Centrifugo-thermopneumatic fluid control for valving and aliquoting applied to multiplex real-time PCR on off-the-shelf centrifugal thermocycler

M. Keller, ^{a,b} S. Wadle, ^{a,b} N. Paust, ^{a,b} L. Dreesen, ^b C. Nuese, ^b O. Strohmeier, ^{a,b} R. Zengerle^{a,b,c} and F. von Stetten^{a,b}

^aHahn-Schickard, Georges-Koehler-Allee 103, 79110 Freiburg, Germany

^bLaboratory for MEMS Applications, IMTEK – Department of Microsystems Engineering, University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, Germany

BIOSS – Centre for Biological Signalling Studies, University of Freiburg, Schaenzlestr. 18, 79104 Freiburg, Germany

Electronic supplementary information (ESI)

Experimental Details S1: Influence of centrifugal and capillary pressures on CTP siphon valving

In the moment of gas volume enclosure (Fig. 3B in manuscript), the initial gas volume V_0 is slightly changed to volume $V_{g,T0}$ by the centrifugal $\Delta p_{cent,T0}$, capillary $\Delta p_{cap,T0}$ and partial vapor $p_{vap,T0}$ pressures. According to eqn (5) (manuscript), this isothermal volume change at constant temperature T_0 can be expressed as follows,

$$V_{g,T0} = \frac{p_0 V_0}{(\Delta p_{cent,T0} + \Delta p_{cap,T0} + p_0 - p_{vap,T0})}$$
(S1A)

The capillary pressure inside a rectangular shape with three sides of cyclic olefin copolymer (COP) and one side of polyolefin (PO) may be expressed as

$$p_{cap} = \sigma \left(\frac{2\cos\theta_{COP}}{w} + \frac{\cos\theta_{COP}}{d} + \frac{\cos\theta_{PO}}{d} \right)$$
(S1B)

where σ is the surface tension, Θ_{COP} and Θ_{PO} are the contact angles on COP and PO, respectively, w is the width and d is the depth of the corresponding microfluidic structure. The centrifugal pressure may be expressed as

$$p_{cent} = \frac{1}{2}\omega^2 \rho (r_{max}^2 - r_{min}^2)$$
(S1C)

where ρ is the density (here 1000 kg/m³), r_{max} is the radially most outer and r_{min} is the radially most inner position of the liquid and ω the angular velocity for 400 RPM here given as

$$\omega = 2 \pi \frac{400}{60} \tag{S1D}$$

The advancing (inside the overflow siphon channel and the volume buffer + connection channel) and the receding contact angles (inside the inlet chamber) were measured at room temperature to be 87.7 ° and 45.3 ° on PO and 69.9 ° and 13.0 ° on COP, respectively, the surface tension was determined to be 43.0 mN/m, p_0 = 98360 Pa and p_{vap} was calculated using eqn (3) (manuscript, A = 8.07131, B = 1730.63, C = 233.426) with a starting temperature T_0 of 25 °C and target temperature T of 90 °C. Applied dimensions are depicted in Fig S1A.



Fig. S1A Dimensions of channels and chambers of the centrifugo-thermopneumatic siphon valve as well as radii of liquid menisci.

Substituting $V_{g,T0}$ of eqn (S1) into eqn (5) (manuscript), ΔV_g of eqn (5) (manuscript) may be divided by ΔV_g of eqn (6) (manuscript, $V_{g,T0} = V_0$), which yields a ratio of 1.017. Since contact angles and surface tensions were measured at room temperature and capillary pressures are expected to reduce with temperature increase, this ratio may be considered a

worst case assumption. Thus, ΔV_g of eqn (5) (manuscript) differs from ΔV_g of eqn (6) (manuscript) only by 1.7 % at most, which justifies to use eqn (6) for the calculation of CTP siphon valving for the demonstrated system.

Experimental Details S2: Influence of centrifugal and capillary pressures on CTP two-stage aliquoting

During saturation of the enclosed gas volume inside a reaction cavity, overpressure is released *via* bubbles leaving the gas volume (Fig. 4B in manuscript). Here, the widening of the narrow connection channel entering the metering finger serves as capillary valves for the gas phase. Thus, the maximum pressure inside the reaction cavity that can build up before a bubble is released, is the sum of the atmospheric pressure p_0 (98360 Pa), the centrifugal pressure $\Delta p_{cent,T0}$ of the liquid plug inside the corresponding metering finger and the capillary burst pressure $\Delta p_{cap,burst}$ of the capillary valve that can be described using eqn (S1B) (ESI Experimental Details S1) as follows,

$$\Delta p_{cap,burst} = \Delta p_{cap,T_0} = \sigma \left(\frac{2}{w} + \frac{1}{d} + \frac{\cos \theta_{PO}}{d} \right)$$
(S2A)

where Θ_{PO} is the receding contact angle of the liquid on polyolefin (45.3°, measured at room temperature) and w is the width (400 µm) and d is the depth (180 µm) of the narrow channel. The centrifugal pressure $\Delta p_{cent,TO}$ may be calculated using eqn (S1C) (ESI Experimental Details S1) by applying 46.1 mm as r_{min} and 53.1 mm as r_{max} (metering finger #8, Fig. 5A, manuscript). At the end of volume contraction during cool down, the originally enclosed gas volume inside the reaction cavity is vented again and thus at atmospheric pressure ($\Delta p_{cent,T} = \Delta p_{cap,T} = p_{vap,T} = 0$). Using a surface tension of 43.0 mN/m (measured at room temperature) and p_{vap} calculated using eqn (3) (manuscript, A = 8.07131, B = 1730.63, C = 233.426) with a starting temperature T_0 of 90 °C and target temperature T of 30 °C, ΔV_g of eqn (5) (manuscript) may be divided by ΔV_g of eqn (6) (manuscript), which yields a ratio of 1.001. Since contact angles and surface tensions were measured at room temperature and capillary pressures are expected to reduce with temperature increase, this ratio may be considered a worst case assumption. Thus, ΔV_g of eqn (5) (manuscript) differs from ΔV_g of eqn (6) (manuscript) only by 0.1 % at most, which justifies to use eqn (6) for the calculation of CTP two-stage aliquoting for the demonstrated system.

Table S1: Sequences of pre-stored primers and probes (provided by QIAGEN).

Target	Description	Sequence (5'–3')
Escherichia coli O157:H7	Forward primer	CGA TGA TGC TAC CCC TGA AAA ACT
containing the intimin (eaeA) gene,	Reverse primer	TAT TGT CGC TTG AAC TGA TTT CCT C
GenBank accession no. U32312	Hydrolysis probe	6-FAM-CGT TGT TAA GTC AAT GGA AAA CCT G-BHQ-1

Video S1: Stationary images of a rotating GeneSlice, recorded by the strobe RGQ.

The video shows the initial CTP siphon valving of the sample volume and its subsequent CTP two-stage aliquoting. In the top left the time in ms is indicated. In the lower left corner first the rotational frequency (constant 400 RPM) and later the temperatures modelled by the RGQ software are displayed.



Figure S1: Position of thermo couples of a temperature measurement system for temperature measurement inside an additional *GeneSlice*. The measurements inside the inlet chamber are discussed in ESI Experimental Details S4.

Experimental Details S3: Robustness analysis for reaction cavity volume

In the design process, a robustness analysis for the reaction cavity volume was carried out based on the following worst case assumptions: The temperature T_{max} at the start of cool down, which is predetermined by CTP siphon valving, was measured to be above 83.6 °C in all experiments (when setting the RGQ to 90 °C). In a worst case, room temperature was assumed not to be higher than 40 °C and aliquoted liquid volume not higher than 20 µl + 10 %, which yields a minimum required air volume inside the reaction cavity of 38.1 µl (eqn (6)). Thus, the reaction cavity volume was dimensioned to be 39 µl for robust functionality.

Experimental Details S4: Volume expansion during centrifugo-thermopneumatic siphon valving

To characterize the volume expansion inside the inlet chamber of the CTP siphon valve the same experimental setup and data processing was applied as described in the manuscript section "Thermal and fluidic characterization of centrifugo-thermopneumatic two-stage aliquoting". Temperature was measured inside the inlet chamber in the gas (1) and liquid (2) phase (*cf.* ESI Figure S1 for exact positions). The gas volume inside the inlet chamber was calculated on every tenth point of image acquisition (from execution of the run to triggering of valving) by measuring the filling height of the liquid inside the inlet chamber. Substitution of the determined gas volume and T_{RT} = temperature of gas (1) phase at the time of $V_{g,TRT}$ measurement) and numerically solving for *T* yields a back calculated temperature under the assumption that gas expansion of CTP siphon valving follows eqn (6) (manuscript). The back calculated temperature is plotted with the measured temperatures of the gas (1) and liquid (2) phase inside the inlet chamber in Fig. S4A.



Fig. S4A Temperatures measured in the gas (red) and liquid (blue) phase inside the inlet chamber of an additional *GeneSlice* (inlet chamber not sealed gastight). Solving eqn (6) (manuscript) for the temperature at different times of gas volume expansion measurement inside the gas-tight inlet chamber of the processed *GeneSlice* yields a back calculated temperature (black).

As can be seen from Fig. S4A, the back calculated temperature using eqn (6) (manuscript) first closely follows the temperature of the gas phase. Then, the temperature is within the range of the measured temperatures inside the gas and liquid phase and finally rises higher than the measured values. Deviations of the back calculated from the measured values are likely to be caused by differences of the processed and the additional *GeneSlice*: Since insertion of the thermo couples of the TMS into the additional *GeneSlice* did not allow gas-tight sealing of the inlet chamber, the sample liquid volume stayed inside the inlet chamber of the additional *GeneSlice* throughout the entire temperature measurement. In contrast, in the processed *GeneSlice*, portions of the sample volume are actuated out of the inlet chamber, thereby changing thermal masses inside the inlet chamber. Taking into account such differences, it may be assumed, that CTP siphon valving is described by eqn (6) (manuscript) and deviations are caused by inaccuracy of the temperature measurement method. **Experimental Details S5: Examination of fluidic resistance**

To compensate for minute liquid volumes that are transferred to downstream metering fingers by rising bubbles during saturation, the residual time of liquid inside the feeding channel may be elongated by a fluidic resistance. The fluidic resistance is used as concatenation of the CTP siphon valve and the CTP two-stage aliquoting structure (Fig. 4A). As the residual time of liquid inside the feeding channel is expected to influence the aliquoting result, aliquoting results of, otherwise identical, *GeneSlices* with (A) no, (B) a "medium" (0.5 x 0.5 x 51.9 mm), and (C) a "high" fluidic resistance (0.5 x 0.5 x 110.1 mm) are compared to find an appropriate fluidic resistance. Quantification was carried out according to the manuscript section "Thermal and fluidic characterization of centrifugo-thermopneumatic two-stage aliquoting".

The comparison of the quantified aliquoted volumes of *GeneSlices* with (A) no, (B) a "medium", and (C) a "high" additional fluidic resistance is given in Table S5A and depicted in Fig. S5A.

Table S5A Aliquoting performance characteristics of centrifugo-thermopneumatic (CTP) siphon valving and subsequent CTP two-stage aliquoting with (A) no, (B) a "medium", and (C) a "high" fluidic resistance in-between both fluidic unit operations.

Aliquoted volume	Additional resistance		
	(A) no	(B) "medium"	(C) "high"
Mean per version [µl]	17.5	18.2	18.5
Inter-GeneSlice CV [%]	3.8	5.5	5.5
Mean intra-GeneSlice CV [%]	8.2 ± 6.9	4.2 ± 1.8	5.2 ± 1.6



Fig. S5A Mean aliquoted volume per reaction cavity and corresponding standard deviation of centrifugo-thermopneumatic (CTP) siphon valving and subsequent CTP two-stage aliquoting with (A) no, (B) a "medium", and (C) a "high" fluidic resistance in-between both fluidic unit operations. In addition, linear fits per version indicate the tendency of volume increase towards downstream reaction cavities.

In all cases the determined mean aliquoted volume per version remains below the nominal volume of 20 µl, which is caused by minor liquid transfer from one metering finger to the downstream adjacent one during the saturation phase (Fig. 4B). If this volume loss remains uncompensated by remaining liquid inside the feeding channel, the mean aliquoted volume per finger increases from 16.7 to 20.3 µl in version A with increasing finger number (except for finger #1 (16.9 µl), which receives a small refill from the siphon channel). Utilizing a simple linear fit of mean aliquoted volumes per finger (Fig. S5A), the tendency of volume increase towards downstream reaction cavities for each version can be examined by the slope of the corresponding fitting curve. The slope of 0.4 ± 0.4 µl/finger for version (A) with no additional fluidic resistance reduces to 0.2 ± 0.2 µl/finger for version (B) with a "medium" additional fluidic resistance and reaches a well-equilibrated state with 0.0 ± 0.2 µl/finger with (C) a "high" additional fluidic resistance. Simultaneously, the mean aliquoted volume per version of 18.5 and 18.2 µl for version (C) and (B), respectively, in contrast to 17.5 µl for version (A), is closer to the nominal volume of 20 µl. The version with the medium fluidic resistance was chosen as appropriate concatenation offering a sufficient aliquoting performance and still low (theoretical) probability of nonspecific adsorption of polymerase or target DNA on the resistance micro-channel surfaces ¹.

Experimental Details S6: Post-processing of real-time PCR data

Real-time PCR data was *post*-processed using the RGQ Series Software (version 2.1.0 build 9, QIAGEN): The raw reporter signals (FAM) were normalized to the passive reference dye (ROX) resulting in reporter normalized values (R_n). Subsequently, R_n values were background normalized including slope correction and applying the dynamic tube option. Using the normalized fluorescence curves, fluorescence thresholds (F_q) of 0.012 and 0.023 RFU were applied in the RGQ Series Software for experimental series (I) and (II), respectively, to obtain quantification cycle (C_q) values (Fig. S6A).



Fig. S6A Exemplary real-time PCR curves obtained from four *GeneSlices* within the same run (1000 copies/reaction cavity). Setting a fluorescence threshold allows determination of a quantification cycle value for each curve.

For experimental series (II) a joint standard curve per dilution series was obtained using C_q values of the inter-run calibrator (1000 copies/reaction) and applying the interplate calibration of GenEx (version 6.0.1.612, MultiD Analyses AB, Goeteborg, Sweden). Also, the PCR efficiency and its standard deviation were determined using the corresponding function of GenEx.

References

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