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Microfluidic droplet-based PCR instrumentation for high-throughput gene expression profiling and biomarker discovery

Christopher J. Hayes [a,b,*], Tara M. Dalton [a]

[a] Stokes Institute, Department of Mechanical, Aeronautical and Biomedical Engineering, University of Limerick, Limerick, Ireland
[b] Department of Life Sciences, University of Limerick, Limerick, Ireland

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ABSTRACT

PCR is a common and often indispensable technique used in medical and biological research labs for a variety of applications. Real-time quantitative PCR (RT-qPCR) has become a definitive technique for quantitating differences in gene expression levels between samples. Yet, in spite of this importance, reliable methods to quantitate nucleic acid amounts in a higher throughput remain elusive. In the following paper, a unique design to quantify gene expression levels at the nanoscale in a continuous flow system is presented. Fully automated, high-throughput, low volume amplification of deoxynucleotides (DNA) in a droplet based microfluidic system is described. Unlike some conventional qPCR instrumentation that use integrated fluidic circuits or plate arrays, the instrument performs qPCR in a continuous, micro-droplet flowing process with droplet generation, distinctive reagent mixing, thermal cycling and optical detection platforms all combined on one complete instrument. Detailed experimental profiling of reactions of less than 300 nl total volume is achieved using the platform demonstrating the dynamic range to be 4 order logs and consistent instrument sensitivity. Furthermore, reduced pipetting steps by as much as 90% and a unique degree of hands-free automation makes the analytical possibilities for this instrumentation far reaching. In conclusion, a discussion of the first demonstrations of this approach to perform novel, continuous high-throughput biological screens is presented. The results generated from the instrument, when compared with commercial instrumentation, demonstrate the instrument reliability and robustness to carry out further studies of clinical significance with added throughput and economic benefits.

Significance statement

Differential gene expression levels have been shown to greatly influence many biological conditions in both normal and abnormal circumstances and can provide valuable information into tissue and cell developmental behaviour in response to environmental stimuli. The microfluidic droplet technology described greatly enhances the ability to analyse the expression levels of hundreds of genes across hundreds of samples in a continuous flow regime while using reduced volumes of both sample and reagents. Reactions are carried out on a single high throughput instrument which provides reliable sensitivity and quantitative precision for differentiating gene expression variability between biological samples. Characterising these expression level changes is critical for multiple pathway analysis, biomarker classification and drug discovery.

1. Introduction

The polymerase chain reaction (PCR) [1] is the dominant method of choice for quickly generating a sufficient amount of identical genetic material for analysis in biological investigations. The need higher throughput with an increased level of specificity coupled with reduced volumes of reagents is a dominant factor for the PCR and gene expression profiling in particular. Existing methodologies such as microarrays [2,3] and digital PCR [4,5] (dPCR) are extremely powerful tools for studies in that they allow one to probe virtually the entire transcriptome to give an overall picture of gene expression behaviour [6,7] and genetic mutations [8]. These technologies are well established and allow for the screening for multiple potential biomarkers and drug targets. However, limitations in dynamic range make the detection of transcripts in low abundance problematic [9] and their results for any given gene are often ambiguous due to system noise interference [10]. Commercial dPCR microfluidic platforms such the BioMark (Fluidigm) and OpenArray (Life Technologies) use microfluidic technology to generate and analyse partitioned samples and enable high throughput gene expression

* Corresponding author at: Stokes Institute, University of Limerick, Limerick, Ireland. Tel.: +353 61202449; fax: +353 61202393.
E-mail address: christopher.hayes@ul.ie (C.J. Hayes).

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measurement [11,12]. This facilitates thousands of assays to be performed in parallel. Thermal cycling and fluorescence detection is performed on integrated fluidic circuits and plates which are high throughput and use low starting quantities but are sometimes limited by their fixed format and the necessity to pre amplify targets which at times has been suggested to introduce amplification bias [13]. More recently the SmartChip system (Wafergen) targets the gap between hybridisation-based microarray technology and PCR [14]. The platform comprises a nanodispenser module and a cycler, which performs PCR thermal cycling and data collection. Integrated systems like this that enable a flexible workflow for high density gene expression profiling allow for more genetic analysis to be performed with more variation. Further to this, technological innovations in the fields of DNA sequencing address many of these issues and provide an unprecedented level of information for the discovery and validation of novel RNA biomarkers [15]. However much of their limitations and possible pitfalls of real in-depth transcriptome sequencing are not well known and tools to analyse the wealth of information to its maximum are also limited. Therefore it has become common practice to check the results of a genome-wide study with RT-qPCR which has excellent sensitivity, dynamic range, and reproducibility and is widely regarded as a "gold standard" measurement [7]. Considering the current use of qPCR for molecular microbiological testing in the clinical laboratory, high-throughput RT-qPCR devices are also likely to be at the forefront of transcript-based diagnostics in the near-future [16].

Utilising microfluidic approaches provides numerous advantages for DNA amplification [17–19] and other diagnostic applications [20] such as economies of scale, parallelisation and automation, and increased sensitivity and precision that comes from small volume reactions. The advantage of microfluidics is not only the ability to perform high-throughput sample analysis but also the ability to perform experiments out of the range of existing technologies [21,22] Furthermore, microliter to nanoliter droplet volumes allow several thousand PCRs to be performed in parallel [23,24] or a continuous-flow based approach in which the temperature is kept constant over time at specific locations in the system, and the sample is moved between the individual temperature zones for cycling [25,26]. Additionally, the challenge associated with realising the desired economies of scale in microfluidic devices is to simultaneously reduce the number of pipetting steps while amortising the sample volume from each pipetting step over a large number of independent assays [27]. The type of instrument that the end user chooses ultimately depends on the specific application(s) that will be carried out. Many microfluidic systems for PCR [24,28,29] which have been developed offer many advantages such as reduced reaction volumes, higher thermal cycling speed and decreased reagent and mastermix consumption. These droplet based microfluidic systems are created by two immiscible phases, typically aqueous droplets held within a non-aqueous carrier fluid such as silicone oil [30]. This creates a wrapped droplet which acts as a bioreactor in this system. The droplets produced are usually in the micrometre range and can be produced at rates of tens of thousands per hour. Wrapped droplets in small channels also allow fluid flows with no dispersion; therefore contamination will not be left in the tube for the next droplet to pick up. In addition, when such droplets are surrounded by an immiscible fluid this can prevent contact between the surfaces of the channels and the sample within the droplet, eliminating adverse effects due to the large surface to volume ratios. The instrumentation developed in this paper expands on the approach of using microfluidic droplets, acting as distinct miniature reactors continuously flowing through the system, from which it will be possible to quantify gene expression levels in DNA samples. The instrument is a four-line system which allows for a variation in experiments to be carried out in parallel (Fig. 1). The entire process, from sample preparation to data acquisition, is performed on one instrument and is automated to ensure speed and consistency in measurement. As this platform is based on a continuous flowing basis, a large number of reactions will be generated with small reaction volumes leading to a fundamental

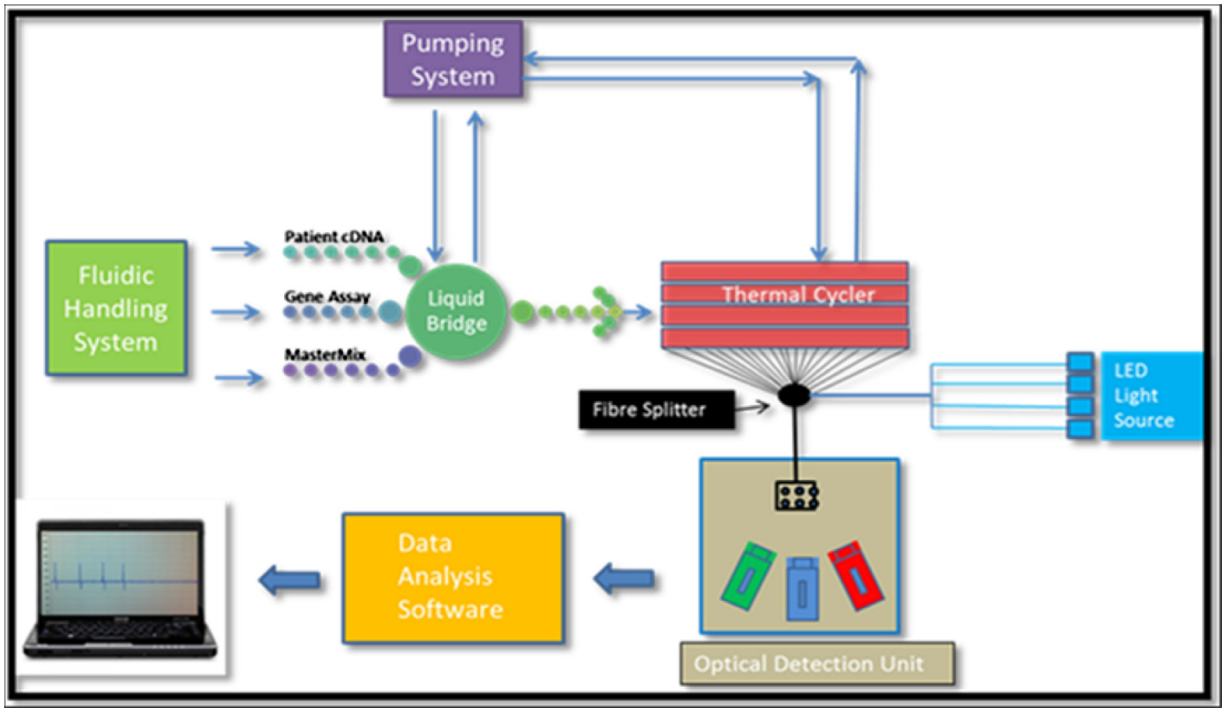

Fig. 1. Graphical schematic of the GEI (Gene Expression Instrument) design showing primary components and overall process flow. The main components of the instrument comprises of liquid bridge technology, fluidic handling systems, an integrated pumping system, a dual temperature thermal cycler, an optical detection platform and a data analysis software platform.

requirement to design a system that is capable of manipulating such small samples. Fluidic manipulation and monitoring techniques that have been developed are presented showing the mixing of individual droplets of DNA, reagents and Taq polymerase occurring in a controlled micro-environment. The mechanisms for the generation of the micro-reactor droplets are also presented. The system has been developed to be reusable without carryover contamination i.e. the unwanted transfer of molecular material from one reaction to another that may ultimately lead to a false positive [31] by encapsulating the droplets by immiscible oil. The final reaction size of the reaction droplet is approximately 300 nl. Thermal cycling is performed in a flowing serpentine cycler which gives superior performance because of the relative size of the droplets therefore resulting in more sensitive experiments. The inclusion of fluorescent probes in the PCR mix permits the amplification process to be monitored within individual droplets at specific locations. A comprehensive data modelling system allows for the signals to be interpreted and expression ratios to be determined.

2. System design and methodology

2.1. Pumping system

Precise fluidic transport is essential to achieve successful continuous flow PCR. The production of precise droplet sizes and correct dwell time of a droplet in a thermal zone is dependent on the flow rate control. Fluidic transport is provided by an active integrated method using micro-annular gear pumps (HNP Mikrosysteme MZR®-2905 series). The pumps provide a flow rate by displacing fluid between and internal and external rotor. The main advantages of this device include high precision, low volume dosage, compact design and an integrated microcontroller. With the ability to operate at a flow rate in the range of 0.003–18 ml/min coupled with a low pulsation of <6%, the micro-annular pumps are ideal for the continuous, stable convection of droplets through the system. It allows the combination of fluidic transport and modulation to achieve active control of droplet generation and enables independent control of droplet generation frequency by adjusting the pumping frequency and droplet size by flow conditions [32]. There are four micro-pumps in the system, each one individually controlled at precise micro-litre flow rates and volumetric displacements. Flow rate control is achieved using a LabVIEW control algorithm. Manifolds are incorporated into the fluidic layout to ensure pumping stability is maintained throughout the system. The manifolds also ensure the prevention of air or any residual content from entering the pump and system at any stage. Two way and three way valves (Lee Control Valves) in the pumping system allow for precise control and high speed direction control of the fluid. The valves are simple in design yet allow for the rapid control of the oil through individual ports and pathways. Flow control sensors (Sensiron ASL1600-10 and ASL1600-20) monitor the flow rates between the pumps and the manifolds and relay real-time flow rates to the central control system which in turn adjusts the pump speeds to counteract peaks in flow rate and keep the system overall flow steady and controlled. A priming system is also incorporated into the platform which allows for the entire purging of the system at the beginning and end of each experiment removing any air bubbles or possible contamination for further tests. A sheathing system is also incorporated into the system which continuously replenishes an oil supply that flows over the aspiration tips in the dipping heads. This allows for the movement of the dipping heads in air between the home position trays and the well plates. This sheathing system also replenishes the oil in the well plates and overlay of the sample. Priming of the system is dependent on the reversal of two of the system pumps, one which draws and the other which forces the liquid through the system at an increased velocity. A total system prime of the liquid bridge, thermal cycler and remaining components can be completed within 8 min. A filtered oil supply is located in a tank within a basement compartment of the instrument alongside and waste oil recycling tank. Each reservoir is 8.25 l in capacity which is equal to +150 h of the instrument continuously running, highlighting an important walk-away automation aspect.

2.2. Droplet generation

The first step in the microfluidic life cycle of a droplet is its production. The majority of microfluidic droplet production is achieved using either active or passive methods [31]. The active method for generation of droplets involves the use of an external factor such as an electric field for droplet generation. Two techniques that fall in this category are di-electrophoresis (DEP) [33] and electro-wetting on dielectric (EWOD) [34,35]. A limitation of these two techniques is in the control of interfacial instabilities where the surface wettability (contact angle) can be difficult to manipulate and control. In passive methods, the droplet generation depends on the geometry and dimensions of the device such as T-junctions and flow focusing methods [36]. A uniform, evenly spaced, continuous stream of droplets can be achieved by these methods. However, there are many limitations with these methods such as droplet polydispersity in droplet streams and flexibility in manipulation of droplets. These boundaries confine the application of these methods to very precise environments which have high shear rates for droplet production. High flow rates can result in undesirable pressure drops in a continuous flow system.

In the instrumentation outlined, micro-scale extruded polytetrafluoroethylene (PTFE) capillary tubing (ID ~ 152 μm, OD ~ 787 μm) is integrated into dipping heads that are attached to modular three-axis robotic stages (Festo Motor Controllers CMMS-AS). The hydrophobic PTFE tubing allows for strong wetting of the surface walls by the carrier fluid and also the internal tolerances on the capillaries prevent the droplets from sticking to the walls on imperfections as seen similar PCR designs [37]. Also, in previous work on this type of PCR, cross-contamination between droplets was attributed to droplet instability and the formation of small satellite droplet [24] To inhibit this, when droplets are produced they are transported in the micro-capillary tubing by the carrier fluid where the interfacial properties of the carrier fluid coupled with the constant flow of fluid into the system separating the droplet trains means carry-over contamination risks are reduced (Fig. 2). Also, due to the biphasic flow in the capillary, a liquid film exists between the droplets and capillary wall. The oil film prevents the aqueous droplets from wetting the wall of the tube, preventing contamination of the flow conduit [38].

The dipping heads versatility allow for access to each individual well on a variety of plates including 384-microplates (Greiner 384 well plates, clear polystyrene wells flat bottom). Droplets are created by moving the dipping heads from an oil overlay in the microplate to the sample for a specific time. The sample is withdrawn and the dipping head moves back to the oil overlay, creating the droplet. On the sample/single tip side, four individual dipping heads aspirate identical volumes of DNA sample while on the multilumen side, a bundle of four dipping heads aspirate the reagent and Mastermix volume. This allows versatility in the instruments robotic movements and allows the sample side to remain in one well while the multilumen bundle head moves from well to well in a second plate assaying for the genes of interest (GOIs). This process can also be reversed where the multilumen head remains in the well while the single tip side moves from well to well. The sequence of movements is determined by individual experimental processes and desired qPCR analysis. The stages programmed sequence allows dipping into the solutions and aspirate predefined

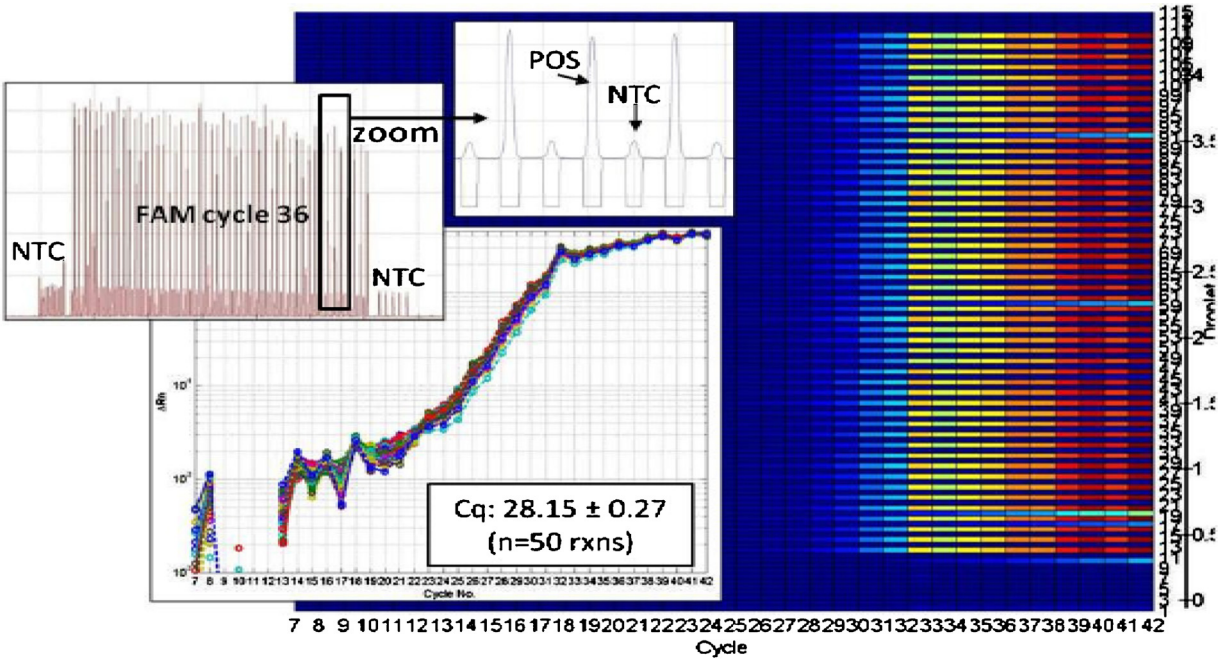

Fig. 2. The system was tested for crossover contamination interspersed dipping evaluation by alternating between NTC reactions ($n = 65$) and template nanodroplet reactions ($n = 50$) in single dipping mode demonstrated no cross contamination. NTCs contain the primers and mastermix but do not contain any template. The reporter dye (FAM) fluorescence at cycle 36 is highlighted on the left of the figure showing clear distinctions between positive reactions and NTCs. The amplification plot (Cq = 28 ± −0.7) and signal intensity heat map (cycle vs. droplet number) are also shown. There is no observable contamination between different droplets and furthermore amplification is consistent for each of the reaction droplets.

quantities of DNA and reagents. The droplet volume is determined by the dwell time of the dipping head and the volumetric flow rate. Accurate and precise incremental dipping accounts for the reduced time the aspiration tip is in the sample due to the reduced height level of sample. The two phase nature of microfluidic flow necessitates a means of combining fluids.

To combine and mix the sample with the reagents within one droplet, which may then be used as a microfluidic biochemical reactor, liquid bridge dispensing that has been developed [39] is exploited. A liquid bridge consists of an isothermal mass of liquid held by interfacial tension between two opposing capillaries. Liquid bridge dispensers operate by continuously creating and rupturing this bridge. The bridge comprises a first inlet port which is at the end of a capillary, two narrower inlet ports each perpendicular to the first port, an outlet port which is at the end of a capillary and a chamber for the carrier fluid (Fig. 3). Initially the system is primed with density-matched oil. The inlet and outlet ports are of diameter 300 μm. The chamber is ~5 mm in diameter and ~3 mm in depth. The oil is density-matched with the reactor droplets such that a neutrally buoyant environment is created within the chamber [40]. Oil between the reactor droplets continuously replenishes the oil in the chamber. This causes the droplets to assume a stable capillary suspended spherical form upon entering the chamber. The first and second droplets remain suspended at the end of the capillary until the third droplet enters the system causing the formation of an unstable funicular liquid bridge allowing the spherical shape to quickly rupture and span the gap between the ports, forming an axisymmetric liquid bridge. This method of periodic, stable mixing allows for the creation of the micro-reactor droplets with DNA sample and reagents for functional experimentation. Highly consistent, low volume micro-reactors can be generated [41] and droplet size can be changed as required. The process is continuous, allowing sample droplets to be carried uninterruptedly into the reaction zone while new drops are aspirated from the sample plate. There are no consumable parts owed to the presence of a liquid film wrapping and separating the droplets, eliminating the high cost per experiment associated with some commercial qPCR technologies.

2.3. Thermal cycling device

A large body of research [17] has focused on developing faster thermal cycling technologies for PCR. To carry out the PCR, thermal control of the reaction droplet is a crucial step to maximise the full efficiency of the amplification process. A non-uniform temperature field may lead to low amplification efficiency of nucleic acids and even non-specific PCR products due to insufficient annealing temperature of the PCR process [42]. The majority of microchip systems utilise fabricated micro-, nano-, or picoliter reservoirs for conventional thermocycling [43–46]. Some of the reported drawbacks of on-chip amplification are difficulty in creating parallel reactions, difficulty in adjusting the number of cycles once design is complete, and the high cost of such devices [47]. Furthermore, the number of micro-reactors is fixed, reducing experimental flexibility and limiting the maximum sample volume that can be processed. An interesting principle which has been exploited is the dynamic reactor, where a continuous flow of samples and reagents is passed through three zones, which are kept at constant temperatures optimised for denaturation, annealing, and elongation of the DNA fragments. The principle was first described by Nakano et al. [48] where a Teflon capillary was routed through three constant temperature baths to provide 30 cycle amplification. The four line continuous flow thermal cycler design outlined in this paper takes from that principle and is characterised by two independent temperature zones with no cyclic hold times inspired in part by the observation that much of the canonical 20–40 cycle denature (90–98 °C), anneal (50–65 °C), and elongation (70–80 °C) temperature sequence can be abbreviated without sacrificing performance [42].

The AB 7900HT Fast Real-Time PCR System [49], a PCR platform manufactured by Applied Biosystems, was used as a benchmark instrument. The AB 7900HT specifies a temperature uniformity of ±0.5 °C and accuracy of ±0.25 °C. This temperature control

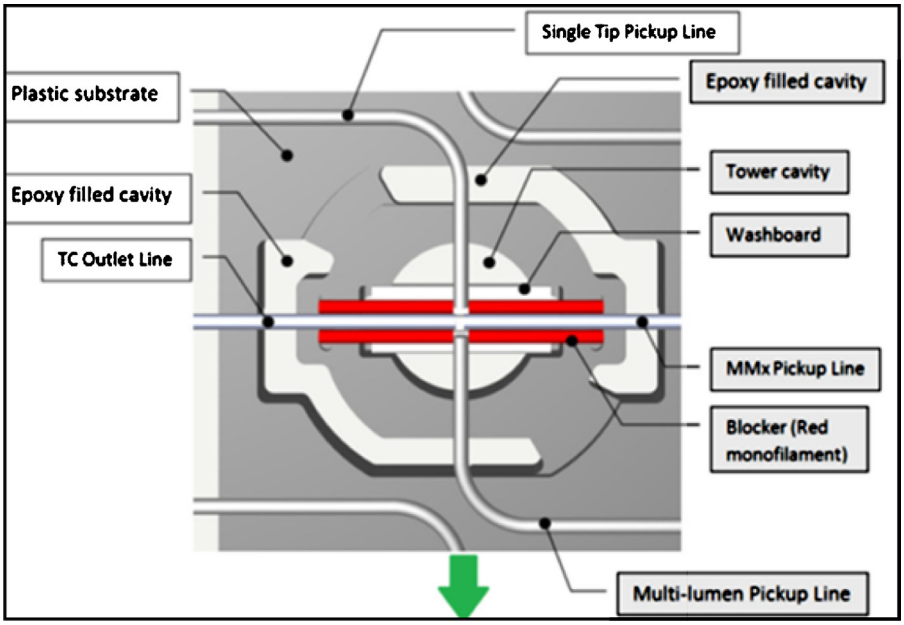

Fig. 3. Liquid bridge design. The illustration shows the internal modules of the liquid bridge including the bridge housing and the individual pick-up lines for the sample and chemistries along with the TC outlet capillary where the combined micro-reactor droplet is conveyed to the serpentine thermal cycler.

was replicated on the instrument design as Applied Biosystems chemistries and protocols are used. Two thermally insulated blocks are arranged in parallel and are separated by an air gap of approximately 1 mm to reduce heat transfer convection rates between the two surfaces. The static blocks/heaters are divided into two thermal zones, a 95 °C denaturisation region and a 60 °C annealing region with incorporated ~1 mm serpentine channels (Fig. 4). Heating elements (Minco Flexible Thermofoil™ heaters) are applied to the blocks which allow for heating from steady state to elevated control temperature in order to maximise test speed and throughput. These thin, flexible components consist of an etched-foil resistive heating element laminated between layers of flexible insulation. The two temperature zones are thermally insulated to reduce temperature fluctuations along the length and because this gives superior temperature control due to the reduced heat dissipation capacity. The micro-droplet enters the thermal cycling system in a single capillary and travel through the 95 °C section for initial denaturisation of 10 min prior to PCR cycling. Similar designs have been demonstrated in literature where sample is flowed through fused silica tubing which is in turn wound helically around a cylindrical device subdivided into discretely heated, constant temperature segments [50] and which was later expanded upon by others [51] with the integration of analysis systems to detect amplification. In the system outlined, the micro-reactor droplet is conveyed through the two zones and follows a serpentine shape which provides for one cycle of PCR for each length of travel along the serpentine design. Since the blocks are isothermal, there is no unnecessary ramping time as seen with most PCR platforms that operate by repeatedly heating and cooling blocks. The elimination of unnecessary ramping time has the potential of reducing the overall thermal cycling times compared to conventional thermal cyclers. In total, the sample undergoes 40 thermal cycles before exiting the thermal cycler for sample collection. The time for each cycle is approximately 1 min (15 s denaturisation/45 s annealing) and determined by the internal diameter of the tubing (Zeus Inc.: ID ~ 584 μm, OD ~ 1040 μm) and the flow rate (~11 μl/min) of the PCR through the system. The serpentine channel length ratio was designed to yield a dwell time duration ratio of 1:3 for denaturation and annealing. From entering the system to exit, the droplet spends a total of 48 min in the thermal cycler. Thermocouple readers providing a feedback loop to a LabVIEW control program maintain a steady temperature profile. Deviation in temperature over the surface of a

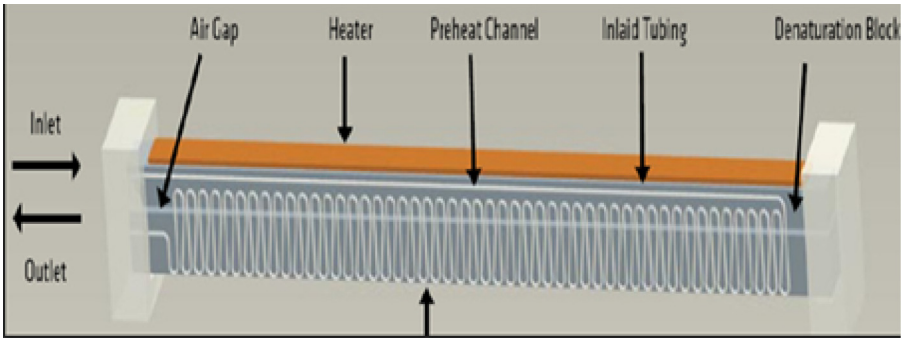

Fig. 4. Thermal cycler image showing individual components and heating elements. Deviation in temperature over the surface of a heating block has not been observed, indicating that employing a highly heat conducting material and efficient thermal insulation provides a homogenous temperature over the block. The reactor droplet is conveyed from the inlet through the preheat channel before undergoing one cycle of PCR upon travel back through tubing (serpentine shape) as it passes through the two thermal zones. Droplets spend ~15 s in the 95 °C denaturisation zone and ~45 s in the 60 °C annealing zone. The instrument platform is optimised for Life Technologies Taqman chemistry which eliminates the need for a 72 °C extension zone as is typical with conventional thermal cyclers. The droplets then exit at the outlet.

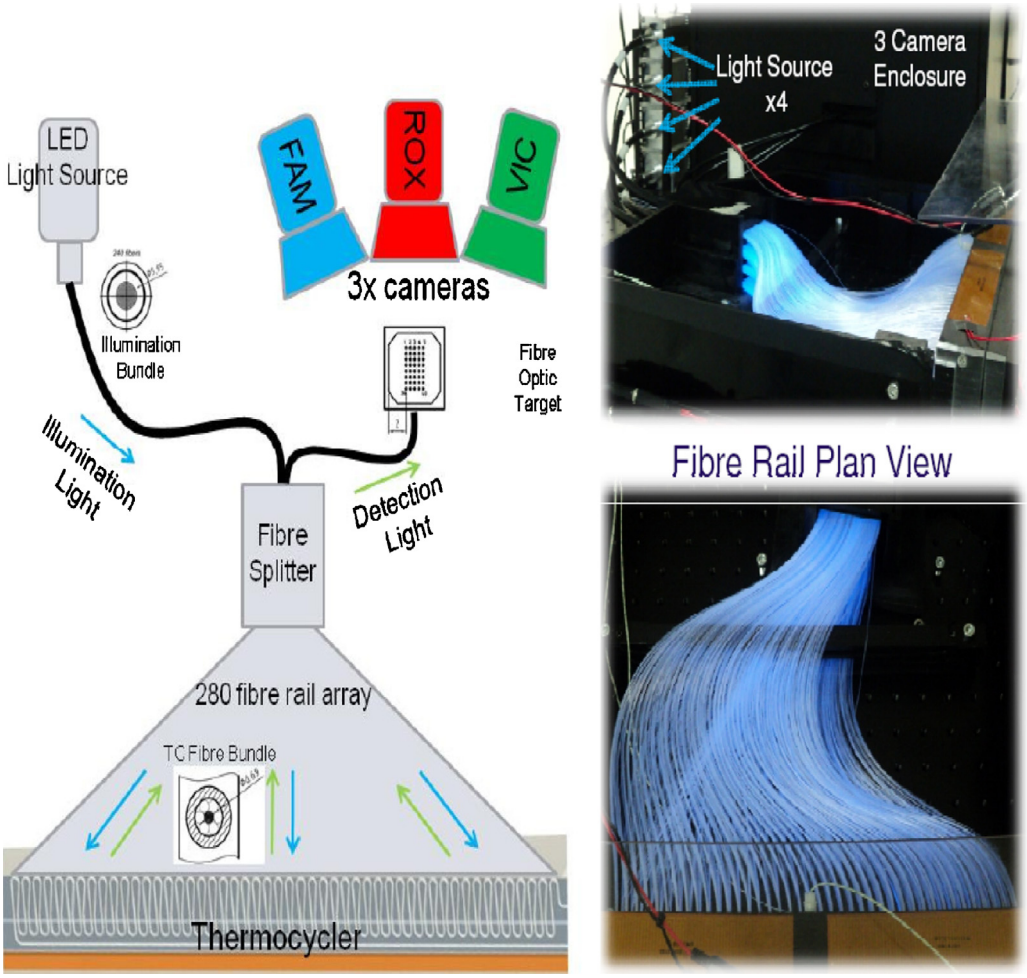

Fig. 5. Illustration of the optical detection platform showing the individual components designed for optimum fluorescence analysis of each micro-droplet as it is conveyed through the system. The design and orientation of the fibre bundle can be seen where the illumination lights are radially positioned around a central detection core. The LED light source, fibre splitter, tiled array and high speed detection camera can also be seen.

heating block has not been observed and fluctuations are monitored by four integrated thermocouple wires (k-type) along the heating blocks. This indicates that employing a high heat conducting material and efficient thermal insulation provides a homogenous temperature over the block. The droplets are fluorescently interrogated on each cycle by the optical detection platform.

2.4. Optical detection platform

The bio-fluorescence detection platform (Fig. 5) analyses the microfluidic reactor droplets with superior accuracy, sensitivity and efficiency for the qPCR in comparison with current methodologies. In this system, qPCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of a specified wavelength and detect the fluorescence emitted by the excited fluorochrome (Table 1).

Table 1
Spectral intensities of the three fluorophores used on the instrument for calibration and normalisation during experiments.

Dye	Excitation maximum (N m)	Excitation minimum (N m)
FAM	494	518
VIC	538	552
ROX	587	607

The detection system is capable of simultaneously detecting, tracking, exciting and detecting three different fluorophores from each individual droplet. To do this, back reflectance probes are integrated into the thermal cycler at 40 locations throughout one length of travel of the capillary through the unit. Each probe, along the 40 points, consists of seven highly sensitive optical fibre cores each measuring 200 μm in diameter. To illuminate the droplet as it passes the probe, six fibre cores are arranged in a circular orientation to fully disperse the light throughout the droplet ensuring maximum illumination. A blue light-emitted diodes (LED) (470 nm) is implemented as an excitation source and this emits at a specific wavelength to excite the fluorochrome being used. The central single core, also 200 μm in diameter, detects the fluorescence from the each droplet as it passes the fibre. In total, forty excitation/detection fibre combinations are distribution per line and arrayed into a coherent bundle to a fibre splitter. Fluorescence emission is then transmitted back to a tiled array (Fig. 6) into which the fibres are positioned in a matrix orientation where it is analysed by three high-resolution digital cameras (Hamamatsu Digital CCD, ORCA-03G) which each have a resolution of 1.37 million pixels and are capable of capturing at a frame rate of 43 frames/s. The cameras are calibrated to analyse individual fluorescence using a signal intensity mapping system (set to detect 6-FAM, VIC and ROX dyes) enabling multiplexed analysis for the targets within the sample. Output signals are recorded and processed with a computer using in-house written software described in the next section.

Fig. 6. High speed cameras (1/3 shown) are focused on the tiled array to detect fluorescence from the dyes. The application of masks over the matrix of fibres determines the focus of the cameras ensuring precise fluorophore variations.

2.5. Data analysis modelling

Unlike end point PCR, real time PCR allows quantification evaluation at any point in the amplification process. To quantify the fluorescent intensity signal from the reaction relayed to the tiled array, the three detection cameras are calibrated to detect at a set fluorescence for each of the dyes in the reaction. Modelling software analyses the raw fluorescence (Rn) from the 6-FAM reporter and this is then normalised against the ROX reference dye. The fluorescence produced by the sample which is detected by the system is proportional to the amount of initial targets for the amplification reaction [52]. The passive ROX dye is found in mastermixes and acts as an internal reference to normalise fluorescence signals. Normalisation is required to correct for fluctuations from droplet to droplet caused by changes in the concentration of the reaction or overall volume. Also the ROX signal normalisation corrects variations in optical detection scanning. The resulting signal (ΔRn) is plotted against the number of cycles on a logarithmic scale. A threshold for fluorescent detection is set above the background levels and the number of cycles necessary for the fluorescence to exceed the background level is called the Ct (threshold cycle) or, as described in the MIQE guidelines [53], the Cq (quantification cycle). The Cq is determined from a log-linear plot of PCR signal versus the cycle number. The background levels are normally determined from the average of the first no-template-control (NTC) droplet in a run. This is subtracted from the subsequent ROX normalised value. Once the signal passes the Cq then, in theory, the sequence of the DNA target doubles every cycle but is dependent on the efficiency of amplification. For the experiment outlined in this paper, threshold values of 0.1 and 0.2 were analysed. For studies of groups of genes carried out on the platform, the differences in gene expression levels are calculated using the $2^{-\Delta\Delta Cq}$ method where the amount of target is normalised to the endogenous control and relative to the calibrator.

3. Device characterisation

The main components for each of the PCRs are the Taqman Gene Expression Master Mix and the Taqman 20× Gene Expression Assays (Life Technologies, Carlsbad, CA, USA). The Master Mix contains NTPs, magnesium chloride, ROX passive reference dye and buffer. The gene expression assays contained the forward and reverse primers and the sequence specific probes. Microplates (Greiner Bio 384-well flat clear bottom polystyrene) which have volumetric capacity of 112 μl were used. To permit continuous sampling of multiple wells without air ingress, the plates were modified by milling out a section which reduced the well capacity to approximately 40 μl. With this, the dipping head can withdraw sample from one well, lift out of the well to an oil overlay and access another well. cDNA dilutions were prepared and then overlaid with the silicone-based oil in the remaining well volume that prevents the sample from being environmentally contaminated and also act as a reservoir for fluid circulation during experimentation. This continuous oil flow into the system also prevents the unwanted introduction of disturbing air bubbles into the system. The MasterMix and Assay combination were prepared in a second microplate and are placed on the instrument plate holding carriage. Volumes can be modified to suit individual experiments depending on genetic focus and volumes available. The dead volume or volume that cannot be aspirated from the bottom of the well, for each experiment is 10 μl. This results in a usable volume of 10 μl (10,000 nl) available on the cDNA sample side and 20 μl (20,000 nl) available on the assay-mastermix side. Using the volumes mentioned allows for 100 replicate experiments to be carried out on a single gene-assay expression analysis. This is a significant reduction in comparison to the commercial instrument (AB 7900 HT) volume where 50 μl cDNA sample, 25 μl of assay, 250 μl of mastermix would be required for approximately 100 reactions. For the experiments in this paper, 20 μl combined volume of cDNA sample and H_2O (6 μl:14 μl) and 30 μl combination of assay-mastermix-H_2O (2.25 nl, 22.5 nl, 5.25 nl) were prepared. The instrument dipping heads move into position and the sample and reagent aspirating tips, initially located in the oil reservoir at the upper part of the plate, are moved vertically to the bottom of the defined well(s), n, and volumes of 35 nl/100 nl of sample/H_2O and 15 nl/150 nl of assay/Mastermix combination are aspirated into the system. Replicates are performed and the tips are then moved vertically from the well plate and displaced laterally to the next sample or assay well, $n+1$. Meanwhile, the individual droplets combine at the liquid bridge to form a 300 nl reaction before being conveyed to the heating section for thermal cycling and optical detection system for analysis. Droplets are continuously being generated and combined into individual reactions which emphasis the high throughput aspect of the system. Also included in each run are no template controls (NTCs) at the beginning and end of each of the replicates to ensure there is no system contamination.

4. Results

The PCR mixtures used Taqman® assays. Taqman® assays are probe based assays, permitting sequence specific fluorescence detection. Firstly, to demonstrate the developed instrumentation and limits of overall system performance, an initial evaluation was carried out to determine the expression of a candidate internal control gene at various concentrations. Complimentary DNA (cDNA) synthesised (using kit manufacturer protocols) [54] from a colon carcinoma epithelial cell (HCT116) population was assessed for β_2-Microglobulin (β2M) (Reference Sequence: NM_004048.2) expression. β2M is an endogenous control assay which allows relative gene expression quantification in cDNA samples when used with other gene expression assays. Expression data from genes of interest are normalised against reference genes to correct for the initial amount of starting material in order to determine expression differences with disease or in response to treatment [55]. However, reference gene expression may vary depending upon the cell type analysed and experimental conditions [56]. To accurately determine the efficiency of a PCR in the system, a four/five log dilution

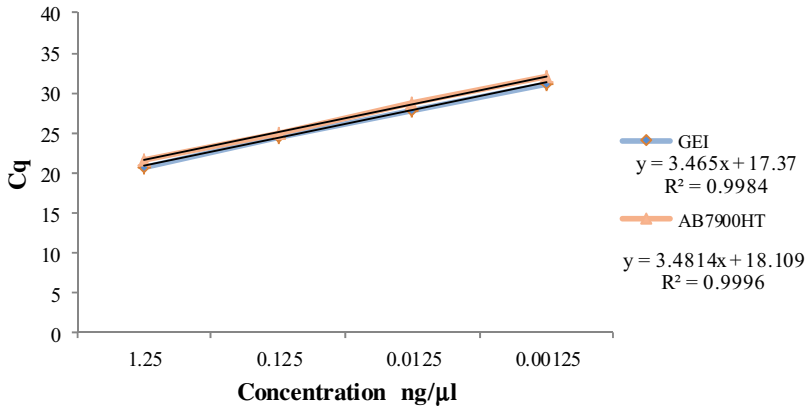

Fig. 7. Real-time PCR standard curves: the real time PCR standard curve is graphically represented as a semi-log regression line plot of Cq value vs. log of input nucleic acid.

Table 2
Ct values for RACK1 and PGK1 expression in HCT116 colon cancer cell line cDNA. Averages of the replicates (AB7900HT three-well average and GEI triplicate average across the four instrument lines) were used when comparing instruments.

	Instrument							
	AB7900HT	GEI						
	μ (3-well)	Line 1	Line 2	Line 3	Line 4	μ (4 lines)	σ (4 lines)	CV on Ct (%)
RACK1	18.56	19.8	20.1	19.2	19.5	19.65	0.3354	1.71
PGK1	20.61	21.8	22	21.8	21.5	21.77	0.1785	0.82

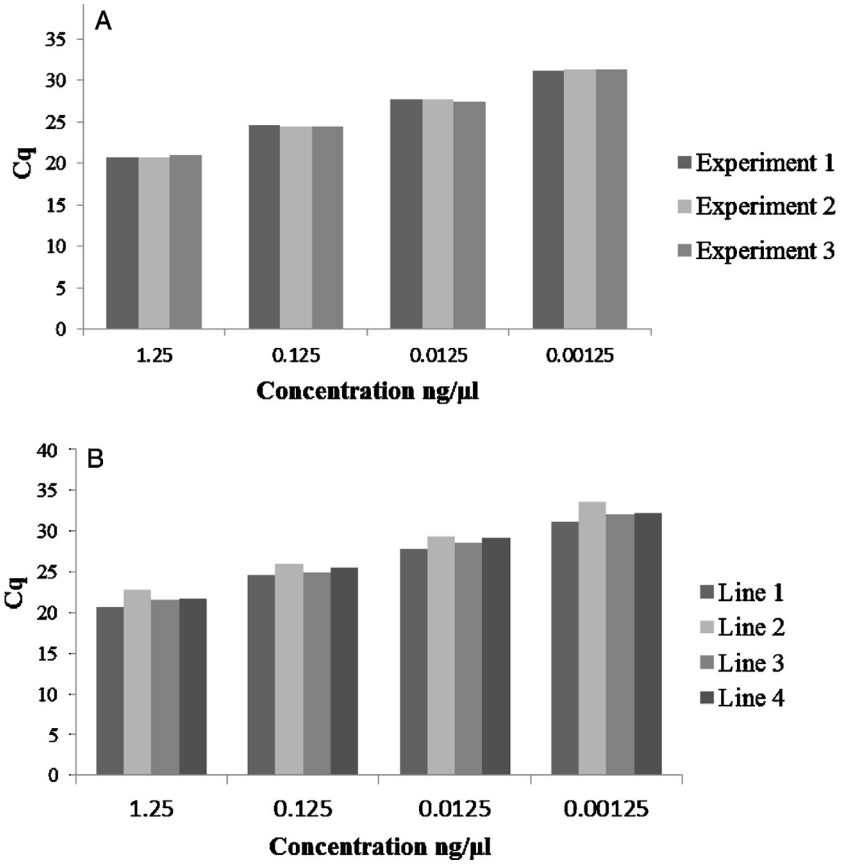

Fig. 8. HCT116 cDNA vs. β2M expression tests. (A) Graphical representation of data across one instrument test line for four dilutions repeated three times. (B) Repeated serial dilution using matching sample and reagents across the four lines on instrument showing minimal line to line variance. The four lines allows for a variation of experiments to be carried out in parallel. The instrumentation can be up scaled or downscaled to modify the number of lines depending on individual experimental requirements.

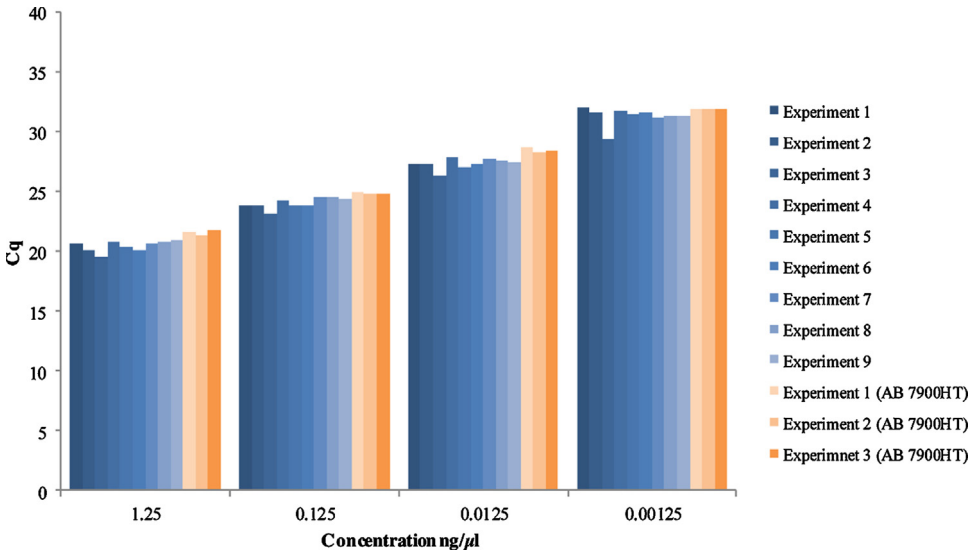

Fig. 9. Reproducibility tests performed on instrument showing Cq values generated for β2M expression serial dilution in a HCT116 colon cancer cell line. The experiment was repeated nine times (experiments 1–9) on the GEI and the Cq values generated show concordance over the nine tests. Experiment 3 (GEI) produced a reduced value due to an incorrect setting being applied to a camera in the optical detection platform. This cumulative dataset for the nine tests can be compared to three tests using the same cDNA, assays and mastermix ran in parallel on the AB7900HT. The results again show good concordance and minimal differences in Cq values.

series was performed. A slope of 3.32 ± 10% reflects an efficiency of 100% ± 10%. A PCR with lower efficiency will have lower sensitivity. Here, serial dilutions were in 10-fold reductions from 1.25 ng/μl to 0.00125 ng/μl. Cq values for β2M which are obtained corresponded well with the data from the commercial instrumentation (Fig. 7). The value for two experiments can be seen to be +5% of 3.32. Another critical parameter that was assessed is the R^2 values generated for PCR efficiency. R^2 values indicate good PCR reproducibility in both experiments. When the R^2 is 1, the value of Y (Cq) can be used to accurately predict the value of X. An R^2 value = >0.99 provides good confidence in correlating two values.

Further serial dilution tests assessing possible line to line variance of the developed instrumentation in comparison to the AB7900HT wells are shown (Fig. 8). Experimental precision was measured from the standard deviation of the results generated from the instrument. To properly evaluate the reproducibility of the serial dilution experiment, it was repeated nine times using the same cDNA, reagents and mastermix on both instruments. Results for a series of experimental tests run on the instrument with the same procedural set up are shown in Fig. 9. Reproducible amplification, no cross-contamination and detection of low concentrations were demonstrated on numerous consecutive sample drops. Amplification curves for this experiment are found in Supplementary Information 1.

Subsequent experiments targeted the Receptor for Activated C Kinase 1 (RACK1) (Reference Sequence: NM_006098.4) and Phosphoglycerate Kinase 1 (PGK1) (Reference Sequence: NM_000291.3) genes, again using cDNA synthesised from the same colon carcinoma cell line (HCT116) as the template and Taqman® chemistry for detection. RACK 1 was chosen for this experiment because it has been shown [57] to be expressed intensely in colon cancer cells and PGK1 because of its stability showing no or only minimal variations in expression levels in similar experiments [58]. Results comparing the expression of these genes, compared to concurrent tests on the AB7900HT using identical experimental preparation conditions, can be seen in Table 2. The mean (μ), standard deviation (σ) and coefficient of variation (CV) across 4 lines are also shown. The closeness of data points to the mean affect the standard deviation. Experimental amplification plots are available in Supplementary Information 2 and 3.

5. Discussion

Gene expression profiling using microfluidic technologies are and innovative and promising area for molecular research. Microfluidic tools are being increasingly used in major biological studies for analysing a large number of samples simultaneously while discreet controls within the system allow for a stable micro-environmental condition to be maintained throughout the process. Along with allowing sensitive and robust analysis at lower cost, microfluidics also offers several superior tools with regard data output rates. Standard current throughput for the instrument at 25 second dips over 4 lines equates to 576 reactions per hour. For a standard operator 8 h working day, this will allow a single operator to produce data for 4608 reactions. This amount of reactions is equal to 12 standard 384 plates at drastically less cost in a shorter time frame. Reducing the time between dips will increase the overall instrument throughput. For the study presented, data provides good accuracy and repeatability and shows a clear, concise fold change over the 4 order of logs for the β2M amplification.

Ultimately, this technology may represent a useful tool for carrying out large scale gene expression profiling of samples of various carcinomas and pathological diseases. Each sample could be assayed for gene expression panels specific to the carcinoma and statistical analysis of combined patient +/ expression data could output a small set of genes for the purpose of developing robust diagnostic and prognostic tools such as prediction of the diagnostic category of patients or the estimation of survival. In comparison to the AB7900HT, the instrumentation described has distinct advantages. DNA sample volumes required are significantly reduced which is becoming an increasingly important factor in molecular diagnostics due to the shortage of high-quality tissue samples. Also each reactor droplet uses less reagents and master mix leading to less overall cost per analysis and allows much more experiments to be carried out per batch order (Table 3).

Additionally experimental times are much lower and the instrument can carry out many more experimentation reactions in comparison to a standard well-format run in the same time frame. The advantages of microfluidics for gene expression analysis are obvious. Compared with typical pipette-based lab-scale equipment

Table 3
Typical volumetric reductions for reactions are shown with a focus on total volume reaction, sample, reagent, mastermix and water use for both instruments.

	GEI	AB7900HT	Change per reaction
Reaction	300 nL	5 μL (5000 nL)	−4700 nL
Sample	35 nL	500 nL	−465 nL
Assay	15 nL	250 nL	−235 nL
MasterMix	150 nL	2500 nL	−2350 nL
H_2O	100 nL	1750 nL	−1650 nL

that use multi-microlitre to millilitre volumes of fluid [59], the microfluidic instrument described is optimised for nanoliter volumes. In addition, flow within the microtube is laminar rather than turbulent due to its size and thus favours a highly predictable and controllable flow. The droplets can be moved, mixed, monitored and then disposed of with the minimum risk of carry over contamination. These are valuable attributes for a high throughput genetic diagnostics system, where the screening of hundreds of discrete samples is required. Overall the system is an example of a droplet based platform that has efficiently integrated large numbers of functional components within a single instrument to perform complex molecular diagnostic experiments.

6. Conclusions

The use of microfluidics for genetic analysis and gene expression studies has advanced substantially in recent years. Here, a microfluidic gene expression instrument that performs real-time qPCR in a continuous flowing process and demonstrates the highest quality results using nanoliter volumes is described. The technology produces more data at substantially less cost in a shorter time frame than comparable market instrumentation. Also, in comparison to microarrays and lab-on-chip technologies that only provide a single function, this microfluidic system provides an integrated platform that has all the necessary components to analyse the gene expression levels from sample to results. A variety of experimental processes can be performed which benefit from the high level of control over the droplets within the microfluidic system and the ability to perform multiple reactions using a variety of assays and reagents in a continuous, contaminant free environment using interspersed dipping programmes. Using small volumes enhances the overall instrument sensitivity and expedites the assay performance while independent robust micro-droplets conveyed by precise fluidic control prevent sample to sample contamination or sample dilution. Automated, continuous instrument control reduces contaminate risks and experimental times and significantly reduces overall labour and process costs. In conclusion, droplet based microfluidic technology represents a promising tool that is likely to transform the way scientists address large scale experimentation. It is hoped that the instrumentation outlined will assist in the development of further microliter and nanoliter microfluidic platforms which will progress molecular diagnostic advances in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bdq.2015.04.003

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