

Droplet microfluidic platform for fast and continuous-flow RT-qPCR analysis devoted to cancer diagnosis application

I. Hajji^{a,b}, M. Serra^{a,b}, L. Geremie^{a,b}, I. Ferrante^{a,b}, R. Renault^{a,b}, J.-L. Viovy^{a,b}, S. Descroix^{a,b}, D. Ferraro^{a,b,c*}

^a Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, CNRS UMR168, Paris, France

^b Institut Pierre-Gilles de Gennes, Paris, France

^c Dipartimento di Fisica e Astronomia G. Galilei, Università di Padova, via Marzolo 8, 35131 Padova, Italy

*corresponding author: Davide Ferraro (davide.ferraro@unipd.it); Stephanie Descroix (stephanie.descroix@curie.fr)

Abstract

RT-qPCR represents a key method in cancer diagnostic, however the constant increase in patients and cancer biomarker panels to screen requires the implementation of faster approaches allowing smaller reagents and samples volumes consumption. To fulfil these needs, we present here a fully automated droplet microfluidics platform that couples a specifically designed thermal system with a fluorescent excitation/detection module. Additionally, the droplet generation and merging approaches allows the analysis of multiple samples and genes with no risk of contamination. This platform has been initially validated by investigating HER2 overexpression in two cancer cell lines starting from total RNA samples; these results have been successfully compared with those obtained by a commercially available machine, showing no limitation in terms of number of processed samples as well as reducing 200 times the analysis costs per patient. Finally, we have demonstrated its capability in performing fast RT-qPCR, raising the throughput of analysis to a hundred samples in less than 20 minutes.

1 Introduction

Recent progresses in genomics led to a deep knowledge of tumour cells genetic characteristics, allowing to identify mutations specific to a particular cancer subtype and, in some cases, to devoted therapies[1]. This approach, defined as precision medicine[2] by the Food and Drug Administration (FDA), is nowadays a common practice in cancer diagnostics and clinics. A typical example for this methodology is the status of HER2 (Human Epidermal growth factor Receptor 2) gene in breast cancer, which presents an overexpression (HER2+ status) in 15-20% of primary breast cancers[3] and is associated with an aggressive tumour. However, recently the patients' outcome has been drastically improved by the administration of targeted treatments (e.g. Trastuzumab and Pertuzumab)[4].

The number of identified mutations is continuously increasing, thus the physicians must deal with larger gene panels to be analysed requiring higher sample amount, as well as larger reagents volumes leading to higher costs. To face these needs, new technologies must be developed allowing fast and multigene analysis of patient tumour while consuming a minimal amount of biological material. Current gold standards to achieve precision medicine diagnosis of breast cancer are Immunohistochemistry (IHC), Fluorescent In Situ Hybridization (FISH) and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)[5]. All these techniques are based on the

38 screening of specific proteins or nucleic acids expressed in the cancer cells, which are known to be markers for a
39 specific cancer subtype or associated with potential targeted therapy. Despite RT-qPCR methodology is quantitative,
40 highly reliable and more labour-saving than other strategies[6], it remains rather expensive, notably due to the cost
41 of reagents and because all the PCR-based diagnosis approaches require rigorous procedures and environments, in
42 order to avoid contamination[7,8] and sample degradation, which is particularly critical for RNA analysis[9].
43 Therefore, the implementation of these bioanalytical methods in a microfluidic format has a great potential to
44 overcome current limitations, as already attested by the approaches developed so far. The pioneer work of Quake et
45 al.[10] presented a system for quantitative RT-PCR based on integrated PDMS valves, currently commercialized as
46 IFC module by Fluidigm. Unfortunately, this disposable and expensive device processes a fixed number of samples
47 per run (48 or 96), therefore it is not well-adapted to a clinical environment, where the samples number varies on a
48 daily basis and the cost per run cannot be optimized. Droplet microfluidics[11,12] has the potential to bypass these
49 limitations, guaranteeing stable compartmentalization without complex valve systems and, especially, using reusable
50 devices since the droplets containing the samples never touch and contaminate the channel walls[13].

51 Nowadays, droplet microfluidics is well established for droplet-digital PCR protocols[14–16] (ddPCR), which
52 consists in single molecule or cell encapsulation in droplet, thermal-cycling amplification and final endpoint
53 detection. However, despite ddPCR allows to achieve single molecule analysis, required for example in circulating
54 tumour DNA detection[17], it provides comparable results in term of sensitivity regarding the quantification of the
55 expression level of target genes[18,19] that, as introduced above, represents the key aspect in current diagnostic
56 strategies. Additionally, high level of multiplexing remains rather problematic due to false positive results[20,21].
57 Therefore, considering also its higher cost[18], ddPCR is still limited to research purposes and it is not yet diffused
58 towards clinical laboratories[22], in which, common RT-qPCR technology is widely used. Unfortunately, droplet
59 microfluidic devices devoted to the latter protocol are much less common. This is partially due to some technological
60 challenges: differently from ddPCR, requiring one single endpoint detection[23,24], qPCR needs accurate and
61 comparable fluorescent detection after each thermal cycle. Some examples reporting qPCR in droplets circumvent
62 this issue by parking the droplets in a specific area of a microfluidic chamber[25–29] or on a EWOD device[30],
63 performing the fluorescent analysis on stationary compartments[31]. Such approaches strongly limit the number of
64 processed samples per run. Moreover, these devices are typically coupled with a fluorescent microscope, preventing
65 their use away from an equipped laboratory and increasing the total cost for the experimental setup[25,26,32].

66 An interesting approach has been proposed by Hatch et al.[33] demonstrating continuous flow qPCR in droplets,
67 generated by a T-junction. Despite this work represents the first proof-of-concept in the field, it suffers from several
68 limitations. The sample preparation step is manually performed off-chip, strongly limiting the automation and the
69 eventual capability for multiple gene analysis. Then, besides the large droplet volume (μL range), the optical module
70 shows low excitation light homogeneity, leading to important issues in the fluorescent detection. The latter has been
71 solved by drastically increase the fibers numbers and using high-sensitivity cameras[34], thus increasing the overall
72 complexity and cost of the setup.

73 In this work, we present a droplet microfluidic platform, named Drop-qPCR, in which droplets, transported in a
74 reusable capillary, alternatively flow through two areas kept at different constant temperatures[35], while the
75 fluorescent detection is performed by a devoted system obtained by widespread and low-cost technologies: 3D
76 printing, LEDs and a CMOS USB-camera. Additionally, Drop-qPCR allows automated analysis of different

77 combinations of samples and genes, thus guaranteeing perfect compatibility with conventional diagnostic protocols.
78 In particular, the implemented droplet generation approach allows to sequentially produce pairs of droplets (sample
79 and Master Mix) that spontaneously merge exploiting their difference in interfacial tension[36]. To evaluate the
80 potential of the Drop-qPCR platform, it has been successfully compared with a commercial RT-qPCR equipment,
81 showing the capability of performing analysis with throughput competitive with the current microfluidic ultrafast-
82 qPCR system[32], which however is not based on droplets microfluidics. Finally, this platform implements for the
83 first time the complete RT and qPCR protocol, thus starting from total RNA samples which, as mentioned before,
84 are very delicate and prone to degradation. This represents an important skill for its implementation in routine clinical
85 practice.

86 In the following, we first describe the technological development of the platform and its characterization. Then, Drop-
87 qPCR is validated i) by determining the expression level of the HER2 gene starting from total RNA samples extracted
88 from cancer cell lines and ii) by comparing the obtained results with a commercially available qPCR system. Finally,
89 we evaluate and discuss the analytical throughput achievable by the developed technology, compared to state-of-the-
90 art microfluidic strategies.

91

92 **2 Materials and Method**

93 The Drop-qPCR platform is composed by several modules. Some of them have been developed during this work
94 and will be described in the “Results and discussion” section, while others that are commercial or have already
95 been presented in previously published works, will be introduced in the following.

96 The droplet generation system, presented in[37], is composed by an arm-robot (Rotaxys, by Cetoni) coupled with
97 two syringe pumps (Nemesys, by Cetoni) mounting two syringes (2.5mL and 250 μ L, by SGE) and two
98 customized pinch valves[38]. Aqueous phase droplets are thus generated by pipetting in fluorinated oil (FC40,
99 by 3M) with 2% w/w of a fluorinated surfactant (1H, 1H, 2H, 2H-perfluoro-1-decanol, by Fluorochem) and
100 transported in PTFE capillary (0.3mm/0.6mm inner/outer diameter, by Sigma Aldrich). **Notably, the use of a
101 fluorinated oil (FC-40) mixed with fluorinated surfactant assures a complete wetting of the fluorinated capillary,
102 preventing any cross-contamination during the pipetting[37]. This will be additionally confirmed by the qPCR results
103 from the setup validation.** Liquids used for droplet generation (aqueous and oil phases) are stored in a commercial
104 384-wells microtiter plate (AB1384Y, by Thermofisher).The different parts of the RT and qPCR modules, that are
105 detailed in the Results and Discussion paragraph, have been produced by micro-milling using the Mini-Mill
106 machine (by Minitech) or 3D printing, by using acrylonitrile butadiene styrene (ABS) plastic and DL-260 resin
107 (by DWS Systems). uPrint SE Plus (by Stratasys) and DigitalWax028J+ (by DWS Systems) printers are used for
108 the two materials in order to obtain low and high resolution parts[39], respectively.

109 For temperature control and monitoring, Peltier modules (CP2-127-06L, by Laird Technologies) controlled by
110 three independent PID boards (TC M PCB by Electron Dynamics) are coupled with three thermal sensors (PT100,
111 by RS Pro) placed in correspondence to the heated parts. All the surfaces in contact that require a good heat
112 exchange are firstly covered with thermal paste (by RS Pro). The temperature characterisation is performed with
113 small-sized thermocouples (0.23mm, IT-24P, by PHYMEP) inserted in the PTFE capillary.

14 For the fluorescence detection part, several optical components have been used: LEDs (CP7P-GZHX-1, by
15 OSRAM), aspheric and cylindrical lenses (354140-A and LJ1598L1-A, by Thorlabs), filters (excitation 485/20
16 nm and emission 530/43nm, by Semrock) and a macro-lens (MLV7000, by Navitar) installed on a camera
17 (acA2500-60um, by Basler). The acquisition framerate is set in between 20fps and 40fps according with the
18 applied flowrate, in order to assure a correct visualization of the flowing droplets. The spectral filters are chosen
19 in agreement with the Taqman probe used during the qPCR experiments (FITC filter set).

20 Finally, the total RNA samples used during the RT-qPCR analysis are extracted from two human cell lines SKBR3
21 (ATCC® HTB-30™) and MCF7 (ATCC® HTB-22™) by spin columns method (RNeasy Mini Kit by Qiagen). The
22 Master Mixes, containing primers for the two investigated genes (HER2 and Actin- β , or ACTB) and Taqman probe,
23 are prepared using the CellsDirect™ One-Step qRT-PCR Kit (by Thermo Fisher Scientific) as indicated in ESI (see
24 Note S1), for both in-droplet experiments and commercial qPCR machine (SmartCycler II, by Cepheid). Each RT-
25 qPCR analysis is repeated at least twice for each sample. The kit has been chosen because it allows both RT and
26 qPCR from the same reaction solution. In detail, the PCR requires incubation times at two temperature conditions
27 (denaturation at $T=95^{\circ}\text{C}$, annealing and elongation at $T=60^{\circ}\text{C}$).

28 **3 Results and discussion**

29 **3.1 Drop-qPCR platform development**

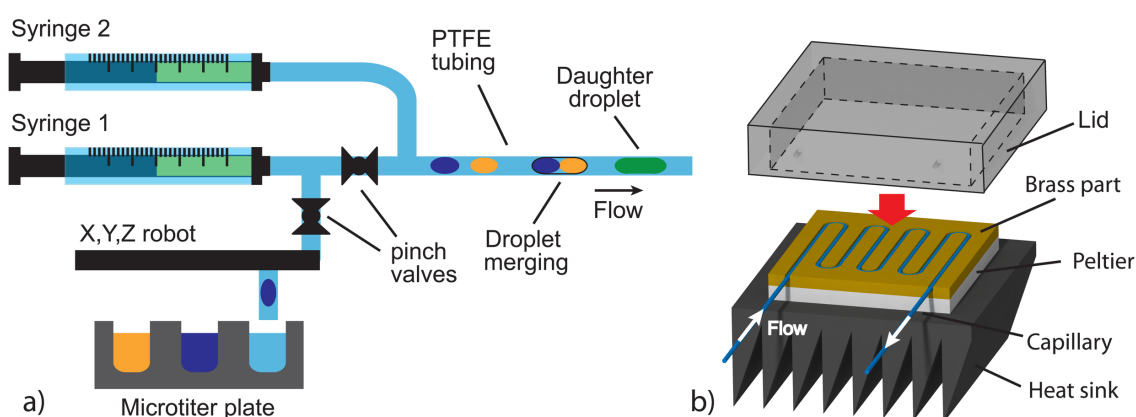
30 The droplet microfluidic platform is composed by three sequential modules for the implementation of three
31 different tasks: i) droplet generation, ii) Reverse Transcription (RT) and iii) qPCR. The first module, already
32 presented in[37,40], allows the pipetting of deterministic trains of confined droplets (between 50 and 300nL,
33 volume polydispersity <2%) from solutions stored in a conventional microtiter plate (see Figure 1a). This
34 approach provides high flexibility in programming the droplet content, which can be tuned by adjusting the
35 pipetting sequence. **Additionally, the microtiter plate and capillary used ensure a minimal waste of reagent that
36 stay on the bottom of the well of 200nL (see Note S2 in ESI).** Moreover, due to a combination of pinch valves
37 and PDMS devices[37], droplets generation can be performed while other droplets previously prepared are
38 flowing in the microfluidic device for the analysis. This allows to achieve a throughput which is not limited by
39 the droplet generation. Additionally, due to the combination of oil and capillary used, possible contaminations
40 between the different wells of the microtiter plate during the droplets sampling are prevented, as demonstrated
41 in[37]. Finally, this system enables decoupling the droplets generation and their processing in the platform,
42 allowing: i) the parallelization between samples pipetting and analysis; ii) the control of the carrier liquid
43 flowrate, keeping constant the droplets size and the production rate.

44 During the experimental protocol, pairs of droplets (volumes of 100nL), containing respectively total RNA
45 sample and RT-qPCR Master Mix, are continuously generated. In parallel, to fulfil the clinical requirements,
46 negative controls are also analysed during the experiments. These can be easily implemented in the Drop-qPCR
47 platform by pipetting a water droplet instead of the total RNA sample. To induce the sample and the Master Mix
48 droplets merging, we have taken advantage of the interfacial tension difference between each droplet and the
49 carrier fluid ($12.8\pm 0.5\text{mN/m}$ and $2.9\pm 0.2\text{mN/m}$, respectively). **As described in[36], when a confined droplet is
50 transported by a carrier fluid, its speed is a function of the interfacial tension (γ) between the two liquids; notably,**

51 lower γ leads to higher speed. Therefore, this effect has been exploited to induce a spontaneous droplets contact
52 and thus, their merging after a constant travelled distance (15cm \pm 1cm, see also ESI in [36]). Notably, being based
53 on the different interfacial tensions of the droplets with the oil phase, this approach ensure 100% merging efficiency,
54 as experimentally observed.

55 After the merging, the resulting daughter droplets flow in the second module devoted to the RT reaction, at a
56 flowrate of 120nL/s. This module is designed to incubate the flowing droplets at a fixed temperature for a desired
57 time, at 50°C for 11 minutes in this case. Therefore, as shown in Figure 1b, we have developed a RT module
58 composed by a brass part presenting a groove on its surface with a serpentine design that acts as capillary holder.
59 In order to assure the desired homogeneous temperature on the capillary, the brass plate is fixed on the Peltier
60 module which is coupled with a heatsink for the temperature dissipation. Finally, an ABS 3D-printed lid is placed
61 all around the module to guarantee thermal insulation from the external environment[41].

62 After the RT, droplets containing the resulting cDNA continuously flow in the third module dedicated to PCR
63 amplification and real-time fluorescence detection. This module allows both temperature cycling and
64 fluorescence measurements after each cycle and for each droplet. These two sections will be separately discussed
65 in the following.



57
58
59 *Figure 1: a) Module for droplet generation and flow (module 1). Two pinch-valves connected with two syringe pumps guarantee a continuous*
60 *pairs of droplets generation starting from solutions stored in a microtiter plate. The first droplet (yellow) contains the RNA sample and the*
61 *second (blue) the Master Mix for the RT-qPCR. After the generation, droplets are transported in the capillary towards the 2nd module of the*
62 *platform; during the flow, each pair spontaneously merges resulting in a daughter droplet (green) ready for the RT protocol. (b) Scheme of*
63 *the RT module (module 2): droplets experience a constant temperature (50°C) flowing in the capillary placed inside the brass part and then,*
64 *they are transported to the 3rd module for the qPCR amplification.*

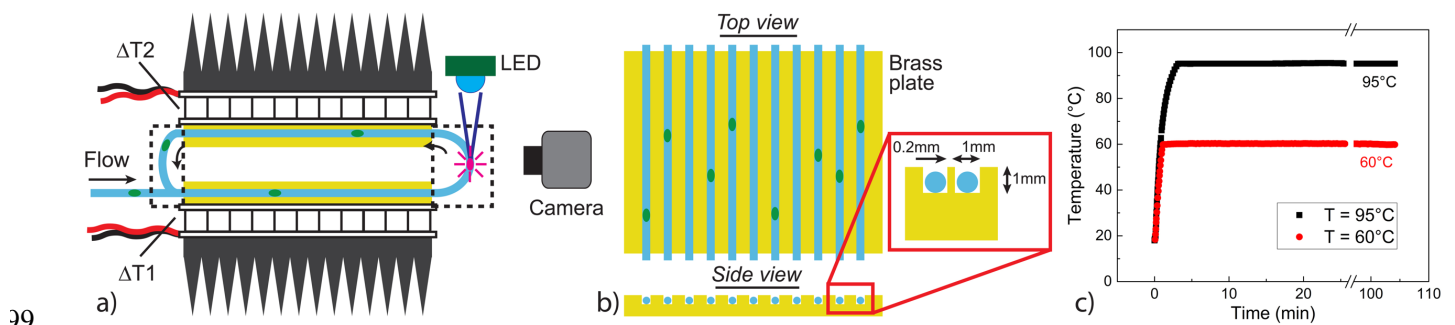
75 **qPCR module: Thermal cycling**

76 As shown in Figure 2a, the third module of the platform is composed by two heating parts facing each other,
77 independently assembled and controlled, as for the RT module. The same capillary (approximately 5m long) is
78 thus rolled-up in between the two parts in order to let the flowing droplets experience two temperatures in
79 alternate manner. This arrangement allows the production of a more compact device than using the common flat
80 configuration[15]. More details about the capillary installation are reported in ESI (see Note S3 and Figures
81 S1,S2). Additionally, in order to guarantee a homogeneous temperature around the capillary, we have designed
82 the brass plates with 45 straight and parallel pockets on the surfaces, having squared cross section (see Figure

33 2b). Then, the two faced heating parts are held together by two screwed 3D printed structures, reported as dashed
34 rectangles in Figure 2a. In detail, a first U-shaped structure (left side in Figure 2a) fixes the plates position and
35 keeps them at a mutual distance of 1cm. Then, a second structure (right side in Figure 2a) presents a comb-like
36 shape in which the capillary is passed through, as shown in Figure 3a. These 45 vertical windows, aligned with
37 the pockets on the brass parts, assure the optical accesses required for the fluorescence measures.

38 Once the thermocycling part is assembled, we have verified the temperature homogeneity and stability by
39 inserting different thermocouples in the capillary hosted by the brass plates. As shown in Figure 2c, the device
40 takes about 4 minutes to reach the desired temperature and presents an average oscillation of 0.2°C. These are
41 crucial requirements for a proper DNA amplification; in fact, enzymes and primers involved in the annealing step
42 are designed to work at specific temperatures and any divergence may lead to low amplification efficiency
43 (<90%)[42,43].

44 Therefore, by keeping the two brass parts at the temperatures required for the amplification protocol, droplets
45 experience the 45 temperature cycles just flowing in the capillary. In detail, the experiment is designed to keep
46 them 25s at both 60°C and 95°C by applying a constant flowrate of 120nL/s, which allows to perform a complete
47 PCR cycle in approximately 1 minute. **These times have been calculated as the ratio between, the width of the**
48 **brass plate multiplied by the capillary section, and the flowrate.**



102 *Figure 2: qPCR module – thermal cycling part. a) The capillary (light blue) is rolled-up in between two brass plates (yellow) kept at defined*
103 *temperatures; b) the plates present straight and parallel pockets holding the capillary in a homogeneous temperature environment. c)*
104 *Temperatures monitoring on both brass plates: denaturation part (95°C, black dots) and annealing-extension (60°C, red dots). These*
105 *measurements are performed by thermocouples placed in a capillary installed in the device and the time=0min corresponds to the device*
106 *starting.*

106 **qPCR module: Fluorescence optical measurement**

107 Typical qPCR curves are obtained in a commercial machine by performing a fluorescence measure of the sample
108 between each amplification cycle. Therefore, the same approach has been applied in the microfluidic device: the
109 detection is done in between every droplet turn in the capillary, which corresponds to a PCR cycle.

110 The fluorescence measure is performed through the observation windows integrated in the 3D printed part as
111 previously described (Figure 3a). More in detail, the excitation light is focused on the capillary's portions passing
112 through the observation windows and the subsequent droplet fluorescence emission is collected by the camera placed
113 in front of it (see Figure 2a). Thus, knowing the droplet generation order, the qPCR curve is easily obtained for each
114 one, by plotting its fluorescence intensity versus the turn (or cycle) number. Notably, camera and macro-lens have
115 been chosen (see Material and Methods) in order to assure a field of view large enough to observe all the 45
116 observation windows in the same frame.

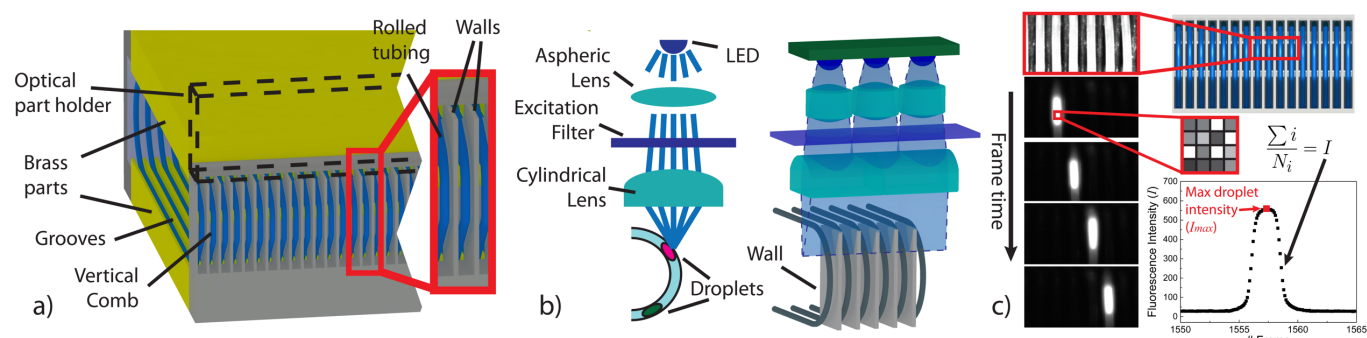


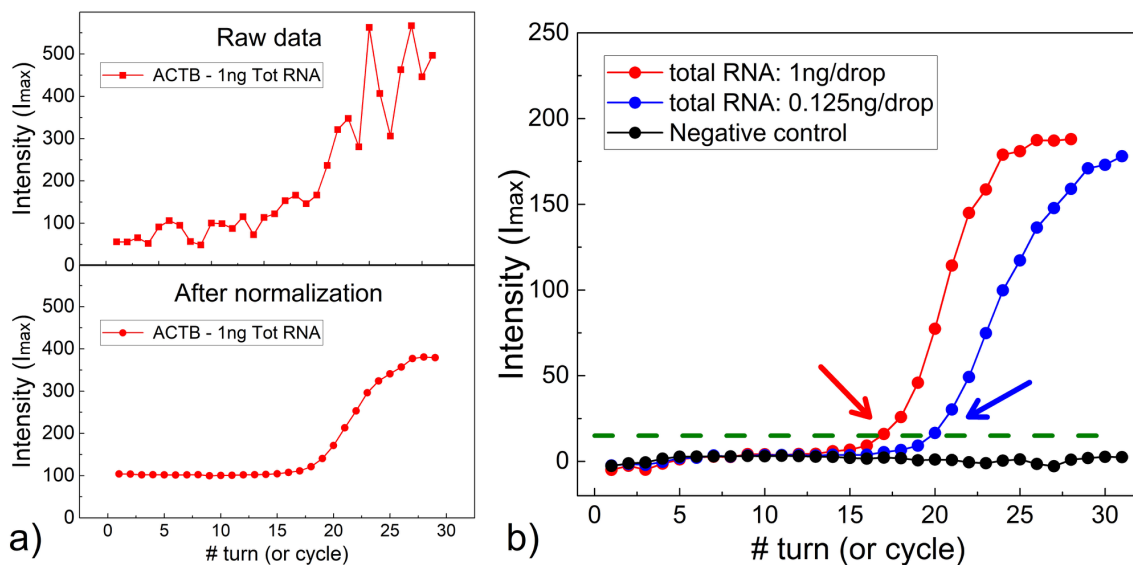
Figure 3: scheme of the module 3. a) A 3D printed comb-like structure (grey) fixes the positions of the capillary on which the optical fluorescence detection takes place. This part is designed to be aligned with the pockets of the brass plates and to avoid light pollution between capillary turns during the detection. b) Scheme of the optical path followed by the LED light in order to obtain a beam homogeneously focused on a line. Optical components are designed to irradiate specific areas of the capillary, which are imaged by an external camera. c) Image processing workflow: for all the images, a ROI is defined for each capillary turn and its average pixel intensity value (I) is constantly monitored; when the droplet crosses the detection window, a peak is observed and its maximum is taken as the fluorescence intensity value of the droplet (I_{max}). To better visualize the droplet in the figure, in the images' sequence, a saturated fluorescent image has been considered. See Movie S1 to view an example of an acquisition of a quantitative experiment.

17 Since the fluorescence emission is proportional to the excitation light intensity, in order to compare the signals
 18 coming from the different observation windows, thus from different PCR cycles, a homogenous irradiation is
 19 required. This has been achieved by combining a custom-made optical setup and a post-processing signal analysis
 20 algorithm. In detail, the setup is composed of a series of optical components aligned by a devoted micro-milled
 21 structure, which is installed on top of the comb-like structure (see dashed line in Figure 3a and Figures S3,S4 in ESI).
 22 As reported in Figure 3b, the light emitted by an array of 13 LEDs (peak emission centre 465 nm) is collected by
 23 13 aspheric lenses; each LED-lens couple is placed in contact between each other in order to collect all the light
 24 emitted by the LED. Being this distance smaller than the focal length of the aspheric lenses used (1.45mm), a
 25 divergent light is obtained which is, at first, narrowed by a FITC excitation filter (485/20 nm), then focalized by
 26 cylindrical lenses. The distance between the aspheric and cylindrical lenses has been experimentally optimized
 27 at 4.5 mm, in order to collect all the light. Therefore, this optical structure is designed to collect the initial LED
 28 light and to produce an elongated and homogenous beam, minimizing light loss, as typically obtained by using
 29 conventional diffuser. Finally, an emission FITC filter (530/43nm) is installed in between the macro-lens and the
 30 camera, to increase the signal/noise ratio. In this way, it is possible to continuously record the entire region
 31 exposed to the excitation beam and to visualize the fluorescence emission of the droplets flowing in the capillary
 32 (see Movie S1 in ESI). Importantly, since every portion of the capillary is embedded in the comb-like structure
 33 (see Figure 3a), the walls act as optical insulators between the different observation windows (pictures of the
 34 setup are reported in Figure S4 in ESI).

35 The acquired images are then analysed by a dedicated Matlab program. In each frame, 45 regions of interest
 36 (ROIs, $170 \times 170 \mu\text{m}^2$) are univocally associated to each of the 45 capillary portions, corresponding to the 45
 37 amplification cycles, as shown in Figure 3c. Therefore, for each frame the system allows to monitor in parallel
 38 all the ROIs by measuring the fluorescence signal; the average pixel value (I) in the ROI is plotted as function of
 39 the acquisition time. Initially, a constant background value is measured, then, when the droplet crosses the ROI,
 40 a peak is observed as shown in Figure 3c and in Movie S1 (see ESI). The peak maximum I_{max} is the representative
 41 value for the fluorescence intensity of a droplet in a given ROI, thus for the corresponding thermal cycle. Notably,

42 by using a Taqman probe, which presents an initial detectable fluorescence signal, all droplets are discerned
 43 starting from the first cycle therefore, being confined and preserving their generation order, they are also
 44 univocally identified by their ordinal number.

45 In order to evaluate the homogeneity of the excitation/emission light, a droplet containing a fluorescence
 46 calibration solution (400nM Taqman probe) is flown in the capillary recording its emission intensity from each
 47 ROI. A light uniformity higher than 82% is measured (see Figure S5 in ESI). Despite this represents a good result
 48 that does not involve customized devoted lenses[44,45], it is found to be not high enough to compare the
 49 emissions from the different 45 capillary's portions. However, since the unwanted light modulation represents a
 50 systematic error, a signal analysis post-processing has been applied to compensate this effect. In details, the signal
 51 from each ROI is systematically normalized by the emission intensity measured by the calibration solution, on
 52 the same ROI. In detail, 5 droplets of the calibration solution are flown in the capillary and the fluorescent signals
 53 from each ROI are acquired; the average values are then used to normalize all the measured intensities from the
 54 corresponding ROIs. Figure 4a reports two examples of qPCR curves before (top) and after (bottom) the
 55 normalization, respectively. Therefore, by plotting the normalized droplet intensity as function of the ROI number,
 56 we can achieve a conventional qPCR amplification curve of the corresponding sample. Finally, from this type of
 57 curves, the cycle threshold (or Ct) associated to the analysed sample can be easily extracted as for standard qPCR
 58 machines: the background probe signal is subtracted, resulting in curves aligned to the zero intensity, then the
 59 fluorescence threshold is chosen within the range of exponential phases of the amplification curves[46–49]. As a
 60 first proof of concept, Figure 4b reports data obtained with the Drop-qPCR platform for a sample of total RNA
 61 extracted from SKBR3 cell line and a negative control. These results prove the feasibility of the Drop-qPCR
 62 approach both in terms of DNA amplification and fluorescence detection. **Finally, since the number of cycles**
 63 **required for observing the qPCR amplification curve depends on the initial quantity of targets, if less than 45**
 64 **cycles are sufficient, the unnecessary last ROIs can be simply ignored during the data analysis, without requiring**
 65 **any adjustment in the setup nor extending the duration of analysis.**



56
 57 *Figure 4: a) Plot of the fluorescence intensity (I_{max}) for each droplet as a function of the capillary turn number, corresponding to the*
 58 *amplification cycle, before (top) and after (bottom) the normalization; data are related to ACTB gene of Total RNA sample (1ng) from MCF7*
 59 *cell lines (red). b) Examples of normalized and shifted qPCR curves obtained for the ACTB gene from the total RNA samples extracted from*
 60 *MCF7 cell lines at 1ng/drop (red dots) and 125pg/drop (blue dots), and negative control (black dots). The intersections between the threshold*
 61 *(green dashed line) and the amplification curves give the Ct values for each sample (red and blue arrows).*

73 3.2 RT-qPCR analysis

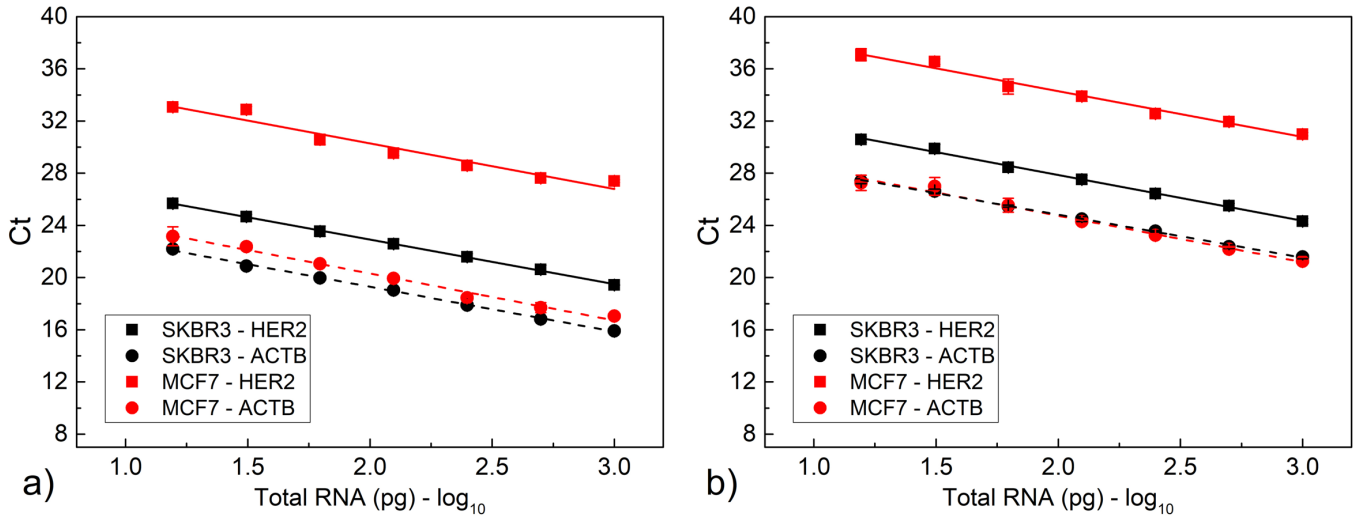
74 To further evaluate the Drop-qPCR platform, analyses have been performed using total RNA samples extracted
75 from two cell lines: SKBR3 and MCF7, that are known to overexpress and non-overexpress HER2,
76 respectively[37]. In detail, these samples have been analysed in order to obtain calibration (or standard) curves
77 for this gene, which is particularly relevant for breast cancer, using ACTB as reference gene. Such dual gene
78 analysis allows to analyse the obtained data by the ΔC_t method[50], which is typically applied to compare the gene
79 expression across different samples or experimental conditions. Notably, for each calibration curve the droplets
80 train is composed by 8 pairs of droplets: 7 for the two-fold dilutions of RNA (between 1ng/drop and 15pg/drop)
81 and one negative control (DEPC-treated water). This range of concentration is lower than the typical quantities used
82 for routine quality check of clinical samples (6 to 25ng per assay)[37] and the lowest calibration point (15pg/drop)
83 corresponds to the average total RNA amount in a single cell.

84 Figure 4a reports the calibration curves for HER2 and ACTB genes in SKBR3 and MCF7 cell lines obtained using
85 the Drop-qPCR. Each data point is the average result of the repeated experiments and the corresponding standard
86 deviation is considered as the error bar. Coefficients of variation (CVs) are found always lower than 2.5%, showing
87 an excellent repeatability. In order to evaluate the performance of the Drop-qPCR approach, the fit parameters, the
88 coefficient of determination R^2 , as well as the resulting PCR efficiencies, are summarised in Table 1. The calibration
89 curves obtained for the total RNA extracted from the two cell lines (Figure 4a and Table 1) present a linear trend
90 ($R^2 > 0.95$) and amplification efficiencies higher than 90%. These aspects are particularly important since they
91 demonstrate that: i) the device design guarantees optimal and homogeneous thermal actuation and reliable
92 fluorescence detection; ii) the results for the two investigated genes, can be analysed by ΔC_t method. In fact, the
93 latter can be applied only if the amplification efficiencies of the genes are comparable. Figure 4a also shows that the
94 platform allows to clearly distinguish between 2-fold serial dilutions, which corresponds to the resolution limit of
95 conventional qPCR assay[51]. Furthermore, considering the gene of interest for clinics (HER2), we observe that the
96 MCF7 curve is shifted upward with respect to the SKBR3 one, indicating the expected overexpression of SKBR3.
97 This is also shown by the resulting ΔC_t values reported in the Table 1 ($\Delta C_{t_{MCF7}} > \Delta C_{t_{SKBR3}}$), confirming that Drop-
98 qPCR allows to clearly differentiate between the two sample populations whatever the initial quantity of mRNA and
99 down to single-cell level. **Finally, since experiments are performed pipetting from the highest to lowest
100 concentrations, plus a negative control, these results confirm that any cross-contaminations between the wells of the
101 microtiter plate or droplets happened.**

102 Furthermore, the performances of our device have been further evaluated by doing a systematic comparison between
103 the data obtained by the Drop-qPCR and a commercially available qPCR system (SmartCycler II, by Cepheid). This
104 comparison has been performed in the same conditions: genes, cell lines and initial quantity of total RNA. The
105 obtained results show that the data by the Drop-qPCR platform and the SmartCycler are perfectly comparable (see
106 Figure 4b and Table 1). In fact, both PCR efficiencies and ΔC_t values for the two cell lines correspond within the
107 propagated errors. This demonstrates that the droplet microfluidic platform provides an accurate quantification as the
108 commercial system, despite the cost reduction in terms of reagents (Master Mix volumes reduction of 200 times).
109 Finally, comparing Figures 4a and 4b, a decrease of about 6 Ct values is observed using the droplet platform. Since
110 the fluorescence intensity of a molecule is proportional to its concentration, this effect is probably due to the total

11 RNA confinement in droplet, as already reported in micrometric size reaction chamber[52,53]. This may lead to the
 12 capability in detecting gene typically poorly expressed.

13



14

15 *Figure 5: calibration curves obtained using the (a) droplet microfluidic device and the (b) commercial system (SmartCycler), using Total RNA*
 16 *sample extracted from MCF7 (red dots) and SKBR3 (black dots) cell lines, to determine HER2 (squared dots) and ACTB (circle dots)*
 17 *genes expression level. Error bars are not visible if smaller than associated data point. The solid and dashed lines are the linear fits of data related*
 18 *to the HER2 and ACTB genes, respectively. The parameters of the fits are reported in Table 1.*

19 *Table 1: Comparison of the results obtained in the droplet microfluidic platform and in the conventional qPCR machine. Investigated factors*
 20 *include fit parameters (Slope, Intercept and R²) of curves reported in Figure 4. PCR efficiencies and ΔCt.*

			Slope	Intercept	R ²	PCR Eff.	ΔCt
Drop-qPCR	SKBR3	HER2	-3.42±0.04	28.75±0.08	0.998	96%	3.6±0.2
		ACTB	-3.44±0.06	25.18±0.14	0.997	95%	
	MCF7	HER2	-3.49±0.35	36.29±0.76	0.946	93%	9.8±0.8
		ACTB	-3.60±0.17	26.50±0.36	0.986	90%	
SmartCycler	SKBR3	HER2	-3.51±0.09	34.89±0.20	0.996	93%	3.5±0.2
		ACTB	-3.30±0.07	31.42±0.14	0.998	101%	
	MCF7	HER2	-3.51±0.21	41.30±0.45	0.980	93%	9.4±0.6
		ACTB	-3.56±0.17	31.86±0.37	0.987	91%	

21

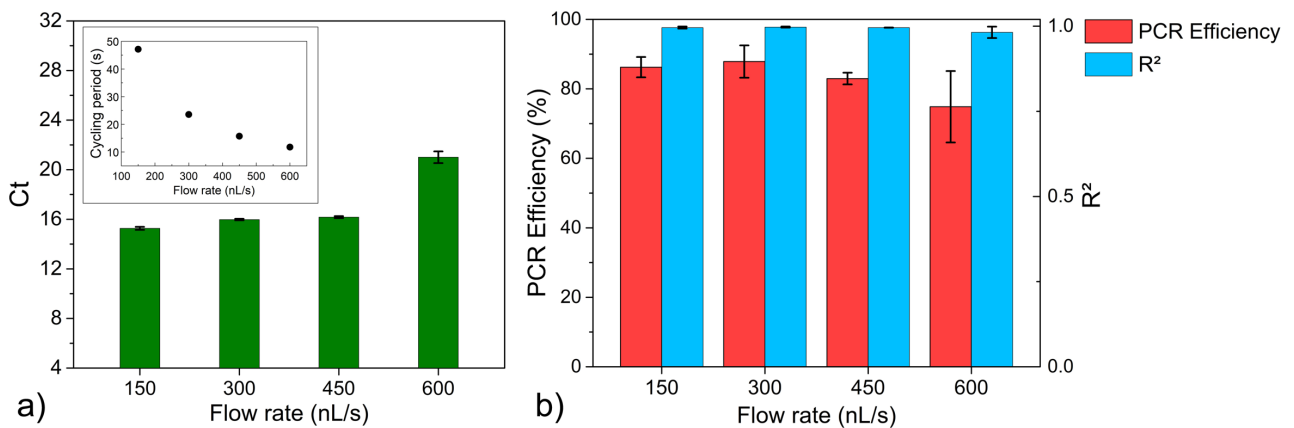
22 3.3 Fast RT-qPCR evaluation

23 To investigate the analytical throughput of the Drop-qPCR platform, the workflows described above have been tested
 24 increasing the flowrate of the carrier oil between 150 and 600nL/s. In this way, droplets spend different residence
 25 times on the heating brass parts. **Knowing the oil flowrate and the device geometry, the times required to perform**
 26 **one turn in the capillary, or one PCR cycle (cycling period), ranges between 47 and 12 seconds (see inset Figure 6a),**
 27 **corresponding to a residence time between 18.8 and 4.7 s at each temperature.**

28 In order to quantitatively compare the different throughput conditions, Ct and amplification efficiency[47] are
 29 evaluated for a fixed total RNA quantity in 5 replicates (1ng of total RNA extracted from MCF7 cells for ACTB
 30 gene). **Figure 6a** reports the Ct values obtained as function of the flowrate. They are constant (average Ct=15.8) with
 31 CV<0.8% up to 450nL/s, while a higher Ct (=21.0) showing higher variability (CV=2.24%) is found at 600nL/s; this

32 indicates the upper speed limit of the protocol. Similarly, **Figure 6b** shows that PCR efficiencies and the related R^2
 33 are constant within the error bars and present small CV (<5.3% and CV<0.1%, respectively) up to 450nL/s, while
 34 they decrease and are associated with higher variabilities (CV=13.7% and CV=1.7% respectively) at 600nL/s.
 35 According to the heat exchanger model presented in[54], droplets experience a complete thermalization in less than
 36 2 seconds for all investigated droplets' speeds. Since the shortest residence time on the brass part per cycle (for
 37 600nL/s) is 4.7 seconds, the decrease in RT-qPCR performance at this flowrate is probably limited by the kinetics of
 38 the reactions: DNA denaturation (95°C) and/or annealing/elongation (60°C). According to general guidelines for
 39 nucleic acid amplification, the elongation time is the slowest and the limiting process. Similar limitation in time are
 40 also found in[32]. It is also worth noting that to get constant Ct values using the commercial qPCR machine
 41 (SmartCycler), at least 3 times longer cycling period have to be applied. This can be partially explained by the longer
 42 thermalization required for the larger reaction volume (25 μ L) and by the temperature ramp needed by the machine
 43 during the PCR cycles (see Note S4 and Figure S6 in ESI for more details).
 44 Altogether, these results demonstrate that the Drop-qPCR platform allows performing the qPCR protocol (45 cycles)
 45 in less than 12 minutes (corresponding to a flowrate of 450nL/s and a cycling period of 15.7s), which not only is
 46 much faster than commercially available machines, but it is also consistent with the actual state-of-the-art in the
 47 monophasic microfluidic devices (cycling period: 14s)[32]. However, the latter is strongly limited by the number of
 48 samples that can be analysed per run; in fact, in order to parallelize the analysis for different samples, several
 49 disposable microfluidic chips must be employed. Differently, our platform allows sequential analysis of different
 50 samples, without presenting drastic limitation in the throughput. Additionally, once the droplets are generated and
 51 stored in the capillary at a **mutual distance of 14 mm (corresponding of 1 μ L of oil)**, the time required for the total
 52 RT-qPCR analysis of 1 or hundred samples, differ of less than 5 minutes (15min for 1 sample and 20min for 100
 53 samples).

54



55

56 *Figure 6: a)Cts, b)PCR Efficiency and related R^2 obtained for different flowrate conditions and thus, different cycling periods related to the*
 57 *qPCR (see a, inset). The results are consistent up to 450nL/s.*

58

59

50 **4 Conclusion**

51 In this work, we present a novel microfluidic platform implementing samples and reagents merging, the entire
52 RT and qPCR protocols, starting from total RNA samples. We demonstrated its ability to discriminate cells with
53 different gene expression levels in perfect agreement with a commercial system, and despite a higher sensitivity
54 and a 200-fold reduction in reagents volume. The latter allow fulfilling clinical laboratories needs in order to,
55 respectively, i) increase the number of genes that can be screened starting from identical sample quantity and ii)
56 reduce the cost per analysis, as it is mainly based on reagents consumption. Moreover, the Drop-qPCR was
57 designed fully-automated and versatile to customize the number and the combinations of samples/genes, without
58 the need for a trained user nor an external pipetting robot. Thus, leading to further cost reduction.

59 However, the increase in the gene panel per patient raise a major question of analysis duration. For that purpose,
60 we showed that compared to other state-of-the-art systems, our platform presents the unique capacity of
61 performing 30 qPCR cycles in less than 8 minutes for a single sample and in less than 12.5min for a hundred
62 samples. Consequently, the Drop-qPCR is perfectly adapted to both small and large number of samples with fast
63 analysis time, and thus being highly suitable for diagnosis applications.

64 Considering that the highest achievable throughput is limited by the kinetic of the PCR reaction, optimization in
65 the reaction buffer solution [32,55] can represent important perspectives for further timing improvements of this
66 technology. Finally, the lower RNA amount considered for the calibration curve corresponds to the one of a
67 single cell (about 15pg/drop), thus future outlooks include the coupling of this technology with a single cell-
68 encapsulation device [56].

69

70

71

72 , thus validating its suitability and potentialities for routine cancer screening in clinics. Notably, the results
73 obtained are in perfect agreement with a commercial system, despite a strong reduction of both reagent and
74 sample volumes required (up to 200 times). **The latter is a crucial aspect in clinical laboratory in order to i)
75 increase the number of genes that can be analysed starting from the same sample quantity and ii) reduce the cost
76 per analysis, asnyrhfhngbf mainly based on the reagents consumption.**

77 **Additionally, Drop-qPCR has been designed to be a versatile tool allowing the automated screening of a
78 customizable number and combinations of samples and genes, leading to a further reduction of costs in term of
79 alternative pipetting robot or long manual operation performed by trained user.** Then, the platform allows the
80 application of the same pipeline used in diagnostic, which consists in multiple analysis per sample and negative
81 control experiments. Moreover, to face the increasing number of gene panels to screen, the platform is able to
82 perform hundred reactions in less than 20 minutes, presenting at the same time the unique capability of being
83 suitable for both small and large number of samples, resulting therefore, perfectly suitable for diagnostic
84 applications.

85 Since the highest achievable throughput is limited by the kinetic of the PCR reaction, optimization in the reaction
86 buffer solution[32,55] can represent important perspectives for further improvements of this technology. Finally,
87 the lower RNA amount considered for the calibration curve corresponds to the one of a single cell (about 15pg/drop),
88 thus future outlooks include the coupling of this technology with a single cell-encapsulation device[56].

00 **References**

- 01 [1] J.A. Ludwig, J.N. Weinstein, Biomarkers in Cancer Staging, Prognosis and Treatment Selection, *Nat. Rev.*
02 *Cancer.* 5 (2005) 845–856. doi:10.1038/nrc1739.
- 03 [2] L.A. Garraway, J. Verweij, K. V Ballman, Precision oncology: an overview., *J. Clin. Oncol.* 31 (2013) 1803–
04 5. doi:10.1200/JCO.2013.49.4799.
- 05 [3] A.C. Wolff, M.E.H. Hammond, D.G. Hicks, M. Dowsett, L.M. McShane, K.H. Allison, D.C. Allred, J.M.S.
06 Bartlett, M. Bilous, P. Fitzgibbons, W. Hanna, R.B. Jenkins, P.B. Mangu, S. Paik, E.A. Perez, M.F. Press,
07 P.A. Spears, G.H. Vance, G. Viale, D.F. Hayes, American Society of Clinical Oncology, College of American
08 Pathologists, Recommendations for human epidermal growth factor receptor 2 testing in breast cancer:
09 American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update.,
10 *J. Clin. Oncol.* 31 (2013) 3997–4013. doi:10.1200/JCO.2013.50.9984.
- 11 [4] E.A. Perez, A.C. Dueck, A.E. McCullough, M.M. Reinholz, K.S. Tenner, N.E. Davidson, J. Gralow, L.N.
12 Harris, L.A. Kutteh, D.W. Hillman, R.B. Jenkins, B. Chen, Predictability of adjuvant trastuzumab benefit in
13 N9831 patients using the ASCO/CAP HER2-positivity criteria., *J. Natl. Cancer Inst.* 104 (2012) 159–62.
14 doi:10.1093/jnci/djr490.
- 15 [5] D. Tvrdík, L. Staněk, H. Skálová, P. Dunder, Z. Velenská, C. Povýšil, Comparison of the IHC, FISH, SISH
16 and qPCR methods for the molecular diagnosis of breast cancer, *Mol. Med. Rep.* 6 (2012) 439–443.
17 doi:10.3892/mmr.2012.919.
- 18 [6] J. Jacquemier, F. Spyrtatos, B. Esterni, M.-J. Mozziconacci, M. Antoine, L. Arnould, S. Lizard, P. Bertheau,
19 J. Lehmann-Che, C.B. Fournier, S. Krieger, F. Bibeau, P.-J. Lamy, M.P. Chenard, M. Legrain, J.-M.
20 Guinebretière, D. Loussouarn, G. MacGrogan, I. Hostein, M.C. Mathieu, L. Lacroix, A. Valent, Y.M. Robin,
21 F. Revillion, M.L. Triki, A. Seaume, A.V. Salomon, P. de Cremoux, G. Portefaix, L. Xerri, S. Vacher, I.
22 Bièche, F. Penault-Llorca, SISH/CISH or qPCR as alternative techniques to FISH for determination of HER2
23 amplification status on breast tumors core needle biopsies: a multicenter experience based on 840 cases, *BMC*
24 *Cancer.* 13 (2013) 351. doi:10.1186/1471-2407-13-351.
- 25 [7] P.S. Bernard, C.T. Wittwer, Real-time PCR technology for cancer diagnostics, *Clin. Chem.* 48 (2002) 1178–
26 1185. doi:48.
- 27 [8] S. Mocellin, C.R. Rossi, P. Pilati, D. Nitti, F.M. Marincola, Quantitative real-time PCR: A powerful ally in
28 cancer research, *Trends Mol. Med.* 9 (2003) 189–195. doi:10.1016/S1471-4914(03)00047-9.
- 29 [9] S. Fleige, M.W. Pfaffl, RNA integrity and the effect on the real-time qRT-PCR performance, *Mol. Aspects*
30 *Med.* 27 (2006) 126–139. doi:10.1016/J.MAM.2005.12.003.
- 31 [10] J.S. Marcus, W.F. Anderson, S.R. Quake, Parallel picoliter RT-PCR assays using microfluidics, *Anal. Chem.*
32 78 (2006) 956–958. doi:10.1021/ac0513865.
- 33 [11] S.-Y. Teh, R. Lin, L.-H. Hung, A.P. Lee, Droplet microfluidics, *Lab Chip.* 8 (2008) 198.
34 doi:10.1039/b715524g.
- 35 [12] M.T. Guo, A. Rotem, J.A. Heyman, D.A. Weitz, Droplet microfluidics for high-throughput biological assays,
36 *Lab Chip.* 12 (2012) 2146. doi:10.1039/c2lc21147e.

- 37 [13] C.N. Baroud, F. Gallaire, R. Dangla, Dynamics of microfluidic droplets, *Lab Chip*. 10 (2010) 2032.
38 doi:10.1039/c001191f.
- 39 [14] K.D. Dorfman, M. Chabert, J.-H. Codarbox, G. Rousseau, P. de Cremoux, J.-L. Viovy, Contamination-Free
40 Continuous Flow Microfluidic Polymerase Chain Reaction for Quantitative and Clinical Applications, *Anal.*
41 *Chem.* 77 (2005) 3700–3704. doi:10.1021/AC050031I.
- 42 [15] Y. Schaerli, R.C. Wootton, T. Robinson, V. Stein, C. Dunsby, M.A.A. Neil, P.M.W. French, A.J. deMello,
43 C. Abell, F. Hollfelder, Continuous-Flow Polymerase Chain Reaction of Single-Copy DNA in Microfluidic
44 Microdroplets, *Anal. Chem.* 81 (2009) 302–306. doi:10.1021/ac802038c.
- 45 [16] Y. Zhang, P. Ozdemir, Microfluidic DNA amplification—A review, *Anal. Chim. Acta.* 638 (2009) 115–125.
46 doi:10.1016/J.ACA.2009.02.038.
- 47 [17] V. Taly, D. Pekin, L. Benhaim, S.K. Kotsopoulos, D. Le Corre, X. Li, I. Atochin, D.R. Link, A.D. Griffiths,
48 K. Pallier, H. Blons, O. Bouché, B. Landi, J.B. Hutchison, P. Laurent-Puig, Multiplex Picodroplet Digital
49 PCR to Detect KRAS Mutations in Circulating DNA from the Plasma of Colorectal Cancer Patients, *Clin.*
50 *Chem.* 59 (2013) 1722–1731. doi:10.1373/CLINCHEM.2013.206359.
- 51 [18] R. Yang, A. Paparini, P. Monis, U. Ryan, Comparison of next-generation droplet digital PCR (ddPCR) with
52 quantitative PCR (qPCR) for enumeration of *Cryptosporidium* oocysts in faecal samples, *Int. J. Parasitol.* 44
53 (2014) 1105–1113. doi:10.1016/J.IJPARA.2014.08.004.
- 54 [19] R. Arvia, M. Sollai, F. Pierucci, C. Urso, D. Massi, K. Zakrzewska, Droplet digital PCR (ddPCR) vs
55 quantitative real-time PCR (qPCR) approach for detection and quantification of Merkel cell polyomavirus
56 (MCPyV) DNA in formalin fixed paraffin embedded (FFPE) cutaneous biopsies, *J. Virol. Methods.* 246
57 (2017) 15–20. doi:10.1016/J.JVIROMET.2017.04.003.
- 58 [20] D. Dobnik, D. Štebih, A. Blejec, D. Morisset, J. Žel, Multiplex quantification of four DNA targets in one
59 reaction with Bio-Rad droplet digital PCR system for GMO detection, *Sci. Rep.* 6 (2016) 35451.
60 doi:10.1038/srep35451.
- 61 [21] B.T. Lau, C. Wood-Bouwens, H.P. Ji, Robust Multiplexed Clustering and Denoising of Digital PCR Assays
62 by Data Gridding, *Anal. Chem.* 89 (2017) 11913–11917. doi:10.1021/acs.analchem.7b02688.
- 63 [22] J.F. Huggett, S. Cowen, C.A. Foy, Considerations for digital PCR as an accurate molecular diagnostic tool.,
64 *Clin. Chem.* 61 (2015) 79–88. doi:10.1373/clinchem.2014.221366.
- 65 [23] B. Shu, C. Zhang, D. Xing, Highly sensitive identification of foodborne pathogenic *Listeria monocytogenes*
66 using single-phase continuous-flow nested PCR microfluidics with on-line fluorescence detection,
67 *Microfluid. Nanofluidics.* 15 (2013) 161–172. doi:10.1007/s10404-013-1138-4.
- 68 [24] J. Chen, Z. Luo, L. Li, J. He, L. Li, J. Zhu, P. Wu, L. He, Capillary-based integrated digital PCR in picoliter
69 droplets, *Lab Chip*. 18 (2018) 412–421. doi:10.1039/C7LC01160A.
- 70 [25] P. Mary, L. Dauphinot, N. Bois, M.-C. Potier, V. Studer, P. Tabeling, Analysis of gene expression at the
71 single-cell level using microdroplet-based microfluidic technology, *Biomicrofluidics.* 5 (2011) 024109.
72 doi:10.1063/1.3596394.
- 73 [26] N.R. Beer, E.K. Wheeler, L. Lee-Houghton, N. Watkins, S. Nasarabadi, N. Hebert, P. Leung, D.W. Arnold,
74 C.G. Bailey, B.W. Colston, On-Chip Single-Copy Real-Time Reverse-Transcription PCR in Isolated Picoliter
75 Droplets, *Anal. Chem.* 80 (2008) 1854–1858. doi:10.1021/AC800048K.

- 76 [27] Y. Zhu, Y.-X. Zhang, W.-W. Liu, Y. Ma, Q. Fang, B. Yao, Printing 2-dimensional droplet array for single-
77 cell reverse transcription quantitative PCR assay with a microfluidic robot., *Sci. Rep.* 5 (2015) 9551.
78 doi:10.1038/srep09551.
- 79 [28] K. Matsuda, A. Yamaguchi, C. Taira, A. Sueki, H. Koeda, F. Takagi, M. Sugano, T. Honda, A novel high-
80 speed droplet-polymerase chain reaction can detect human influenza virus in less than 30 min, *Clin. Chim.*
81 *Acta.* 413 (2012) 1742–1745. doi:10.1016/J.CCA.2012.06.026.
- 82 [29] M. UEHARA, K. MATSUDA, M. SUGANO, T. HONDA, A New High-Speed Droplet-Real-Time
83 Polymerase Chain Reaction Method Can Detect Bovine Respiratory Syncytial Virus in Less than 10 Min, *J.*
84 *Vet. Med. Sci.* 76 (2014) 477–480. doi:10.1292/jvms.13-0357.
- 85 [30] A. Rival, D. Jary, C. Delattre, Y. Fouillet, G. Castellan, A. Bellemin-Comte, X. Gidrol, An EWOD-based
86 microfluidic chip for single-cell isolation, mRNA purification and subsequent multiplex qPCR, *Lab Chip.* 14
87 (2014) 3739–3749. doi:10.1039/C4LC00592A.
- 88 [31] M. Serra, D. Ferraro, I. Pereiro, J.-L. Viovy, S. Descroix, The power of solid supports in multiphase and
89 droplet-based microfluidics: towards clinical applications, *Lab Chip.* 17 (2017) 3979–3999.
90 doi:10.1039/C7LC00582B.
- 91 [32] T. Houssin, J. Cramer, R. Grojsman, L. Bellahsene, G. Colas, H. Moulet, W. Minnella, C. Pannetier, M.
92 Leberre, A. Plecis, Y. Chen, Ultrafast, sensitive and large-volume on-chip real-time PCR for the molecular
93 diagnosis of bacterial and viral infections, *Lab Chip.* 16 (2016) 1401–1411. doi:10.1039/C5LC01459J.
- 94 [33] A.C. Hatch, T. Ray, K. Lintecum, C. Youngbull, Continuous flow real-time PCR device using multi-channel
95 fluorescence excitation and detection, *Lab Chip.* 14 (2014) 562–568. doi:10.1039/C3LC51236C.
- 96 [34] C.J. Hayes, T.M. Dalton, Microfluidic droplet-based PCR instrumentation for high-throughput gene
97 expression profiling and biomarker discovery, *Biomol. Detect. Quantif.* 4 (2015) 22–32.
98 doi:10.1016/J.BDQ.2015.04.003.
- 99 [35] Y. Schaerli, R.C. Wootton, T. Robinson, V. Stein, C. Dunsby, M.A.A. Neil, P.M.W. French, A.J. deMello,
00 C. Abell, F. Hollfelder, Continuous-Flow Polymerase Chain Reaction of Single-Copy DNA in Microfluidic
01 Microdroplets, *Anal. Chem.* 81 (2009) 302–306. doi:10.1021/ac802038c.
- 02 [36] D. Ferraro, M. Serra, D. Filippi, L. Zago, E. Guglielmin, M. Pierno, S. Descroix, J.-L. Viovy, G. Mistura,
03 Controlling the distance of highly confined droplets in a capillary by interfacial tension for merging on-
04 demand, *Lab Chip.* 19 (2019) 136–146. doi:10.1039/C8LC01182F.
- 05 [37] D. Ferraro, J. Champ, B. Teste, M. Serra, L. Malaquin, J.-L. Viovy, P. de Cremoux, S. Descroix, Microfluidic
06 platform combining droplets and magnetic tweezers: application to HER2 expression in cancer diagnosis.,
07 *Sci. Rep.* 6 (2016) 25540. doi:10.1038/srep25540.
- 08 [38] D. Ferraro, M. Serra, I. Ferrante, J.-L. Viovy, S. Descroix, Microfluidic valve with zero dead volume and
09 negligible back-flow for droplets handling, *Sensors Actuators B Chem.* 258 (2018) 1051–1059.
10 doi:10.1016/J.SNB.2017.12.002.
- 11 [39] P. Juskova, A. Ollitrault, M. Serra, J.-L. Viovy, L. Malaquin, Resolution improvement of 3D stereo-
12 lithography through the direct laser trajectory programming: Application to microfluidic deterministic lateral
13 displacement device, *Anal. Chim. Acta.* 1000 (2018) 239–247. doi:10.1016/J.ACA.2017.11.062.
- 14 [40] T.D. Mai, D. Ferraro, N. Aboud, R. Renault, M. Serra, N.T. Tran, J.-L. Viovy, C. Smadja, S. Descroix, M.

- 15 Taverna, Single-step immunoassays and microfluidic droplet operation: towards a versatile approach for
16 detection of amyloid- β peptide-based biomarkers of Alzheimer's disease, *Sensors Actuators B Chem.* 255
17 (2018) 2126–2135. doi:10.1016/j.snb.2017.09.003.
- 18 [41] D. Ferraro, Y. Lin, B. Teste, D. Talbot, L. Malaquin, S. Descroix, A. Abou-Hassan, Continuous chemical
19 operations and modifications on magnetic $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles confined in nanoliter droplets for the
20 assembly of fluorescent and magnetic $\text{SiO}_2 @\gamma\text{-Fe}_2\text{O}_3$, *Chem. Commun.* 51 (2015) 16904–16907.
21 doi:10.1039/C5CC07044A.
- 22 [42] D.Y. Wu, L. Ugozzoli, B.K. Pal, J. Qian, R.B. Wallace, The Effect of Temperature and Oligonucleotide
23 Primer Length on the Specificity and Efficiency of Amplification by the Polymerase Chain Reaction, *DNA*
24 *Cell Biol.* 10 (1991) 233–238. doi:10.1089/dna.1991.10.233.
- 25 [43] S. Taylor, M. Wakem, G. Dijkman, M. Alsarraj, M. Nguyen, A practical approach to RT-qPCR—Publishing
26 data that conform to the MIQE guidelines, *Methods.* 50 (2010) S1–S5. doi:10.1016/J.YMETH.2010.01.005.
- 27 [44] J.-W. Pan, C.-M. Wang, H.-C. Lan, W.-S. Sun, J.-Y. Chang, Homogenized LED-illumination using microlens
28 arrays for a pocket-sized projector, *Opt. Express.* 15 (2007) 10483. doi:10.1364/OE.15.010483.
- 29 [45] G. Wang, L. Wang, F. Li, D. Kong, Design of optical element combining Fresnel lens with microlens array
30 for uniform light-emitting diode lighting, *J. Opt. Soc. Am. A.* 29 (2012) 1877.
31 doi:10.1364/JOSAA.29.001877.
- 32 [46] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl,
33 G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication
34 of quantitative real-time PCR experiments., *Clin. Chem.* 55 (2009) 611–22.
35 doi:10.1373/clinchem.2008.112797.
- 36 [47] R.G. Rutledge, C. Côté, Mathematics of quantitative kinetic PCR and the application of standard curves,
37 *Nucleic Acids Res.* 31 (2003) 93e – 93. doi:10.1093/nar/gng093.
- 38 [48] J.L. Gevertz, S.M. Dunn, C.M. Roth, Mathematical model of real-time PCR kinetics, *Biotechnol. Bioeng.* 92
39 (2005) 346–355. doi:10.1002/bit.20617.
- 40 [49] M. Kubista, J.M. Andrade, M. Bengtsson, A. Forootan, J. Jonák, K. Lind, R. Sindelka, R. Sjöback, B.
41 Sjögreen, L. Strömbom, A. Ståhlberg, N. Zoric, The real-time polymerase chain reaction, *Mol. Aspects Med.*
42 27 (2006) 95–125. doi:10.1016/j.mam.2005.12.007.
- 43 [50] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative CT method, *Nat. Protoc.* 3
44 (2008) 1101–1108. doi:10.1038/nprot.2008.73.
- 45 [51] S.C. Taylor, G. Laperriere, H. Germain, Droplet Digital PCR versus qPCR for gene expression analysis with
46 low abundant targets: from variable nonsense to publication quality data, *Sci. Rep.* 7 (2017) 2409.
47 doi:10.1038/s41598-017-02217-x.
- 48 [52] A.K. White, M. VanInsberghe, O.I. Petriv, M. Hamidi, D. Sikorski, M.A. Marra, J. Piret, S. Aparicio, C.L.
49 Hansen, High-throughput microfluidic single-cell RT-qPCR, *Proc. Natl. Acad. Sci.* 108 (2011) 13999–14004.
50 doi:10.1073/pnas.1019446108.
- 51 [53] M. VanInsberghe, H. Zahn, A.K. White, O.I. Petriv, C.L. Hansen, Highly multiplexed single-cell quantitative
52 PCR, *PLoS One.* 13 (2018) 1–18. doi:10.1371/journal.pone.0191601.
- 53 [54] D.W. Green, R.H. Perry, *Perry's chemical engineer handbook*, 8th edition, 2007.

- 54 [55] J.S. Farrar, C.T. Wittwer, G. Reed, J. Cherry, Extreme PCR: efficient and specific DNA amplification in 15-
55 60 seconds., *Clin. Chem.* 61 (2015) 145–53. doi:10.1373/clinchem.2014.228304.
- 56 [56] D.J. Collins, A. Neild, A. deMello, A.-Q. Liu, Y. Ai, The Poisson distribution and beyond: methods for
57 microfluidic droplet production and single cell encapsulation, *Lab Chip.* 15 (2015) 3439–3459.
58 doi:10.1039/C5LC00614G.
- 59

50 **Acknowledgment**

51 This work was supported by the French National Research Agency (ANR) as part of the “Investissements d’Avenir”
52 program (reference: ANR 10-NANO 0207), by the Labex and Equipex IPGG (references: 10-LABX-0031 and 10-
53 EQPX-0034), by ERC “Cello” (FP7-IDEAS-ERC-321107) and by the ARC foundation for young researcher
54 fellowship (DF). IH was supported by PhD founding from Fondation Pierre-Gilles de Gennes. MS was supported by
55 a Curie Institute International PhD fellowship.

56 **Conflict of interest**

57 DF, MS, J-LV and SD are co-inventors of a patent application filed by Institut Curie and CNRS, encompassing some
58 of the technologies described in this article.