The characterization of four gene expression analysis in circulating tumor cells made by Multiplex-PCR from the AdnaTest kit on the lab-on-a-chip Agilent DNA 1000 platform

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Abstract

Introduction: Nowadays, on-a-chip capillary electrophoresis is a routine method for the detection of PCR fragments. The Agilent 2100 Bioanalyzer was one of the first commercial devices in this field. Our project was designed to study the characteristics of Agilent DNA 1000 kit in PCR fragment analysis as a part of circulating tumour cell (CTC) detection technique. Despite the common use of this kit a complex analysis of the results from a long-term project is still missing.

Materials and methods: A commercially available Agilent DNA 1000 kit was used as a final step in the CTC detection (AdnaTest) for the determination of the presence of PCR fragments generated by Multiplex PCR. Data from 30 prostate cancer patients obtained during two years of research were analyzed to determine the trueness and precision of the PCR fragment size determination. Additional experiments were performed to demonstrate the precision (repeatability, reproducibility) and robustness of PCR fragment concentration determination.

Results: The trueness and precision of the size determination was below 3% and 2% respectively. The repeatability of the concentration determination was below 15%. The difference in concentration determination increases when Multiplex-PCR/storage step is added between the two measurements of one sample.

Conclusions: The characteristics established in our study are in concordance with the manufacturer's specifications established for a ladder as a sample. However, the concentration determination may vary depending on chip preparation, sample storage and concentration. The 15% variation of concentration determination repeatability was shown to be partly proportional and can be suppressed by proper normalization.

Key words: lab-on-a-chip devices; capillary electrophoresis; multiplex PCR; circulating tumour cells; Agilent DNA 1000 kit

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Introduction

The Agilent 2100 Bioanalyzer is an on-a-chip capillary electrophoresis system. It represents a perfect compromise in DNA separation methods. It is a bench-top device which is smaller and less expensive than classic capillary electrophoresis. It is easier to handle and more time-efficient than agarose gel electrophoresis (1,2). Moreover, the Agilent 2100 Bioanalyzer provides both size and concentration determination of PCR fragments. For these reasons the Agilent 2100 Bioanalyzer has established a place in many molecular biology applications (3-6).

Despite the frequent research use of the Agilent 2100 Bioanalyzer, only a few papers exist which focus on the technical aspects of this device or discuss its characteristics in specific applications. Basic statistical data for well-to-well, chip-to-chip and day-to-day measurements were summarized

by Panaro *et al*. They used commercially available DNA fragments and PCR products for the evaluation of the characteristics of the DNA 7500 LabChip (7). Later on, the accuracy and reproducibility of DNA fragment measurements on the DNA 500 LabChip were assessed by Jabasini *et al*. (8). Throughout the years, the characteristics of measurements on the Agilent 2100 Bioanalyzer have been published in works presenting its usage in new applications (9-12).

The Agilent DNA 1000 kit can be used as a final step in the determination of circulating tumour cells (CTC) in the blood of patients with castration-resistant prostate cancer made by AdnaTest technique. The final result of this analysis depends on the presence of PCR fragments of the expected size in a concentration above the threshold given by the manufacturer (13). The aims of this study were i) to evaluate the data obtained during two years of CTC research made by this technique and ii) to establish the characteristics of the method used for the PCR fragment detection. The trueness and precision of PCR fragment size determination by the Agilent DNA 1000 kit in this specific application will be determined based on the data collected in the first phase of the study. Moreover, additional experiments will be performed to determine the precision (repeatability, reproducibility) and robustness of PCR fragment concentration determination.

Materials and methods

Subjects

The study describes the PCR fragment size and concentration determination performed with the commercially available Agilent DNA 1000 reagent kit (Agilent Technologies, USA, Santa Clara) on the 2100 Bioanalyzer (Agilent Technologies, USA, Santa Clara). The blood of 33 castration-resistant prostate cancer patients was analyzed at the time of diagnosis and in the course of therapy (altogether 60 samples) to determine the CTC presence by AdnaTest (AdnaGen, Germany, Langenhagen). Obtained data were used for the evaluation of the Agilent DNA 1000 kit and 2100 Bioanalyzer properties. The study was approved by the Ethics Committee of the General University Hospital in Prague. All the patients signed their written informed consent to participation in the study.

Methods

All of the samples were prepared through use of the AdnaTest ProstateCancer Select and Detect kits for the detection of CTCs. Every step was made according to the manufacturer's protocol (13,14). CTCs were enriched from 5 mL of whole (EDTA) blood by magnetic beads coated with anti-EpCAM and anti-HER2 antibodies. Immunomagnetically separated mRNA from CTCs' enriched fraction was reversed transcribed to cDNA. Subsequently, there occurred Multiplex-PCR (Multi-PCR) with the primers for internal control (Actin-120 bp) and epithelial/prostate-specific markers (Epidermal growth factor receptor (EGFR)-163 bp, Prostate specific antigen (PSA)-357 bp, Prostate specific membrane antigen (PSMA)-449 bp). The presence and concentration of PCR products were evaluated by the reagent kit Agilent DNA 1000 on 2100 Bioanalyzer. If at least one of the PCR fragments was of a higher concentration than 0.30 ng/μL the sample was evaluated as CTC positive, concentration between 0.15-0.30 ng/μL was evaluated as borderline and a concentration of under 0.15 ng/μL was evaluated as CTC negative. The cDNA and PCR products from each sample were stored at -20 °C to allow for a repeat of the analysis. The positive controls used in the experiments were part of the AdnaGen ProstateCancerDetect kits. They contained the DNA of all four monitored genes.

The detection of the PCR fragments takes place on disposable chips which consist of a plastic cover with 16 wells and a glass chip. The fragment separation takes place in the channels micro fabricated into the glass chip which is filled with a gel/dye mixture before the analysis. The fluorescent intercalating dye enables the detection of the PCR fragments by a semiconductor laser (630 nm) integral to the 2100 Bioanalyzer (5). The maximum capacity per chip is 12 samples. The remaining four wells placed on the chip serve as a ladder well, priming well, gel/dye reserve and waste well. All the sam-

ple wells and the ladder well are filled with the Marker mix (internal control) into which 1 µL of sample or ladder is added (15-17). The presence of internal markers (lower marker 15 bp, 4.2 ng/µL; upper marker 1500 bp, 2.1 ng/µL) in each well enables a comparison of results even between different chips. The size of PCR fragment is determined from the molecular size ladder which is measured on each chip. The quantification of the fragments is based on the comparison of the peaks with the upper marker which is measured in each well (15).

The Agilent DNA 1000 Kit designed for the analysis of DNA fragments from 25-1000 bp was used in our work. Manufacturer's specifications for the size determination accuracy and reproducibility of this product are relative standard deviation (RSD) of 10% and 5% for a ladder as a sample, respectively. The specifications for the concentration determination accuracy and reproducibility are 20% RSD and 15% RSD for a ladder as a sample, respectively (17). The whole procedure from the CTCs selection until chip preparation was conducted in the laminar hood. Positive and negative controls from Multi-PCR and negative control from RT were run on each chip. From one to nine samples were measured in the remaining wells. A dsDNA 1000 setting was used in 2100 Expert software (Agilent Technologies, USA, Santa Clara).

The results of the size determination of all four PCR fragments (Actin, EGFR, PSA, PSMA) from all the measurements performed during the two years of the CTC project were recorded. All together, 31 positive control and 101 patient sample measurement were performed in the study. However, not all patient samples contained all monitored fragments. Actin as a control fragment present in each measurement was measured 101 times, EGFR 15 times, PSA 69 times and PSMA 31 times. The number of measurements is higher than the total number of the patient samples since some of the samples were measured several times. The additional experiments exploring the repeatability, reproducibility and robustness of the concentration determination of the Agilent DNA 1000 kit on the 2100 Bioanalyzer were performed. The outline of these experiments is depicted in Figure 1.

Statistical analysis

The averages and the standard deviations (SD) of PCR fragment size and concentration determination were calculated. Relative standard deviation (RSD) was calculated as a quotient of SD divided by the average of PCR fragment size/concentration:

$$\text{RSD [\%]} = (\text{SD} / \text{Average}) \times 100.$$

Average RSD was calculated in those cases in which more measurements of samples/controls in appropriate conditions were taken for example for PCR fragment concentration repeatability, reproducibility and robustness determination. The RSDs of all PCR fragments from each sample/control measurement were taken and the average RSD and its SD were counted to show the overall variability between the measurements.

For a better comparison of the results between chips the absolute values of the concentration of each PCR product in the sample was used. The concentration of each fragment was normalized by dividing its actual concentration by the sum of concentrations of all fragments measured in the sample (well). The normalization of the concentrations for all fragments was calculated by the formula:

$$C_{normalized} = C_{Actin/EGFR/PSA/PSMA} / (C_{Actin} + C_{EGFR} + C_{PSA} + C_{PSMA}).$$

Statistical functions from Microsoft Office Excel 2007 were used for the calculations.

Results

The precision and the trueness of PCR fragment size determination

The precision and trueness of PCR fragment size determination were determined based on all four PCR fragment size measurements collected during the two years of CTC research. The evaluated data are presented in Table 1. The results from patient samples and positive controls are evaluated both separately and altogether. The RSDs for the size determination of the PCR fragments did not exceed 2%.

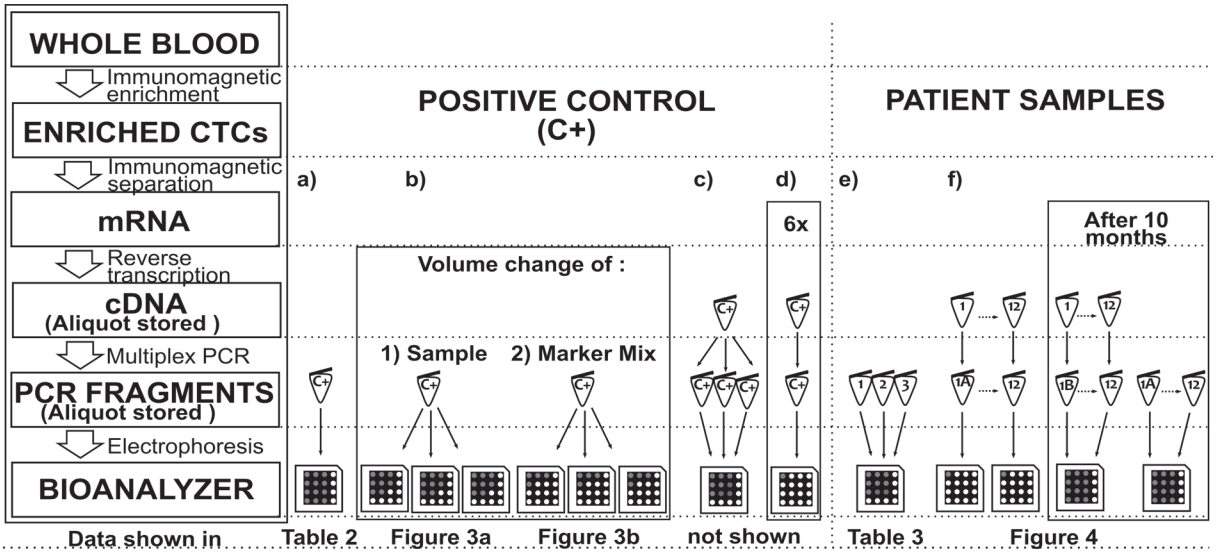

Figure 1. A scheme of the experiments investigating the characteristics of the concentration determination by the Agilent 1000 DNA kit on the 2100 Bioanalyzer.
a) Repeatability: One positive control stored at -20°C after Multi-PCR was slowly thawed, vortexes and briefly spun down. One µL was put into each of the twelve wells on one chip.
b) Robustness: 1) Sample volume changed: 0.8 µL; 0.9 µL; 1.0 µL; 1.1 µL; 1.1 µL; 1.0 µL; 0.9 µL; 0.8 µL of one positive control was put into wells number 1, 2, 3, 4, 5, 6, 7, 8 on one chip instead of the standard 1 µL respectively. The measurement was repeated three times on different chips. 2) Marker mix volume changed: 4.5 µL; 4.8 µL; 5.0 µL and 5.2 µL of the well-mixed Marker mix put into different wells on one chip with 1 µL of positive control. The measurement was repeated three times on different chips.
c) Inter-Multi PCR repeatability: Multi-PCR with one positive control from AdnaTest ProstateCancerDetect kit was run in three separate test tubes in the same thermo cycler run using the same master mix. The concentration of each PCR product was measured in three different wells on one chip.
d) Inter-Multi PCR reproducibility: One positive control from the AdnaGen ProstateCancerDetect kit was used in six different Multi-PCRs run, measured on six different chips.
e) Repeatability: Three different samples (frozen after Multi-PCR) were measured in triplets on one chip.
f) Reproducibility: Twelve cDNA samples (obtained after RT) frozen for 10 months were thawed. The Multi-PCR was repeated. New PCR products (1B-12 after 10 months) were measured on one chip. Frozen mixtures of these samples generated by the first Multi-PCR were re measured on the second chip (1A-12 after 10 months) and compared with the previous results (1A-12).

The difference between the average sizes of the PCR fragments determined by our measurement and the data quoted by the manufacturer (Table 1) was lower than 3% (13).

The repeatability of PCR fragment concentration determination

Positive control

The RSD of the concentration determination was established based on the twelve measurements of one positive control (Figure 2). The RSD was under 15% and after the normalization it was decreased to 5% (Table 2). By the normalization of the concentrations we discovered that DNA fragments in the positive controls were in a consistent ratio after the Multi-PCR: Actin 24%, EGFR 49%, PSA 18%, PSMA 10% (± 3).

Patient samples

The repeatability was established based on three patient samples measurement in triplicates. The average RSD of the PCR fragments with a concentration over 0.3 ng/µL was 17 ± 3% and 2 ± 2% before and after the normalization respectively. The RSDs for the borderline results (concentration be-

Table 1. The obtained average sizes of specific PCR fragments for control and patient samples measured during the two years research by the Agilent DNA 1000 kit on the 2100 Bioanalyzer in order to obtain presence of CTCs - precision study.

Number of measurements		Positive controls (N = 31)			Patient samples (N = 101)*			All results (N = 132)†		
PCR fragment	Manufacturer's quoted size [bp]	average size [bp]	SD [bp]	RSD	average size [bp]	SD [bp]	RSD	average size [bp]	SD [bp]	RSD
Actin	120	117	1	0.81%	117	1	1.25%	117	1	1.16%
EGFR	163	163	1	0.77%	163	2	1.00%	163	1	0.84%
PSA	357	357	2	0.68%	356	3	0.96%	356	3	0.88%
PSMA	449	444	6	1.29%	442	6	1.45%	443	6	1.37%

bp - base pair; SD - standard deviation; RSD - relative standard deviation; EGFR - epithelial growth factor receptor; PSA - prostate specific antigen; PSMA - prostate specific membrane antigen

*Not all samples from patients contained all monitored fragments. Only Actin as a control fragment was present in each measurement. Consequently, the number of measurements is different for each fragment: $N_{Actin} = 101$, $N_{EGFR} = 15$, $N_{PSA} = 69$, $N_{PSMA} = 31$. The number of measurements is higher than the total number of patients since some of the samples were measured several times.

† The number of measurements is the sum of $N_{Positive\ controls}$ and $N_{Actin/EGFR/PSA/PSMA}$.

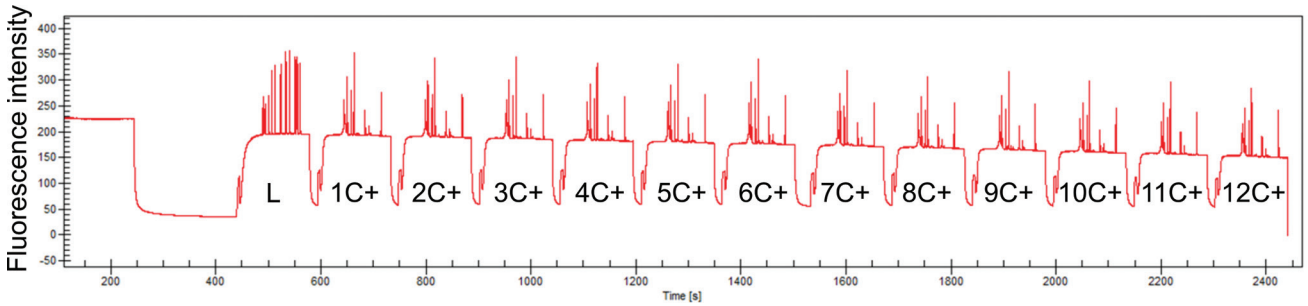

Figure 2. Baseline record of the whole Agilent DNA 1000 chip analysis on 2100 Bioanalyzer. Ladder well is measured first followed by 12 wells filled with the same positive control. The fluorescence intensity is decreasing during the measurement.
L - ladder, 1C +…12C + - 12 measurements of one positive control.

Table 2. The average concentration of specific PCR fragments obtained by repeated measurement (N = 12) of one positive control sample on one Agilent DNA 1000 chip.

	Obtained concentrations of positive control measurements before normalization			Obtained concentrations of positive control measurements after normalization		
PCR fragment type	Average concentration [ng/µL]	SD [ng/µL]	RSD	Average concentration [none]	SD [none]	RSD
Actin	2.89	0.29	10%	0.27	<0.01	2%
EGFR	5.54	0.48	9%	0.51	0.01	1%
PSA	1.77	0.21	12%	0.16	0.01	3%
PSMA	0.64	0.05	7%	0.06	<0.01	5%

bp - base pair; SD - standard deviation; RSD - relative standard deviation; EGFR - epithelial growth factor receptor; PSA - prostate specific antigen; PSMA - prostate specific membrane antigen.

tween 0.15-0.3 ng/μL) were from 3% to 38% and from 10 to 31% before and after the normalization respectively (Table 3). These results are marked with stars in Table 3. After the normalization the highest RSDs remained in the PCR fragments with the borderline concentrations.

The robustness of PCR fragment concentration determination

The robustness study was performed by measurement of different volume of positive control (0.8-1.1 μL) in the different well position (1-8) (Figure 3a). The average RSD of the measurements of all four PCR fragments in all positions was 17 ± 2%. After the normalization the RSD decreased below 4%. No decreasing trend in the concentration determination was observed in the wells 5-8 in comparison with the wells 1-4 with the same sample volume.

The experiment with the changed Marker mix volume from 4.5 to 5.2 μL resulted in an average RSD of 9 ± 3% (Figure 3b). After the normalization the RSD fell to under 2%.

The repeatability and reproducibility of PCR fragment concentration determination after Multi-PCR repetition

Positive control

In the inter-Multi-PCR repeatability analysis the average RSD was 19 ± 10% before and 11 ± 8% after the normalization. For the inter-Multi PCR reproducibility, the average RSD was 37 ± 16% before and 18 ± 11% after the normalization.

Patient samples

The reproducibility in time (10 months) was studied for cDNA and Multi-PCR product storage. Al-

Table 3. The concentration of patient samples (N = 3) measured in triplets on one Agilent DNA 1000 chip. The comparison of differences (RSDs) between measured concentration before and after the normalization.

	Obtained concentrations of patient samples before normalisation				Obtained concentrations of patient samples after normalisation			
	C_1 [ng/μL]	C_2 [ng/μL]	C_3 [ng/μL]	RSD	C_1 [none]	C_2 [none]	C_3 [none]	RSD
Sample ID		**39**				**39**		
Actin	3.98	3.05	3.12	15%	0.28	0.29	0.27	3%
EGFR	0.12	0.09	0.08	22%	0.01	0.01	0.01	11%‡
PSA	8.87	6.54	7.34	16%	0.63	0.62	0.64	2%
PSMA	1.09	0.81	0.87	16%	0.08	0.08	0.08	1%
Sample ID		**52**				**52**		
Actin	3.78	5.64	3.87	24%	0.22	0.23	0.21	4%
EGFR	0.20	0.25	0.11	38%	0.01	0.01	0.01	31%‡
PSA	13.02	17.89	13.84	17%	0.76	0.74	0.77	2%
PSMA	0.19	0.32	0.21	29%	0.01	0.01	0.01	10%‡
Sample ID		**58**				**58**		
Actin	2.42	1.81	2.72	20%	0.29	0.27	0.29	5%
EGFR	0.12	0.08	0.11	20%	0.01	0.01	0.01	12%‡
PSA	5.54	4.66	6.22	14%	0.67	0.69	0.67	2%
PSMA	0.21	0.21	0.22	3%	0.03	0.03	0.02	14%‡

bp - base pair; RSD - relative standard deviation; EGFR - epithelial growth factor receptor; PSA - prostate specific antigen; PSMA - prostate specific membrane antigen; $C_{1,2,3}$ – PCR fragment concentration measured in the 1st, 2nd and 3rd measurement.
‡ indicates borderline (0.15 - 0.3 ng/μL) concentration of the PCR fragment.

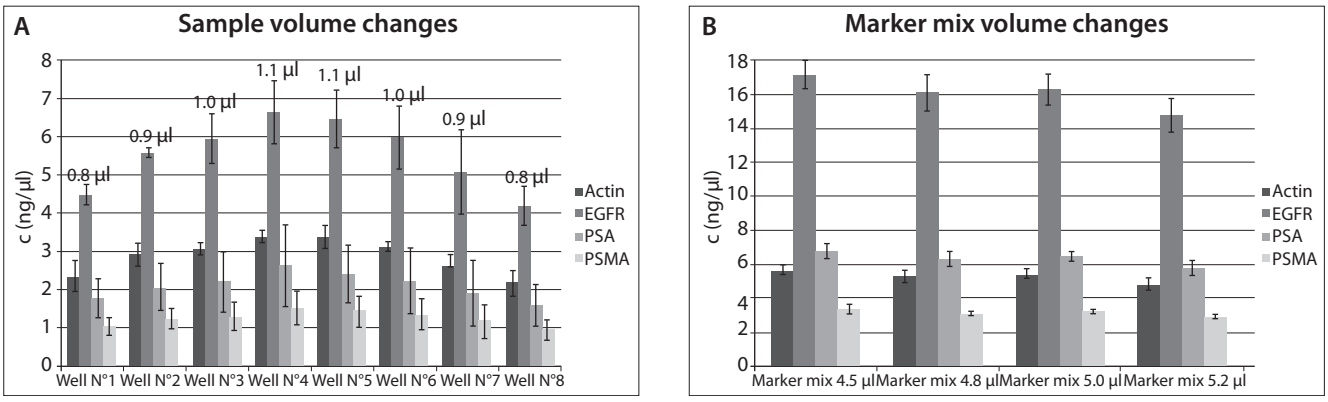

Figure 3. The obtained average concentrations (with SD) of specific PCR fragments measured in positive control for (a) different sample volumes (0.8-1.1 µL) and well locations (well number 1-8) (b) different Marker mix volumes (4.5-5.2 µL) - Robustness study.
EGFR - epithelial growth factor receptor; PSA - prostate specific antigen, PSMA - prostate specific membrane antigen

though the evaluation of results as positive/borderline/negative was consistent even after long storage, the average RSD of the results was 40 ± 20% before and 21 ± 20% after the normalization. There was a significant decrease in the concentration between the original and the repeated Multi-PCR results (Figure 4). The average RSD for the original and the second measurement of the products from the same Multi-PCR was 17 ± 14% before and 7 ± 6% after the normalization.

The influence of magnetic beads presence on the measurement on the Agilent DNA 1000 platform

During the measurements on 2100 Bioanalyzer due to the transfer of magnetic beads form the sample to the chip, specific peaks, called spikes may appear (18). Sometimes, they can influence the measurement by interfering with the upper marker. These specific peaks appeared several times during our measurements. Results are shown in Figure 5.

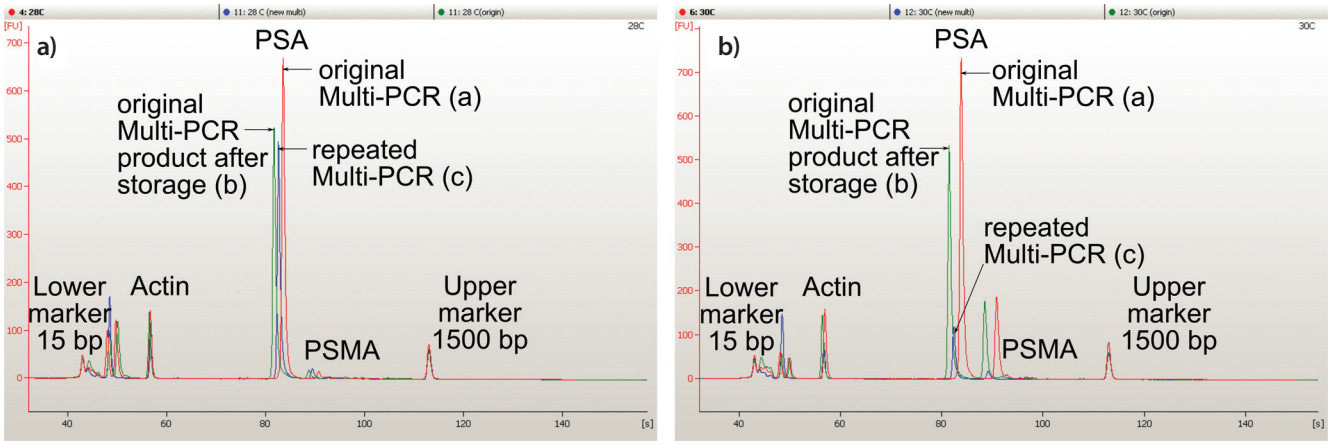

Figure 4. Overlaid electropherograms of two patient samples measured after the original Multi-PCR (a), the 10 months storage of original Multi-PCR product (b) and the repeated Multi-PCR (c) for two different samples. The time between the original and the second Multi-PCR/measurement was 10 months for both samples.
EGFR - epithelial growth factor receptor; PSA - prostate specific antigen; PSMA - prostate specific membrane antigen.

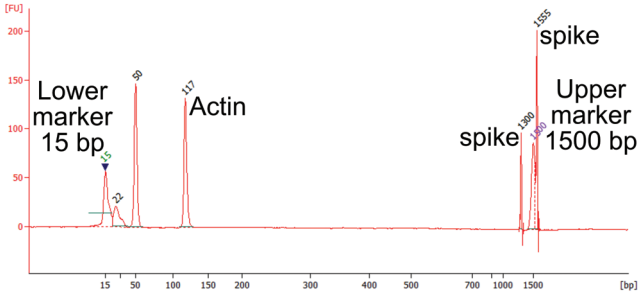

Figure 5. Spike overlapping with the upper marker observed in one patient sample measurement. The change in the upper marker peak area influences the concentration determination of PCR fragment (Actin) in this sample. Lower and upper markers are present in each well and enable the PCR fragment size and concentration determination.

Discussion

The analysis of the data collected throughout two years of research combined with the additional experiments gave us a picture of the characteristics of the PCR fragment analysis by the Agilent 1000 DNA kit on the 2100 Bioanalyzer.

The PCR fragment size determination gave very satisfactory results. The RSD was up to 2% and there was no difference between the samples and positive controls (Table 4). The standard deviation (SD) of the size determination slowly increases with increasing length of the PCR fragments (Table 1). It results from the principle of the electrophoresis - the longer the fragment the lower the resolution. The trueness of PCR fragment size determination was under 3%.

Table 4. Summary of all method characteristics determined in the study based on the results obtained from the two years CTC research (PCR fragment size measurement) and additional experiments (PCR fragment concentration measurement).

	Purpose of testing		Sample type	Number of measured samples	Average RSD before the normalization	Average RSD after the normalization	Data shown in
Obtained characteristics for PCR fragment size determination	Precision		Positive control	31	under 2%	-	Table 1
	Trueness			31	under 3%	-	Table 1
	Precision		Patient samples	101§	under 2%	-	Table 1
	Trueness			101	under 3%	-	Table 1
Obtained characteristics for PCR fragment concentration determination	Repeatability		Positive control	12	15%	5%	Table 2
	Robustness	Sample volume		3 × 8	17 ± 2%	2 ± 1%	Figure 3a
		Marker mix volume		3 × 4	9 ± 3%	1 ± 1%	Figure 3b
	Inter Multi-PCR Repeatability			3 × 3	19 ± 10%	11 ± 8%	Not shown
	Inter Multi-PCR Reproducibility			6 × 1	37 ± 16%	18 ± 11%	Not shown
	Repeatability		Patient samples	3 × 3	17 ± 3%	2 ± 2%	Table 3
	Reproducibility for PCR product storage‖			1 × 1 × 12	17 ± 14%	7 ± 6%	Figure 4
	Reproducibility for cDNA storage‖			1 × 1 × 1 × 12	40 ± 20%	21 ± 20%	Figure 4

CTC - circulating tumour cell; PCR – polymerase chain reaction; RSD - relative standard deviation

§Not all samples from patients contained all monitored fragments. Only Actin as a control fragment was present in each measurement. Consequently, the number of measurements is different for each fragment: $N_{Actin} = 101$, $N_{EGFR} = 15$, $N_{PSA} = 69$, $N_{PSMA} = 31$. The number of measurements is higher than the total number of patients since some of the samples were measured several times.

‖Samples were stored for 10 months in -20 °C.

Small differences between the predicted sizes of the PCR fragments and the average sizes obtained from the measurements were mentioned in the previous studies (3,19). These can be caused by the small changes between standard curves of migration time *versus* DNA size which are determined from the DNA sizing ladder on each chip (20) or by the changes in the fluorescent dye binding which may affect the fragment mobility (21). Our results are in agreement with other studies, which also reported the PCR fragment size determination by the Agilent as true and precise (7,8,11). However, to the best of our knowledge we are the first to have evaluated data from patient samples acquired during a long-term project. The sizing trueness and precision measured on patient samples in our study are within the range stated by the manufacturer for the ladder as a sample (17).

The RSD of the concentration determination repeatability around 15% (Table 4) is consistent with Alberice *et al.* (22). It also confirms on the patient samples the manufacturer's data which were measured with the ladder as a sample (17). After the normalization we can compare the ratio of individual markers in the sample. Since the normalization significantly reduces the RSD (Table 2) we know that the differences in the concentrations between different wells are proportional. That proportional error could be generated by manual pipetting required during the loading of the chip.

According to our experiment with method robustness the change in sample volume influences the resulting concentration (Figure 3a). When the volume of the Marker mix is changed, the concentration of the DNA fragments is, as expected, inversely proportional to the quantity of the Marker mix in the well (Figure 3b). However, even these changes in tens of percent in sample or Marker mix volume do not lead to an RSD much higher than the 15%, which is present in normal measurement. Unlike in previous work (8), results of our study indicate that the manual pipetting is not the main cause of the 15% variance in concentration determination, otherwise such major pipetting errors have to be present in every chip preparation procedure.

A continual decrease and deterioration of the signal observed during our measurements was also considered to be a possible cause of this 15% variance (Figure 2). The baseline decrease can be caused by a decrease in fluorescent dye concentration caused by its constant migration toward the waste well or by its photo bleaching caused by the laser beam. Since there is only one detection/separation channel on the chip this could be also one reason for a worsening baseline (16,23). The dedicated measurements did not prove the impact of the well position on the concentration determination (Figure 3a). Despite the fact that the difference in sample migration times on one chip follow one pattern (15) the changes in sample concentration within one chip seem to be irregular and differ between the chips. As showed by the normalization the 15% variation is a proportional error. The data for the Bioanalyzer shows that the changes in concentration are connected with the change in the sample/marker signal ratio. In our view, this change can be generated by the principle of the sample injection to the detection/separation channel (2,23). Based on this assumption, the concentration variation within 15% cannot be eliminated by the user.

For the concentration determination repeatability, the RSDs in the patient samples are higher than RSDs in the positive controls because of the presence of the low concentration PCR fragments. The repeatability of the quantification of PCR fragments in a concentration under 0.3 ng/μL is poor (Table 3.). The measurement is influenced by noise, baseline vibration and Multi-PCR conditions. If PCR fragments are present only in these concentrations, the manufacturer recommends retesting the patient after a few weeks because of the high probability of false results (13). One clarification option is to repeat the Multi-PCR several times and statistically evaluate the results.

As expected, the differences in PCR fragment concentration between the separate Multi-PCRs were higher than those of the intra-Multi PCR. This may have been caused by minute differences in PCR conditions and reaction efficiency. However, the average RSD fewer than 30% before and 20% after the normalization may be considered acceptable.

For the positive control inter-Multi PCR reproducibility, the slight differences in master mix and subtle variations in PCR conditions, even if a validated thermo cycler and calibrated pipettes are used, have to be taken into account. Also the use of different chips, the storage and the repeated freezing and thawing of the positive control can play a role. The high RSD measured in the inter-Multi-PCR experiment showed that the long storage of single-stranded cDNA at -20 °C may lead to its degradation. The degradation of the samples differs from sample to sample (Figure 4). A small shift in the peak position is present because the DNA fragment migration is calculated for each chip and may marginally differ (20). The Multi-PCR repeated with the stored cDNA results in different PCR fragment concentration than the original Multi-PCR. Interestingly, the RSD remained at about 20% even after the normalization (Table 4). This indicates that the storage also influenced the proportional representation of the PCR fragments in the Multi-PCR product. The manufacturer advises against storing the cDNA for more than four weeks (14). On the other hand, double-stranded PCR fragments originating from Multi-PCR can be successfully measured again even after several months of storage. To conclude, the RSD of the PCR fragment concentration determination increases with every additional analytical/storage step added between the two measurements (Table 4).

Finally, we would like to highlight our experience of spikes, which appeared several times during our measurements. They can be cause by the presence of magnetic beads in the sample, against which the manufacturer warns (17). The problem may arise when spike interfere with the upper marker (Figure 5) from which the concentration determination of PCR fragments is derived. In that case the repeated measurement of the sample on 2100 Bioanalyzer is necessary. From our two years experience the beads in the sample usually do not cause major problems. However, it is better to retain them in a PCR-tube by use of a magnetic holder when sample is put on the chip to prevent the necessity of measurement repetition.

In conclusion, the Agilent DNA 1000 kit used on 2100 Bioanalyzer represents a useful tool for determining the size and concentration of PCR fragments. In particular, the size determination is true and precise even for the data acquired during a long-term project. As regards concentration determination, it is necessary to anticipate a result variation of up to 15% and for the concentrations below 0.3 ng/μL even higher variation. In the Multi-PCR analysis, an important thing to be taken in account is the possible influence of the storage of cDNA as well as mRNA on the results. We suggest future users of this method to think about the possibility of evaluating results based on relative rather than on the absolute concentration of PCR fragments, especially for those cases in which comparison between the samples is more important than the precise concentration.

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Potential conflict of interest

None declared.

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