Curve Analysis, as assay times are less than half that of typical direct hybridization and SSR assays. Therefore Melting Curve Analysis provides an excellent tool for agricultural marker assisted selection from both an economic and throughput perspective.
The combination of the Roche LightCycler® 480 and the Innovadyne Nanodrop Express is a powerful platform for high throughput SNP detection.

Reference

Bo-Keun, H, Boerma, H.R. 2008 High-throughput SNP Genotyping by Melting Curve Analysis for Resistance to Southern Root-knot Nematode and Frogeye Leaf Spot in Soybean. J. Crop Sci. Biotech. 2008 11 (2): 91-100

About Roche

Headquartered in Basel, Switzerland, Roche is one of the world's leading research-focused healthcare groups in the fields of pharmaceuticals and diagnostics. As the world's biggest biotech company and an innovator of products and services for the early detection, prevention, diagnosis and treatment of diseases, the Group contributes on a broad range of fronts to improving people's health and quality of life. Roche is the world leader in in-vitro diagnostics and drugs for cancer and transplantation, and is a market leader in virology. It is also active in other major therapeutic areas such as autoimmune diseases, inflammatory and metabolic disorders and diseases of the central nervous system.

About Innovadyne Instruments

Innovadyne instruments provide robust fluidics solutions to life science researchers for HTS, PCR, bead dispensing, cell dispensing, protein crystallography, MALDI plate spotting, and C50 applications. Our proprietary isolated-solenoid non-contact dispense technology offers tools that enable increases in throughput, sensitivity and flexibility for assays, while significantly lowering reagent and other related costs.

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