Lab on a Chip

Electronic Supplementary Information (ESI)

Two-stage tuberculosis diagnostics: Combining centrifugal microfluidics to detect TB infection, Inh and Rif resistance at the point of care with subsequent antibiotic resistance profiling by targeted NGS

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S1: Two-stage TB diagnostic workflow.

The required equipment for the first stage as PoC solution is shown in Fig. S1. Figure S1A shows the Rhonda player, a portable, electric power-operated benchtop device with dimensions of 233 mm (width) x 296 mm (height) x 414 mm (depth) with a centrifugal microfluidic test cartridge with cover in the direction of insertion. Two cartridges, a silica bag and two inlet caps are packed in an aluminium bag to provide low humidity and dark conditions for storage. These contents of the aluminium bag provided to the user are shown in Figure S1B.

A) Rhonda player and cartridge with cover

B) Cartridges packed in an aluminium bag with silica bag and inlet caps

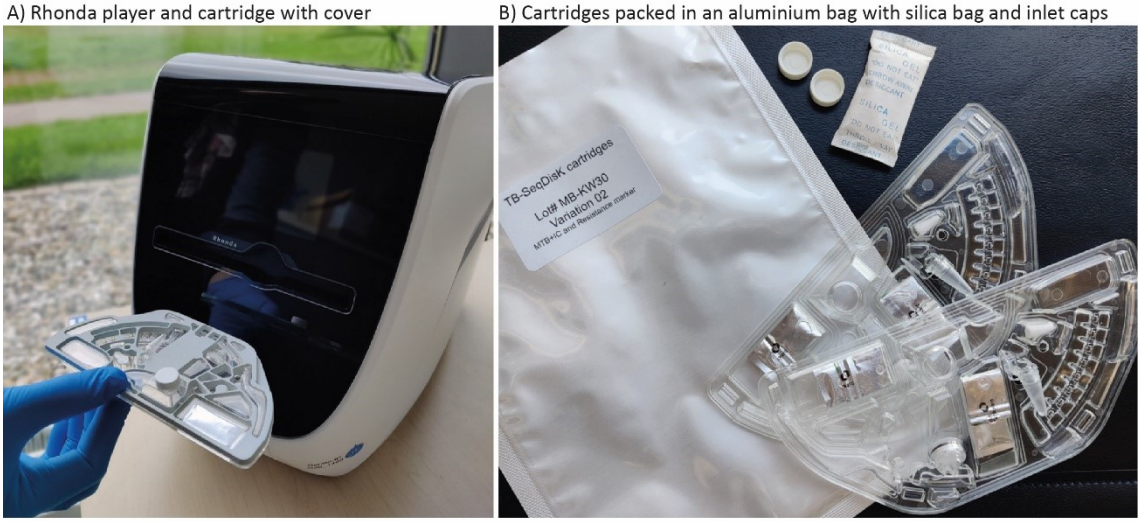


Figure S1: Photos of the presented centrifugal microfluidic PoC system. A) Rhonda player and cartridge with attached cover. B) Two cartridges, a silica bag, two inlet caps and the aluminum bag in which the cartridges are packed.

S2: Material & Methods

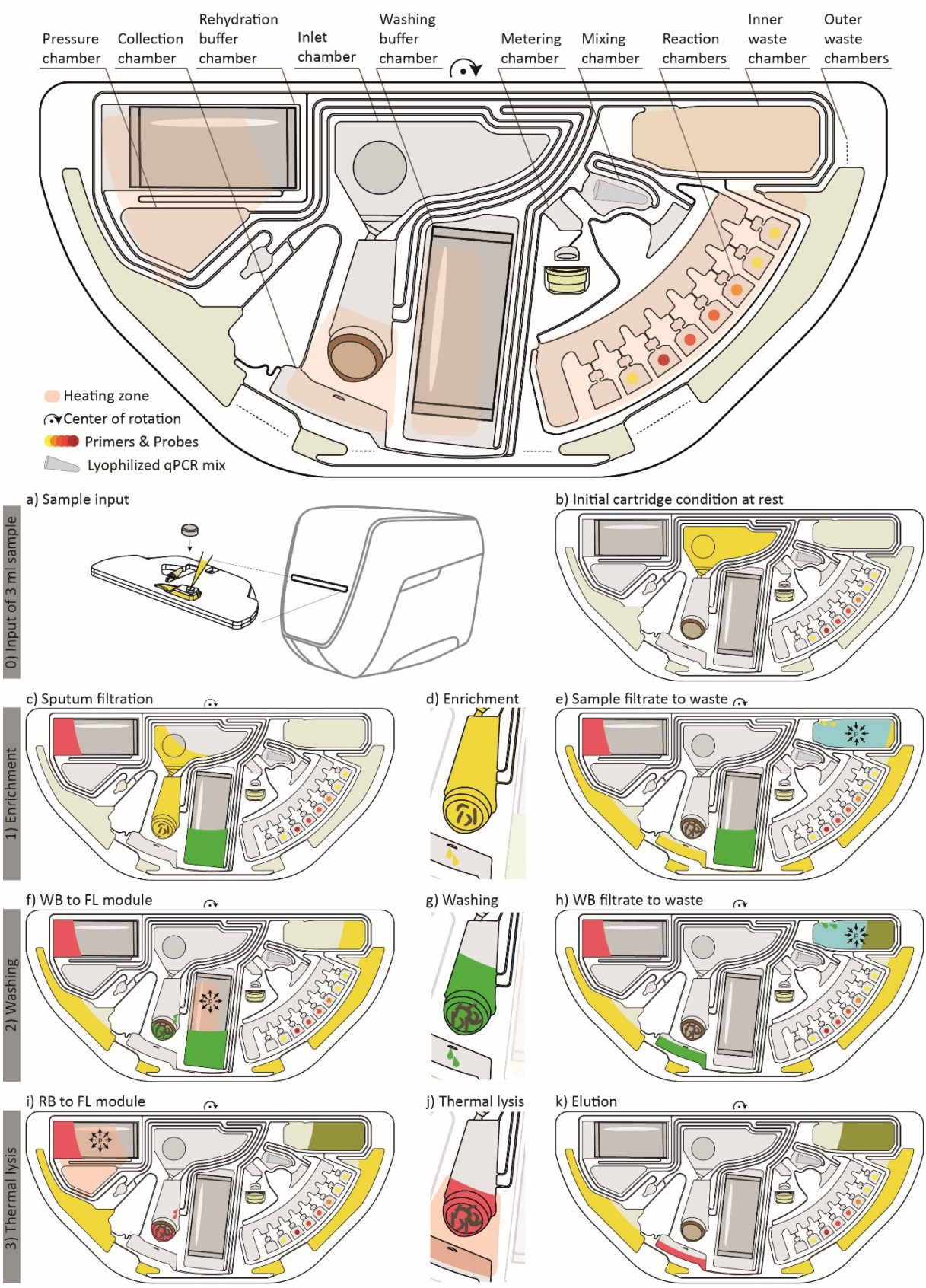
Table S1: Scheme of the qPCR for resistance detection, with the subsequent decision according to the workflow.

Wild type sequence	SNP sequence	$\Delta C_T = C_T$(wild type) - C_T(SNP)	MTBC detected	Resistance detected	Decision for next step
-	-	No ΔC_T	No	No	Patient release
+	-	No ΔC_T	Yes	No	Standard treatment
+	+	High ΔC_T	Yes	No	Standard treatment
+	+	Low ΔC_T	Yes	Yes	DNA solution send for tNGS

S3: Microfluidic implementation.

A detailed insight into the centrifugal microfluidic cartridge automation concept is shown in Fig. S2. The heating zones in red in respect to the cartridge are depicted on the top. In the following smaller images, the heating zones are only displayed when they are activated to provide a better overview. After inactivation and liquefaction of the sputum sample, 3 ml of sample is added to the cartridge, which is then closed with an inlet cap (Fig. S2-a). After manually inserting the cartridge into the player, the cartridge is automatically retracted, placed and pressed on the turntable of the player, and fixed in place via a vacuum (Fig. S2-b). With a sequential increase of the frequency first to 50 Hz and then gradually to 70 Hz, an initial sample filtration and thus MTBC enrichment in the filter is performed (Fig. S2-c/d). The collection chamber, which collects the filtrate, has a capacity of 600 µl. As more sample volume is filtered, an overflow mechanism distributes the sample filtrate to the outer waste chambers, which are all interconnected (Fig. S2-e). The remaining sample filtrate in the collection chamber is then transferred to the inner waste chamber entirely located on a heating zone. Here, vapor pressure assisted pumping is applied. In brief, the heating zone below the waste chamber is heated, the air inside the chamber expands and is displaced by air bubbles through the liquid in the collection chamber to the periphery of the fluidic network. During the subsequent cooling of the heating zone, negative pressure is created by the contraction of the trapped air in the waste chamber and liquid is drawn into the waste chamber. This process can be repeated sequentially. However, from the second pumping cycle onwards, an additional vapor pressure is generated, which significantly increases the pressure differences and thus the transferable liquid volumes. An additional filtration and pumping step is performed to ensure, that entire sample is filtered and the collection chamber is completely cleared (Fig. S2-frequency and temperature protocol-e*-e**). Once the collection chamber is emptied and all sample filtrate has entered the waste, the heating zone below the washing buffer chamber is heated to transfer the washing buffer (WB) to the filter and lysis module. Again, the trapped air expands and additional vapor pressure builds up, creating a considerable overpressure that overcomes the centrifugal pressure and displaces 600 µl of wash buffer from the wash buffer chamber into the filter and lysis module (Fig. S2-f). At high frequency of 70 Hz, the wash buffer is perfused through the filter to remove residual interfering substances from the original sample (Fig. S2-g). The wash buffer filtrate is again pumped into the inner waste by vapor pressure assisted pumping (Fig. S2-h). In the following stage, the heating zone below the rehydration buffer chamber is heated and the rehydration buffer (RB) is transferred to the filter and lysis module by overpressure, as previously described for the wash buffer (Fig. S2-i). During this transfer, the pressure chamber is heated and the air inside expands. In contrast to the enclosed air volume of the buffer chambers, the air here relaxes into the periphery of the fluidic network and no significant overpressure is generated. The liquid volume of the rehydration buffer covers the entire filter and is retained by the filter during thermal lysis at a frequency of 10 Hz at a set temperature of 100 °C for 3 min (Fig. S2-j). Because of the applied contact heating, the temperature gradient inside the liquid is significant[1]. This means that the liquid in close proximity of the heating zone is warmer than on the outer side, where cooling takes place at the chamber surface due to the rotation. Therefore, the high lysis temperature is necessary to ensure sufficient heat input into the liquid on the entire filter and thus to all bacteria. The mycobacterial DNA is released by the lysis and subsequently flushed through the filter at 70 Hz (Fig. S2-k). This step completes the sample preparation from the sputum sample, making the MTBC DNA available for subsequent analyses in- and outside of the cartridge.

Next, the heating zones below the reaction chambers and the pressure chamber are heated up. The trapped air expands and is displaced by air bubbles through the liquid in the collection chamber to the periphery of the fluidic network. With cooling the heating zones again, the air contracts and negative pressure draws liquid to the metering and mixing chamber. First, liquid fills the metering chamber of the DNA sample tube interface and subsequently the mixing chamber (Fig. S2-l). The transferred liquid in the mixing chamber experiences negative pressure on both liquid interfaces: on the meniscus in the siphon resulting from the reaction chamber air volume and on the interface in the mixing chamber itself. The negative pressure in the mixing chamber is generated partly by the connection to the air volume of the pressure chamber and partly by the connection via a resistance channel to the air volume of the reaction chamber structure. This keeps the liquid in the mixing chamber and prevents premature liquid transfer to the reaction chambers. In this way, a transfer of 70 µl into the metering chamber and subsequently of 150 µl into the mixing chamber can be realized (Fig. S2-m). By heating the zone below the reaction chambers the lyophilized, pre-stored qPCR reagents are dissolved by combined bubble and shake mode mixing[1,2] (Fig. S2-n). Subsequent cooling of the corresponding heating zone primes the siphon and, by increasing the frequency, the centrifugal pressure transfers the qPCR mix completely to the reaction chamber structure (Fig. S2-o&p). For each reaction chamber, 20 µl are metered (Fig. S2-q) and finally transferred into the reaction chambers by high frequencies (Fig. S2-r). The individual pre-stored primer and probes are dissolved (Fig. S2-r) and geometric and colorimetric multiplex qPCR is performed with temperature cycling and real-time read out (Fig. S2-s&t). The first two and the last reaction chamber are functionalized with primer and probes for the detection of MTBC, an internal control (IC), Mycobacterium avium complex (MAC) and nontuberculous mycobacteria (NTM). The chambers in between contain primer and probe mixes for the detection of resistances against Inh and Rif. Mutations were classified and divided into different primer mixes. One mix contains primers and probes for both isoniazid and rifampicin associated mutations that can be distinguished by the fluorescent detection channel. The other two mixes contain only primers and probes for rifampicin associated mutations. After completion of the microfluidic process, the detachable sample tube filled with enriched MTBC DNA can be removed and used for subsequent analyses, e.g. by NGS.



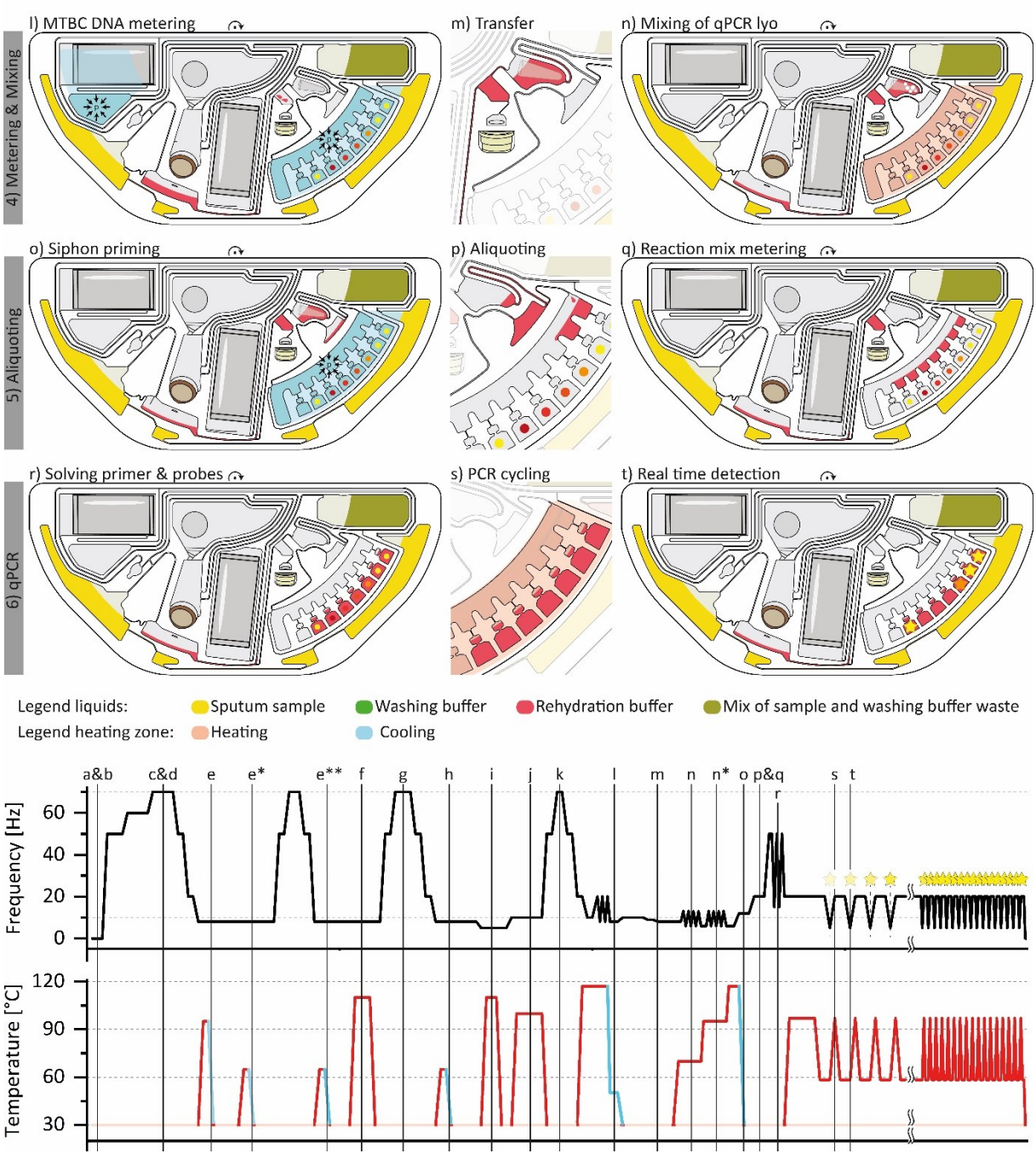


Figure S2: Illustration of the detailed liquid manipulation processes on the centrifugal microfluidic cartridge. Top: Centrifugal microfluidic cartridge with labling of fluidic chambers and illustration of all heating zones in relation to the chambers. Middle: Detailed microfluidic workflow with (0) input of the 3 ml sample, (1) enrichment, (2) washing, (3) thermal lysis, (4) metering and mixing, (5) aliquoting and (6) qPCR. Bottom: Frequency and temperature protocol. Which individual heating zone(s) are activated according to the temperature protocol can be seen in the correspondingly illustrated cartridges from the middle section of this figure. Whenever cooling is relevant for liquid transport it is depicted in blue.

Six cartridge runs were evaluated fluidically to demonstrate reproducible functionality. To avoid contamination during sample preparation (main manuscript Figure 2B - Sample preparation), it is essential that all filtrate of sample or washing buffer is removed into the waste chambers (outer and inner waste chambers) before the next step begins. This is the case for 100 % of the evaluated cartridges after the enrichment and the washing step and exemplarily shown for one run in Fig. S3 in the first two lines. This results in an averaged inner waste chamber volume after elution of 975 µl $\pm$ 40 µl calculated from the inner waste chamber meniscus (Fig. S3-k). The deviation of inner waste level filling originates from different filter flow behaviour. If the entire sample is filtered during the first sample filtration step, more liquid is stored in the outer waste chambers. In contrast, if a portion of the sample remains on the filter and is filtered during the second filtration step (Fig. S2-frequency protocol-e*-e**), this volume is additionally transferred to the inner waste chamber.

The averaged transferred DNA solution to the mixing chamber is calculated to be 171 µl $\pm$ 13 µl from the mixing chamber meniscus (Fig. S3-m), and the number of reaction chambers filled averages 6.75 $\pm$ 0.46 (Fig. S3-q&r). This indicates that the reaction chambers are likely larger than initially designed with 20 µl. However, fluidic and biological reliability and robustness against slide volume variations are proven with the data set of the analytical sensitivity in the following Table S2.

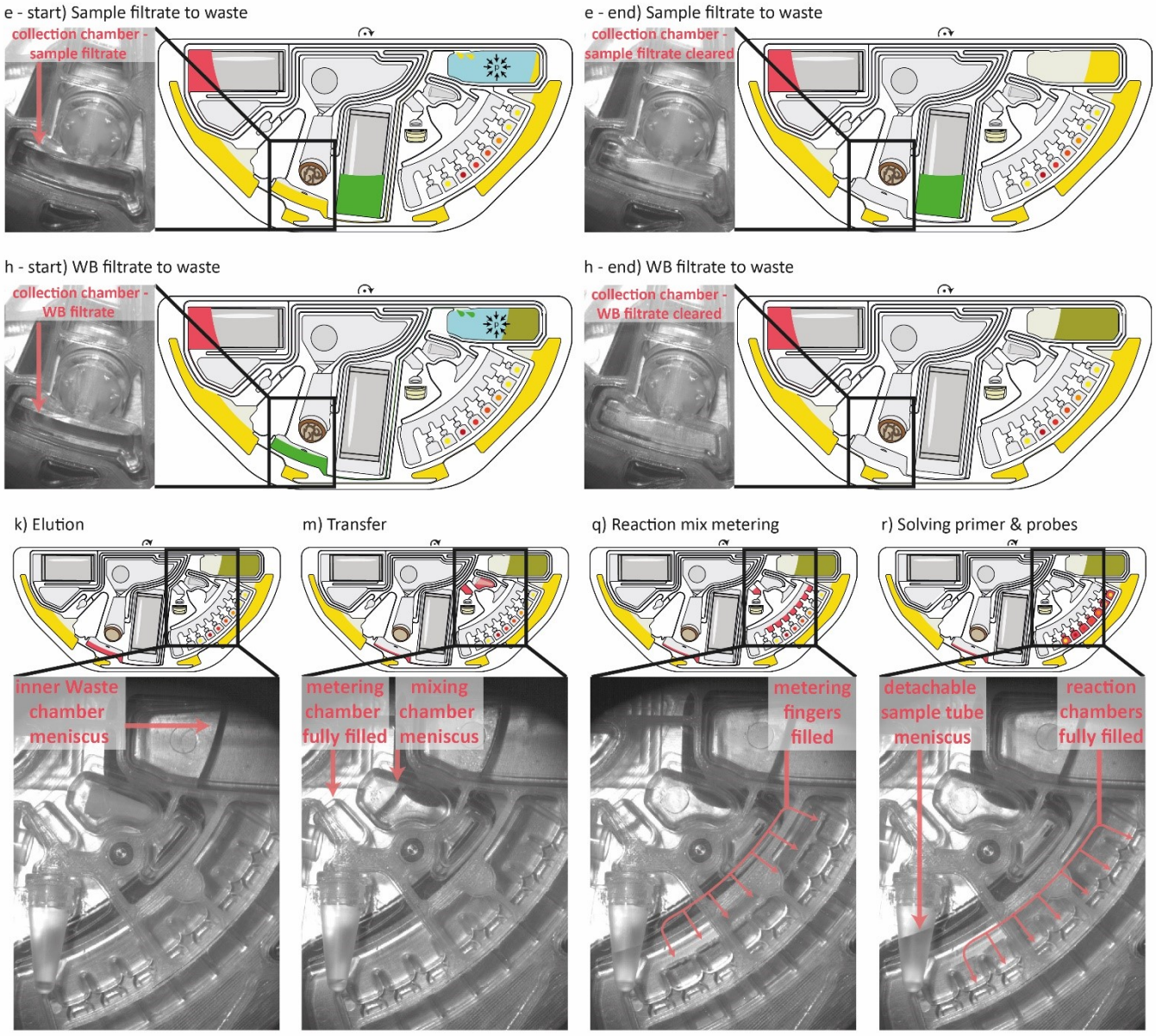


Figure S3: Stroboscopic pictures for the evaluation of the reproducibility of the fluidic protocol. First line: Transfer of sample filtrate to waste (Figure S2e). Second line: Transfer of washing buffer filtrate to waste (Figure S2h). Third line: left: inner waste chamber fill level after elution (Figure S2k), middle-left: fill level of metering and mixing chamber after transfer (Figure S2 m), middle-right: filled metering finger (Figure S2q), right: seven filled reaction chambers (Figure S2r).

S4: Analytical sensitivity.

The CT values of analytical sensitivity data set are shown in Table S2 for the MTBC and IC qPCR with the corresponding standard deviations between the reaction chambers. The IC qPCR is set to achieve CT values around 30 to confirm a valid cartridge run. This is particularly important for the cartridge runs were the MTBC detection no longer provides a positive result.

Table S2: CT values for the cartridge analytical sensitivity data set for the MTBC and IC qPCR with standard deviation between the reaction chambers.

CFU/ml	Replicates	C_T value MTBC qPCR	Standard deviation	C_T value IC qPCR	Standard deviation
11,475	1	27.6	0.2	30.1	0.3
	2	29.1	0.3	30.4	0.0
	3	27.9	0.5	29.5	0.1
	4	27.7	0.4	30.0	0.0
573	1	30.7	0.7	31.4	0.9
	2	30.4	0.5	30.8	0.1
	3	30.9	0.4	30.5	0.4
	4	32.0	0.7	30.8	0.1
	5	34.9	1.7	36.2	1.1
255	1	31.8	0.5	30.0	0.3
	2	33.4	0.4	29.5	0.2
	3	32.8	1.0	29.6	0.6
127	1	32.2	0.2	30.3	0.2
114	1	32.7	0.1	30.7	1.6
	2	36.3	-	35.3	-
	3	32.3	0.1	30.5	0.3
57	1	36.5	0.3	34.8	0.5
	2	34.5	0.3	29.7	1.3
	3	35.2	0.1	30.8	0.1
	4	34.5	1.1	29.2	2.4
	5	35.2	0.0	31.2	0.0
25	1	36.1	0.5	30.8	0.2
	2	-	-	30.4	0.2
	3	36.3	-	30.4	0.6
	4	40.9	-	33.5	-
11	1	-	-	30.7	0.4
	2	41.4	-	30.6	1.4
	3	-	-	30.7	0.0
	4	-	-	30.6	0.4
	5	-	-	30.8	0.8
5	1	-	-	30.7	0.8
	2	-	-	30.7	0.2
	3	-	-	31.7	0.8
	4	-	-	28.2	1.9

S5: tNGS.

The relative enrichment of MTBC DNA by the cartridge processing was evaluated by comparing MTBC DNA gained from the cartridge with a reference of direct DNA extraction. The relative MTBC DNA enrichment of the cartridge is based on the combination of liquefaction and filtration method. On the one hand, the harsh liquefaction reagent already lyses a portion of human cells and gram-negative bacteria cells and releases their DNA. On the other hand, the MTBC bacteria are inactivated but not lysed at this stage. The released human and other bacterial DNA is therefore flushed through the filter during the sample filtration, while the MTBC bacteria are retained and thus relatively enriched.

Cartridge samples were processed by the standard cartridge protocol: liquefaction and inactivation, cartridge processing and removal of the detachable MTBC DNA sample tube. The reference samples were liquefied and inactivated with the same reagent. Both sample types were then processed with the NukEx Mag RNA/DNA kit from gerbion on the M32 robot from Biocomma. Using these two methods, replicate of spike-in sputum samples with 11,475 CFU/ml and 573 CFU/ml were sequenced by WGS and evaluated with Kraken. Kraken is a sequence classification tool that uses exact-match database queries to analyse the sequences present in a sample[3]. Table S2 shows the classification and percentage of human, bacterial and MTBC DNA sequences. All samples processed with the cartridge show a reduced percentage of human DNA in comparison to the reference samples, whereas the percentage of bacterial DNA is clearly increased. When searching for MTBC DNA, the cartridge samples provide results, while in the reference samples the amount of MTBC DNA disappears in the background.

The coverage analysis of the sequenced patient samples from the cartridge with corresponding CT values from MTBC qPCR is shown in Table S3. Out of the 21 patient samples, four failed to achieve a coverage of at least 10 reads and 75% allele frequency of at least 95% of the target regions. Failed samples all featured CT values of 29 or higher.

Table S3: Percentage increase of MTBC DNA in comparison to human DNA achieved with the cartridge processing in comparison to reference samples.

		Replicate	Total reads	Kraken human [%]	Kraken bacteria [%]	Kraken MTBC [%]	Qubit [ng/µl]
11,475 CFU/ml	Cartridge	1	397,663	46.3	21.9	0.09	3.12
		2	808,239	44.3	24.3	0.07	3.82
		3	1,282	4.3	7.6	0.77	3.26
		4	924,331	48.9	20.6	0.09	6.82
	Ref	1	4,191,676	91.0	0.1	0	2.00
		2	4,256,355	90.7	0.1	0	1.91
573 CFU/ml	Cartridge	1	1,618,088	60.8	14.4	0.03	3.64
		2	1,031,957	65.1	11.3	0.04	5.38
		3	527,614	48.1	21.1	0.03	2.76
		4	1,020,576	59.8	14.8	0.04	6.54
		5	4,177,237	74.4	5.8	0.01	3.48
	Ref	1	3,838,423	91.1	0.1	0	2.40
		2	4,025,434	90.5	0.1	0	1.99
		3	3,895,860	90.8	0.1	0	1.75
		4	3,834,385	90.9	0.2	0	2.02

Table S4: Coverage of target regions with sequence information.

Patient sample ID	C_T values from MTBC qPCR detection on the cartridge	Percentage of reads mapped to target regions	Target regions covered by reads [bp]	Percentage of target regions covered by reads	Mean coverage depth	Target regions with at least 10 reads and 75% allele frequency coverage [bp]	Percentage of target regions with at least 10 reads and 75% allele frequency	tNGS status
1	31.5	62.4%	75,880	60.0%	186.5	64,657	51.1%	*failed*
2	35.3	1.9%	33,612	26.6%	128.2	12,112	9.6%	*failed*
3	34.0	0.7%	21,245	16.8%	106.8	13,758	10.9%	*failed*
4	31.9	36.0%	125,507	99.2%	788.6	122,607	96.9%	*successful*
5	28.5	42.5%	125,657	99.3%	1099.9	123,403	97.5%	*successful*
6	24.9	72.2%	125,874	99.5%	2260.1	124,474	98.4%	*successful*
7	23.5	90.5%	126,140	99.7%	2622.9	124,762	98.6%	*successful*
8	24.4	81.7%	125,613	99.3%	858.0	122,838	97.1%	*successful*
9	26.2	54.7%	125,359	99.1%	997.0	123,341	97.5%	*successful*
10	21.6	90.6%	126,208	99.7%	2769.2	124,642	98.5%	*successful*
11	24.5	85.2%	126,096	99.6%	2279.4	124,524	98.4%	*successful*
12	24.6	70.3%	125,921	99.5%	1964.3	124,272	98.2%	*successful*
13	23.9	75.5%	125,923	99.5%	2333.7	124,345	98.3%	*successful*
14	21.1	89.9%	126,338	99.8%	2640.3	124,306	98.2%	*successful*
15	21.2	90.7%	126,256	99.8%	2633.9	124,208	98.2%	*successful*
16	27.9	78.0%	125,751	99.4%	2098.8	124,073	98.1%	*successful*
17	27.8	76.1%	126,258	99.8%	1453.5	123,580	97.7%	*successful*
18	25.8	77.1%	125,852	99.5%	1670.1	123,840	97.9%	*successful*
19	29.7	22.8%	73,204	57.9%	208.5	64,498	51.0%	*failed*
20	23.4	74.6%	126,041	99.6%	1820.8	123,990	98.0%	*successful*
21	24.3	70.4%	125,957	99.5%	2007.7	123,897	97.9%	*successful*

ESI References

1. Hess JF, Kotrová M, Calabrese S, et al. Automation of Amplicon-Based Library Preparation for Next-Generation Sequencing by Centrifugal Microfluidics. Anal Chem 2020; 92: 12833–12841.
2. Hin S, Paust N, Keller M, et al. Temperature change rate actuated bubble mixing for homogeneous rehydration of dry pre-stored reagents in centrifugal microfluidics. *Lab Chip* 2018; 18: 362–370.
3. Salzberg SL and Wood DE. Releasing the Kraken. *Front Bioinform* 2021; 1: 808003.