A MICROFLUIDIC PLATFORM FOR PERSONALIZED CANCER DIAGNOSTICS BY PADLOCK PROBES LIGATION AND CIRCLE-TO-CIRCLE AMPLIFICATION

Annika Ahlford[1], Alvaro J. Conde[2], David Sabourin[3], Jörg P. Kutter[3], Mats Nilsson[1], Martin Dufva[3] and Monica Brivio[1,3*]

[1]Rudbecklaboratoriet, Uppsala University, SWEDEN
[2]Universidad Nacional de Tucumán, ARGENTINA
[3]DTU Nanotech, Technical University of Denmark, DENMARK

ABSTRACT

We present a microfluidic system built from modular platform elements intended for detection of the seven most common mutations in the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene by highly selective padlock probes ligation combined with circle-to-circle amplification (C2CA) and fluorescence detection. Padlock probes assays have been successfully established and the feasibility of the ligation-based procedure on a microfluidic format has been evaluated in PMMA chambers. The assays are currently being integrated in the microfluidic system described in this paper.

KEYWORDS: Personalized Medicine, Ligation-based, Mutations, Integrated System.

INTRODUCTION

A limitation of cancer therapy is the inability to predict treatment response. Although molecular cancer therapies have improved treatment outcome, they are expensive and only benefit a small fraction of patients. The costs and efficacy of cancer therapy could therefore considerably be improved through personalized medicine. Drugs based on monoclonal antibodies are used to block the epidermal growth factor receptor (EGFR/HER1) to reduce downstream cell signaling that promote tumor development. These drugs only have an effect in patients that have a normal EGFR signaling pathway. Many genes encoding important factors downstream in the pathway are, however, mutated in tumors. The *KRAS* gene is, e.g., altered in about 30% of frequent cancers. Mutant forms of *KRAS* are unaffected by upstream EGFR receptor inhibition by the drugs, which leads to permanent signaling and proliferation of tumor cells. Detection of mutations in these genes, including *KRAS*, is therefore vital to improve cancer therapy.

MOLECULAR METHOD

In previous studies we have shown that ligation-based assays, using padlock probes, are attractive for mutation detection [1]. Detection signals generated by padlock probe ligation can be amplified by rolling-circle amplification (RCA) [2]. By counting individual labeled RCA products, a digital measurement of specifically detected DNA molecules is obtained [3]. Detection sensitivity is greatly improved by applying an exponential version of RCA, called circle-to-circle amplification (C2CA) [4]. A schematic representation of the C2CA protocol is reported in Figure 1.

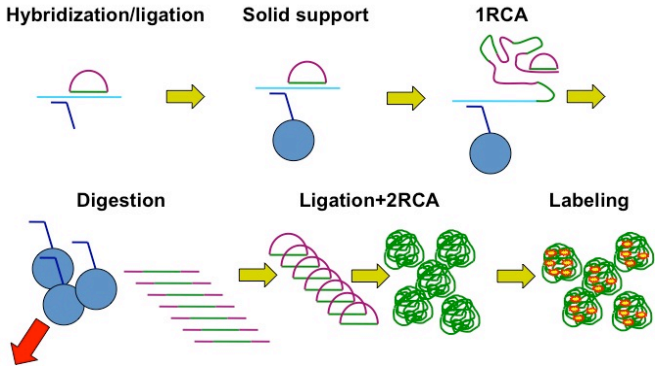

Figure 1: Schematic representation of the C2CA protocol. Upon recognition of the target sequence (light blue) the padlock probe (pink-green) is circularized and enzymatically ligated. The target-padlock complex is captured at the surface of magnetic beads and the target is enzymatically amplified using the padlock as a template. RCA products are restriction digested and the beads are discarded. The monomers are re-circularized and used as templates for the second RCA. Second generation RCA products are fluorescently labeled and analyzed.

EXPERIMENTAL

Ten padlock probe assays have been designed and established for wild type alleles and mutations in codons 12 and 13 of the *KRAS* gene. Padlock probe sequences along with their respective *KRAS* target are shown in Table1. Blue sequences de-

note target specific recognition arms including each variable allele. Red, green and purple sequences are universal backbone parts for digestion, circularization and 2nd RCA (see Figure 1). Preliminary tests, to evaluate the feasibility of the proposed molecular procedure on a microfluidic format, have been performed in 15 uL and 30 uL chambers fabricated by micromilling in PMMA substrates (not shown here) bonded to a cover lid by UV-assisted bonding [5]. Samples as well as reagents and products were loaded on-chip and recovered by means of micropipettes.

Table 1. Padlock probe assays

Assay name	*KRAS target*		*Padlock probe sequence*
wt1	**G**GT	GGC	AGCTCCAACTACCACAAAGTCGATAGTCACGGCTACT-TGTATGAGCTCCTCAGTACTTGCCTACGCCACC
mut12.1	**A**GT	GGC	AGCTCCAACTACCACAAAGCCAAACATGTGCATTGAG-TGTATGCAGCTCCTCAGTATCTTGCCTACGCCACT
mut12.2	**C**GT	GGC	AGCTCCAACTACCACAAAGCCAAACATGTGCATTGAG-TGTATGCAGCTCCTCAGTACTTGCCTACGCCACG
mut12.3	**T**GT	GGC	AGCTCCAACTACCACAAAGCCAAACATGTGCATTGAG-TGTATGCAGCTCCTCAGTATCTTGCCTACGCCACA
wt2	G**G**T	GGC	CAGCTCCAACTACCACAAAGTCGATAGTCACGGCTACT-TGTATGCAGCTCCTCAGTACTTGCCTACGCCAC
mut12.4	G**A**T	GGC	CAGCTCCAACTACCACAGCCAAACATGTGCATTGAG-TGTATGCAGCTCCTCAGTACTCTTGCCTACGCCAT
mut12.5	G**C**T	GGC	CAGCTCCAACTACCACAGCCAAACATGTGCATTGAG-TGTATGCAGCTCCTCAGTACTCTTGCCTACGCCAG
mut12.6	G**T**T	GGC	CAGCTCCAACTACCACAGCCAAACATGTGCATTGAG-TGTATGCAGCTCCTCAGTACTCTTGCCTACGCCAA
wt3	GGT	G**G**C	CACCAGCTCCAACTACCACAAAGTCGATAGTCACGGCTACT TGTATGCAGCTCCTCAGTACTTGCCTACGC
mut13	GGT	G**A**C	CACCAGCTCCAACTACAGCCAAACATGTGCATTGAG-TGTATGCAGCTCCTCAGTAGCACTCTTGCCTACGT

RESULTS AND DISCUSSION

Microscale technology is promising for improved cancer therapy providing rapid, automated, highly accurate and sensitive tests at low cost. Here, we present a microfluidic platform for DNA analysis of *KRAS* (Figure 2). A first microfluidic chip prototype with three interconnected chambers (Figure 3) for padlock probe ligation and C2CA amplification has been developed. Target selection and RCA are performed in the first chamber using a solid support. The first generation RCA products are restriction digested and eluted to the second chamber where the second RCA is performed. Second generation RCA products are fluorescently labeled in the third chamber prior to off-chip detection [3]. Flow control is provided by modular valves and micropumps [6], which are interfaced to the C2CA chip on a modularly integrated platform (Figure 2). Actuation, control and programming of the micorpump and valves is performed using LEGO® motors and controllers. Internal sensors allow control of motor rotation to within 1°.

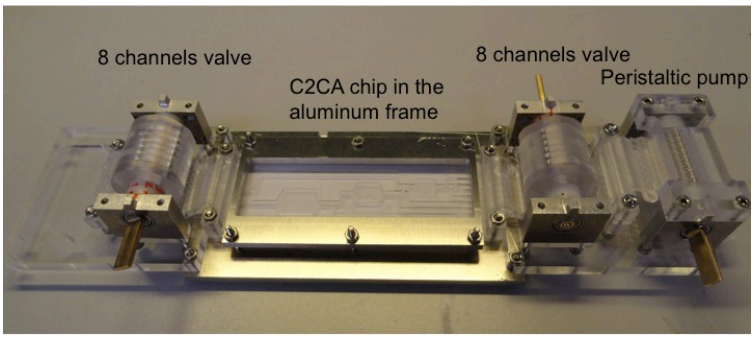

Figure 2. Modular microfluidic system. The basic building blocks (i.e. pumps, valves and baseplate) were previously reported [6].

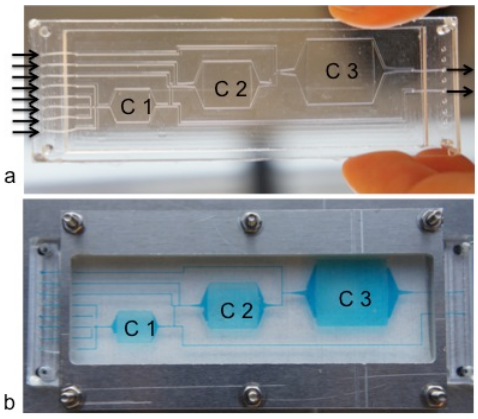

Figure 3. Microfluidic C2CA chip. (a) Chambers (C1, C2 and C3) and channels are casted in PDMS and sealed with a glass microscope slide. (b) The PDMS/glass chip assembled in a metal frame is fitted on a heating block for temperature control.

Molecular assays have been developed for the detection of the seven most common mutations in codons 12 and 13 of the *KRAS* gene. Protocols for all enzymatic reaction steps including target recognition, amplification and digestion (illustrated in

Figure 1) along with labelling and variant detection, have successfully been performed and optimized in the conventional test-tube format. The histogram in Figure 4 shows the number of counted fluorescent RCA products for each assay using a flow channel and a confocal microscope. The protocols have next been transferred and adapted to a microfluidic assay format. This implementation includes efforts to maintain and improve assay sensitivity, the evaluation of different solid supports, and slight modifications of reagent mixtures and volumes and order of reaction steps. Preliminary results of the C2CA-based detection of wild type KRAS carried out on-chip with manual handling are shown in Figure 5, providing the feasibility of the method. A lower RCA product count observed for the on-chip samples, as compared to parallel tube experiments analyzing the same DNA sample, however indicates the need for optimization of the microfluidic procedure.

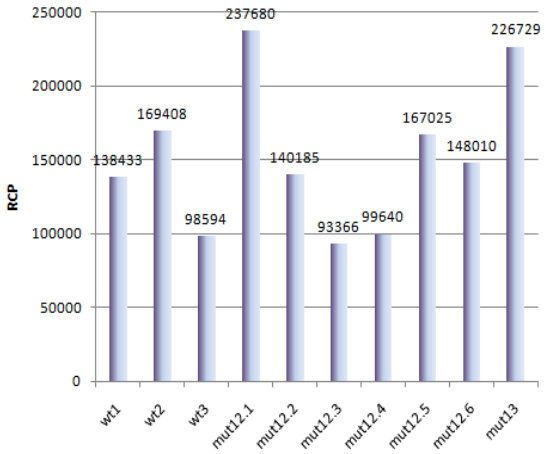

Figure 4: Histogram showing RCA product (RCP) count on y-axis for each of the ten padlock probe assays on the x-axis.

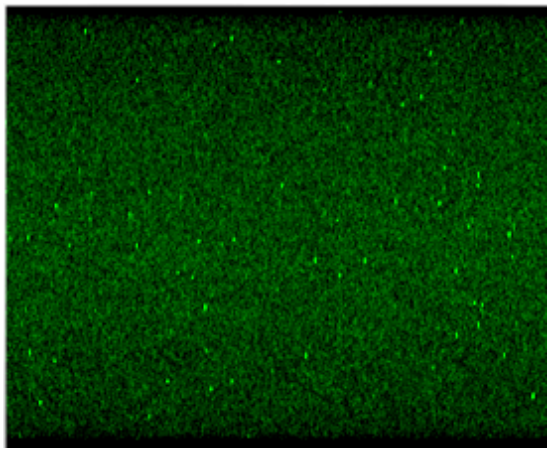

Figure 5: Confocal micrograph of RCA products (bright dots) generated in a PMMA chip with manual handling.

CONCLUSION

Here, we present a microfluidic platform for point-of-care DNA analysis of *KRAS*. We have designed a microfluidic chip for C2CA-based mutation detection. The modular approach and use of a disposable chip and a re-usable handling and control unit makes the system highly flexible. After successfully establishing biochemical assays and developing a microfluidic chip, the entire assay is being validated within the integrated microfluidic system. Our approach combines the advantages of lab-on-a-chip technologies and the versatility of microfluidic devices with the high specificity and sensitivity of ligation-based molecular assays for mutation detection in personalized cancer medicine.

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CONTACT

*M. Brivio, tel: +46-760287181; monica.brivio@nanotech.dtu.dk; monica.brivio@igp.uu.se