# Novel Automated Quantitative Multiplex Platform for Gene Expression Analysis in a Single Reaction Volume

***Presenter:*** ***George Serbedzija, PhD.***
***S. Voronov, B. Stone, A. Leblang, V. Slepnev and M. Verhoef***

***PrimeraDx*** *Mansfield, MA*

## ABSTRACT

**Introduction:**
Gene expression profiling is a powerful tool for a variety of medical and scientific applications. The majority of gene expression panels today rely on quantification of individual genes by real-time PCR. Multiplexing capabilities in real-time PCR are currently limited to the use of fluorescent dye combinations, typically allowing 2-4 simultaneous reporters. Gene expression panels often include transcripts with over a thousand fold difference in expression. Simultaneous quantification of targets with such a high expression difference by the means of traditional real-time PCR is problematic due to notable spectral crosstalk associated with the presence of multiple dyes in one reaction. Here, a real-time PCR-based 16-plex single-tube assay for gene expression measuring biomarkers currently used in prediction of heart transplant rejection is described.

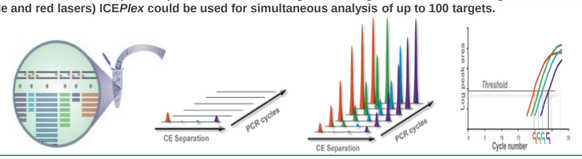
**Materials and Methods:**
All target amplicons were designed to have unique size and screened to minimize the probability of primer-primer and primer-amplicon interactions. RNA extracted from samples was reverse transcribed and split for use with 16 single-plex ABI TaqMan assays and with one single-tube 16-plex assay. The multiplex assay was designed such that the 9 genes were FAM-labeled and 7 genes were TYE-labeled. Using a fully automated platform, the multiplex PCR reactions were sampled and subjected to CE separation and detected using a two laser system in real-time during the PCR amplification. The amplification curve of each amplicon was reconstructed by compiling the electropherogram peak areas over successive PCR cycles. A "simulated sample" comprised of a mix of 16 plasmids encoding target genes mimicking averaged donor sample concentrations was used for linearity assessment.

**Results and Discussion:**
The multiplex assay demonstrated linear response over 3 logs of dynamic range using individual plasmids with target genes and at least 2 logs using simulated blood samples. Effective copy number determined across all 16 genes by TaqMan single-plex assays and the single- tube 16-plex 2-color gene expression assay using a panel of blood donor samples showed good correlation in the results.

**Conclusions:**
The proposed single-tube multiplexing approach provides greatly simplified setup, reduced sample volume requirement and increased throughput for running gene expression panels.

## PrimeraDx Technology

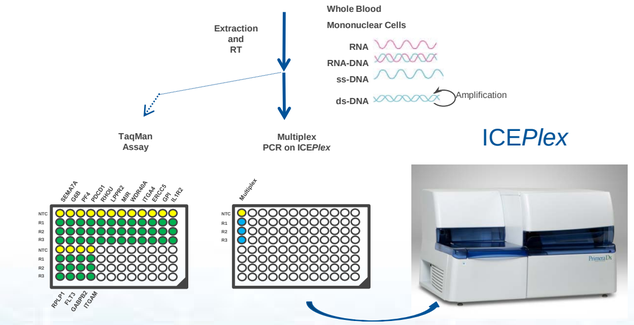
The technology is based on novel functional integration of Polymerase Chain Reaction (PCR) and capillary electrophoresis (CE). It uses size-based approach for quantitative multiplexing of dozens of targets in a single reaction. In the assay each nucleic acid target is amplified as a DNA fragment of a unique size. Every other cycle during the PCR amplification, direct electrokinetic injection is performed from the PCR, introducing amplified targets into the separating capillary. The size - and therefore identity of amplified PCR product is precisely measured by capillary electrophoresis, which is performed concurrently with PCR amplification allowing real-time amplification, separation and quantification. Analysis of the peak areas is performed over consecutive cycles allowing measurement of the threshold cycle and, therefore, the determination of the initial copy number of each nucleic acid target. Typically, primers for the assay are designed to produce 5-10 bases spacing between amplified targets. One of the primers in a specific pair is fluorescently labeled, enabling detection of amplified products. The technology does not use additional fluorogenic probes. Instrument system utilizing our technology, called ICE*Plex*™ , can separate up to 50 nucleic acid targets in a single color channel. Using dual laser excitation (blue and red lasers) ICE*Plex* could be used for simultaneous analysis of up to 100 targets.



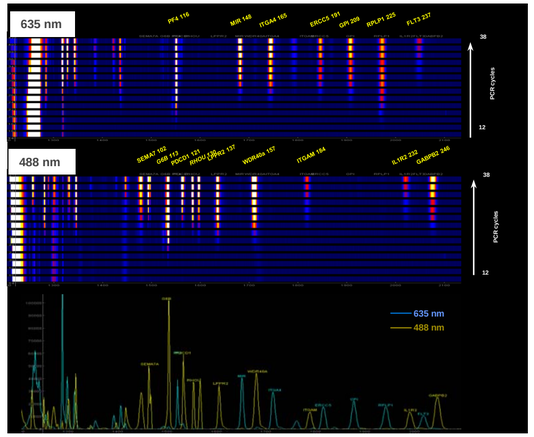

## Method

RNA extracted from donor's samples was reverse-transcribed and analyzed by either (1) combination of 16 single-plex ABI Taqman® assays or (2) by one single-tube multiplex assay.

In the multiplex assay, all amplicons were designed to have unique size.
Multiplex assay was performed in the ICE*Plex* system.
ICE*Plex* system automatically perform downstream data processing, which consist of alignment of successive electropherograms, peak assignment, peak quantification and Ct computation.



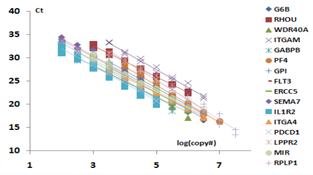

## ICE*Plex* raw data output




Top: Digital images from a single capillary showing the accumulation of PCR product over multiple cycles. Signal detected in 635nm and 488nm color channels. Note appearance and growth of signal from individual targets through amplification course.

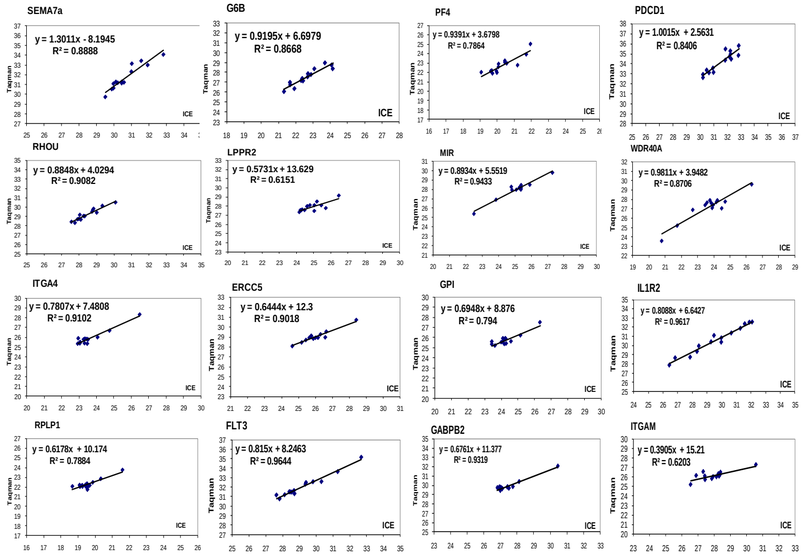
Bottom: PCR endpoint electropherogram showing overlay of both color channels

## Analytical measurement range




Multiplex assay exhibit linear response to target input on at least 5 orders of concentrations. Range of concentrations chosen for this test is determined by the expression level of a given gene in donor's samples

## Correlation Between ICE*Plex* and Taqman assay




Blood samples from 15 individual donors was assayed by a combination of 16 singleplex ABI Taqman assays or by one ICE*Plex* assay. Plots above show correlation of ICEPlex and TaqMan results for individual genes and show good correlation of the results from both platforms.

TaqMan and ICEPlex assays cover different exon boundaries for some of the genes in the panel. Less than perfect correlation for some of the genes, like LPPR2 and ITGAM may be related to contribution from different splice forms present in the donor's blood.

## Conclusions

- Proposed approach provides quantitative multiplexing for running gene expression panels, resulting in simplified setup, reduced sample volume requirement and increased throughput
- Multiplex assay exhibits linear response that covers at least 5 logs of dynamic range using individual targets and includes concentrations of the analytes present in donor blood samples
- Single-tube multiplex assay demonstrates good correlation in the results with existing real-time methods

PrimeraDx
Multiply Your Results