MULTIPLEX DETECTION OF *KRAS* POINT MUTATIONS FROM TUMOR CELL DNA ON A CENTRIFUGAL MICROFLUIDIC CARTRIDGE (GENESLICE) FOR CHOICE OF PERSONALIZED CANCER THERAPY

O. Strohmeier[1,2*], S. Laßmann[3,4,5,6], B. Riedel[3,6], M. Werner[3,5,6], D. Mark[1], R. Zengerle[1,2,4] and F. von Stetten[1,2]

[1] HSG-IMIT, Villingen-Schwenningen, Germany; [2] Laboratory for MEMS Applications, IMTEK - Department of Microsystems Engineering, University of Freiburg, Germany;
[3] Inst. of Pathology, University Medical Center Freiburg, Germany; [4] BIOSS – Centre for Biological Signalling Studies, University of Freiburg, Germany; [5] Comprehensive Cancer Center Freiburg, Germany;
[6] German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), Heidelberg, Germany

ABSTRACT

A novel **centrifugal microfluidic cartridge** "GeneSlice" enables **automated, parallel detection** of seven point mutations on the ***KRAS* gene** [1], a highly relevant **biomarker** for **choice of personalized cancer therapy**. **Time-to-result is approx. 2 hours**, significantly shorter than **approx. 20 hours for the state-of-the-art** sequencing approach. Primers and probes were preloaded, making the disposable cartridge **ready-to-use**. Feasibility and reproducibility have been demonstrated by genotyping DNA from 6 *KRAS* cell lines and two human colorectal carcinomas. 23 / 24 genotyping experiments for cell line DNA and 5 / 5 experiments from human specimens resulted in correctly genotyped mutations. Genotyping results predict the success of antibody based cancer therapy.

KEYWORDS: Centrifugal microfluidics, LabDisk, genotyping, KRAS, microfluidic

INTRODUCTION

The *KRAS* gene is an important biomarker to predict the success of state-of-the-art antibody based cancer therapy targeting the epidermal growth factor receptor (EGFR). A mutated KRAS gene is associated with poor therapy response. Primarily relevant are seven point mutations on codons 12 / 13 of exon 2. Genotyping of these mutations is usually conducted by costly and time consuming sequencing requiring labor intensive DNA preparation and expensive equipment. As a fast and cost efficient alternative, an allele-specific real-time PCR assay has been introduced recently [1]. We demonstrate the integration of this assay into a low-cost, disposable centrifugal microfluidic cartridge called "GeneSlice" to enable process automation and to reduce time-to-result. The GeneSlice is depicted in *Figure 1*.

THEORY

Allele-specific real-time PCR exploits differences in amplification efficiency. If a primer fully matches the target DNA, the C_q value is lower compared to the C_q value resulting from a mismatching primer. For each of the seven *KRAS* point mutations and the wildtype, a perfectly matching allele-specific forward primer was designed and prestored in one of the eight amplification chambers of the GeneSlice along with a mutual reverse primer and a hydrolysis probe. A mutation was assumed to be present, if the difference in C_q between the wildtype (detected in amplification chamber #1 of the GeneSlice) and a specific mutation (detected in the amplification chambers #2 - #8) was below a cut-off of 9 – 10 cycles (*Figure 2*).

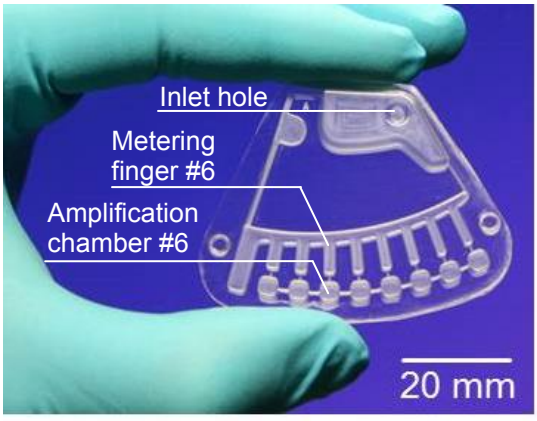

Figure 1: Photograph of one GeneSlice. Each GeneSlice features an inlet hole for loading the sample into an inlet chamber that is connected to an aliquoting structure with 8 metering fingers. Each metering finger is connected to an amplification chamber #1 to #8.

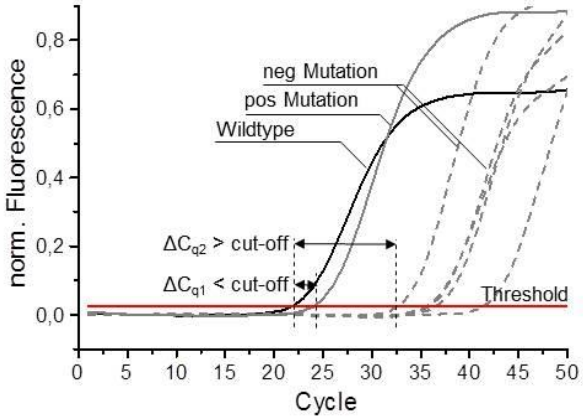

Figure 2: Graphical depiction of allele-specific PCR and cut-off values. A mutation was assumed to be present if the $\Delta C_q = (C_{q,mutation} - C_{q,wildtype})$ was below a cut-off value of 9 to 10 C_q values depending on the mutation [1].

EXPERIMENTAL

All GeneSlices used in this work were fabricated by the HSG-IMIT Lab-on-a-Chip Design- & Foundry Service [2]. In short, polymer foils (COP ZF 14, Zeon Chemicals, USA) were structured using the µTSL process [3,4]. Each amplification chamber #1- #8 was loaded with one allele-specific forward primer, mutual reverse primer and FAM labeled hydrolysis probe according to *Lang* et al. [1]. The GeneSlices were sealed by a pressure sensitive adhesive (Cat. No. 900320, HJ Bioanalytik, Germany). For processing, 1 to 4 GeneSlices are placed in a custom made holder, thereby up to 4 patient samples can be processed independently and in parallel. 90 µL mixture of DNA sample and PCR mastermix (QIAGEN QuantiTect, Germany) were pipetted into the inlet hole and the holder was placed in a commercially available real-time PCR thermocycler (Rotor-Gene 2000, Corbett Research acquired by QIAGEN, Germany). A hardware modification of the thermocycler allows changing the spin speed between 6.6 Hz (default) and 27.2 Hz [3]. At 6.6 Hz, the liquid is routed from the inlet radially outwards and aliquoted into eight 10 µL subvolumes (*Figure 3*). The aliquots are then transferred into the amplification chambers at 27.2 Hz, where the prestored primers and hydrolysis probes are rehydrated. After fluidic processing, amplification and real-time fluorescence detection can be started.

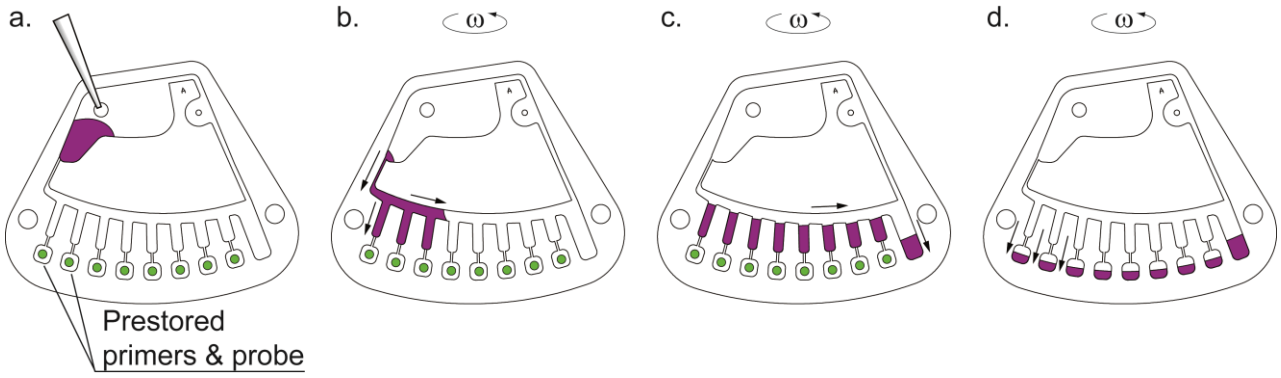

Figure 3: Depiction of the fluidic processflow. (a) 45 µL mastermix (containing the polymerase), 36 µL PCR grade water and 9 µL of sample DNA are premixed and pipetted into the inlet hole. (b) Rotation at 6.6 Hz forces the liquid radially outwards. (c) The liquid fills the metering fingers, supernatant is gated into the waste. (d) Acceleration to 27.2 Hz triggers the transport of the aliquots into the amplification chamber where prestored primers and hydrolysis probes are rehydrated and real-time amplification is performed.

RESULTS AND DISCUSSION

Since the mutational status is assessed by a difference in C_q values between amplifications in different amplification chambers, it is a prerequisite that PCR performance is comparable in each amplification chamber throughout one GeneSlice. Therefore, two GeneSlices were prepared with primers and probes to measure intra-chip variation of C_q values by amplification of *KRAS* wildtype in all eight amplification chambers. Standard deviation of all eight C_q values was 0.13 (GeneSlice 1) and 0.26 cycles (GeneSlice 2) what is negligible compared to the ΔC_q cut-off of 9 – 10 cycles required for assessment of mutation (*Figure 4*). Thus, the allele-specific *KRAS* assay is suitable for integration into the GeneSlice.

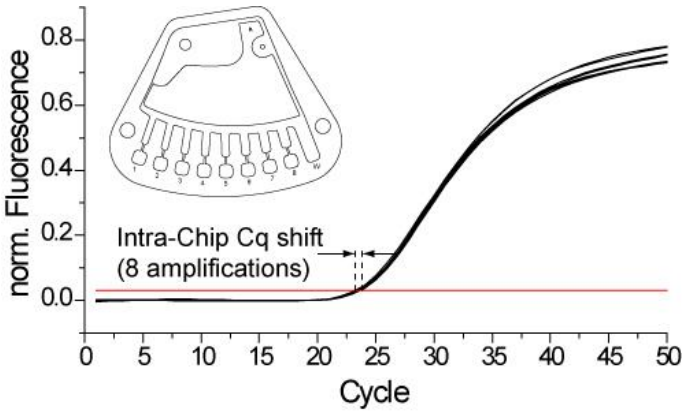

Figure 4: Amplification plots from wildtype KRAS in all eight amplification chambers of GeneSlice 1 for evaluation of intra-chip C_q variation.

Reproducibility was evaluated by genotyping DNA from 6 colorectal cancer cell lines (SW 480, LS 174T, HT 29, DLD 1, HCT 1 and CaCo2; all ATCC, LGC Standards, Germany) with known *KRAS* mutation status. Each experiment was conducted in replicates of 4. In 23 out of 24 experiments the results from the genotyping on GeneSlices was concordant with standard qPCR (ABI7900HT real-time PCR system) and sequencing (ABI 3100 sequencer). One out of four experiments for the CaCo2 cell line resulted in a wrong positive Gly12Cys mutation instead of the wild type. Since the other 3 replicate experiments resulted in correctly genotyped wildtype for this cell line, the most probable explanation for the wrong positive detected mutation might be cross contamination during preparation of the template DNA or a mistake in preparation of the GeneSlice e.g. due to prestorage of primers and probe for wildtype *KRAS* in the amplification chamber dedicated for the detection of the Gly12Cys mutation. Additionally, DNA from two human colorectal carcinomas has been genotyped in replicates of three showing full consistency with sequencing. Patient A was diagnosed as wildtype *KRAS*, hence antibody treatment can be considered as therapy. Patient B was diagnosed to carry a Gly13Asp mutation what may result in poor response to antibody treatment. One experiment was excluded due to cross contamination (*Table 1*).

Table 1: Results from genotyping of human colorectal carcinoma DNA on the GeneSlice. Patient A was genotyped as wildtype KRAS (one experiment excluded due to cross contamination). Patient B was genotyped to carry Gly13Asp mutation. Thus, antibody therapy for Patient B is not appropriate.

			GeneSlice Amplification Chamber								
			#1	#2	#3	#4	#5	#6	#7	#8	
Sample		Mutation	Wild Type	Gly 12Ser	Gly 12Arg	Gly 12Cys	Gly 12Asp	Gly 12Ala	Gly 12Val	Gly 13Asp	AB-therapy recommended
Pat. A	1	Wildt.	+	-	-	-	-	-	-	-	yes
	2		+	-	-	-	-	-	-	-	yes
Pat. B	1	Gly13Asp	-	-	-	-	-	-	-	+	no
	2		-	-	-	-	-	-	-	+	no
	3		-	-	-	-	-	-	-	+	no

CONCLUSION

Application of the centrifugal microfluidic GeneSlice for genotyping of *KRAS* point mutations on tumor cell DNA is suggested to accelerate decision making in personalized cancer therapy. The ready-to-use GeneSlice with the integrated allele-specific real-time PCR assay enables a short turnaround time (approx. 2 hours), full automation and ease of use compared to labor intensive sequencing (~ 20 hours) what is the current gold standard in diagnosis. As an advantage, the GeneSlices can be processed in a commercially available real-time PCR thermocycler with minor hardware modification. Intra-chip C_q variation was measured to be negligible (variations of 0.13 and 0.26 cycles between 8 amplifications). Additionally, 28/29 genotyping experiments (23/24 from cell line DNA; 5/5 from human colorectal cancer DNA) resulted in correctly genotyped *KRAS*. Hence, the GeneSlice might be a helpful tool for routine diagnostics and is currently evaluated in a broader molecular biological study.

ACKNOWLEDGEMENTS

We gratefully acknowledge financial support from the Baden-Württemberg Foundation for funding this project ("Amplidisk" - MST II – 17).

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CONTACT

*O. Strohmeier, tel: +49-761-203 73232; oliver.strohmeier@hsg-imit.de