Chapter 2

Assessment of a Fully Automated High-Throughput DNA Extraction Method from Formalin-Fixed, Paraffin-Embedded Tissue for *KRAS,* and *BRAF* Somatic Mutation Analysis

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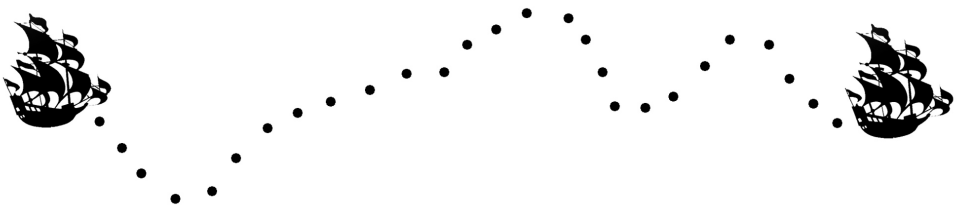

Abstract
Preoperative biopsies or imbedded cytological cells will become more and more a primary source of tissue for molecular diagnostic analyses as a result of novel neo-adjuvant treatment regimens for several cancer types. Furthermore there is a growing need to examine metastatic cancer tissue. Hence, nucleic acids need to be reliably isolated and analyzed from small amounts of formalin-fixed and paraffin-embedded (FFPE) tissue. The limited numbers of (tumor) cells in these samples make high quality and sensitive DNA isolation challenging. Also demands for faster turnaround times are growing. Therefore, we evaluated a fully automated DNA/RNA isolation system and compared this with a manual, classical routine molecular pathology method. We compared the quality of the isolates from both tissue cores and micro-dissection for detection of hotspot mutations in *KRAS*, *BRAF* applying hydrolysis probe assays. In addition we determined whether the automated method decreases the hands-on-time and turnaround times in routine molecular pathology workflow.
In conclusion, the automated method delivers high quality DNA from both small FFPE tissue cores and micro-dissected tissue material. In comparison to classical methods, less than 50% of starting tissue was sufficient as input for micro-dissection. Turnaround times decreased significantly and 50% less hands-on time was needed.

Introduction
Increasing numbers of cancer biomarkers have been implemented in molecular tumor diagnostics worldwide [1–4]. Mutations in *KRAS* predict for resistance to monoclonal antibody therapy in colon cancer patients [5]. In *BRAF,* the V600E and the rarer V600K variant are found in the majority of cutaneous melanoma, making the patients eligible for treatment with vemurafenib (PLX4032). [6] A subset of Non_Small_Cell_Lung_Cancers may harbour activating mutations in the *EGFR* kinase domain and might thereby respond to certain tyrosine kinase inhibitors [2]. Thus, based on these test results for *KRAS, BRAF and EGFR*, patients may be selected for guided treatment. Furthermore, a delay in the start of treatment of a cancer patient might influence the patient's life expectation (e.g., for the treatment of lung cancer with tyrosine kinase inhibitors). In addition, the amount of cancer material that is available for testing is decreasing as a result of the introduction of neo-adjuvant treatment protocols and the growing need to examine metastatic cancer tissue. Less invasive sampling procedures [7] may lead to little amounts of material.
The starting point in biomarker testing in pathological specimen is the efficient isolation of nucleic acids. These can be isolated from formalin-fixed, paraffin-embedded (FFPE) tissue from whole tumor sections, micro-dissected material, tissue cores or imbedded cytological material [8,9]. In FFPE tissue DNA degradation has already taken place resulting in a negative contribution to the quantity and quality of the DNA. [10,11] Several manual and semi-automated methods have been described for DNA extraction from FFPE tissue [12,13]. DNA quality and quantity obtained with these different techniques is variable. This

variability is primarily due to the quality of the material that has been used and not because of the quality of the isolation technique [14]. Nevertheless, all techniques described thus far require many hours of hands-on time and include operator- to-operator variation that might contribute to less reproducible and robust results [15]. Part of the hands-on time is due to the manual micro-dissection and lysis of the tumor tissue. However, deparaffinization is a crucial, time consuming step that can impact the quality and quantity of the extraction [16]. Moreover, this process often includes the use of toxic reagents such as xylene [17]. Additionally, in many cases, and specifically in micro-dissection, the final DNA yield is low and of reduced quality, thereby requiring additional steps such as whole genome amplification or other pre-amplification steps in order to obtain sufficient DNA for further molecular testing. [18–20]. Therefore, there is an increasing demand for fully automated, optimized and time-saving methods for the high quality DNA extraction from limited amounts of material.

Here we describe DNA extraction using a fully automated DNA/RNA extraction system which can process 48 tissue samples in 3 hours 15 min using silica-coated magnetic nanoparticles. The process integrates both lysis and deparaffinization by hydrophobic adsorption instead of offline xylene based deparaffinization [21,22]. We investigated if the quality of the isolated DNA from tissue cores and micro-dissected tissue obtained with this newly described method compares to our classical method. We also evaluated if the method decreases the turnover (turnaround) time for our most common molecular assays.

We determined that the fully automated method delivers high quality DNA from small tissue cores and micro-dissected material as compared to our classical method. For micro-dissection we found that only 20%- 50% of starting material was needed for the fully automated method when compared to the classical method. When the DNA is used in hydrolysis probes assays we achieved 24 hours faster turnover (turnaround) time with 50% less hands-on time being required.

Material and Methods

ETHICS STATEMENT

All samples used in this study were handled according to the medical ethical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences (www.federa.org, accessed October 27, 2010). According to these guidelines, the specific need for the ethics committee's approval was not necessary for this study because all human material used in this study has been anonymized.

TEST MATERIAL

This study included formalin-fixed, paraffin-embedded tissue samples obtained from micro- dissected tissue from slides (10 µm) and tissue cores (0.3 mm diameter and variable length) of different tissue types (Supplementary table 1). Hematoxylin-eosin staining was performed on tissue sections to visualize presence of tumor cells. These were used to guide micro-dissection on hematoxilin-stained duplicate slides and to determine the area of the tissue cores.

DNA EXTRACTION

The classical method

Classical DNA extraction from FFPE material was performed according to the method described by de Jong [23]. In brief, the FFPE sections on slides or tissue cores were deparaffinized by two xylene and ethanol washing steps (process includes centrifugation and incubation steps as well). The sections and cores were collected in various amounts of PK1 buffer depending on the amount of material and then incubated overnight at 56°C in the presence of proteinase K and Chelex beads. If the volume of the PK1 buffer was under 15 µl no Chelex beads were added. The following day the samples were further incubated at 100°C for 10 minutes, centrifuged and then the supernatant was transferred to a clean tube.

The fully automated method

The fully automated DNA extraction from FFPE tissue (Tissue Preparation System with VERSANT Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY) has been described previously, [21,22] In this method micro-dissected tissue or tissue cores were directly transferred into 1.5 ml tubes (Sarstedt, Nümbrecht, Germany) and subjected to automated total nucleic acid extraction. Samples were heat lysed in 150-µL FFPE buffer at 80°C for 30 minutes with shaking. After cooling, enzymatic lysis was carried out at 65°C for 30 minutes with proteinase K. Any residual tissue debris was then removed by the nonspecific binding to silica-coated iron oxide beads followed by subsequent magnetic separation. Deparaffinized and clarified lysates were transferred to new tubes and nucleic acids were bound to fresh silica-coated beads under chaotrophic conditions. Beads were washed 3 times and total nucleic acids were eluted with 100 µL of elution buffer at 70°C.

MOLECULAR ANALYSIS

Hydrolysis probes assays were performed as described elsewhere [7]. In this method 10µl qPCR reactions contained 5µl mastermix (FastStart Universal Probe Master, Roche Diagnostics, Almere, The Netherlands), 1µl of 10x primer and hydrolysis probe solutions and 2µl DNA solution or sterile water. qPCR was performed in a sealed 384 well plate in a qPCR instrument (CFX384, Bio-Rad, Veenendaal, The Netherlands), with an initial denaturation step of 10 minutes at 95°C follow by 40 cycles of 15 seconds at 92°C, 60 seconds at 60°C and 10 seconds at 72°C. In the experiments described below we used 8 different assays, 7 for *KRAS* p,G12S, p,G12R, p.G12C, p.G12D, p.G12A, p.G12V and p.G13D and one for the *BRAF* p.V600E variant.

Results

For molecular diagnostic analyses of hotspot mutations in *KRAS* and *BRAF* on DNA isolated from small tissue cores or micro-dissected tissue sections hydrolysis probes assays are often used.. As described, DNA isolation from FFPE tissue sections is possible with a fully automated system in the routine laboratory [22,24]. Since in our laboratory we isolate DNA in ~ 60% of the cases

from tissue cores, our first assessment of the fully automated system was to determine if DNA could be isolated from 0.3 mm tissue cores taken from tumor fields of FFPE tissue blocks. We used 3 tissue cores from 4 colon tumor / normal pairs, respectively for the classic and automated isolation methods as described in the material and methods. Final DNA was collected in 100 µl PK1 for the classic and 100 elution buffer for the automated method. To check for the quality of the material we performed the 8 hydrolysis probes assays with 2 µl of a 1:5 diluted stock of both eluates (Supplementary table 1). All 64 data-points were plotted in a scatterplot (Figure 1A). The mean Cq of the "Classic samples was 31.45 with a standard deviation of 1.55. The automated method had a mean Cq of 32.23 with a standard deviation of 1.96 demonstrating that Cq values obtained with the Classic method are in the same range as the Cq values obtained with the automated method.

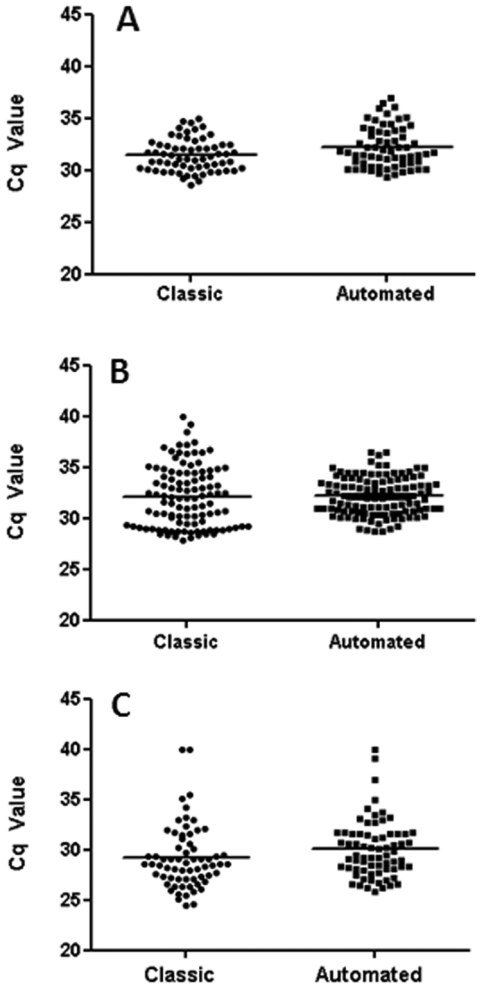

Figure 1. Scatter plots of hydrolysis probes assays Cq values over isolation type. Plot A shows the Cq values obtained in DNA isolated from tissue cores in equal amounts of DNA obtained with the classic and fully automated method with 8 different hydrolysis probes assays for *KRAS* and *BRAF*. Plot B shows the Cq values obtained with equal amounts of micro-dissected DNA in 8 different hydrolysis probes assays for KRAS and BRAF. Plot C shows the Cq values obtained with a BRAFV600E Hydrolysis probes assay on samples in two different, however comparable sets of samples tested in two different time intervals in molecular diagnostics

Micro-dissection.

In our laboratory micro-dissection for tumor cell enrichment, where 8 to 10 consecutive 10 micron sections are used as starting material, is required in about 40% of the cases. Deparaffinized and stained tumor containing fields are scraped off the different slides and pooled. This process, at about 5 minutes per slide, adds up to approximately 1 hour hands-on time.

We determined the threshold for the minimal input of the automated system by micro-dissecting in duplicate 1, 2 and 4 mm² tissue from deparaffinized and stained sections of a single 10 micron slide of a colon and a lung specimen. DNA was extracted with the automated method and eluted in a final volume of 100µl. Two µl of the eluate was used in the *KRAS* and the *BRAF* hydrolysis probes assay. Mean Cq values of 31.97 +/- 1.6, 31.45 +/- 1.5 and 29.93 +/- 1.3 were observed for the 1, 2 and 4 mm² tissue sections, respectively (Supplementary table 2). This demonstrates that as little as 1 mm² micro dissected tumor material of a single 10 µM slide can produce enough DNA to perform 50 qPCR reactions when using the automated extraction method. In the colon cancer specimen a *KRAS* c.34G>A mutation was clearly detectable in the 1, 2 and 4 mm² micro-dissected tissue sections. Remarkably the wild type allele tended to disappear in the 1 mm² .This may possibly be explained by the loss of the wild type allele or preferential amplification of the mutant allele. (Figure 2)

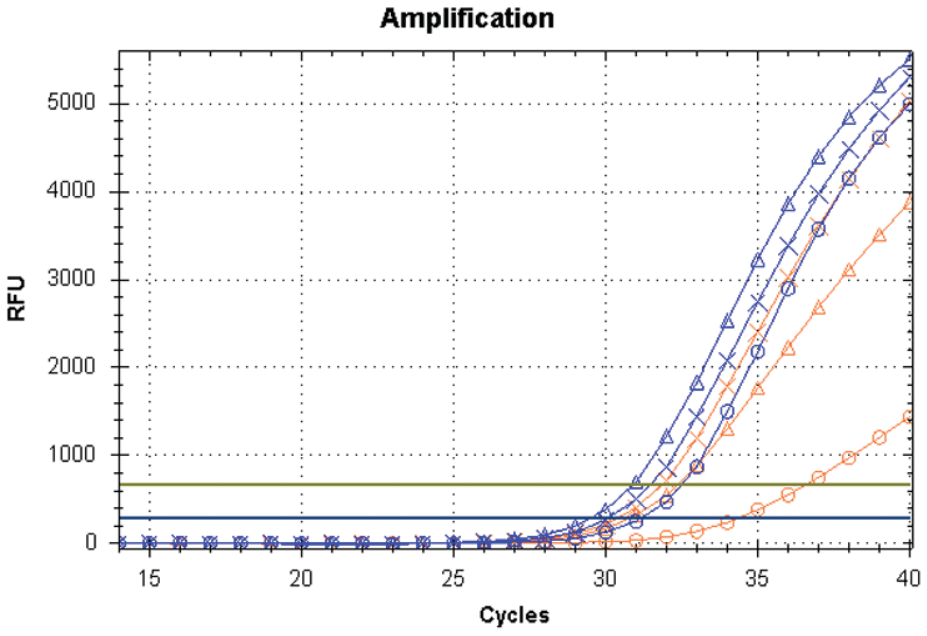

Figure 2. Minimal input testing in the fully automated system. Wild type (orange) and mutant (blue) amplification curves of 1mm² (Circle), 2mm² (Triangle) and 4mm² (Cross) micro-dissected tissue parts originating from one single 10 µM slice of a lung tumor harboring a *KRAS* c.34G>A p.G12S mutation.

Subsequently, micro-dissection was performed on 14 different tissue type specimen to enrich for tumor cells (Supplementary Table 3). Like in routine diagnostics, eight sections per specimen were used for the classic isolation with micro-dissection. These were compared to two sections per specimen for micro-dissection with the automated DNA isolation, thus 2x1 mm^2 or more tissue was available for processing. The classical isolates were eluted in PK1 varying from 12.5 to 75 µl depending on the amount of tumor material present (Supplementary table 3). DNA isolates from the classical method was diluted five times in sterile water prior to hydrolysis probes assays while 2 µl of undiluted DNA obtained with the automated method was always used. Eight assays detecting *KRAS* and *BRAF* hotspot mutations were performed and Cq values were compared. The mean Cq for the classical method was 32.10 +/- 2.9 and the automated method had a mean Cq of 32.18 +/- 1.9. This indicated that although 4 times less tissue was used for the automated method similar Cq's were obtained. (Figure 1B).

These results demonstrate that the automated method leads to at least the same quality DNA and detection rates of mutations as compared to the manual method while workload can be reduced and quicker turnover (turnaround) times can be achieved. In the classical protocol, micro dissecting ten replicates for each of 14 samples requires up to 7 hours hands-on time resulting in a total time including DNA extraction of about 28 hours before isolated DNA is available for assay (Figure 3). With the automated approach, micro dissecting only two replicates for each of 14 samples requires up to 2 hours hands-on time resulting in a total time including DNA extraction of about only 6 hours before isolated DNA is available for assay (Figure 3).
To determine if the automated approach will have a positive effect on both the hands-on time and turnover (turnaround) time we performed the automated approach for four consecutive weeks. Although the initial experiments demonstrated that micro-dissection on two slides generally yielded sufficient DNA for each test, we used 5 slides for micro-dissection in order to always guarantee sufficient DNA concentrations, accounting for very small tumors and/or much degraded tissues. Using 5 slides for microdissection still reduces the workload by half when compared to the classical method. To demonstrate that the hydrolysis probe assays perform equally well with DNA from both methods we compared the overall results from the four week interval with a previous four week interval in which samples were isolated with the classical method. In the first time interval, DNA from 66 samples was isolated using the classical method and Cq values for the *KRAS* and V600E assays were measured. In the second time interval, the identical assay was performed on 70 independent samples for which DNA was isolated using the fully automated method. In this way we compared a consecutive, representable series of DNA from tumor tissue cores or microdissected tumors from different tissue types (Supplementary Table 4). For the *BRAF* V600E assay the results are shown in figure 1C. The Mean Cq for the classical method was 29.20 +/- 3.14 and 30.03+/-2.88 for the automated method. This indicated that both methods compared well despite the different

amount of input DNA. (Figure 1C). For the 7 *KRAS* assays comparable results were obtained (Supplementary Figure 1).

Discussion

We demonstrated that a fully automated DNA isolation method is an excellent tool to obtain hands-on time reduction and lower turnover (turnaround) times in the daily practice of molecular tumor diagnostics. In an ideal situation the use of the fully automated system allowed for molecular test results to be delivered to the clinic about 24 hours earlier than when the classical DNA isolation method was used. However, it still remains to be seen if this gain in time can also be achieved in daily practice. (Figure 3)

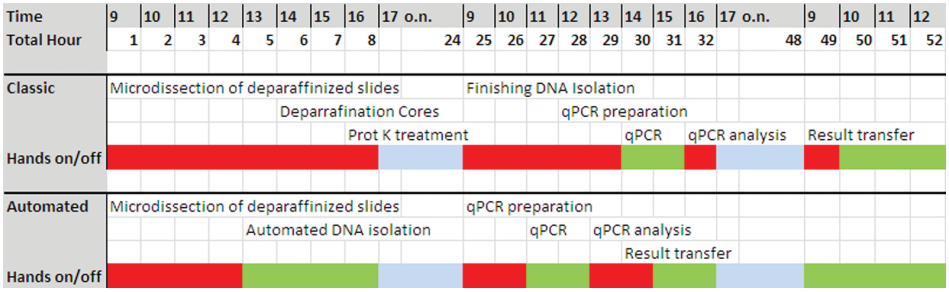

Figure 3. Laboratory implications for the fully automated method. Time evaluation of hands-on (red bar) and hands-off (green bar) workflow from tissue section to first molecular results. In the classical approach we start on Monday morning with the micro-dissection of 10 slides per sample request. One or two technicians work through this process until about the middle of the afternoon. In contrast, with the automated approach, micro-dissection is performed on only five tissue sections, and the work is finished around noon. Consequently, 50% of the hands-on time is saved. With the classical approach, on Monday afternoon the whole tissue sections or tissue cores are prepared and deparaffinized and an overnight Proteinase-K step is initiated. In the automated approach, the technician(s) finish the micro-dissection and start the DNA isolation around noon on Monday. At this point they can walk away from the system. The entire extraction is finished on the same day. On the following day (Tuesday) hours of hands-on time can be saved because the technician can immediately start with the qPCR processes instead of finishing the DNA isolation. This then results in the transfer of the results to the clinicians on Tuesday afternoon instead of Wednesday morning. In the near future it might be even possible to start the qPCR reaction on Monday afternoon which makes it possible to have the results reported to the clinic on Tuesday before noon. These results show that hands-on time, from tissue slide to first molecular results, can be reduced by approximately 50% . In addition, it is likely that the turnaround time can be further reduced to less than 24 hours in the near future. To make this process transparent to a broader public the department of Pathology made a video presentation of this process which can be viewed on http://www.scivee.tv/node/39348 (accessed February 24, 2012).

The isolated DNA is suitable for mutation detection by high throughput processes like routine hydrolysis probes assays. We demonstrated that in small deparaffinized tissue cores DNA of at least the same quality and quantity as with the classic method can be isolated. In many cases where micro-dissection is required the automated system provides significant added value in the whole process. Although deparaffinization and staining has already been performed before actual micro-dissection takes place at least 50% hands-on time can be saved by the fact that only 2mm^2 of material from a single 10µm slide is actually required for good quality DNA. Further, the consumption of rare and precious patient material is dramatically reduced. Thus, the automated extraction method can also decreases the burden on a patient by allowing for the isolation of DNA from minimal biopsies or other very small tissue fragments instead of larger tissue resections obtained by invasive surgery. DNA isolated with the automated system using nano-bead technology promises to be of sufficient quality and quantity for use in additional applications. It also potentially avoids pre-amplification protocols like whole genome amplification which again saves hands-on time, turnover (turnaround) time and costs.

The first results of using DNA obtained from the fully automated system in Sanger sequencing demonstrate that the overall quality of the sequences is higher than in the classical process (assessed by internal quality score; data not shown). Consequently, extra DNA treatment with whole genome amplification procedures can probably become obsolete for this application. Further validation of the automated extraction method should be performed for other types of assays such as Microsatellite Instability testing, clonality typing, MLPA, Maldi-tof, SNP arrays and high throughput and deep sequencing.

We conclude that the fully-automated IVD extraction system delivers sufficient and high quality DNA from precious FFPE tissue cores and micro-dissected tissue material. It significantly reduces the amount of starting tissue and labor and turnaround time. The automated and standardized extraction procedure can contribute to less operator-to-operator variability and reduces contamination risk between samples. In addition, the flexibility of the automated system including the ability to process between 1 and 48 samples per run and to select different protocols for both DNA and RNA while using the same reagents and protocol makes it very amenable for current and future high-throughput molecular laboratories.

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Conflict of Interest statement

RvE declares minor competing interests by being invited by Siemens Healthcare Diagnostics to deliver a presentation at a user meeting (travelling expenses paid). LS, HM,TvW declare no conflict of interests. Siemens Healthcare Diagnostics provided the necessary kits to evaluate the system in our laboratory, and supported to the preparation of this manuscript.

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