

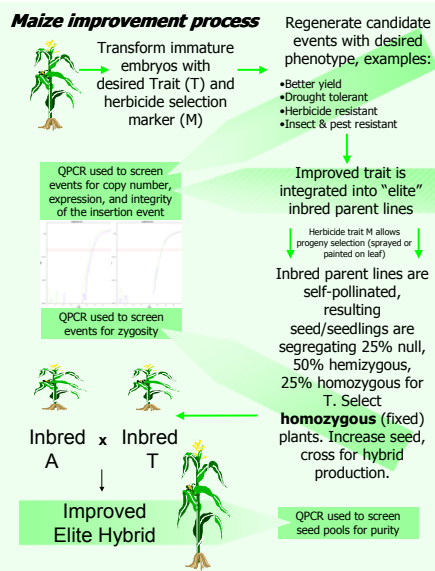
# Quantitative PCR in a high-throughput agricultural biotechnology setting



**MEYER S, LENDERTS B, LEYSENS N, PENNINGTON-COPE N, PETERSBURG T, PIETILA J, THOMPSON L, and HONDRED D (sandra.meyer@pioneer.com)**  
**Analytical and Genomics Technologies, Pioneer Hi-Bred, International, a DuPont Company, USA**

## ABSTRACT

Quantitative PCR analysis has proven to be a valuable tool in the process of development of improved maize hybrids. Copy number and zygosity quantification, SNP detection, and relative gene expression results are generated using optimized, simplified procedures and robust robotic systems. An overview of our QPCR and data analysis process is presented, along with future goals and directions for process enhancement.



## Method optimization for high-throughput agricultural biotechnology's needs

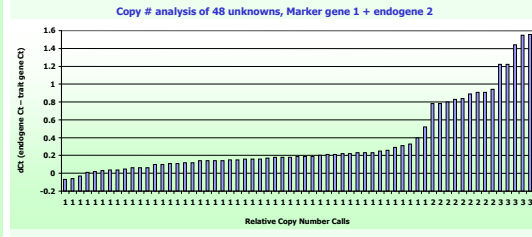
Process step	Enhancements achieved
Sample and data management	Integrated sample tracking, analysis software and results archiving.
Assay optimization & validation	Simple two-step system yields ddCt validated assays with few exceptions. <ul style="list-style-type: none"> <li>Simplified, lower cost DNA prep.</li> <li>Robust, reliable endogenous control gene in duplexed assays.</li> <li>Robotic liquid and plate handling systems with ABI 7900HT.</li> <li>Graphical-interface automated scoring software.</li> <li>Statistical methods for data analysis ensure data quality.</li> </ul>
Production-level analysis	<ul style="list-style-type: none"> <li>Identification of simple integration events by copy number analysis and event integrity analysis of progeny.</li> <li>Relative expression analysis adds value to other measurements of event performance.</li> </ul>
Event management	

**Future enhancements**

- Application of simplified, lower-cost DNA prep and QPCR analysis method to more assays.
- Eliminate most gel-based analysis by implementation of high-throughput real time assays.
- Implementation of endpoint zygosity assays (using allele discrimination) for many events.

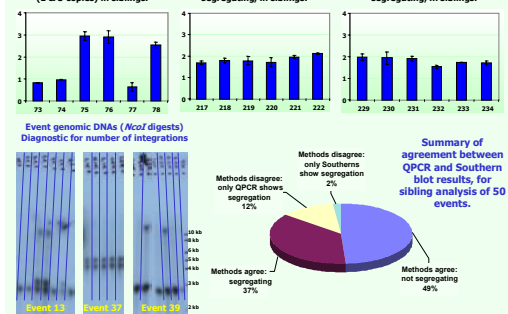
## Copy number analysis

- Accuracy best for 1, 2 and 3 copy samples. Can always distinguish 1X from 2X copies (as in zygosity analysis).
- Need 1 copy population (or controls) in each analysis for calibration of copy number.



## Event integrity analysis

- Southern analysis is traditionally used to identify transgenic events with multiple transgenic insertion sites, which may segregate apart during hybrid line creation.
- QPCR can be used to identify if there are segregating siblings within an event, as well as the copy number of the individual sibling plants.
- Methods Test: Southern analysis and QPCR comparison for 50 events. Events 13 and 37, below, are examples in which both analysis methods agreed. Event 39 is an example in which QPCR does not show segregation, while the Southern is inconclusive.



## Managing high-throughput demands

- Quantitative PCR assay requests have been doubling in number every two years (since 1998).
- Number of lab personnel has stayed the same.

Productivity has kept pace with demand through improved sample handling and efficiencies in analysis thanks to our **Integrated Laboratory Information Management System**

### Sample Tracking And Request System (STARS)

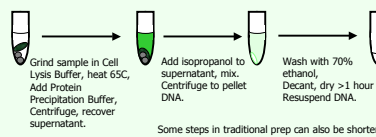
- All analyses requested through STARS
- All sample plates bar code labeled
- LIMS on the lab side manages steps of...
  - DNA prep
  - Assay set up
  - Analysis
- Results finalization

### Proprietary QPCR analysis software

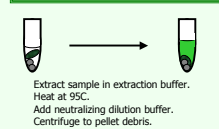
- Imports raw text file exported from ABI7900
- Performs data reduction, statistical analysis of ddCt data, and relative copy number or zygosity calculations
- Aligns results from multiple analyses and generates composite scores

## Streamlined DNA preps

### Traditional DNA preps (~2 hrs)



### Rapid DNA preps (~45 min.)



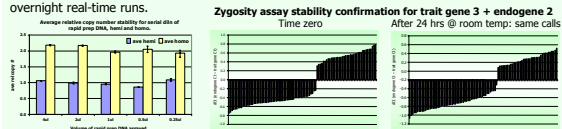
### Cost comparison

Extraction + PCR process	Costs per 96 well plate
Traditional prep + Applied Bio Universal Master Mix	\$ 76.02
Rapid prep + RS PCR Mix	\$ 61.42

Does not include the cost of the detection reagents such as primers and probes, labor, or pipette tips.

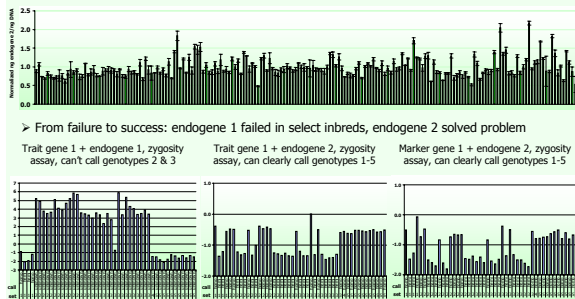
## Simplified duplex assay optimization & validation

- Optimize primer concentrations for both duplex partners for most sensitive Ct's, using equimolar forward & reverse primers at 150, 300, 600 and 900nM. Optimize probe concentration (100, 150 or 200nM) only if delta Rn is less than 0.7.
- Validate best duplex conditions using serial dilution of positive DNA, input simulates the range of concentrations in unknowns. Demonstrate delta Ct and relative copy number stability.
- Ex below shows validation of rapid DNA prep assay of trait gene 3 duplexed with endogene 2.
- Stability of the assay is confirmed at 24 hours, so that zygosity plates may be queued for overnight real-time runs.

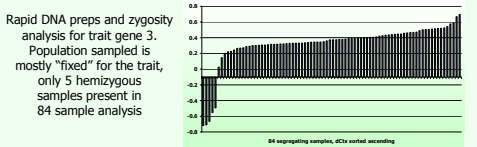
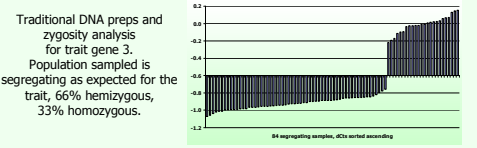


## Improved duplexed assays with reliable endogene

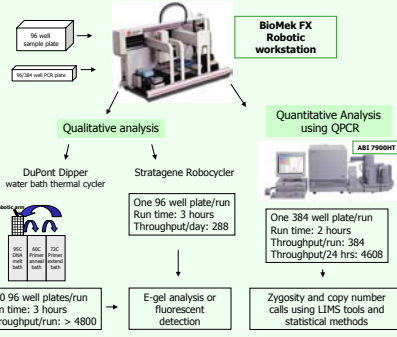
- Proprietary bioinformatics used to identify candidate sequences.
- 10 assays were tested on a panel of 23 diverse inbred genotypes (not shown).
- Endogene 2 assay was tested on 192 diverse inbred genotypes (below). Uniformity desired.



## Zygosity analysis



## PCR analysis with integrated robotics



## Expression analysis

- RNA is isolated using a scalable, aqueous, commercial kit, 96-well format.
- 18s rRNA endogenous control is primer-limited in the ddCt validated, duplexed assay for trait gene relative expression.
- One-step QRT-PCR using gene-specific primers.
- Relative expression = 2<sup>-(dCt calibrator ave - dCt unknown)</sup>

