Electronic Supplementary Material (ESI) for Lab on a Chip.

The MRD Disk: Automated Minimal Residual Disease Monitoring by Highly Sensitive Centrifugal Microfluidic Multiplex qPCR

ESI

Methods and materials

Multiplex Mediator Probe PCR panel

The Mediator Probe PCR (MP PCR) is a real-time PCR principle, where the fluorescence signal generation is independent from the DNA target sequence.[1] This offers great potential to standardise assays with variable targets, as it is required in MRD quantification with patient-individual target sequences, since this techniques features a constant and similar quenching efficiency for all targets. In contrast to conventional real-time PCR signal generation using sequence-specific hydrolysis probes, the MP PCR uses target specific but label-free probes (mediator probe) and target independent fluorogenic oligonucleotides (universal reporter) as depicted in Fig. 1a. Universal reporter molecules, available in five different colour classes, can easily be exchanged (Fig. 1b). Consequently, the DNA amplification process is hardly affected by exchanging the fluorogenic reporters.

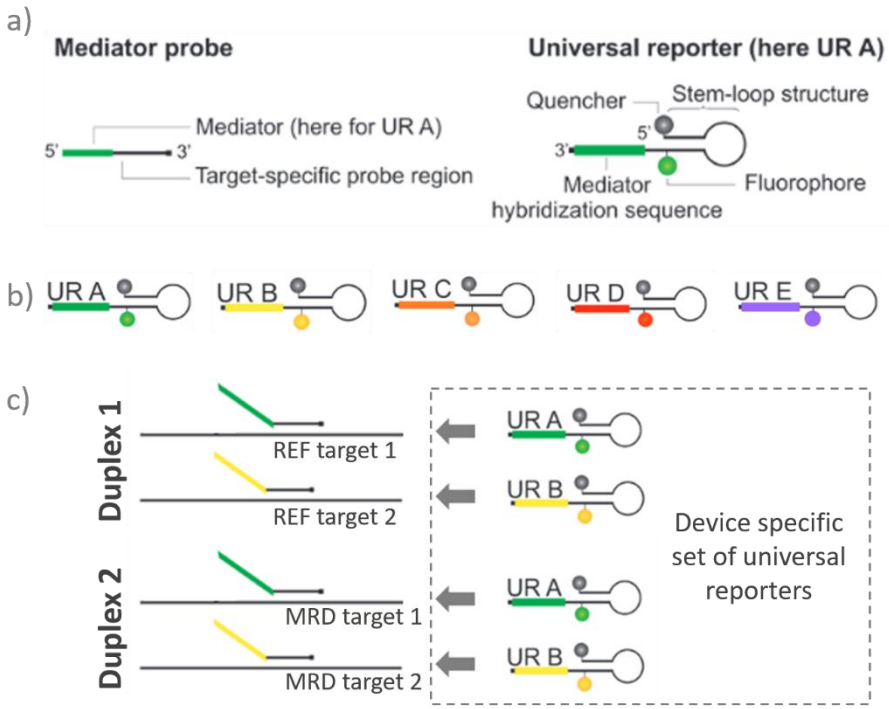
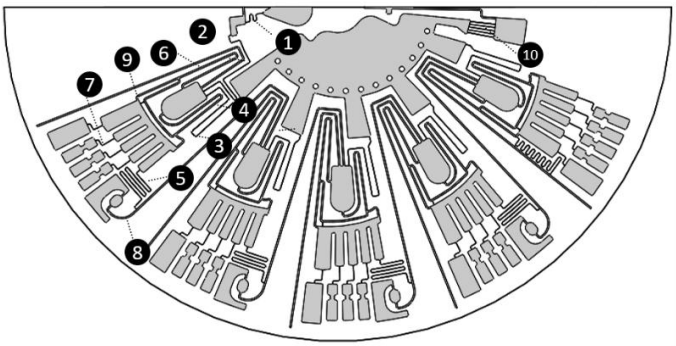


Figure 1: a) Schematic explanation of mediator probe and universal reporter for target-independent signal generation in Mediator Probe PCR. b) Panel of available universal reporter molecules from green to crimson spectrum. c) Concept for colorimetric duplexing. A constant set of only two different universal reporters, here exemplary shown as green and yellow, will be used for both duplex systems and for all patients. (Adapted from BioTechniques (2016) 61(3), 123-128 with permission of Future Science Group[2])

Cartridge design: Dimensions



#	Description	Dimension w x h x l [µm]
1	Sample transfer channel	60 x 40 x 7,000
2	Sample delay channel	60 x 40 x 28,000
3	Buffer transfer channel	60 x 40 x 28,000
4	TCR valving siphon	300 x 200 x 31,000
5	TCR valving flow restriction vent	60 x 40 x 36,000
6	TCR valving coupled siphon	300 x 200 x 35,000
7	Dilution transfer channel	60 x 40 x 5,000
8	Inward pumping channel	300 x 200 x 59,000
9	Waste connection channel	60 x 40 x 700
10	Capillary array	60 x 40 x 5,000

Figure 2: Overview of all critical geometries in the MRD Disk layout. Non-displayed fluidic structures, such as the second serial dilution structure or the structures for automated aliquoting, are designed with identic geometric parameters.

Cartridge design: Combined load-and-vent inlet

A previously reported design attempt with multiple serial dilutions on a single LabDisk lacked of robustness, resulting in several failed dilutions.[3] The undesired wetting of venting channels during liquid loading in the disk was identified as cause of disturbances in the fluidic logic (Fig. 3a). To improve robustness of the disk loading process a new principle for LabDisk loading is introduced, termed as combined load-and-vent inlet (Fig. 3b). After liquid dispensing through an inlet hole (I), a cylindrical DNA filter is pushed inside the inlet hole (II). By a conical shape of the inlet hole a press-fit between filter and LabDisk is created (III). The press-fit assures a leakage-tight fit of the filter as well as mechanical fixation of the filter during rotation. In such a design the inlet hole fulfils two functions at once: Interface for pipette based liquid loading and venting interface for fluidic functionality. As a result of the combined load-and-vent inlet the venting channels in inlet chambers become obsolete. Wetting liquids can still creep into outlet channels, but this is less critical concerning disturbances in the fluidic logic (Fig. 3c). DNA filters of different suppliers were compared. As a compromise between small enough for acceptable filter footprint and large enough for user-friendly handling the filter 9-120-02-0 (Ahn Biotechnologie, Germany) with a diameter of D = 2.4 mm and a height of h = 3.5 mm was chosen. Based on this geometry, a conical press-fit of the inlet is designed as shown in Fig. 4.

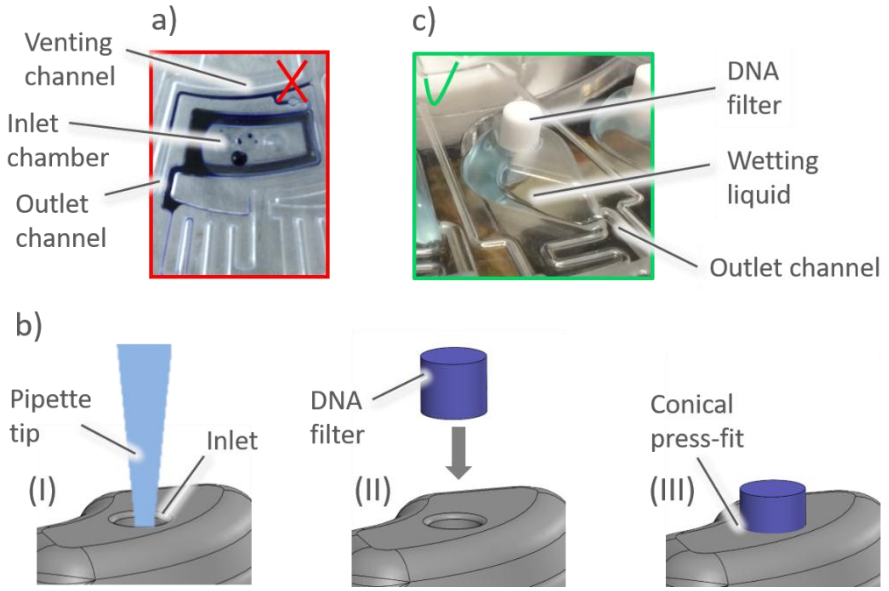


Figure 3: a) Capillary wetting of venting channels during pipetting can cause disturbances of the fluidic logic during disk processing. b) The combined load-and-vent inlet allows for robust disk loading in three steps. (I) Liquid is dispensed inside the inlet chamber by pipetting. (II) A cylindrical pipette tip DNA filter is pushed into the inlet. (III) By conical press-fit the DNA filter is fixed inside the inlet hole. c) The combined load-and-vent inlet allows an inlet chamber design without dedicated venting channel. The wetting liquid is shown to only reach the outlet channel, which is considered as less critical than reaching a venting channel.

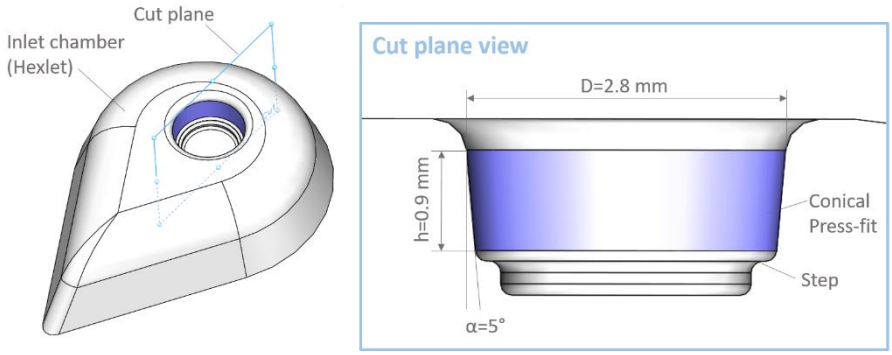


Figure 4: CAD model of the combined load-and-vent inlet, designed for a DNA filter with D=2.4 mm in diameter and a height of h=3.5 mm. The increased diameter (D=2.8 mm) compensates wall thickness of polymer foil (expected to be ~0.2 mm after micro-thermoforming). The angular wall with a slope of 5° assures conical press-fit. The height of 0.9 mm was shown to provide sufficient mechanical support to the filter during centrifugation at frequencies up to 80 Hz and temperature stress. The step at the bottom is avoiding contact between the DNA filter and the liquid meniscus inside the chamber, in order to prevent wetting of the DNA filter.

Setup for assay integration and automated qPCR: LabDisk Player optical specifications

Table 1: Overview of the five different fluorescence channels in a LabDisk Player. The filtered bandwidths are comparably large (Rotor-Gene Q: typically ± 5 nm), leading to potentially physical crosstalk in colour multiplexing.

Fluorescence channel	Excitation [nm]	Detection [nm]
green	470 ± 15	520 ± 10
yellow	520 ± 10	570 ± 15
orange	560 ± 15	625 ± 20
red	625 ± 25	680 ± 5
crimson	650 ± 25	720 ± 15

Setup for assay integration and automated qPCR: Mediator extension assay

In the mediator extension assay, the fluorogenic reporter is directly activated by the mediator. In contrast to Mediator Probe PCR this principle of signal generation works in absence of target DNA template. The short mediator oligonucleotide, with a length of 19-20 bases, does not require cleavage of a probe sequence.[4]

For crosstalk determination experiments the proposed cycling protocol of Lehnert et al. is used for mediator extension assay in a Rotor-Gene Q device.[4] For universal reporter characterisation experiments the mediator extension assay needs to run directly inside the LabDisk Player. For this purpose the published cycling protocol is adapted to the LabDisk Player: Hot start 300 sec at 93 °C, followed by 45 cycles of 8 sec denaturation at 93 °C and 30 sec annealing and extension at 62 °C. The 10 µl reaction mixes are composed of the HotStar TaqPlus 2 x Mastermix (QIAGEN, Netherlands) and final concentrations of 5 mM MgCl2 (QIAGEN, Netherlands), 100 nM of universal reporter (biomers.net, Germany) and 0.1 µg µl 1 BSA Molecular Biology Grade (New England BioLabs, US). For signal measurement, 150 nM mediator (biomers.net, Germany) is added. The NTC reactions for noise measurement are prepared identically, except from replacing mediators by water.

Multiplex Mediator Probe PCR assays: Preliminary comparison of assays in a Rotor-Gene Q thermos-cycler

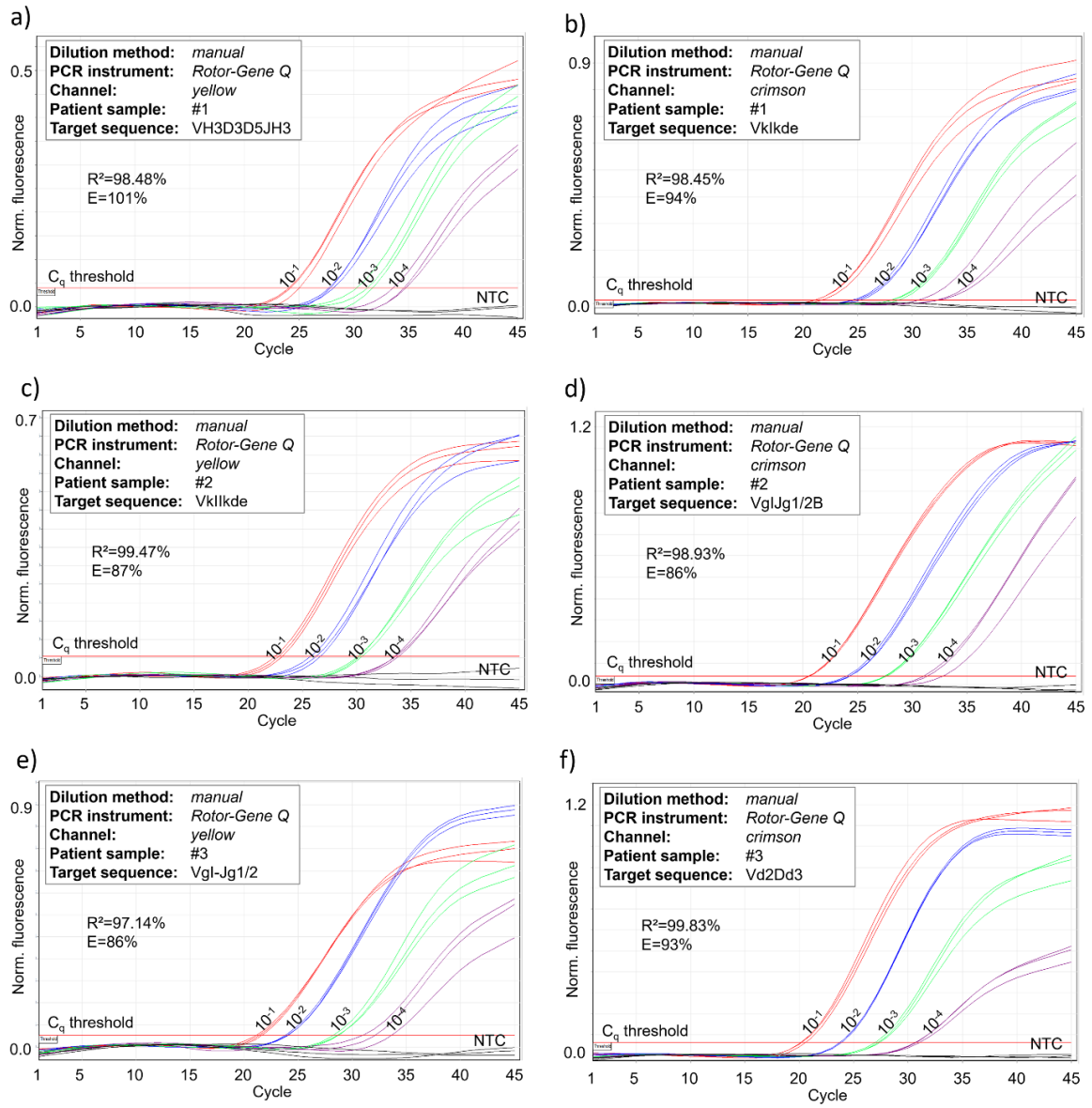


Figure 5: Preliminary testing of three different duplex assays of three different patients in a Rotor-Gene Q thermo-cycler. As main evaluation criterion, the PCR efficiency E and the standard curve linearity R² were evaluated. Patient #1 duplex with MRD targets VH3D3D5JH3 and VkIkde was selected because of highest PCR efficiencies in average.

Multiplex Mediator Probe PCR assays

Table 2: Multiplex Mediator Probe PCR Albumin assay: Oligonucleotide and target sequences (C3 = C3-Spacer; Q2 = BHQ-2; BMN = BMN-Q-535; Underlined: Primer binding positions; Underlined and bold: Mediator probe binding position; Small letters: Universal reporter binding position).

Oligonucleotide / target	Description	Sequence (5'-3')
Fw primer	Forward primer	TGAAACATACGTTCCCAAAGAGTTT
Rv primer	Reverse primer	TCTCCTTCTCAGAAAGTGTGCATAT
MP	Mediator probe	cagttcggtcagtgaac**aATGCTGAAACATTCACCTTCCATGCAG**-C3
UR	Universal reporter	BMN-GACCGGCCAAGACGCGCCGGT(dC-HEX)tgttcactgaccgaactgGAGCA-C3
Target	Albumin	TGAAACATACGTTCCCAAAGAGTTT**AATGCTGAAACATTCACCTTCCATGCAG**ATATATGCACACTTTCTGAGAAGGAGA

Table 3: Multiplex Mediator Probe PCR Betaglobin assay: Oligonucleotide and target sequences (C3 = C3-Spacer; Q2 = BHQ-2; BMN = BMN-Q-535; Underlined: Primer binding positions; Underlined and bold: Mediator probe binding position; Small letters: Universal reporter binding position).

Oligonucleotide / target	Description	Sequence (5'-3')
Fw primer	Forward primer	CTGACACAACTGTGTTCACTAGC
Rv primer	Reverse primer	TATTGGTCTCCTTAAACCTGTCTTG
MP	Mediator probe	gaaggcaggtaggctc**aACCTCAAACAGACACCATGGTG**-C3
UR	Universal reporter	Q2-GACGCGTAGTACAGAACGCGT(dC-Cy5.5)TGTTCAGtgagcctacctgccttc-C3
Target	Betaglobin	CTGACACAACTGTGTTCACTAGC**AACCTCAAACAGACACCATGGTG**CACCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATA

Table 4: Multiplex Mediator Probe PCR VH3D3D5JH3 assay: Oligonucleotide and target sequences (C3 = C3-Spacer; Q2 = BHQ-2; BMN = BMN-Q-535; Underlined: Primer binding positions; Underlined and bold: Mediator probe binding position; Small letters: Universal reporter binding position).

Oligonucleotide / target	Description	Sequence (5'-3')
Fw primer	Forward primer	GGGAGGTCCCTTCGAGACT
Rv primer	Reverse primer	AAGAGACGGTGACCATTGTCC
MP	Mediator probe	ctccagttcggtcagtgaa**cTGGGGGATAAGGAAATGCTTTTGATATCTGG**-C3
UR	Universal reporter	BMN-GACCGGCTAAGACGCGCCGGT(dC-HEX)TgttcactgaccgaactggagCA-C3
Target	VH3D3D5JH3	GGGAGGTCCCTTCGAGACT**CTGGGGGATAAGGAAATGCTTTTGATATCTGG**GGCCAAGGGACAATG GTCACCGTCTCTT

Table 5: Multiplex Mediator Probe PCR VkIkde assay: Oligonucleotide and target sequences (C3 = C3-Spacer; Q2 = BHQ-2; BMN = BMN-Q-535; Underlined: Primer binding positions; Underlined and bold: Mediator probe binding position; Small letters: Universal reporter binding position).

Oligonucleotide / target	Description	Sequence (5'-3')
Fw primer	Forward primer	GCATAATAGTTACCCCCAAGGC
Rv primer	Reverse primer	GAGGTCAGACTCCAAATAGTGGATATG
MP	Mediator probe	caggtaggctcactgaa**cCCCTCTCTAGTGGCAGCCC AGGGCG**-C3
UR	Universal reporter	Q2-GACGCGTAGTACAGAACGCGT(dC-Cy5.5)Tgt tcagtgagcctacctgCCTTC-C3
Target	VkIkde	GCATAATAGTTACCCCCAAGGC**CCCCTCTCTAGTGGC AGCCCAGGGCG**ACTCCTCATGAGTCTGCAGCTGATTT TTGCCATATCCACTATTTGGAGTCTGACCTC

Gold standard assays

The mastermix is based on the Invitrogen Platinum Taq DNA Polymerase Kit (Thermo Fisher Scientific, US) and prepared for a final reaction volume of 20 µl. Final concentrations in the mastermix are as follows: MgCl2 (Thermo Fisher Scientific, US) at 5 mM, BSA B9000S (New England Biolabs, US) at 2.5 µg µl-1, dNTP Set GEN-009-250 at 200 µM (Rapidozym, Germany) forward and reverse primers (TIB Molbiol, Germany) at 500 nM, probe (TIB Molbiol, Germany) at 100 nM and 10x buffer as well as Platinum polymerase (Thermo Fisher Scientific, US) according to the supplier information.

The DNA containing eluates are mixed in a 1:3 ratio with mastermixes, resulting in the same absolute number of 100,000 cells per reaction as during Multiplex MP PCR experiments. Here, the manual preparation of serial dilutions is carried out as dilution of DG sample in BC sample, before adding the dilutions to the PCR mastermixes.

The gold standard assays are cycled and analysed in a StepOne Plus (Thermo Fisher Scientific, US) real-time block thermo-cycler. After a hot start phase (300 sec at 94 °C) a number of 40 (reference target) or 50 (MRD targets) cycles is performed. While denaturation temperature is fixed (8 sec at 94 °C) the optimal annealing/extension temperature can vary between 65 and 71 °C (23 sec). Fluorescence signal acquisition is carried out in the FAM channel (emission = 470 nm; detection = 520 nm).

Analysis of qPCR data is carried out in the StepOne Plus software by guidelines of the Charité - Universitätsmedizin Berlin reference laboratory, as applied in clinical routine testing of ALL patient MRD samples.

Results and discussion

Fluidic automation: Combined load-and-vent inlet

Based on this new inlet principle the observed fails of serial dilutions, as reported before,[3] were not observed anymore. While the earlier disk layout with conventional inlet and venting channel design failed in 2 out of 9 serial dilutions (n = 3 disks), the here introduced combined load-and-vent inlet was successful in 12 out of 12 serial dilutions (n = 6 disks). To realise this new principle, each pipetting inlet needs to be equipped with a DNA filter after liquid loading. For the MRD Disk design this results in nine filters to be inserted after pipetting (Fig. 8). For designs with substantially more inlets the cost of DNA filters as well as manual effort for filter insertion need to be considered. Here, the obtained fluidic robustness by introduction of the combined load-and-vent inlet is an essential step to enable upcoming disk testing in a clinical environment.

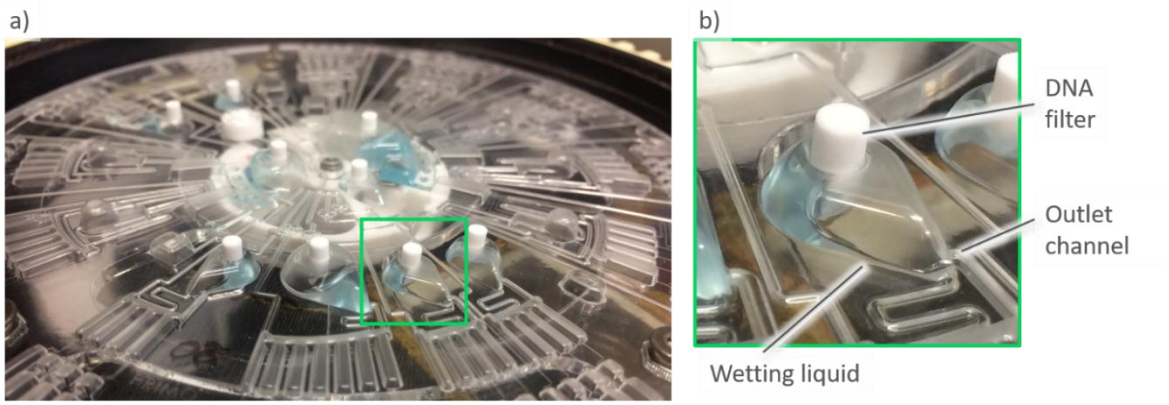


Figure 6: a) Photography of a MRD Disk, filled with all nine different reagents. b) Detail view: As a result of the combined load-and-vent inlets, that make the venting channels of earlier disk designs superfluous, the capillary flow of liquids is now less critical, as it only reaches the chamber outlet channel.

Fluidic automation: Temperature- and frequency-protocols

Table 6: MRD Disk temperature- and frequency protocol (RT: Room temperature).

Step	Description	Frequency [Hz]	Acceleration / Deceleration [Hz/s]	Hold time [s]	Temperature [°C]
Part A: Automated aliquoting					
1	Reagent loading	0	0	n.a.	RT
2	Aliquoting	80	20	5	RT
3	Transfer (5 cycles)	80 / 50	20	1	RT
Part B: Automated serial dilution and qPCR					
1	Reagent loading	0	0	n.a.	RT
2	Pre-aliquoting	15	20	1	RT
3	Aliquoting (2 cycles)	5 / 15	20	1	RT
4	Transfer	80	20	60	RT
Iterative dilution part – one cycle per dilution stage (5 cycles)					
5	Mixing by shake-mode (40 cycles)	15 / 30	20	0	65
6	TCR valving	15	20	1	45 (Ramp: ≥ -1 K/s)
7	Centrifugo-pneumatic aliquoting	20	20	5	45
8	Metering and pneumatic loading	80	20	60	45
9	Pneumatic pumping	15	5	1	45
10	Clearing siphon	80	20	5	45
11	Thermal pumping	15	5	1	65
Automated qPCR (45 cycles)					
12	Denaturing	40	10	8 (1st cycle: 120)	93
13	Annealing + Extension	40	10	30	62
14	Readout	0	10	1	62

Fluidic automation: Metering accuracies

Table 7: Metering accuracies of PCR reaction chambers in different parts of the MRD Disk.

High-dynamic-range serial dilution: $V_{reaction}$ [µl]

#1	#2	#3	#4	#5	#6	**AVG**	**SD**
9,9	10,1	10,1	10,1	10,1	10,3	**10,1**	**0,1**

Mid-dynamic-range serial dilution: $V_{reaction}$ [µl]

#1	#2	#3	#4	#5	#6	**AVG**	**SD**
10,1	10,1	10,2	10,0	9,9	9,9	**10,0**	**0,1**

Triplet: $V_{reaction}$ [µl]

#1	#2	#3	#4	#5	#6	**AVG**	**SD**
10,1	10,1	10,2	10,1	10,1	10,2	**10,1**	**0,1**

Sextuplet: $V_{reaction}$ [µl]

#1	#2	#3	#4	#5	#6	**AVG**	**SD**
10,4	10,2	10,0	10,0	10,2	10,4	**10,2**	**0,2**

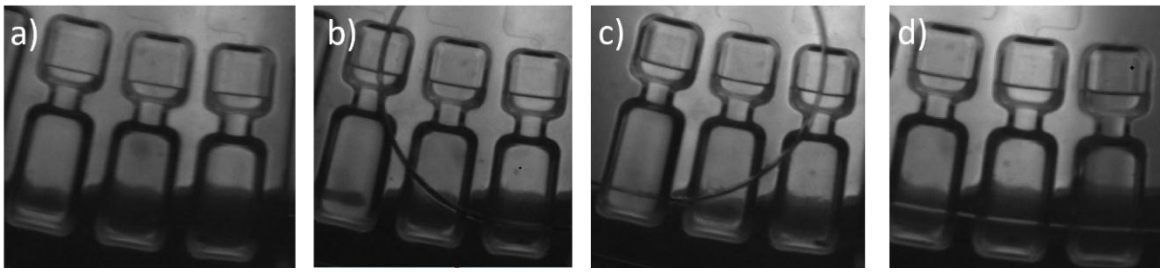


Figure 7: Exemplary stroboscopic images used for determination of metering accuracies in PCR reaction chambers. a) High-dynamic-range serial dilution structure. b) Mid-dynamic-range serial dilution structure. c) Triplet aliquoting structure. d) Sextuplet aliquoting structure.

Automated multiplex qPCR on disk: PCR curves

Evaluating the obtained PCR curves, the reference targets albumin (Fig. 10a) and beta-globin (Fig. 10b) show sigmoidal amplification curves for all mid-dynamic-range dilution replicates from $1x10^{0}$ to $1x10^{-2}$, as well as the corresponding FU sample quantification triplet with an expected concentration of $1x10^{-1}$. The automated high-dynamic-range dilutions from $1x10^{1}$ to $1x10^{-5}$ as well as the quantifications of both MRD target sequences, VH3D3D5JH3 (Fig. 10c) and VkIkde (Fig. 10d) also show the expected sigmoidal amplification curves. Even the $1x10^{-5}$ replicates, containing theoretically 1 target DNA template in a background of 100,000 unspecific DNA molecules, were amplified in a high number of replicates. As explained in the MIQE guidelines for PCR data, it can be explained by stochastic limitations, that not all single-copy replicates were amplified.[5] The quantifications of the MRD target FU samples match the expected DNA concentration of $5x10^{-4}$ with high accuracy. The Cq threshold crossing of some water NTCs were individually checked in raw data and can all be disregarded, as the curves rise very late (Cq > 35) and show a linear signal increase, uncommon for a PCR amplification. Furthermore, none of the buffy coat NTC curves (specificity control) showed a signal increase.

An evaluation of all twelve PCR curve plots reveals a systematic relation: In all three runs, the replicate curve variations of beta-globin (reference target in crimson) and VH3D3D5JH3 (MRD target in yellow) are lower than those of albumin (reference target in yellow) and VkIkde (MRD target in crimson). Consequently, the observed curve variations are not influenced by the fluorescence channel or the universal reporter. In addition, as always one crimson and one yellow result belong to one identic serial dilution, the observed PCR curve variations are not caused by the MRD Disk dilution method. Consequently, the results indicate a target dependent influence on the PCR quality, not related to the microfluidic automation. In summary, the PCR curves show the success of both, assay integration as well as automated serial dilution.

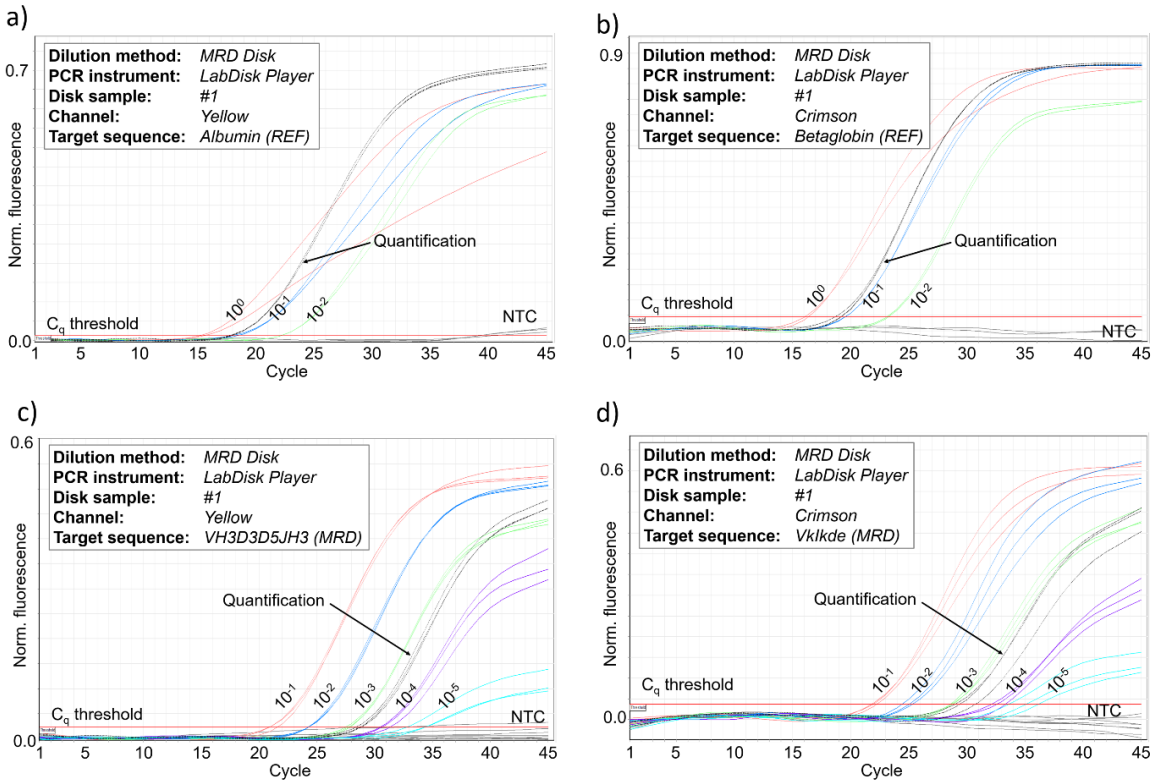


Figure 8: Results of MRD Disk sample #1: Four different target sequences are quantified simultaneously and fully automated. The qPCR curves show two duplex assays for automated reference target and MRD target quantification, carried out in disk sample #1. a) Reference gene Albumin dilution and quantification of follow-up sample (concentration 10^{-1}). b) Reference gene Betaglobin dilution and quantification of follow-up sample (concentration 10^{-1}). c) MRD target VH3D3D5JH3 dilution and quantification of follow-up sample (concentration $5x10^{-4}$). d) MRD target VkIkde dilution and quantification of follow-up sample (concentration $5x10^{-4}$).

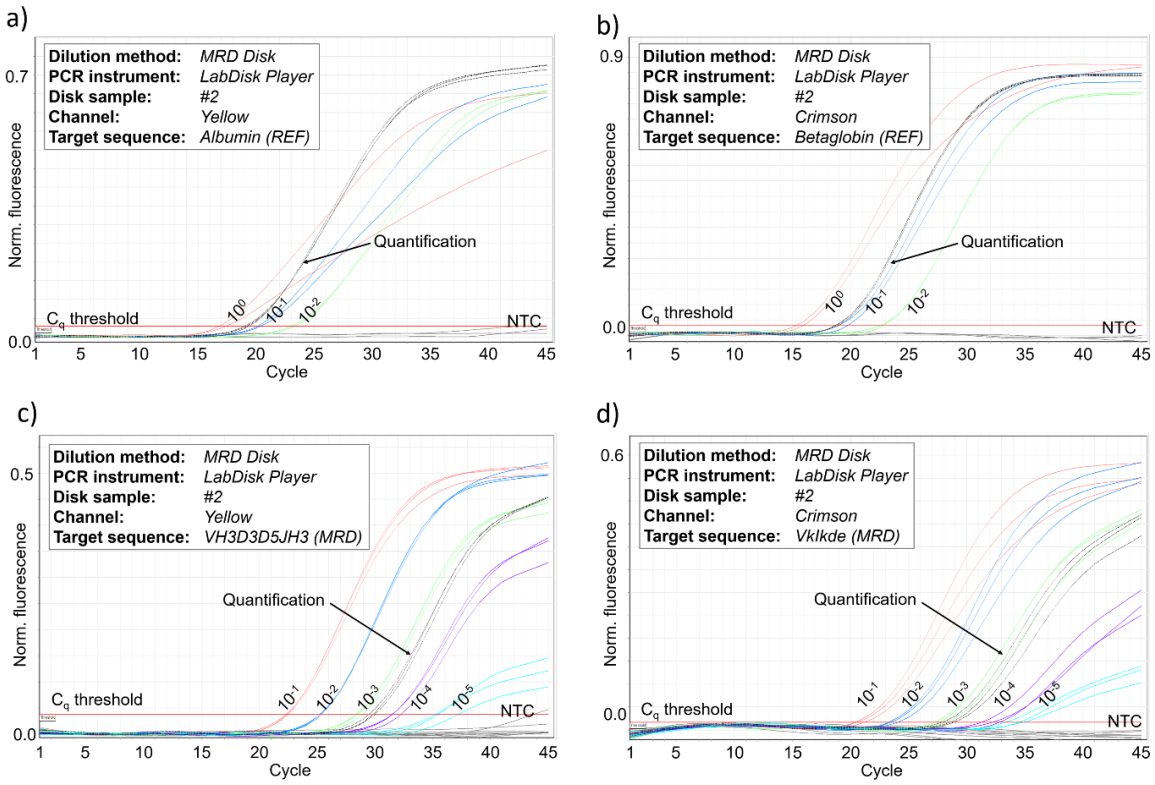


Figure 9: qPCR curve plots of MRD Disk sample #2.

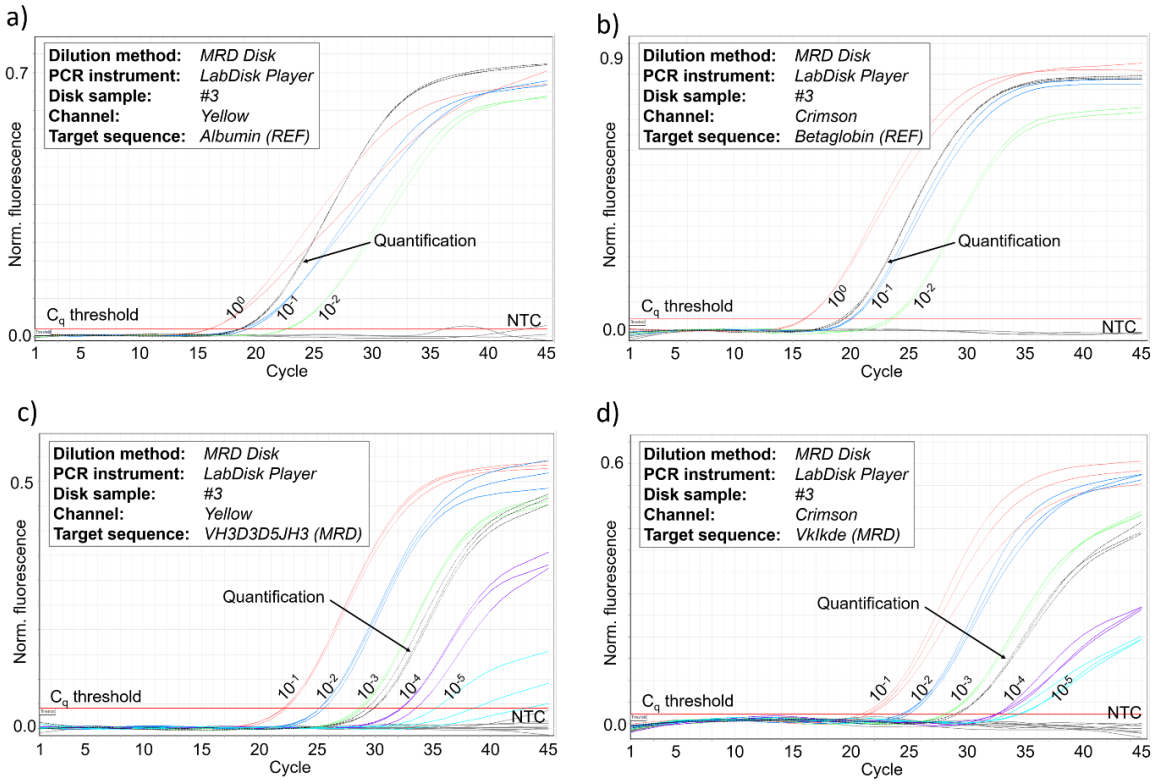


Figure 10: qPCR curve plots of MRD Disk sample #3.

References

1 B. Faltin, S. Wadle, G. Roth, R. Zengerle and F. von Stetten, *Clinical chemistry*, 2012, **58**, 1546–1556.

2 S. Wadle, M. Lehnert, F. Schuler, R. Köppel, A. Serr, R. Zengerle and F. von Stetten, *BioTechniques*, 2016, **61**, 123–128.

3 P. Juelg, M. Specht, E. Kipf, M. Lehnert, C. Eckert, N. Paust, R. Zengerle and T. Hutzenlaub, *22nd International Conference on Miniaturized Systems for Chemistry and Life Sciences, Kaohsiung, Taiwan*, **2018**, 2388–2389.

4 M. Lehnert, E. Kipf, F. Schlenker, N. Borst, R. Zengerle and F. von Stetten, *Anal. Methods*, 2018, **10**, 3444–3454.

5 S. A. Bustin, V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele and C. T. Wittwer, *Clinical chemistry*, 2009, **55**, 611–622.