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Automated high multiplex qPCR platform for simultaneous detection and quantification of multiple nucleic acid targets

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Quantitative PCR (qPCR) using real-time detection of amplification is limited to a small number of targets within a single reaction. The ICE*Plex* system, using our scalable target analysis routine (STAR) technology, was developed to provide an automated, high multiplexing PCR solution. ICE*Plex* combines PCR thermal cycling with dynamic, sequential amplicon separation by capillary electrophoresis and two-color quantitative detection in a single integrated system. In contrast to probe-based qPCR, ICE*Plex* directly measures amplicon accumulation through incorporation of labeled primers. Three orders of magnitude of optical detection range and at least 7 logs of detectable target concentration range are demonstrated. The system can separate more than 50 amplicons per color channel, ranging from 100 to 500 bases, providing broad multiplexing capabilities for a wide spectrum of nucleic acid amplification applications. ICE*Plex* can be used for analysis of viral DNA or RNA targets, detection of genetic variants, and for reverse-transcriptase PCR gene expression panels.

Real-time PCR has become the gold standard for molecular diagnostics. The method offers excellent sensitivity and specificity and is adaptable to a simple automated instrument platform (1–3). These advantages have driven broad adoption of real-time PCR for numerous diagnostic applications in infectious disease, oncology, and genetic diseases (4). Recently, a new generation of tests based on quantification of expression levels of a panel of genes has emerged as a promising diagnostic and prognostic tool (5). However, the performance requirements of these assays have uncovered the limited multiplexing capabilities of current real-time PCR platforms (6,7). These are attributable to two main issues. The first is inherent to the current detection technology used in real-time PCR, in which different fluorescent dyes are used to distinguish distinct nucleic acid targets. A significant overlap in fluorescent dye excitation and emission spectra leads to a complex computational problem, particularly when the targeted nucleic acids differ in their abundance. This limits multiplexing to three or four quantifiable color channels (6,7). Second, sequence-based interactions, commonly referred to as "multiplex interference" (4,8), can be problematic. Several methods have been developed to address these deficiencies. However, they are based on parallel individual (i.e., singleplex) amplifications in miniaturized reaction wells (9). As these are no longer truly multiplex assays, larger amounts of nucleic acid are required in the reaction mixture, and small reaction volumes lead to lower sensitivity. In addition, preloaded assay formats lack flexibility in customization of assay design and per-sample cost, and time requirements are expected to be significantly higher compared with multiplex reactions. As a consequence, these limitations restrict broad application of these methods for diagnostic purposes. We have previously described scalable target analysis routine (STAR) technology as an alternative real-time multiplexing approach (8). The technology is based on the continuous sampling of PCR reactions containing fluorescently labeled primers during sequential cycles of amplification. This is followed by size-based detection of amplified products by capillary electrophoresis (CE) and reconstruction of the amplification kinetics using real-time PCR algorithms to quantify the amount of material in the initial sample. Using STAR technology, as well as an innovative approach to primer design, we have significantly improved multiplexing capabil-

ities over limits previously associated with chemistries using fluorogenic probes, such as TaqMan. With the addition of our automated bench top system, ICE*Plex*, we have created an innovative platform enabling simultaneous quantification of multiple targets from a single reaction. Herein we describe performance characteristics of this platform, which integrates direct electrokinetic injection from a PCR amplification reaction mixture with real-time CE separation and detection.

Materials and methods

System description

ICE*Plex* comprises several key elements (Supplementary Figure 1): an operating infrastructure with computer control, a PCR thermal cycler (TC), robotics and fluidics, and a CE system. The software elements include the user interface, consumables management, instrument control, data analysis, and reporting.

The TC heating block accommodates standard 96-well PCR plates and is temperature controlled in four zones to ensure uniformity. It incorporates a weight-calibrated and spring-loaded hold-down lid to provide uniform clamping and thermal contact between the plate and the block. The lid is perforated to allow insertion of the cannulae from the CE cartridge, for sampling from each well during cycling.

The system computer controls a three-axis robotic motor system, the fluidic pumps, and valves necessary to fill capillaries with gel and to pump CE buffer and system decontamination solution. All of these functions are preprogrammed and occur without operator intervention. The system monitors fluid levels and alerts the operator to replenish consumables and empty the waste reservoir when necessary.

The CE system itself consists of a prefilled disposable 384-well CE plate, a 96-well idle tray, a capillary cartridge, a gel and buffer filling system, and two high voltage power supplies.

The optics module houses two solid state lasers (488 and 639 nm excitation) and a spectrophotometer with a charge-coupled device (CCD) camera for simultaneous laser-induced fluorescence detection of two dye colors in each capillary. The emission photons are detected by an ~3 × ~25 mm CCD camera (Hamamatsu, Bridgewater, NJ, USA) that is thermoelectrically cooled.

Depicted in Supplementary Figure 2 is the CE cartridge, which is replaceable and houses the capillary array. An integral chip included in the cartridge body collects data on capillary configuration, serial number, expiration date, and usage. After each CE cycle, the capillary array is automatically purged of used gel and refilled with fresh gel. The cartridge contains 48 (and up to 96) electrodes that complete the electrical circuit. The capillaries periodically contact the PCR enabling the electrokinetic injection from the sample, followed by CE separation of the amplicons. Within the cartridge, the capillaries are arranged into an optical window where the laser impinges for excitation of the fluorescently labeled amplicons. The window is designed to eliminate capillary-to-capillary crosstalk by incorporating opaque barriers between the capillaries.

Table 1. ViraQuant™ Assay targets

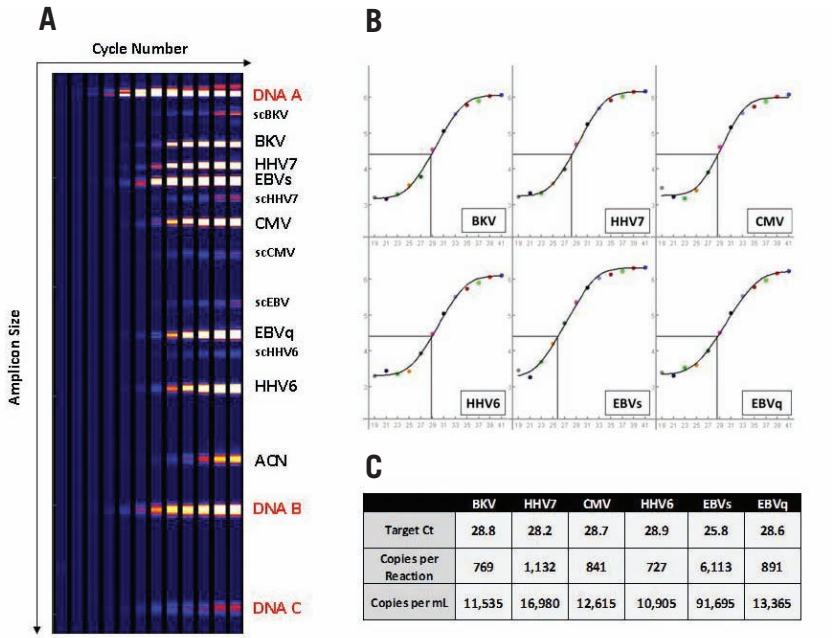
Target	Amplicon Size (bp)	Primer sequences
BKV	135	FAM-5'-GCTTGATCCATGTCCAGAGTCTTCA
scBKV[a]	121	5'-GGAAGGAAAGGCTGGATTCTGAGAT
HHV7	146	FAM-5'-ATATTGTGCCTTGCAGCTCTATGTTTCTC
scHHV7[a]	161	5'-ACCGAGATGCGGCTTTTATAGTTGA
CMV	172	FAM-5'-TCCGGCGATGTTTACTTTATCAACC
scCMV[a]	187	5'-CCGTGATAAAACACAAACTGGCAAA
EBVq	225	FAM-5'-AATGACTCCAACACCTCCGTCTCTC
scEBV[a]	210	5'-GACTAATGTGGTGGGGGCTATGGTA
EBVs	153	FAM-5'-AGAACCCAGACGAGTCCGTAGAAGG 5'-CTACCCCAGGTTCCTGTGAAAAGCAAGA
HHV6	250	FAM-5'-TCCCAATTGTCTAGCATGTTCTCCA
scHHV6[a]	234	5'-GTGTTTACGGTGCATGTGCAATTTT
Extraction Control[b]	283	FAM-5'-TGCTTTTGTAATTGGCTTCTGACCA 5'-CTACCCCAGGTTCCTGTGAAAAGCAAGA

[a]Sensitivity control (sc). [b]*Autographa californica* nucleopolyhedrovirus was used as extraction control.

PCR assays and real-time CE

To demonstrate the capability of ICE*Plex*, an in-house prototype multiplex assay, the ViraQuant assay, which simultaneously detects and quantifies viral loads of cytomegalovirus (CMV), Epstein-Barr virus (EBV), BK virus (BKV), and human herpes viruses-6B (HHV6) and -7 (HHV7) along with an extraction control, was developed. For EBV detection, a dual approach was followed to allow accurate virus particle quantification while maximizing detection sensitivity. For this purpose, two different genomic regions were targeted: one primer pair, designed for virus particle quantification and designated EBVq, amplifies an unique region of the EBV genome and a second primer pair, designed for sensitivity and designated EBVs, targets a genomic repetitive DNA element with a strain-specific copy number variation of 5–11 copies/genome (10). Amplicon sizes and primer sequences for the various targets are shown in Table 1. Primers for the ViraQuant assay were designed with melting temperatures (T_m) of approximately 60°C for all targets and controls in the assay. In order to minimize the potential for primer-dimer formation in a multiplex reaction, primer candidate sequences were screened with in-house developed software. This software analyzed all primer candidates for sequence interactions and output problematic individual primer pairs. Potential sequence interactions were ranked according to strength, expressed in ΔG values, and location (i.e., either internally or at the 3′-end of one of the primers, the latter having a higher probability for sequence extension during PCR). Primer candidates with values of ΔG < -6 and extendable 3′- ends were eliminated from the primer candidate pool. This approach generated in silico matched primers for use in multiplex reactions.

PCR reactions contained 2× Qiagen Multiplex PCR Master mix with HotStarTaq DNA polymerase (Qiagen, Valencia, CA, USA), gene-specific primer pairs (250–600 nM), where one primer of each pair was labeled with 6-carboxyfluorescein (FAM), and the plasmid clones of quantification calibrators and detection sensitivity controls (SCs). The SCs varied in size from their cognate wild-type targets (Table 1), and were amplified using the identical set of wild-type target primers, included at approximately 100 copies per reaction. In the absence of a viral target, the amplification of the SC ensures proper functioning of its cognate primer pair.



	BKV	HHV7	CMV	HHV6	EBVs	EBVq
Target Ct	28.8	28.2	28.7	28.9	25.8	28.6
Copies per Reaction	769	1,132	841	727	6,113	891
Copies per mL	11,535	16,980	12,615	10,905	91,695	13,365

Figure 1. Performance of the ViraQuant assay on the ICE*Plex* platform. A single-tube, multiplex reaction was performed on the ICE*Plex* system for each of the viral targets (CMV, EBV, BKV, HHV6, and HHV7). See Materials and methods for additional details. A sample containing the extracted viral nucleic acids and extraction control (ACN), along with sensitivity controls (sc) and calibrators (DNA A, B, and C), was amplified in a multiplex format in a reaction volume of 50 µL. Reaction samples (PCR amplicons) were separated by CE and analyzed by ICE*Plex* analysis software generating digital electropherograms with cycles of amplification presented as a heat plot in panel A. (B) Amplification curves were automatically reconstructed for each target from integrated fluorescent peak areas (y-axes, log scale) versus cycle number (x-axes). The analysis software uses the log-linear range at a set fluorescence value to determine the C_t for each target. (C) Initial concentrations of each of the targets per reaction and per milliliter of the original sample were calculated automatically by the ICE*Plex* analysis software using the equation shown in Materials and methods section.

Thermal cycling parameters included an initial 15-min hot-start activation of the HotStarTaq DNA polymerase, followed by 95°C for 30 s, 62°C for 90 s, and 72°C for 60 s for each of 41 cycles. Sampling for CE was performed 12 times during alternate cycles, beginning with cycle 19 and concluding with cycle 41.

Quantification calibrators consisted of a set of three engineered plasmids, designated DNA A, DNA B, and DNA C, each producing amplicons of unique sizes (113 bp for DNA A, 304 bp for DNA B, and 350 bp for DNA C) from an identical primer pair and each added at a predetermined concentration of 50,000 copies for DNA A, 5000 copies for DNA B, and 1000 copies for DNA C. These were used to generate internal calibration threshold cycle (C_t) values for every sample by comparing the amounts of each calibrator input with the resulting C_t. The amplification curves for each target analyte amplicon were reconstructed using ICE*Plex* analysis software by quantifying the electropherogram peak areas over successive PCR cycles (8). C_t values for each target in a sample were then determined and compared with the calibrator C_t values, to arrive at the number of target copies initially present in each reaction: $N_t = N_c/E_c^{[\Delta Ct]}$ [Where N_t = number of test targets; N_c = number of calibrators; E_c = efficiency of doubling, which here = 2.0; and $\Delta C_t = C_t(\text{test}) - C_t(\text{ref})$].

A plate map, indicating the position and identity of each sample, the assay to be performed, and the results to be reported was constructed using the graphical user interface. The run was initiated following loading of the sample plate, containing PCR mixtures covered with mineral oil, and the CE plate was placed into the instrument. The run, which is fully automated, consisted of an initial denaturation cycle followed by a series of complete cycles of denaturation-annealing-extension, sufficient to amplify any input targets to just below their initial detectability. Next, the metal cannulae of the CE cartridge were immersed directly into the PCRs during the course of one denaturation step. Electrokinetic injection of negatively charged molecules, including DNA amplicons, into the capillaries was performed by applying a fixed voltage during this immersion (11). A very small fraction (estimated < 0.002%) of the sample is transferred per injection. As the TC proceeded to the next annealing step, the cannulae were removed from the reaction wells and transferred to a separate disposable CE plate containing CE running buffer. A fixed voltage of 9kV was then applied for the duration of the electrophoretic separation. Following separation, the instrument automatically purged the separation gel and rinsed the cannulae tips. At this point the TC had gone through two cycles and was entering the denaturation step of a third cycle. The CE cannulae were then reintroduced into the PCRs for the next electrokinetic sampling, which thereafter occurred every other cycle. The run was completed in slightly over 4 h, which included data analyses and reporting.

The capillary array was automatically decontaminated between runs to prevent run-to-run carryover of amplification products.

Measurement of ICE*Plex* optical detection range

A series of purified random sequence 10-mer oligonucleotides, 5′-labeled with either FAM or TYE-665 (an analog of Cy5; Integrated DNA Technologies, Coralville, IA, USA) at differing concentrations, ranging from 1×10^{13} down to 1×10^{7} molecules/µL (16.6 µM to 16.6 pM) were electrokinetically injected. The areas of the peaks from these oligonucleotides were measured down to background levels.

Measurement of ICE*Plex* target concentration operating range

The operating range was tested using the ViraQuant assay. Serial dilutions of a single plasmid clone of the BKV target gene (pBK), from 1×10^{8} to 10 copies/reaction, were amplified in triplicate in individual wells and quantified using a 48-capillary CE cartridge. The polynomial fit test for linearity was used following Clinical and Laboratory Standards Institute guidelines (12).

Measurement of ICE*Plex* target size operating range

The range of detectable amplicon sizes was determined by utilizing the Multiplex Ligation-dependent Probe Amplification (MLPA) (13) kit for breast cancer markers (SALSA MLPA kit P078-B1 Breast Tumor; MRC-Holland, Amsterdam, The Netherlands) and genomic DNA from human Jurkat cells (New England BioLabs, Ipswich, MA, USA) following the manufacturer's instructions. The MLPA kit generates 52 amplicons from a variety of genes, spanning the size range of 100 to 500 bp, along with additional control amplicons from 96 bp down to 64 bp. The fragments are separated by 5 to 10 bp with the spacing increasing for the larger

molecules. The reaction end points were placed into the instrument and electrokinetically injected to establish the separation capabilities of the system.

Demonstration of ICE*Plex* two-color capability

A series of primer pairs, with target T_m values of approximately 60°C, was developed to create a 16-target, two-color multiplex assay derived from the AlloMap gene expression test from XDx (Brisbane, CA, USA). Nine genes were targeted using FAM-labeled primers, and seven genes were targeted using TYE-labeled primers.

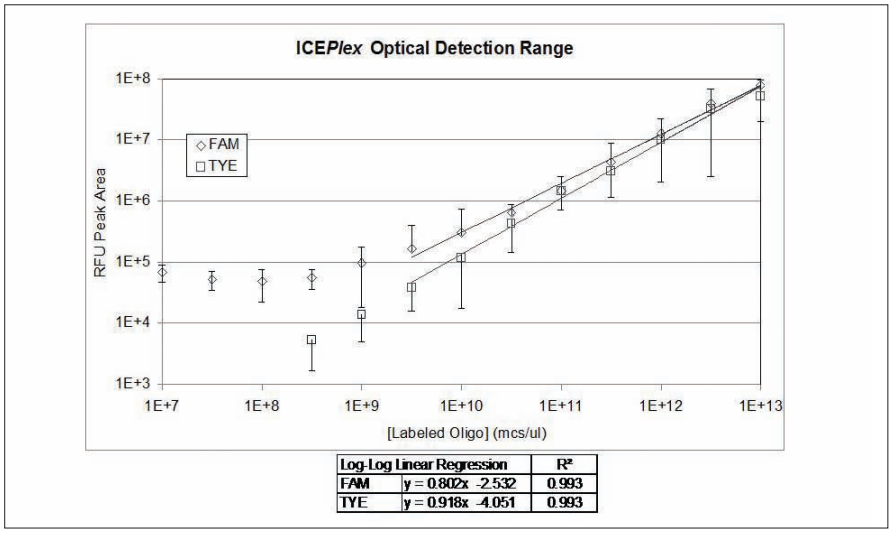
Donor whole blood was collected into BD vacutainer CPT heparin tubes (Becton Dickinson, Franklin Lakes, NJ, USA) to separate white blood cells. These were then lysed, and the RNA extracted using QIAshredder and RNeasy extraction kits from Qiagen following the manufacturer's instructions. cDNAs were generated with random hexamer priming and Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Final products were amplified with the above described primers along with 2× Qiagen Multiplex PCR Master Mix with HotStarTaq DNA polymerase and analyzed on the ICE*Plex* system.

Comparison of ICE*Plex* to real-time PCR TaqMan data

Data generated with the ICE*Plex* system using the ViraQuant assay was compared with that using the TaqMan method and the 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), with primer/probe kits either from Applied Biosystems or from Argene (North Massapequa, NY, USA). Target genes for CMV and EBV were cloned into the pUC19 vector (New England Biolabs). When viral particles were assayed, they were spiked into negative human plasma from 1×10^2 to 5.5×10^5 particles/mL and extracted using the NucliSENS easyMAG system (bioMérieux, Durham, NC, USA).

Tests for potential cross-contamination

The ViraQuant assay was used to assess the potential for cross-contamination between samples. A checkerboard arrangement was used with alternating negative and positive controls in 48 sample wells using a 48-capillary CE cartridge. Upon completion of the run, the ICE*Plex* cartridge was automatically decontaminated by immersing the cannulae into the onboard decontamination solution in the idle tray for 10 min with periodic swirling. This was followed by a run of 48 negative reactions to confirm the absence of potential well-to-well and run-to-run carryover contamination.



Log-Log Linear Regression		R^2
FAM	y = 0.802x -2.532	0.993
TYE	y = 0.918x -4.051	0.993

Figure 2. ICE*Plex* optical detection range. Oligonucleotides with either FAM or TYE fluorescent labels were diluted in half-log steps and electrokinetically injected ($n = 12$) for separation and detection on the ICE*Plex* system.

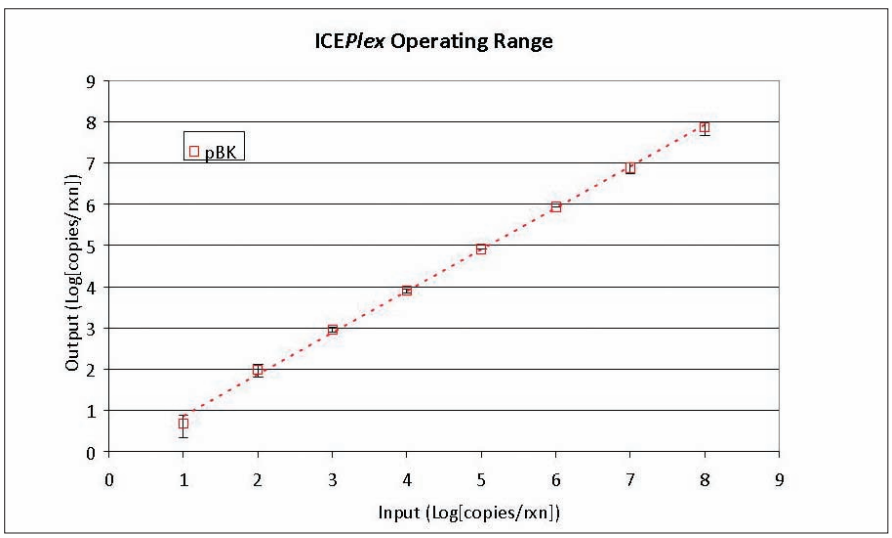


Figure 3. ICE*Plex* operating range. Plasmids containing a cloned BK virus target gene (pBK) were mixed with PCR reagents and amplified, separated, and quantified on the ICE*Plex* system. The log-linear range was from 10^2 to 10^7 copies/reaction (rxn).

Results and discussion

Performance characteristics of the prototype ViraQuant assay on ICE*Plex*

STAR technology is based on monitoring PCR amplification by sampling the PCR at sequential cycles and detecting the fluorescent signals during real-time CE separation (8). Using the ICE*Plex* system, CE resolves and identifies PCR amplicons designed to be of different sizes, thus allowing high numbers of amplicons to be detected simultaneously, while at the same time increasing confidence in result interpretation considering expected versus detected amplicon sizes. Images are continuously and regularly collected at the detection window to monitor the varying peak signals generated from fluorescently labeled migrating amplicons. As the areas of these peaks increased over consecutive PCR cycles, they are used to construct amplification curves for each amplicon. Similar to real-time PCR amplification, a C_t value is determined from an amplification curve for each of the targets while in the exponential phase of PCR amplification. Multiple calibrator controls of predetermined copy numbers are included in each assay to quantify the initial abundance of all targets through a comparison of C_t values between targets and calibrators.

This process of multiplex amplification and quantification is illustrated in Figure 1 for the prototype multiplex assay, ViraQuant, implemented on the ICE*Plex* platform. It depicts the digital image electropherogram (Figure 1A) showing the increase in fluorescent signal for each of the five viral targets, along with sensitivity and calibrator controls, as a function

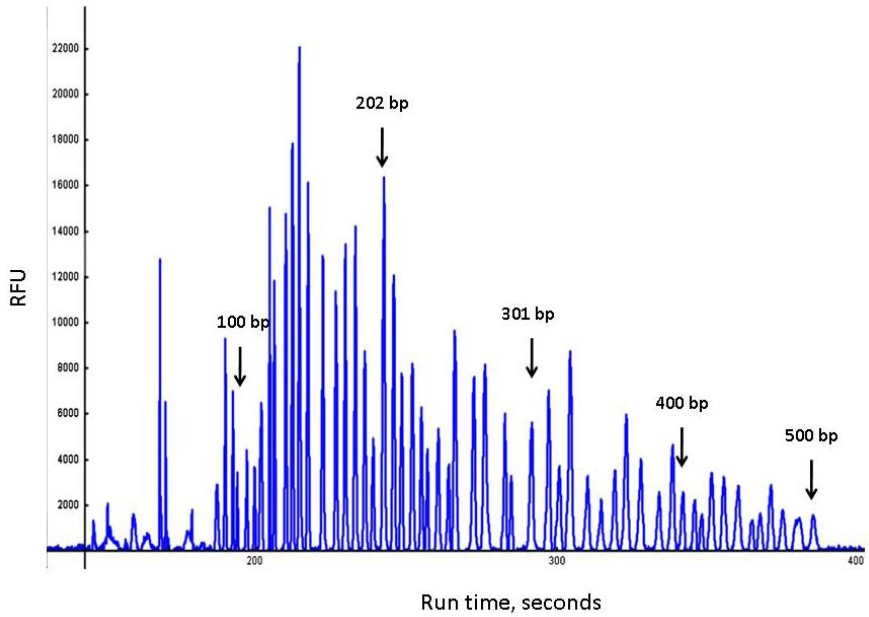


Figure 4. ICE*Plex* target size range. Human Jurkat cells as the source of DNA were assayed for 52 amplicons using the MLPA kit. Fragment sizes are highlighted in bp (arrows), and peak height is shown as RFU (y-axis) in the screen-shot taken from ICE*Plex*.

of sequential sampling and CE separations over increasing cycle number. From these fluorescent intensity values, amplification curves were generated for each target (Figure 1B), from which C_t values were determined. The number of copies for each of the five viral targets initially present in the reaction was then quantified according to the formula presented in the Materials and methods section (Figure 1C).

ICE*Plex* optical detection range

Figure 2 depicts the optical operating range of the ICE*Plex* system, which demonstrates its ability to detect both FAM and TYE signals in excess of three orders of magnitude of relative fluorescence units (RFU). This broad range of log-linear sensitivity permits quantitative detection of a wide range of target concentrations over many PCR cycles. The log-log linear regression slope for the FAM signal was 0.80, and greater than 0.91 for TYE. The difference in slopes suggests minor discrepancies in signal acquisition or filtering that is specific for the two wavelengths in use.

ICE*Plex* target concentration operating range

A 10-fold dilution series of the pBK plasmid clone was used to assess the target operating range (Figure 3). The assay exhibited linear performance across all concentrations, with an overall slope of 0.9865, and a DNA target detection range extending at least seven orders of magnitude (1×10^8 to 10 copies/reaction). When the polynomial fit method was applied to these log transformed data (9), the pBK targets were shown to be log-linear over five orders of magnitude, from 10^2 to 10^7 copies/reaction [slope (s.e.) = 0.9865 (0.0143), intercept (s.e.) = - 0.0164 (0.0699), r^2 = 0.9971, s.e. residuals = 0.1009].

ICE*Plex* target size operating range

Since the ICE*Plex* system uses size differences to distinguish and track specific amplicons, it is essential to evaluate the amplicons size operating range. Figure 4 demonstrates that the ICE*Plex* system was capable of resolving up to at least 52 amplicons over the size range of 100 to 500 bp when injected directly from PCR buffer. At the smaller sizes, the fragments were separated by 5 bp, whereas the largest two amplicons were spaced 10 bp apart.

The MLPA system was expected to yield approximately equal peak areas for most of these amplicons when using control DNA (13). According to the vendor however, heights of amplicon peaks can decrease as much as 3-fold with increasing fragment size. This reduction was estimated by comparing the peak heights at the smaller sizes compared with the largest sizes, and indeed this effect was seen with the ICE*Plex* system. However, the peaks also became somewhat broader, and when peak areas were compared with their corresponding fragment sizes, there was a minimal negative correlation with an r^2 value of only 0.145 (data not shown).

ICE*Plex* two-color capability

To increase the number of potential multiplex reactions on the ICE*Plex* system, two different dyes were used concurrently. Figure 5 shows the simultaneous measurement of 16 amplicons from the AlloMap panel using both FAM and TYE channels. The data indicate that the two-color capabilities of the system permit close approximation of amplicon peaks, permitting increased "density" of different amplicons within a constrained range of sizes.

Comparison of ICE*Plex* with real-time PCR TaqMan data

Detection outcomes using the ICE*Plex* system were compared with several TaqMan assays from Argene, an alternative real-time technology. Viral particles were spiked into target-free plasma, and the extracted DNAs were used for testing. For EBV, the prototype viral assay using ICE*Plex* was more sensitive than TaqMan, yielding a log-linear slope of 1.46 (r^2 = 0.988). The ICE*Plex* CMV results were slightly lower than those using the TaqMan approach (slope = 0.90, r^2 = 0.984), potentially due to slight variations in the detected amplification efficiencies of the two systems that use different primer and probe sets. Overall, the ICE*Plex* system yielded results comparable with the TaqMan platform.

ICE*Plex* cross-contamination tests

Using the ViraQuant assay, the potential for well-to-well cross-contamination was examined by alternating negative and positive samples in a checkerboard arrangement in a full 48-well PCR plate. This was repeated multiple times on two different instruments, and none of the 168 negative wells (24 per plate from seven runs) generated any positive signal. This confirmed that the traditional oil-covered system used in the ICE*Plex* system was efficient in preventing well-to-well contamination. In order to examine the possibility of run-to-run carryover from the high levels of end-point amplicons, another full 48-well PCR plate alternating negative and positive samples in the opposite checkerboard arrangement was run immediately after the previous experiment. The arrangement of each subsequent plate alternated, such that a negative sample always followed the location of a positive in the previous run. Under the cycling and sampling conditions used, none of the 24 negative wells per plate showed a positive signal above the limit of detection, which ranged from 10 to 40 copies/reaction of the five viral targets.

Currently available molecular diagnostic platforms either are quantitative with limited multiplexing capability or are capable of multiplexing thousands of genes but significantly lacking in sensitivity and quantitative ability (2–4). Based on STAR technology (8), ICE*Plex* is designed to be an

automated, high-throughput molecular testing platform integrating PCR thermocycling and CE separation, to allow quantitative multiplexing of dozens of targets in a single reaction well. Similar to real-time PCR, STAR uses the concept of kinetic PCR (14), wherein the quantification is based on the number of cycles required for a target amplicon to reach a preselected threshold (C_t). Here however, detection and quantification are achieved by sampling through electrokinetic injection of the DNA samples directly from PCRs into the CE device. In contrast to TaqMan real-time PCR, STAR does not require fluorogenic probes and secondary labeling. Rather, direct fluorescently labeled PCR products are separated and detected by CE. The use of two-color detection further expands the number of targets that can be measured from a single sample.

The optical detection range of the ICE*Plex* system is over three orders of magnitude (Figure 2), and its DNA target detection range covers at least seven orders of magnitude (Figure 3). Thus, the system has the capability of detecting target molecules over a wide range of input concentrations. Furthermore, the ability to separate more than 50 DNA fragments from a single mixture (Figure 4), and use of multiple color channels for detection (Figure 5) demonstrate that ICE*Plex* is an extremely versatile platform. Additionally, this high level of multiplex capability permits the inclusion of a variety of internal molecular controls, in addition to the desired target analytes, to ensure accurate and quantitative results.

STAR unites two well-characterized technologies, PCR and CE, and ICE*Plex* effectively automates the process via a TC module, a CE module, electrokinetic injection, and a straightforward and innovative software interface. By using a direct labeling approach, some of the complexities of designing multiplex assays that have been encountered with more commonplace probe-based chemistries, such as TaqMan, have been overcome. It should be possible to use the ICE*Plex* system for a variety of applications, including detection and quantification of microorganisms and fusion genes, SNP genotyping, as well as gene expression and microRNA profiling. Thus, the ICE*Plex* technology is applicable to many fields of investigation, from basic research and clinical testing, to environmental monitoring, biohazard detection, and bioterrorism surveillance. We are currently exploring a number of different technological approaches and applications to fully realize the potential of this novel molecular platform.

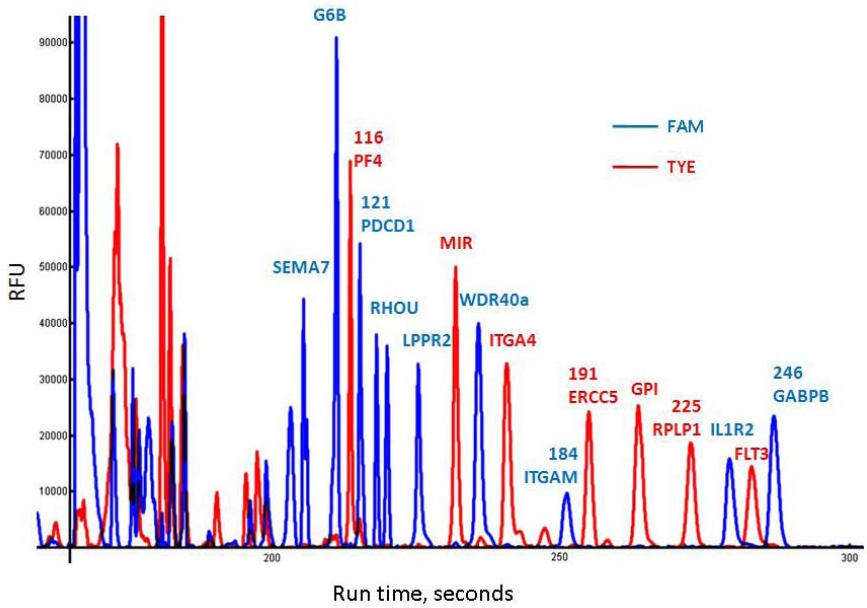


Figure 5. ICE*Plex* two-color capability. Human RNA was extracted from donor whole blood, cDNA was generated, and the material was then analyzed for expression markers derived from the AlloMap 16-target panel developed by XDx. The RFU signals from mixtures of amplicons incorporating primers with two colors, FAM in blue and TYE in red, are shown as a function of CE separation run time in seconds.

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Competing interests

PrimeraDx owns patents on and is the commercial manufacturer of the ICE*Plex* instrument system.

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Supplementary Material For:

Automated high multiplex PCR platform for simultaneous detection and quantification of multiple nucleic acid targets

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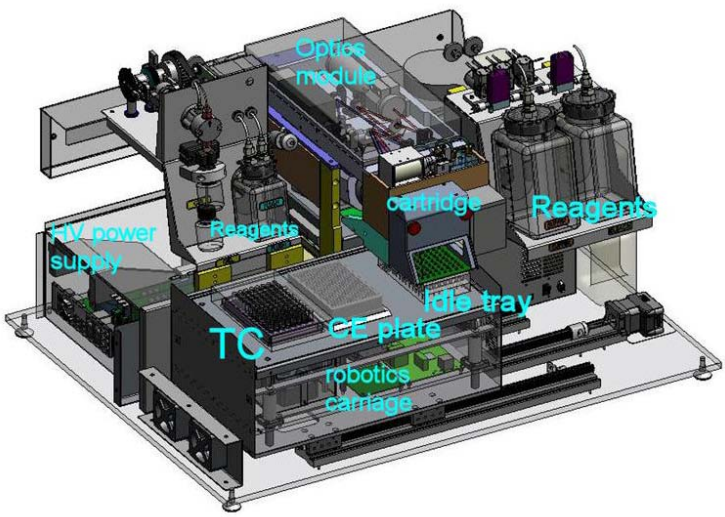
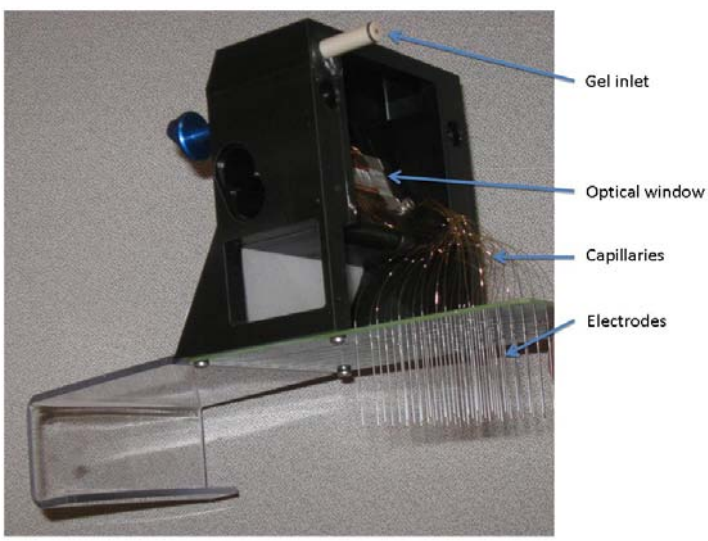


Supplementary Figure 1. ICE*Plex* system subcomponent view. The TC, CE, and idle plate (left to right) are on top of the robotic carriage.



Supplementary Figure 2. CE cartridge. The replaceable cartridge in a 48-capillary configuration, with gel inlet, optical window, and electrodes is shown.