Non-invasive detection and analysis of circulating nucleic acid biomarkers

Inaugural Dissertation

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Annette Nocon
from Dormagen

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supervised by the Department for Plant Biochemistry and
the Urological Research Laboratory at
Heinrich Heine University Düsseldorf

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Supervisor: Prof. Dr. Andreas Weber, Department for Plant Biochemistry, Heinrich Heine University Düsseldorf
Co-supervisor: Prof. Dr. Wolfgang A. Schulz, Urological Research Laboratory, Heinrich Heine University Düsseldorf
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Summary

Background

Nucleic acids in human blood plasma were discovered in 1948, but it took a long time until research resumed and the meaning for cancer and prenatal diagnostics as well as further application fields became apparent. So-called circulating cell-free nucleic acids (=ccfNA) are present in blood and other body fluids, but their origin is still controversially discussed. To that effect, different sources and release mechanisms into blood circulation have been published to date. According to their specific characteristics, the integration of circulating cell-free DNA (=ccfDNA) as a tool for “liquid biopsy” or a biomarker in general into clinical practice is very challenging. Therefore, preanalytical as well as analytical procedures not only have to be optimized, but also standardized for possible future clinical use. The most important characteristics, which still had to be considered during this PhD thesis, were the strong fragmentation and very low concentration of ccfDNA in plasma.

Experimental approach

After the establishment of optimal preanalytical conditions, a new automated DNA extraction method for the enrichment of ccfDNA was developed on the QIAsymphony SP instrument. The QIAamp Circulating Nucleic Acid (=cNA) Kit was always used as manual reference method. Using the QIAsymphony circulating DNA (=cDNA) Protocol at least the same DNA recovery should be achieved. Most experiments were performed with plasma of healthy donors, but, as a tool for clinical practice, the successful extraction of DNA from clinical samples had to be demonstrated. Fetal DNA was therefore isolated from maternal plasma samples and tumor DNA was recovered after processing cancer patient samples. To be able to discriminate between male fetal or tumor and “normal” (maternal or healthy) DNA background, a gene localized on the Y-chromosome as well as specific DNA mutations were targeted. The final goal was not only to show the applicability for clinical samples, but also the compatibility with different analysis methods, so quantitative real-time PCR assays, BEAMing digital PCR technology, pyrosequencing, Qubit measurement, Agilent virtual gel electrophoresis and targeted sequencing were performed.

Results

The newly developed automated protocol and the QIAamp cNA Kit were comparable. Although DNA recovery using the reference method was higher for clinical samples the fraction of fetal or tumor DNA was very similar in most cases. So, if there was a DNA loss for the newly developed automated protocol, it should not only be specific for fetal or tumor DNA, but also be related to the “normal” DNA background. Looking at the results of Agilent virtual gel electrophoresis, DNA size distribution was almost the same between healthy individuals, maternal plasma samples and patients with cancer or other diseases. The main fraction mostly occurred in the length of mononucleosomes. Consequently, there was no obvious difference concerning DNA fragment length between plasma DNA from clinical samples and healthy individuals.

Conclusion

The newly developed automated protocol will help towards standardization and provides a good basis for the analysis of existing and the discovery of new biomarkers to push progress in the field of ccfNA as a non-invasive to minimally invasive diagnostic or prognostic tool.

Zusammenfassung

Hintergrund

Nukleinsäuren wurden bereits 1948 in menschlichem Blutplasma entdeckt, aber erst sehr viel später wurden die Forschungsarbeiten wiederaufgenommen und die Bedeutung für die Krebs- und Pränataldiagnostik, sowie weitere Anwendungsbereiche erkannt. So genannte zirkulierende, zellfreie Nukleinsäuren kommen in Blut und anderen Körperflüssigkeiten vor, aber ihr Ursprung ist immer noch umstritten. Aus diesem Grund wurden bislang unterschiedliche Quellen und Freisetzungsmechanismen von zirkulierenden, zellfreien Nukleinsäuren in die Blutzirkulation veröffentlicht. Aufgrund ihrer besonderen Eigenschaften, ist ihr klinischer Einsatz als Werkzeug für eine Biopsie aus Flüssigkeit (hier: Plasma) oder als Biomarker im Allgemeinen eine große Herausforderung. Dabei müssen präanalytische, sowie analytische Abläufe nicht nur optimiert, sondern auch standardisiert werden, um eine zukünftige klinische Nutzung zu ermöglichen. Die wichtigsten Eigenschaften, die stets während meiner Dissertation berücksichtigt werden mussten, waren die starke Fragmentierung und die äußerst geringe Konzentration von zirkulierender zellfreier DNA in Plasma.

Experimenteller Ansatz

Nach der Etablierung optimaler präanalytischer Bedingungen, wurde eine neue automatisierte DNA-Extraktionsmethode auf einem bestehenden Gerät, dem QIAsymphony SP, entwickelt, um zirkulierende, zellfreie DNA anreichern zu können. Das QIAamp Circulating Nucleic Acid (=cNA) Kit wurde immer als manuelle Referenzmethode verwendet. Bei Verwendung des QIAsymphony circulating DNA (=cDNA) Protokolls sollte mindestens die gleiche DNA-Ausbeute erzielt werden. Die meisten Experimente wurden mit Plasma von gesunden Einzelspendern durchgeführt, aber um die zukünftige klinische Eignung zu bestätigen, musste auch die erfolgreiche Extraktion von DNA aus klinischen Patientenproben gezeigt werden. Dafür wurde fötale DNA aus maternalen Plasmaproben isoliert und Tumor-DNA wurde nach Verarbeitung von Plasmaproben, die von Krebspatienten stammen, gewonnen. Um zwischen männlicher fötaler, bzw. Tumor-DNA und normalem (maternalem/ gesundem) Hintergrund unterscheiden zu können, wurden ein Y-Chromosom spezifisches Gen, sowie bestimmte DNA-Mutationen nachgewiesen. Dabei sollte nicht nur die Anwendbarkeit für klinische Proben, sondern auch die

Kompatibilität mit unterschiedlichsten Analysemethoden gezeigt werden. So wurden quantitative Real-time PCR, BEAMing als digitale PCR-Methode, Pyrosequenzierung, Qubit-Messungen, sowie Agilent virtuelle Gelelektrophorese und die gezielte Sequenzierung von bestimmten Genen durchgeführt.

Ergebnisse

Das neu entwickelte automatisierte Protokoll und das QIAamp cNA Kit waren vergleichbar. Obwohl die DNA-Ausbeute bei Verwendung der Referenzmethode für klinische Proben höher war, war der Anteil an fötaler und Tumor-DNA in den meisten Fällen vergleichbar. Wenn also ein DNA-Verlust für das neu entwickelte automatisierte Protokoll auftrat, wirkte sich dieser nicht spezifisch auf die Ziel-DNA (hier: fötale und Tumor-DNA), sondern in gleichem Maße auch auf den normalen DNA-Hintergrund aus. Bei Betrachtung der Ergebnisse nach virtueller Gelelektrophorese mittels Agilent, scheint sich die Größenverteilung innerhalb zirkulierender, zellfreier DNA zwischen gesunden Einzelspendern, maternalen Plasmaproben und Proben von Patienten mit Krebs und anderen Erkrankungen, kaum zu unterscheiden. Der Hauptteil war im Längenbereich von Mononukleosomen vertreten. Folglich gab es keinen offensichtlichen Längenunterschied von Plasma-DNA-Fragmenten aus Proben von Patienten oder gesunden Individuen.

Schlussfolgerung

Das neu entwickelte automatisierte Protokoll sollte für die geplante Standardisierung hilfreich sein, eine gute Grundlage für die Analyse bestehender und die Entdeckung neuer Biomarker liefern und möglicherweise den Fortschritt im Bereich zirkulierender, zellfreier Nukleinsäuren als nicht- bis minimal invasives diagnostisches und/ oder prognostisches Werkzeug, bzw. Hilfsmittel fördern.

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1. Introduction

1.1. Circulating cell-free nucleic acids

1.1.1. History

Cell-free nucleic acids in human blood plasma were first discovered in 1948 by Mandel and Métais [1]. In the beginning, this finding did not attract much attention. Eighteen years went by until Tan *et al.* found DNA and anti-DNA antibodies in serum of patients suffering from systemic lupus erythematosus [2]. Again, it took a long time until this work was resumed and further studies were published concerning that disease [3, 4]. Then, more and more diseases were examined over the years. For instance, serum DNA concentration in cancer patients was analyzed [5] and elevated serum DNA levels were found in patients suffering from rheumatoid arthritis [6]. More than a decade later, in 1989, Stroun *et al.* asserted that DNA from plasma of cancer patients primarily originates from cancer cells [7]. In 1994, Sorenson *et al.* were able to detect specific gene mutations within plasma and serum DNA from pancreatic cancer patients [8] and, in the same year, further mutations were found in plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukemia [9]. The idea of using circulating cell-free DNA (=ccfDNA) as a tumor biomarker for diagnosis, prognosis or therapy monitoring was born. Two years later, microsatellite alterations were found in serum DNA of head and neck cancer patients [10] and, in 1997, Kopreski *et al.* showed the presence of mutant DNA in plasma and serum from colorectal cancer patients [11]. These and other studies were the beginning of promising research into circulating nucleic acids as disease biomarkers and the analysis of many other cancer types and genes using DNA from blood samples followed.

In 1997, the presence of fetal DNA in maternal plasma and serum was demonstrated by Lo and colleagues [12]. They used plasma from women, who were carrying a male fetus and detected Y-chromosome-specific gene sequences. One year later, Lo *et al.* quantified fetal DNA in plasma and serum of pregnant women. Fetal DNA was detectable in maternal serum and its concentration increased during the course of pregnancy. As a result, fetal DNA could be applicable as target for minimally invasive prenatal diagnosis [13]. In 1999, Lo *et al.* found increased fetal DNA levels in preeclamptic women [14] and prenatal rhesus D (=RhD) genotyping was demonstrated using fetal DNA from plasma of RhD negative

pregnant women [15]. These and other discoveries were the beginning of many promising developments in non-invasive to minimally invasive prenatal diagnosis.

Besides DNA, circulating cell-free RNA had also become an interesting analyte. Wieczorek and colleagues had already detected RNA in proteolipid complexes in the serum of cancer patients [16] in 1987; however, it took more than ten years until the clinical value became apparent. Circulating cell-free RNA was connected to nasopharyngeal carcinoma [17] and malignant melanoma in 1999 [18]. In 2000, the presence of fetal RNA in maternal plasma was presented [19] and, in 2003, a specific mRNA in maternal plasma showed, similar to fetal DNA, higher concentrations in blood samples of pregnant women suffering from preeclampsia [20]. Besides plasma and serum from cancer patients or pregnant women, RNA was also found in blood samples of patients suffering from other diseases like *diabetes mellitus*, where higher rhodopsin mRNA levels were detected [21]. Among circulating RNA, not only mRNA was analyzed, but also, even though a bit later, microRNAs, which were discussed as potential biomarkers in maternal serum for fetal congenital heart defects [22] and as stable blood-based markers for cancer detection [23]. One possible reason for their stability in blood samples is that RNA as well as DNA can be enclosed by so-called exosomes, nanovesicles which are released from cells [24]. They were, for example, detected in plasma from ovarian cancer patients [25] and in maternal serum [26].

1.1.2. Origin of circulating cell-free nucleic acids

Circulating cell-free nucleic acids (=ccfNA) are present in blood and other body fluids like saliva [27], urine [28, 29], cerebrospinal fluid [30], peritoneal fluid [31], bronchoalveolar lavage fluid [32] or amniotic fluid [33]. They can be found in healthy donors, pregnant women, cancer patients as well as patients suffering from other diseases. In plasma or serum of pregnant women, fetal as well as maternal DNA can be detected. In cancer patients, tumor and non-tumor DNA is present in blood and other body fluids. The use of circulating nucleic acids as biomarkers for various diseases as well as prenatal diagnosis is intensely investigated. However, the origin of ccfNA is still controversially discussed regarding the different sources or mechanisms of release, for example.

Circulating cell-free nucleic acids in cancer patients are released from lymphocytes or other nucleated cells as well as tumor cells [34]. Murtaza and colleagues compared tissue biopsies and matched plasma samples from two patients and discovered the genome-wide

representation of the tumor-genome in plasma. [35]. Apoptotic and necrotic cells are discussed to be an important source of ccfDNA. [36]. Apoptotic and necrotic cells in vitro as well as apoptotic and necrotic liver injury in mice, was associated with characteristic DNA patterns shown after gel electrophoresis. In the case of apoptosis, DNA fragment size was about 180 bp or multiples thereof, whereas cell death caused by necrosis led to long DNA fragments of about 10,000 bp and more. These characteristic patterns of DNA fragments were also found in the plasma of cancer patients. The oligonucleosomal DNA ladder is typical for apoptotic cell death, where chromatin degradation by caspase-activated DNase takes place. Arends *et al.* published a ladder pattern of oligonucleosomes formed by apoptotic cell DNA. In contrast, DNA of normal cells could not be separated on agarose gels [37]. Heitzer and colleagues compared the size distribution of DNA fragments from healthy individuals and cancer patients. They saw an enrichment of single and multiple nucleoprotein complexes among circulating cell-free plasma DNA [38]. In a further study, elevated nucleosomal levels in patients suffering from systemic inflammation and sepsis were found [39]. Nevertheless, necrosis does not always result in DNA of high molecular weight. Diehl *et al.* suggested the release of circulating DNA fragments from necrotic neoplastic cells, engulfed by macrophages [40]. Jin-Jung Choi described, that co-cultures of necrotic cells and macrophages led to molecular weights of DNA lower than that of intact cellular DNA [41]. Zheng Dong and colleagues described the appearance of a specific ladder pattern of (oligo)-nucleosome lengths during necrosis [42]. However, larger DNA fragments (maybe caused by necrosis) were also detected in patients with pancreatic ductal adenocarcinoma, a pancreatic neuroendocrine tumor or chronic pancreatitis [43]. For this reason, it seems to be hard to discriminate between apoptotic and necrotic DNA only by size distribution. Apoptosis and necrosis belong to the most frequently discussed mechanisms for the release of ccfDNA. Although apoptosis is supposed to be lost in proliferating cancer cells, Tjoa and colleagues presented the induction of apoptosis by oxidative stress and it is known that uncontrolled proliferation of cancer cells leads to oxygen limitation [44]. On the other hand, higher plasma DNA concentrations in patients suffering from cancer and having large or advanced tumors support a release of DNA by necrosis. Nevertheless, at the same time, plasma DNA levels decreased in most patients after radiation therapy, although necrotic cell death should be induced that way [5]. Leon *et al.* tried to explain the DNA decrease by radiation caused arrest of cell proliferation. Anyhow, it is hard to explain the presence of ccfDNA only by cell lysis or cell death. Sorenson calculated that about 850 tumor cells would be needed for a tumor-derived DNA

concentration of 5 ng/mL, but the detected tumor cell level is usually lower [45]. Consequently, further sources and mechanisms for the release of ccfDNA might exist. Using cell cultures, the active and spontaneous release of preferably newly synthesized DNA was described and also led to the already mentioned specific nucleosomal ladder pattern [46]. Stroun *et al.* additionally discussed the active release of ccfDNA into blood circulation [47]. Furthermore, elevated DNA levels could also be explained by DNase activity, which was lower in blood samples of patients with prostate tumors compared to healthy individuals resulting in less DNA degradation [48]. In summary, it might be concluded that "the presence of tumour DNA in the plasma is probably the result, in variable proportions, of the different mechanisms which produce leakage or excretion of DNA" [49].

Regarding the blood samples of pregnant women, besides fetal cells, circulating cell-free fetal DNA is also present [50]. Sekizawa *et al.* reported that almost half of the fetal nucleated erythrocytes, detected in maternal plasma, undergo apoptotic cell death [51]. Therefore, fetal DNA in maternal plasma may originate from fetal cells [52]. Furthermore, fetal cells as well as ccfDNA are both increased in pregnant women with complications like preeclampsia [14, 53] or trisomy 21 [54, 55]. However, fetal cell levels are lower than the amount of circulating cell-free fetal DNA in most cases [56] and no increase of fetal DNA fraction was detected after storage of whole blood samples for 24 hours [57]. This indicates that at least one further source for circulating cell-free fetal DNA has to exist for which the placenta is often mentioned as a major source. Wataganara and colleagues discovered that there is no correlation between placental volume and fetal DNA level [58]. Hence, fetal DNA may be released by a controlled and size-independent process. Apoptosis seems to be the main mechanism of release. It was asserted that apoptosis induced by oxidative stress causes the release of fetal DNA from syncytiotrophoblasts [44] and, besides fetal DNA, placenta-specific mRNA is also present in maternal plasma, which supports the idea that ccfNA originate from the placenta [59]. Additionally, in women with a diagnosis of anembryonic gestation, fetal gender was determined by the analysis of ccfDNA in maternal plasma [60].

After using whole genome sequencing for the analysis of plasma DNA from pregnant women, Lo *et al.* suggested that, according to the nucleosomal fragmentation pattern, DNA fragments originate by nuclease cleavage of DNA from apoptotic cells [61]. These apoptotic cells could be of fetal or placental origin.

Therefore, there are different sources and release mechanisms of ccfNA in samples of patients suffering from cancer and other diseases, pregnant women and healthy individuals. The following figure (Fig. 1) gives a short overview concerning the origin of ccfNA.

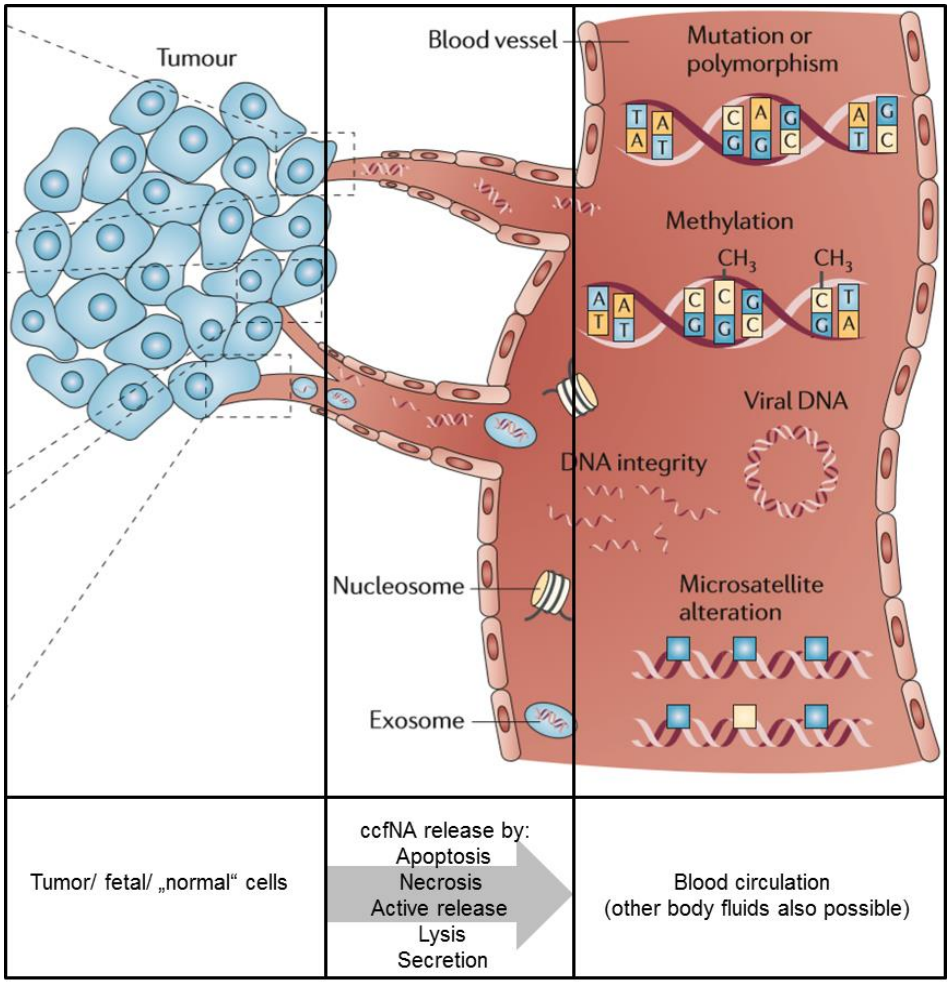


Figure 1 Origin of circulating cell-free nucleic acids [62]
Circulating cell-free nucleic acids are present in the plasma, serum, urine and other body fluids of patients suffering from cancer and other pathological disorders, pregnant women and healthy subjects. Necrosis, apoptosis, secretion, lysis and an active release are discussed as possible mechanisms for DNA release into extracellular body fluids. Circulating cell-free nucleic acids can be enclosed by so-called exosomes and DNA is mainly present in the form of mononucleosomes. DNA integrity, the occurrence of viral nucleic acids, microsatellite alterations, methylation status as well as mutations and polymorphisms can be used as diagnostic or prognostic biomarkers.

1.1.3. Diagnostic applications of circulating nucleic acids

Circulating cell-free nucleic acids can be used as biomarkers in prenatal diagnosis, cancer research and diagnosis and for the diagnosis and progression monitoring of many other diseases. The most important advantage over existing analytical methods is their

minimally to non-invasive nature, because they can be obtained from blood and other body fluids (“easy-to-draw-samples”).

Quantitative and qualitative alterations of ccfDNA in plasma and serum were analyzed for diagnostic applications in cancer. Higher DNA levels were detected in plasma and serum of patients suffering from colon [63], lung [64-66], breast [67, 68], gastric [69] and esophageal [70] cancer compared to control individuals, for example. Shapiro *et al.* even proved that the average plasma DNA concentration in patients with benign gastrointestinal diseases was lower than in cancer patients [71]. Although mean plasma ccfDNA concentrations often differ between cancer patients and healthy individuals or patients suffering from other diseases, it is hard to define a cut-off concentration for discrimination between these groups. The ranges of individual ccfDNA concentrations sometimes overlap in healthy individuals and patients. The plasma ccfDNA concentration can also be elevated in healthy individuals under physiological stress or in patients affected by inflammation, trauma or sepsis [72-75]. In a review, DNA concentrations between 122 and 462 ng/mL in plasma and 153 and 549 ng/mL in serum of breast cancer patients were described, whereas it was between 3 and 63 ng/mL and 63 and 318 ng/mL, respectively, in plasma and serum of healthy individuals [72]. Consequently, there is not always a significant difference between ccfDNA levels of patients and healthy individuals and a differentiation against non-malignant diseases is often much more difficult [76, 77]. Furthermore, the relationship between DNA level and clinicopathological features like tumor size, stage or metastasis is uncertain. For colorectal cancer, higher DNA levels in cancer patients than in healthy individuals were discovered, but without a correlation to tumor size, site or the clinical course of disease [78, 79], while in another study DNA levels were correlated with pathological stage [80], Gleason score, surgical margin status and extraprostatic extension in blood samples from prostate cancer patients [81]. With regard to lung cancer patients, different opinions concerning a possible correlation of ccfDNA level with survival exist, so in some studies a negative correlation with relapse-free or overall survival was reported [82, 83] while others did not see any correlation [84, 85]. For colorectal cancer patients, DNA levels decreased in tumor-free patients and increased when recurrence or metastasis developed [63] and in one study even the detection of minimally residual disease after surgical resection was possible [40].

An integrity index can be applied when physically characterizing ccfDNA. It is the ratio between the concentrations of long (possibly related to necrosis) and short (possibly

related to apoptosis) DNA fragments. Wang and colleagues suggested that the DNA integrity index is increased in plasma of cancer patients [86]. Besides the measurement of DNA levels and the calculation of integrity index, further molecular alterations like gene mutations, gene methylations, microsatellite alterations or the detection and analysis of viral and mitochondrial DNA can be present in tumor-derived ccfDNA. All of these alterations should be specific to enable the differentiation between circulating cell-free tumor and non-tumor DNA.

One of the most studied genes is the *KRAS* oncogene, which is mutated in colon, pancreatic and lung tumors, for example [87]. In colorectal cancer, *KRAS* mutations were present in tumor tissue in 40-50% of patients and in 25-30% they were also detectable in matched plasma and serum samples [88]. In another study, Kopreski *et al.* found *KRAS* mutations in plasma from 83% of patients with a mutant tumor [89]. Furthermore, they also detected *KRAS* mutations in plasma of patients with a normal colonoscopy, making these mutations possibly suitable for early diagnosis and screening. As already mentioned above, *KRAS* can also be mutated in other cancer types like lung cancer. While Camps *et al.* saw no difference in non-small cell lung cancer (=NSCLC) patients between mutant and wild type *KRAS* regarding patient characteristics, response rates, progression-free or overall survival [90], mutant *KRAS* in serum or plasma of NSCLC patients was associated with survival in two other studies [91, 92], just as in a further large study including 180 lung cancer patients where plasma DNA was correlated with *KRAS* mutation and poor prognosis [93]. In colorectal cancer patients treated with cetuximab (a therapeutic monoclonal antibody against *EGFR*, the epidermal growth factor receptor for immunotherapy in oncology), *KRAS* mutations were detected in blood samples as early as 10 months before disease progression was documented by radiography [94]. Besides *KRAS*, there are many other genes analyzed in different cancer types. In many cases, gene panels instead of single genes are used to improve sensitivity or specificity. Consequently, more than one gene is analyzed to increase the probability of mutation detection in tested samples (sensitivity) or to improve specificity by assessing samples as mutant if at least two genes result in a mutant signal. In one study, a panel of the most frequent mutations in colorectal cancer (*APC*, *KRAS*, *TP53*, *PIK3CA* and *BRAF*) appeared to be a more universal marker for monitoring of the clinical course of disease than currently used protein biomarkers [95]. Many chemotherapeutics are targeted to specific cancer pathways. Genes like *KRAS*, *BRAF*, *EGFR* or *p53* [96, 97] play important roles there, because gene products like kinases are components of signaling pathways for cell

cycle regulation. That is why the mutation status of these and other genes can be important for therapy response and monitoring. In lung cancer patients, the detection of *EGFR* mutations in plasma might help predicting disease progression, disease-free intervals and drug resistance, for example [96-98].

Epigenetic changes are constant alterations in cancer cells and influence early carcinogenesis and progression [99]. In 1999, serum DNA from non-small cell lung cancer patients was analyzed and, for the first time, deviant promoter hypermethylation of tumor suppressor genes was found [100] and developed to one of the most promising advances in cancer diagnosis. Similar to gene mutations, gene panels were often used to increase sensitivity [101-103]. Early stage ovarian cancer was detected with 82% sensitivity using *RASSF1A*, *BRCA1*, *APC*, *DAPK* and *CDKN2A* hypermethylation. Since these genes were not hypermethylated in non-neoplastic tissue, peritoneal fluid or serum of control subjects, specificity was 100% [104]. Another example for an epigenetic biomarker is *p16* gene methylation belonging to the most frequent used gene methylations in patients with liver, lung and breast cancer [100, 105 and 106].

Further biomarkers exist besides the described DNA level, integrity index and genetic as well as epigenetic changes. As a result, loss of heterozygosity and microsatellite instability detected by the analysis of microsatellite alterations [10, 107-115] and viral integration in cancer patients [62, 72, 116-121] can also be used. Furthermore, specific alterations can be detected in mitochondrial DNA from tissue and blood samples of cancer patients [122] and it may have some advantages. The mitochondrial genome is shorter and more simply organized, making a screening easier and more cost-effective. They have a high number of copies per cell, enabling a much more sensitive detection and they are not only present in blood [122, 123], but also in body fluids like saliva [124] and urine [125] from early stage cancer patients. Mitochondrial DNA can be found in the plasma of healthy individuals [126], but it is also related to tumor development and progression [127] and point mutations, deletions, insertions and quantitative changes [128] were found in breast, colon, liver, head and neck as well as lung tumors [129], for example.

Besides ccfDNA, RNA is also an important analyte in cancer diagnosis. *hTERT* belongs to the most frequently analyzed mRNAs in plasma. It is overexpressed in many tumors [130-137], but circulating cell-free RNA in general is not only tumor-specific. It is also present in healthy individuals or patients suffering from trauma [74, 138]. *hTERT* levels in plasma can be correlated with tumor stage, making it a proper tool for cancer screening, monitoring

[134] and therapy response [137]. Cytokeratin 19 mRNA combined with mammaglobin even led to higher detection sensitivity than ccfDNA [139]. Serum metastasin mRNA, on the other hand, is a good indicator for survival [140]. In addition to mRNAs, aberrant miRNA expression was correlated with colon [141-144], lung [145-148], breast [149], prostate [23], gastric [150] and esophageal [151] cancer as well as lymphoma [152]. Mitchel *et al.* [23] and Chen *et al.* [141] reported the possible utility of miRNA as a serological biomarker for solid tumors. miR-21, which is perhaps the most frequently analyzed miRNA, is overexpressed in many tumor types [153]. Nevertheless, miR-1, 10b, 17-92, 24, 92a, 122, 141, 155, 195, 221 and 375 and others are also possible biomarker candidates [154]. As shown for other nucleic acid biomarkers, panels should result in higher diagnostic values [155]. A correlation between four plasma miRNAs (miR-486, 30d, 1 and 499) and patient survival was detected in a large study with 303 patients [145]. The results of these studies emphasize the potential clinical value of circulating miRNAs.

The aim of prenatal testing, including screening and diagnosis, is the identification of fetal chromosomal and genetic disorders prior to delivery. Fetal RhD status or gender can also be determined. Maternal age, biochemical and ultrasound markers are considered for present screening procedures. If fetal abnormalities occur, invasive methods like chorionic villus sampling and amniocentesis follow to allow further diagnosis [156]. The disadvantage of the aforementioned invasive methods is that a significant risk of fetal loss exists [157]. Circulating cell-free fetal DNA from blood samples or other body fluids could be a non-invasive to minimally invasive alternative to existing procedures. Despite its very low concentration in maternal sample material such as plasma, fetal DNA can be detected very early in pregnancy [158]. Fetal fraction (proportion of fetal DNA in total circulating DNA) can be about 10% in early pregnancy and increases during pregnancy progress [159]. Nevertheless, fetal DNA is removed by two days postpartum so that DNA of previous pregnancies cannot interfere [160]. The first application for non-invasive or minimally invasive prenatal testing was fetal sex assessment by the detection of Y-chromosome-specific gene sequences like Dys14 [12] or SRY [161]. Fetal gender can be determined after gestational week 13 using sonography [162], while the analysis of circulating cell-free fetal DNA enables a determination between gestational week 7 and 20. Test performance using circulating cell-free fetal DNA thereby increases with gestational age according to rising ccfDNA concentrations [163]. Another application of non-invasive or minimally invasive prenatal testing is fetal RhD status determination in RhD negative women. An RhD positive fetus can then lead to alloimmunization in future pregnancies,

possibly causing severe hemolytic disease in appropriate women [164]. With fetal RhD status determination, immunoglobulin prophylaxis [165] can be administered only in cases where it is really required. RhD detection within circulating cell-free fetal DNA was first reported by Lo *et al.* [166] and Faas *et al.* [167]. Furthermore, circulating cell-free fetal DNA can be used for paternity determination by short tandem repeat or single nucleotide polymorphism (=SNP) analysis [168-170]. The quantification of paternal-specific gene sequences can also be used for the determination of fetal DNA fraction [171] or twin zygosity [172]. In addition, higher circulating cell-free fetal DNA levels in maternal serum have been associated with preeclampsia [173] and an increased risk of preterm labor [174] as well as in pregnancies with intrauterine growth restriction [175]. Using circulating cell-free fetal DNA, trisomy 21, 18, 13 and other chromosome aneuploidies can be detected by digital PCR [176, 177] or massively parallel sequencing [178, 179], for example. The maspin gene, which is methylated in maternal blood cells and hypomethylated in the placenta, was the first universal marker for fetal ccfDNA allowing a sex- and polymorphism-independent determination of the fetal DNA fraction by analysis of DNA methylation in this gene [180].

While circulating nucleic acids are an important diagnostic analyte in fetal medicine and oncology, they play a role in the diagnostics of other diseases too. In most cases, the concentration of ccfNA was determined and used as a biomarker. Lo *et al.* revealed increased ccfDNA levels after acute blunt trauma [181], analyzed the DNA concentration in burn victims [182] and observed increased DNA levels in plasma of patients suffering from sepsis [183]. Saukkonen *et al.* suggested that infection in general causes elevated ccfDNA levels [184]. Furthermore, the ccfDNA amount was increased in blood samples of patients with acute stages of myocardial infarction compared to heathy individuals [185]. A correlation between ccfDNA level and the severity of stroke was detected in stroke patients [186]. Donor-derived ccfDNA was found in the plasma of liver [187], heart [188] and kidney [189] transplantation recipients. These donor organ-specific nucleic acids were analyzed as a potential marker for graft rejection and in some studies, cell-free urine was also used as sample material besides plasma or serum and urinary mRNA was reported, for example [190-192]. With sickle cell disease, ccfDNA levels were higher in patients with acute pain than at steady state [193]. Additional diseases include autoimmune disorders like systemic lupus erythematosus and prion diseases [194], but plasma ccfDNA levels also increased in healthy individuals after chronic excessive resistance training [195] or even long distance running [73].

1.2. Liquid biopsy

The term “liquid biopsy” refers to a specific application in the field of cancer treatment. Liquid biopsy is non-invasive to minimally invasive depending on whether blood or other body fluids like urine are used as sample material. According to its less invasive character compared to standard biopsy sampling, it shows a higher applicability as a clinical tool for early diagnosis within the scope of a preventive screening, prognosis, therapy response or monitoring as well as companion diagnostics, for example. Genetic alterations such as point mutations, loss of heterozygosity and viral integration as well as epigenetic changes, e.g. the hypermethylation of tumor suppressor genes are informative parameters, which can be found in blood and other body fluids of cancer patients, for the molecular characterization of tumors. They serve as biomarkers and are intensely tested for cancer treatment. One important challenge in the course of cancer treatment in clinical practice is to overcome the development of tumor resistance that is often observed using molecular cancer characterization [196]. Resistance can be caused by mutations present in only a small subset of the tumor tissue, which is possibly not covered by biopsy sampling. Using blood and other body fluids for mutation detection might enhance the chance of catching the target molecules of interest. While a tissue biopsy provides only a part of the tumor genome, the entire one (including metastases) can be found in the mentioned liquid sample material. The mutation status of certain tumor suppressor genes is usually determined by tumor tissue analysis after biopsy sampling and these biopsies possibly do not contain the mutation of interest (Fig. 2).

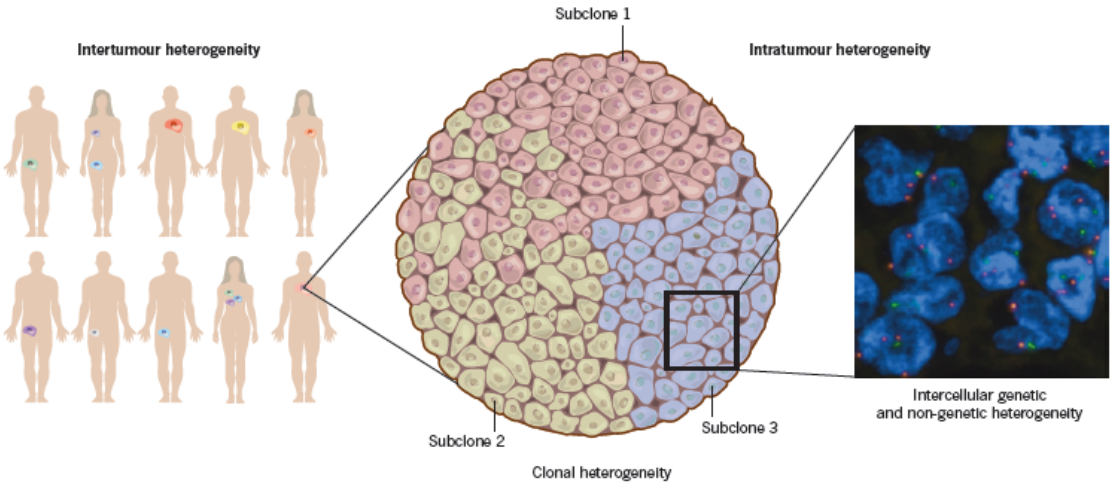


Figure 2 Intertumor and intratumor heterogeneity [197]
Genetic and non-genetic heterogeneity-like variation of, for example, chromosome copy number, somatic point mutations or epigenetic modifications exists.

In the case of anti-*EGFR* antibody therapy, mutant *KRAS* DNA was detected in serum samples of *KRAS* wild type colorectal cancer patients after five to six months of treatment [198]. Gerlinger and colleagues demonstrated intratumor heterogeneity by exome sequencing for many tumor suppressor genes [199]. Many mutations can be used as biomarkers to decide if specific targeted inhibitors are reasonable for the treatment of cancer patients [200]. Furthermore, tumor evolution may cause a change in specific aberrations of the primary tumor, metastases or relapses [201]. Circulating cell-free tumor DNA would be a potential alternative tool for biopsy sampling, enabling non-invasive to minimally invasive tumor genotyping at different time-points of disease. For combined *KRAS* and *BRAF* DNA mutation detection in plasma samples of metastatic colorectal cancer patients, 96% concordance between plasma and tumor tissue was achieved [198].

In terms of prenatal diagnostics, it is known that circulating cell-free fetal DNA is rapidly removed after delivery [202], although intact fetal cells can be detected in blood samples for many years post-partum [203]. The stability of ccfDNA in cancer patients is comparable. Diehl *et al.* estimated the half-life of circulating tumor DNA after surgery as 114 minutes [204].

1.3. Impact of preanalytical procedures

Preanalytical procedures have an important impact on the quality and quantity of ccfNA and influence the success of biomarker analysis as well as detection sensitivity this way. Specific characteristics of ccfDNA make research in this field very challenging. There is a very low concentration of DNA in plasma, serum, urine and other body fluids. In one study, it was only 21 ng/mL (mean and median value) in plasma of healthy individuals [205]. The concentration of ccfDNA can be elevated in different disease situations. In prostate cancer, for example, DNA level was between 11 and 1.746 ng/mL in plasma of patients with localized disease and between 108 and 5.556 ng/mL in plasma of patients with metastases [205], but only some molecules of used biomarkers can be present within these ccfDNA levels. For example, the mutation rate of the *APC* (=*adenomatous polyposis coli*) gene can be as low as 0.01% [40]. Target molecules like the mentioned *APC* gene mutations can be further diluted by genomic DNA released from blood cells during e.g. blood sample storage, because genomic DNA usually does not contain the genetic and epigenetic biomarker molecules of interest. However, ccfNA are not only very low concentrated; they are also strongly fragmented and mainly present in the form of mononucleosomes [206].

Dinucleosomes or trinucleosomes as well as high molecular weight DNA (approx. 10,000 bp) can also occur in some cases. Diehl *et al.* reported that in most cases DNA containing mutations from stool as well as plasma were even smaller than 150 bp [207]. Consequently, different fragment sizes can be included among ccfNA, whereas proportions differ between samples and not all fragment sizes are always represented.

As sample material, both plasma and serum are used to analyze circulating, cell-free DNA from blood samples and in most cases DNA levels are higher in serum than in plasma samples [208]. Houfflin Debarge *et al.* compared plasma, serum and blood cells as sample material for fetal DNA extraction in 2000. They reported higher fetal DNA recoveries from plasma compared to serum and “fetal gender could not reliably be determined from DNA extracted from maternal nucleated blood cells” [209]. Higher DNA concentrations in serum than in plasma samples were detected, even if whole blood was immediately processed. Since whole blood cells released DNA during the clotting process and serum DNA level increased after storage, the higher DNA concentration found in serum samples mainly seemed to be genomic DNA from breaking cells after blood withdrawal. That is why the authors recommended avoiding the use of serum for the monitoring of ccfDNA concentrations [210, 211]. The dilution of ccfDNA by genomic DNA can decrease detection sensitivity. Although loss of heterozygosity or microsatellite shift was observed in 80% of primary tumors from colorectal cancer patients, none of these biomarkers was detected in serum [212]. Two possibilities to remove genomic DNA contamination caused by cell lysis are high-speed centrifugation or filtration [213].

However, there is not only a difference between sample materials like plasma and serum. There are many blood collection tubes using different anticoagulants. EDTA, for example, leads to better blood cell stability during storage than citrate and heparin [214]. Furthermore, an anticoagulant like heparin is known to interact with DNA polymerases by binding magnesium ions, which are necessary for PCR reactions [215, 216]. To enable the storage of whole blood samples for more than two hours at room temperature, a special blood collection tube exists, the 10 mL Streck Cell-free DNA BCT (cat. no.: 218961; Streck, La Vista, Nebraska, USA). It prevents the degradation of cell-free DNA in plasma and the included formaldehyde-free preservative stabilizes white blood cells. As a result, genomic DNA is not released and high-quality cell-free DNA can be isolated. Sample stability is guaranteed by Streck for up to 14 days at room temperature, allowing convenient sample collection, transport and storage. It was compared against K_3EDTA, for example [217].

Besides the storage of fresh samples, the influence of plasma freezing was also tested. Serum and amniotic fluid were stored at -20°C and the fetal DNA amount in serum samples decreased by a factor of about 0.66 genome equivalents/mL per month [218]. In another study, the amount of isolated DNA as well as DNA from stored plasma samples had a decrease of approximately 30% per year [219]. As a consequence, storage conditions should be similar between different samples to make a comparison possible.

Many various methods are used for the extraction of ccfNA, with significant differences between them regarding extraction efficiency and repeatability [220]. Wang M. *et al.* compared two DNA extraction methods and found a higher sensitivity for mutated *KRAS* DNA detection using a modified Guanidine/Promega Resin procedure; however, the QIAamp DNA Blood Midi Kit led to higher DNA yields in most cases. Accordingly, total DNA amount is higher, but there is a loss of short DNA fragments, possibly containing mutated *KRAS* DNA. This difference in size distribution of isolated DNA fragments was displayed by gel electrophoresis [221]. In a further study, the QIAamp DNA Blood Midi Kit was compared to a new DNA extraction method and lower DNA yields were reached using the QIAamp DNA Blood Midi Kit [222]. Although the QIAamp DNA Blood Midi Kit was not optimized for the extraction of small DNA fragments, it was often used for the extraction of ccfDNA from plasma and serum [223-225]. A modified phenol-chloroform method led to a higher amount of tumor DNA from plasma of NSCLC patients than the QIAamp MinElute Virus Spin Kit [226]. By comparing four DNA extraction methods Mazurek and colleagues determined that detection sensitivity of *KRAS* mutation and cancer-associated HPV16 differed and could be improved using the right method [227]. In yet another study, the QIAamp cNA Kit as well as the QIAamp DNA Blood Mini Kit was compared with the NucleoSpin Plasma XS and FitAmp plasma/serum DNA isolation Kit. The QIAamp cNA Kit and the QIAamp DNA Blood Mini Kit had the highest DNA extraction efficiencies, while the QIAamp cNA Kit and the NucleoSpin Plasma XS were able to especially isolate smaller DNA fragments [228]. Therefore, the choice of the DNA extraction method has a great influence on the amount as well as the size of isolated ccfDNA and, furthermore, on the detection sensitivity for further analysis. It also might have an impact on concordance between mutation status in tumor tissue and matched ccfDNA plasma samples. Besides Kit systems, also salting out [220], chromatography resins [220, 229], magnetic beads [230], guanidine thiocyanate [231] and a Triton/Heat/Phenol protocol [232] were used for DNA extraction. There are also different extraction possibilities for other ccfNA like miRNAs. In one study the miRNeasy Serum/Plasma Kit led to the highest yields compared

to the miRvana microRNA Isolation Kit and the QIAamp cNA Kit [233]. According to this, the choice of extraction method also strongly depends on the target molecule within ccfNA and might differ between DNA, mRNA and miRNA, for example.

For this PhD thesis, ccfDNA was extracted from plasma, whereas preferably high sample volumes were processed. The QIAamp cNA Kit led, as mentioned before, to comparably high DNA extraction efficiencies, enabling the isolation of especially smaller DNA fragments. Furthermore, this Kit was optimized for the extraction of ccfDNA from plasma, serum and urine allowing for sample input volumes up to 5 mL. As a result, this manual DNA extraction method was chosen as the gold standard or reference for the development of a new automated protocol in which strongly fragmented and very low concentrated ccfDNA was extracted from preferably high plasma volumes.

1.4. Goal of the thesis project

The special characteristics of ccfDNA described before causes specific needs concerning preanalytical procedures like the DNA extraction method, for example. Many different manual Kit solutions, optimized for the extraction of ccfDNA, already exist, but automation is still needed for a broad applicability, including high throughput laboratories and other institutions relying on automation as well as to enhance comparability between different research groups or clinical sites. Since the development of a new instrument would be very time-consuming and cost-intensive, a new DNA extraction method should be developed that could be combined with a liquid handling instrument that is already available (in this case the QIAsymphony SP). State of the art DNA extraction methods are usually based on silica chaotrope chemistry. The maximal processing volume for DNA extraction is limited by the processing volume enabled by the extraction instrument, i.e., the maximum liquid volume that can be processed at once in any given step of the extraction procedure, e.g., the tube size of sample preparation cartridges, which provides a total volume of about 3 mL. Using silica chaotrope based chemistry, a high ratio of reagents to sample volume of 2 to 3 is required. To enable a preferably high sample input volume for improvement of detection sensitivity during analysis, reagent volumes have to be reduced as far as possible. Consequently, not only a new DNA extraction method had to be developed, but also a new chemistry that allows for a low reagents-to-sample volume ratio, so the following three main parts were defined for this PhD thesis:

1. Preanalytics
2. Enrichment of ccfDNA and automation of a new DNA extraction method
3. Application to diagnostic assays

Preanalytics has an important influence on the amount and quality of ccfNA and therefore influences the final results of the biomarker analysis. As a result, optimal preanalytical conditions, including all procedures from blood withdrawal to plasma storage, were first established. Always bearing preanalytics in mind, a new DNA extraction method was developed for the enrichment of ccfDNA realized by the processing of preferably large sample volumes keeping final elution volumes as small as possible. The DNA extraction method was based on a new chemistry enabling the reduction of reagent volume to increase the possible sample volume to a maximum. This way automation of the developed protocol was possible using an already existing QIAGEN instrument, the QIAsymphony SP. The mentioned special characteristics of ccfDNA were always considered in the course of protocol development and optimization. The aim was to achieve DNA recoveries, which were comparable to the QIAamp cNA Kit at least. It is one currently used manual standard method for the extraction of ccfNA. Furthermore, as a proof of principle for the diagnostic applicability, the compatibility with different analysis methods as well as with clinical samples was tested. Fetal and tumor DNA, including mutations and other gene variations, for example that could be used as biomarkers, were successfully extracted.

One reason for the development of an automated DNA extraction method was the strong need for standardization and improvement of generic preanalytical tools and procedures for in vitro diagnostics. The lack of standardization concerning the handling of circulating, cell-free nucleic acids is an often mentioned problem that makes comparisons between the results of different laboratories difficult. The total number of patients, the sample material and DNA extraction and analysis methods vary between studies [234]. Study designs, including selection of patient or control groups as well as methods for extraction and quantification of ccfNA are often different [77, 213, 225, 235 and 236].

Although it was indicated that detection sensitivity can be additionally increased by higher sample volumes [237], in some studies sample volumes as low as 200 µL were used as total sample input material [238]. Preferably high sample volumes were used during this thesis project. It was already possible to manually process up to 5 mL of plasma, but a new

automated large volume DNA extraction method had to be developed because it is strongly needed for standardization and regular use in clinical daily routine. It was optimized according to the specific characteristics of ccfDNA and the manual QIAamp cNA Kit, which is widely used in the field, served as reference system. Combined with different analysis methods and showing the applicability for clinical samples, the findings and developments of this PhD thesis project should help towards progress in standardization and improvement concerning in-vitro diagnostic procedures. The idea was to provide a proper basis for subsequent biomarker research and the development of molecular diagnostic tests based on the analysis of ccfDNA. There is also a good chance to reach high detection sensitivities at the end, but only if the starting material for the chosen analysis method has a preferably high quality. Many previous studies focused on the analysis of existing or the detection of new nucleic acid biomarkers and often disregarded the importance of preanalytics. To that effect, sometimes the question is if already tested biomarkers are really not suitable for clinical application or if only disadvantageous preanalytical procedures have been chosen, resulting in inconclusive study outcomes. Furthermore, research progress is facilitated by making results comparable between different laboratories.

2. Materials and Methods

2.1. Samples

In most cases, plasma was used as sample material. Whole blood was drawn into 10 mL BD Vacutainer® Plus Plastic K_2EDTA blood collection tubes (cat. no.: 367525; Becton Dickinson, Heidelberg, Germany). K_2EDTA was used as the anticoagulant. A centrifugation step at 3,000 rpm was performed for 15 min. at room temperature to separate the blood plasma from blood cells. Blood plasma was pipetted without disturbing the buffy coat (layer between erythrocytes and blood plasma, mostly containing leucocytes and platelets). To avoid a release of genomic DNA from blood cells, plasma separation took place as soon as possible after blood withdrawal. Plasma samples were stored at -80°C until processing. After thawing, a second centrifugation step at 16,000 x g was performed for 10 min. at 4°C to precipitate disruptive cryo-precipitates as well as residual cell debris and genomic DNA. The influence of high-speed centrifugation speed is described in chapter 3.1.2. In some cases, fresh plasma without prior freezing was used to

analyze a possible difference concerning DNA extraction efficiency using the newly developed automated protocol.

2.1.1. Healthy individuals

For most experiments described here, blood from healthy donors (volunteers at QIAGEN) was used after obtaining written informed consent and review board approval. Due to the in-house blood donation, all required steps from blood withdrawal to plasma storage were easily controlled, ensuring comparable handling conditions between samples and the required quality standard.

2.1.2. Clinical samples

To show the applicability of the automated DNA extraction protocol, which was developed during this PhD thesis, for clinical use, different experiments were performed, including pregnant women, cancer patients and non-cancer patients. The aim was to successfully extract DNA from clinical plasma samples and achieve DNA recoveries comparable to those of healthy individuals.

2.1.2.1. Pregnant women

Maternal plasma samples that were provided by “Praenatal-Medizin und Genetik” (Prenatal Medicine and Genetics) in Düsseldorf were used for one experiment. Plasma from pregnant women contains ccfNA of maternal as well as fetal origin, which should be comparably extracted. Table 1 shows the respective week of gestation for the ten female patient samples used.

Table 1 Week of gestation for each maternal plasma sample
Week of gestation was between 11+6 and 15, whereas no information was available for Patient 10.

Sample	Week of gestation
1	12+3
2	13+0
3	12+3
4	13+1
5	11+6
6	11+6
7	12+2
8	15
9	12
10	N/A

2.1.2.2. Cancer patients

Most samples for experiments dealing with clinical applicability were collected from cancer patients. Plasma from 24 colorectal cancer patients was provided by Sysmex Inostics GmbH in Hamburg. Table 2 shows the tumor stage after TNM or AJCC classification.

Table 2 Sample overview of the colorectal cancer patients from Sysmex Inostics GmbH (Hamburg)
Plasma samples of 24 colorectal cancer patients, provided by Sysmex Inostics GmbH in Hamburg were classified and pTNM as well as overall AJCC stage were determined. Pathological classification after surgery was performed for the categories tumor, lymph nodes and metastases (=pTNM). R is an additional category describing the success of tumor removal (R0= no tumor after surgery; R1= residual tumor, only detectable by microscopy; R2= macroscopic tumor or metastases). The overall stage (0-IV) is determined due to the most common TNM staging system from the American Joint Committee on Cancer (=AJCC), whereas higher numbers stand for more advanced tumors with a worse prognosis concerning the outcome.

Sample	Colorectal cancer type	pTNM stage				AJCC stage
		T	N	M	R	
1	Moderately differentiated adenocarcinoma	4	0	1	2	IV
2	Moderately differentiated mucinous adenocarcinoma	3	2	1	2	IV
3	Undifferentiated small cell carcinoma	3	2	1	2	IV
4	Poorly differentiated mucinous adenocarcinoma	3	2	1	2	IV
5	Moderately differentiated adenocarcinoma	3	2	1	2	IV
6	Moderately differentiated adenocarcinoma	3	1	1	2	IV
7	Moderately to poorly differentiated adenocarcinoma	4	2	1	2	IV
8	Moderately differentiated adenocarcinoma	3	2	1	2	IV
9	Moderately differentiated adenocarcinoma	3	1	1	2	IV
10	N/A	-	-	1	-	IV
11	Poorly differentiated adenocarcinoma	3	2	1	2	IV
12	Moderately differentiated adenocarcinoma	3	0	1	2	IV
13	Poorly differentiated mucinous adenocarcinoma	2	2	1	2	IV
14	Moderately differentiated adenocarcinoma	3	2	1	2	IV
15	Moderately differentiated adenocarcinoma	2	2	1	2	IV
16	Moderately differentiated adenocarcinoma	3	1	1	2	IV
17	Poorly differentiated mucinous adenocarcinoma	3	0	1	2	IV
18	Moderately differentiated adenocarcinoma	3	0	1	2	IV
19	Poorly differentiated adenocarcinoma	3	2	1	2	IV
20	Poorly differentiated adenocarcinoma	3	2	1	2	IV
21	Moderately differentiated adenocarcinoma	3	1	0	0	III
22	Moderately differentiated adenocarcinoma	3	1	0	0	III
23	Moderately differentiated adenocarcinoma	3	1	0	0	III
24	Moderately differentiated adenocarcinoma	3	1	0	1	III

The Surgical Research Laboratory at the University Hospital in Düsseldorf provided plasma from 91 cancer patients, whereas samples were collected before and after surgery for 51 of them (for three cancer patients even two samples before or after surgery exist). A sample overview can be found in Table 3.

Table 3 Overview of the cancer patient samples from the Surgical Research Laboratory at the University Hospital in Düsseldorf

Cancer type	Patient number
Pancreatic	14
Head and neck	21
Colon	24
Liver	6
Kidney	2
Thyroid	2
Esophagus	5
Stomach	5
Gall bladder	6
Lung	1
Breast	1
Ovarian	1
Major duodenal papilla carcinoma	1
Multiple endocrine neoplasia	1
Non-Hodgkin lymphoma	1

2.1.2.3. Patients suffering from non-cancer diseases

Plasma samples from 15 non-cancer patients, displayed in Table 4, were provided as the control group for the cooperation with the Surgical Research Laboratory at the University Hospital in Düsseldorf.

Table 4 Overview of the non-cancer patient samples from the Surgical Research Laboratory at the University Hospital in Düsseldorf

Non-cancer disease	Patient number
Chronic pancreatitis	2
Solid pseudo-papillary neoplasia of the pancreas	1
Adrenal adenoma	1
Hyperplasia pancreas tail	1
N/A	10

In addition, another study was performed with the Department of Pathology at the Medical University in Graz. Table 5 gives an overview of the collected samples.

Table 5 Overview of non-cancer patient samples from a laboratory at the Medical University in Graz

Non-cancer disease	Patient number
Non-alcoholic steatosis	2
Non-alcoholic steatohepatitis	2
HCV	2
Dermatological diseases	4

A study protocol for plasma preparation (further described in the attachment) for the plasma separation in Graz and Düsseldorf was prepared according to the defined workflow

from blood withdrawal to plasma storage. As it was not possible to immediately perform plasma separation after blood withdrawal, whole blood samples were stored at 4°C for up to two hours after inverting each tube eight times. In Düsseldorf, blood samples were collected in 6 mL BD Vacutainer® Plus Plastic K_2EDTA blood collection tubes (cat. no.: 367873; Becton Dickinson, Heidelberg, Germany). Plasma samples from Hamburg had already been collected before the cooperation was planned, but they took comparable blood withdrawal conditions into account. For blood withdrawal in Hamburg, 9 mL Sarstedt Monovette K_3EDTA whole blood tubes (cat. no.: 02.1066.001; Sarstedt, Nümbrecht, Germany) were used. Transport from the hospitals to the laboratory as well as storage until plasma preparation were performed on ice. Blood separation tubes (cat. no.: PAA535710; PAA Laboratories GmbH, Cölbe, Germany) were used for plasma preparation. Sarstedt Monovette tubes were slightly inverted and transferred into blood separation tubes. Centrifugation for 10 min. at 1,000 x g and room temperature followed. The plasma layer above the erythrocytes was removed and transferred into 2 mL cryo-tubes. The cryo-tubes were stored at -20°C until the end of a working day and were then transferred to -80°C freezers for long-term storage. The time of the call from hospital after blood withdrawal and the arrival at the laboratory were documented for each sample.

2.2. Nucleic acids

The collected blood plasma contains native ccfNA, which are extracted and analyzed afterwards. During this PhD thesis, I focused on ccfDNA. Besides native DNA, however, additional spike-in systems were used as the control of extraction efficiency. They were therefore added to samples for later extraction and analysis.

2.2.1. “Fragment” spike-in

Three different DNA fragments (75, 200 and 1,000 bp long) from the pEGFP-C1 plasmid were synthesized by preparative PCR to be able to analyze the possible size-dependent behavior of both DNA extraction methods (the QIAamp cNA Kit and the QIAsymphony cDNA Protocol). The fragment spike-in system was already used in my diploma thesis, where the existing set of 200 and 1,000 bp fragments was extended by the synthesis of a 75 bp long fragment. A detailed description can be found there [239a]. The named sizes were chosen according to the naturally occurring size range of circulating plasma DNA, mainly occurring in the form of mononucleosomes [206]. In addition, they were used as an

internal control of the DNA extraction efficiency by adding defined amounts to plasma samples and calculating the recovery at the end. Furthermore, they were spiked into elution buffers to be able to analyze the PCR-inhibitory effect solely caused by reagents without the influence of plasma components. Using them as spike-in in PBS (equivalent to a plasma sample), the whole workflow of the automated DNA extraction method was analyzed without effects caused by plasma. The GFP sequence does not occur in the human genome. For this reason, human nucleic acids cannot interfere by using quantitative real-time PCR for GFP fragment detection. The used primers are specific for each fragment and all three fragments occur from different, non-overlapping sections within the GFP sequence. The amplicon size of all three DNA fragments for quantitative real-time PCR after DNA extraction is nearly equal to enable the comparable amplification efficiency between all fragments. The details about the DNA fragments (the primer sequences for preparative and quantitative real-time PCR as well as the location within the GFP sequence and the location of target sequences for quantitative real-time PCR) can be found in the appendix of my diploma thesis [239b].

2.2.2. "*KRAS*" spike-in

"*KRAS*" spike-in systems were used to simulate mutated tumor DNA. First, it was tested in the form of single-stranded DNA and then so-called gBlocks were ordered. gBlocks are artificial double-stranded DNA fragments, consisting of a specific DNA sequence and available from Integrated DNA Technologies, Inc., Coralville, USA. In each case the DNA sequence corresponds with the *KRAS* c.34G>A (G12S) mutation. This gene mutation results in an amino acid substitution at position 12 in *KRAS*, from a glycine (G) to a serine (S) [240] and is detected by the later described BEAMing digital PCR technology.

2.3. Oligonucleotides

All primers and probes for quantitative real-time PCR assays (described later in chapter 2.9.5.2.) were ordered from Biolegio BV (Nijmegen, the Netherlands). All probes are HPLC purified, while primers are only salt-free. Primers/probes for pyrosequencing came from Integrated DNA Technologies, Inc. (Coralville, Iowa, USA).

2.4. Chemicals

Most of the buffers and solutions used are compounds of commercialized QIAGEN Kits. The others were prepared using chemicals from Merck, Sigma Aldrich or Invitrogen.

2.5. Kit systems

- QIAamp Circulating Nucleic Acid Kit
  [cat. no.: 55114; QIAGEN GmbH, Hilden, Germany]
- QIAsymphony DSP Virus/Pathogen Midi Kit
  [cat. no.: 937055; QIAGEN GmbH, Hilden, Germany]
- EpiTect Fast DNA Bisulfite Kit (50)
  [cat. no.: 59824; QIAGEN GmbH, Hilden, Germany]
- therascreen *KRAS* RGQ PCR Kit (24) CE
  [cat. no.: 870011; QIAGEN GmbH, Hilden, Germany]
- qBiomarker™ Somatic Mutation PCR Array Human Head & Neck Cancer
  [cat. no.: SMH-037ARE-2; QIAGEN GmbH, Hilden, Germany]
- Cancer Comprehensive 384HC Mutation PCR Array
  [cat. no.: SMH-3007AE; QIAGEN GmbH, Hilden, Germany]
- GeneRead DNA QuantiMIZE Array Kit
  [cat. no.: NGQA-002; QIAGEN GmbH, Hilden, Germany]
- Human Comprehensive Cancer Panel (GeneRead DNAseq Targeted Panels V2)
  [cat. no.: NGHS-501X; QIAGEN GmbH, Hilden, Germany]
- GeneRead Library I Core Kit
  [cat. no.: 180432; QIAGEN GmbH, Hilden, Germany]
- GeneRead DNA I Amp Kit
  [cat. no.: 180455; QIAGEN GmbH, Hilden, Germany]
- GeneRead Adapter 1 Set 12-plex
  [cat. no.: 180984; QIAGEN GmbH, Hilden, Germany]
- GeneRead Size Selection Kit
  [cat. no.: 180514; QIAGEN GmbH, Hilden, Germany]
- QIAquick PCR Purification Kit (50)
  [cat. no.: 28104; QIAGEN GmbH, Hilden, Germany]
- GeneRead Library Quant Array
  [cat. no.: 180611; QIAGEN GmbH, Hilden, Germany]
- Agencourt AMPure XP Kit
  [cat. no.: A63880; Beckman Coulter GmbH, Krefeld, Germany]
- Agilent High Sensitivity DNA Kit
  [cat. no.: 5067-4626; Agilent, Santa Clara, California, USA]

- Qubit® dsDNA HS Assay Kit
  [cat. no.: Q32851; Life Technologies Corporation, Carlsbad, California, USA]
- PyroMark Q24 Advanced CpG Reagents (4 x 24)
  [cat. no.: 970922; QIAGEN GmbH, Hilden, Germany]
- PyroMark PCR Kit (200)
  [cat. no.: 978703; QIAGEN GmbH, Hilden, Germany]
- ExoRNeasy Serum/Plasma Maxi Kit (50)
  [cat. no.: 77064; QIAGEN GmbH, Hilden, Germany]
- ExoQuick serum exosome precipitation solution (5 mL)
  [cat. no: EXOQ5A-1; System Biosciences, Palo Alto, California, USA]

2.6. Buffers

- PBS: Dulbecco's Phosphate Buffered Saline
  [cat. no.: D8537; Sigma Aldrich, St. Louis, Missouri, USA]
- RNase-Free Water (12 x 1.9 mL)
  [cat. no.: 129112; QIAGEN GmbH, Hilden, Germany]
- Buffer TE= 10 mM TRIS-HCl + 1 mM EDTA, pH 8.0
- Buffer ATL (200 mL)
  [cat. no.: 19076; QIAGEN GmbH, Hilden, Germany]
- PyroMark Wash Buffer (conc., 200 mL)
  [cat. no.: 979008; QIAGEN GmbH, Hilden, Germany]
- PyroMark Denaturation Sol. (500 mL)
  [cat. no.: 979007; QIAGEN GmbH, Hilden, Germany]
- Component of the RNase-Free DNA Set (50)
  [cat. no.: 79254; QIAGEN GmbH, Hilden, Germany]: Buffer RDD
- Component of the PAXgene Blood RNA Kit (50)
  [cat. no.: 762174; QIAGEN GmbH, Hilden, Germany]: Buffer BR4
- Proteinase K (2 mL)
  [cat. no.: 19131; QIAGEN GmbH, Hilden, Germany]
- Components of the QIAsymphony DSP AXpH DNA Kit
  [cat. no.: 937156; QIAGEN GmbH, Hilden, Germany]: AXpH lysis, elution and neutralization buffer as well as beads
- 2x QuantiTect Multiplex PCR Master Mix from the QuantiTect Multiplex PCR Kit (40)

[cat. no.: 204541; QIAGEN GmbH, Hilden; Germany]

- 2x QuantiTect Multiplex PCR noROX Master Mix from the QuantiTect Multiplex PCR noROX Kit (40)
  [cat. no.: 204741; QIAGEN GmbH, Hilden, Germany]
- 5x QuantiTect Virus Master Mix from the QuantiTect Virus Kit (50)
  [cat. no.: 211011; QIAGEN GmbH, Hilden; Germany]
- Component of the Qproteome Albumin/IgG Depletion Kit
  [cat. no.: 37521; QIAGEN GmbH, Hilden, Germany]: Qproteome Albumin/IgG depletion resins
- 20 mM glycine (pH 2)
- 40 mM K_2EDTA
- 100% ethanol
- 80% ethanol
- SDS
- Nonidet P40
- TritonX-100
- Sodium chloride
- Guanidinium thiocyanate (=GTC)
- Hydrochloric acid
- Acetic acid
- 5 mM/40 mM/327 mM/1 M citric acid
- 0.12 M/1 M sodium acetate/acetic acid
- 100 mM TRIS-HCl + 154 mM sodium chloride (pH 7.2)
- 35 mM TRIS-HCl + 0.1% TritonX-100 (pH 7.2)
- 35 mM MES + 0.1% TritonX-100 + 300 mM sodium chloride (pH 5.8/6.7)
- 35 mM MES + 0.1% TritonX-100 (pH 5.8/6.7)
- 35 mM TRIS-HCl (pH 7.5/8.5)
- 100 mM TRIS-HCl + 154 mM sodium chloride (pH 8.5)
- 100 mM MOPS + 154 mM sodium chloride (pH 7.9)
- 20 mM TRIS base
- 20 mM TRIS base + 40 mM/80 mM sodium chloride

- 10 mM/40 mM sodium hydroxide
- Polyacrylic acid (=PAA)
- 5 mM magnesium chloride
- 154 mM ammonium chloride
- 20 mM/80 mM/160 mM potassium chloride

2.7. Instruments

- Eppendorf centrifuge 5417 C
  [cat. no.: 5417 000.315; Eppendorf, Hauppauge, New York, USA]
- QIAvac 24 Plus vacuum manifold
  [cat. no.: 19413; QIAGEN GmbH, Hilden, Germany]
- Vacuum pump
  [cat. no.: 84020; QIAGEN GmbH, Hilden, Germany]
- Eppendorf thermomixer comfort
  [no.: 5355 000.011; Eppendorf, Hauppauge, New York, USA]
- GFL water bath
  [cat. no.: 1003; GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany]
- Sigma laboratory centrifuge with rotor 4K15
  [discontinued, replaced by 4-16KS; Sigma Laborzentrifugen GmbH, Osterode, Germany]
- LABINCO BV overhead rotational shaker Model LD-79
  [cat. no: 79000; LABINCO BV, Breda, the Netherlands]
- Magnetic tube racks (50, 15 and 1.5 mL tubes) for manual bead separation
  [no further details available]
- Real-time cycler:
  Rotor-Gene Q 5plex HRM Platform
  [cat. no.: 9001580; QIAGEN GmbH, Hilden, Germany]
  Applied Biosystems 7900HT Fast Real-Time PCR System
  [cat. no.: 4329001; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA]
- MJ Research PTC-200 Thermal Cycler
  [discontinued; MJ Research Inc., St. Bruno, Quebec, Canada]
- NanoDrop 2000 UV-Vis Spectrophotometer
  [Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA]

- QIAsymphony SP instrument
  [cat. no.: 9001297; QIAGEN GmbH, Hilden, Germany]
- 2100 Electrophoresis Bioanalyzer Instrument
  [cat. no.: G2939AA; Agilent Technologies, Santa Clara, California, USA]
- Qubit® 2.0 Fluorometer
  [cat. no.: Q33216; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA]
- PyroMark Q24 Advanced System
  [cat. no.: 180611; QIAGEN GmbH, Hilden, Germany]
- PyroMark Q24 Vacuum Workstation 220 V
  [cat. no.: 9001518; QIAGEN GmbH, Hilden, Germany]
- PyroMark Vacuum Prep Filter Probe 100
  [cat. no.: 979010; QIAGEN GmbH, Hilden, Germany]
- PyroMark Q24 Cartridge 3
  [cat. no.: 979202; QIAGEN GmbH, Hilden, Germany]
- Illumina MiSeq Desktop Sequencer
  [Illumina Inc., San Diego, California, USA]

2.8. Anion exchange beads

The new automated DNA extraction protocol, which was developed during this PhD thesis, is based on magnetic particles that enable the intended automation of DNA extraction on a proper instrument featuring, for example, magnetic rod covers for a magnetic particle based DNA extraction method. All tested bead types consist of a magnetic core covered with an anion exchange surface, forming the so-called bead. The surface molecules differ in function and detailed structure depending on the used bead type. As such, the following bead types were tested:

2.8.1. Type A beads

Their surface consists of functional groups chosen such that DNA binding to beads is weakened and elution simplified. Lower pH values are therefore sufficient to elute bead-bound nucleic acids completely.

2.8.2. Type B beads

The surface of these bead types is composed only of functional groups, which cause a stronger binding power and, consequently, higher pH values are necessary to successfully elute nucleic acids from beads.

2.8.3. Type C beads

Type C beads show a similar binding and elution behavior like type B beads, even though functional groups on their surface are different.

Purchasable type C beads are stored in a specific suspension for storage and they are used in the QIAsymphony DSP AXpH DNA Kit [cat. no.: 937156; QIAGEN GmbH, Hilden, Germany]. In-house produced type A and B beads are usually stored in RNase-free H_2O.

2.9. Molecular biological methods

2.9.1. Extraction of nucleic acids

2.9.1.1. QIAamp Circulating Nucleic Acid Kit

The QIAamp cNA Kit was developed for the extraction of ccfNA from up to 5 mL plasma, serum and urine. Lysis as well as binding-, wash- and elution-steps were performed on QIAamp Mini Spin Columns with silica membranes on a vacuum manifold. Denaturation of samples during the lysis step took place at 60°C. Proteinase K and buffer ACL were used for nucleic acid release and inactivation of DNases and RNases. Afterwards, buffer ACB was added to adjust optimal binding conditions between nucleic acids and silica membranes. Specific salt and pH conditions were adjusted to get rid of proteins and other contaminants after three wash steps. The ethanol concentration was increased in each wash step up to 100% at the end. Finally, nucleic acids were eluted in 20-150 µL AVE and detected by PCR or other downstream enzymatic reactions afterwards. For a 5 mL plasma protocol, according to the Kit handbook, 500 µL Proteinase K were pipetted into 50 mL centrifuge tubes. Then 5 mL plasma and 4 mL buffer ACL containing 5.6 µg Carrier-RNA were added. Carrier-RNA consists of short RNA fragments, used for e.g. the saturation of tube plastics to prevent unspecific DNA binding. Pulse vortexing (15 times) and an incubation for 30 min. at 60°C followed, then 9 mL buffer ACB was added and samples were transferred into tube extenders on QIAamp Mini Spin Columns after pulse vortexing and incubation for 5 min. on ice. The samples passed through membranes by vacuum and three wash steps were performed using 600 µL ACW1, 750 µL ACW2 and 750 µL 100% ethanol. The QIAamp Mini Spin Columns were then placed into new 2 mL collection tubes and centrifuged for 3 min. at 14,000 rpm. They were transferred into new 2 mL collection tubes and dried for 10 min. at 56°C with an open lid to remove residual ethanol, which has

a negative influence on the quantitative real-time PCR assay. QIAamp Mini Spin Columns were placed into 1.5 mL collection tubes and AVE was pipetted to the center of the membranes. After an incubation time of 3 min., centrifugation for 1 min. at 14,000 rpm was performed.

For the extraction of ccfDNA from urine samples the proper protocol of the Kit handbook was used.

2.9.1.2. QIAsymphony circulating DNA Protocol

The automated extraction method for ccfDNA was newly developed for the QIAsymphony SP instrument. Using this instrument, up to 96 samples could be automatically processed in one run. The QIAsymphony SP instrument for sample preparation can be combined with the QIAsymphony AS instrument for assay setup enabling automation of a whole workflow. Figure 3 shows such a QIAsymphony SP/AS instrument.

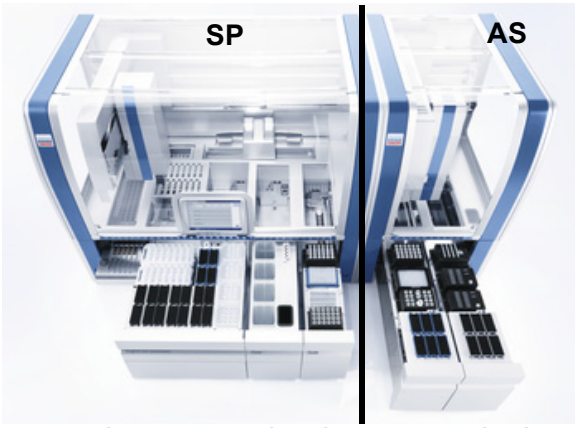


Figure 3 QIAsymphony SP/AS instrument [QIAGEN image database]
The QIAsymphony SP instrument (left side) can be extended by an AS module. All consumables needed for sample preparation and assay setup are loaded onto drawers.

To be able to perform DNA extraction automatically, samples were transferred into special sample tube carriers shown in Figure 4.

Figure 4 QIAsymphony Sample Tube Carrier [QIAGEN image database]
The QIAsymphony Sample Tube Carrier is provided for different sample tubes. BD Falcon Polystyrene Round-Bottom Test Tubes (17x100 mm, 14 mL) were used as shown in the picture for the experiments described in this PhD thesis. The tubes were filled with plasma and the carrier was placed on the instrument.

After starting the automated protocol, samples were pipetted by the robotic system into so-called sample preparation cartridges (=SPC) for DNA extraction. SPC are special tube plates for the execution of sample preparation, displayed in Figure 5.

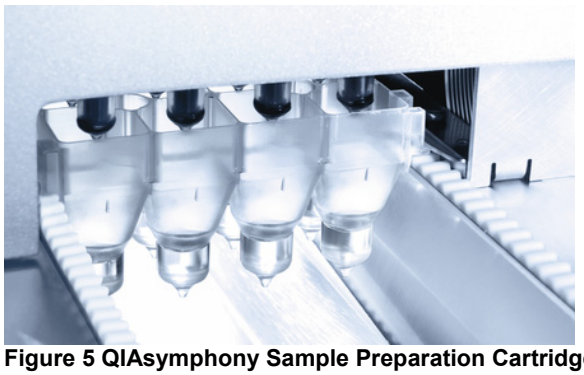


Figure 5 QIAsymphony Sample Preparation Cartridges [QIAGEN image database]
All steps for DNA extraction were performed in SPC. No more than 3 mL should be filled to avoid contamination between samples of different tubes. They were placed on the conveyer belt to move the ready reaction mix under the extractor for mixing and later bead separation by magnetic rods.

The principle of the QIAsymphony SP instrument is described in Figure 6.

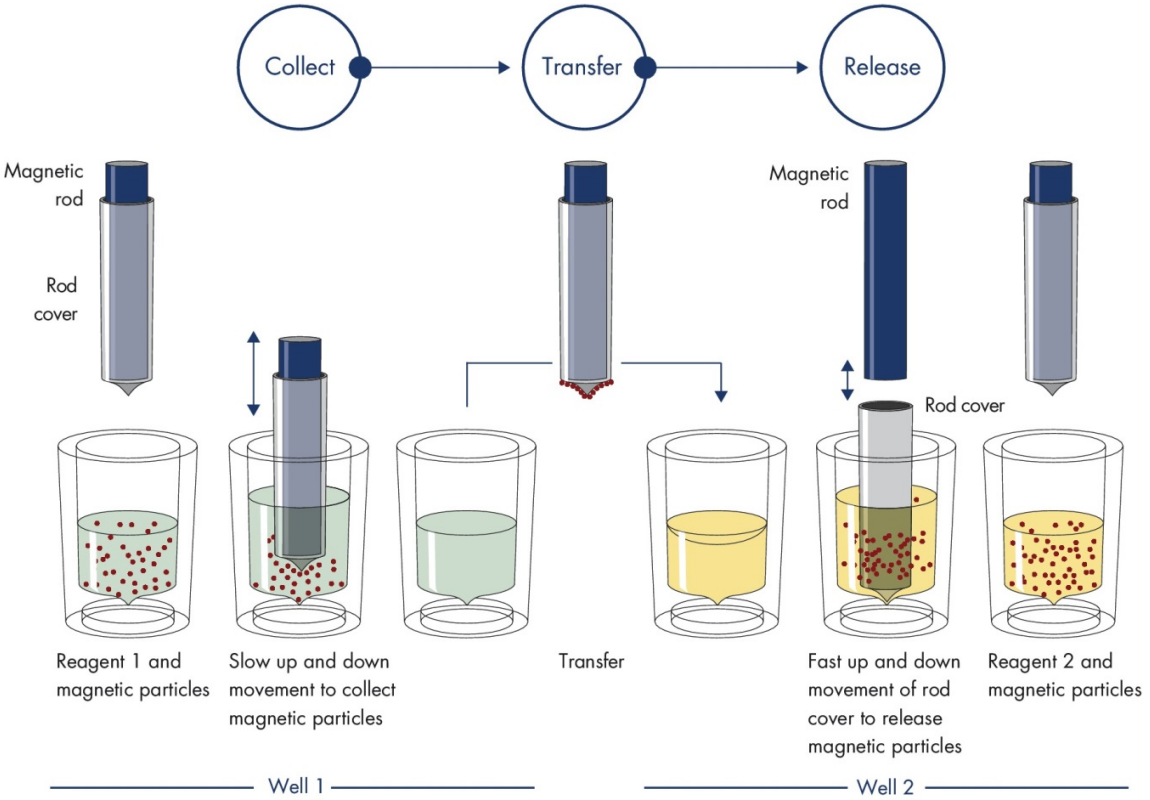


Figure 6 QIAsymphony Principle [QIAGEN image database]
The QIAsymphony principle for DNA extraction is based on magnetic particles, which were put into each reaction mix. Rod covers (=RC) without magnetic rods were used for mixing. In combination with the magnetic rods, magnetic particles were collected and transferred into the next reaction mix. RC were moved into the solution and beads were released for mixing again by removing the magnetic rod.

Depending on the buffers used, up to 2.9 mL plasma could be processed. Magnetic beads, binding buffer and Proteinase K (only in some cases) were added and so-called “Mag-Mix” began. “Mag-Mix" is the mixing by RC, performing periodic magnetic bead separation. The beads were then separated and transferred into a new tube of the SPC containing the wash buffer. After mixing, the beads were separated again and transferred into the second wash buffer (in some cases repetition with a third wash buffer followed). Elution was then performed by mixing and, after bead separation, eluted target molecules (in this case DNA) were transferred into an elution microtube rack (EMTR).

A new large volume DNA extraction method was developed using the described QIAsymphony SP instrument. According to the fact that the maximal processing volume for DNA extraction is 3 mL, not only a new protocol, but also a new chemistry had to be developed to reduce reagent volumes and enable preferably high sample volumes for DNA extraction.

2.9.1.3. QIAsymphony Custom Protocol

The QIAsymphony Custom (or customized) Protocol was developed as an interim solution for the automated processing of high sample volumes. It is based on an existing protocol version for the QIAsymphony SP instrument, the “Virus Cellfree 1000” protocol using the QIAsymphony DSP Virus/Pathogen Midi Kit, which was changed enabling the DNA extraction of sample volumes up to 4 mL. It was only an interim solution because of its lower throughput: 24 instead of 96 samples can be processed in one QIAsymphony SP run using one Kit and run-time is high compared to the newly developed protocol.

2.9.2. DNA bisulfite conversion

DNA had to be converted for the methylation analysis used by pyrosequencing (further described in chapter 2.9.5.4.). Two possible methods were tested.

2.9.2.1. Manual EpiTect Fast DNA Bisulfite Kit

According to the Kit handbook, bisulfite reactions were pipetted into 200 µL PCR tubes and 40 µL of low concentrated DNA samples (1-500 ng) were mixed with 85 µL Bisulfite Solution as well as 15 µL DNA Protect Buffer. The Bisulfite Solution is highly concentrated and enables a complete DNA conversion of unmethylated cytosine residues into uracil within 30 minutes. The DNA Protect Buffer, which contains a pH indicator dye as mixing control and confirms that the correct pH value is adjusted, prevents DNA fragmentation at high temperatures and low pH value. In addition, DNA denaturation is caused by heating, resulting in single-stranded DNA, which is needed for a complete cytosine conversion. The cycling conditions are shown in Table 6.

Table 6 Thermal cycler conditions for DNA bisulfite conversion

Step	Time [min.]	Temperature [°C]
Denaturation	5	95
Incubation	10	60
Denaturation	5	95
Incubation	10	60
Hold	∞	20

PCR tubes were centrifuged and the bisulfite reactions were transferred into 1.5 mL microcentrifuge tubes. Buffer BL (310 µl), containing 10 µg/mL Carrier-RNA, was added to bind converted single-stranded DNA to the QIAamp Mini Spin Column membranes. Solutions were vortexed and centrifuged, then 250 µL ethanol (96-100%) was added and the solution was mixed by pulse vortexing for 15 seconds and centrifuged briefly. The mixture was transferred into spin columns and centrifuged at maximum speed for 1 minute. Flow-through was discarded and spin columns were placed back into the collection tubes. Residual Bisulfite Solution was removed from the membrane by 500 µL BW, which was pipetted onto the membrane. After centrifugation at maximum speed for 1 min., flow-through was discarded and spin columns were placed back into the collection tubes. For desulfonation, 500 µL buffer BD was added and incubation for 15 min. at room temperature followed after closing spin column lids. After centrifugation and discarding the flow-through, two further wash steps using buffer BW were performed as previously described to further desalinate the DNA. For the final wash step, 250 µL ethanol (96-100%) were used and after centrifugation the spin columns were placed into new 2 mL collection tubes, centrifuged again and incubated with open lids in fresh collection tubes for 5 min. at 60°C using a heating block to remove any residual liquid. To elute DNA from the membrane, 15 µL buffer EB was added and the spin columns were incubated in 1.5 mL microcentrifuge tubes for 1 min. at room temperature and then centrifuged for 1 min. at 12,000 rpm.

2.9.2.2. Automated QIAsymphony DNA Bisulfite Conversion Protocol

The manual EpiTect Fast DNA Bisulfite Kit was automated on the QIAsymphony SP instrument, thus developing a new protocol and adapting the chemistry. In-house magnetic anion exchange beads (type B) were used instead of silica membranes. The QIAamp cNA Kit eluate was used as the sample input material for the automated QIAsymphony DNA Bisulfite Conversion Protocol after using sodium hydroxide for DNA denaturation.

2.9.3. Exosome isolation

Exosomes were isolated to separate nucleic acids, enclosed by exosomes from the free circulating part (“naked” nucleic acids or nucleic acids associated to proteins). The different nucleic acid fractions and their influence on DNA extraction should be determined this way. Two different methods were tested.

2.9.3.1. ExoQuick exosome precipitation solution

Using ExoQuick, exosomes were separated by pelleting. After plasma centrifugation at 3,000 x g for 15 min., 504 µL ExoQuick exosome precipitation solution was added to a 2 mL plasma sample (supernatant after centrifugation) and mixed by flicking the tube, followed by incubation for 30 min. and centrifugation at 1,500 x g for 30 min. at 4°C. The supernatant was removed and the residual solution was spun down by a further centrifugation step at 1,500 x g for 5 minutes. The pellet should contain the desired plasma exosomes.

2.9.3.2. ExoRNeasy

The ExoRNeasy Serum/Plasma Maxi Kit was optimized for RNA extraction, making the final eluate not applicable for the planned DNA analysis afterwards. Consequently, only binding and the first wash step were performed according to the handbook. Four mL of XBP was added to each 4 mL plasma sample and mixed by inverting the tube five times. The mixture was put on spin columns and centrifuged at 500 x g for 1 minute. Flow-through was kept for further analysis, then 10 mL XWP was added and centrifuged at 5,000 g for 5 minutes. Flow-through was also used for analysis. Ideally, only exosomes were bound to spin column membranes and free DNA should be found in flow-through after binding or wash step.

2.9.4. Protein depletion from plasma samples as pretreatment

Protein depletion was tested as pretreatment to make plasma samples more compatible for the newly developed automated DNA extraction method on the QIAsymphony SP instrument (QIAsymphony cDNA Protocol). Therefore, two different methods were tested.

2.9.4.1. Qproteome Albumin/IgG depletion resins

They are based on monoclonal antibodies, binding albumins and immunoglobulins with high affinity and specificity. Two 50 mL tubes containing the depletion resins were centrifuged for 1 min. at 500 x g and supernatant was discarded. Twenty-five mL PBS was filled instead and centrifugation was performed. After discarding the supernatant, this step was repeated using another 25 mL PBS, then 4.5 mL plasma was added into each tube and the tubes were incubated for 20 min. at 4°C. After centrifugation, the supernatant (=plasma after first protein depletion) was used as input material for a second protein depletion to remove residual proteins. Before the depletion resins were used again, they

had to be washed twice with 25 mL 20 mM glycine (pH 2) and five times using 25 mL PBS. Centrifugation for each wash step was performed for 1 min. at 500 g again.

2.9.4.2. Type D beads

Type D beads are coupled with different polyacrylic acids to form a negative charge on their surface. The aim is to bind positive charged proteins and other plasma components without losing DNA. Therefore, 1 mg magnetic type D beads were added per mL of plasma. After bead separation, supernatant was used as the sample input material for the QIAsymphony circulating DNA Protocol (=QIAsymphony cDNA Protocol). Like the other bead types described under 2.8. (A and B), they consist of a magnetic core and are usually stored in RNase-free H_2O.

2.9.5. Analysis of nucleic acids

Different methods were used for the analysis of extracted plasma ccfNA.

2.9.5.1. Qubit 2.0 Fluorometer

The Qubit dsDNA HS assay was performed using the Qubit 2.0 Fluorometer. This assay is highly sensitive for double stranded DNA with concentrations between 10 pg/µL and 100 ng/µL. It can be performed at room temperature and the signal is stable for up to three hours. The assay was started by diluting the Qubit dsDNA HS reagent 1:200 in a Qubit dsDNA HS buffer. Qubit working solution (190 µL) was required for the two standards, 195/199 µL were used for samples (sample volume depends on sample concentration -- 5 µL for clinical samples after DNA extraction and 1 µL for clinical samples before library construction). As such, 190 µL of working solution were pipetted into 0.5 mL tubes and 10 µL of each standard was added. The mixture was vortexed for two to three seconds without creating bubbles. The needed volume for samples was mixed with the working solution the same way and all of the tubes were incubated for 2 min. at room temperature. First, standards were measured for calibration and then the DNA concentration of samples was determined. Further details can be found in the corresponding handbook.

2.9.5.2. PCR

Polymerase chain reaction (or PCR) was used for DNA amplification. Certain DNA sequences were selectively enriched, even if target concentrations were low. Amplification was performed by *Taq* polymerase. A “hot start” led to more specificity, because the *Taq*

polymerase had to be initially activated at 95°C so that no amplification beyond a PCR run took place. Two specific oligonucleotide primers were designed for each target region, binding at the 3' end of one of the complementary DNA strands. All DNA fragments appeared in full-length before PCR amplification. After the first amplification cycle, one end of each of the complementary strands was terminated by the corresponding primer. From the second cycle, the opposite end was also defined by the respectively other primer. Each PCR cycle can be divided into three steps: denaturation, annealing and elongation. Ideally, the specific target DNA fragment is exponentially accumulated (2^n, whereas n is the number of PCR cycles). Replacing *E.coli* DNA polymerase with a thermostable polymerase from the thermophilic bacterium *Thermus aquaticus* (=*Taq*), it was no longer necessary to add fresh enzyme after the denaturation step of each cycle. Simultaneously, sensitivity, specificity and yield were improved, making the amplification of longer DNA target sequences possible [241].

Quantitative real-time PCR/assays

Instead of analyzing the accumulation of PCR product by electrophoresis, quantitative real-time PCR was introduced. DNA concentration was determined with accuracy and high sensitivity in a closed-tube system by fluorescence measurement. Many detection methods were developed. Quantitative real-time PCR was performed here using SYBR Green as intercalating fluorescent dye or probe based assays. Probes are single-stranded fluorescently-labeled oligonucleotides. The oligonucleotides used are hydrolysis probes, which are degraded after binding during amplification by the 5'-3' exonuclease activity of the *Taq* polymerase, so fluorescence is measured after elongation and is proportional to the amount of amplified PCR product. Using TaqMan probes (Fig. 7), each probe contains a donor (at the 5' end) and acceptor fluorophore (at the 3' end). The acceptor fluorophore is also called the quencher, because it quenches the fluorescence emitted from the donor by its close proximity. After degradation, the distance between donor and quencher increases and results in fluorescence generation [242].

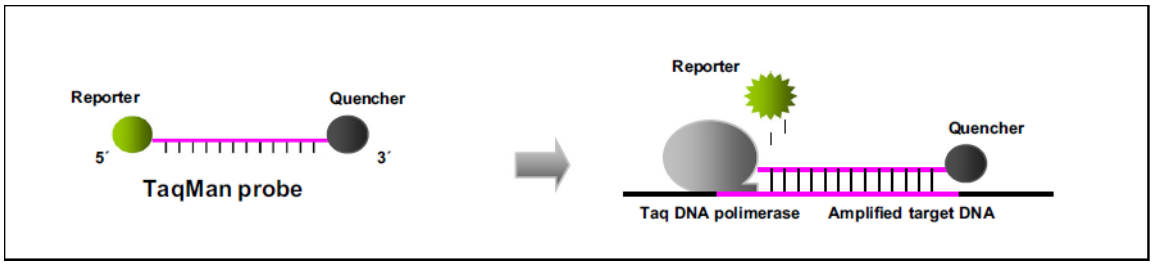


Figure 7 TaqMan probe: structure and mechanism [242]
TaqMan probes are provided with a donor and acceptor fluorophore called reporter and quencher. Both fluorophores are released through the exonuclease activity of the *Taq* polymerase and the reporter fluorescence signal is no longer suppressed.

Standards can be used for DNA quantification. The standard contains a known template amount, which leads to certain cycle of threshold (=Ct) values. Consequently, the amplification signal of an unknown sample can be quantified by comparison to a standard curve [243].

Different assays were used for the quantification of ccfDNA. Most assays were performed on an Applied Biosystems 7900HT Fast Real-Time PCR System using a 384 well plate format. The Rotor-Gene Q instrument was only used for the experiment shown in Figure 30 as well as combined with the therascreen *KRAS* RGQ PCR Kit and GeneRead DNA QuantiMIZE Array Kit. The qPCR components and cycling conditions remained unchanged, but Cy5 (in combination with BHQ 3) instead of Bodipy was used as fluorescence dye for the probe of the 66 bp amplicon of the 18S ribosomal DNA assay. 20 µL served as PCR reaction volume. All components are listed in the following table (Tab. 7).

Table 7 qPCR components

Component	Volume per reaction [µL]
2x QuantiTect Multiplex PCR Master Mix	10
Primer Probe Mix 1	1
Primer Probe Mix 2	1
Primer Probe Mix 3	1
RNase-free H_2O	Variable
Template DNA	Variable
Total PCR volume	20

In these cases, only a single or duplex assay (targeting one or two DNA sequences) is performed, one or two primer/probe mixes are needed and more RNase-free H_2O is pipetted. The used primer/probe mixes are concentrated 20-fold. Sixteen µM primer and 4 µM probe are used for the 18S ribosomal DNA and Dys14 target sequences. A lower

primer concentration of only 8 µM is sufficient for the fragment assay. Extracted male human DNA is used in 5,000, 500, 50, 5 and 0.5 copies per reaction as the quantification standard for the 18S and Dys14 target sequences. For the GFP fragments, the spike-in solution is used as the highest standard, which corresponds to 50,000 copies per reaction. The other standards (12,500, 3125, 781 and 195 copies per sample) are generated by 1:4 dilutions. In each case, RNase-free H_2O is used as the "No Template Control" (=NTC) and for standard row dilutions.

The cycling conditions are described in Table 8.

Table 8 qPCR cycling conditions

Stage	Repetition	Temperature [°C]	Time [min.]
1	Hold	96	20:00
2	45	94	01:00
		60	02:00

To reduce PCR-inhibitory effects, activation of *Taq* polymerase was changed, increasing temperature and incubation time. The initial setting (95°C and 15 min.) was only used for the experiment presented in Figure 30. For detection of ccfDNA, the following three assays were used:

- 18S ribosomal DNA duplex assay
  A 66 and 500 bp long amplicon were detected using the 18S ribosomal DNA duplex assay.
- Dys14/18S duplex assay
  For the Dys14/18S duplex assay, the 66 bp amplicon of the 18S assay was combined with the Dys14 target sequence, which can be used to calculate the fetal fraction in blood samples of women with a male fetus, because of its Y-chromosome location.
- Fragment triplex assay
  The fragment triplex assay was designed to quantify the spike-in system, consisting of 75, 200 and 1,000 bp GFP DNA fragments. 200,000 copies were usually added per sample. The amplicon size is almost equal between fragments.

An additional DNA assay was used for DNA quantification after the performance of bisulfite conversion protocols. The primer pair used targets a DNA sequence without any

cytosine residues enabling DNA quantification before and after bisulfite treatment. The cytosine free fragment (=CFF) target was analyzed using a SYBR Green Master Mix, which was prepared as shown in Table 9.

Table 9 PCR components for the CFF DNA qPCR assay

Component	Volume per reaction [µL]
2x QuantiTect Multiplex PCR Master Mix	10
Forward Primer (100 µM)	0.08
Reverse Primer (100 µM)	0.08
10x SYBR Green	0.3
RNase-free H_2O	4.54
Template DNA	5
Total PCR volume	20

A PCR run was performed using the following cycling conditions (Tab. 10).

Table 10 PCR cycling conditions for the CFF DNA qPCR assay

Stage	Repetition	Temperature [°C]	Time [°C]
1	Hold	95	10:00
2	40	94	00:15
		55	00:30
		72	00:30
3	Hold	72	10:00

All primer/probe sequences of the described quantitative real-time PCR assays are listed in the following table (Tab. 11).

Table 11 Details about quantitative real-time PCR assays

Assay	NCBI reference sequence	Amplicon size [bp]	Primer/ probe	Sequence 5' – 3'	Length [nt]	Dye - BHQ
18S ribosomal DNA duplex	NR_003286.2	66	Forward	GCCGCTAGAGGTGAAATTCTTG	22	5' Bodipy – BHQ 3'
			Reverse	CATTCTTGGCAAATGCTTTCG	21	
			Probe	ACCGGCGCAAGACGGACCAGA	21	
		500	Forward	GTCGCTCGCTCCTCTCCTACTT	22	5' FAM – BHQ 3'
			Reverse	GGCTGCTGGCACCAGACTT	19	
			Probe	CTAATACATGCCGACGGGCGCTGAC	25	
Dys14/18S duplex	NG_027958.1	67 (Dys14)	Forward	GAGCAGGCGTGGGTACTATTG	21	5' FAM – BHQ 3'
			Reverse	GTCTGCTGCTCGGCATCAC	19	
			Probe	CCTGCATGCGGCAGAGAAACCC	22	
	NR_003286.2	66 (18S)	Forward	GCCGCTAGAGGTGAAATTCTTG	22	5' Bodipy – BHQ 3'
			Reverse	CATTCTTGGCAAATGCTTTCG	21	
			Probe	ACCGGCGCAAGACGGACCAGA	21	
Fragment triplex	U55763.1	75 (75 bp GFP fragment)	Forward	ACCGCATCGAGCTGAAGG	18	5' Bodipy – BHQ 3'
			Reverse	TGTAGTTGTACTCCAGCTTGTGCC	24	
			Probe	TCGACTTCAAGGAGGACGGCAACA	24	
		63 (200 bp GFP fragment)	Forward	CACATGAAGCAGCACGACTTC	21	5' FAM – BHQ 3'
			Reverse	GGTGCGCTCCTGGACGTA	18	
			Probe	TCAAGTCCGCCATGCCCGAAG	21	
		73 (1,000 bp GFP fragment)	Forward	CTGCTGCCCGACAACCA	17	5' Joe – BHQ 3'
			Reverse	TGTGATCGCGCTTCTCGTT	19	
			Probe	TACCTGAGCACCCAGTCCGCCCT	23	
CFF	NC_000013.11	84	Forward	TAAGAGTAATAATGGATGGATGATG	25	-
			Reverse	CCTCCCATCTCCCTTCC	17	

Therascreen *KRAS* RGQ PCR Kit

The therascreen *KRAS* RGQ PCR Kit is a real-time qualitative PCR assay to detect seven somatic mutations within the human *KRAS* oncogene using Scorpion and ARMS technologies. Scorpion technology (Fig. 8) is based on primer-probes (=oligonucleotides, which combine a primer and a probe in one molecule). Using hairpin primer-probes, a hairpin secondary structure is included, whereas the loop sequence specifically binds to the target DNA. Six nucleotides at the 5' end of the probe are complementary to the 3' end region and two fluorophores are attached to each end (reporter at the 5' end and quencher at the 3' end). The 5' end of a primer is linked to the 3' end of a hairpin structure by a hexaethylene glycol blocker. This blocker prevents primer extension by polymerase activity. After binding of primer-probe, DNA extension from the 3' end of the primer takes place and the specific probe sequence binds to the complementary region of the newly amplified DNA after denaturation. The hairpin is opened and reporter and quencher are separated, resulting in a fluorescent signal proportional to the amount of amplified PCR product [242]. Figure 8 displays the described mechanism.

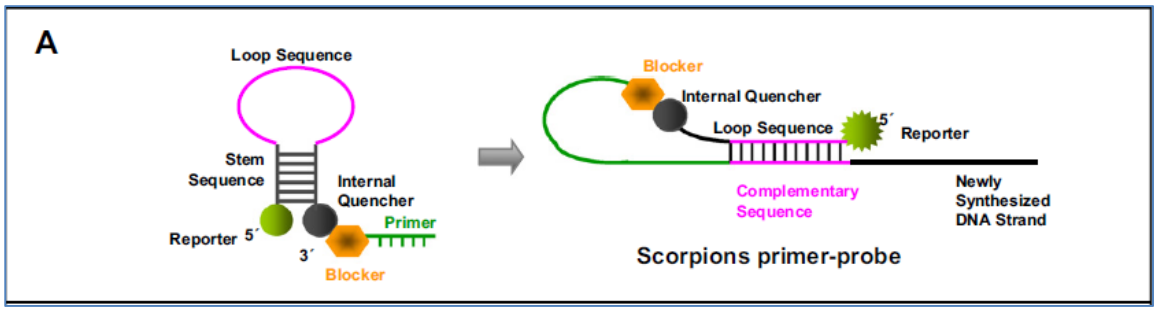


Figure 8 Scorpion primer-probes: structure and mechanism [242]

For Amplification, Refractory Mutation System (=ARMS) technology the 3' nucleotide of PCR primers differs and can either bind mutant or wild type DNA sequences. Sometimes more than one nucleotide is varied at the 3' end [244].

The detection limit was 0.8% mutant in the background of wild type DNA. In addition to the seven somatic mutations in codons 12 and 13 of exon 2 of the *KRAS* oncogene, a wild type control in exon 4 was detected. It was used for DNA quantification and determination of mutation status. An internal control (a non-*KRAS* target sequence to control PCR efficiency and analyze potential PCR inhibition) was included in each of the eight reaction mixes. The *KRAS* Positive Control (PC) composed of synthetic oligonucleotides for each Kit mutation is a further control for mutation detection. Finally, RNase-free H_2O is used as NTC to detect potential contamination, which might have an influence on assay results. Two templates are available for the PCR run: the "therascreen *KRAS* QC Locked Template" (for DNA sample assessment) and the "therascreen *KRAS* Locked Template" (for detection of *KRAS* mutations), which contain all PCR run parameters and calculate the results. In this PhD thesis, only the "therascreen *KRAS* Locked Template" was used for the detection of *KRAS* mutations and no previous DNA quality check by "therascreen *KRAS* QC Locked Template" was performed because of the limited eluate volume. After activation of *Taq* DNA polymerase for 15 min. at 95°C, 40 cycles were performed, including denaturation for 30 seconds at 95°C as well as annealing and extension for 1 min. at 60°C. Mutation status was calculated from the ΔCt, which results from the subtraction of the control assay Ct value from the mutation assay Ct value (ΔCt = [mutation assay Ct value] – [control assay Ct value]). Reaction mix tubes, RNase-free H_2O and positive control were thawed for at least 1 hour at room temperature (15–25°C), then the tubes were mixed by inverting (10 times). Master mixes were prepared in microcentrifuge tubes according to the following table (Tab. 12).

Table 12 Preparation of assay master mixes

Assay	*KRAS* DNA mutation	Reaction mix volume [µL]	*Taq* DNA polymerase volume [µL]
Control (=CTRL)	-	19.76*(n+1)	0.24*(n+1)
12ALA	G12A	19.76*(n+1)	0.24*(n+1)
12ASP	G12D	19.76*(n+1)	0.24*(n+1)
12ARG	G12R	19.76*(n+1)	0.24*(n+1)
12CYS	G12C	19.76*(n+1)	0.24*(n+1)
12SER	G12S	19.76*(n+1)	0.24*(n+1)
12VAL	G12V	19.76*(n+1)	0.24*(n+1)
13ASP	G13D	19.76*(n+1)	0.24*(n+1)

They were dispensed according to the described layout in the handbook and 5 µL RNase-free H_2O was immediately added to the appropriate tubes. The tubes were closed and 5 µL of DNA sample was pipetted. After closing them, at least 5 µL of positive control was added to the remaining tubes. After closing, all tubes were inverted four times and placed into the Rotor-Gene Q PCR instrument to start the run.

BEAMing digital PCR technology

Besides the therascreen *KRAS* RGQ PCR Kit, BEAMing digital PCR technology (Fig. 9) was also used for the analysis of *KRAS* DNA mutation status, which was performed by Sysmex Inostics GmbH in Hamburg. A59T, Q61L and Q61H were tested in addition to the mutations already described for the therascreen assay (Tab.12). BEAMing digital PCR technology is based on four components: Beads, Emulsion, Amplification and Magnetics. Figure 9 gives a short overview of the technology.

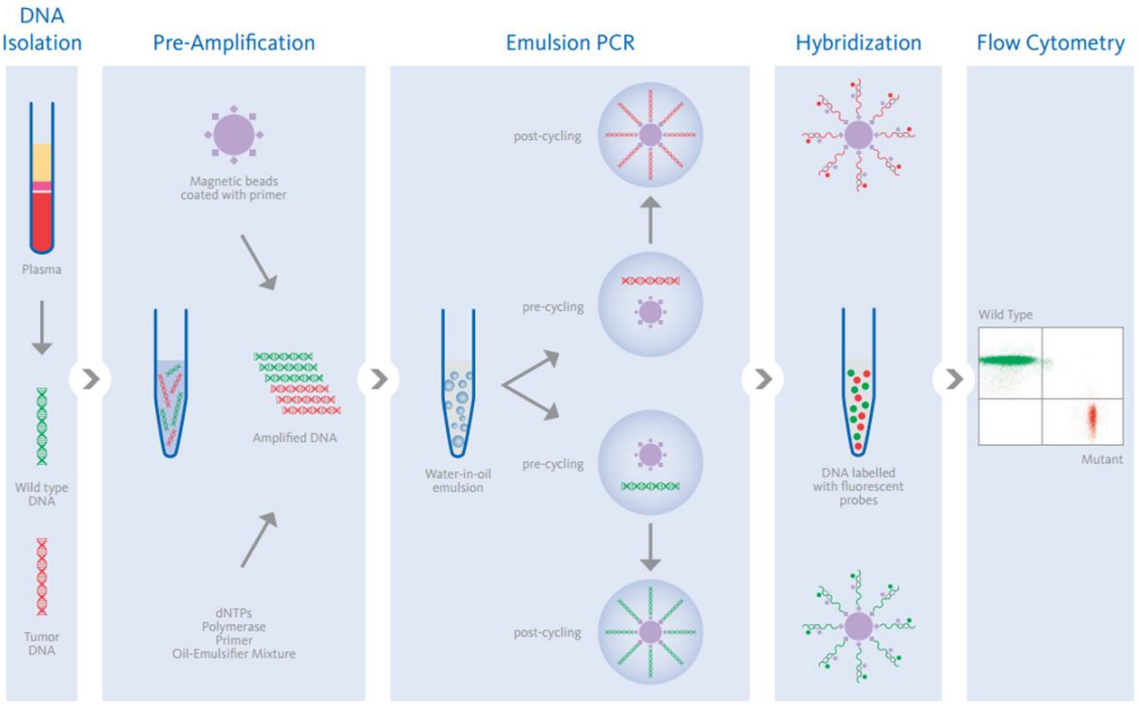


Figure 9 BEAMing digital PCR technology [245]
Magnetic bead based emulsion PCR combined with flow cytometry enables mutant tumor DNA detection with a high sensitivity of ≥0.01%. For BEAMing (=**B**eads, **E**mulsions, **A**mplification and **M**agnetics) a DNA population is transformed into a bead population coated with thousands of copies of one DNA sequence.

Extracted DNA consisted of tumor (mutant) and non-tumor (wild type) DNA sequences, whereas the mutant DNA fraction was usually underrepresented. First, a pre-amplification of the target region was performed using PCR. The target region was exon 2 of the *KRAS* oncogene. Afterwards a nested PCR was performed. In addition to sample DNA, process controls for wild type plasma (=Wt-Pl), negative controls (=NegKo) and mutant controls (=MutKo) were processed. Amplification was controlled by agarose gel electrophoresis. PCR products after pre-amplification were quantified by fluorescence measurement using standard DNA of known concentration and diluted to a specific concentration. After pre-amplification of the target region, water-in-oil emulsions were generated and emulsion PCR was performed using the pre-amplified DNA fragments and magnetic beads coated with primers. Ideally, one DNA molecule and one bead should be available per droplet. Pre-amplified DNA was normalized to a specific concentration. After amplification of wild type as well as mutated DNA sequences, micro-emulsions were broken by the addition of a specific breaking buffer to release beads coupled with amplified DNA. Beads were then magnetically separated and the supernatant was discarded. This step was repeated twice. Hybridization with specific fluorescent probes followed to allow for differentiation using flow cytometry. The probe mix always contained universal, wild type and mutant probes. The

probe mix was added and an allele-specific hybridization reaction on a PCR cycler was performed. Beads were then separated again and the supernatant was removed. Then beads were washed and resuspended in a specific buffer for flow cytometric analysis. The so-called gate was used to define the beads, which had to be analyzed and to separate the required single beads from duplicates and triplicates. Single extended beads successfully covered with DNA were further divided into universal, wild type and mutant beads. Only wild type and mutant signals were used to finally determine the mutant fraction [246, 247]. An example for a result after flow cytometry is shown in Figure 10.

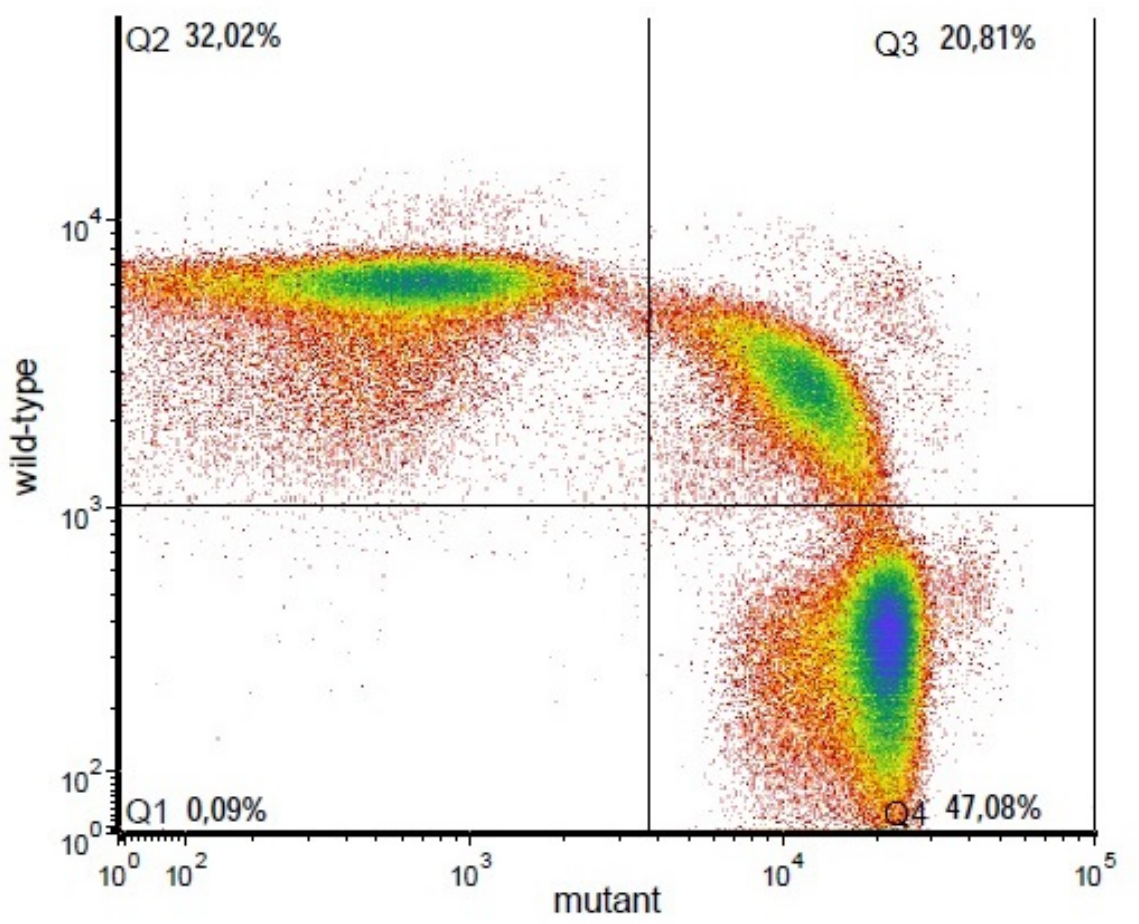


Figure 10 Wild type versus mutant beads
Result after flow cytometry for the DNA extracted with the QIAamp cNA Kit from Patient 10 are shown (Figure 54). Wild type (Q2) and mixed signals (Q3) can be distinguished from extended mutant beads (Q4).

In this case, the mutant fraction was 47.08%.

LINE-1 (=long interspersed element-1) quantitative real-time PCR assay is always used for DNA quantification before pre-amplification. *LINE*-1 is a repetitive DNA retrotransposon and constitutes approximately 17% of the human genome [248].

2.9.5.3. Agilent 2100 Electrophoresis Bioanalyzer Instrument

The Agilent High Sensitivity DNA Kit was used for sizing and quantitation of DNA fragments by virtual gel electrophoresis on a chip using the Agilent 2100 Electrophoresis Bioanalyzer instrument. First, the gel-dye mix was prepared. High sensitivity DNA dye concentrate and high sensitivity DNA gel matrix were equilibrated for 30 min. to room temperature, then 15 µL of high sensitivity DNA dye concentrate was added to a high sensitivity DNA gel matrix vial. The solution was mixed, centrifuged and transferred to the spin filter. After centrifugation at 2,240 x g for 10 min., the cleaned gel-dye-matrix was loaded on the chip. Nine µL was pipetted in the proper well and the chip priming station was used to push it into the capillaries of the chip. The residual wells for the gel-dye mix were then filled with 9 µL each. In all the other wells, not including the gel-dye mix, 5 µL of marker were pipetted and 1 µL of high sensitivity DNA ladder (well 12) or sample (well 1-11) was added. A typical Agilent chip for DNA analysis is shown in Figure 11.

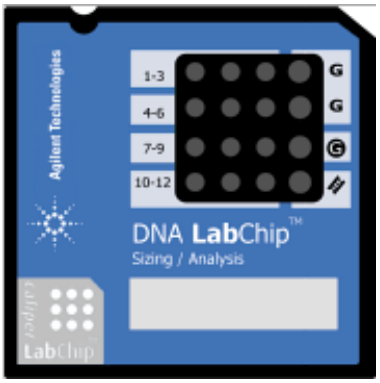


Figure 11 Agilent chip [249]

The chip was vortexed for 1 min. at 2,400 rpm and was run on the Agilent 2100 Bioanalyzer instrument within the next 5 minutes.

2.9.5.4. Pyrosequencing

The PyroMark Q24 Advanced instrument and reagents were used for pyrosequencing. The aim was to determine the methylation status of the *KRT23* gene analyzing its CpG islands. Methylation status of the *KRT23* gene was analyzed using the primer/probes sequences described in Table 13.

Table 13 Details about the *KRT23* pyrosequencing assay

Assay	NCBI reference sequence	Amplicon size [bp]	Primer	Sequence 5' – 3'	Length [nt]
KRT23	NG_012287.1	194	PCR forward	GTGGTGAAGGATAGGGAGAT	20
			PCR reverse (biotinylated)	CCAAAAAATAAAACAAAACTCAAC	24
			Pyrosequencing	GAAGGATAGGGAGATG	16

Using the PyroMark Q24 Advanced instrument for pyrosequencing, CpG methylation analysis was performed with assays based on larger sequencing read lengths. Pyrophosphate was released, inducing ATP generation, by inserting nucleotides through DNA polymerases into the analyzed DNA strand. ATP caused a luciferase reaction. The detected light signal was proportional to the number of integrated nucleotides. Nucleotides, which were not integrated as well as ATP were degraded through apyrase after each reaction. Additionally, single-stranded binding protein prevented secondary structures in DNA templates and primers. dATPαS instead of dATP was used, because it can be recognized by DNA polymerase but not by luciferase [250]. After thawing PCR reagents at room temperature, PCR reaction mix was set up according to the following table (Tab. 14).

Table 14 Reaction composition using PyroMark PCR Master Mix

Component	Volume per reaction [µL]
2x PyroMark PCR Master Mix	12.5
10x CoralLoad Concentrate	2.5
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
RNase-free H_2O	5
Template DNA	4
Total PCR volume	25

0.5 µL of each primer (forward and reverse) were pipetted to reach the final concentration of 0.2 µM. 4 µL of template DNA after bisulfite treatment were used, so 5 µL RNase-free H_2O were needed to get a total volume of 25 µL. The master mix was mixed by pipetting and transferred to PCR tubes to add the template DNA. The cycling conditions, as shown in Table 15, were used.

Table 15 Optimized cycling protocol when using PyroMark PCR Master Mix

Stage	Repetition	Temperature [°C]	Time [min.]
1	Hold	95	15:00
2	45	94	00:30
		56	00:30
		72	00:30
3	Hold	72	10:00

To perform a run on a thermal cycler instrument, the PCR product was immobilized to Streptavidin Sepharose beads. The beads were homogenized and 2 µL were mixed with 40 µL Binding Buffer and 28 µL H_2O. Seventy µL of the mixture and 10 µL of the biotinylated PCR product were pipetted into the wells of a 24-well PCR plate. The wells were closed using an adhesive foil and the plate was mixed for 10 min. at 1,400 rpm and room temperature. The reagents were then loaded into the PyroMark cartridge. Volume information was taken from the pre-run information report of the predefined run setup. The filled cartridge was placed on the instrument and the sequencing primer was diluted with Annealing Buffer to a final concentration of 0.375 µM. Twenty µL were put into the wells of a PyroMark Q24 plate. Using a vacuum workstation, the beads were captured, washed in 70% ethanol, denatured and washed with a certain buffer to be finally released into plate wells containing primers. After heating for 5 min. at 80°C, the plate was placed on the instrument to start the run immediately. Data analysis was performed according to the PyroMark Q24 Advanced User Manual. Detailed instructions were available in the PyroMark Q24 Advanced Software User Guide of the PyroMark Q24 Advanced Software. After opening the run file with the corresponding software, wells were selected to show CpG analysis results. The methylation percentage was displayed in the form of a pyrogram. Analysis reports were selected from the “Reports” menu.

2.9.5.5. Sequencing

Targeted sequencing using a GeneRead DNAseq Targeted Panel V2 was performed on the Illumina MiSeq Sequencer. The Human Comprehensive Cancer Panel was used in this case. A specific DNA amount of a certain integrity/quality was needed for PCR setup, which was determined by using the GeneRead DNA QuantiMIZE Array Kit. The PCR reaction was prepared according to Table 16.

Table 16 PCR component mix

Component	Volume [µL] for each sample	Volume [µL] for empty positions
RNase-free H_2O	68	48
GeneRead qPCR SYBR Green ROX FAST Master Mix	72	48
Template DNA	4	-
Total PCR volume	144	96

Each mix contained the volume for all replicates of one sample, including the DNA template. Twenty µL were pipetted into the predetermined Rotor-Gene tubes, which were

closed and placed in the Rotor-Gene Q instrument. The cycling conditions are displayed in Table 17.

Table 17 Cycling conditions

Stage	Repetition	Temperature [°C]	Time [min.]
1	Hold	95	10:00
2	40	94	00:15
		60	00:30

DNA was diluted according to the results of the GeneRead DNA QuantiMIZE Array. For the Human Comprehensive Cancer Panel 4*4 µL were added to each of the four PCR reactions, containing 16 µL of the PCR mix, which was prepared on ice according to Table 18.

Table 18 Preparation of PCR mix for each primer mix pool of the 4-pool panel

Component	Volume per sample [µL]
5x GeneRead DNAseq Panel PCR Buffer	4.4
2x Primer mix pool	11
GeneRead Hot Start *Taq* DNA polymerase (6 U/µL)	1.5
RNase-free H_2O	0.7
Total volume	17.6

Using a thermal cycler, the PCR program was started according to Table 19.

Table 19 PCR program

Stage	Repetition	Temperature [°C]	Time [min.]
1	Hold	95	15:00
2	Determined by QuantiMIZE Array	95	00:15
		60	08:00
3	Hold	72	10:00
4	Hold	4	∞

The number of cycles was also determined by the GeneRead DNA QuantiMIZE Array. After the described enrichment-PCR, it was followed by sample pooling and purification, so all four reactions for each sample were combined and transferred into Axygen® 1.7 mL MaxyClear Microtubes (Corning Inc.; Corning, New York, USA). Seventy-two µL AMPure XP beads were added for purification and the solution was vortexed and incubated for 5 min. at room temperature. After a short pulse-spin, beads were separated on a magnetic rack and, as soon as the solution was clear (approx. 5 min.), the supernatant (140 µl) was transferred into new tubes. This step was repeated with 128 µL AMPure XP beads, this

time keeping the beads with bound target DNA and discarding the supernatant. Then 80% ethanol (400 µl) was added to the tubes, which were still on the magnetic tube rack. After incubation for 30 seconds at room temperature, ethanol was discarded. This step was repeated and the beads were centrifuged and separated again to remove any residual ethanol. The beads were then dried for 15 min. after opening the tube lids on the magnetic tube rack. Twenty-eight µL of RNase-free H_2O was added to elute target DNA from the beads and, after vortexing and centrifugation, beads were separated on a magnetic tube rack and 25 µL of the supernatant were transferred to clean Axygen® 1.7 mL MaxyClear Microtubes (Corning Inc.; Corning, New York, USA). The DNA amount was determined by Agilent 2100 Bioanalyzer using the High Sensitivity DNA Kit as well as by Qubit measurement, as a possible alternative. Library construction then started with the end repair of DNA. Therefore, 10-200 ng DNA was needed for optimal efficiency of library construction. The reaction mix contained 20.5 µL PCR-enriched DNA, 2.5 µL End-Repair Buffer and 2 µL End-Repair Enzyme Mix. These components were mixed by pipetting and incubated for 30 min. at 25°C and 20 min. at 75°C in a thermal cycler, followed by A-addition. Twenty-five µL end-repaired DNA as well as 3 µL A-addition Buffer and 3 µL Klenow Fragment were mixed by pipetting and incubated for 30 min. at 37°C and 10 min. at 75°C in a thermal cycler. Afterwards, adaptor ligation was performed. Thirty-one µL DNA, 45 µL ligation buffer, 2.5 µL Adaptor, 4 µL T4 DNA ligase and 7.5 µL RNase-free H_2O were mixed. For each of the twelve samples another adaptor was used. After mixing, incubation for 10 min. at 25°C was started. The thermal cycler had to be used without a heated lid. The GeneRead Size Selection Kit served for cleanup of adaptor-ligated DNA. DNA was transferred into Axygen® 1.7 mL MaxyClear Microtubes (Corning Inc.; Corning, New York, USA) and 360 µL buffer SB1 was added. After mixing, the sample was applied to MinElute Spin Columns and centrifuged for 1 min. at 14,000 rpm. The DNA was bound and flow-through discarded. Steps after addition of 360 µL buffer SB1 were repeated twice; this time to wash bound DNA using 700 µL of 80% ethanol instead of SB1. Empty MinElute Spin Columns were then centrifuged again for 1 min. at 14,000 rpm to dry membranes for the removal of residual ethanol and placed into 1.5 mL microcentrifuge tubes. Ninety µL buffer TE was added, incubated for 1 min. and centrifuged for 1 min. at 14,000 rpm. Buffer SB1 (360 µl) was mixed with the flow-through and applied to the same spin columns,

which were placed into new 2 mL collection tubes, followed by centrifugation for 1 min. at 14,000 rpm and discarding the flow-through. The two wash steps, as already described above, were then performed using 700 µL of 80% ethanol in each case. An additional centrifugation step without liquid followed and the spin column was placed in clean 1.5 mL microcentrifuge tubes. Ninety µL buffer EB was used for elution and centrifugation was started after an incubation time of 1 minute. AMPure XP beads were used for fine size selection. Seventy-two µL were added to the DNA solution, mixed and centrifuged for 5 minutes. The tube was pulse-spun and placed on a magnetic rack for bead separation. The clear supernatant (155 µl) was transferred to new tubes. Thirty-six µL beads were added, mixed, incubated and separated again, as described before, but this time the supernatant was discarded and 400 µL of 80% ethanol was pipetted and removed after an incubation time of 30 seconds. After repeating this wash step, the tubes were spun and placed on the magnetic tube rack and residual ethanol was completely removed. Additionally, beads were dried with open lids on a magnetic rack for 10 minutes. DNA was eluted from beads with 19 µL of RNase-free H_2O. After the known procedure for bead separation, 17 µL of the clear supernatant was used for PCR amplification. Therefore, 25 µL HiFi PCR Master Mix, 1.5 µL Primer Mix, 17 µL DNA as well as 6.5 µL RNase-free H_2O were mixed and amplification was started using cycling conditions shown in Table 20.

Table 20 Cycling conditions for PCR amplification of purified DNA library

Stage	Repetition	Temperature [°C]	Time [min.]
1	Hold	98	02:00
2	10	98	00:20
		60	00:30
		72	00:30
3	Hold	72	01:00
4	Hold	4	∞

The QIAquick PCR Purification Kit was used for cleanup of PCR reactions. Buffer PB (250 µl) was mixed with PCR reaction, applied to QIAquick columns and placed in 2 mL collection tubes. After centrifugation for 1 min. at 13,000 rpm, flow-through was discarded. The step was repeated with 750 µL buffer PE. The empty column was then centrifuged again and placed into clean Axygen® 1.7 mL MaxyClear Microtubes (Corning Inc.; Corning, New York, USA). Thirty µL buffer EB was added for elution and the tubes were centrifuged after an incubation time of one minute. The sample library was quantified by

GeneRead Library Quant Array. Two dilutions of the sample library (1:2,000 and 1:20,000) were prepared according to the handbook using buffer EB. The components of the PCR reaction mix are listed in Table 21.

Table 21 PCR reaction mix for standard or sample library (96-well plate format)

Component	Volume [µL] for standard and NTC	Volume [µL] for sample library
RNase-free H_2O	240	32
GeneRead qPCR SYBR Green Master Mix	240	42
Template (sample library)	-	10

Ten µL were pipetted into each plate well referring to a specific layout. The plate was closed and centrifuged for 1 min. at 1,000 rpm and room temperature. After programming the cycling conditions (Tab. 22), PCR run was started on an Applied Biosystems 7900HT Fast Real-Time PCR System.

Table 22 Cycling conditions

Stage	Repetition	Temperature [°C]	Time [min.]
1	Hold	95	10:00
2	30	95	00:15
		60	00:30
		72	02:00

Each library was diluted to 10 nM according to the results of the GeneRead Library Quant Array. They were then combined in equal amounts, mixed well and used as input material for sequencing on the Illumina MiSeq instrument. Sequencing was performed by Holger Wedler and his team (QIAGEN GmbH, Hilden, Germany) and with his help the resulting data were analyzed. Data analysis was performed according to the “User Guide for GeneRead Data Variant Analysis.” First, “http://ngsdataanalysis.sabiosciences.com” was opened and the read files in FASTQ format were uploaded, whereas files had to contain reads from only one sample. Job type (single), analysis mode (somatic mode), panel identifier (GeneRead panel) and sample information (paired-end reads and individual samples) were entered under “variant calling jobs.” Analysis was started by clicking the “Create Analysis Job” button and, after “Job Done Successfully” was shown, files for analysis were available.

3. Results

3.1. Sample material

Circulating cell-free DNA can be found in blood samples and other body fluids. For this PhD thesis, ccfDNA was mostly extracted from plasma of healthy individuals. To make comparison between different samples possible, blood withdrawal and plasma preparation was held as equal as possible for each experiment. Furthermore, each preanalytical step was optimized according to the specific characteristics of ccfDNA to prevent any DNA loss during DNA extraction, DNA degradation during storage of whole blood and plasma samples, the release of genomic DNA by blood cells and the dilution of target molecules. Since the newly developed automated QIAsymphony cDNA Protocol has no real lysis step like the silica chaotrope based manual DNA extraction method using the QIAamp cNA Kit, preanalytical procedures influencing the DNA nature might have an even stronger effect on protocol performance. The analysis of different preanalytical procedures is described in the following section. Since the general influence of preanalytics should be tested, only the QIAamp cNA Kit was used as the gold standard in most experiments to determine optimal preanalytical conditions for the development of the automated DNA extraction protocol.

3.1.1. Blood sample collection

Many different systems exist for blood withdrawal. Each company (BD, Sarstedt and others) offers its own solution and, according to the variety of possible applications, many blood collection tubes are available that differ in their included substances. We decided to use plasma instead of serum to have a lower genomic DNA background [211] and used potassium EDTA as the anticoagulant, because it not only stabilizes blood samples but also does not interfere in later analyses [214]. Therefore, K_2- and K_3EDTA whole blood collection tubes were used. Shearing forces during blood withdrawal may have a negative influence on blood cell stability, possibly activating the release of genomic DNA. As a result, aspiration and vacuum based whole blood collection tubes were compared and a hollow needle was tested against a Multifly hollow needle with a lower inner diameter and a longer passage distance.

With regard to blood sample collection, three different blood withdrawal systems were tested in cooperation with the Clinical Trial Center of the Medical University in Graz, Austria. About 60 mL blood was taken from 15 healthy individuals using

- 2*10 mL BD Vacutainer K_2EDTA tubes combined with a hollow needle
- 2*10 mL BD Vacutainer K_2EDTA tubes combined with a Multifly hollow needle
- 2*9 mL Sarstedt Monovette K_3EDTA tubes combined with a Multifly hollow needle

After blood withdrawal by aspiration (Sarstedt) or vacuum (BD), blood collection tubes were centrifuged at 1,900 x g for 15 min. at room temperature for plasma preparation and plasma samples were stored frozen. For DNA extraction using the QIAamp cNA Kit, samples were thawed at room temperature, transferred into 15 mL tubes and centrifuged at 16,000 x g for 10 min. at 4°C. The supernatant was transferred into a new tube without disturbing the pellet. Three mL plasma was processed for most samples. In cases with less than 3 mL plasma, 2.4 mL plasma was filled with 600 µL PBS for sample preparation. Carrier-RNA was not used, because it could interfere with later analysis methods, which were not certain at the beginning of the experiment. DNA was ultimately eluted in 60 µL AVE. 200,000 copies of the fragment spike-in system (75, 200 and 1,000 bp fragments of the pEGFP-C1 plasmid) were added as internal control (further detailed information can be found in chapter 2.2.1.). These DNA fragments can be independently detected from naturally occurring human DNA. Human DNA concentration deviates between samples and healthy individuals. In contrast, non-human DNA fragments can be added in defined levels to detect possible DNA losses during DNA extraction. Furthermore, the influence on different DNA fragment sizes can also be tested. The 18S ribosomal DNA assay was used to quantify native DNA. Results for the fragment assay are shown in Figure 12.

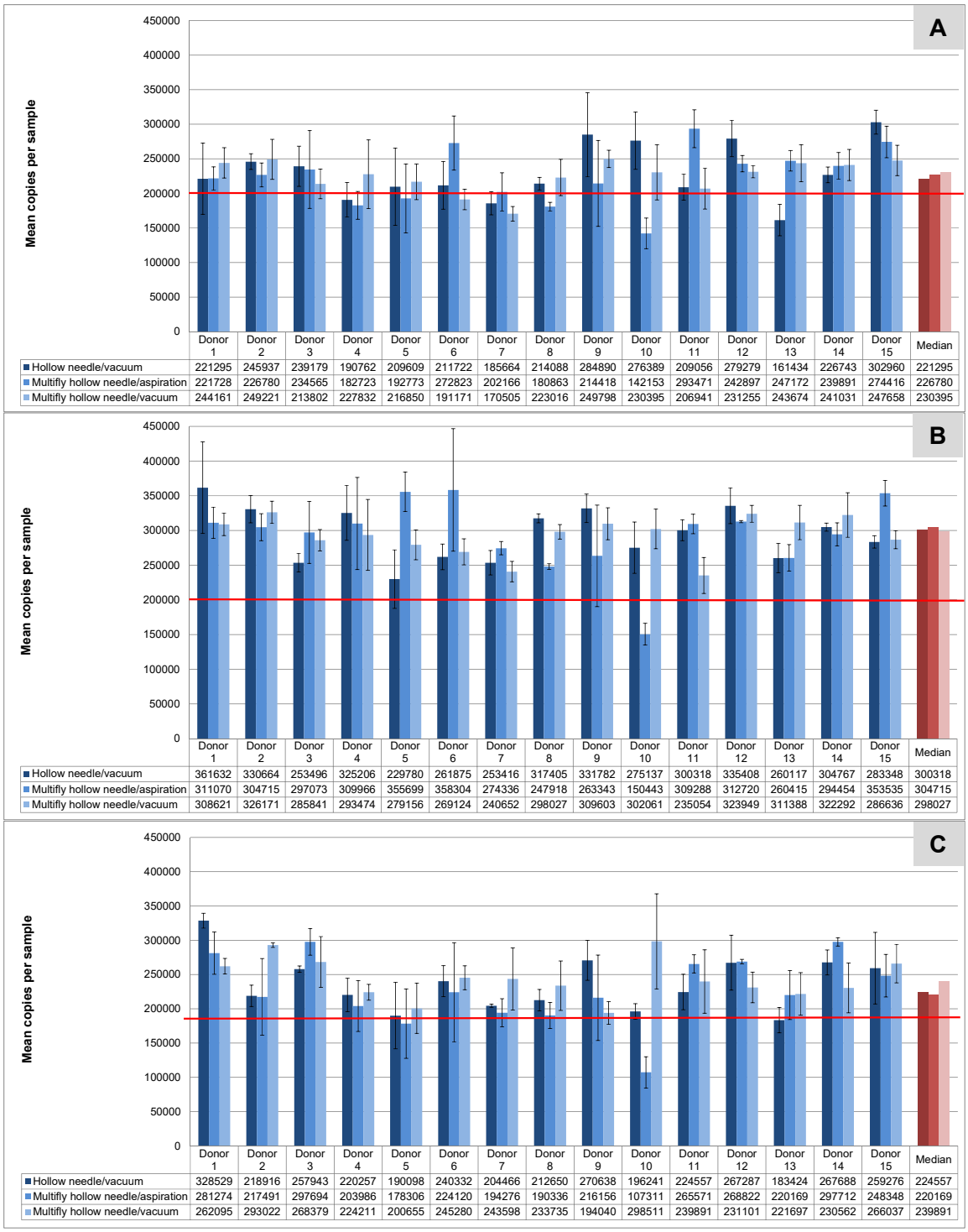
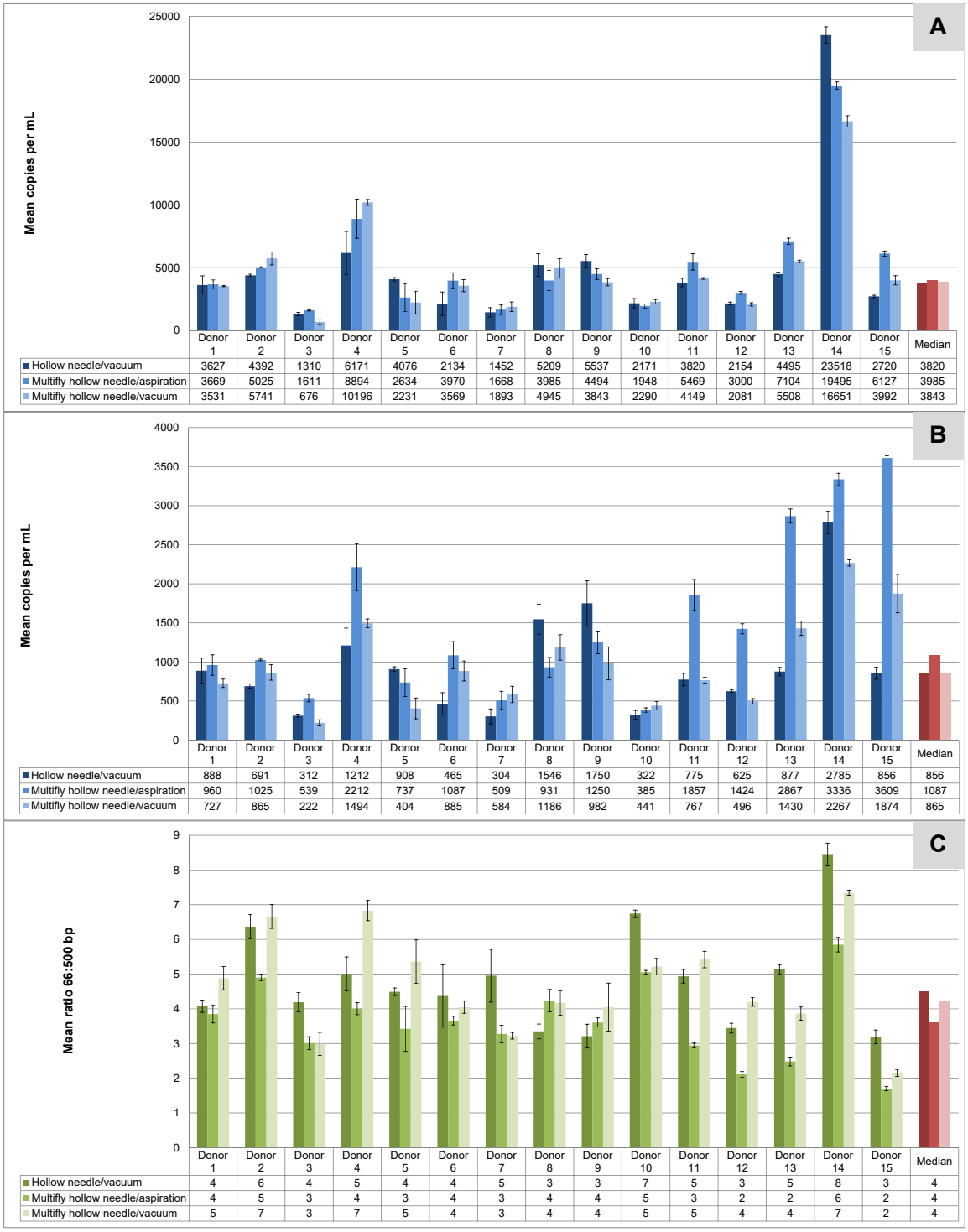


Figure 12 Comparison between different blood withdrawal procedures (GFP DNA)
The GFP DNA fragment assay was performed and mean copies per sample were calculated. Three different blood withdrawal procedures were compared for 15 individual healthy donors. Ideally, the added 200,000 copies per sample should be detected. The mean copies per sample are displayed for each condition in the form of blue bars and median values were additionally calculated (red bars). Results are shown for the 75 bp (**A**), 200 bp (**B**) and 1,000 bp (**C**) DNA fragments. Using one-way analysis of variance (= ANOVA), differences between tested conditions were evaluated as non-specific (JMP software).

The spiked DNA fragments were detected for all tested conditions. Median values between different blood withdrawal methods were very comparable between the different blood withdrawal systems apart from Donor 10, who showed a lower DNA recovery using the Sarstedt Monovette system. Fig. 13 shows the results for the 18S ribosomal DNA assay.



	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Donor 9	Donor 10	Donor 11	Donor 12	Donor 13	Donor 14	Donor 15	Median
Hollow needle/vacuum	3627	4392	1310	6171	4076	2134	1452	5209	5537	2171	3820	2154	4495	23518	2720	3820
Multifly hollow needle/aspiration	3669	5025	1611	8894	2634	3970	1668	3985	4494	1948	5469	3000	7104	19495	6127	3985
Multifly hollow needle/vacuum	3531	5741	676	10196	2231	3569	1893	4945	3843	2290	4149	2081	5508	16651	3992	3843

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Donor 9	Donor 10	Donor 11	Donor 12	Donor 13	Donor 14	Donor 15	Median
Hollow needle/vacuum	888	691	312	1212	908	465	304	1546	1750	322	775	625	877	2785	856	856
Multifly hollow needle/aspiration	960	1025	539	2212	737	1087	509	931	1250	385	1857	1424	2867	3336	3609	1087
Multifly hollow needle/vacuum	727	865	222	1494	404	885	584	1186	982	441	767	496	1430	2267	1874	865

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Donor 9	Donor 10	Donor 11	Donor 12	Donor 13	Donor 14	Donor 15	Median
Hollow needle/vacuum	4	6	4	5	4	4	5	3	3	7	5	3	5	8	3	4
Multifly hollow needle/aspiration	4	5	3	4	3	4	3	4	4	5	3	2	2	6	2	4
Multifly hollow needle/vacuum	5	7	3	7	5	4	3	4	4	5	5	4	4	7	2	4

Figure 13 Comparison between different blood withdrawal procedures (18S DNA)
The 18S ribosomal DNA assay was performed and mean copies per mL sample were calculated. Three different blood withdrawal procedures were compared for 15 individual healthy donors. Results for the short 66 bp (**A**) and long 500 bp (**B**) amplicon of the 18S ribosomal DNA assay are shown here. Mean copies per mL sample are marked in blue, while the red bars represent median values. Results of the 18S ribosomal DNA duplex assay were also used to calculate the ratio between the 66 and 500 bp amplicon (Diagram **C**). Individual mean ratio values are displayed (green bars) and median was built for each condition (red bars). The lower the mean ratio value is, the more long DNA fragments, detected by the 500 bp amplicon, are included in tested samples. Significance of differences between mean copies per mL plasma of tested conditions was calculated using Dunnett's multiple comparison method (JMP software). The condition "Multifly hollow needle/ vacuum" was defined as control group. While differences using the 18S 66 bp assay were not significant (one-way ANOVA), the condition "Multifly hollow needle/ aspiration" led to significant higher 500 bp DNA levels (p= 0.0037**). Consequently, ratio values for this condition were significant lower (p= 0.0003***).

According to the different plasma sample input volumes, mean copies were calculated per mL plasma. DNA recovery was significant higher using the Sarstedt Monovette system only for the long 500 bp amplicon. This difference was also noticeable in the ratio between the 66 and 500 bp amplicon where ratio values were significant lower for the Sarstedt Monovette system.

3.1.2. Blood processing

Blood samples should be ideally processed within two hours to prevent dilution of ccfDNA by genomic DNA contamination from breaking blood cells. In addition, residual cells and cell debris as well as a big part of genomic DNA can be pelletized by high-speed centrifugation [213] of the generated plasma samples for 10 min. at 16,000 x g and 4°C. The influence of the mentioned high-speed centrifugation step was analyzed by comparing the mean copies per sample (18S ribosomal DNA assay) for eight individual healthy donors, for which blood was processed either using or not using the high-speed centrifugation step (Fig. 14). Indeed, the total DNA amount, especially for the long fragment, was higher when samples were not centrifuged at 16,000 x g.

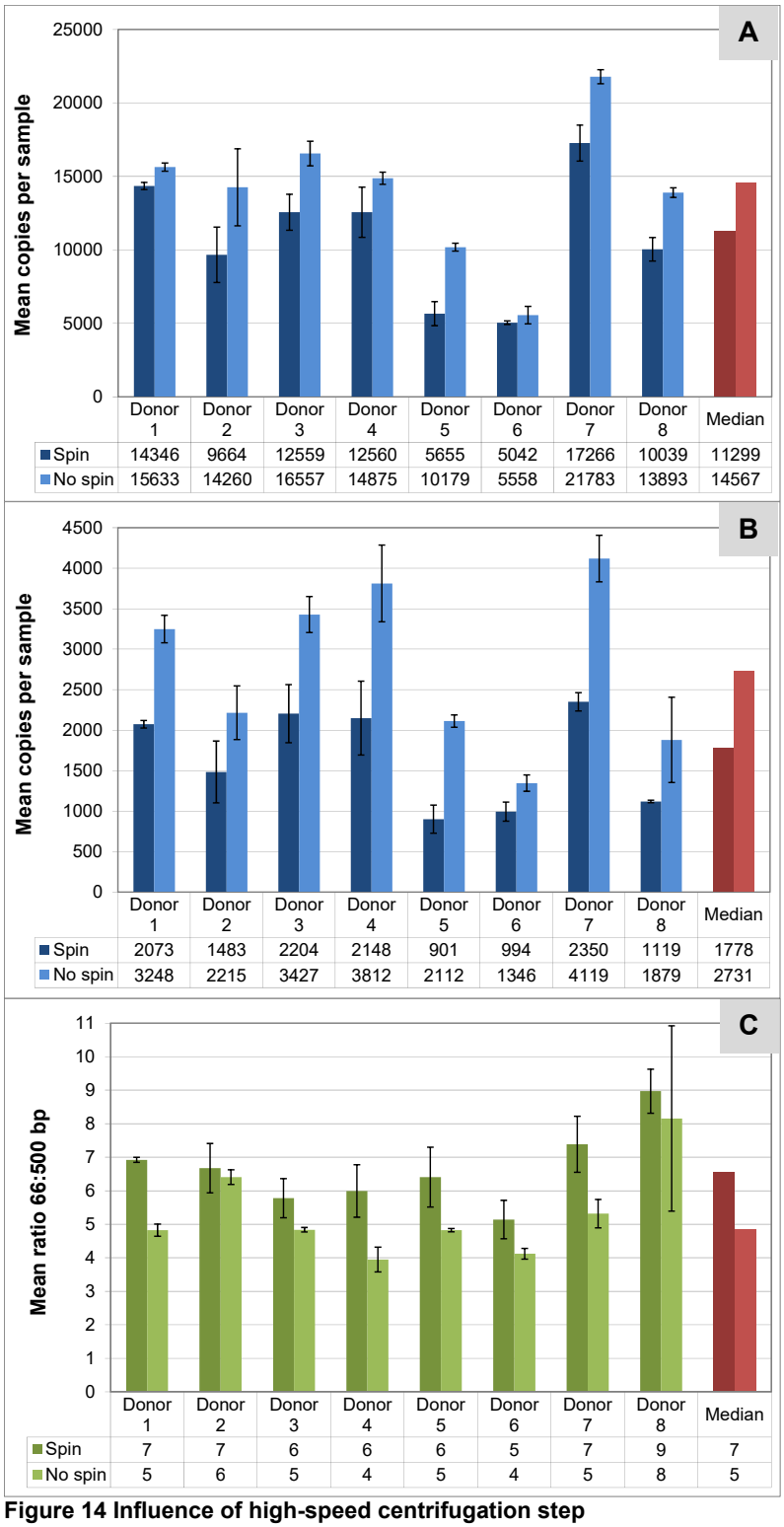


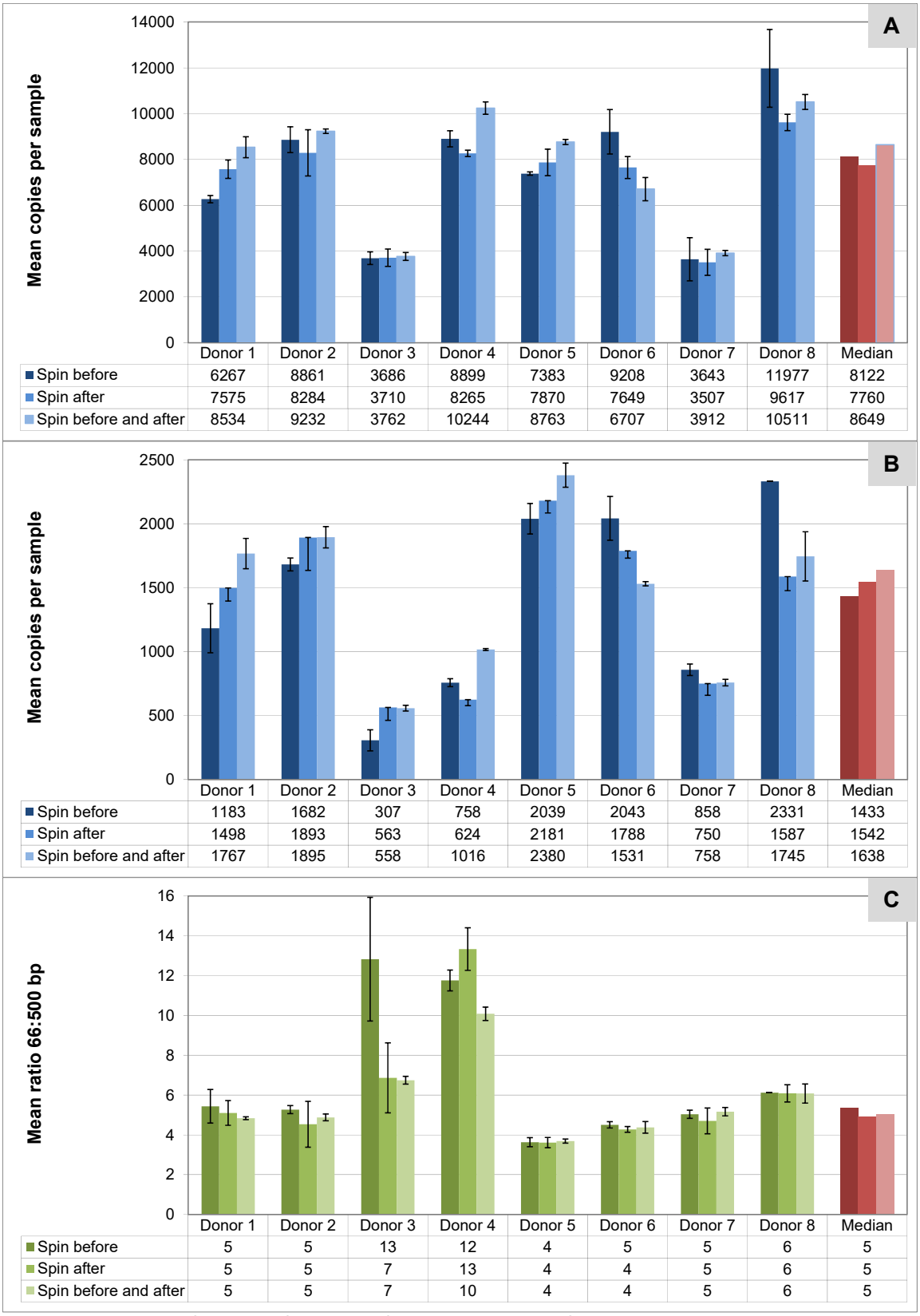
Figure 14 Influence of high-speed centrifugation step

The 18S ribosomal DNA assay was used to determine the mean copies per sample for the short 66 bp (**A**) and 500 bp (**B**) amplicon. The influence of a high-speed centrifugation step (=Spin) was analyzed for eight healthy individuals. The ratio between 66 and 500 bp amplicons of the 18S ribosomal DNA assay was built to calculate the fraction of longer (≥500 bp) DNA fragments (see Diagram **C**). Paired two-tailed t-test (Excel) was used to

calculate significance levels, which were p= 4.63563*10^-7*** (66 bp), p= 3.06712*10^-8*** (500 bp) and p= 4.54071*10^-5*** (ratio).

Again, mean copies per sample are presented for the 18S ribosomal DNA assay. As expected, total DNA amount varied between healthy individuals and, for the short 66 bp amplicon, it was always higher without the performance of a high-speed centrifugation step. Looking at the 500 bp amplicon, this difference was even bigger, which was supported by 66:500 bp ratio values. They were, except for Donor 2, higher for the tested condition with high-speed centrifugation step, so fewer long DNA fragments (≥500 bp) seemed to be present in the eluate.

In most cases, blood samples of healthy employees from our in-house blood donations were used and plasma was preferably generated within two hours after blood donation, reducing the genomic DNA background as much as possible. But, as a precaution and to have comparable conditions if blood samples from other studies are used, it was decided to generally include the high-speed centrifugation step in the plasma preparation process. As a next step, it was tested if this centrifugation should be performed before, after or both before and after freezing at -80°C for storage. This was analyzed for both the QIAamp cNA Kit and the QIAsymphony cDNA Protocol, but according to the intended analysis of a possible general preanalytical influence, only results for the manual DNA extraction method as the gold standard are shown (Fig. 15).



	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Median
Spin before	6267	8861	3686	8899	7383	9208	3643	11977	8122
Spin after	7575	8284	3710	8265	7870	7649	3507	9617	7760
Spin before and after	8534	9232	3762	10244	8763	6707	3912	10511	8649

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Median
Spin before	1183	1682	307	758	2039	2043	858	2331	1433
Spin after	1498	1893	563	624	2181	1788	750	1587	1542
Spin before and after	1767	1895	558	1016	2380	1531	758	1745	1638

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Median
Spin before	5	5	13	12	4	5	5	6	5
Spin after	5	5	7	13	4	4	5	6	5
Spin before and after	5	5	7	10	4	4	5	6	5

Figure 15 Time-point for the performance of a high-speed centrifugation step

Mean copies per sample were determined using the 18S ribosomal DNA assay. The high-speed centrifugation step was performed before, after or before and after freezing of plasma samples, which were processed using the QIAamp cNA Kit. Results are shown for eight healthy individuals. Diagram **A** shows the results for the short 66 bp amplicon and results of the 500 bp amplicon are displayed in Diagram **B**. In addition, the ratio between 66 and 500 bp amplicons was calculated to determine the fraction of short (<500 bp) and long (≥500 bp) DNA fragments (see Diagram **C**). Using one-way ANOVA, differences between tested conditions were evaluated as non-specific (JMP software).

Mean copies per sample varied between conditions and healthy individuals, but on average there seemed to be no big difference between tested conditions within the sample. Looking at the 66:500 bp ratios, similar results were achieved. Furthermore, DNA recovery using the QIAsymphony cDNA Protocol compared to the QIAamp cNA Kit seemed to be unaffected by the tested plasma treatment (data not shown). However, tested plasma was prepared within two hours and without disturbing the buffy coat layer. The genomic DNA or blood cell background was consequently low.

All plasma samples used during this PhD thesis were stored at -80°C before processing, so the general influence of freezing on performance of the newly developed automated DNA extraction protocol was additionally tested using frozen and fresh plasma samples as sample input material. As a result, the freezing of plasma samples even seemed to improve DNA recovery using the QIAsymphony cDNA Protocol compared to the QIAamp cNA Kit (data not shown).

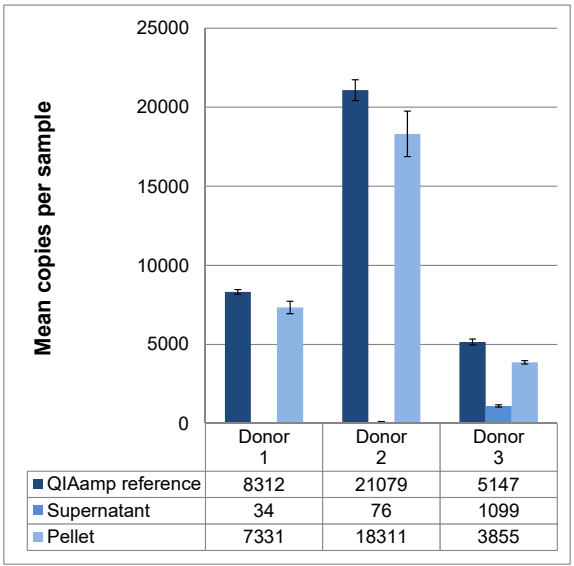
3.1.3. Plasma analysis

During the development of the new automated DNA extraction protocol on the QIAsymphony SP instrument, substantial differences concerning DNA recovery between plasma samples of individual donors were noticed. Some plasma samples seemed to be more critical for automated DNA extraction than others. One possible explanation might be the different nature of ccfDNA. Circulating cell-free DNA is supposed to be mainly present in the form of mononucleosomes [206], but an association to other proteins, an inclusion in so-called exosomes [24] or an occurrence in a “naked” form is also possible. In the following experiments, it was tried to analyze plasma by exosome isolation. This way, two different fractions of ccfNA would be differentiated:

1. Nucleic acids enclosed by so-called exosomes
2. “Free” nucleic acids

The following experiments thereby focused on DNA. First, ExoQuick exosome precipitation solution (see chapter 2.9.3.1.) was used to separate exosomes by pelletizing. The pellet,

which is supposed to contain the desired exosomes, was resuspended in 2 mL RNase-free H_2O and the suspension as well as the supernatant after pelletizing, possibly including "free" or not by exosomes enclosed nucleic acids, were processed using the QIAamp cNA Kit. However, the signal of the resuspended pellet was almost equal to total DNA recovery from 2 mL plasma using the QIAamp cNA Kit, indicating that exosomes were not selectively isolated (Fig. 16).



	Donor 1	Donor 2	Donor 3
QIAamp reference	8312	21079	5147
Supernatant	34	76	1099
Pellet	7331	18311	3855

Figure 16 Exosome precipitation
Mean copies per sample were calculated for three healthy individuals, detecting the 66 bp amplicon of the 18S ribosomal DNA assay. The QIAamp cNA Kit was used as a reference to determine the total amount of ccfDNA. Exosomes were pelletized using ExoQuick exosome precipitation solution. The supernatant was supposed to include ccfDNA. The supernatant and the pellet, resuspended in buffer PBS, were processed using the QIAamp cNA Kit to isolate ccfDNA.

Total DNA amount seemed to be pelletized instead.

Consequently, another method for selective exosome isolation was tested. This time it was based on exosome binding using specific spin columns and reagents from the exoRNeasy Serum/Plasma Maxi Kit, but the protocol was optimized for RNA extraction and final eluate was not applicable for DNA analysis. Therefore, the flow-through after exosome binding as well as the wash flow-through were kept and used as input material for the QIAamp cNA Kit, which also served as reference to determine the total DNA amount of the tested plasma sample. The flow-throughs after the binding and wash steps were analyzed to look if only exosomes and not the ccfDNA were bound to spin column membranes. Fresh and frozen plasma samples were compared, because exosomes could be broken by freezing. Furthermore, fresh plasma was tested with and without the 16,000 x g centrifugation step

because of a possible exosome precipitation by high-speed centrifugation. Plasma from two further donors was processed to include individual effects. In addition, binding buffer (XBP) and wash buffer (XWP) were spiked with extracted human control DNA and compared with buffer PBS to analyze any possible inhibitory effects of the buffer on the QIAamp cNA Kit. The flow-through after binding (buffer XBP) and wash step (buffer XWP) as well as the buffers themselves were processed with the QIAamp cNA Kit to extract present ccfDNA. Ideally, the difference between mean copies per sample for fresh plasma without the 16,000 x g step including or not including a DNA spike-in should correspond with results of the buffer systems, which only contain the spike-in DNA. Although about 50% of spiked DNA was recovered from buffer XBP as well as XWP, no DNA was detected in flow-through after the exosome binding or wash step (data not shown). As a result, total DNA instead of only exosomes was bound to spin column membranes.

It was also tested if nature of circulating DNA, which is present in plasma samples and other naturally occurring components like proteins, has an influence on DNA recovery using the QIAsymphony cDNA Protocol compared to the QIAamp cNA Kit. Male human plasma DNA was therefore compared to DNA, extracted from the same male human plasma volume using the QIAamp cNA Kit and spiked into PBS (the whole eluate). In cases with lower DNA recovery from human plasma, spiked PBS samples led to better automated protocol performance (Fig. 17).

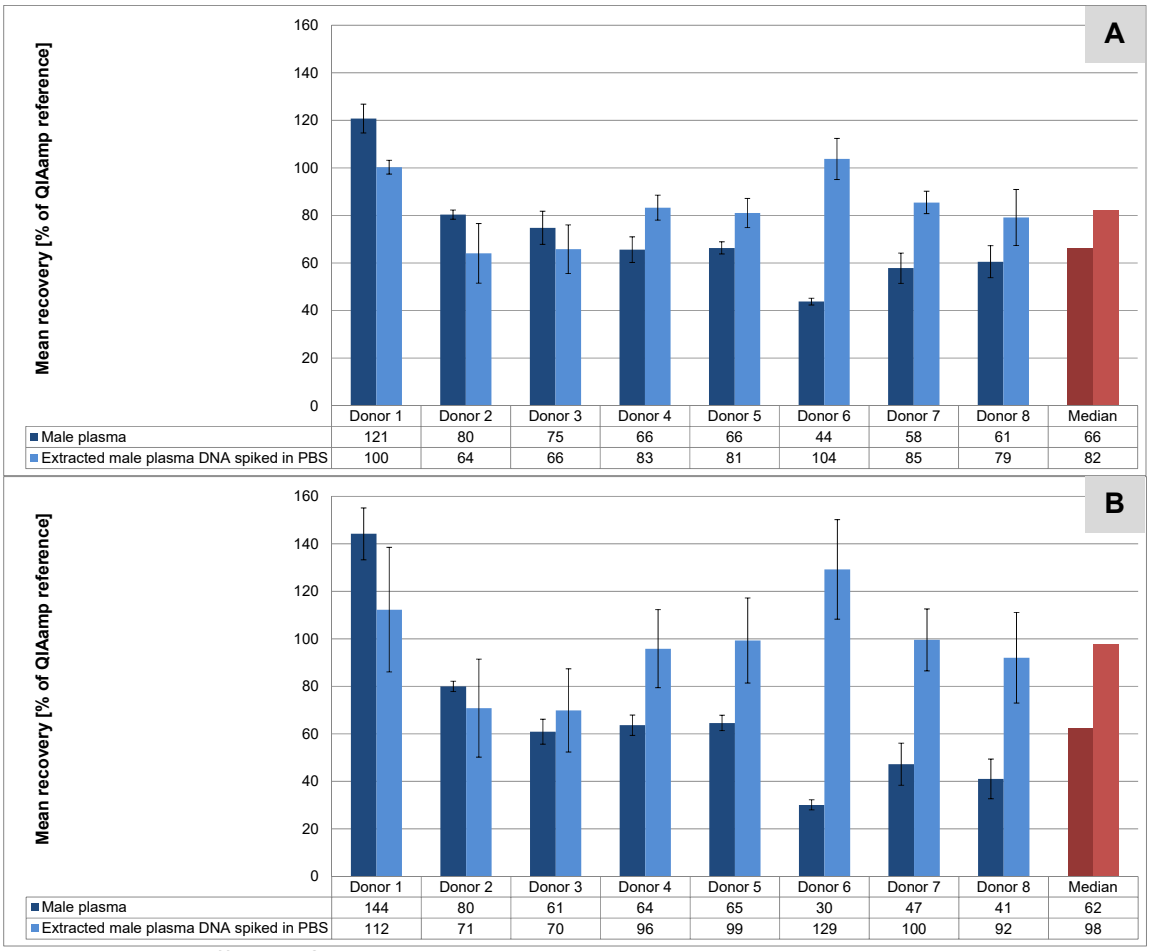
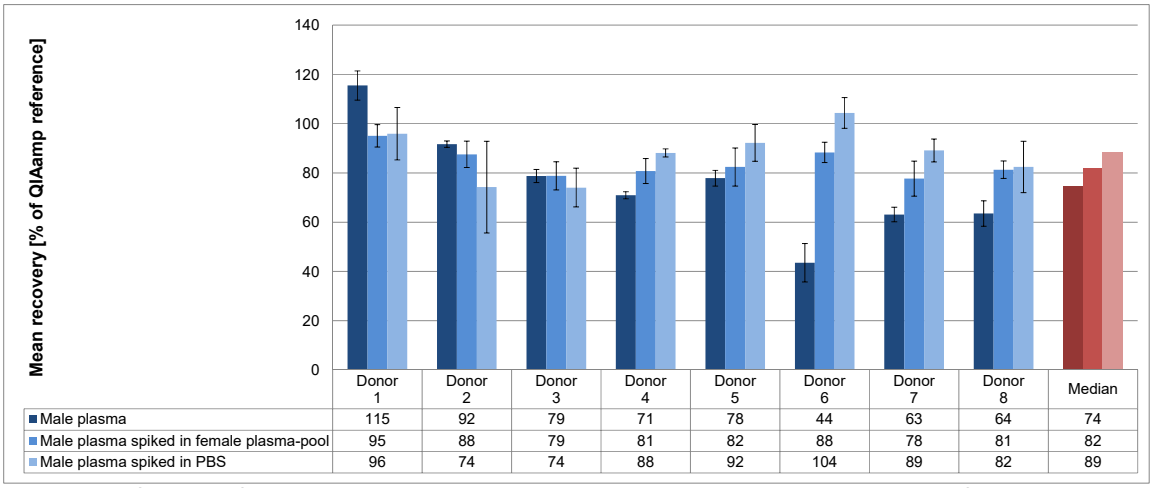


Figure 17 Plasma effect on QIAsymphony cDNA Protocol
The mean DNA recovery for the QIAsymphony cDNA Protocol compared to the QIAamp cNA Kit is shown for eight healthy individuals. The 18S ribosomal DNA assay was used for DNA detection (**A** = results for the 66 bp amplicon, **B** = results of the 500 bp amplicon). Male plasma was processed using the QIAamp cNA Kit. In addition, DNA was extracted from male plasma and spiked into buffer PBS before DNA isolation by QIAamp cNA Kit was performed.

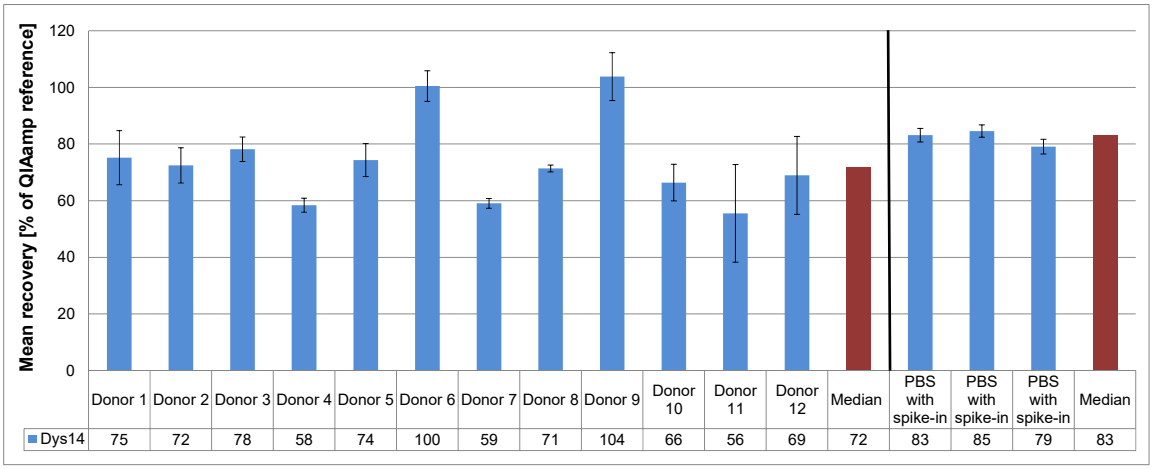
To be able to differentiate between the effect of DNA nature and naturally occurring components like proteins, extracted male human DNA was additionally spiked into female plasma. Again, in cases with lower DNA recovery from male human plasma, results were better for spiked samples. Accordingly, spiked PBS samples tended to lead to higher DNA recoveries than spiked female pooled plasma samples (Fig. 18).



	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Median
Male plasma	115	92	79	71	78	44	63	64	74
Male plasma spiked in female plasma-pool	95	88	79	81	82	88	78	81	82
Male plasma spiked in PBS	96	74	74	88	92	104	89	82	89

Figure 18 Influence of plasma components and DNA nature on automated protocol performance
The mean DNA recovery for the QIAsymphony cDNA Protocol compared to the QIAamp cNA Kit is shown for eight healthy individuals. The Dys14 DNA assay was used for the detection of male human DNA. Male plasma was processed using the QIAamp cNA Kit. In addition, DNA was extracted from male plasma and spiked into a female plasma-pool and buffer PBS before DNA isolation by QIAamp cNA Kit was performed.

To test if the plasma effect on DNA recovery varies between samples of different individual donors, extracted male human DNA of one plasma pool was spiked into plasma samples of twelve individual female donors as well as into PBS. Female DNA of the twelve healthy individuals used was extracted to at least 100% compared to the QIAamp cNA Kit (data not shown), so there was no critical plasma sample. Recovery of spiked male DNA was about 80% in PBS and varied between the twelve healthy individuals (Fig. 19).



	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Donor 9	Donor 10	Donor 11	Donor 12	Median	PBS with spike-in	PBS with spike-in	PBS with spike-in	Median
Dys14	75	72	78	58	74	100	59	71	104	66	56	69	72	83	85	79	83

Figure 19 Influence of individual plasma components on DNA extraction
The mean DNA recovery for the QIAsymphony cDNA Protocol compared to the QIAamp cNA Kit is shown for eight healthy individuals. The Dys14 DNA assay was used for the detection of male human DNA. This time extracted DNA from male human pooled plasma was spiked into samples of individual female donors as well as PBS and was processed using the QIAamp cNA Kit afterwards.

Although the twelve individual plasma samples did not seem to be critical, DNA recovery varied and the median value was about 72%, so plasma components as well as DNA

nature might influence the DNA recovery of the QIAsymphony cDNA Protocol. However, by spiking extracted DNA into PBS, DNA recovery of the QIAsymphony cDNA Protocol also did not reach 100% compared to the QIAamp cNA Kit. As a result, plasma components as well as the availability of “free” DNA fragments are not the only reasons for the occurring DNA loss using the newly developed automated protocol.

3.1.4. Urine as alternative sample material

Besides blood samples, other body fluids are often used as sample material for biomarker analysis depending on the present disease. For urological and gynecological cancer examinations, for example, urine can be used as alternative and non-invasive sample material [251]. For initially planned translational experiments, which were unfortunately discontinued, DNA should be extracted from urine samples of prostate cancer patients. Therefore, sample stability was tested at the beginning to find optimal conditions for sample collection and ensure high quality sample material. The requirements were comparable to those of plasma samples. Degradation of ccfDNA as well as the release of genomic DNA from cells was to be prevented, so urine stability was analyzed under the following four storage conditions at:

- Room temperature
- 4°C
- 4°C after the addition of 40 mM K_2EDTA
- 4°C after ATL addition (according to the urine protocol of the QIAamp cNA Kit)

Time-point 0 (T_0) was the reference value. It was determined by immediately extracting DNA from a fresh urine sample using the urine protocol of the QIAamp cNA Kit according to the handbook. DNA was then extracted after one, two, three and four hours of storage (T_1-T_4). The results for T_X:T_0 as well as the 66:500 bp ratio are shown in those experiments. The 18S ribosomal DNA assay was used for the calculation (Fig. 20).

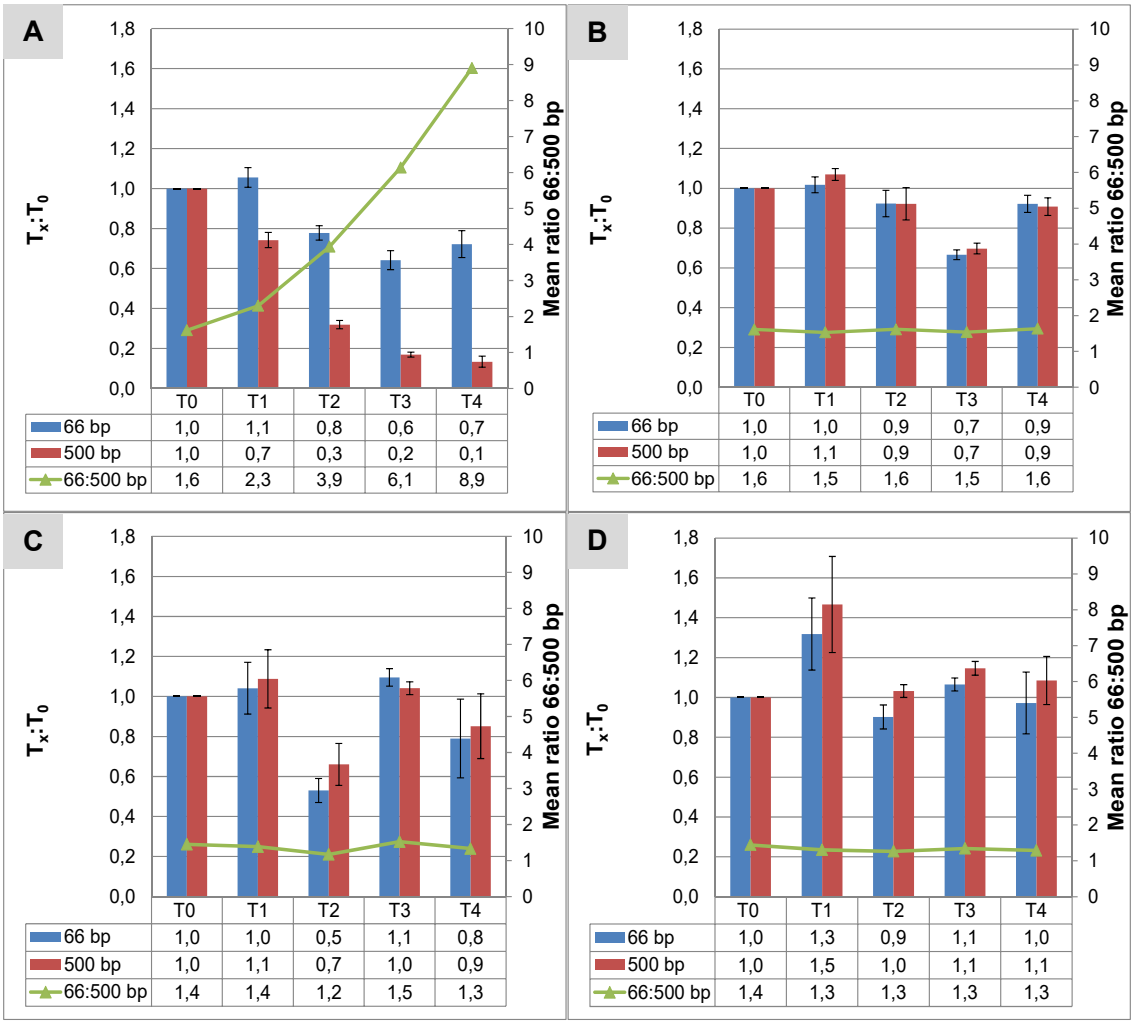


Figure 20 Test of the DNA stability in urine

Mean copies per sample were determined using the 18S ribosomal DNA assay. Pooled urine was therefore processed using the urine protocol of the QIAamp cNA Kit at five different time-points. T_0 was achieved by processing the urine pool immediately after donation. T_1 to T_4 were processed after one to four hours. Each time-point (T_1 to T_4) was divided with T_0. If the ratio is =1, the DNA amount remains unchanged. If values >1 occur, DNA concentration increases and DNA is degraded for values <1. Blue bars show the results of the short 66 bp amplicon and the 500 bp amplicon is displayed in red. In addition, the ratio between the 66 and 500 bp amplicon was calculated to analyze a potential genomic DNA release (green line). Four different storage conditions were compared: room temperature (**A**), 4°C (**B**), 4°C after the addition of 40 mM K_2EDTA (**C**) and 4°C combined with buffer ATL (**D**). For the ATL condition, no further ATL was added for the QIAamp cNA Kit preparation.

DNA amount decreased during storage at room temperature and the strong increase of the 66:500 bp ratios indicated a worse availability of longer DNA fragments. At 4°C, results were already comparable between different time-points. Apart from T_3, DNA amount remained almost unchanged and the 66:500 bp ratios was nearly equal for all tested time-points. Because the further two conditions using K_2EDTA and ATL were also performed at 4°C, results for the 66:500 bp ratio were very similar. For both conditions one time-point

showed some deviation, but the other values were very comparable and stayed nearly unaffected.

The experiment was therefore repeated using K_2EDTA at room temperature and adding two further time-points after five and 24 hours (overnight). Figure 21 shows the results for the 18S ribosomal DNA assay.

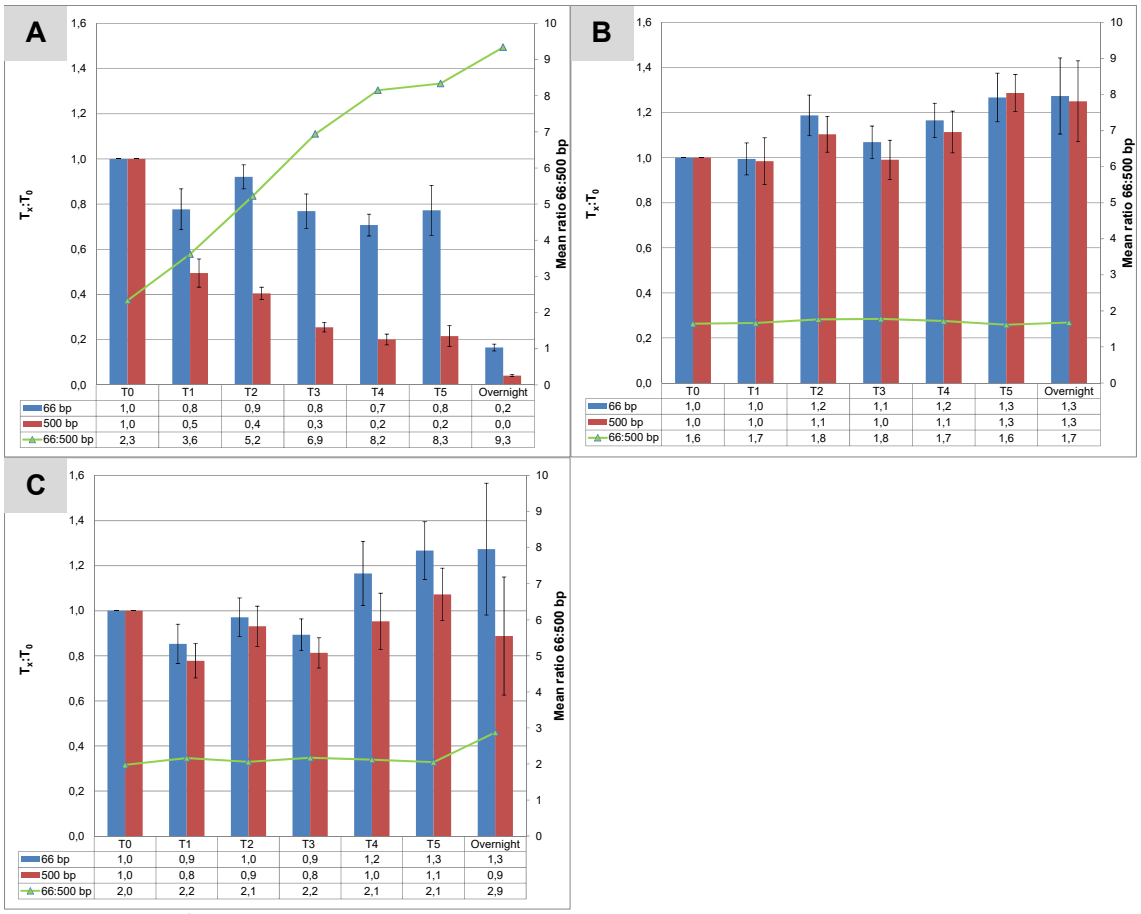


Figure 21 Test of the DNA stability in urine (repetition)
Mean copies per sample were determined using the 18S ribosomal DNA assay. Pooled urine was processed, but two additional time-points were added for storage (5 hours and approx. 24 hours). Tx:T0 as well as the ratio between the 66 and 500 bp amplicon are shown. The results for urine storage at room temperature (**A**), at 4°C (**B**) and at room temperature after the addition of 40 mM K_2EDTA (**C**) can be seen here.

At room temperature, the same results can be seen as before. This time 40 mM K_2EDTA was added for storage at room temperature to be able to recognize a possible stabilizing effect of the reagent and, indeed, results were comparable to storage at 4°C (Fig. 21).

But looking at the results of the GFP DNA fragment spike-in system (Fig. 22), 40 mM K_2EDTA tended to lead to a better stability of circulating cell-free (and in this case “naked”) DNA than storage at 4°C.

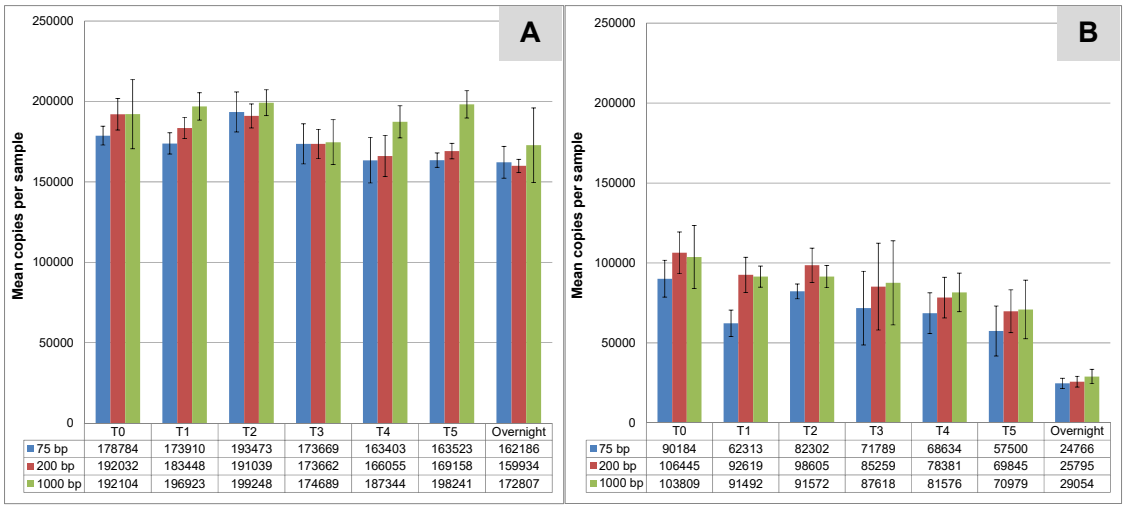


Figure 22 Test of DNA stability in urine (40 mM K_2EDTA at RT versus 4°C)
200,000 copies of the GFP DNA fragments (75, 200 and 1,000 bp) were spiked into plasma samples and detected using quantitative real-time PCR. Mean copies per sample of each fragment are shown for the different time-points. 40 mM K_2EDTA was added for storage at room temperature (see Diagram **A**) and compared against storage at 4°C (see Diagram **B**).

Since the occurrence of “naked” DNA fragments comparable to the GFP spike-in system cannot be totally excluded and storage at 4°C cannot always be guaranteed, 40 mM K_2EDTA should be used to prevent DNA degradation as well as genomic DNA release from breaking cells.

Optimal preanalytical conditions for the extraction and later analysis of ccfNA were defined, including blood sample collection as well as blood processing procedures. Furthermore, plasma was analyzed to determine the influence of plasma components and DNA nature on recovery after DNA extraction, but DNA loss also occurred for the QIAsymphony cDNA Protocol compared to the QIAamp cNA Kit reference when extracted DNA was spiked into PBS. Consequently, further reasons for DNA loss have to exist. Keeping the tested optimal preanalytical conditions in mind, a new DNA extraction method for ccfDNA was developed and automated on the QIAsymphony SP instrument.

3.2. Automated DNA extraction

Different manual DNA extraction methods, optimized for ccfNA from plasma and other body fluids, like the commercially available and widely used QIAamp cNA Kit already exist. A new DNA extraction method has now been developed, which is applicable for automation on an existing instrument, the QIAsymphony SP. Preferably high plasma volumes were used as sample input material for the automated DNA extraction method to improve the detection sensitivity and probability of catching the target molecules of

interest. The availability of total and especially ccfDNA was increased that way and, simultaneously keeping the elution volume as small as possible, a strong enrichment of target molecules was achieved.

3.2.1. Interim solution

Based on demand from customers to increase both sample throughput and sample tracking security, for example, an existing protocol for the QIAsymphony SP instrument (the “Virus Cellfree 1000” protocol using the QIAsymphony DSP Virus/Pathogen Midi Kit) was therefore modified and tested as an interim solution to provide customers with a DNA extraction method until the newly developed protocol would be available. The customized protocol was neither tested in detail, nor validated or verified as it is planned for the newly developed protocol during product development. Furthermore, the newly developed protocol will have a higher sample throughput by processing 96 instead of 24 samples per run using one Kit and with a lower run-time. Up to 1 mL plasma can usually be processed with the “Virus Cellfree 1000” protocol. Consequently, the binding step was performed up to four times, allowing for the processing of 4 mL plasma, but these four binding steps were performed successively using one bead aliquot, which was transferred from one binding reaction to the next (Fig. 23).

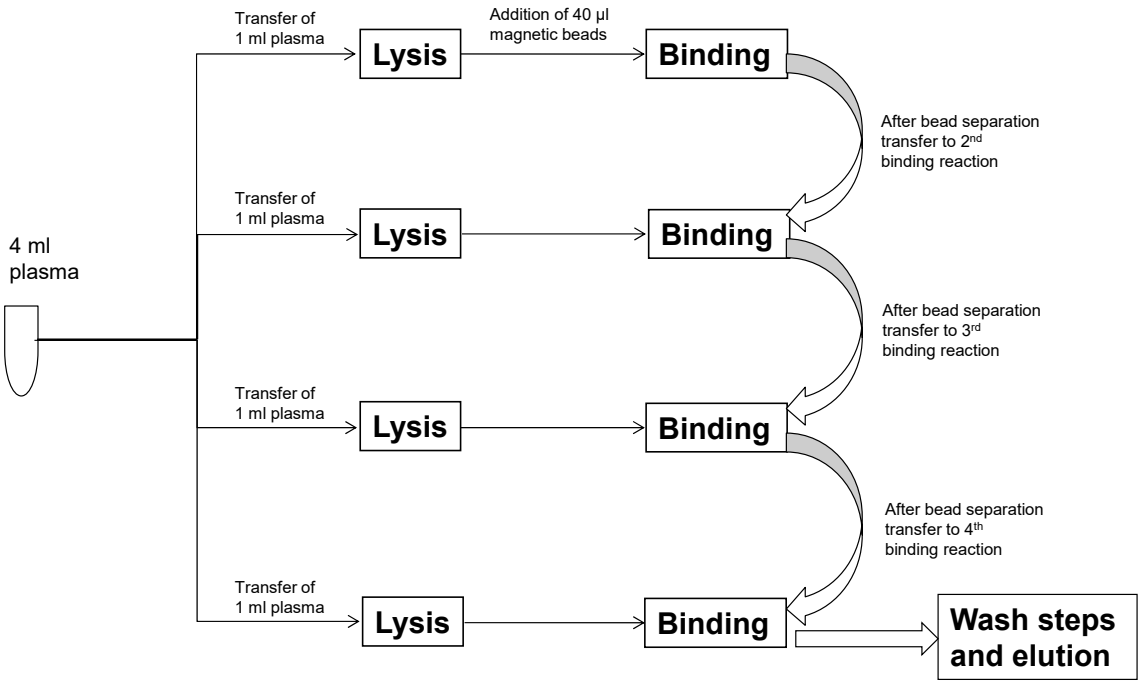
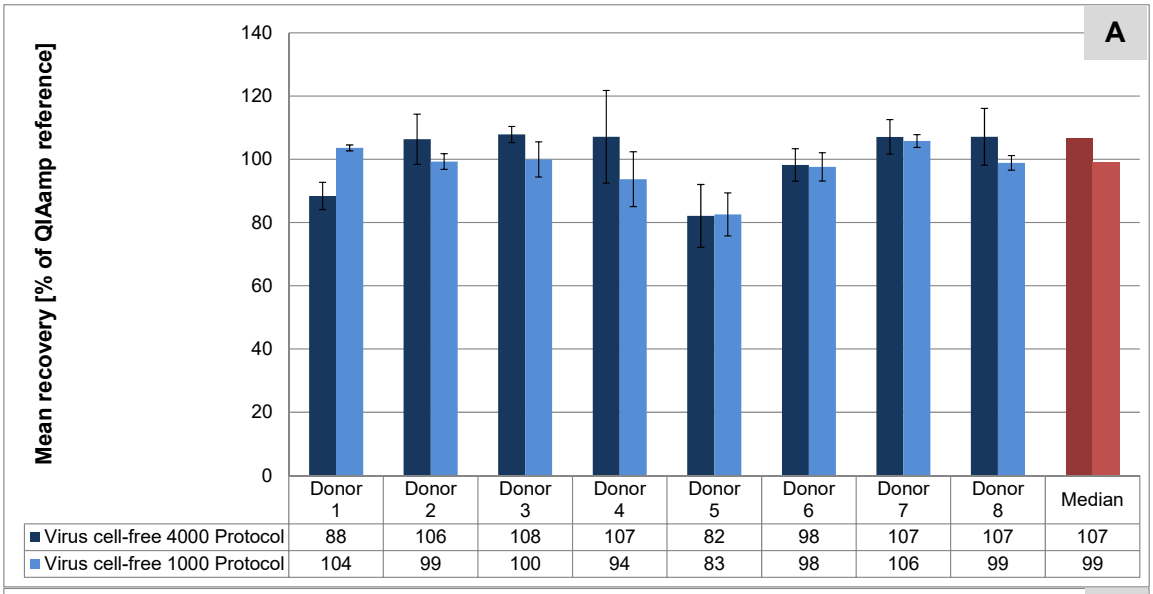


Figure 23 Principle of the QIAsymphony Custom Protocol
The principle of the QIAsymphony Custom Protocol is exemplified using the version for the processing of 4 mL plasma. The total sample volume was divided into 4*1 mL. Each mL was pipetted into the reaction mix for lysis. After the lysis step, magnetic beads were added to the first mix for DNA binding, then beads were separated and transferred into the second binding reaction. This step was repeated twice and, after the performance of the last binding step, wash steps and elution followed. One bead aliquot was used for all binding batches.

Lysis time was shortened and other changes were implemented in cooperation with QIAGEN GmbH's application laboratory in Hilden. The aim was to achieve approx. 100% DNA recovery compared to the QIAamp cNA Kit and to get the same DNA amount per mL plasma using the large volume (here 4 mL) version of the “Virus Cellfree 1000” protocol or the 1 mL standard protocol. Results for the 18S ribosomal DNA assay are shown in Figure 24. At least 80% DNA recovery was achieved for the eight individual healthy donors.



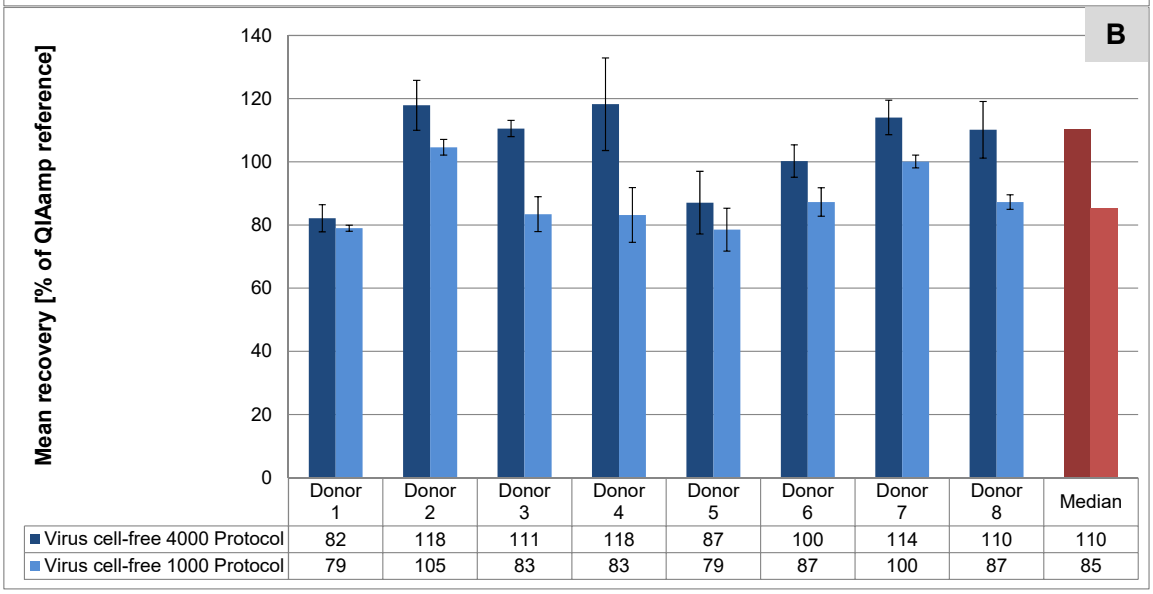


Figure 24 QIAsymphony Custom Protocol
The mean DNA recovery of the QIAsymphony Custom Protocol is shown here. The QIAamp cNA Kit was used as reference (=100%). Two protocol versions (1 and 4 mL) were tested with plasma samples from eight healthy individuals. Diagram **A** displays the results for the short 66 bp amplicon and results for the long 500 bp amplicon are presented in Diagram **B**. Difference between the two tested protocols was significant (p= 7.2643*10^-6) using the 18S 500 bp assay (Excel t-test).

The customized protocol was further improved with respect to DNA recovery and now is available for interested QIAsymphony users.

3.2.2. Newly developed protocol

A new protocol for the QIAsymphony SP instrument was developed to be able to process more samples in a shorter time. By reducing the reagent volume, up to 2.9 mL plasma could be processed in one instrument run. In-house produced magnetic anion exchange

beads were used and a new DNA extraction chemistry as well as a proper protocol were developed.

The protocol had to be changed further to enable the processing of higher plasma volumes (>2.9 mL). By splitting the plasma volume in two equal aliquots, up to 5.8 mL plasma (2*2.9 mL) could be used for DNA extraction. These high plasma volumes were handled in two alternating reaction batches, so the first batch was pipetted and binding by “Mag-Mix” began. As soon as the second batch was ready for mixing, both binding batches were mixed in turn, whereas change took place every few minutes. After the first batch was mixed completely, the beads were separated and transferred into the second batch for the rest of binding time. The beads were pooled during binding step and the following steps were performed as mentioned above for the 2.9 mL protocol version using one bead aliquot. Binding performance is described in Figure 25.

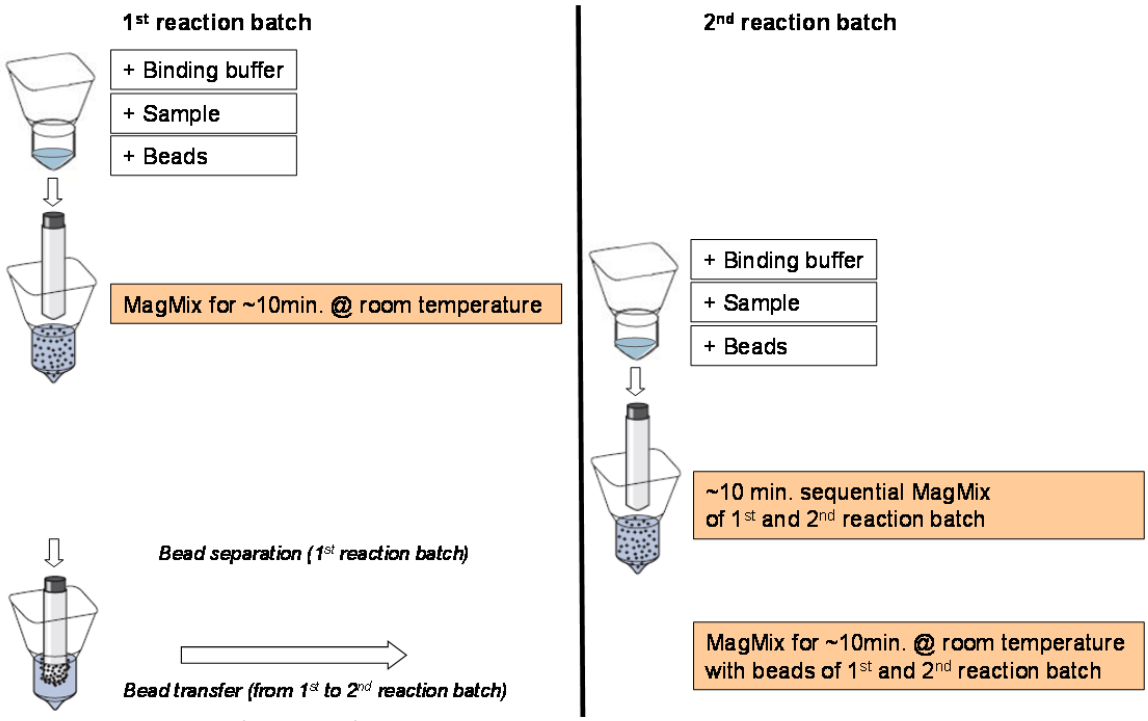


Figure 25 Binding performance for the automated large volume processing
Up to 2.9 mL plasma could be processed in one batch depending on the reagent volume. If higher sample volumes were needed, a second batch for sequential binding had to be included.

Some protocol versions contained an additional Proteinase K step (later called the RDD step). DNA, which was bound to beads, was eluted in a mixture of buffer RDD and Proteinase K after the binding step and rebound using 5 mM citric acid. The already mentioned wash steps were then followed.

3.2.2.1. Protocol development

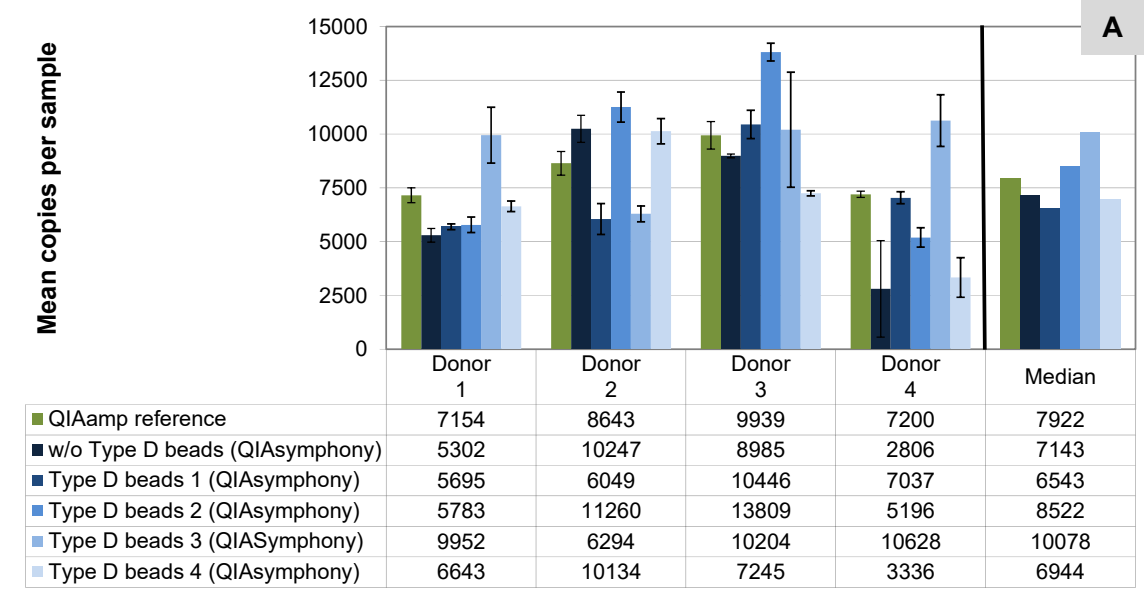
First, the protocol itself was developed and all development steps were performed manually. According to the fact that the reagent volume had to be reduced, an anion exchange instead of the usually used silica chaotrope based chemistry was required. The main challenges of the first early protocol version, which resulted from my diploma thesis [239c], were the improvement of DNA recovery compared to the QIAamp cNA Kit and to remove PCR-inhibitory contamination from eluates to make them compatible for different downstream applications. As a result, different buffers for DNA binding, wash and elution steps were tested, buffer volumes were changed, times and the way of mixing were varied and various magnetic beads were compared. In the following part, the results of the most important experiments during protocol development are shown. Compared to the QIAamp cNA Kit, there was no real lysis step. Neither chaotrope buffers nor Proteinase K, detergents or heat treatment were used at the beginning, so DNA was extracted from comparable native plasma samples. DNA extraction was based on in-house developed magnetic beads with an anion exchange surface. These beads allowed for the best and most reproducible DNA recovery compared to other concentration technologies that were tested during my diploma thesis [239d]. The advantage of their magnetic nature was that they could be automated on proper robots, so low pH values were adjusted to bind DNA from plasma. Wash steps had to be modified to be able to remove PCR-inhibitory contaminants like proteins without simultaneously losing DNA. For some applications, it might be interesting to remove longer DNA fragments to concentrate the target molecules of choice within a specific size fraction. The elution step was very challenging. The elution buffer had to be PCR compatible and stringent enough to elute DNA completely. These and other facts were always considered during protocol development, so the following steps were tested.

DNA binding

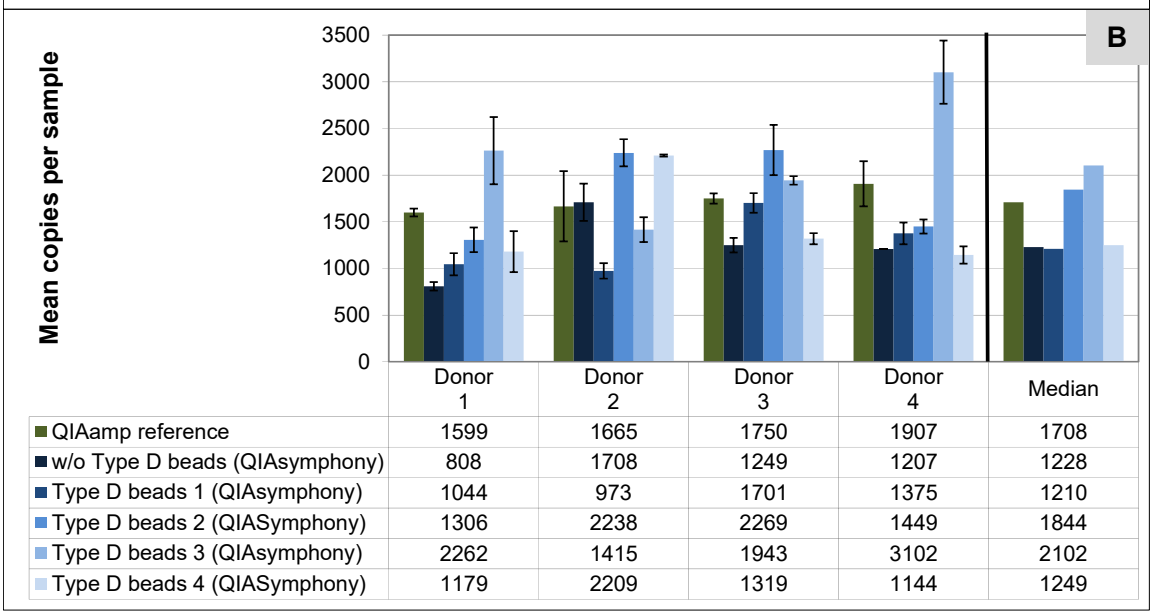
One possible reason for the low DNA recovery could be an insufficient DNA binding to beads. Plasma proteins and other components might hinder DNA binding to beads through saturation of bead surface or covering DNA target molecules. It was tried to improve the binding step performing a kind of lysis to loosen the DNA binding to proteins by the replacement of binding sites or protein denaturation prior to the binding reaction. As the initial binding buffer, 75 µL 1 M sodium acetate/acetic acid buffer (pH 4) was used for 3 mL plasma. 1% SDS, 10 mM sodium chloride or 1500 µL AXpH buffer for lysis were added,

but this way almost no DNA was recovered at the end (data not shown). SDS concentration was then lowered to 0.1% or 300 µL Proteinase K was used, but again no improvement concerning DNA recovery was achieved (data not shown). Finally, GTC (100, 200, 300, 400 and 500 mM) was added for a possible protein denaturation or lysis, but it also led to no further improvement in DNA recovery (data not shown).

Although DNA might be bound to proteins under the available conditions, it was tried to remove them prior to DNA extraction by using two different methods. First, type D beads were tested to remove proteins and possible other plasma components inhibiting binding or later analysis methods like quantitative real-time PCR, thus improving DNA recovery. Type D beads have a negative charged surface to bind positive charged proteins and other plasma components without losing DNA. Four different versions were tested (varying in concentration and surface chemistry). Results differ between the tested four healthy individual donors and, looking at the median values, type D beads 2 and 3 were able to improve DNA recovery compared to the standard QIAsymphony protocol without type D beads (Fig. 26).



	Donor 1	Donor 2	Donor 3	Donor 4	Median
QIAamp reference	7154	8643	9939	7200	7922
w/o Type D beads (QIAsymphony)	5302	10247	8985	2806	7143
Type D beads 1 (QIAsymphony)	5695	6049	10446	7037	6543
Type D beads 2 (QIAsymphony)	5783	11260	13809	5196	8522
Type D beads 3 (QIASymphony)	9952	6294	10204	10628	10078
Type D beads 4 (QIAsymphony)	6643	10134	7245	3336	6944



	Donor 1	Donor 2	Donor 3	Donor 4	Median
QIAamp reference	1599	1665	1750	1907	1708
w/o Type D beads (QIAsymphony)	808	1708	1249	1207	1228
Type D beads 1 (QIAsymphony)	1044	973	1701	1375	1210
Type D beads 2 (QIASymphony)	1306	2238	2269	1449	1844
Type D beads 3 (QIAsymphony)	2262	1415	1943	3102	2102
Type D beads 4 (QIASymphony)	1179	2209	1319	1144	1249

Figure 26 Type D beads for removal of PCR-inhibitory contaminants
The 18S ribosomal DNA assay was performed and mean copies per sample were calculated for the short 66 (**A**) and long 500 bp amplicon (**B**). The QIAamp cNA Kit was used as reference again and the QIAsymphony cDNA Protocol was performed after plasma pretreatment with four different type D bead versions as well as without these additional beads. Therefore, plasma from four healthy individuals was used.

Using the Qproteome Albumin/IgG depletion resins, proteins should be removed from the plasma samples of four healthy individuals. But after protein depletion, DNA was also removed and it was neither present in the supernatant after binding, nor the final eluate. To be able to exclude PCR-inhibitory effects of the eluate as a possible reason for the low DNA recovery, one part of the eluate was processed with the QIAamp cNA Kit as cleanup

procedure, but results remained unchanged compared to the direct use of the eluate for PCR analysis (data not shown).

Without attention to included proteins or other plasma components, it was tried to improve binding step by changing the composition of the binding mixture. The idea was to enhance bonding force, increase the amount of possible binding sites or reduce bead loss for further improvement. The binding buffer and its amount were varied. While the use of 25 µL acetic acid or hydrochloric acid led to a decrease of DNA recovery, comparable results to the initial version were achieved using 1,000 µL 0.12 M sodium acetate/acetic acid for 3 mL plasma. As there was no further improvement, the current buffer still remained unchanged (data not shown). Doubling of the binding buffer volume as well as diluting plasma samples with PBS did not improve DNA recovery either (data not shown). As the binding process is based on the magnetic anion exchange beads, different types and concentrations of beads were tested. Using the type B beads 3, 4.5 and 6 mg led to comparable results. In some cases, DNA recovery increased with higher bead concentration. DNA recovery was slightly improved for 7.5 mg beads (data not shown). These and other bead types are usually stored in RNase-free H_2O. Type A and B beads were compared. Both bead types led to almost equal results, but looking at the results of the GFP DNA fragment spike-in system, there was a loss of short 75 bp DNA fragments using type A beads.

The reason why it was first tried to improve type A beads concerning the recovery of small 75 bp fragments was the lower binding strength allowing for lower pH values or salt concentrations during elution step, but type B beads led to similar results compared to the type A beads and no additional bead pretreatment was needed to achieve high DNA recovery of all fragment sizes (data not shown). Therefore, type B beads were chosen for future experiments.

Up to 25% of 18S ribosomal DNA was detected by analyzing the supernatant after binding (Fig. 27).

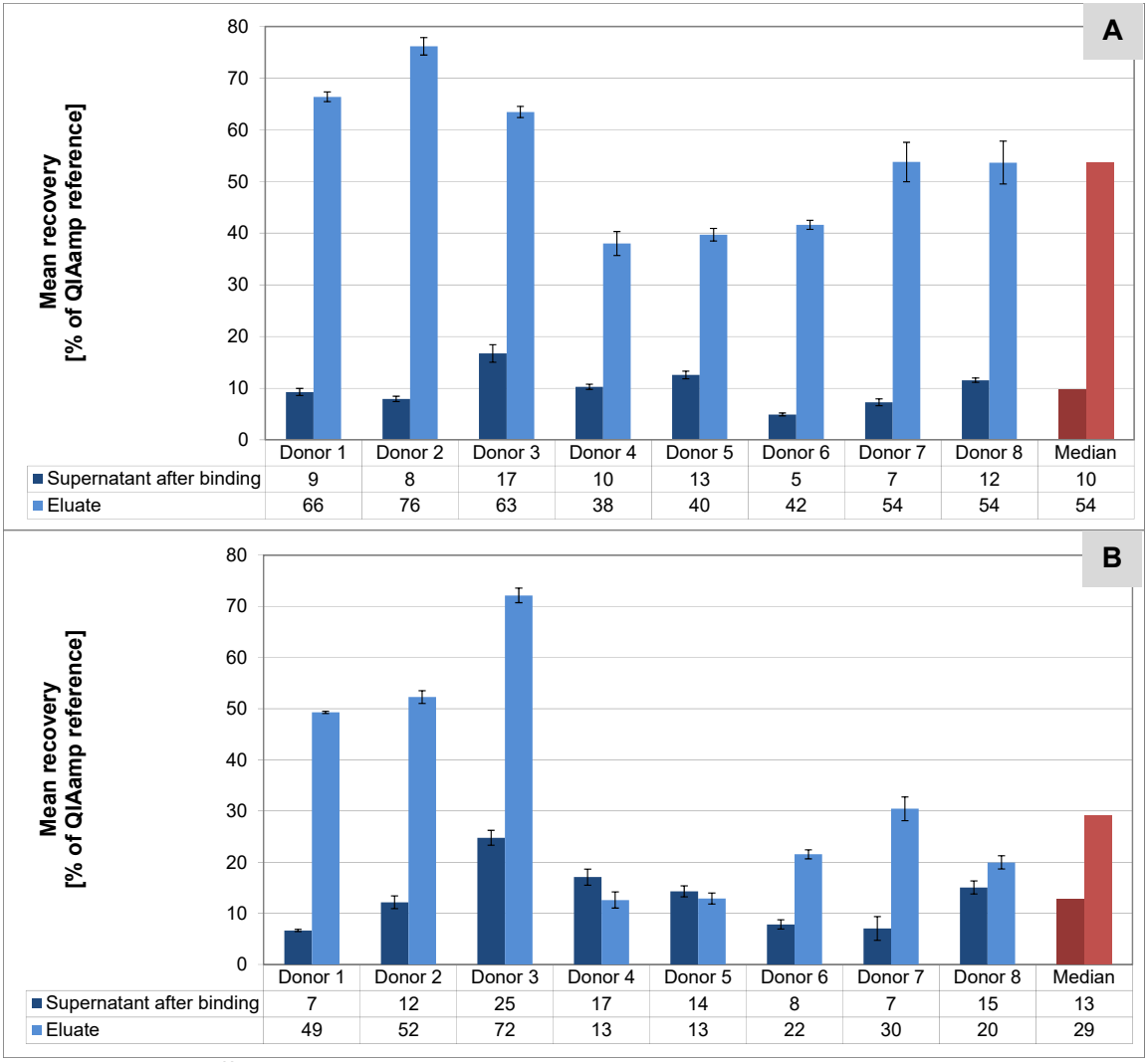


Figure 27 Binding efficiency
To analyze the binding efficiency of the QIAsymphony cDNA Protocol, the supernatant after binding was analyzed in addition to the eluate from plasma samples from eight healthy individuals. Results are shown in the form of mean DNA recovery compared to the QIAamp cNA Kit reference. The 18S ribosomal DNA assay (A = 66 bp and B = 500 bp amplicon) was used for quantitative real-time PCR assay.

DNA loss during the binding step was comparable (5-25%), but up to 62% less DNA was recovered within the final eluate looking at the 66 bp amplicon of the 18S ribosomal DNA assay. DNA recovery was even lower for the 500 bp amplicon. So, DNA was not completely bound, but it could not be the only reason for DNA loss during extraction process because total DNA loss was often higher.

Wash

Wash steps were included to remove possible contaminants from plasma, which were also bound to the beads. Using small elution volumes of ≤150 µL, wash steps were needed for a successful DNA extraction at the end (Fig. 28).

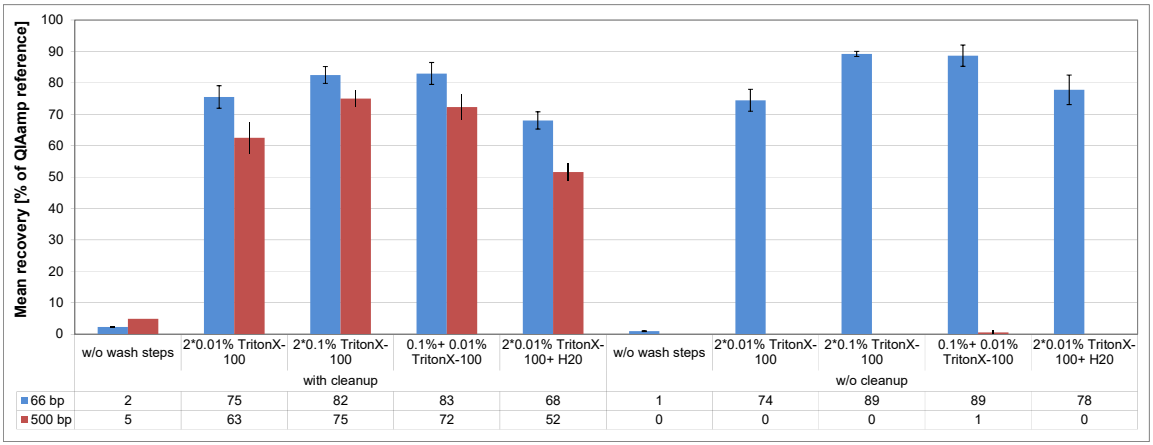
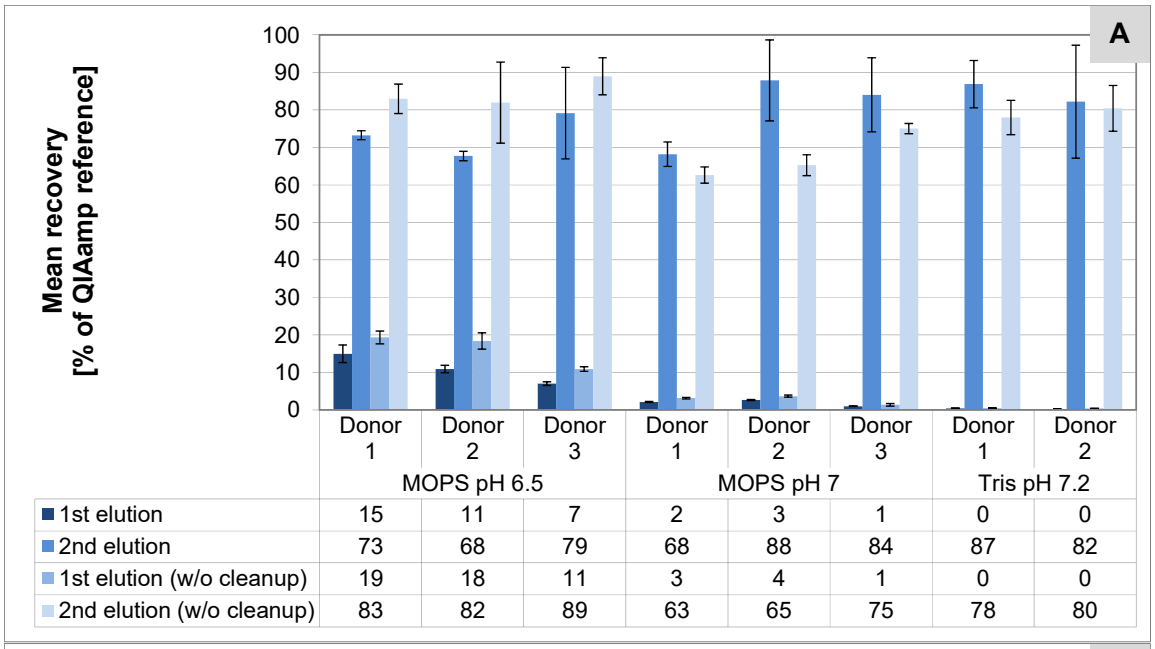


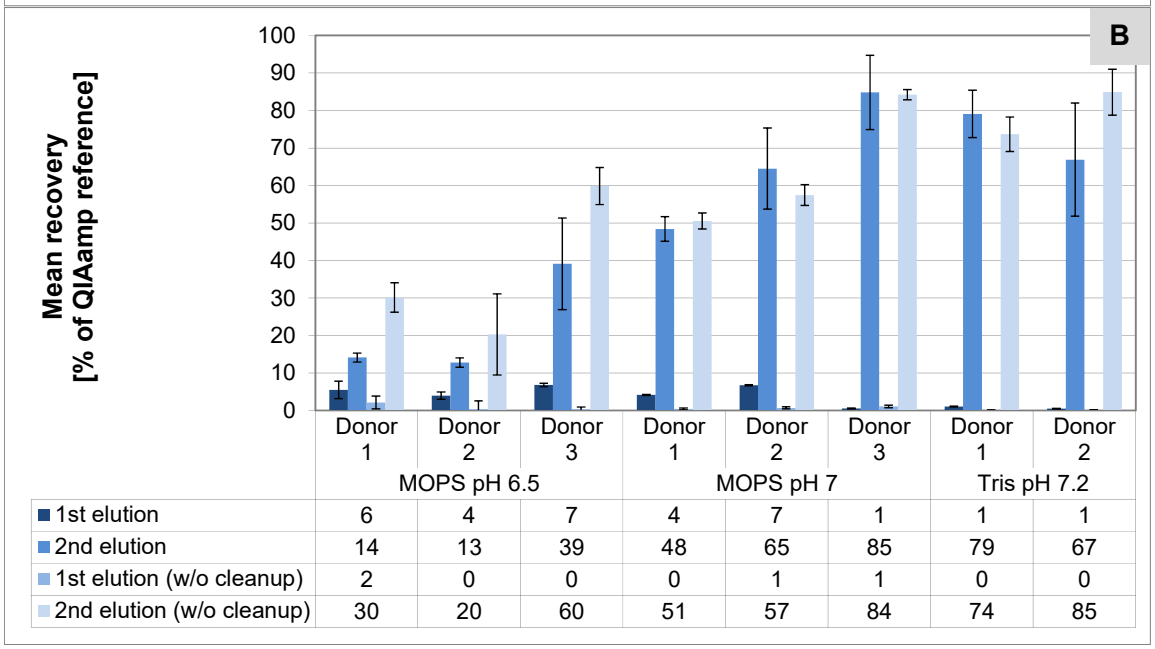
Figure 28 Wash buffers
For the QIAsymphony cDNA Protocol, mean DNA recovery compared to the QIAamp cNA Kit was determined using the 18S ribosomal DNA assay. Five different wash conditions were performed, whereas eluates were used for quantitative real-time PCR directly or after performing the QIAamp cNA Kit as a cleanup. The protocol version without wash steps was compared to three versions including two wash steps in each case. Both wash steps were performed using 0.01% TritonX-100, 0.1% TritonX-100 or 0.1% TritonX-100 for the first wash step followed by 0.01% for the second one. Furthermore, 0.01% TritonX-100 was tested in combination with RNase-free H_2O for a third wash step.

Without including wash steps, almost no DNA was recovered, even if an eluate cleanup using the QIAamp cNA Kit was performed. Consequently, at least one further reason for DNA loss had to exist besides plasma contaminants. Perhaps, residual binding buffer (pH 4) decreased the pH value of the eluate, worsening DNA elution. The idea of implementing a cleanup procedure for the residual conditions that includes wash steps was to make eluates more compatible to downstream applications like quantitative real-time PCR by reducing their PCR-inhibitory effect. No improvement of DNA recovery was achieved for the 66 bp amplicon of the 18S ribosomal DNA assay using the named cleanup; however, detection of the 500 bp amplicon was only possible through cleanup performance. Consequently, longer DNA fragments (≥500 bp) also were successfully recovered, but quantitative real-time PCR seemed to be more sensitive for longer amplicons, which are possibly more prone to PCR inhibition by protein binding, for example. For all conditions including wash steps, 68 to 83% were achieved for the 66 bp amplicon when a cleanup protocol was used for purification. Without a cleanup, 74 to 89% were reached. No DNA

was detected without a cleanup for the 500 bp amplicon, but 52 to 97% DNA recovery was possible with cleanup. Therefore, the use of 0.1% TritonX-100 as wash buffer seemed to lead to higher DNA recoveries than 0.01%. A third wash step with 300 µL RNase-free H_2O did not lead to any further improvement. The idea of using pure RNase-free H_2O was to prevent the carry-over of detergents into the eluate, which might have a negative influence on PCR performance. The first and second wash steps were usually performed with a buffer volume of 1,000 and 300 µL to gradually reduce the wash buffer volume and minimize the loss of beads bound to the plastics of SPC tubes. Performing these two wash steps, elution volume was reduced from an initial 1,000 µL to 150 µL. In addition, another more stringent wash step was tested. It was like a pre-elution of proteins and other plasma contaminants at lower pH value without losing target DNA. At that time, 150 µL 100 mM TRIS-HCl + 154 mM sodium chloride at pH 8.5 was used to elute DNA molecules from beads, so the same buffer at pH 7.2 as well as MOPS buffer with the same molarity and sodium chloride concentration at pH 6.5 and 7.0 were tested. The lowest DNA loss was achieved using 150 µL 100 mM TRIS-HCl + 154 mM sodium chloride at pH 7.2 (Fig. 29).



1st elution	15	11	7	2	3	1	0	0
2nd elution	73	68	79	68	88	84	87	82
1st elution (w/o cleanup)	19	18	11	3	4	1	0	0
2nd elution (w/o cleanup)	83	82	89	63	65	75	78	80



1st elution	6	4	7	4	7	1	1	1
2nd elution	14	13	39	48	65	85	79	67
1st elution (w/o cleanup)	2	0	0	0	1	1	0	0
2nd elution (w/o cleanup)	30	20	60	51	57	84	74	85

Figure 29 Test of a pre-elution step
Mean DNA recovery was determined again. A third wash or pre-elution step was integrated and tested for two or three healthy individuals. 150 µL 100 mM TRIS + 154 mM sodium chloride at pH 7.2 as well as 100 mM MOPS + 154 mM sodium chloride at pH 6.5 and 7.0 were used. The first eluate after pre-elution as well as the second final eluate were analyzed by 18S ribosomal DNA assay directly before and after performing the QIAamp cNA Kit as cleanup (**A** = 66 and **B** = 500 bp amplicon).

300 µL of pre-elution buffer after wash steps as well as pre-elution buffer instead of wash buffer with and without 0.1% TritonX-100 (1,000 and 300 µL) were then tested. Replacing the wash buffer and adding 0.1% TritonX-100 led to the highest DNA recovery within the

final eluate, while a third wash step, using the same buffer, did not lead to any further improvement (data not shown).

Elution

The initial elution buffer was 100 mM TRIS-HCl + 154 mM sodium chloride (pH 8.5) and type B beads were used. It was tried to create more suitable conditions for PCR reactions by changing the elution buffer. At the same time, complete DNA elution from beads should be guaranteed. Therefore, elution was performed without salt by increasing the pH value, but experiments led to different results. In one case, DNA recovery was improved by salt addition, while it was unchanged in another experiment (data not shown). DNA recovery was further improved by increasing the pH value of the initial elution buffer from 8.5 to 9 as well as by adding 0.02 or 0.04% polyacrylic acid (data not shown). Polyacrylic acid was supposed to replace DNA from binding sites, but only low concentrations were tested because it is also known to show a PCR-inhibitory effect (according to unpublished data from QIAGEN GmbH). Furthermore, TRIS buffer with 5 mM magnesium chloride or QuantiTect Multiplex PCR Master Mix were used for elution. Magnesium is described to improve PCR efficiency [252] and, in the form of magnesium chloride, it should replace sodium chloride for elution. Since the QuantiTect Multiplex PCR Master Mix contains TRIS buffer at an acceptable pH value for elution, it was also tested as a buffer for DNA elution. Nevertheless, both alternative elution buffers led to a worse DNA recovery compared to the standard elution buffer (data not shown). DNA recovery also decreased using a lower TRIS molarity as well as potassium chloride or ammonium chloride (tested for the same reason as magnesium chloride) instead of sodium chloride for elution (data not shown). Using type A beads, 100 mM MOPS + 154 mM sodium chloride (pH 7.9) led to comparable results regarding type B beads and the initial elution buffer (data not shown).

Elution time was then raised from 20 to 40 min., but results were ambiguous. DNA recovery was improved for individual healthy donors 1 and 2, while the detection signal for Donor 3 got nearly lost (data not shown).

To achieve a high DNA target enrichment within the eluate by processing large sample volumes, the eluate volume had to be as small as possible and therefore reduced, so it was possible to elute DNA in 150 µL buffer volume after proper wash steps were implemented. These 150 µL were used in most experiments, but, as shown in Figure 30,

elution in 100 µL, 80 µL and 60 µL was also successfully tested using the manual protocol version.

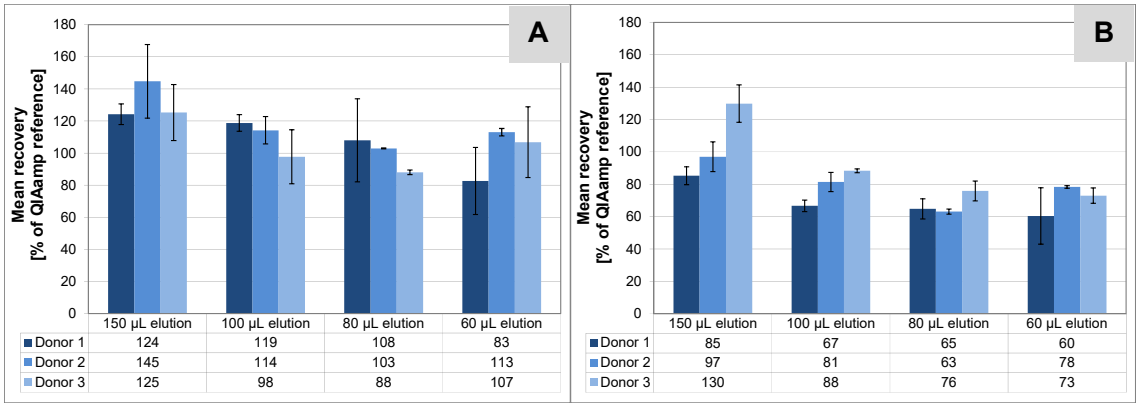
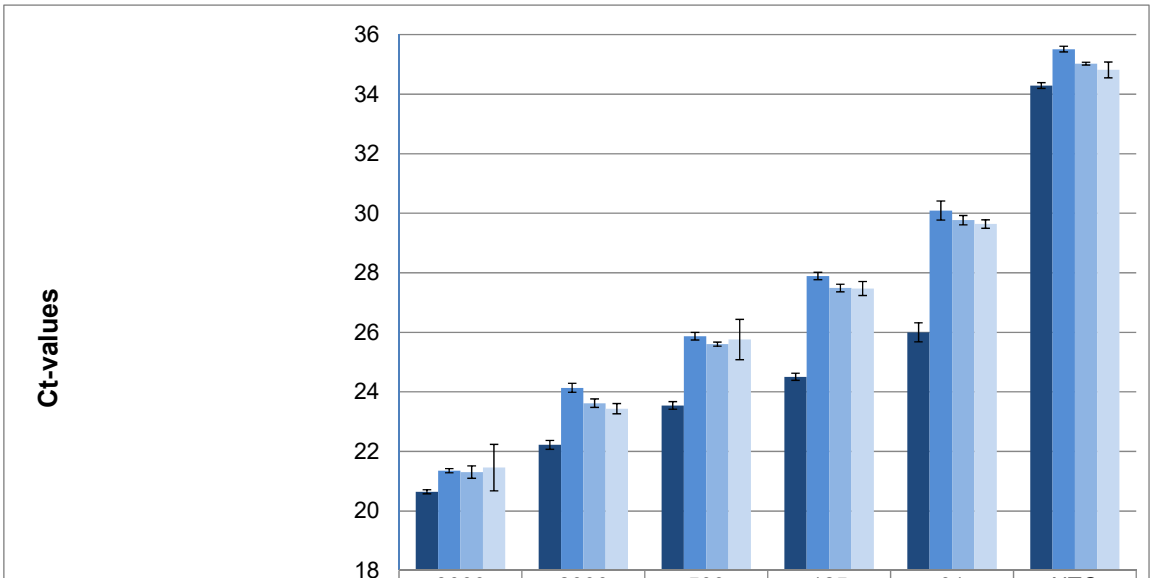


Figure 30 Elution volume
Four different elution volumes were compared for three healthy individuals. Mean DNA recovery was calculated for the QIAsymphony cDNA Protocol compared to the QIAamp cNA Kit detecting the 66 (**A**) and 500 bp (**B**) amplicon of the 18S ribosomal DNA assay. The 80 and 60 µl elution volume was significant different, when the 150 µl elution volume was defined as control group. P-values were 0.0046** and 0.0066** for the 66 bp amplicon and <0.0001*** and 0.0001*** detecting the 500 bp amplicon (Dunnett's method of JMP software). Additionally, the 100 µl elution volume was significant different compared to the 150 µl standard elution volume using the 18S 500 bp assay (p= 0.0030**).

Results were comparable between the 66 and 500 bp amplicon of the 18S ribosomal DNA assay. The highest decrease in DNA recovery was detected after reducing the elution volume from 150 to 100 µL, but a further reduction down to 80 and 60 µL led to similar results. In most cases, total DNA recovery was lower for the long 500 bp amplicon of the 18S ribosomal DNA assay. This could be caused by PCR inhibition for the long amplicon or a preferred elution of smaller DNA fragments.

Regarding the elution buffer, it was not only important to elute DNA completely. As already mentioned, the elution buffer also had to be compatible concerning different downstream applications enabling DNA detection and analysis, so various elution buffers were tested concerning the applicability for quantitative real-time PCR. They were spiked with the previously described 75, 200 and 1,000 bp GFP DNA fragments or extracted male human DNA of a known concentration, which were afterwards detected by quantitative real-time PCR. Using the QuantiTect Multiplex PCR Master Mix, results were nearly comparable between different TRIS molarities, salt concentrations and pH values of the elution buffer. Higher molarity and salt concentration tended to result in higher Ct values only in some cases. This calls attention to the robustness of the used PCR chemistry (data not shown). The difference between RNase-free H_2O and possible elution buffers was more obvious

using the QuantiTect Virus Master Mix, which allows for higher DNA template volumes (Fig. 31).



	8000	2000	500	125	31	NTC
H2O	20,65	22,22	23,54	24,51	26,00	34,29
100 mM Tris, 154 mM NaCl, pH 9	21,35	24,14	25,87	27,89	30,09	35,51
50 mM Tris, 80 mM NaCl, pH 9	21,31	23,62	25,60	27,49	29,77	35,02
50 mM Tris, 80 mM KCl, pH 9	21,46	23,44	25,76	27,47	29,64	34,81

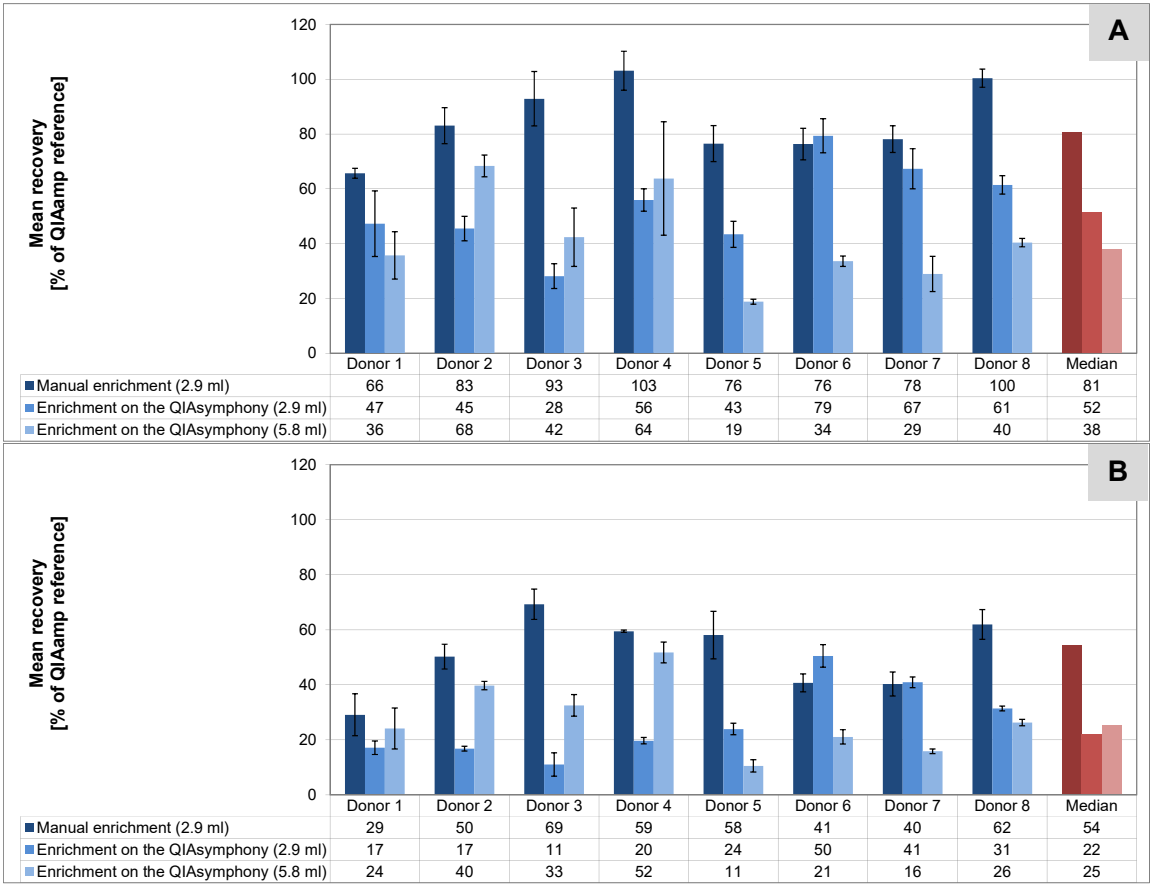
Figure 31 Test of elution buffers according to PCR-inhibitory effects
Using the QuantiTect Virus noROX Master Mix, up to 70% template volume can be used. Extracted human male DNA was added to three different elution buffers and RNase-free H_2O served as reference. Ideally, Ct differences between dilutions should be 2. Ct values above the reference possibly indicate a PCR-inhibitory effect.

The lowest Ct values were achieved using RNase-free H_2O, but tested elution buffers led to comparable results.

3.2.2.2. Automation

After the development of a manual protocol version, automation should take place, so the protocol had to be transferred to the QIAsymphony SP instrument. The aim was to reach a comparable or better performance compared to the manual QIAamp cNA Kit, so in a first try the QIAamp cNA Kit was compared against a small (2.9 mL) and large (5.8 mL) volume automated QIAsymphony protocol as well as the manual version. In all cases, except Donor 6, there was a clear difference between the manual and automated protocol version using 2.9 mL plasma and higher DNA recoveries were reached extracting plasma DNA manually. Furthermore, lower DNA recoveries were reached by increasing the sample

input volume of the automated QIAsymphony protocol, but difference was not significant (Fig. 32).



	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Median
Manual enrichment (2.9 ml)	66	83	93	103	76	76	78	100	81
Enrichment on the QIAsymphony (2.9 ml)	47	45	28	56	43	79	67	61	52
Enrichment on the QIAsymphony (5.8 ml)	36	68	42	64	19	34	29	40	38

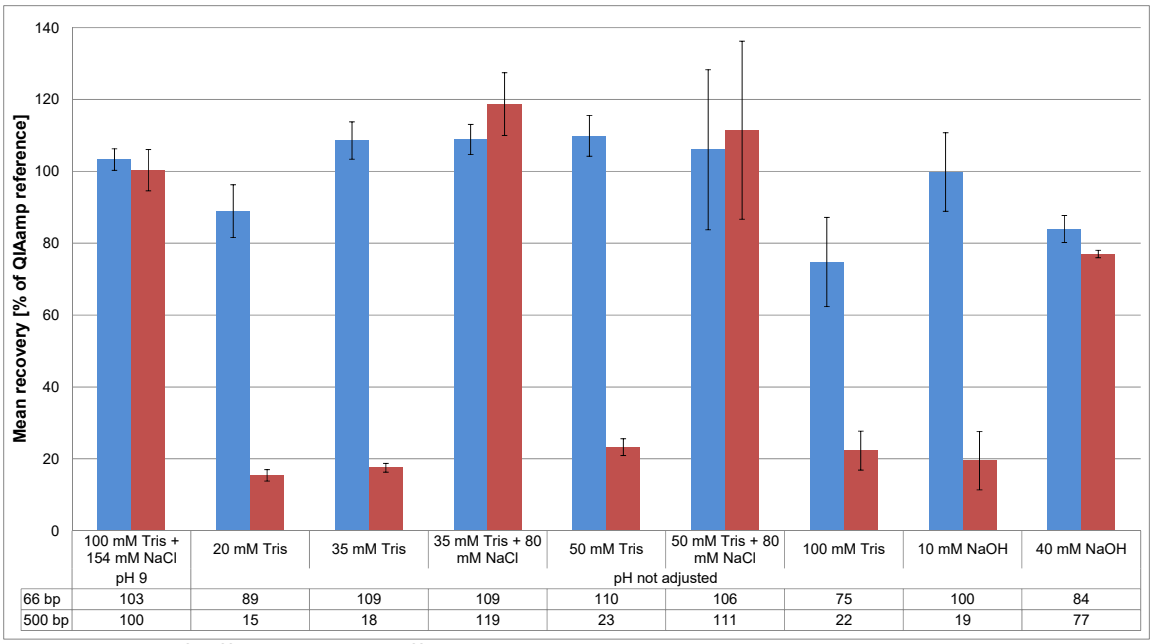
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Median
Manual enrichment (2.9 ml)	29	50	69	59	58	41	40	62	54
Enrichment on the QIAsymphony (2.9 ml)	17	17	11	20	24	50	41	31	22
Enrichment on the QIAsymphony (5.8 ml)	24	40	33	52	11	21	16	26	25

Figure 32 Binding efficiency
Looking at the mean DNA recovery of the 66 (**A**) and 500 bp (**B**) amplicon of the 18S ribosomal DNA assay, the manual version of the newly developed protocol was compared with two automated DNA enrichment procedures on the QIAsymphony SP instrument (early versions of the QIAsymphony cDNA Protocol). Therefore, 2.9 and 5.8 mL plasma of eight healthy individuals were used as sample input material. The QIAamp cNA Kit served as reference again. Significance of differences between tested protocol versions was calculated using Dunnett's method (JMP software). The manual protocol for DNA enrichment from 2.9 mL plasma was defined as control group. For both DNA fragments of the 18S DNA assay, p-values were <0.0001*** for automated protocols.

First, the binding step was analyzed. The QIAsymphony run was stopped after the binding process and DNA was extracted from the supernatant after binding using the QIAamp cNA Kit. The manual and automated protocol versions were compared and showed there was a significant DNA loss during binding using the automated protocol version (data not shown). As a result, mixing with the magnetic rods for the binding step and different ways were tested to get mixing or binding conditions that were comparable to the manual procedure using the overhead rotational shaker. The initial adjustment for mixing during the binding step was the so-called "Mag-Mix." For Mag-Mix, beads were separated by magnetic RC

every 10 seconds for about 10 seconds and mixed again with RC to prevent bead sedimentation. For a first test, binding time was increased from 20 to 40 min. and bead separation was completely removed from the mixing procedure. However, although DNA loss was lowered by 40 min. of Mag-Mix, it was still higher than by using the manual protocol version (data not shown). Then slow mixing without Mag-Mix and mixing on lysis station by swinging round were tested, but 40 min. of Mag-Mix once again led to a better performance (data not shown). A two-fold amount of beads as well as Mag-Mix with less frequent bead separation only caused a better DNA binding in some cases (data not shown). As a result, Mag-Mix for 40 min. seemed to be the best adjustment among tested versions. It was tested for the large volume protocol, but led to a worse performance than the 2.9 mL one (data not shown). Although there was still a significant DNA loss in some experiments, Mag-Mix for 40 min. was used for binding and further steps were considered.

Wash steps remained unchanged and different elution buffers were tested. It was tried to reduce TRIS molarity and salt concentration again by increasing the pH value at the same time. Finally, 20 mM TRIS base (without additional adjustment of pH value) appeared to be the best, but short DNA fragments were preferably recovered using this buffer. To obtain a comparable recovery of longer DNA fragments, 35 mM TRIS base + 80 mM sodium chloride was suitable (Fig. 33).



	100 mM Tris + 154 mM NaCl pH 9	20 mM Tris	35 mM Tris	35 mM Tris + 80 mM NaCl	50 mM Tris	50 mM Tris + 80 mM NaCl	100 mM Tris	10 mM NaOH	40 mM NaOH
66 bp	103	89	109	109	110	106	75	100	84
500 bp	100	15	18	119	23	111	22	19	77

Figure 33 Test of different elution buffers

Mean DNA recovery of the QIAsymphony cDNA Protocol compared to the QIAamp cNA Kit was once again calculated using the 18S ribosomal DNA assay. Nine elution buffers were tested in two experiments. TRIS molarity, pH value and sodium chloride concentration were varied. Additionally, two sodium hydroxide concentrations were used for DNA elution.

With regard to the 66 bp amplicon, all tested elution buffers led to comparable results. A lower DNA recovery was only achieved using 100 mM TRIS base. Salt or high pH values were needed to achieve DNA recoveries which were comparable to the initial elution buffer (100 mM TRIS-HCl + 154 mM sodium chloride, pH 9) for the 500 bp amplicon. Higher bonding forces between DNA and beads according to the DNA fragment length are one possible reason. The longer DNA fragments are, the stronger they are bound to the beads due to a bigger binding surface.

According to the elution buffer change from 100 mM TRIS-HCl + 154 mM sodium chloride, pH 9 to 35 mM TRIS base + 80 mM sodium chloride, wash buffers were adapted to 35 mM TRIS-HCl + 0.1% TritonX-100 (pH 7.2), minimizing the influence of wash buffer on elution buffer pH value. Elution volume was reduced down to 60 µL without any critical DNA loss through manual protocol performance, but a reduction from 150 to 100 µL was not possible on the QIAsymphony instrument without losing DNA (data not shown). As a result, further optimization concerning bead amount and other protocol steps or conditions were needed.

DNA recoveries of up to 100% compared to the QIAamp cNA Kit were already achieved during the automated protocol development, but there were some donors (in this case Donor 10) who showed lower DNA recoveries, as displayed in the following results (Fig. 34).

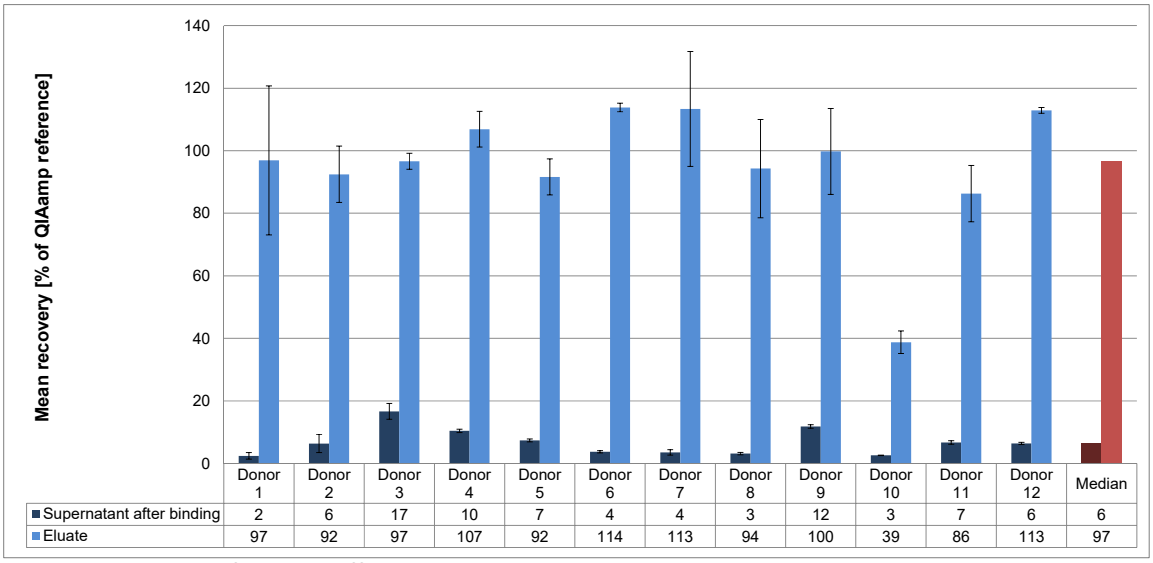


Figure 34 Analysis of binding efficiency using the automated protocol
Mean DNA recovery of the short 66 bp long amplicon was determined for the supernatant after binding as well as the eluate after plasma processing with the QIAsymphony cDNA Protocol. Twelve healthy individuals were regarded and the QIAamp cNA Kit served as reference again.

Furthermore, the elution volume had to be reduced and eluates should be more compatible concerning downstream applications like quantitative real-time PCR.

Automation of the developed DNA extraction protocol was possible, but still had to be further optimized.

3.2.2.3. Optimization

Further optimization was needed and in the following experiment not only the supernatant after binding was analyzed, but also DNA, which was still bound to the beads after elution step. Therefore, a second elution was performed using strongly alkaline AXpH elution buffer, which was neutralized with hydrochloric acid to a pH value of about 9 before quantitative real-time PCR performance. Most DNA got lost during the binding step, but a certain part was still bound to beads after elution (Fig. 35).

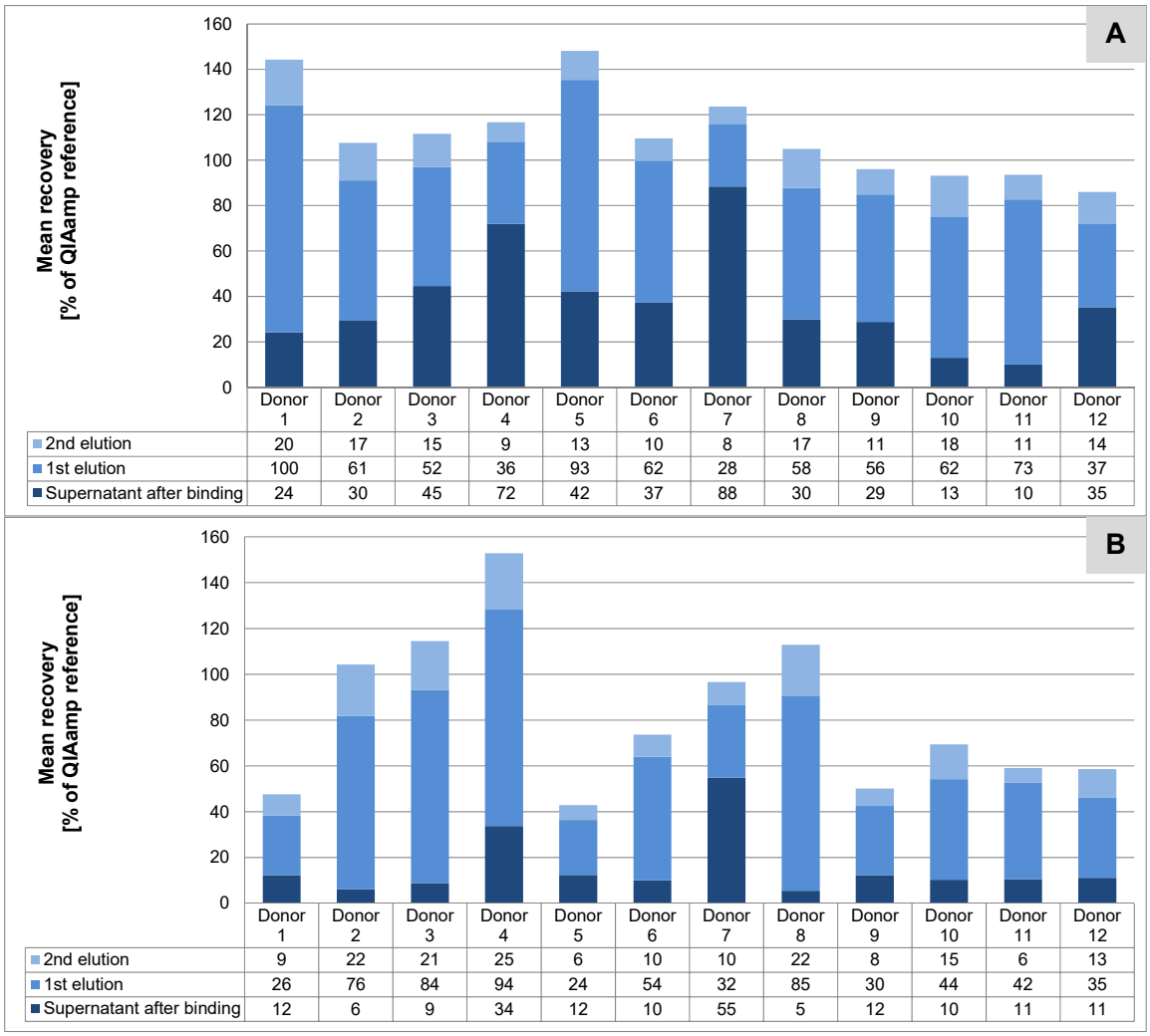


Figure 35 Analysis of DNA loss

Mean DNA recovery of the short 66 bp long amplicon of the 18S ribosomal DNA assay was calculated for twelve individual donors. The supernatant after binding with sodium acetate/acetic acid buffer (**A**) and citric acid (**B**) as well as the first standard eluate were analyzed. A second elution buffer (AXpH elution buffer) was used to remove any residual DNA bound to beads.

In addition to the standard binding buffer (sodium acetate/acetic acid buffer), the same amount (75 µL for 3 mL plasma sample) of 1 M citric acid (pH 2.5) was used for binding. DNA loss during binding process was reduced this way, while DNA recovery within the eluate decreased. One reason for the decreased DNA recovery within the eluate could be the observed loss of beads after the binding step. The marked tubes of the SPC, displayed in Figure 36, contain the binding mixture using citric acid buffer.

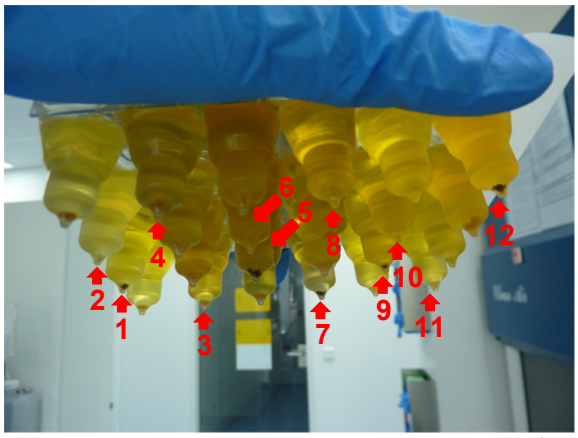


Figure 36 Bead pellets in the binding mixtures of sample preparation cartridges
The sample preparation cartridges after bead separation at the end of the binding step are displayed here. They contain plasma, binding buffer and residual magnetic beads on the tube bottoms (the supernatant after DNA binding).

After magnetic bead separation and the bead transfer into wash buffer, the supernatant after binding was discarded and bead pellets as well as beads on tube sides remained in the SPC tubes (see Figure 37). DNA was probably also bound to these residual beads and was not available for further DNA extraction.

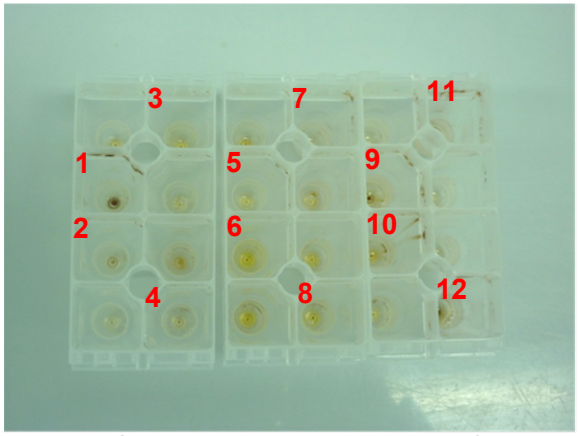


Figure 37 Sample preparation cartridges after removal of binding supernatant
Residual beads can be seen on tube bottoms and frames after removal of the supernatant after binding (plasma and binding buffer after bead separation).

To generate cleaner eluates, it was tried to combine the newly developed protocol with a silica chaotrope based protocol in the form of the already mentioned “Virus Cellfree 1000” protocol. After binding, DNA was eluted in 1,000 µL and this volume was used as the sample input volume for the subsequent “Virus Cellfree 1000” protocol. However, although both protocols worked separately, their combination led to almost no DNA recovery (data not shown). More stringent wash conditions were then tested using different sodium chloride concentrations for wash steps. As a result, the pH value was simultaneously

lowered to prevent any DNA loss, so 35 mM MES + 0.1% TritonX-100 at pH 5.8 was used and different sodium chloride concentrations were tested. In each case, the second wash step was always performed without salt to prevent its carry-over into the eluate. As shown in Figure 38, 200 mM sodium chloride seemed to increase DNA recovery without losing any DNA.

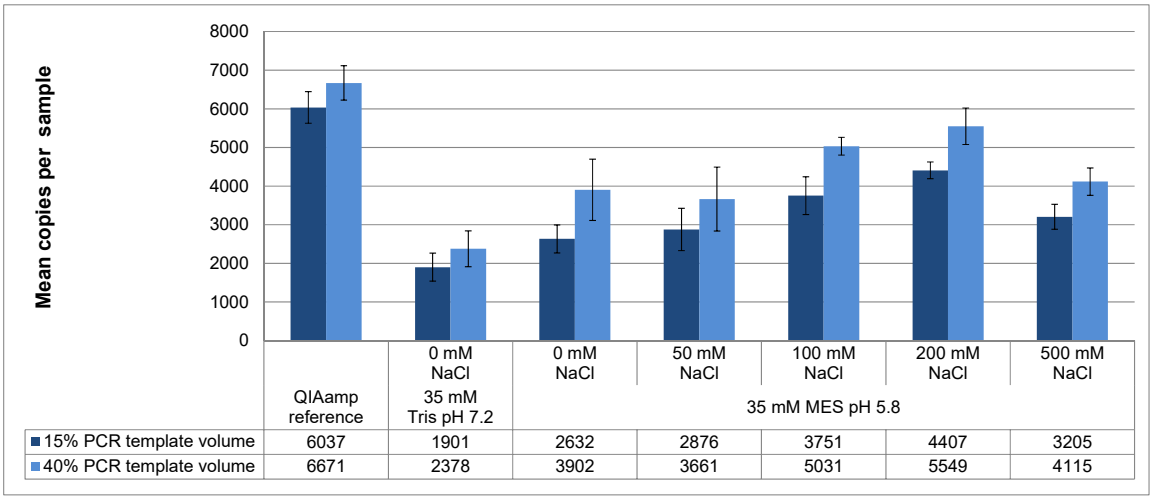


Figure 38 Effect of sodium chloride for wash step
Mean copies per sample for the short 66 bp amplicon of the 18S ribosomal DNA assay are shown here. The QIAamp cNA Kit served as reference. To analyze PCR-inhibitory effects, 15 and 40% template volume were used. If PCR inhibition occurred, mean copies per sample should be lower using higher PCR template volumes. Six different wash buffers were tested. 35 mM TRIS-HCl (pH 7.2) and MES (pH 5.8) containing 0.1% TritonX-100 were used. As a result, MES buffer was analyzed without and with 50, 100, 200 and 500 mM sodium chloride for the first wash step. The second one was always performed without any salt.

Furthermore, detergent was added to plasma samples again. This time TritonX-100 and Nonidet P40 were used in different concentrations, but combined with citric acid as the binding buffer. Because of the occurring deviations, it was hard to say if DNA recovery was really increased (data not shown). Since TritonX-100 tended to result in higher DNA recoveries, different concentrations were further tested and 0.5% TritonX-100 led to slightly higher DNA recoveries than the other tested concentrations (data not shown).

The effectiveness of Proteinase K was one important finding after the protocol was transferred to projects for product development. It had already been tested before in different concentrations and at different temperatures, but now, combined with other buffers, a positive effect was achieved. This revealed the dependencies between buffers and components of all protocol steps. Changing one step necessitated testing all of the other ones.

As a result, protocol version 1 (further described in the attachment) was tested against four extended protocol versions using Proteinase K. Proteinase K was thus pipetted

1. Before the binding step (100 µL)
2. During the binding step (100 µL)
3. Manually to the plasma sample tubes before the instrument run was started (300 µL)
4. Manually to the plasma sample tubes before the instrument run was started (100 µL) and after binding in a separate step (=RDD step)

At this time, the last protocol version (4.) led to the highest DNA recoveries (further details can be found under protocol version 2 in the attachment). Results are shown in Figure 39.

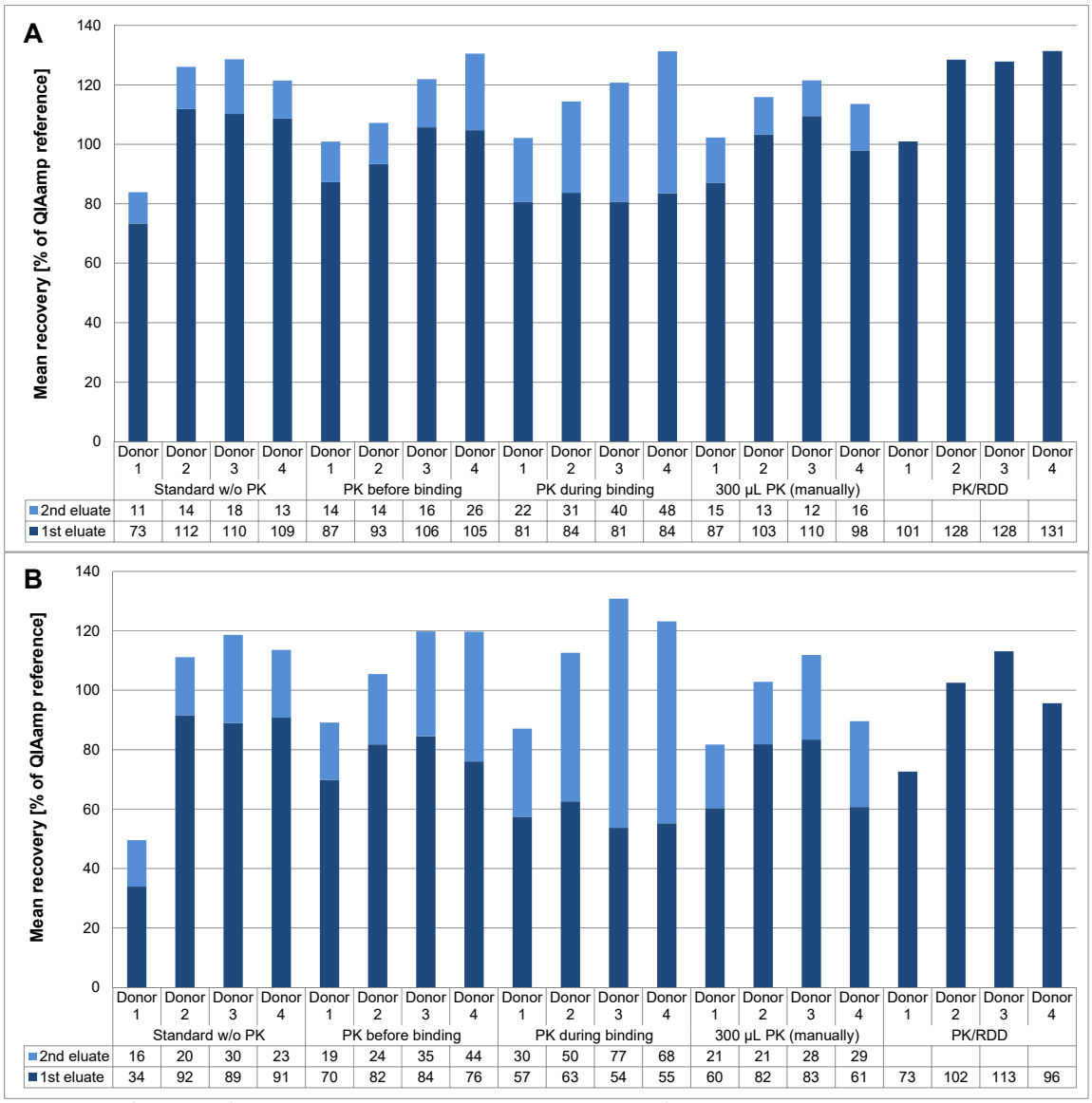
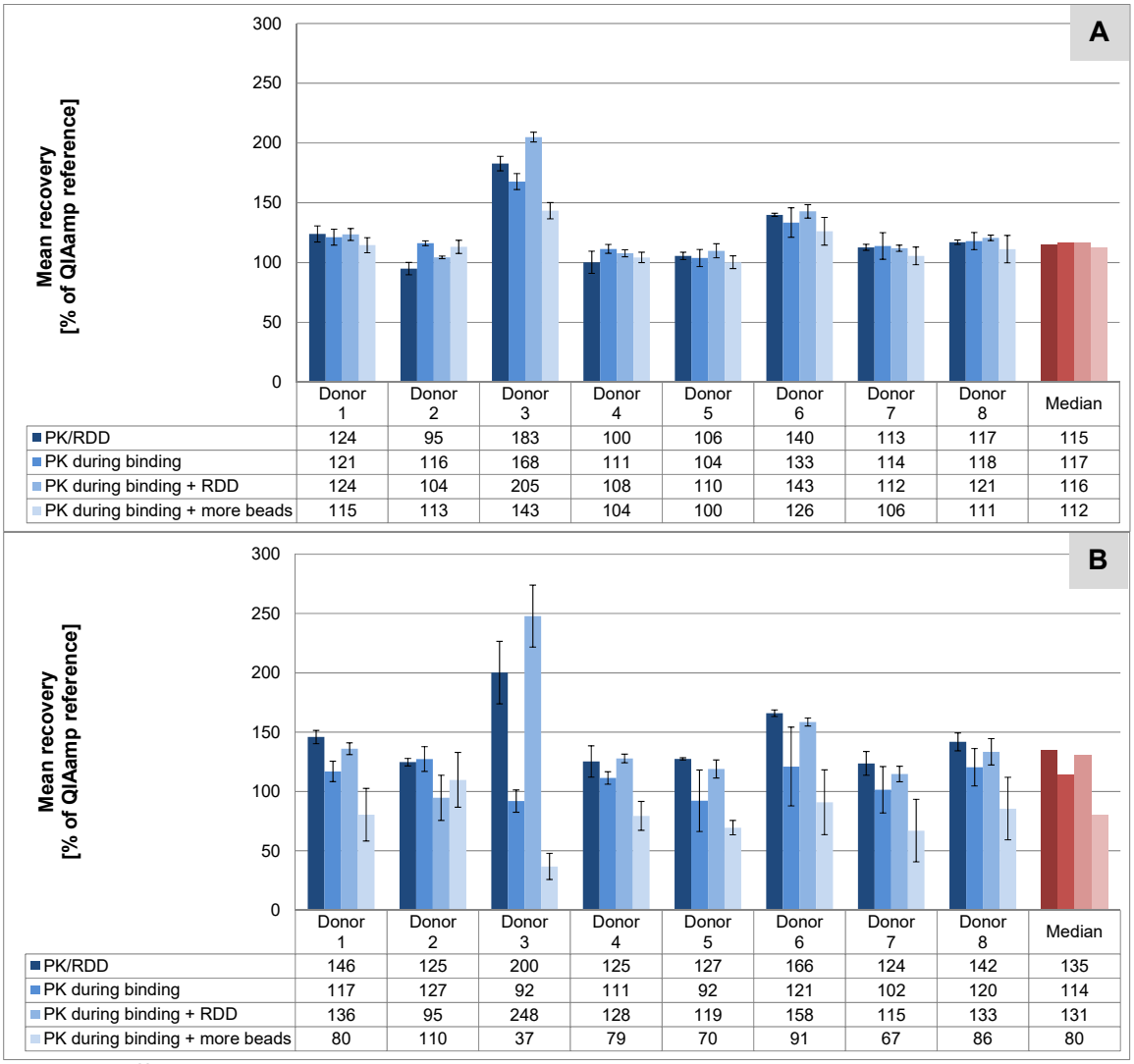


Figure 39 Influence of Proteinase K on automated protocol performance
The mean DNA recovery of the QIAsymphony cDNA Protocol compared to the QIAamp cNA Kit reference was determined using the 18S ribosomal DNA assay (A = 66 bp and B = 500 bp amplicon). Each condition was tested with plasma from four healthy individuals and, except version “PK/RDD”, a second elution using AXpH elution buffer followed after the performance of the first standard one to elute residual DNA from beads. The protocol versions differed in the Proteinase K treatment. The “Standard” version did not include a Proteinase K step, while for the other versions Proteinase K was added before binding, during binding, manually to the sample and manually to the sample combined with an additional Proteinase K step later using the buffer RDD (=PK/RDD version).

This recovery was increased by adding 200 mM sodium chloride for binding (data not shown). Even higher DNA recoveries, which could not be increased further using more beads, were reached by further optimizing the QIAsymphony protocol version 1 for 2 and 4 mL plasma and adding a RDD step (see protocol version 3 in the attachment) (Fig. 40).

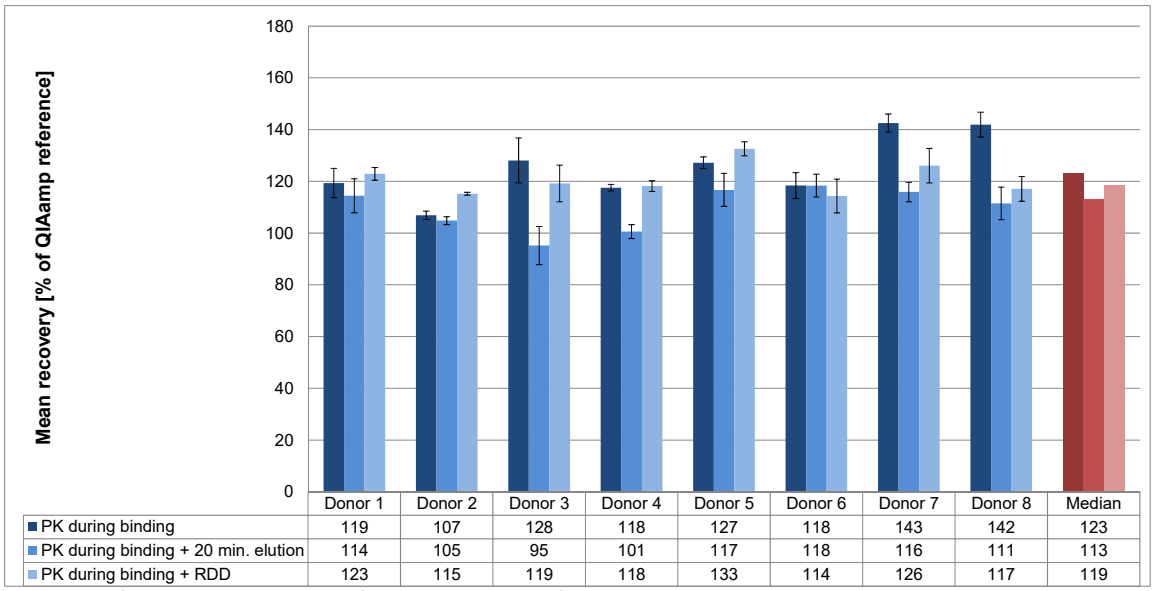


	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Median
PK/RDD	124	95	183	100	106	140	113	117	115
PK during binding	121	116	168	111	104	133	114	118	117
PK during binding + RDD	124	104	205	108	110	143	112	121	116
PK during binding + more beads	115	113	143	104	100	126	106	111	112

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Median
PK/RDD	146	125	200	125	127	166	124	142	135
PK during binding	117	127	92	111	92	121	102	120	114
PK during binding + RDD	136	95	248	128	119	158	115	133	131
PK during binding + more beads	80	110	37	79	70	91	67	86	80

Figure 40 Different protocol versions using Proteinase K
Mean DNA recovery compared to the QIAamp cNA Kit is presented again here. The 18S ribosomal DNA assay was used to detect the 66 (**A**) and 500 bp (**B**) amplicon. Plasma samples from eight healthy individuals were processed with the QIAsymphony cDNA Protocol. The "PAX" protocol version from the experiment described above was compared with an improved version of the standard Proteinase K protocol. This standard Proteinase K protocol was extended by an additional Proteinase K/RDD treatment or more beads were used (3 instead of 2 mg). Significance of differences between tested conditions was calculated using Dunnett's method (JMP software). The addition of Proteinase K during binding was defined as control group. While differences were not significant using the 18S 66 bp assay, they led to a high significance of all tested conditions detecting the 500 bp fragment ($p < 0.0001$***).

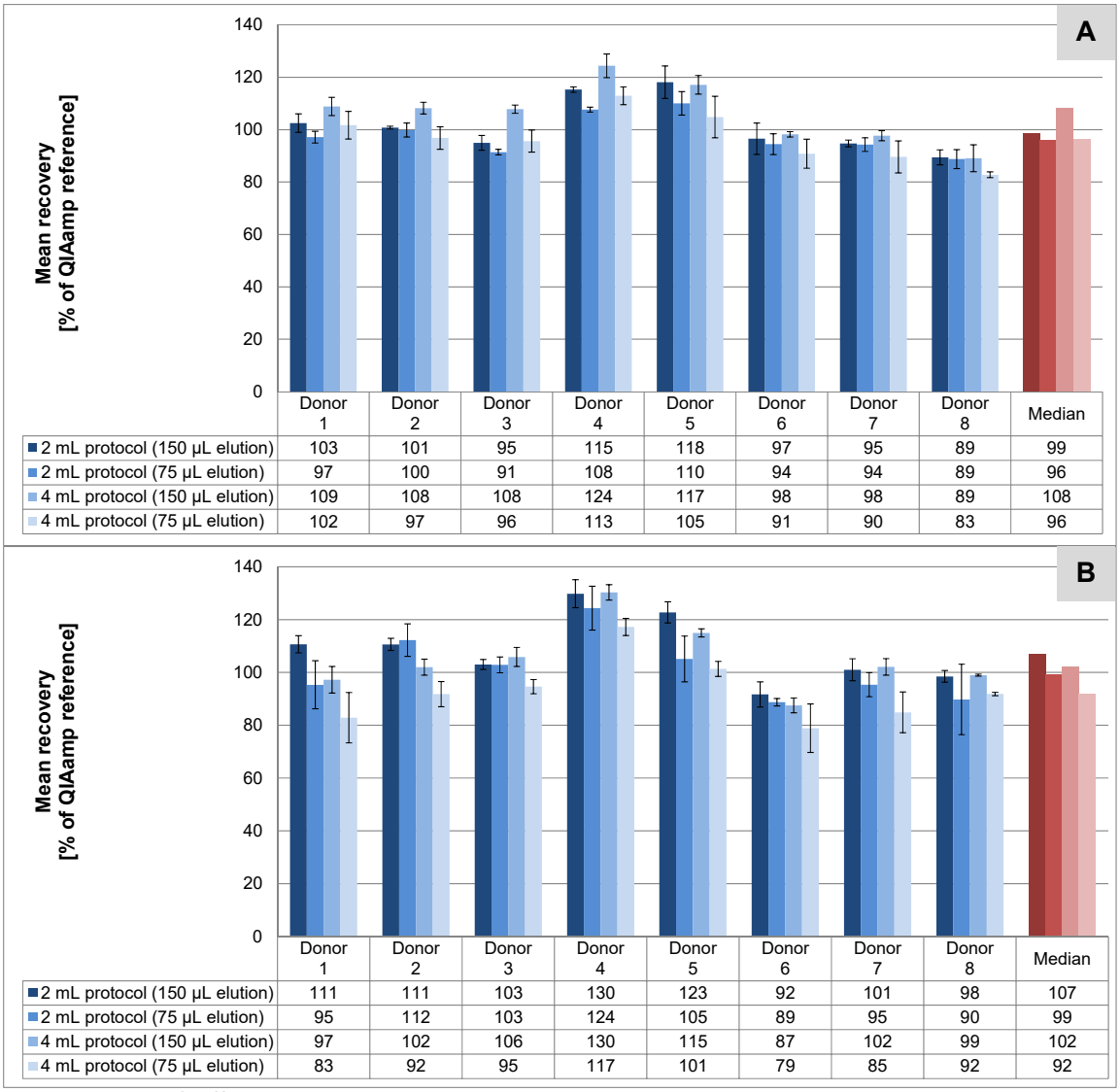
After further improvement of the initial protocol version 1 (see protocol version 4 in the attachment), the additional RDD step became redundant and was removed. An extended elution time did not seem to have any beneficial effect. The additional RDD step as well as the extended elution time even led to lower DNA recoveries compared to the QIAamp cNA Kit (Fig. 41).



	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Median
PK during binding	119	107	128	118	127	118	143	142	123
PK during binding + 20 min. elution	114	105	95	101	117	118	116	111	113
PK during binding + RDD	123	115	119	118	133	114	126	117	119

Figure 41 Comparison between further versions of the automated protocol
The mean DNA recovery of the short 66 bp amplicon of the 18S ribosomal DNA assay was determined for eight healthy individuals. The standard Proteinase K protocol was further improved and tested against a protocol version with a longer elution time (20 instead of 5 min.) or including the additional Proteinase K/RDD step. Significance of differences between tested conditions was calculated using Dunnett's method (JMP software). The addition of Proteinase K during binding was defined as control group again. It was different compared to PK during binding and an extended elution time ($p < 0.0001$***) as well as PK during binding and an additional RDD step ($p = 0.0432$*).

This time 1,000 µL 35 mM MES + 0.1% TritonX-100 + 300 mM sodium chloride at pH 5.8 and 300 µL 35 mM MES + 0.1% TritonX-100 at pH 5.8 were used for the first and second wash step. A third wash step was then added using 35 mM TRIS-HCl at pH 7.5 and DNA was eluted in 40 mM sodium hydroxide instead of 16.7 mM sodium hydroxide for elution to enable a further increase of DNA recovery. The pipette order was changed, so plasma sample was added last into the binding mixture to prevent possible cross-contamination without any negative influence on DNA recovery (data not shown). Protocol versions using 2 and 4 mL as the sample input volume as well as 75 and 150 µL elution volume were comparable and led to about 100% DNA recovery compared to the QIAamp cNA Kit (Fig. 42).



	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Median
2 mL protocol (150 µL elution)	103	101	95	115	118	97	95	89	99
2 mL protocol (75 µL elution)	97	100	91	108	110	94	94	89	96
4 mL protocol (150 µL elution)	109	108	108	124	117	98	98	89	108
4 mL protocol (75 µL elution)	102	97	96	113	105	91	90	83	96

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Median
2 mL protocol (150 µL elution)	111	111	103	130	123	92	101	98	107
2 mL protocol (75 µL elution)	95	112	103	124	105	89	95	90	99
4 mL protocol (150 µL elution)	97	102	106	130	115	87	102	99	102
4 mL protocol (75 µL elution)	83	92	95	117	101	79	85	92	92

Figure 42 Test of different sample and elution volumes
The mean recovery was calculated for eight healthy individuals using the 18S ribosomal DNA assay for the detection of the 66 (**A**) and 500 bp (**B**) amplicon. The final standard Proteinase K protocol was tested with 2 and 4 mL plasma as the sample input material and two different elution volumes (75 and 150 µL) were used. Significance of differences between tested conditions was calculated using Dunnett's method (JMP software). The QIAsymphony cDNA protocol with 4 mL sample input and 150 µl elution volume was defined as control group. While differences using the 18S 66 bp assay were significant for lower elution volumes (p< 0.0001***), lower sample input volumes (p= 0.0390*) as well as by reducing sample input and elution volume at the same time (p= 0.0002***), detecting the 500 bp DNA fragment of the 18S assay, only the single reduction of elution volume led to a significant difference (p<0.0001***).

The wash buffers were then changed one last time to ideally increase the release of proteins and other plasma components during wash step without losing DNA. 35 mM MES + 0.1% TritonX-100 with and without 300 mM sodium chloride were still used, but this time at pH 6.7. The third wash step was performed with 35 mM TRIS-HCl at pH 8.5. Besides the buffers, the protocol adjustments were optimized as well, so by raising the number of

magnetic bead separation steps after elution from 3 to 10, it was possible to prevent the bead carry-over into the eluate. The latest versions of the newly developed QIAsymphony cDNA Protocol were used for the proof of principle described in chapter 3.4.

3.3. Automation of DNA Bisulfite Conversion

For the initially planned translational experiments mentioned in 3.1.4, DNA bisulfite conversion for methylation analysis was supposed to be automated. In addition to the newly developed DNA extraction protocol, it was tried to automate the EpiTect Fast DNA Bisulfite Kit for the subsequent methylation analysis of ccfDNA. With the development of the QIAsymphony cDNA Protocol, the basis for the automation of further protocols and methods was established as demonstrated by the successful automation of the Bisulfite Kit. Four µL 3 M sodium hydroxide were therefore added to 36 µL of the eluate (TE buffer) after QIAamp cNA Kit performance for DNA denaturation, because sample heat treatment was limited according to the possible maximum temperature using the QIAsymphony SP instrument. The primary protocol version was developed and optimized according to the manual Kit. The total denatured sample volume was 40 µL. An initial protocol version, applicable for planned automation on the QIAsymphony SP instrument in theory, was developed in cooperation with the responsible team at QIAGEN. 40 µL eluate were transferred via the QIAsymphony from sample tubes in SPC and 15 µL DNA protection buffer as well as 105 µL bisulfite solution was automatically added, followed by an incubation for 40 min. at 80°C to perform bisulfite conversion of single-stranded DNA. Due to the use of magnetic anion exchange beads instead of silica membranes, buffers for the binding, wash and elution steps had to be adapted. To adjust optimal DNA binding conditions using magnetic anion exchange beads, 1,000 µL H_2O as well as 65 µL beads were pipetted after incubation and binding started. After 20 min. at room temperature, beads were separated and transferred into 485 µL wash buffer (20 mM TRIS-HCl + 0.1% TritonX-100 at pH 7). After mixing for about 5 min., 500 µL buffer BD was added and incubation for 15 min. at room temperature began for DNA desulfonation. For buffer BD preparation, H_2O instead of ethanol was added. Ethanol is needed for silica chaotrope based methods like the manual EpiTect Fast DNA Bisulfite Kit, but pH value of buffer BD is the only crucial factor when using an anion exchange based protocol. The binding conditions were then adjusted again using 656 µL 1% acetic acid. After 20 min., the beads

were separated and three wash steps were performed using 2*485 µL 20 mM TRIS-HCl + 0.1% TritonX-100 (pH 7). DNA was eluted in 100 µL 35 mM TRIS base + 40 mM sodium chloride within 20 min. at room temperature. To improve the first protocol version, binding conditions were adjusted using more RNase-free H_2O (1,800 instead of 1,000 µL) to further dilute the salt concentration of the DNA mixture for bisulfite conversion. Furthermore, the bead volume was increased (from initial 65 to 75 µL) and the wash buffers as well as volumes were changed. 35 mM MES + 0.1% TritonX-100 (pH 5.8) was used and 1,000 and 300 µL instead of 2*485 µL were pipetted for the second wash step to gradually reduce the wash buffer volume and minimize the loss of beads bound to the plastic of SPC tubes. Finally, elution buffer volume was raised from 100 to 150 µL to improve elution conditions. The DNA recovery compared to the manual method was improved and further increased after using 2,456 µL 0.7% acetic acid for DNA binding after desulfonation. The CFF quantitative real-time PCR was performed to analyze the possible DNA loss during bisulfite treatment. Looking at the median value, about 115% were reached using the latest version of the QIAsymphony DNA Bisulfite Protocol compared to the EpiTect Fast DNA Bisulfite Kit, but preparation of the EpiTect Fast DNA Bisulfite Kit was not performed under optimal conditions. In addition to heat denaturation, sodium hydroxide was added to the eluates (Fig. 43).

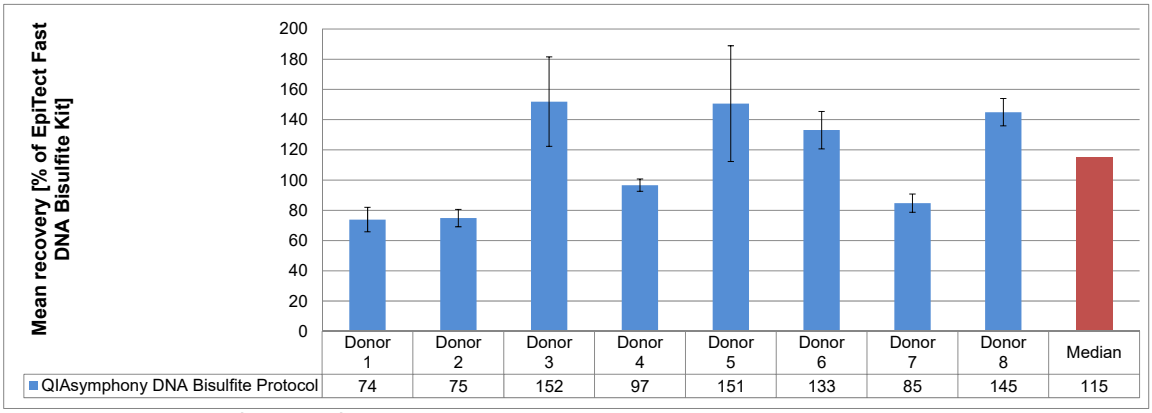


Figure 43 DNA loss after bisulfite conversion
DNA concentration of the QIAamp cNA Kit eluate was determined before and after bisulfite treatment using the EpiTect Fast DNA Bisulfite Kit and the newly developed QIAsymphony DNA Bisulfite Protocol, so the CFF DNA assay was used. DNA recovery of the QIAsymphony DNA Bisulfite Protocol compared to the EpiTect Fast DNA Bisulfite Kit is shown here for eight individual donors.

The usefulness of the newly developed QIAsymphony cDNA Protocol for the automation of further methods (in this case the DNA bisulfite conversion) was demonstrated.

3.4. Proof of principle

The newly developed large volume protocol for DNA extraction on the QIAsymhony SP instrument was optimized using plasma from individual healthy donors from our in-house blood donation for QIAGEN employees. As the protocol was developed for subsequent diagnostic use, it had to be tested with real patient samples. As “proof of principle,” plasma samples from patients (pregnant women, cancer as well as non-cancer patients) were processed using the newly developed automated QIAsymphony cDNA Protocol and the manual QIAamp cNA Kit. As previously mentioned, DNA concentration varies between different samples and donors, so there is no expected DNA level to rate performance of the newly developed automated protocol. That is why the named manual DNA extraction method, which is widely used in the field by many customers, was used as reference for the newly developed automated protocol. The aim was to at least reach comparable results between these two methods.

3.4.1. Prenatal diagnostics

One of the most important and already established application fields is prenatal diagnostics, so the QIAsymphony cDNA Protocol was tested using plasma from pregnant women, provided by “Praenatal-Medizin und Genetik” (Prenatal Medicine and Genetics) in Düsseldorf. In this case, 4 mL plasma was processed with the QIAsymphony cDNA Protocol and 1 mL plasma was used as sample input material for the QIAamp cNA Kit. Results from an early version of the automated protocol are shown in Figure 44 (see protocol version 5 in the attachment). Male fetal DNA was detected targeting a specific region of the DYS14 gene. The fetal DNA fraction was determined by calculating the ratio between Dys14 and the total DNA amount in the form of 18S ribosomal DNA (66 bp amplicon).

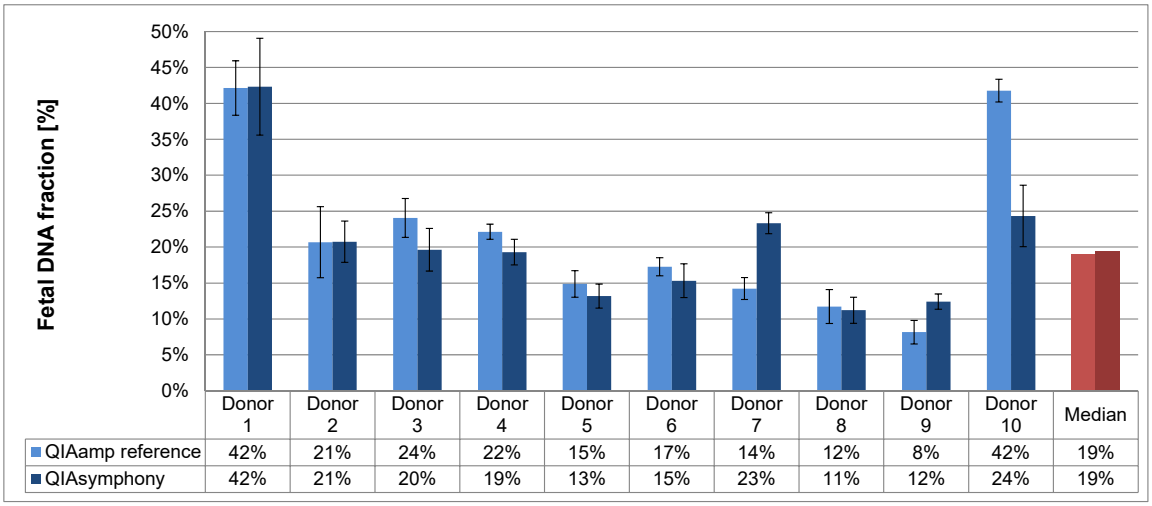


Figure 44 Determination of fetal DNA fraction
For this experiment, plasma samples from ten pregnant women with a male fetus were used. The Dys14/18S 66 bp DNA duplex assay was performed to determine the fetal DNA fraction in maternal plasma. The Dys14 gene is located on the Y-chromosome, so it is specific to the fetus. Total DNA was detected with the help of the 66 bp amplicon of the 18S ribosomal DNA assay. Consequently, fetal DNA fraction was calculated by the ratio between Dys14 and 18S DNA. The fetal DNA fraction is shown for the QIAsymphony cDNA Protocol and the QIAamp cNA Kit.

Total DNA recovery was between 50 and 100% for the QIAsymphony protocol compared to the QIAamp cNA Kit (Fig. 45), but the fetal fraction was very comparable between these two methods (Fig. 44).

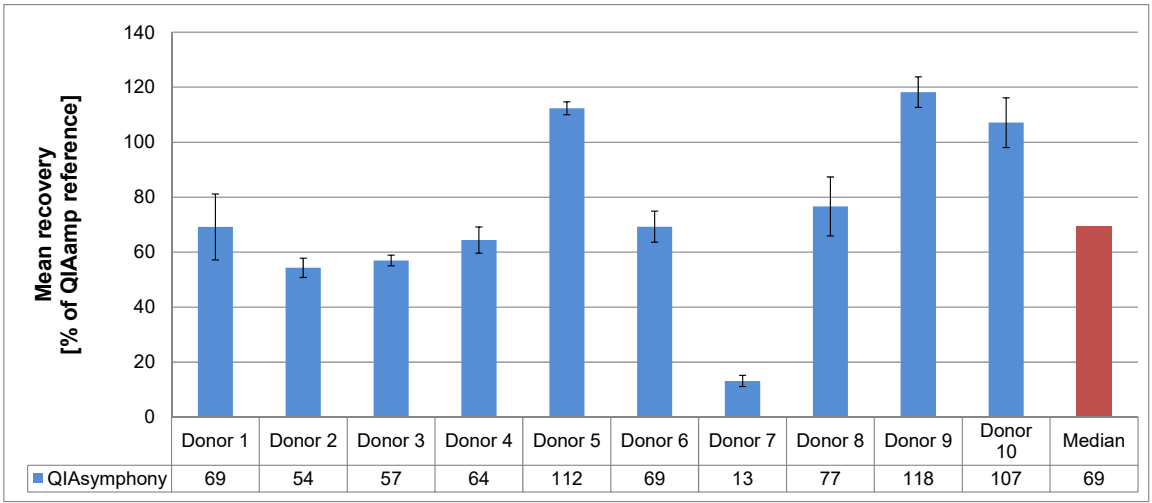


Figure 45 Total DNA recovery in plasma from pregnant women
Mean recovery of total DNA after plasma processing of ten pregnant women with the QIAsymphony cDNA Protocol was determined using the 18S ribosomal DNA assay for the short 66 bp amplicon.

3.4.2. Cancer diagnostics

During this PhD thesis, the focus was on cancer diagnostics. Therefore, two studies were realized in cooperation with Sysmex Inostics GmbH in Hamburg and the Surgical

Research Laboratory of the University Hospital in Düsseldorf. Sysmex Inostics provided plasma samples from 24 colorectal cancer patients, while plasma samples of the 92 samples collected in Düsseldorf were derived from a heterogeneous patient group comprised of many different cancer types. The control group, consisting of patients suffering from non-malignant diseases and healthy donors from QIAGEN blood donations, was comparably small. Matched tissue samples or other reference material was not available. As a result, a “proof of principle” was done, comparing the newly developed QIAsymphony cDNA Protocol with the QIAamp cNA Kit within these two different studies based on clinical samples and using various analysis methods.

3.4.2.1. Cooperation with Sysmex Inostics GmbH in Hamburg

In cooperation with the Sysmex Inostics GmbH in Hamburg, approximately 4 mL plasma from 24 colorectal cancer patients was used to test the newly developed automated protocol against the manual reference method. Besides total DNA recovery, mutation status of the *KRAS* oncogene was determined comparing two different PCR methods.

BEAMing digital PCR technology

One of these PCR methods was BEAMing digital PCR technology. Before clinical samples were analyzed, the compatibility between QIAsymphony eluates and BEAMing digital PCR technology was tested. While amplification and gel electrophoresis before BEAMing analysis as well as digital PCR itself were less sensitive concerning the eluate quality, *LINE*-1 quantitative real-time PCR for total DNA quantification seemed to be prone to PCR inhibition caused by contaminants and the elution buffer compounds. To be able to perform mutation analyses in healthy donors, *KRAS* DNA fragments were spiked into samples, but the first spike-in system failed and almost no spiked DNA fragments were detected by BEAMing digital PCR technology using the QIAamp cNA Kit as the DNA extraction method due to the single-stranded character of the *KRAS* DNA fragment spike-in system used (Tab. 23).

Table 23 Calculation of mutant fraction using BEAMing digital PCR technology
BEAMing digital PCR technology was performed for four healthy individuals. For the QIAsymphony cDNA Protocol, two different elution buffers were tested and compared against the QIAamp cNA Kit reference. Since wild type as well as mutant DNA were measured, the percentage of mutant fraction was calculated. Both used elution buffers, wild type plasma, a negative and a mutant control were also tested.

Sample	Mutant fraction [%]		
	QIAsymphony (35 mM TRIS base + 40 mM sodium chloride)	QIAsymphony (35 mM TRIS base + 80 mM sodium chloride)	QIAamp reference
Donor 3	16.25	9.61	1.04
Donor 4	6.43	7.5	0.54
Donor 5	6.75	9.62	0.95
Donor 6	7.21	8.67	0.82

Control	Mutant fraction [%]
35 mM TRIS base + 40 mM sodium chloride	0.00
35 mM TRIS base + 80 mM sodium chloride	0.00
Wt-Pl	0.00
MutKo_015	11.86
NegKo	0.00

The mutant fraction was (disregarding Donor 3) higher for 35 mM TRIS base + 80 mM sodium chloride. Elution buffers, wild type plasma and negative control did not contain any mutant DNA fragments. The mutant fraction in the mutant control, which should ideally correspond to the mutant fraction in samples, was about 12%.
Furthermore, as displayed in Figure 46, DNA recovery using the *LINE*-1 assay was low.

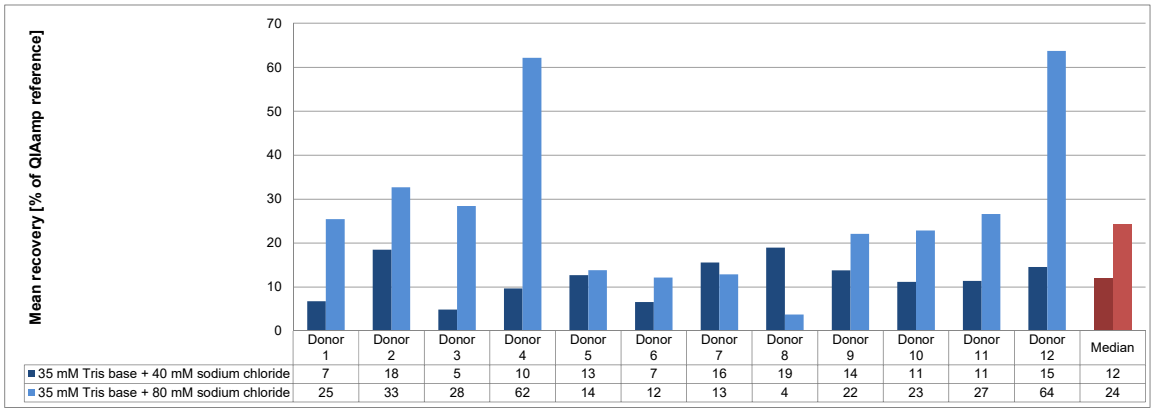
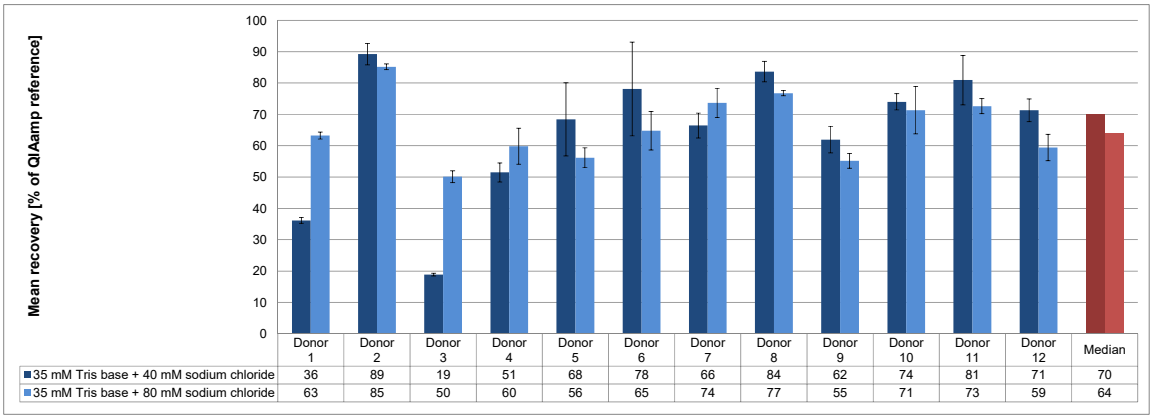


Figure 46 *LINE*-1 quantitative real-time PCR results of Sysmex Inostics GmbH
Plasma samples from twelve healthy individuals were processed with the QIAsymphony cDNA Protocol and two different elution buffers were used. The *LINE*-1 DNA quantitative real-time PCR assay was performed at Sysmex Inostics GmbH. Mean DNA recovery compared to the QIAamp cNA Kit is shown here. Paired two-tailed t-test (Excel) was used to calculate the significance level between tested elution buffers, which was p= 0.0212*.

By detecting ribosomal 18S DNA (66 bp amplicon), up to 70% was reached as the median value for the twelve tested donors, as shown in Figure 47.



	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Donor 9	Donor 10	Donor 11	Donor 12	Median
35 mM Tris base + 40 mM sodium chloride	36	89	19	51	68	78	66	84	62	74	81	71	70
35 mM Tris base + 80 mM sodium chloride	63	85	50	60	56	65	74	77	55	71	73	59	64

Figure 47 Test of two different elution buffers for Sysmex Inostics GmbH
5 mL plasma from twelve healthy individuals (after the addition of 1.4 mL RNase-free H_2O) was processed with the QIAsymphony cDNA Protocol. Therefore, two different elution buffers were used. The 18S ribosomal DNA assay was performed for the calculation of mean DNA recovery compared to the QIAamp cNA Kit. Paired two-tailed t-test (Excel) showed that the difference between tested elution buffers was not significant (p=0.8102).

The DNA recovery after plasma processing with protocol version 6 of the QIAsymphony cDNA Protocol (further described in the attachment) was significantly lower with detecting the 500 bp amplicon of the 18S ribosomal DNA assay (data not shown), which possibly indicates a PCR-inhibitory effect of the eluate, possibly causing the comparably low DNA recovery using the *LINE*-1 assay with a potentially less robust PCR chemistry. Consequently, rather PCR-inhibitory effects than insufficient DNA recovery using the newly developed QIAsymphony cDNA Protocol seemed to be responsible for the declining results after performing the *LINE*-1 quantitative real-time PCR assay for DNA quantification.

As a result, the experiment was repeated using a new spike-in system, the commercially available *KRAS* gBlocks. As expected, the double-stranded DNA fragments were successfully extracted using the QIAamp cNA Kit, but DNA recovery of the newly developed QIAsymphony protocol was still low compared to the QIAamp cNA Kit performing the *LINE*-1 assay for DNA quantification (Fig. 48).

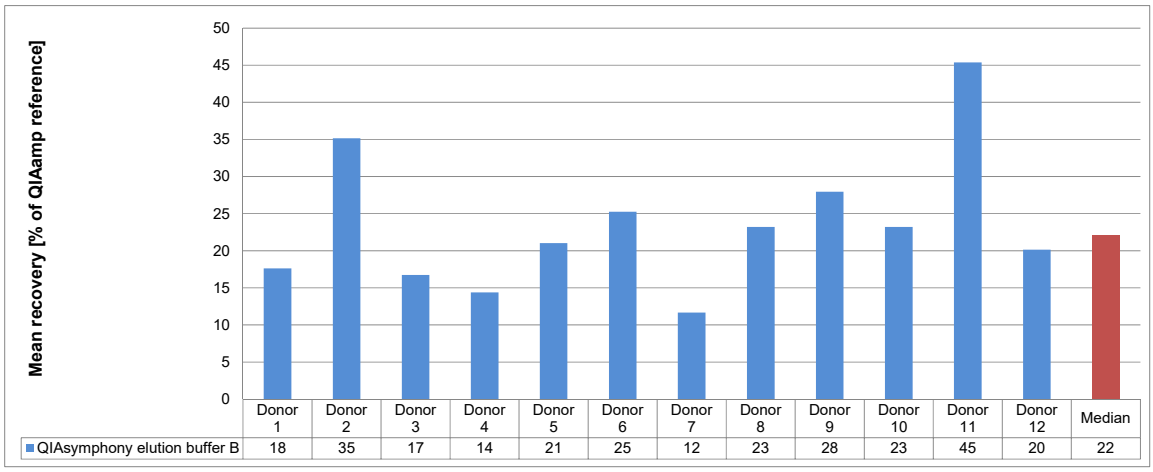


Figure 48 *LINE*-1 PCR (Sysmex Inostics GmbH)
After further optimization of the QIAsymphony cDNA Protocol with 35 mM TRIS base + 80 mM sodium chloride as an elution buffer, the plasma samples from twelve healthy individuals were processed again. The *LINE*-1 DNA quantitative real-time PCR assay was performed at Sysmex Inostics GmbH and mean DNA recovery compared to the QIAamp cNA Kit is displayed here.

Eluates, after performance of protocol version 7 of the QIAsymphony cDNA Protocol, contained slimy pellets, which might be caused by proteins, so the newly developed protocol was further optimized and DNA recovery as determined by *LINE*-1 DNA quantification was improved (data not shown). The most important change was the use of Proteinase K, which was already previously tested, but now, in combination with other buffers, it led to the expected improvement of DNA recovery and purer eluates, so a new version of the QIAsymphony protocol for 4 mL plasma was used (protocol version 8, further described in the attachment). Two different binding buffers were tested:

1. 50 mM MES + 0.1% TritonX-100 + 300 mM sodium chloride (final concentration in binding step) at pH 4.5
2. 40 mM citric acid + 0.1% TritonX-100 + 200 mM sodium chloride (final concentration in binding step) at pH 4.5

Figure 49 shows the results for the 18S ribosomal DNA assay.

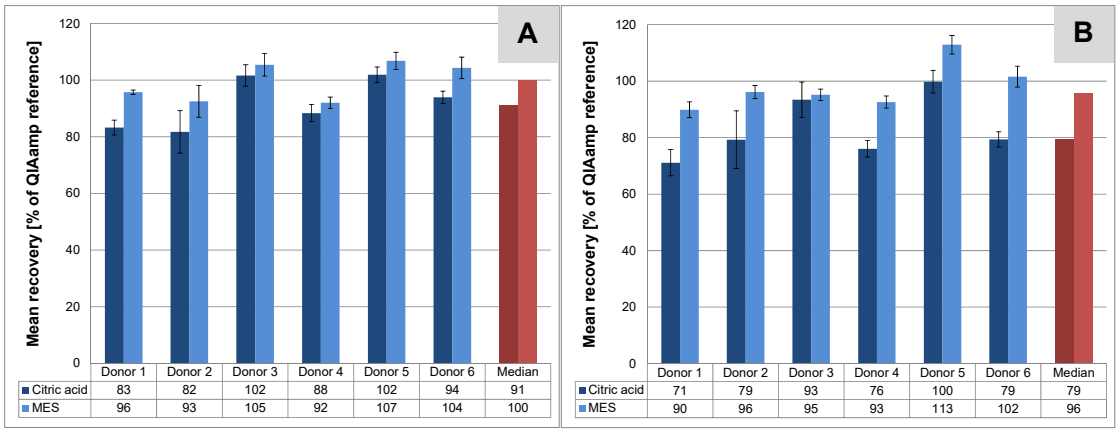


Figure 49 Test of binding buffers for Sysmex Inostics GmbH
Mean DNA recovery of the QIAsymphony cDNA Protocol was determined for six healthy individuals using the 18S ribosomal DNA assay, so two different binding buffers were tested. Results for the 66 (**A**) and 500 bp (**B**) amplicon are shown here. Paired two-tailed t-test (Excel) was used to calculate the significance levels between binding buffers, which were p= 8.26882*10^-6*** (66 bp) and p= 2.6355*10^-6*** (500 bp).

By using MES buffer for binding, the standard QIAsymphony protocol led to similar DNA recoveries compared to the RDD version, which was used before (see results in Figure 40). The RDD step was therefore removed for the next experiment and, according to previous results, only MES was used as the binding buffer. Wash and elution conditions were the same. This time 2 mL plasma was processed using the newly developed QIAsymphony cDNA Protocol and the QIAsymphony Custom Protocol with a “Virus Cellfree 1000” protocol version for 2 mL plasma (Fig. 50).

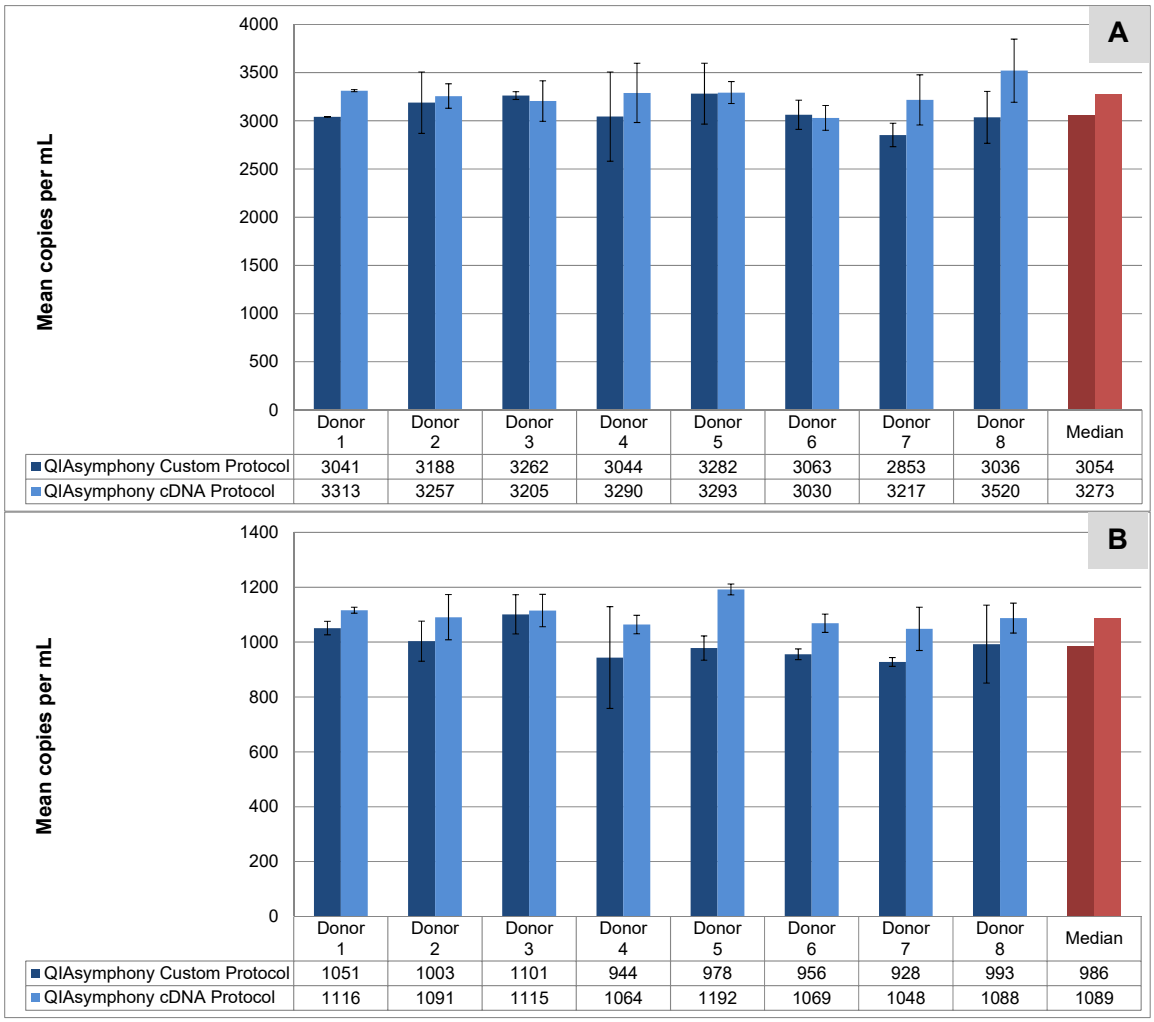


Figure 50 Comparison between the QIAsymphony Custom and cDNA Protocol
The QIAsymphony Custom Protocol was also used as an interim solution by Sysmex Inostics GmbH. As a result, it was compared to the performance of the QIAsymphony cDNA Protocol. The mean copies per mL for eight healthy individuals using the 18S ribosomal DNA assay for quantitative real-time PCR are shown here. Diagram **A** shows the results of the 66 bp amplicon and results for the 500 bp amplicon are shown in Diagram **B**. Paired two-tailed t-test (Excel) was used to calculate significance levels between protocol versions, which were p= 0.0145* (66 bp), p= 0.0001*** (500 bp).

The QIAamp cNA Kit reference led to higher DNA recoveries, but it was processed at Sysmex Inostics GmbH (data not shown) and therefore handling procedures like plasma sample preparation differ from automated methods (QIAsymphony Custom and cDNA Protocol), performed at QIAGEN GmbH. The compatibility of QIAsymphony eluates and BEAMing digital PCR technology was successfully tested and 2*2 mL plasma from 24 colorectal cancer patients, provided by Sysmex Inostics GmbH, was processed using the QIAsymphony cDNA Protocol and the QIAamp cNA Kit. *KRAS* mutation status was determined by BEAMing digital PCR technology. The QIAamp cNA Kit was used according to the handbook with 150 µL AVE for elution. Version 9 was used for the QIAsymphony

cDNA Protocol. Total DNA quantification was performed by Sysmex Inostics GmbH using the *LINE*-1 quantitative real-time PCR assay. Median DNA recovery for all 24 patients was about 90% for the QIAsymphony cDNA Protocol compared to the QIAamp cNA Kit (Fig. 51).

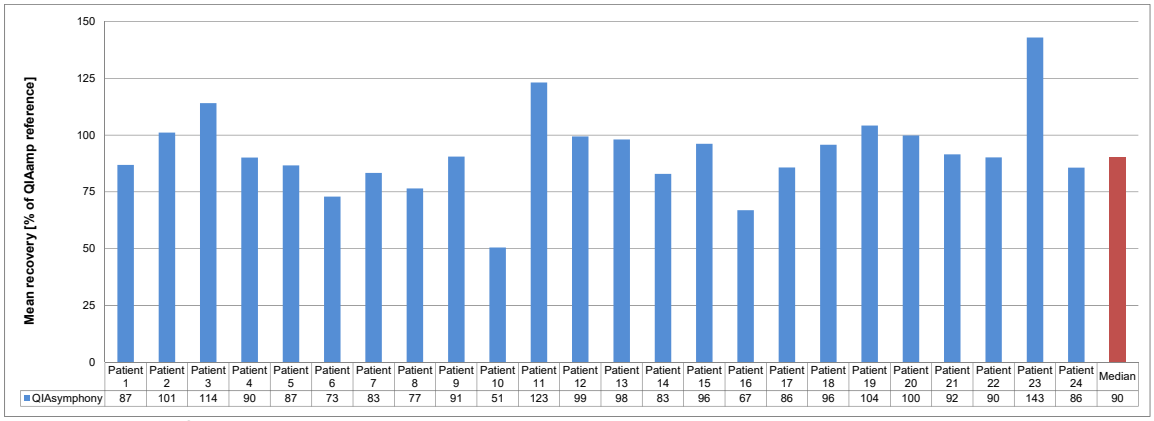
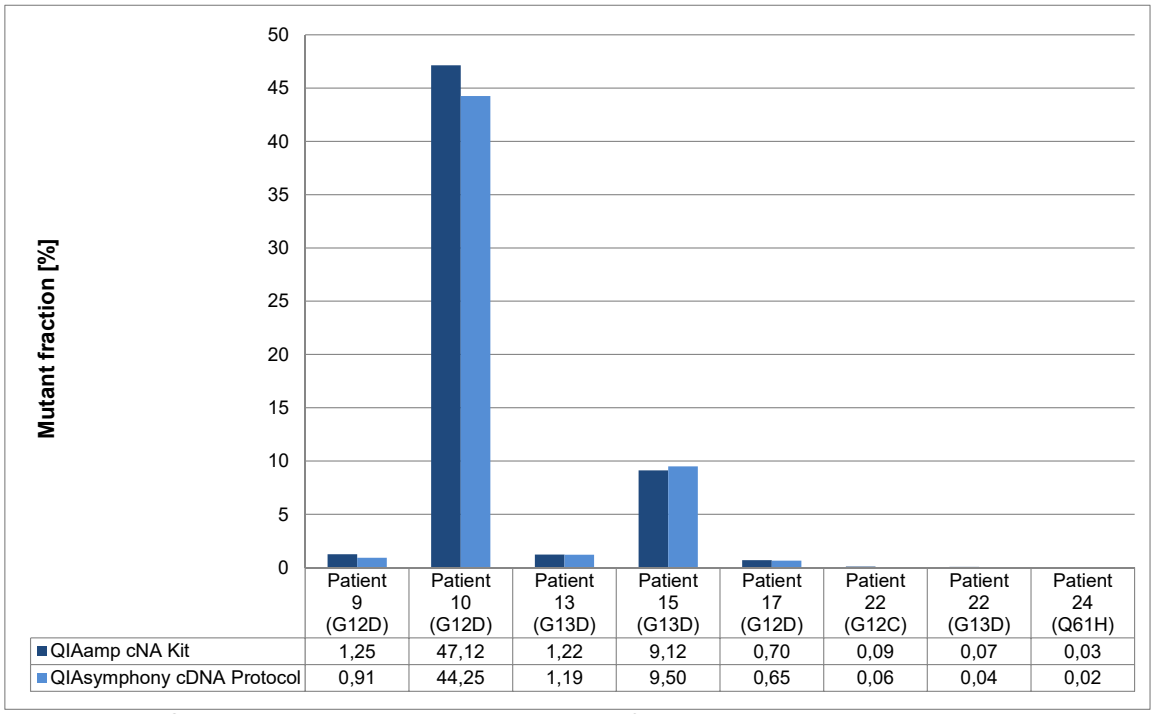


Figure 51 Test of the optimized cDNA Protocol with clinical samples (*LINE*-1 results)
The QIAsymphony cDNA Protocol was further improved and tested with plasma samples from 24 colorectal cancer patients. *LINE*-1 DNA assay was performed at Sysmex Inostics GmbH and mean DNA recovery compared to the QIAamp cNA Kit was calculated.

As displayed in Figure 52, seven of the 24 patients were *KRAS* mutant, whereas Patient 22 showed two different *KRAS* mutations. The mutant fraction ranged from 0.02 to 47% and results were very comparable between both tested DNA extraction methods.



	Patient 9 (G12D)	Patient 10 (G12D)	Patient 13 (G13D)	Patient 15 (G13D)	Patient 17 (G12D)	Patient 22 (G12C)	Patient 22 (G13D)	Patient 24 (Q61H)
QIAamp cNA Kit	1,25	47,12	1,22	9,12	0,70	0,09	0,07	0,03
QIAsymphony cDNA Protocol	0,91	44,25	1,19	9,50	0,65	0,06	0,04	0,02

Figure 52 *KRAS* mutation BEAMing analysis in plasma of colorectal cancer patients

Plasma samples from 24 colorectal cancer patients were processed with the QIAamp cNA Kit and the newly developed QIAsymphony cDNA Protocol. *KRAS* mutation analysis was performed by BEAMing digital PCR technology at Sysmex Inostics GmbH. G12C, G12D, G13D and Q61H were the occurring *KRAS* mutations. The mutant fraction in % is shown here.

Therascreen *KRAS* RGQ PCR Kit

KRAS mutations, which are included in the therascreen *KRAS* RGQ PCR Kit, were also analyzed by the therascreen *KRAS* RGQ PCR Kit as an alternative method to show its possible applicability for ccfDNA from plasma. As such, three of the four different mutation types were analyzed (G12C, G12D and G13D). For all patients, except Patient 22, present mutations determined by BEAMing digital PCR technology were also detected using the therascreen *KRAS* RGQ PCR Kit. Results were comparable between both methods. Mutation G12C was identified for Patient 22 using the QIAamp cNA Kit, but there was not enough eluate left for the QIAsymphony cDNA Protocol. In contrast, the same patient's mutation G13D was tested for both methods, but it was not detectable in any case, taking into account that Patient 22 had the lowest mutant fraction, which was between 0.04 and 0.09%. Results are displayed in Table 24.

Table 24 *KRAS* mutation analysis using the therascreen *KRAS* RGQ PCR Kit
Three different *KRAS* mutations were tested by the therascreen *KRAS* RGQ PCR Kit. Six patients were regarded for which the mutant status was confirmed by BEAMing digital PCR technology. The results were compared between the QIAamp cNA Kit and the QIAsymphony cDNA Protocol. The difference between Ct values of samples and controls (=total amount of the non-mutated *KRAS* DNA fragments) were calculated (the delta Cts). The delta Ct cut-off values are listed in the handbook, but they were determined for DNA from FFPE tissue samples. If delta Ct values are below these cut-off values, a sample is defined as mutant. Otherwise it is wild type. Results marked in red did not result in any Ct values. Results emphasized in green are clearly positive for the tested *KRAS* mutation and results in orange led to Cts above the provided cut-off values.

Patient	*KRAS* mutation	Delta Ct (=Ct_{sample}-$Ct_{control}$)		$Cut\text{-}off_{delta\ Ct}$
		QIAamp cNA Kit	QIAsymphony cDNA Protocol	
9	G12D	(7)	(6.7)	6.6
10	G12D	0.9	1.1	6.6
13	D13D	7.4	7.9	8
15	D13D	3.9	4.4	8
17	G12D	6.3	(8.1)	6.6
22	G13D	N/A	N/A	7.5
	G12C	No eluate	N/A	8

3.4.2.2. Cooperation with the Surgical Research Laboratory of the University Hospital in Düsseldorf

Quantitative real-time PCR

Most patient samples were provided by the Surgical Research Laboratory of the University Hospital in Düsseldorf. Samples from 91 cancer patients were collected and plasma

samples before and after surgery exist for 51 of them. However, the group of cancer patients was very heterogeneous. Furthermore, only plasma samples from 15 non-cancer patients (for three patients, samples before and after surgery exist) were available as controls. Protocol version 10 (see attachment) of the QIAsymphony cDNA Protocol was used. Compared to protocol version 7, the pipette order of the binding step was changed so that plasma was added at the end to prevent cross-contamination. Furthermore, the pH value of wash buffers was raised to eliminate more proteins and generate purer eluates containing less contaminating material. 2*4 mL plasma was processed using the QIAsymphony cDNA Protocol and the QIAamp cNA Kit according to the handbook with elution in 75 µL AVE and without Carrier-RNA. Total DNA recovery was first determined by quantitative real-time PCR.

18S ribosomal DNA assay

One quantitative real-time PCR assay used was the 18S ribosomal DNA assay. In addition to the non-cancer patients, the plasma from 11 healthy donors as well as two aliquots of a plasma pool from QIAGEN blood donations was analyzed. Results were comparable between the short 66 and long 500 bp amplicon. While the median value for DNA recovery compared to the QIAamp cNA Kit was about 100% for healthy donors, only about 80% DNA recovery was reached in most cases for cancer as well as non-cancer patients suffering from non-malignant diseases (Fig. 53).

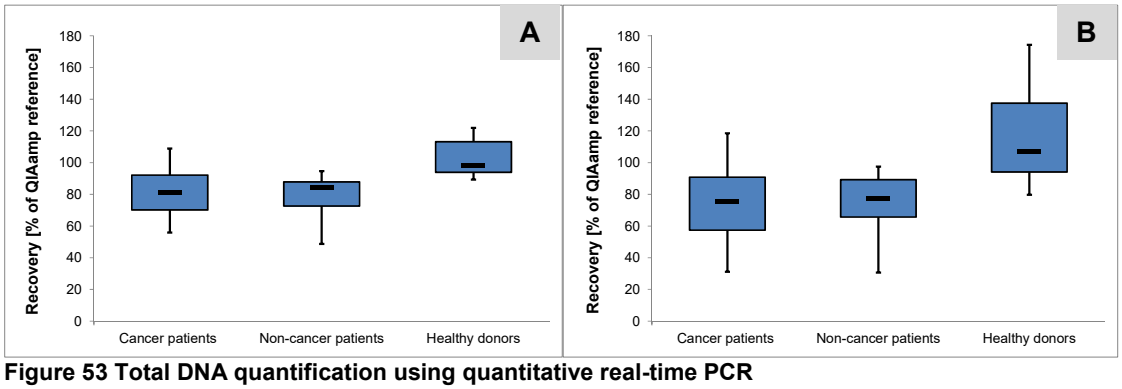


Figure 53 Total DNA quantification using quantitative real-time PCR
Total DNA was quantified using the 18S ribosomal DNA assay (A = 66 and B = 500 bp amplicon); therefore, plasma samples from 91 cancer patients, 15 non-cancer patients (for three patients, samples before and after surgery exist) and 11 healthy individuals as well as two aliquots of a plasma pool were processed with the QIAsymphony cDNA Protocol. Recovery compared to the QIAamp cNA Kit reference is displayed in the form of box plots. The horizontal line within each box represents the median of each sample group (cancer, non-cancer and healthy individuals). The limits of each box mark the 25th and 75th percentiles. Standard deviations show the 5th and 95th percentiles. Significance of differences between tested sample groups was calculated using Dunnett's method (JMP software). Healthy donors were defined as control group. For both amplicons of

the 18S DNA assay (66 and 500 bp), DNA recovery from plasma samples of healthy donors was significant higher than for cancer ($p<0.0001$***) or non-cancer patients ($p<0.0001$***).

After surgery, DNA levels were mostly elevated, so the 66:500 bp ratio was built to analyze possible differences in size distribution and increased, decreased and unchanged ratio values were detected (data not shown). Consequently, increasing DNA concentrations cannot be explained by the release of only short or long (≥500 bp) DNA fragments.

Therascreen *KRAS* RGQ PCR

Total DNA including tumor and non-tumor DNA in plasma of cancer patients was successfully quantified using the 18S ribosomal DNA assay. Mutated *KRAS* DNA was detected for the sole determination of tumor DNA extraction efficiency. Since *KRAS* is mostly mutated in tumor DNA, discrimination between tumor and non-tumor DNA was possible this way, so *KRAS* DNA mutation status was determined using the therascreen *KRAS* RGQ PCR Kit. *KRAS* is known to be strongly mutated in colorectal and pancreatic cancer patients, for example. For this reason, plasma samples for these two cancer types were chosen for PCR analysis. *KRAS* mutant DNA corresponds to tumor DNA, so total and especially DNA of tumor origin was detected by *KRAS* mutation detection to demonstrate the protocol ability for tumor DNA extraction. *KRAS* was chosen because of its high mutation frequency in many cancer types. It was important to enhance the chance of mutation detection in a relative small patient cohort. Sample Ct values were comparably high for pancreatic cancer patients, so only results for colorectal cancer patients are shown (Tab. 25). Four of the 24 patients were clearly mutated, whereas one patient had two different *KRAS* mutations. Four of the six *KRAS* mutations led to comparable results between both tested methods. Only two mutations, for which the calculated delta Ct was above the cut-off value specified for FFPE tissue, were inconsistent between the tested methods.

Table 25 Tumor DNA detection using the therascreen *KRAS* RGQ PCR Kit
The therascreen *KRAS* RGQ PCR Kit enables the detection of seven different *KRAS* mutations. Twenty-four colorectal cancer patients were analyzed. Results were compared between the QIAamp cNA Kit and the QIAsymphony cDNA Protocol. The difference between Ct values of samples and controls (=total amount of the non-mutated *KRAS* DNA fragments) were calculated (delta Cts). The delta Ct cut-off values are listed in the handbook, but they have been determined for DNA from FFPE tissue samples. If delta Cts are below these cut-off values, a sample is defined as mutant. Otherwise it is wild type. Results emphasized in green are clearly positive for the tested *KRAS* mutation and results in orange led to Cts above the provided cut-off values.

Patient	*KRAS* mutation	Delta Ct (=Ct_{sample}-$Ct_{control}$)		$Cut\text{-}off_{delta\ Ct}$
		QIAamp cNA Kit	QIAsymphony cDNA Protocol	
3vB	G12D	3.81	3.78	6.6
8vB	G12D	8.06	N/A	6.6
85vB	G12V	4.14	4.86	7.5
86vB	G12A	6.9	7.66	8
	G12V	1.14	1.16	7.5
100vB	G12A	N/A	10,51	8

Qubit dsDNA quantification

Besides quantitative real-time PCR, total DNA in plasma from cancer patients was also quantified measuring double-stranded DNA using the Qubit fluorometer. Individual results differed between quantitative real-time PCR and Qubit measurement, but median values were comparable. Therefore, about 100% DNA recovery was achieved compared to the QIAamp cNA Kit and about 80% for the cancer patients and non-cancer controls were reached (Fig. 54).

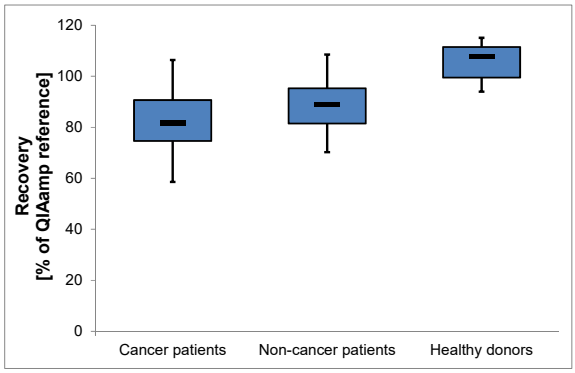


Figure 54 Total DNA quantification by Qubit fluorometer
Total DNA was also quantified by measuring double-stranded DNA using the Qubit fluorometer. Recovery is once again displayed for cancer and non-cancer patients as well as healthy controls in the form of box plots. Significance of differences between tested sample groups was calculated using Dunnett's method (JMP software). Healthy donors were defined as control group. DNA recovery from plasma samples of healthy donors compared to the QIAamp cNA Kit was significantly higher than for cancer ($p<0.0001$***) or non-cancer patients ($p<0.0008$***).

DNA integrity

As already mentioned before, DNA integrity index is supposed to be increased in plasma of cancer patients [86]. So, data shown in Fig. 53 was reanalyzed and total cancer patients were additionally divided into cancer patients whose blood was taken before or after surgery. Results are shown in the following figure (Fig. 55)

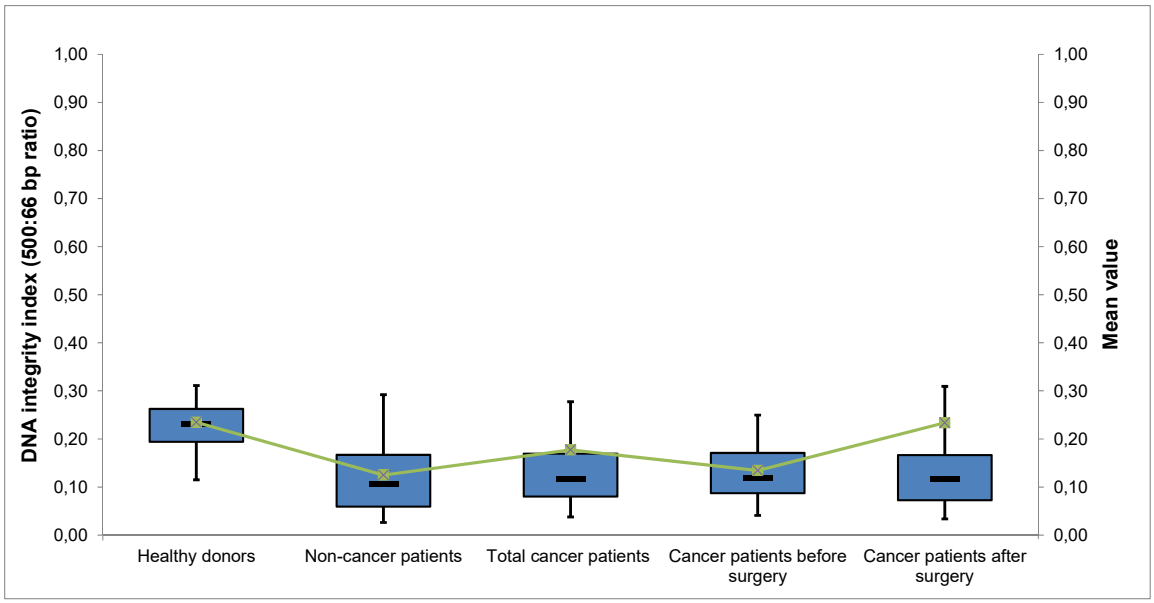


Figure 55 DNA integrity index
Total DNA was quantified using the 18S ribosomal DNA assay detecting the 66 and 500 bp amplicon; therefore, plasma samples from 91 cancer patients (81 with samples collected before and 61 with samples collected after surgery), 15 non-cancer patients (for three patients, samples before and after surgery exist) and 11 healthy individuals as well as two aliquots of a plasma pool were processed with the QIAamp cNA Kit reference. The DNA integrity index is the ratio between the amount of long (represented by the 500 bp amplicon) and short (represented by the 66 bp amplicon) DNA fragments. Ratio values are displayed in the form of box plots. The horizontal line within each box represents the median value of each sample group (cancer, non-cancer and healthy individuals). The limits of each box mark the 25th and 75th percentiles. Standard deviations show the 5th and 95th percentiles. In addition, mean values of each sample group are displayed in green. Using one-way ANOVA, differences between tested conditions were evaluated as non-specific (JMP software).

Agilent virtual gel electrophoresis

It was tried to analyze the size distribution of eluted DNA fragments from cancer patients, non-cancer patients and healthy individuals again, but this time using the Agilent instrument for virtual gel electrophoresis. Results were comparable to quantitative real-time PCR in some cases. If an increase of ratio value was detected by 18S ribosomal DNA assay, the amount of longer DNA fragments (≥500 bp) determined after Agilent virtual gel electrophoresis decreased after surgery. If a decrease in ratio value was seen, the DNA

amount of longer DNA fragments increased and it was nearly unchanged when ratio values before and after surgery were similar. Results from quantitative real-time PCR are shown for DNA from three patient samples in Figure 56.

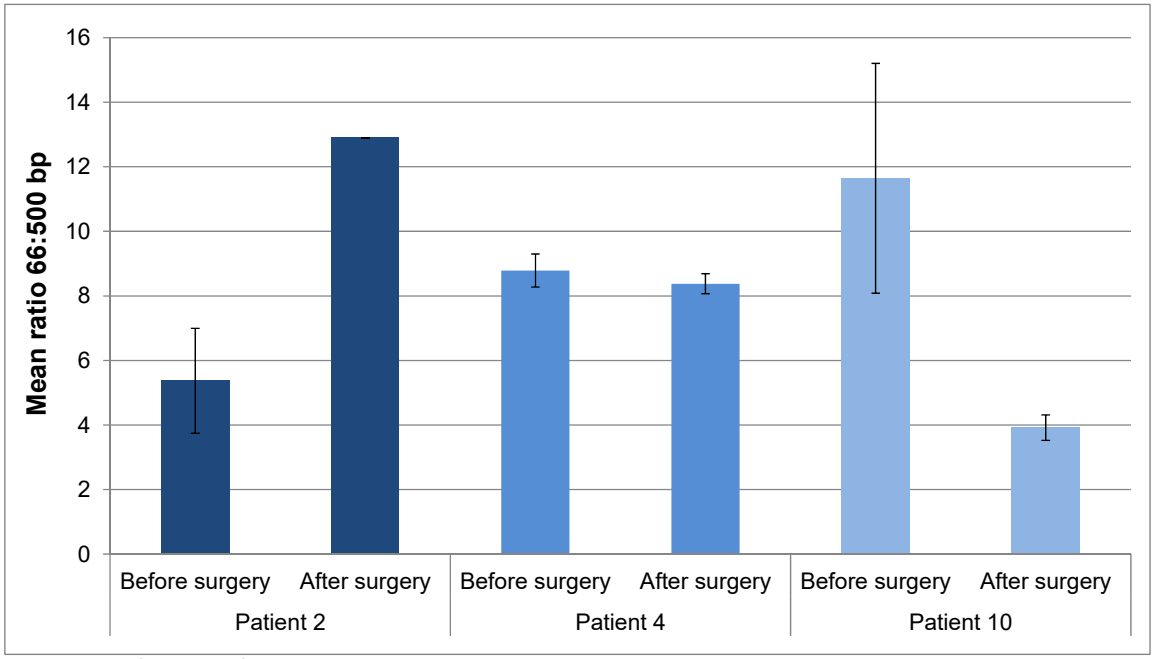
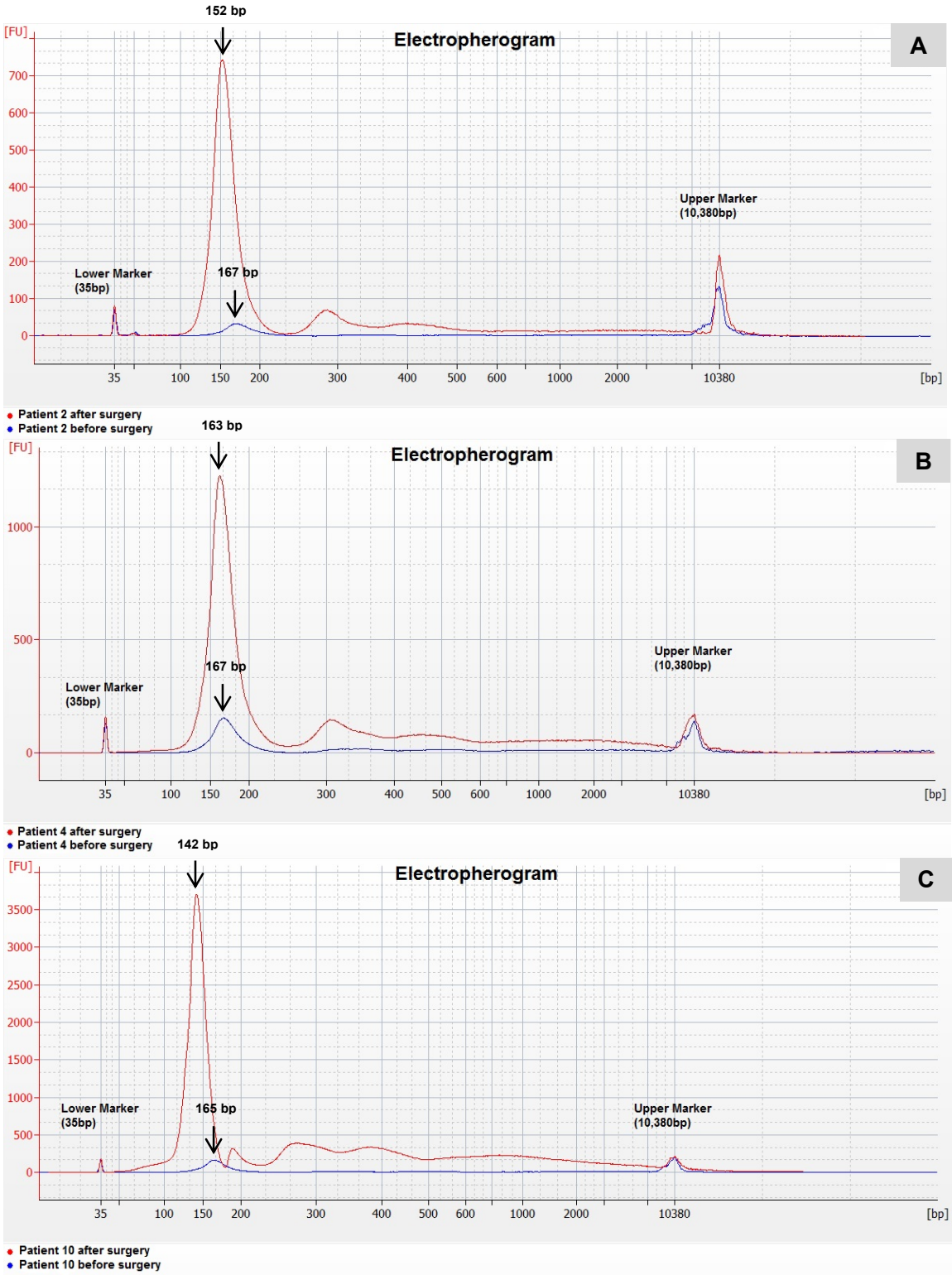


Figure 56 Influence of surgery on DNA concentration
DNA concentration increased after surgery in most cases. Mean ratio values between the 66 and 500 bp amplicon were calculated to analyze if elevated DNA levels were caused by the release of short or long DNA fragments. Results for plasma samples from three patients are shown here and the QIAamp cNA Kit was used for DNA extraction. An increase of ratio value means an increase of short DNA fragments (<500 bp). If ratio values remain unchanged, DNA amount does not change or higher DNA concentrations are caused by short and long DNA fragments in an almost equal way. A decrease of ratio value, on the other hand, can be achieved through elevated levels of mostly long DNA fragments (≥500 bp).

These three patient samples show an increase, unchanged level and decrease of the mean ratio value after surgery. DNA size distribution was also determined by Agilent gel electrophoresis to explain the results of the 66:500 bp ratios (Fig. 57).


152 bp
Electropherogram
A
[FU]
Lower Marker
(35bp)
167 bp
Upper Marker
(10,380bp)
[bp]
Patient 2 after surgery
Patient 2 before surgery
163 bp
Electropherogram
B
[FU]
167 bp
Lower Marker
(35bp)
Upper Marker
(10,380bp)
[bp]
Patient 4 after surgery
Patient 4 before surgery
142 bp
Electropherogram
C
[FU]
Lower Marker
(35bp)
165 bp
Upper Marker
(10,380bp)
[bp]
Patient 10 after surgery
Patient 10 before surgery

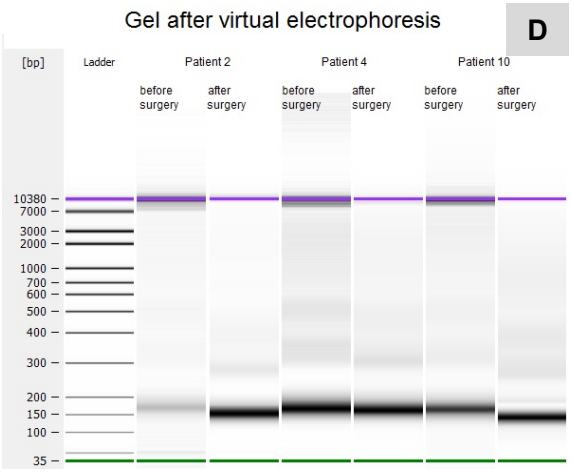


Figure 57 DNA distribution before and after surgery for 3 patient samples (Agilent)
Plasma samples from three patients (before and after surgery) were analyzed using the Agilent 2100 electrophoresis bioanalyzer instrument. **A**, **B** and **C** show the electropherogram after analysis. The lower and upper marker is assigned. The x-axis shows the length of included DNA fragments in bp and on the y-axis fluorescence units (=FU) are shown. The higher the peaks for specific DNA fragments are, the higher the FU value is and therefore the amount of DNA. **A** shows the results for Patient 2, while Patient 4's results are displayed in **B** and those of Patient 10 in **C**. Looking at **D,** another display format can be seen. Since the Agilent 2100 is an instrument for electrophoresis, a virtual gel can be generated. The green and purple lines are the lower and upper markers and the intensity of bands indicates the DNA concentration of plasma samples. The darker the bands the higher the DNA level is.

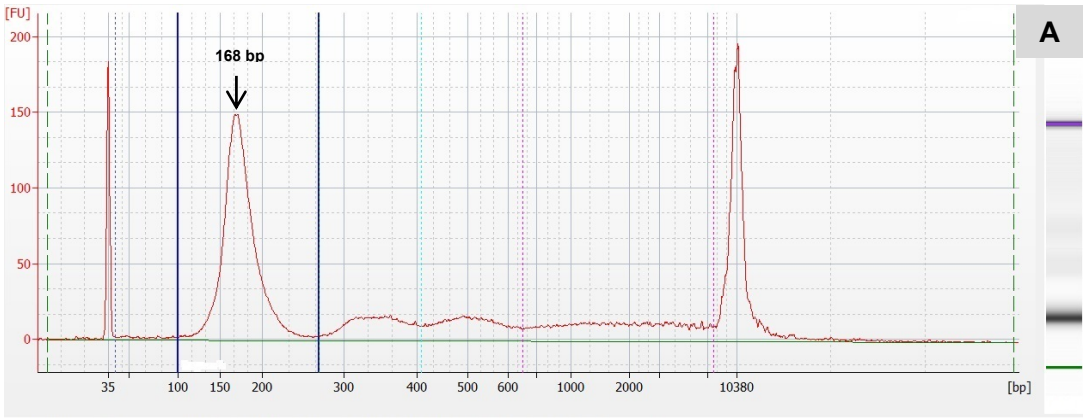
To analyze a possible correlation between the fraction of longer DNA fragments and ratio values, all DNA fragments ≥500 bp had to be regarded within the fraction of longer DNA. Furthermore, it was important to know if the fraction of small DNA fragments (<500 bp) changes at the same time, so the electropherogram was divided into different regions. These regions are listed in Table 26.

Table 26 Agilent results: Differences between different DNA fragment sizes
The results (shown in the electropherogram or the gel after virtual electrophoresis) are listed in the following table for patients 2, 4 and 10 (before and after surgery). DNA fragment sizes were separated into five regions (40-100, 100-270, 270-410, 410-650 and 650-7700 bp or more). The percentage fraction of total DNA as well as the average DNA fragment size and the DNA concentration are listed for each region.

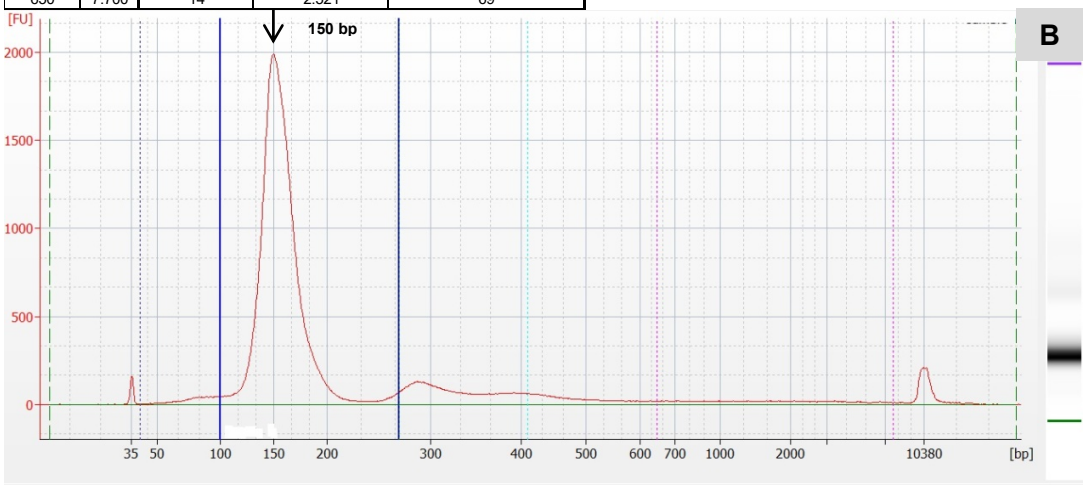
Patient	From bp	To bp	% of total DNA	Average size [bp]	DNA concentration [pg/ µL]
2vB	40	100	4	52	12
	100	270	58	175	171
	270	410	5	348	12
	410	650	6	518	13
	650	9192	24	5.309	40
2nB	40	100	0	68	12
	100	270	79	158	2599
	270	410	11	325	284
	410	650	5	494	110
	650	9307	5	2.716	103
4vB	40	100	0	41	0
	100	270	72	171	855
	270	410	10	345	93
	410	650	7	507	60
	650	9461	11	4.793	78
4nB	40	100	1	78	45
	100	270	69	169	3636
	270	410	13	333	543
	410	650	8	505	293
	650	8594	9	2.360	283
10vB	40	100	1	72	8
	100	270	65	168	589
	270	410	9	340	62
	410	650	7	513	46
	650	9335	15	4.270	80
10nB	40	100	2	83	591
	100	270	64	154	15257
	270	410	15	334	2857
	410	650	8	511	1359
	650	9215	10	2.111	1388

The regions were divided according to the typically occurring peak pattern. The first region may contain strongly fragmented DNA, while mononucleosomes, dinucleosomes and trinucleosomes can possibly be included in regions 2-4 and region 5 possibly consists of residual genomic or necrotic DNA. Region details for patients 2 and 4 can be correlated with ratio values, but proportions between short and longer DNA fragments were comparable for Patient 10 with a decrease in ratio values. Although the amount of longer and very short DNA fragments differs between samples, the main part is always present at a length of about 170 bp (region from 100 to 270 bp). Therefore, it appears to be no difference between samples before surgery (median value= 172 bp), after surgery (median

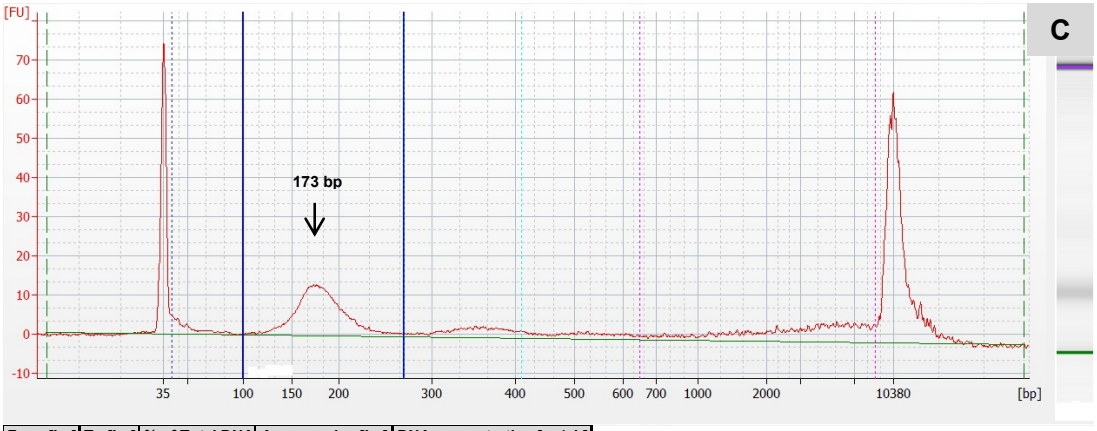
value= 164 bp), from healthy donors (median value= 174 bp) and from non-cancer patients (median value= 169 bp). Furthermore, the median value for the percentage main fraction (100-270 bp) is lower for healthy controls and non-cancer patients than for cancer patients before and after surgery (data not shown). The team of the QIAsymphony circulating DNA project also analyzed plasma from pregnant women. One example is shown in Figure 58.



From [bp]	To [bp]	% of Total DNA	Average size [bp]	DNA concentration [pg/µL]
40	100	2	67	14
100	270	61	175	506
270	410	11	344	74
410	650	10	512	62
650	7.700	14	2.521	69



From [bp]	To [bp]	% of Total DNA	Average size [bp]	DNA concentration [pg/µL]
40	100	2	80	398
100	270	83	157	12990
270	410	8	327	1064
410	650	3	491	322
650	9.133	2	2.536	226



From [bp]	To [bp]	% of Total DNA	Average size [bp]	DNA concentration [pg/µL]
40	100	8	54	31
100	270	48	182	170
270	410	11	348	31
410	650	7	516	17
650	9.461	21	3.946	44

Figure 58 DNA size distributions after using Agilent virtual gel electrophoresis
Plasma samples from a healthy individual (**A**), a patient (**B**) and pregnant women (**C**) were analyzed using the Agilent 2100 electrophoresis bioanalyzer instrument. **A**, **B** and **C** show the electropherogram as well as the virtual gel. The tables below each figure display the respective fraction of the different DNA fragment size regions. All DNA fragments were divided into five regions: 40-100, 100-270, 270-410, 410-650 and 650-7,700 bp or more. Pooled plasma from pregnant women was used for the analysis of fetal and maternal DNA. The fetus was female in each case. Analysis was performed within the QIAsymphony circulating DNA project. Samples were provided by Life Codexx in Konstanz, Germany.

While the DNA concentration varied between different samples, the pattern of DNA size distribution was almost the same. The main fraction was always between 100 and 270 bp and two lower peaks were often seen within the next two regions (270-410 and 410-650 bp).

Sequencing

The last analysis method, which was used for “proof of principle,” was targeted sequencing using the Illumina MiSeq instrument and the “Human Comprehensive Cancer Panel” from the “GeneRead DNAseq Targeted Panels V2.” Using this panel, 160 genes were analyzed by generating 7951 amplicons. The whole target region was 744,835 bp long. Three head and neck cancer patients were selected for analysis, including plasma samples before and after surgery as well as using the QIAamp cNA Kit and the QIAsymphony cDNA Protocol for DNA extraction. First, results for quality control checks on raw sequence data are shown. The results are almost the same for both reading directions (R1 and R2). The “per base sequence quality” is displayed by a quality score. Values between 28 and 40 mean “high quality.” As shown in Figure 59, all of the values are within this “high quality” area and results are very comparable between samples and methods.

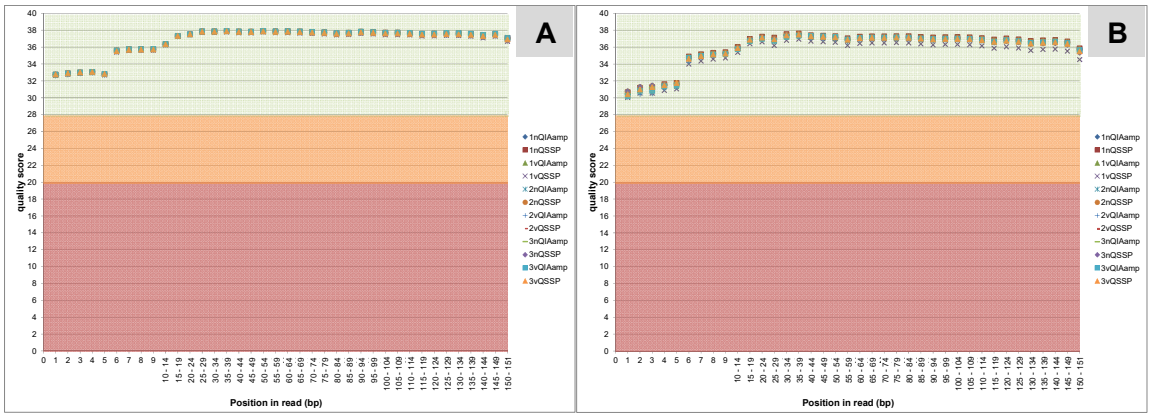


Figure 59 Per base sequence quality
For each reading position, a quality score was automatically calculated by the analysis software. A quality score between 28 and 40 means "high quality." The marked areas below stand for low (orange) and too low quality (red). **A** and **B** show the results for the same samples. They only differ in reading direction R1 and R2. "QIAamp" and "QSSP" are the DNA extraction methods, which were used to process plasma samples of Patient 1-3 before ("v") and after ("n") surgery and describe the QIAamp cNA Kit and the QIAsymphony cDNA Protocol.

The "per sequence GC content," displayed in Figure 60, shows the expected normalized Gaussian distribution and has its maximum at about 50%. In some cases, mean GC content is a little bit lower using the QIAamp cNA Kit.

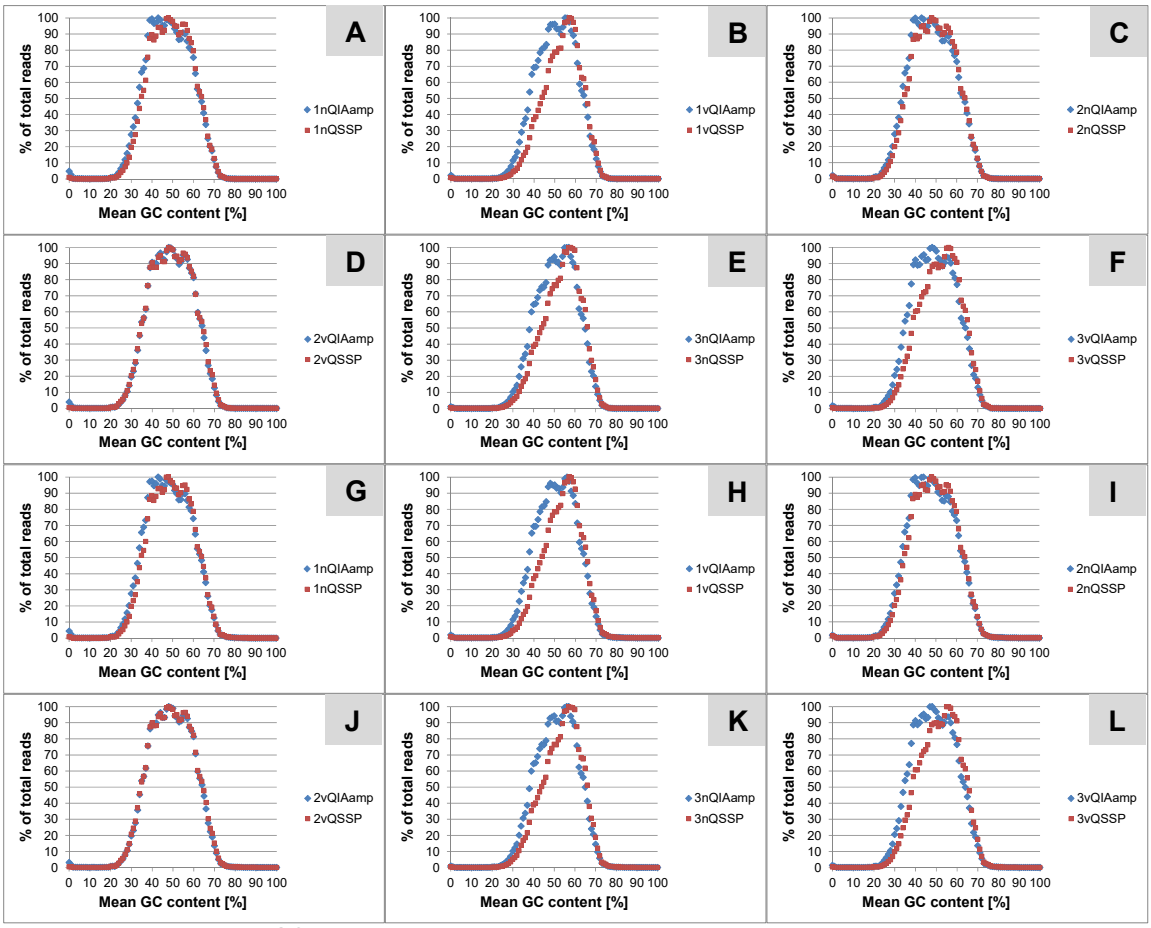


Figure 60 Per sequence GC content
Total read number was different between samples. Consequently, it was set to 100% in each case for normalization and to make results comparable between samples. The read number for a specific GC content was specified as the percentage fraction of total reads. **A**-**F** show the results for reading direction 1 (=R1), while **G**-**L** include the results for reading direction 2 (=R2). Each diagram (**A**-**L**) describes the per sequence GC content of one patient before or after surgery.

As shown in Figure 61, the “per base sequence content” was also comparable between samples and methods.

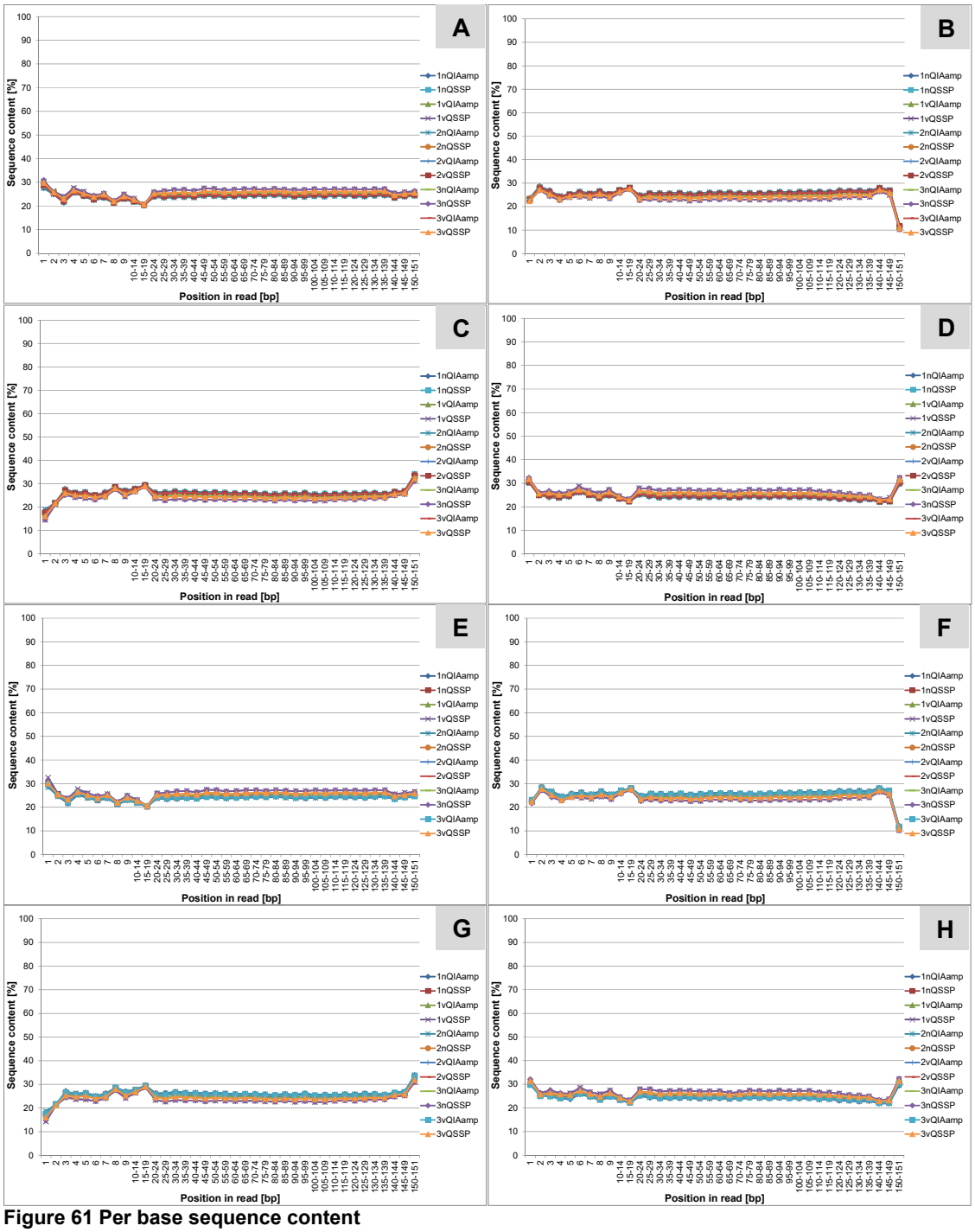


Figure 61 Per base sequence content
The percentage sequence content of guanine (**A** and **E**), adenine (**B** and **F**), thymine (**C** and **G**) and cytosine (**D** and **H**) is shown for each reading position. **A**-**D** contain the results of reading direction 1 and reading direction 2 is included in **E**-**H**.

Thus, all samples successfully passed the quality check and quality as well as GC content and the proportion of individual bases was very comparable between samples and methods. The alignment results were then analyzed. Figure 62 shows the total number of

reads that passed the adjusted filter. Reads smaller than 45 bp were discarded, because they might result from undesired PCR products or poor sequence quality. The number of aligned reads was some lower and further decreased for reads that were aligned in pairs and high quality aligned reads. However, all results were once again very comparable between samples and methods.

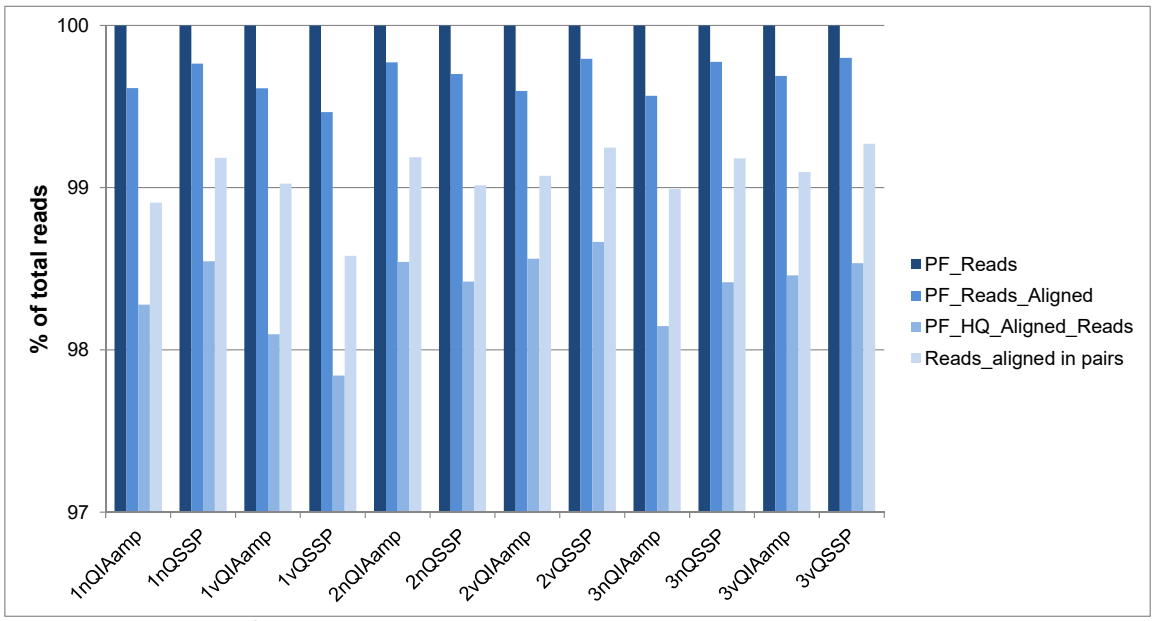
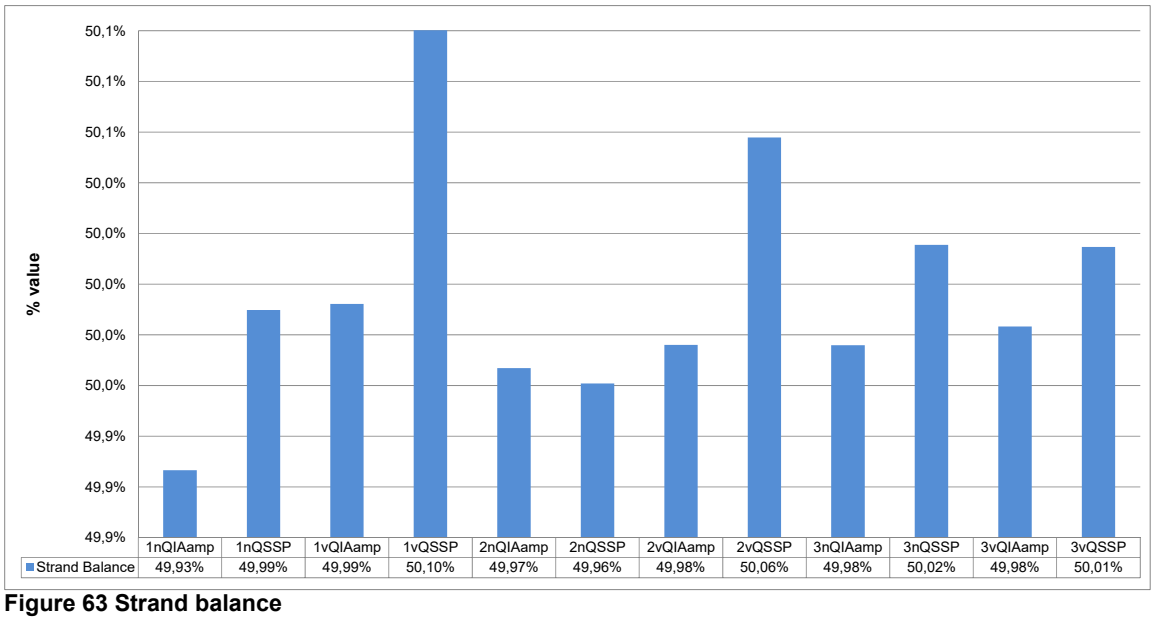


Figure 62 Alignment of reads
Aligned reads can be divided into “pass filter” reads, “pass filter” reads that were aligned, “pass filter” high quality aligned reads and reads that were aligned in pairs (R1 and R2). Total read number of each sample was used for normalization and therefore set to 100%. Each read type was specified as percentage fraction of total reads.

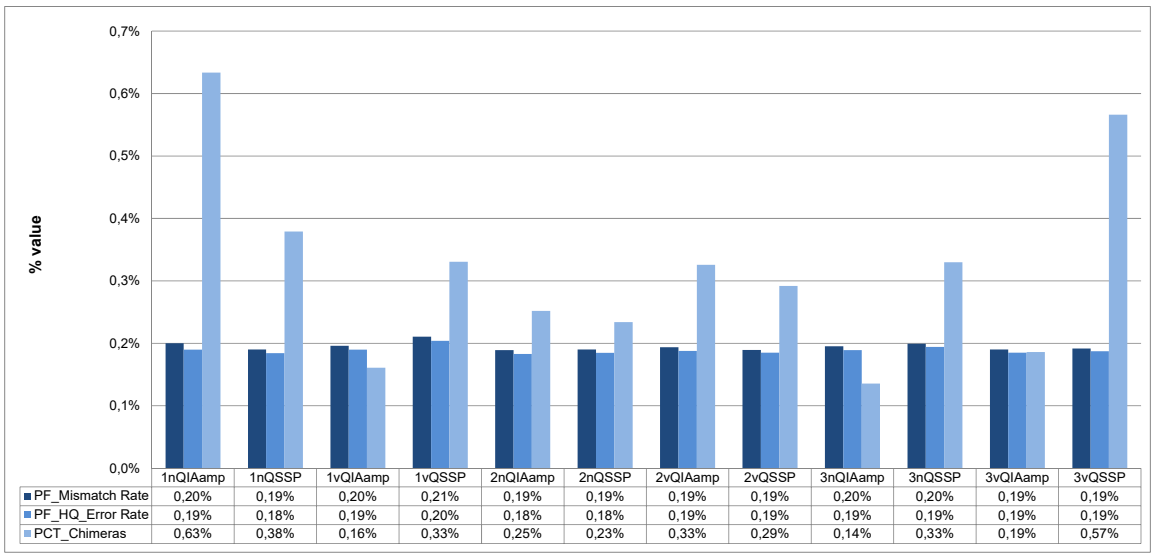
As shown in Figure 63, “strand balance” between forward and reverse strand was given and always about the expected 50%.



	1nQIAamp	1nQSSP	1vQIAamp	1vQSSP	2nQIAamp	2nQSSP	2vQIAamp	2vQSSP	3nQIAamp	3nQSSP	3vQIAamp	3vQSSP
Strand Balance	49,93%	49,99%	49,99%	50,10%	49,97%	49,96%	49,98%	50,06%	49,98%	50,02%	49,98%	50,01%

Figure 63 Strand balance
The balance between forward and reverse DNA strands is displayed here for each condition. Ideally, the percentage value should be 50%.

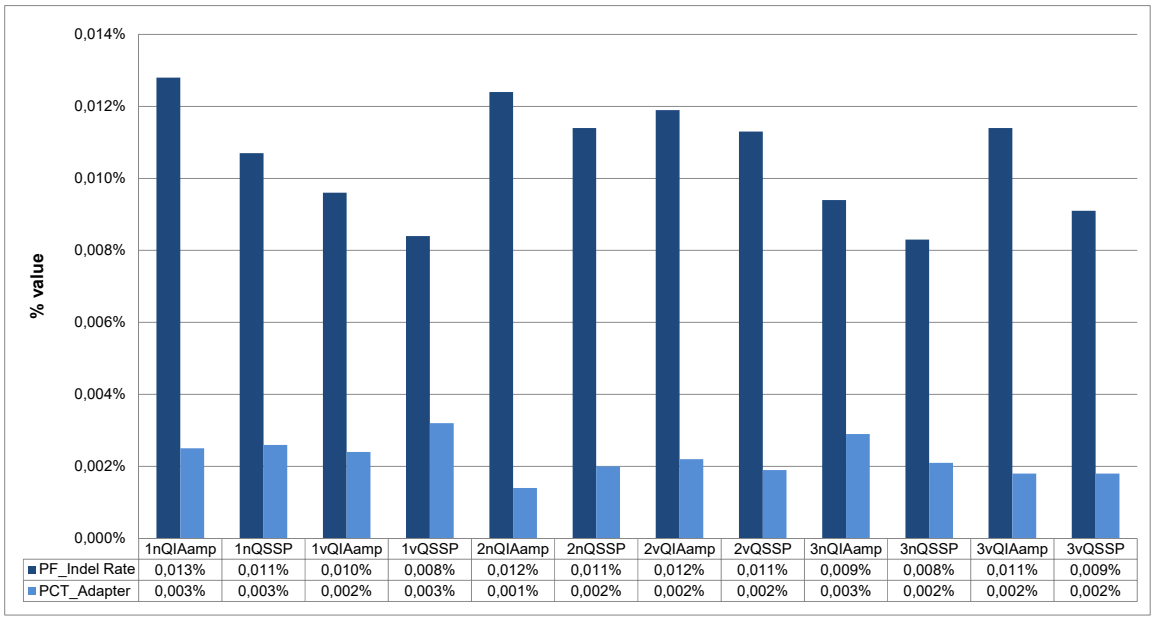
Mismatches and high quality error rates as well as chimeras occurred, but at an acceptable amount. They were comparable between methods and patients before and after surgery (Fig. 64).



	1nQIAamp	1nQSSP	1vQIAamp	1vQSSP	2nQIAamp	2nQSSP	2vQIAamp	2vQSSP	3nQIAamp	3nQSSP	3vQIAamp	3vQSSP
PF_Mismatch Rate	0,20%	0,19%	0,20%	0,21%	0,19%	0,19%	0,19%	0,19%	0,20%	0,20%	0,19%	0,19%
PF_HQ_Error Rate	0,19%	0,18%	0,19%	0,20%	0,18%	0,18%	0,19%	0,19%	0,19%	0,19%	0,19%	0,19%
PCT_Chimeras	0,63%	0,38%	0,16%	0,33%	0,25%	0,23%	0,33%	0,29%	0,14%	0,33%	0,19%	0,57%

Figure 64 Mismatch, error and chimera rate
The percentage rate of “pass filter” mismatches, “pass filter” high quality errors and chimeras are presented here for each condition.

The same was aimed at indels and adapter dimers (Fig. 65).



	1nQIAamp	1nQSSP	1vQIAamp	1vQSSP	2nQIAamp	2nQSSP	2vQIAamp	2vQSSP	3nQIAamp	3nQSSP	3vQIAamp	3vQSSP
PF_Indel Rate	0,013%	0,011%	0,010%	0,008%	0,012%	0,011%	0,012%	0,011%	0,009%	0,008%	0,011%	0,009%
PCT_Adapter	0,003%	0,003%	0,002%	0,003%	0,001%	0,002%	0,002%	0,002%	0,003%	0,002%	0,002%	0,002%

Figure 65 Indel and adapter rate
Indels and adapter dimers were additional possible errors during the sequencing procedure. Percentage values were shown for each condition.

The target region coverage was then checked. The normalized sequence coverage is displayed in Figure 66. Each figure shows one patient before or after surgery and the QIAamp cNA Kit and the QIAsymphony cDNA Protocol are displayed in blue and red, respectively. In most cases, the red line (QIAsymphony) runs below the blue line (QIAamp), so sequence coverage tended to be lower for the QIAsymphony cDNA Protocol. The average read depth (=number of times a base is red) of each condition was defined as 1. According to this, all read depths below the average were calculated by read depth/mean read depth. Looking at the y-axis, the fraction of total target bases in %, which were covered at a certain read depth, was shown. So, at a read depth of 0, 100% of the target bases were covered, because all bases have a read depth of 0 or higher. Ideally, coverage would be even 100% for the average read depth at the end, but coverage decreased with higher read depth. However, results between samples and methods were comparable.

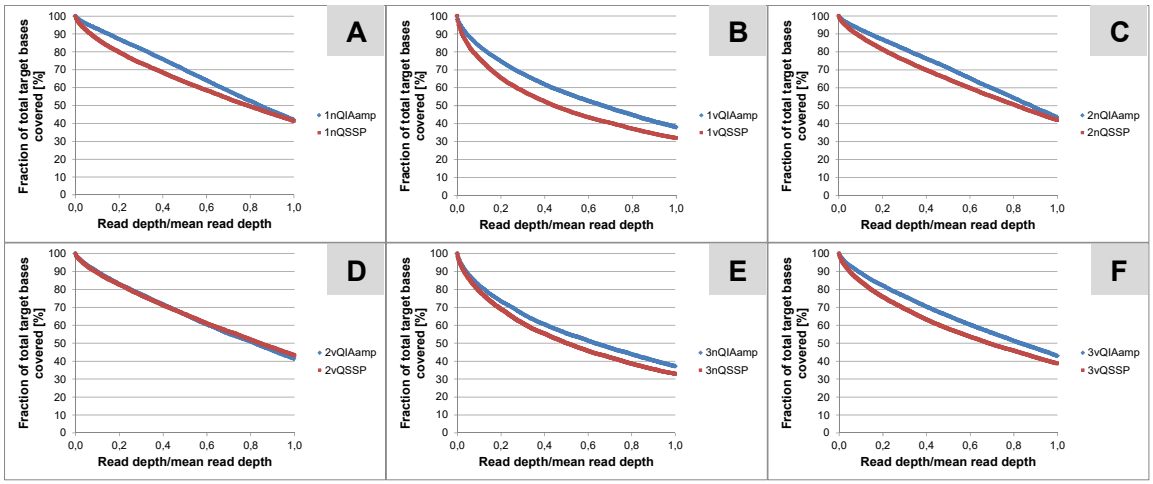


Figure 66 Normalized sequence coverage
Each diagram (**A**-**F**) shows one patient before or after surgery. The QIAamp cNA Kit and the QIAsymphony cDNA Protocol were used for DNA extraction from plasma samples and they are displayed in blue and red. The average read depth (=number of times a base is red) of each condition was defined as 1. According to this, all read depths below the average were calculated by read depth divided with mean read depth. Looking at the y-axis, we see the fraction of total target bases in %, which were covered at a certain read depth. So, at a read depth of 0, 100% of the target bases were covered, because all bases have a read depth of 0 or higher.

If only completely covered target regions were considered and compared between methods for each sample, 96-99% agreed with one other. Looking at the deviating part, in four cases more target regions were covered using the QIAamp cNA Kit, in one case covered target regions between both methods were comparable and in one case more target regions were only covered by the QIAsymphony cDNA Protocol (Fig. 67).

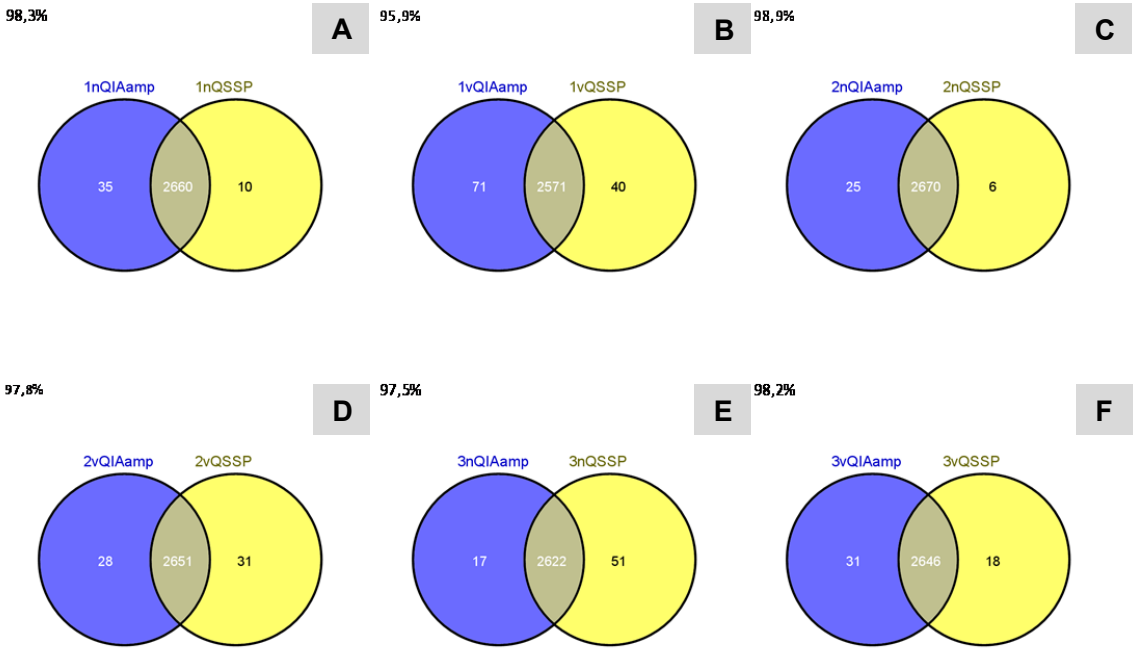


Figure 67 Venn diagrams of completely covered target regions
Only completely covered target regions were considered and compared between methods for each sample. Results are shown in the form of Venn diagrams (**A**-**F**).

Using all called variants for a scatter plot display, it became obvious that the comparison between “before surgery” and “after surgery” (see Fig. 68) led to similar results like the comparison between both tested methods (see Fig. 69). In each case, variants, which were solely called for one of the compared conditions, appeared.

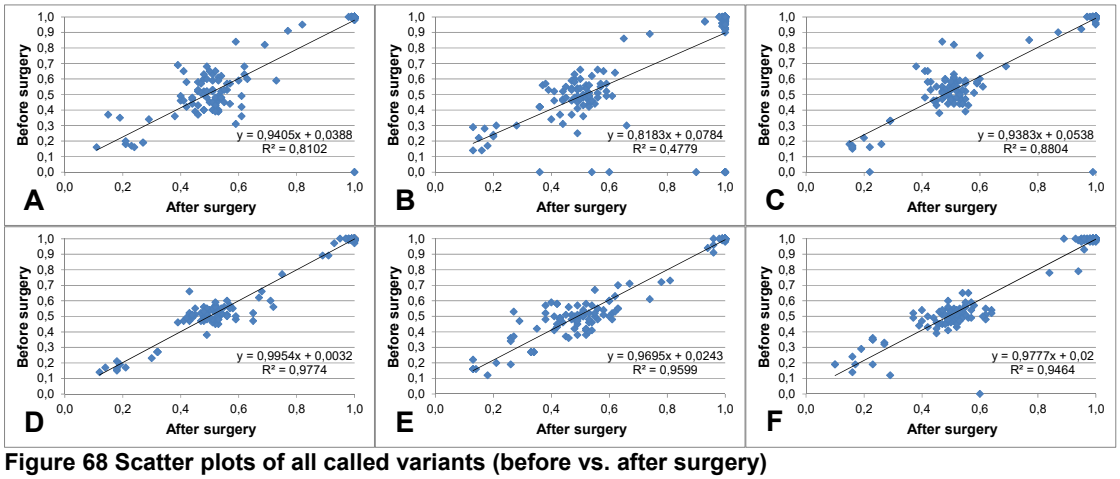


Figure 68 Scatter plots of all called variants (before vs. after surgery)
All called variants from the analysis software are displayed in scatter plots. Samples before and after surgery were compared here for each patient. Therefore, allele frequency values were taken. An allele frequency of 1 means 100%. **A** = QIAamp (Donor 1), **B** = QSSP (Donor 1), **C** = QIAamp (Donor 2), **D** = QSSP (Donor 2), **E** = QIAamp (Donor 3), **F** = QSSP (Donor 3)

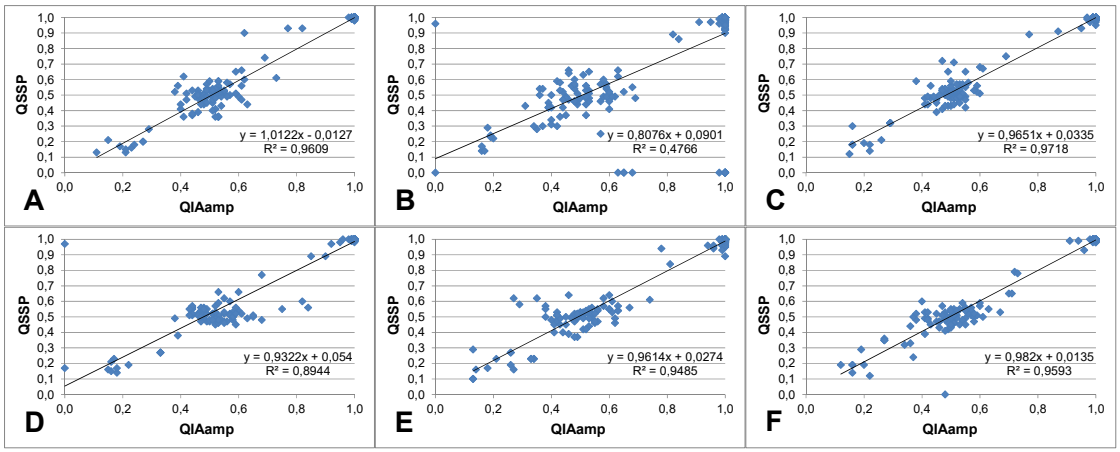


Figure 69 Scatter plots of all called variants (comparison between methods)
All called variants from the analysis software are displayed in scatter plots. DNA extraction methods were compared for each sample here. Therefore, allele frequency values were taken again. **A** = Donor 1 after surgery, **B** = Donor 1 before surgery, **C** = Donor 2 after surgery, **D** = Donor 2 before surgery, **E** = Donor 3 after surgery, **F** = Donor 3 before surgery

Furthermore, certain gene positions were not completely mutated or showed different changes compared to the reference, so if variants were observed for both conditions, the affected allele fraction was often different. However, 86-96% of all variants were present in both of the compared conditions (data not shown). Then, only high quality bases with a phred score of >20 and a coverage of >10 were taken for comparison. Only variants that were called in both methods for at least 10% were further considered and once again displayed in the form of scatter plots (Fig. 70).

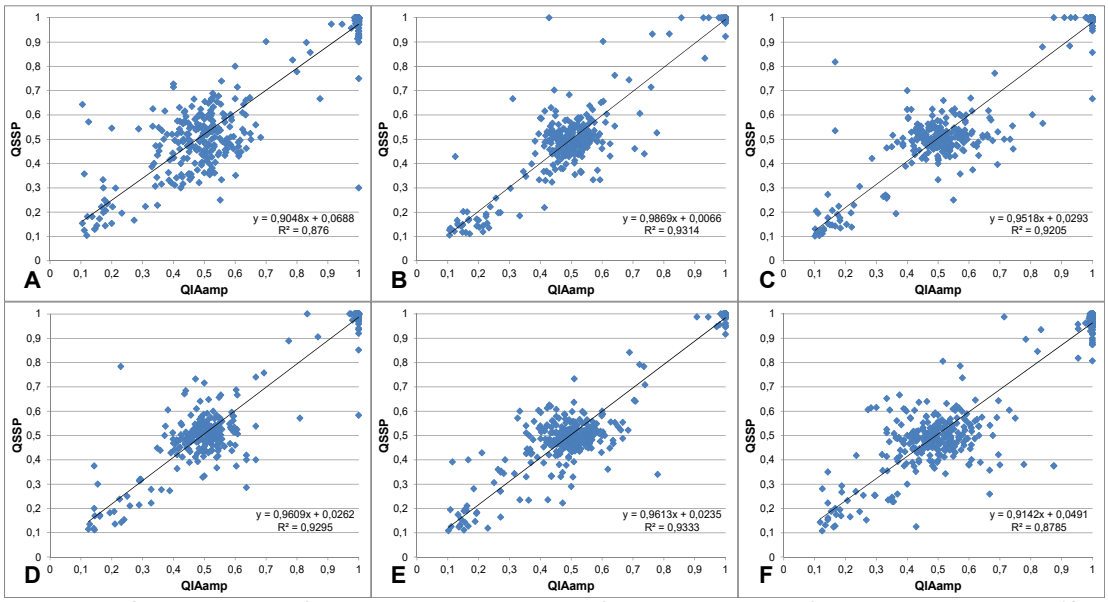


Figure 70 Scatter plots of variants (phred score of >20, coverage of >10 and at least 10% allele frequency in both methods)
Only high quality bases with a phred score of >20 and a coverage of >10 were taken for comparison here. Only variants that were called in both methods for at least 10% were further considered and displayed in the form of

a scatter plot again. The QIAamp cNA Kit and the QIAsymphony cDNA Protocol were compared for each sample (**A**-**F**). **A** = Donor 1 before surgery, **B** = Donor 1 after surgery, **C** = Donor 2 before surgery, **D** = Donor 2 after surgery, **E** = Donor 3 before surgery, **F** = Donor 3 after surgery

Furthermore, common variants were sorted according to their allele frequency in descending order of the QIAamp results and the corresponding QIAsymphony results were displayed (Fig. 71).

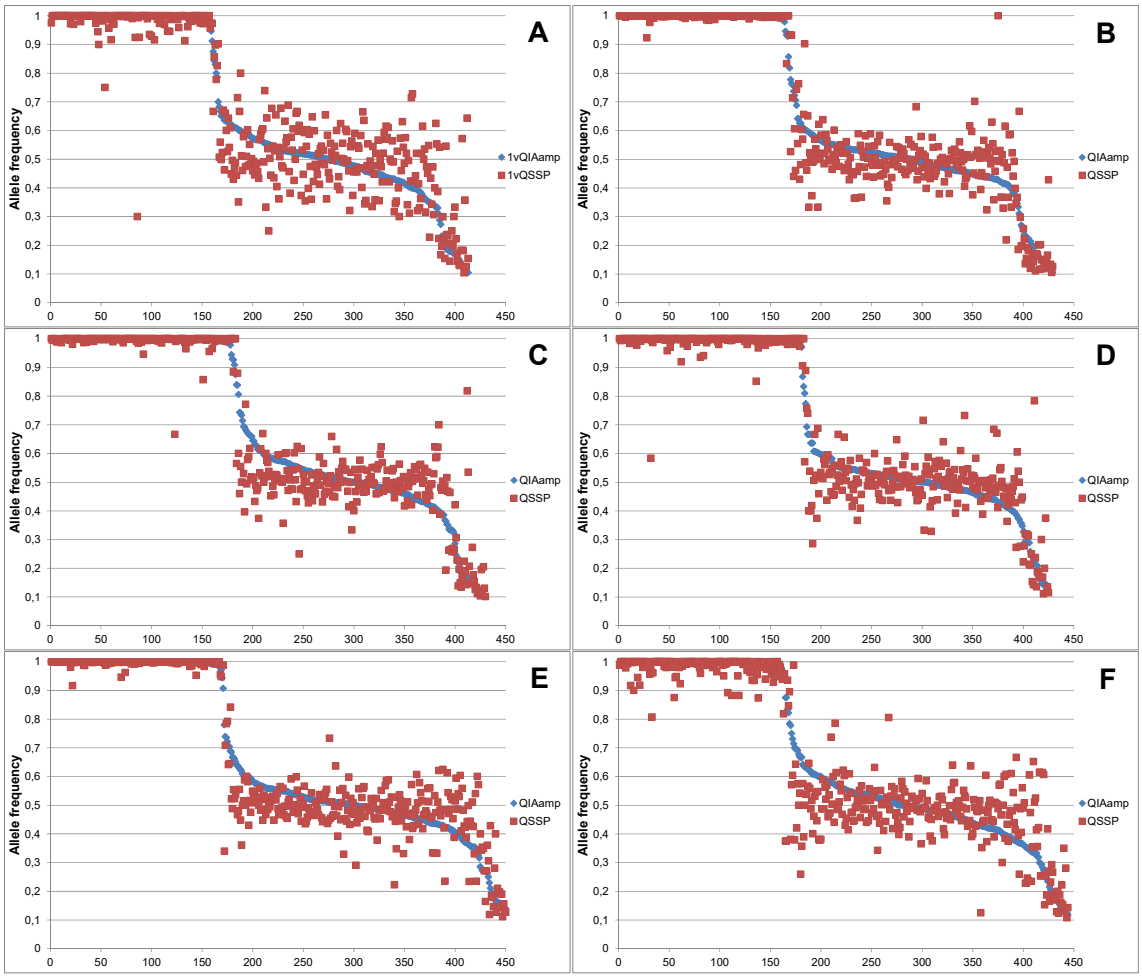


Figure 71 Common variants (>10%) sorted in descending order of QIAamp results
The common variants were sorted according to their allele frequency in descending order of the QIAamp results (in blue). The corresponding QIAsymphony results were also displayed (in red). **A** = Donor 1 before surgery, **B** = Donor 1 after surgery, **C** = Donor 2 before surgery, **D** = Donor 2 after surgery, **E** = Donor 3 before surgery, **F** = Donor 3 after surgery

Looking at these figures and the mentioned scatter plots, it can be seen that results between methods were not equal, but very comparable. Looking at the trend of QIAamp results, values for the QIAsymphony are very similar.

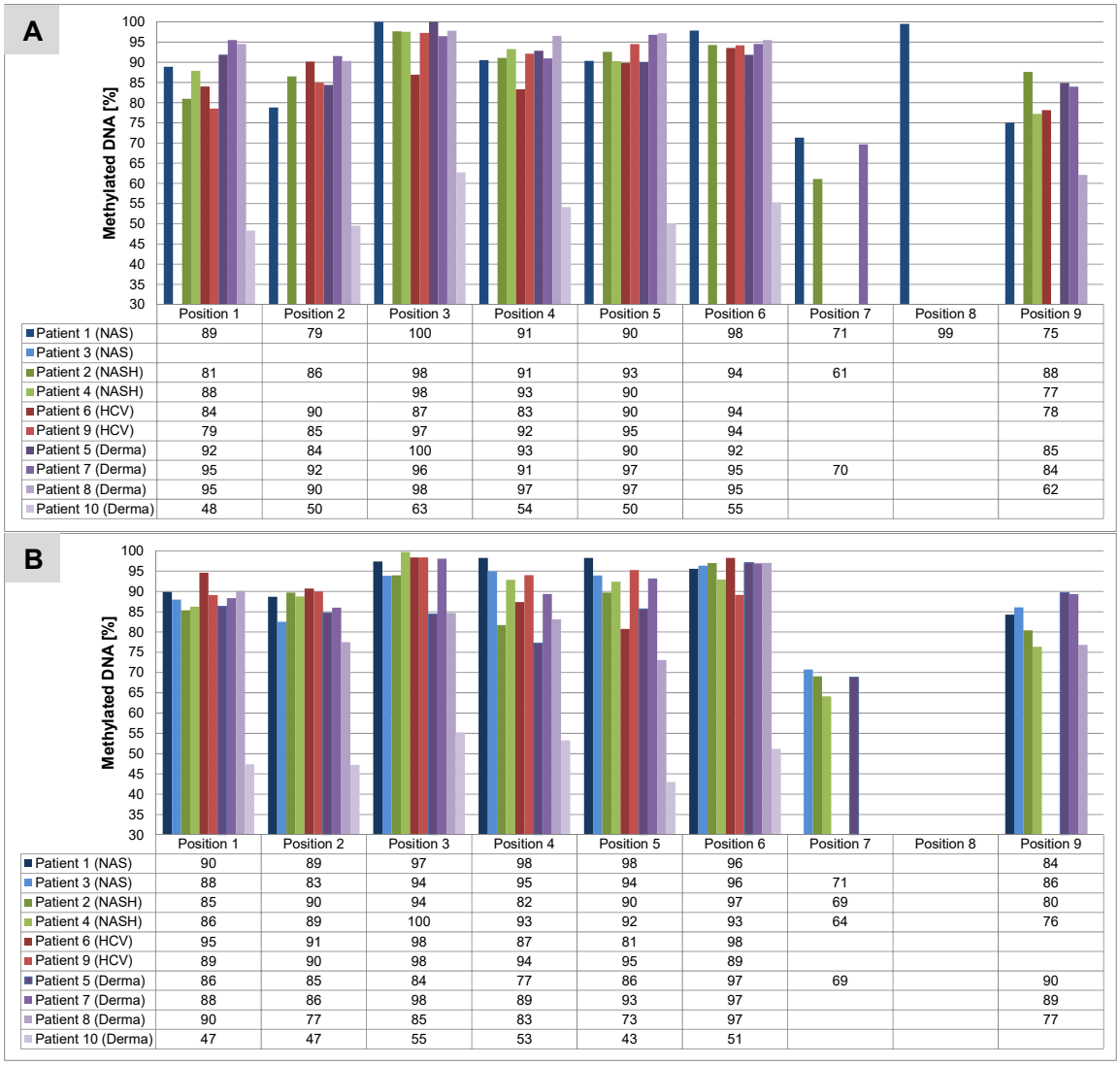
3.4.3. Non-cancer diseases

Plasma samples of patients with non-cancer diseases were not only tested as control, they were also provided by a laboratory of the Medical University in Graz, taken from patients suffering from non-alcoholic steatosis, non-alcoholic steatohepatitis, HCV and dermatological diseases. The *KRT23* DNA methylation marker should therefore be tested. It was supposed to be applicable for the discrimination between patients suffering from steatosis and steatohepatitis by showing decreased DNA methylation patterns in cases of steatohepatitis. Consequently, HCV patients and patients with dermatological diseases served as controls and were expected to lead to comparable results as patients suffering from non-alcoholic steatosis.

Pyrosequencing

To determine *KRT23* DNA methylation status, pyrosequencing was used as a further method for the aimed “proof of principle”. The newly developed QIAsymphony cDNA Protocol was once again compared to the QIAamp cNA Kit. *KRT23* was used as the target sequence and a proper assay for the PyroMark Q24 Advanced instrument was developed. Primers for Pyro-PCR and pyrosequencing were designed and template volume for PCR was optimized. Replicates for bisulfite treatment as well as Pyro-PCR or pyrosequencing were tested. Ten methylation sites were included, but the last one could not be calculated with certainty by the PyroMark Q24 advanced software, so it was tried to implement an additional primer for the last two sites, but no improvement was achieved (data not shown). Deviations and dispensation errors occurred and could not be completely eliminated by changing reagents, instruments and cartridges or by Pyro-PCR optimization. Different primer concentrations as well as annealing temperatures were tested, but no improvement was reached (data not shown). Finally, the QIAamp and QIAsymphony eluates were tested in different concentrations (2 and 4 mL plasma was used as sample material). Results between QIAamp and QIAsymphony as well as between different DNA concentrations were comparable in most cases (data not shown), so clinical samples from a laboratory of the Medical University in Graz were analyzed. The original protocol for DNA methylation analysis was delivered by Dr. Karl Kashofer, but it was for analysis by sequencing, so the protocol was adapted for pyrosequencing. Only 10 patients were available for the planned analysis. 2 mL plasma was processed using the QIAamp cNA Kit according to the handbook with 75 µL AVE elution, but without using Carrier-RNA and the

QIAsymphony cDNA Protocol was performed as described for the 24 colorectal cancer patients, whereas 75 instead of 150 µL elution volume was used. The only difference was the second wash buffer. In this case, buffer BR4 was used instead of 35 mM MES + 0.1% TritonX-100 (pH 5.8). Buffer BR4 contains ethanol and was used to inactivate sample pathogens. 40 µL of the eluates was taken as template for the EpiTect Fast DNA Bisulfite Kit. It was used according to the handbook and DNA was finally eluted in 15 µL buffer EB. For Pyro-PCR or pre-amplification, the appropriate *KRT23* DNA sequence was amplified using an endpoint thermal cycler. Therefore, a proper forward and biotinylated reverse primer were used to generate 194 bp long amplicons. 4 µL of bisulfite converted DNA was used as PCR-template. It was tested in triplicate. Cycling conditions and PCR chemistry were chosen according to the handbook. An additional primer was used for pyrosequencing on the PyroMark Q24 advanced instrument. Although all arguable results as well as clear outliers (marked by the PyroMark software) were removed, deviations between Pyro-PCR triplicates were often >5%, so it was hard to value differences between various methods or diseases. 9 of the 10 methylation sites were included and results seemed to be comparable between all conditions. Only Patient 10 from dermatology showed a significant decrease in DNA methylation (Fig. 72).



	Position 1	Position 2	Position 3	Position 4	Position 5	Position 6	Position 7	Position 8	Position 9
Patient 1 (NAS)	89	79	100	91	90	98	71	99	75
Patient 3 (NAS)									
Patient 2 (NASH)	81	86	98	91	93	94	61		88
Patient 4 (NASH)	88		98	93	90				77
Patient 6 (HCV)	84	90	87	83	90	94			78
Patient 9 (HCV)	79	85	97	92	95	94			
Patient 5 (Derma)	92	84	100	93	90	92			85
Patient 7 (Derma)	95	92	96	91	97	95	70		84
Patient 8 (Derma)	95	90	98	97	97	95			62
Patient 10 (Derma)	48	50	63	54	50	55			

	Position 1	Position 2	Position 3	Position 4	Position 5	Position 6	Position 7	Position 8	Position 9
Patient 1 (NAS)	90	89	97	98	98	96			84
Patient 3 (NAS)	88	83	94	95	94	96	71		86
Patient 2 (NASH)	85	90	94	82	90	97	69		80
Patient 4 (NASH)	86	89	100	93	92	93	64		76
Patient 6 (HCV)	95	91	98	87	81	98			
Patient 9 (HCV)	89	90	98	94	95	89			
Patient 5 (Derma)	86	85	84	77	86	97	69		90
Patient 7 (Derma)	88	86	98	89	93	97			89
Patient 8 (Derma)	90	77	85	83	73	97			77
Patient 10 (Derma)	47	47	55	53	43	51			

Figure 72 DNA methylation analysis using pyrosequencing
Plasma samples of ten patients suffering from non-alcoholic steatosis, non-alcoholic steatohepatitis, HCV and dermatological diseases were processed for DNA methylation analysis of the *KRT23* gene using the QIAamp cNA Kit (**A**) and the QIAsymphony cDNA Protocol (**B**). Within the *KRT23* gene, nine CpG sites were analyzed using the PyroMark Q24 Advanced instrument. The median fraction of methylated DNA in % is shown.

As a result, no difference between plasma samples of non-alcoholic steatohepatitis or steatosis was seen. Instead, the expected decrease of *KRT23* DNA methylation appeared for one of the four patients suffering from dermatological diseases.

4. Discussion

4.1. Impact of sample material

The sample material has an important impact on the analysis of ccfNA. On the one hand, ccfNA in plasma should be stabilized and, on the other hand, an elevation of genomic DNA background should be prevented to not further dilute the low concentrated target molecules of interest. The influence of a high-speed centrifugation step was tested and it was indeed able to reduce the total number of long (≥500 bp) DNA fragments, measured by the 500 bp amplicon of the 18S ribosomal DNA assay. High-speed centrifugation and filtration were already described previously for the removal of genomic DNA contamination caused by cell lysis [213]. There was no difference performing the high-speed centrifugation step before, after, or before and after freezing at -80°C and although cryo-precipitates were visible without centrifugation, they did not seem to have any negative influence on sample preparation. It was then tested influence of typically used blood collection systems. In each case, potassium EDTA was used as an anticoagulant (K_2 and K_3EDTA). It was discussed to lead to a better blood cell stability during storage than citrate and heparin [214] and it should be more applicable for analysis than heparin, for example, which is known to interact with DNA polymerases by binding magnesium ions necessary for PCR reactions [215, 216]. Furthermore, degradation of ccfDNA is prevented by inhibition of DNase activity [253]. Blood withdrawal was performed by aspiration or vacuum, using a hollow needle or Multifly hollow needle. The idea was that each system causes different shearing forces, influencing the blood cell stability and maybe increasing the amount of genomic DNA. Regarding the 18S 66 bp ribosomal DNA qPCR assay, results were very comparable between methods, but using a Multifly hollow needle combined with an aspiration system for blood withdrawal, 500 bp DNA levels as well as ratio value were significantly higher compared to Multifly hollow needle/ vacuum. However, all samples were centrifuged at 16,000 x g before they were used as input material for the QIAamp cNA Kit. As a result, possibly caused genomic DNA background might be partly removed by performing a high-speed centrifugation step [213]. Differences between blood withdrawal methods would potentially be visible without high-speed centrifugation step, but, as this step was also included for clinical samples, it was not necessary to test the effect without performing centrifugation. The only question that had to be answered was what kind of blood withdrawal system should be used for the

collection of patient samples. Besides the blood withdrawal system, sample handling, transport, storage and plasma preparation also have an important influence on genomic DNA background concentration. Despite the provided study protocol for plasma preparation, unavoidable differences between individual samples like storage time until plasma preparation have to be allowed and are possible in clinical daily routine. Not performing the high-speed centrifugation step would mean analyzing preanalytical differences and not only the difference between samples. There are many publications analyzing the stability of whole blood samples during storage. Xue and colleagues saw comparable plasma DNA concentrations for up to 2 hours of whole blood storage, whereas a slight increase of DNA amount was detected after storage for 4 hours and longer [232]. Ideally, plasma should be generated immediately after blood withdrawal, but in daily routine only a time frame of 2 hours was realizable and storage time of whole blood samples was documented for clinical samples. While the stability of whole blood samples was also tested in our group, there was no data concerning alternative body fluids like urine. Since K_2EDTA was already tested by the Research Laboratory for Urology at the University Hospital in Düsseldorf (by Prof. Dr. Schulz and his team), it was used as a stabilizing reagent and compared with other conditions. K_2EDTA and storage at 4°C led to comparable results using the 18S ribosomal DNA assay for detection. Naturally occurring DNA is supposed to be mainly present in the form of mononucleosomes [206], associated to other proteins or enclosed in so-called exosomes [24-26]. This packaging possibly increases DNA stability. Consequently, “naked” DNA represented by the GFP fragment spike-in system could be degraded quite simply and, for the added DNA fragments, storage at 4°C was not sufficient. Furthermore, in many cases storage at room temperature cannot be completely omitted during clinical workflow. As a result, K_2EDTA should always be added for stabilization to guarantee stabilization for all ccfDNA fragment types even at room temperature.

For most experiments blood samples from healthy volunteers were used, where the blood collection workflow was controlled so that blood and plasma of consistent quality was obtained, but clinical samples were also needed. It turned out to be difficult to obtain preferably high sample volumes and already available material was often useless either because serum instead of plasma was collected or whole blood samples were frozen for storage. As previously mentioned, genomic DNA background is higher in serum than in plasma samples [210, 211]. By freezing whole blood samples, included blood cells are destroyed and release genomic DNA. Even if high sample volumes were used, genomic

DNA background would be strongly elevated due to the presence of gDNA from the cellular blood fraction, diluting the target molecules of interest. A new sample collection workflow had to be set up and each step from blood withdrawal to plasma storage was defined. A realistic chance to achieve high detection sensitivities at the end only exist if all preanalytical procedures, which are necessary to obtain high quality starting material for DNA extraction, are considered.

4.2. Nature of circulating cell-free DNA

The newly developed QIAsymphony cDNA Protocol works, as already mentioned, very native. In contrast to silica chaotrope based protocols, there is no real lysis step. Circulating cell-free DNA from plasma samples possibly remains unchanged. Consequently, their nature in blood and other body fluids may play an important role for DNA extraction efficiency using the newly developed protocol. For some healthy individuals, DNA recoveries after automated DNA extraction were comparably low. One idea was that fractions of “naked” or mononucleosomal [206] DNA and DNA enclosed by so-called exosomes [24-26] differ between donors and can be recovered more or less efficiently. Accordingly, it was tried to isolate exosomes selectively using two different methods, but, as a result, whole DNA was extracted from blood plasma. A differentiation between the named DNA fractions was not possible.

Different spike-in experiments were performed to test if the nature of ccfDNA or other plasma components like proteins have a negative influence on protocol performance. First, DNA was extracted from plasma of twelve male healthy individuals using the newly developed automated protocol and then DNA of each donor extracted with the QIAamp cNA Kit was spiked into PBS and was also extracted by the automated protocol. An improvement of DNA recovery was achieved using the PBS condition in plasma samples with lower DNA recoveries (30-66%). Since extracted “naked” DNA as well as a reference system without plasma components was used, it is hard to say which effect might be responsible for the improvement, so extracted male DNA was also spiked into a female plasma pool. This way, DNA recoveries were also increased in critical samples, but they were always a little bit lower than in the PBS reference system. Consequently, “naked” DNA was better isolated by the automated protocol. One reason could be the missing association to histones and other proteins, which hinder the binding to beads or cause a weaker binding strength. DNA is therefore lost during the binding process or wash steps. Alternatively, plasma components like proteins could bind included DNA (“naked” or not) or

magnetic anion exchange beads and prevent DNA binding. Furthermore, the long 500 bp amplicon led to bigger differences between plasma and extracted DNA in female plasma or PBS. Binding of longer DNA fragments to proteins could be stronger or plasma components could have an inhibitory effect on PCR reaction. The amplification of longer amplicons (in this case 500 bp) is much more sensitive than of smaller ones like the 66 bp amplicon of the 18S ribosomal DNA assay. The used GFP fragment spike-in system is also “naked” DNA, so its DNA recovery does not always correlate with the DNA recovery of native DNA. It is often higher for the spike-in system and sometimes independent from tested protocol changes. Using extracted male DNA from pooled plasma, the same spike-in was used for twelve female individual plasma samples. DNA recovery of the QIAsymphony cDNA Protocol varied between 56 and 104% and thus seemed to depend on plasma components, but only 79 to 85% were reached using PBS. DNA recovery is always determined using the QIAamp cNA Kit as reference system. DNA extraction efficiency may differ between different Kit lots and, using quantitative real-time PCR for detection, a certain deviation is always expected including pipette inaccuracy and plate position effects. Therefore, an Applied Biosystems 7900HT Fast Real-Time PCR instrument was tested using the already described 18S ribosomal DNA assay. Eighty copies of extracted male human DNA were applied on a 384 well plate and the 500 bp amplicon was detected after amplification. The maximal Ct difference was 0.89, corresponding to a concentration difference of 1.85 (data not shown). The experiment concerning protein depletion could be an additional indicator for the possible binding of DNA to proteins, because DNA was also removed from plasma samples using albumin/IgG depletion resins. The increase in DNA recovery using the newly developed automated protocol after freezing could be caused by denaturation of proteins or other plasma components, so, using such a “native” protocol, the nature of ccfNA has a great influence on protocol performance and the impact of sample material even gains in importance.

4.3. Development of automated DNA extraction: reasons & challenges

Many different DNA extraction methods exist, but only a few were optimized for the recovery of ccfDNA with specific characteristics like strong fragmentation [206, 207] and very low concentration, especially of targets within low concentrated ccfDNA like mutated

tumor DNA [40]. One possibility to overcome the challenge of highly diluted target molecules and to increase the probability of catching the target molecules of interest is to enrich or concentrate desired ccfDNA fragments. For this DNA enrichment or concentration, preferably high sample input volumes were used, while the elution volume was simultaneously kept as small as possible. Although it was indicated that the detection sensitivity can also be increased by higher sample volumes [237], in some studies as low as 200 µL was used as total sample input material [238]. Manual methods already exist, but automation is strongly needed. Therefore, it is not only required for high through-put laboratories, but also demanded to help towards standardization. The impact of blood processing methods, including the time between sample and DNA extraction, centrifugation conditions and sample material (difference between serum or plasma) was already analyzed [254] and the influence of preanalytical factors of blood sampling and processing on DNA yield was demonstrated [255]. Promising research concerning ccfNA has strongly developed to date, but various research groups exist and the design of experiment is often different [77, 213, 225, 234-236]. One component causing the unwanted diversity, which makes comparison difficult, is the DNA extraction method [220-228]. An automated solution would be applicable in a broader field including clinical daily routine and research at university. Furthermore, automation better allows for compatibility and reproducibility than manual methods, including many individual steps as potential source of errors. Preanalytical as well as analytical procedures have to be standardized “to validate the liquid biopsy as a clinical biomarker in well-designed and sufficiently powered multicenter studies” [256]. Different quantification methods lead to different results depending on the measured target, which can be total or only amplifiable DNA [257]. The potential of liquid biopsy for clinical cancer research has already been recognized so that it is often included in clinical trials, but for successful implementation in clinical practice the development of standardized preanalytical and analytical procedures is strongly needed [256].

Basic research is still needed in the field of ccfNA and the nature of included DNA remains unclear, making the development and optimization of the automated protocol challenging. Furthermore, the DNA extraction method is based on new magnetic particles requiring a new chemistry, which is not yet a part of the QIAGEN portfolio. Each protocol or buffer change possibly entails the test of the following steps, so by changing binding conditions, for example, proteins could be removed from DNA and DNA binding to beads would be

strengthened this way. At the same time, more stringent conditions are needed to elute DNA from beads completely. Consequently, by changing protocol conditions, the nature of ccfDNA and other plasma components is perhaps influenced, causing a specific behavior during DNA extraction. Therefore, the theoretical binding and elution behavior do not always agree with the actual protocol performance.

4.4. Applicability of the automated protocol

DNA bisulfite conversion was automated on the QIAsymphony SP instrument. The EpiTect Fast DNA Bisulfite Kit served as reference method. The used automated protocol was similar to the QIAsymphony cDNA Protocol. Chemistry was adapted according to the new application. The developed protocol for DNA bisulfite conversion was successful and showed the applicability of the newly developed QIAsymphony cDNA Protocol for further methods. Consequently, not just one automated protocol was developed; it can also be used as basis for the automation of other existing manual protocols.

Most experiments for the development of an automated DNA extraction protocol were performed using plasma samples from healthy individuals. The question was if DNA from clinical and maybe more critical sample material could be recovered the same way.

Prenatal diagnostics is the most established field for ccfNA. However, only one experiment was performed to show the applicability of the newly developed QIAsymphony cDNA Protocol for fetal DNA extraction from maternal plasma samples. Although total DNA recovery using an early version of the automated protocol was often lower than results of the QIAamp cNA Kit, fetal DNA concentration was always very comparable between these two methods. As a result, loss of maternal and fetal DNA was almost equal. That means recovery of fetal and total DNA was very comparable. Fetal DNA concentration increases with gestational age, but the initial DNA amount differs between individuals [159]. Consequently, elevated DNA levels can be observed in the course of pregnancy for each woman, but they are not always the same in a specific week of gestation, so fetal DNA concentration cannot always be correlated with the week of gestation looking at different individuals. Furthermore, fetal DNA fraction can also be influenced by physical activity [258], for example.

Most samples were collected from cancer patients. Cancer diagnostics is another important application field for ccfNA. While pregnant women are potentially healthy, plasma from cancer patients could be much more critical. The aim was to show that tumor

DNA can also be successfully extracted compared to the reference method, the QIAamp cNA Kit. Therefore, tumor DNA had to be discriminated from the “normal” DNA background. One way to differentiate between tumor and non-tumor DNA is the detection of tumor-specific mutations. According to the diversity of cancer types and the relative small patient number for each one, high frequent DNA biomarkers were needed to detect mutated and therefore tumor DNA in preferably many plasma samples. First, it was tried to find biomarkers by DNA mutation screening of some patients. Therefore, three head and neck cancer patients were selected due to their mutation diversity with intent to find one DNA mutation or a small panel, occurring in all tested patient samples, applicable to a multiplex assay for quantitative real-time PCR. TP53 DNA mutation therefore belongs to the earliest and most frequently detectable genetic alterations in head and neck squamous cell carcinoma [259, 260]

SABiosciences qBiomarker Somatic Mutation PCR Arrays were used to profile the somatic mutation status of certain genes with detection sensitivities down to 1%. Two arrays were tested for this purpose. First, the qBiomarker™ Somatic Human Head & Neck Cancer Mutation PCR Array was tested for the detection of 83 gene mutations, whereas most can be found within the TP53 gene and the Cancer Comprehensive 384HC Mutation PCR Array, which is a more generic one detecting 321 mutations in 61 genes. But PCR signals were not clear enough to differentiate between mutant and wild type genes. Some gene mutations were included in both tested arrays, but, although the same sample was used, results were different in some cases (data not shown). So, according to the literature and available analysis methods, *KRAS* mutations were chosen for tumor-DNA analysis. *KRAS* belongs to the first two genes whose mutations were studied in plasma from patients suffering from pancreatic [8] and colorectal cancer [11] and its mutation belongs to the most common in pancreatic [261, 262] as well as colorectal cancer patients [263, 264]. It was also confirmed by COSMIC Cancer Browser results [265]. The three biggest patient groups included in the described studies of this PhD thesis suffered from head and neck, pancreatic and colorectal cancer. The therascreen *KRAS* RGQ PCR Kit used covers the seven most frequent mutations in codons 12 and 13 of exon 2. The Kit was developed for DNA from tissue samples, but, for *EGFR* mutation detection, the method was also successfully tested for ccfDNA from plasma samples (therascreen *EGFR* Plasma RGQ PCR Kit (24) CE, cat. no.: 870311; QIAGEN GmbH, Hilden, Germany). To confirm the applicability of the *KRAS* Kit version, it was first tested using plasma samples from colorectal cancer patients provided by Sysmex Inostics GmbH (Hamburg, Germany). This

way the therascreen *KRAS* RGQ PCR Kit was tested against a reference system for *KRAS* mutation detection, the BEAMing digital PCR technology. Since results between these two analysis methods were comparable, the therascreen *KRAS* RGQ PCR Kit was also used for the colorectal cancer patient samples from the Surgical Research Laboratory (from the University Hospital in Düsseldorf). For both studies, the frequency of mutant *KRAS* DNA was lower than expected. *KRAS* mutation frequency was 29% for clinical samples provided by Sysmex Inostics GmbH and 18% for samples coming from the Surgical Research Laboratory at the University Hospital in Düsseldorf, although the rate of *KRAS* mutations was 38% in metastatic colorectal cancer patients [266]. However, according to the small patient group size that was analyzed and the fact that not all tested colorectal cancer patients were metastatic, deviations from the expected value are possible. Looking at the results of BEAMing digital PCR technology, mutant fractions vary between 0.03 and 47% for the QIAamp cNA Kit method. This variation was also described above. In plasma samples from metastatic colorectal cancer patients, for example, *KRAS* and *BRAF* mutation status was determined and led to mutant fractions between 0.5 and 64.1% [198]. In addition to *KRAS* mutation status, *KRT23* gene methylation was analyzed using clinical samples from the Department of Pathology at the Medical University in Graz. *KRT23* is supposed to be highly differentially expressed in steatohepatitis compared to steatosis and a normal liver [267]. It was tried to confirm previous research results concerning the DNA methylation status generated by sequencing analysis using a pyrosequencing approach, so the amplicon size was kept. Since ccfDNA mainly occurs in the form of mononucleosomes (145-200 bp) using an amplicon size of 194 bp, only a small fraction of ccfDNA can be detected. Conditions for pyrosequencing were not optimal, but, by using a smaller amplicon size, possible target molecules would change and a reduction of amplicon size could have an effect on results if the methylation pattern differed between short and long DNA fragments. Besides testing different clinical samples, the applicability of various analysis methods should be demonstrated. In addition to the already mentioned therascreen *KRAS* RGQ PCR Kit, BEAMing digital PCR technology, pyrosequencing, quantitative real-time PCR targeting 18S ribosomal DNA as well as Qubit double-stranded DNA measurement, DNA fragment size distribution using the Agilent instrument and targeted sequencing were performed. The newly developed QIAsymphony cDNA Protocol was always compared to the QIAamp cNA Kit. Results were comparable in most cases. Deviations exist, but they also occur within one method and do not seem to be related to the tested procedure. Furthermore, the size of the control group, consisting of non-cancer

patients and healthy individuals, is too small compared to the cancer patient group size and plasma samples from healthy individuals were not collected at clinical sites. Healthy control samples came, except for one sample, from QIAGEN blood donations. Looking at total DNA quantification by the 18S ribosomal DNA assay or Qubit analysis, results for non-cancer controls and cancer patients were comparable, whereas DNA recovery from healthy individuals was higher. Preanalytical procedures were performed under controlled conditions, but possible influencing effects cannot be completely excluded. In most cases (81%) elevated plasma DNA levels were discovered for cancer patients after surgery. This increase of DNA concentration was already described above. Increased plasma DNA levels were detected in NSCLC patients one week after primary tumor resection; however, it was also observed in patients after orthopedic surgical treatment. Possible reduced plasma DNA levels can be detected later after treatment, so plasma DNA levels of NSCLC patients without disease recurrence decreased during the 3- to 6-month follow-up, whereas an increase was demonstrated in relapsed subjects [65]. Furthermore, mean plasma DNA concentration was higher for NSCLC patients than for healthy controls. Looking at the mean or median values of this PhD thesis, these results were confirmed. In some studies, these significantly higher concentrations of ccfDNA were demonstrated using samples from cancer patients [63-70] and the presence of cancer or disease-free status and relapse was confirmed through quantification [63, 268]. Higher plasma ccfDNA levels in cancer patients were therefore not only detected compared to healthy individuals, but also patients with benign diseases [71]. However, many other studies analyzed DNA abnormalities in addition to the determined DNA concentration to get a useful diagnostic tool [269, 84]. As demonstrated in this PhD thesis, high levels of ccfDNA are also present in blood samples from patients with benign diseases [270] and plasma ccfDNA concentration can also be elevated in healthy individuals under physiological stress or in patients affected by inflammation, trauma or sepsis [72-75]. The pattern of DNA fragment size distribution after using the Agilent instrument was very similar to plasma from cancer- and non-cancer patients as well as pregnant women or healthy individuals. The main part of DNA fragments occurs in the length of mononucleosomes or multiples thereof. It was also previously demonstrated for ccfDNA of healthy individuals and cancer patients [206]. The following figure from a review article illustrates the size distribution of ccfDNA in plasma samples from cancer patients and non-cancer controls.

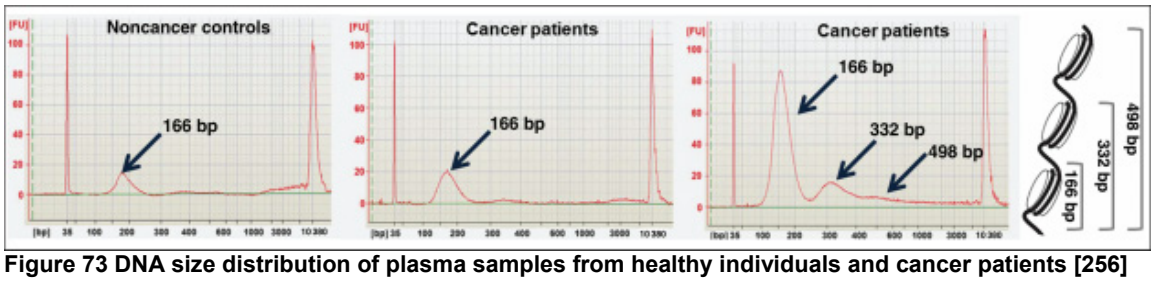


Figure 73 DNA size distribution of plasma samples from healthy individuals and cancer patients [256]
The figure shows three electropherograms after virtual gel electrophoresis using the Agilent 2100 Electrophoresis Bioanalyzer Instrument. Both non-cancer controls and cancer patients show a similar DNA size distribution. Most ccfDNA fragments occur in the length of mononucleosomes (145-200 bp) or multiples thereof.

The detected peak after virtual gel electrophoresis was 166 bp for cancer and non-cancer patients. In experiments for this PhD thesis, the fraction below 100 bp or above 650 bp as well as the total DNA concentration varies between groups and individuals. With regard to cancer patients, DNA level mostly increases after surgery, as it was already described by Szpechcinski A. *et al.* [65]. However, most DNA fragments still seem to be present in the form of mononucleosomes and varied around the described maximum peak value of 166 bp. Maximum peak was 165 or 167 bp for samples of cancer patients before surgery and 142, 152 or 163 after treatment. In plasma samples of healthy individuals, maximum peak was 168 bp and it was 150 bp for non-cancer patients or 173 bp in maternal plasma samples.

The last and maybe one of the most important analysis methods now and in the future, was sequencing. This method enables the simultaneous analysis of many genes using one eluate aliquot. Since sample material is limited in most cases, it is an advantage over other methods like quantitative real-time PCR. Furthermore, in terms of tumor heterogeneity and mutation diversity, it is hard to confine the number of genes that have to be detected. According to the current ethics approval, a targeted approach was performed. Massive parallel sequencing has two important advantages: the entire genome is used without dependence on DNA fragments with specific genomic loci and all circulating DNA fragments independent of size can be detected [160]. Targeted sequencing, exomic sequencing, or whole-genome sequencing were able to detect one mutant DNA fragment within up to 5 mL of plasma [271]. But sequencing is not only a very sensitive method; according to the cost reduction from USD 108,065 per genome in 2009 to USD 4,920 in 2014 and improvement of speed for whole genome sequencing, it will also be a possible method for ccfDNA analysis in the future [272]. TP53 was chosen as the most frequent

gene mutation for analysis [273] of head and neck squamous cell carcinoma patients. Since many different mutations exist for this gene, it is hard to analyze it using quantitative real-time PCR or BEAMing digital PCR technology, so the “Human Comprehensive Cancer Panel” was used. It covers TP53 and 159 other genes by detecting a total of 7951 target sequences. Although the panel was designed for DNA from FFPE tissue, it was tested for ccfDNA from plasma samples. These plasma samples were taken from three head and neck cancer patients before and after surgery using the QIAamp cNA Kit and the QIAsymphony cDNA Protocol for DNA extraction. Ideally, these two methods should lead to comparable results, but, according to the generated data, it is hard to decide whether or not results are comparable. What we certainly know is that they are not only different between methods, but also within one method. Sequencing analysis was limited to the mentioned samples. The whole workflow before DNA sequencing had to be performed manually and DNA library could not be quantified exactly to adjust the required amount as input material resulting in different reading numbers for each sample, whereas the coverage of the DNA target sequence depends on read number. Therefore, many possible sources for variation exist during the mentioned workflow. Nevertheless, results of quality control checks on raw sequence data indicated that sequencing runs were successful and comparable between samples. To get more significant results for variant analysis, not only more samples have to be processed, but also a preferably high number of replicates to distinguish between deviations caused by sample or DNA extraction method and the manual workflow before DNA sequencing. According to this, it is hard to value differences and decide which result is the “right” one. Furthermore, it is not possible to differentiate between variability caused by sample or DNA extraction method and sample preparation before DNA sequencing.

4.5. Possible use of the automated protocol

The applicability of the newly developed automated DNA extraction protocol was successfully tested for clinical plasma samples as well as different analysis methods, so future use in clinical practice seems to be possible. The study results of this PhD thesis showed the feasibility of the newly developed automated DNA extraction protocol on the QIAsymphony SP instrument for clinical plasma samples. The final version of this PhD thesis and the gained knowledge was transferred into two product development projects for further protocol development, optimization and testing. Broad access to the automated protocol can be provided through commercialization. Using this protocol an approximately

50-fold concentration of plasma DNA is possible, enabling higher detection sensitivity and the probability of catching the target molecules of interest. Zuiverloon *et al.* showed an improvement of FGFR3 mutation PCR assay by increasing the sample volume [274]. In addition, large volume DNA extractions with low final elution volumes enabled the detection of even “trace amounts of tumour-specific cfDNA from cancer patients” [275]. Many studies demonstrated the possible use of ccfNA or circulating tumor cells as biomarkers for so-called non-invasive to minimally invasive liquid biopsy. While circulating cell-free nucleic acid biomarkers are already a tool for prenatal diagnostics like the PraenaTest (LifeCodexx AG, Konstanz, Germany) or the Panorama Test (Natera Inc., San Carlos, California, USA) for, e.g., trisomy 21 testing, they are not yet established for patient care in the field of cancer. The lack of standardization as well as the need of well-designed and sufficiently powered multicenter studies is often discussed as the main reasons [256]. The newly developed protocol should help towards standardization and enable required multicenter studies. Many manual solutions for the isolation of ccfNA, which were already specified in 1.3, exist, but automation is strongly needed to make DNA extraction available for preferably all interested persons in research and clinical practice, including high throughput laboratories or sites where manual performance of DNA extraction is not practical for other reasons like lack of staff, for example. Furthermore, automated solutions better allow for compatibility and reproducibility than manual methods including many individual steps as potential error sources, making automation more suitable for standardization. The planned IVD status of the automated protocol provides the basis for using “liquid biopsy” as a clinical tool in cancer diagnostics. Furthermore, automation should improve reproducibility and comparability between different laboratories. Combined with further standardized and optimal preanalytical and analytical procedures, progress concerning the analysis of existing and the development of new biomarkers within ccfNA should be advanced.

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6. Appendix

6.1. Abbreviations

Abbreviation	Meaning
AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
ARMS	Amplification refractory mutation system
Bp	Base pairs
ccfDNA	Circulating cell-free DNA
cDNA	Circulating DNA
CFF	Cytosine-free fragment
cNA	Circulating nucleic acid
Ct	Cycle of threshold
CTRL	Control
EMTR	Elution microtube rack
FU	Fluorescence unit
GFP	Green fluorecent protein
GTC	Guanidinium thiocyanate
LINE-1	Long interspersed element 1
MutKo	Mutationskontrolle (mutation control)
NegKo	Negativkontrolle (negative control)
NSCLC	Non-small cell lung cancer
NTC	No template control
PAA	Polyacrylic acid
PBS	Phosphate buffered saline
PK	Proteinase K
R1/2	Reading direction 1/2
RC	Rod covers
RhD	Rhesus D
SNP	Single nucleotide polymorhism
SPC	Sample preparation cartridge
Taq	*Thermus aquaticus*
Wt-Pl	Wild type plasma

6.2. Study protocol for plasma preparation

Plasma preparation from EDTA whole blood samples for circulating cell-free DNA extraction

Whole blood in blood collection tubes has to be immediately stored at 4°C after blood withdrawal until plasma preparation. Blood collection tubes have to be additionally protected from mechanical influences and plasma has to be prepared within two hours.

1. Four BD Vacutainer® Plus plastic whole blood tubes (cat. no.: 367864) are used for blood withdrawal. Blood collection tubes have to be carefully inverted (eight times) after blood withdrawal and immediately placed into a fridge

2. Check list for plasma preparation has to be filled and documentation for pseudonymization has to be done

3. Informed consent has to be filed after educational advertising

4. Whole blood in blood collection tubes is placed in a centrifuge with swinging bucket rotor and fitting tube containers

5. Blood samples are centrifuged for 15 min. at 1900 x g (approx. 3000 rpm) and room temperature

6. Plasma supernatant is carefully removed after centrifugation using a Pasteur pipette without disturbing the buffy coat layer (3-5 mm plasma above the layer is left behind; a separate pipette is used for each donor). Blood collection tubes should therefore be kept upright and plasma should be slowly removed from above (pipette can be put to two tube positions to get more hold). 2-3 mL plasma can be prepared from a 6 mL blood collection tube

7. Hemolytic tubes are marked respectively and prepared separately. Other abnormalities like clouding are also noted

8. Prepared plasma of the four blood collection tubes is pooled in conical centrifugation tubes (15 mL). Centrifugation tubes are therefore marked with the running donor number

9. Plasma samples are immediately frozen and stored at -80°C

Important: This study protocol has to be completely carried out for each donor. All deviations are documented in the check list.

Checklist always has to be filled completely.

Furthermore, tissue has to be available for each donor (please announce any need accordingly).

6.3. QIAsymphony protocol versions

6.3.1. Protocol version 1

Sample material: 2 mL plasma

Binding buffer: 22 mM citric acid + 0.05% TritonX-100 (pH 2.68)

3 mg type B beads

Wash buffer 1: 1,000 µL 35 mM MES + 300 mM sodium chloride + 0.1% Triton-X100 (pH 5.8)

Wash buffer 2: 300 µL 35 mM MES + 0.1% Triton-X100 (pH 5.8)

Elution buffer: 150 µL 35 mM TRIS base + 80 mM sodium chloride

6.3.2. Protocol version 2

Sample material: 2 mL plasma

100 µL Proteinase K

Binding buffer: 22 mM citric acid + 0.05% TritonX-100 (pH 2.68)

3 mg type B beads

20 µL Proteinase K

180 µL buffer RDD } RDD step

1,800 µL 5 mM citric acid

Wash buffer 1: 1,000 µL 35 mM MES + 300 mM sodium chloride + 0.1% Triton-X100 (pH 5.8)

Wash buffer 2: 300 µL 35 mM MES + 0.1% Triton-X100 (pH 5.8)

Elution buffer: 150 µL 35 mM TRIS base + 80 mM sodium chloride

6.3.3. Protocol version 3

Sample material: 2 mL plasma

Binding buffer: 40 mM citric acid + 0.1% TritonX-100 + 200 mM sodium chloride (pH 4.5)

2 mg type B beads

Wash buffer 1: 1,000 µL 35 mM MES + 300 mM sodium chloride + 0.1% Triton-X100 (pH 5.8)

Wash buffer 2: 300 µL 35 mM MES + 0.1% Triton-X100 (pH 5.8)

Elution buffer: 150 µL 20 mM sodium hydroxide

6.3.4. Protocol version 4

Sample material: 2 mL plasma

Binding buffer: 40 mM citric acid + 0.1% TritonX-100 + 200 mM sodium chloride (pH 4.5)

2 mg type B beads

Wash buffer 1: 1,000 µL 35 mM MES + 300 mM sodium chloride + 0.1% Triton-X100 (pH 5.8)

Wash buffer 2: 300 µL 35 mM MES + 0.1% Triton-X100 (pH 5.8)

Elution buffer: 150 µL 40 mM sodium hydroxide

6.3.5. Protocol version 5

Sample material: 2 mL plasma

Binding buffer: 75 µL 1 M sodium acetate/acetic acid (pH 4)

3 mg type B beads

Wash buffer 1: 1,000 µL RNase free H_2O + 0.1% TritonX-100

Wash buffer 2: 300 µL RNase free H_2O + 0.1% TritonX-100

Elution buffer: 150 µL 35 mM TRIS base + 40 mM sodium chloride

6.3.6. Protocol version 6

Sample material: 2 mL plasma

Binding buffer: 75 µL 1 M sodium acetate/acetic acid buffer (pH 4)

3 mg type B beads

Wash buffer 1: 1,000 µL 35 mM TRIS + 0.1% TritonX-100 (pH 7.2)

Wash buffer 2: 300 µL 35 mM TRIS + 0.1% TritonX-100 (pH 7.2)

Elution buffer 1: 150 µL 35 mM TRIS base + 40 mM sodium chloride

Elution buffer 2: 150 µL 35 mM TRIS base + 80 mM sodium chloride

6.3.7. Protocol version 7

Sample material: 5 mL plasma

6 mg type B beads

Binding buffer: 150 µL 1 M citric acid + 0.05% TritonX-100 (pH 2.5)

Wash buffer 1: 1,000 µL 35 mM MES + 500 mM sodium chloride (pH 5.8)

Wash buffer 2: 300 µL 35 mM MES (pH 5.8)

Elution buffer: 150 µL 35 mM TRIS base + 80 mM sodium chloride

6.3.8. Protocol version 8

Sample material: 4 mL plasma

200 µL Proteinase K

Binding buffer 1: 50 mM MES + 0.1% TritonX-100 + 300 mM sodium chloride (pH 4.5)

Binding buffer 2: 40 mM citric acid + 0.1% TritonX-100 + 200 mM sodium chloride (pH 4.5)

6 mg type B beads

20 µL Proteinase K

180 µL buffer RDD

1,800 µL 5 mM citric acid

} RDD step

Wash buffer 1: 1,000 µL 35 mM MES + 0.1% TritonX-100 + 300 mM sodium chloride (pH 5.8)

Wash buffer 2: 300 µL 35 mM MES + 0.1% TritonX-100 (pH 5.8)

Elution buffer: 150 µL 10 mM sodium hydroxide

6.3.9. Protocol version 9

Sample material: 2 mL plasma

Binding buffer: 300 µL 327 mM citric acid + 1667 mM sodium chloride + 0.82% TritonX-100 (pH 4.5)

2 mg type B beads

Wash buffer 1: 1,000 µL 35 mM MES + 0.1% TritonX-100 + 300 mM sodium chloride (pH 5.8)

Wash buffer 2: 300 µL 35 mM MES + 0.1% TritonX-100 (pH 5.8)

Wash buffer 3: 300 µL 35 mM TRIS (pH 7.5)

Elution buffer: 150 µL 16.7 mM sodium hydroxide

6.3.10. Protocol version 10

Sample material: 4 mL plasma

Binding buffer: 300 µL 327 mM citric acid + 1667 mM sodium chloride + 0.82% TritonX-100 (pH 4.5)

2 mg type B beads

Wash buffer 1: 1,000 µL 35 mM MES + 0.1% TritonX-100 + 300 mM sodium chloride (pH 6.7)

Wash buffer 2: 300 µL 35 mM MES + 0.1% TritonX-100 (pH 6.7)

Wash buffer 3: 300 µL 35 mM TRIS (pH 8.5)

Elution buffer: 75 µL 16.7 mM sodium hydroxide

7. Declaration

I affirm that the graduate thesis has been created by myself independently and without assistance, taking the "fundamentals for protection of good scientific practice at the Heinrich Heine University Düsseldorf" into account.

I further certify that I have neither tried to submit this dissertation to Heinrich Heine University Düsseldorf nor another university and that I have not made any unsuccessful promotion attempts before.

Hamburg, March 2017

X

Annette Nocon

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