A Field Guide for Cancer Diagnostics using cell-free DNA: from Principles to Practice and Clinical Applications.

Anna-Lena Volckmar[1], Holger Sültmann[2*], Anja Riediger[2], Thoas Fioretos[3, 4], Peter Schirmacher[1], Volker Endris[1], Albrecht Stenzinger[1,5*], Steffen Dietz[2]

1 Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany

2 Division of Cancer Genome Research, German Cancer Research Center (DKFZ) and German Cancer Consortium (DKTK), Im Neuenheimer Feld 460, Heidelberg 69120, Germany

3 Department of Clinical Genetics, Lund University, Lund, Sweden

4 Department of Clinical Genetics, University and Regional Laboratories, Region Skåne, Lund, Sweden

5 German Cancer Consortium (DKTK), Partner Site Heidelberg, and German Cancer Research Center (DKFZ), Heidelberg, Germany

*Correspondence to:

Albrecht Stenzinger, Institute of Pathology, University Hospital Heidelberg, Im Neuenheimer Feld 224, 69120 Heidelberg, Phone: +49 06221-56334380, Fax: +49 06221-565251

Holger Sültmann, Cancer Genome Research (B063), German Cancer Research Center (DKFZ) and National Center for Tumor Diseases (NCT), Im Neuenheimer Feld 460, 69120 Heidelberg, Phone: +49 06221- 565934

Funded by: the German Cancer Consortium (DKTK)

Abbreviated Title: Guideline for cfDNA-based Cancer Diagnostics

Keywords: Resistance testing, tumor heterogeneity, ccfDNA, actionable

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/gcc.22517

Abstract

Recently, many genome-wide profiling studies provided insights into the molecular background of major cancer types. The deeper understanding of the genetic alterations and their functional consequences leveraged the discovery of novel therapeutic opportunities, improving clinical management of cancer patients. While tissue-based molecular patient stratification is the gold standard for precision medicine, it has certain limitations: Tissue biopsies are risky invasive sampling procedures and may not represent the entire tumor due to genetic heterogeneity. Complementary characterization of genetic information in the blood of cancer patients can serve as minimal-invasive 'liquid biopsy'. Released by different tissues in patients as well as healthy individuals, fragments of circulating cell-free DNA (cfDNA) are a potential source of diagnostic, predictive or prognostic biomarkers. cfDNA released from primary tumors or metastases (i.e. circulating tumor DNA, ctDNA) represents genomic aberrations in cancer cells. Recent studies have demonstrated technical feasibility and clinical applications of ctDNA including detection of drug targets and resistance mutations, longitudinal monitoring of tumors under therapy and detection of minimal residual disease. However, broad implementation of ctDNA analysis into daily clinical practice requires standardization and validation of pre-analytical and analytical procedures across different technologies. Currently, a variety of pre-analytical procedures for blood processing, isolation and quantification of cfDNA are used. Moreover, several analytical methods and technologies are available, ranging from PCR-based single locus assays to genome-wide approaches, which considerably differ in sensitivity, specificity, and throughput. In this review, we discuss the advantages and limitations of available methodologies and their potential applications in molecular diagnostics.

1. Cell-free circulating DNA and the subpopulation of circulating tumor DNA

In 1948, Mandel and Métais discovered the presence of cell free DNA (cfDNA) in human blood samples[1]. Thirty years later, it was demonstrated that serum and plasma from cancer patients contain higher concentrations of cfDNA than those from healthy individuals[2]. Later, it was found that the former harbor tumor-specific molecular alterations, suggesting that tumor-derived cfDNA, i.e., circulating tumor DNA (ctDNA), can appear in the circulation (Figure 1)[3]. Notably, cfDNA and ctDNA can also be detected in urine of patients with bladder cancer[4-6], colorectal cancer[7, 8], and was recently reported for patients with non-small cell lung cancer (NSCLC)[9, 10]. Other biological materials, such as stool[11, 12], saliva[13], cerebrospinal fluid[14], and pleural fluid[15] can also serve as sources of ctDNA. Despite these findings and the increased attention during the last decade, the exact origin and molecular release mechanism of cfDNA is still not fully understood. It is assumed that release of cfDNA occurs though apoptosis and necrosis of normal as well as malignant cells[16-19]. Moreover, some studies provided evidence for an active release via secretion of extracellular vesicles such as exosomes[20-22].

Initial evaluation of cfDNA size distributions[16] as well as recent next-generation sequencing (NGS) based analyses of cfDNA from pregnant women[23, 24], identified prominent cfDNA fragment lengths of 167 base pairs and multiples thereof, corresponding to the size of DNA in the chromatosome (nucleosome + linker histone H1). This fragmentation pattern suggests a contribution of nuclease cleavage during apoptotic cell death to the release of cfDNA into the blood. Interestingly, tumor-derived DNA molecules are characterized by higher fragmentation than non-neoplastic cfDNA fragments[25]. Shorter ctDNA fragments (between 132 and 145 bp) were observed in xenografted animal models as well as in plasma of cancer patients[25-28]. Although the cause for the occurrence of shorter fragments is currently unknown, it is hypothesized that differences in the nucleosomal patterning between malignant and hematopoietic cells play a role and may reflect the cells or tissues of origin[29]. DNAse activity may also be involved in this fragmentation process. For example, ctDNA molecules from urine and plasma samples vary in their fragment lengths with shorter fragments appearing in urine where more DNAses are abundant than in blood[30]. However, the exact proportion of each process contributing to the total cfDNA pool remains unknown, and the release of DNA is further influenced by physiological processes and disorders, including pregnancy[31], exhaustive exercise[32, 33], trauma[34, 35], inflammation[36], myocardial infarction[37], autoimmune disorders[38], and acute stroke[39, 40].

While in healthy individuals regular apoptotic cell death of lymphoid and myeloid cells as part of hematopoietic homeostasis constitutes the majority of cfDNA[29, 41], the contribution of tumor-derived ctDNA in the blood of cancer patients varies substantially from <0.01% to more than 60% of alleles in the circulation[42-45]. Of note, the amount and relative level of ctDNA in the blood is affected by tumor volume and stage (Figure 2 B), and it appears to be tumor entity-dependent[42]. Other factors such as tumor vascularization, cellular turnover rates (including proliferation and apoptosis) as well as passive DNA release from necrotic cells may further influence the level of ctDNA. In line with these observations, tumor size, predominant histology, necrosis, lymph-node involvement, lymphovascular invasion, high Ki67 proliferation index, and the total amount of cfDNA input were found to be predictive for the detection of ctDNA in plasma of NSCLC patients[46]. Treatment and timing of liquid biopsy sampling during therapy can also affect ctDNA burden: in patients diagnosed with NSCLC, ctDNA levels in blood peak within the first 24 h after start of tyrosine kinase inhibitor therapy but decrease quickly afterwards under successful therapy[47].

Multiregional analysis has demonstrated morphological and molecular heterogeneity between spatially distinct regions across primary tumors and metastases[48-52]. Thus, in general, a single tissue biopsy might not be representative for the entire tumor and complementary liquid biopsies can help to assess genetic heterogeneity of the tumor more comprehensively, especially in the context of resistance mutations emerging under therapy. Originating from multiple lesions, ctDNA harbors the potential to reflect the (sub-) clonal molecular heterogeneity of primary and metastatic disease[46, 53-55] and can inform about genomic aberrations in the absence of tissue biopsies[56] or in cases where the corresponding tumor tissue samples were negative[55, 57]. Moreover, ctDNA profiles depict the heterogeneity of acquired EGFR inhibitor resistance mechanisms in colorectal carcinoma (CRC) and NSCLC patients[58-61]. Although these studies show that ctDNA analysis can detect clonal variations of distinct tumor lesions, it is paramount to further investigate how well clonal populations are preserved and represented in a given cfDNA sample, i.e. how comprehensively ctDNA depicts genetic tumor heterogeneity in a given clinical scenario. Taken together, ctDNA analysis may inform tumor heterogeneity and potentially reveal mutations in very low clonal fractions, but is highly variable between individuals and during the course of disease.

2. cfDNA extraction from blood and other materials

As the amount of ctDNA in blood samples is limited and prone to contamination by high molecular genomic DNA from leucocytes, collection and extraction methods play a crucial role for the detection of tumor specific mutations. For blood storage until analysis, the choice between commonly available EDTA tubes and cell stabilizing tubes should depend on the transportation time: For in-house samples processed within four to six hours after collection, EDTA tubes are sufficient to extract cfDNA[62-64]. Longer periods of storage or transportation warrant the use of tubes containing cell-stabilizing reagents[65-70]. From these tubes, steady cfDNA quality can be obtained after up to one week of storage, independent of the temperatures tested in the analysis (4 °C or room temperature)[71]. Although manufacturers claim that cfDNA and cells are stable within the cell stabilizing tubes for two weeks, one study reported an increase of high molecular DNA after 7 days of storage at 4°C and room temperature[71]. Moreover, another study provides evidence that storage and shipping of blood in Cell-Free DNA BCT tubes (Streck) deviating from room temperature (4°C or 40°C) increases genomic DNA levels[72]. If longer storage periods are required, plasma or cfDNA can be frozen. However, a 30 % degradation of extracted cfDNA as well as cfDNA in plasma has been reported after one year of storage at -20 °C and -80°C, respectively[73]. Significant qualitative or quantitative differences of cfDNA preservation between cell-stabilizing tubes that would affect clinical-grade diagnostics have not yet been observed[64]. Another factor influencing cfDNA quality is transportation as shaking and moving the sample increases cell lysis and thus increases sample contamination with high molecular DNA[66, 74]. To minimize the impact of transportation, tubes should be filled without air bubbles.

Centrifuging whole blood twice, once to separate plasma, the second time to remove cells and cell debris is the current standard procedure. Reported conditions for centrifugation are between 800 - 2000 x g for 10-20 min for the first spin and 2000 – 16000 x g for the second[68, 75]. Additional centrifugation to remove further cell debris is still under debate. The temperature of centrifugation can vary between 4°C and room temperature and does not appear to influence cfDNA quantity or quality[62, 75].

For diagnostic purposes, cfDNA must be extracted with kits or methods dedicated for cfDNA as use of generic DNA extraction methods will lead to a loss or further fragmentation of short cfDNA fragments[76]. Extraction methods for cfDNA differ in several aspects (Figure 1). Many of the commercially available kits bind cfDNA to silica gel membrane-based columns (i.e. QIAamp circulating nucleic acid kit or QIAamp MinElute ccfDNA Kit (Qiagen), NucleoSpin® Plasma XS Kit (Macherey-Nagel) and FitAmp®Plasma/Serum DNA Isolation kit (Epigentek)) or to magnetic beads (i.e. Promega Maxwell RSC kits (Promega), QIAsymphony PAXgene Blood ccfDNA Kit (Qiagen) or MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific)). Other investigators use polymers to bind and later release cfDNA (i.e. Polymer Mediated Enrichment (PME) free-circulating DNA Extraction Kit (Analytik Jena)). Interestingly, some studies reported an improvement of cfDNA quality upon use of automated, magnetic bead-based extraction either with QIAsymphony or Promega Maxwell RSC kits when compared to manual methods[77-81] while others claimed that higher cfDNA yields can be obtained by manual approaches[82]. There may be interaction effects between blood collection tube and extraction method as some manufacturers provide different protocols for EDTA and cell conserving tubes.

Since interaction effects between the method for cfDNA extraction and the method applied for mutation detection may lead to increased artifacts, careful comparison during establishing liquid biopsy procedures is warranted[78, 83]. Different abilities to recover longer or shorter fragments have been described in the literature[84, 85]. Besides biological and pathological parameters reviewed above in chapter 1, the concentration of isolated cfDNA is influenced by the efficiency of the DNA extraction method and the final elution volume of the respective method. In a comparative study, cfDNA concentrations from identical artificially spiked plasma samples extracted at 56 different laboratories revealed wide variations in cfDNA yields ranging from 2.87 to 224.02 ng/ml with a median of 23.15 ng/ml of plasma[76]. While it is still debated whether blood plasma or serum contains more and higher quality cfDNA[67, 86, 87], there is evidence that serum is prone to high-molecular-weight DNA contamination due to delayed processing[88]. For cfDNA-based analytics of primary and metastatic brain tumors it is important to note that cerebrospinal fluid harbors more ctDNA than blood as the blood-brain barrier reduces molecular exchange with the peripheral circulation, total volume is comparably low and contamination by cellular DNA is limited[14, 42]. For cfDNA extraction from material other than blood plasma, different conditions may be required. For example, as cerebrospinal fluid contains much lower numbers of cells compared to blood, the influence of a second centrifugation step on the contamination with high molecular leucocyte DNA might be dispensable[14]. If and how storage conditions for example for blood, urine or cerebrospinal fluid affect mutation detection has not been comprehensively investigated yet.

While some commercial products result in high concentrations of cfDNA, these measurements have to be partially attributed to the carrier RNA used to increase the efficiency of cfDNA extraction. A single study discusses whether the extraction of cfDNA is necessary at all, as the authors noticed a loss of 40% of cfDNA in the buffers and 10% in the column of the QIAmp DNA Blood Mini kit[89]. The use and utility of whole plasma, which is directly subjected to amplification by digital droplet PCR (ddPCR), BEAMing or next generation sequencing (NGS) methods remains to be tested. Of note, an increase of input material does not always lead to a proportional increase of the cfDNA output. Thus, using more plasma for extraction might not significantly increase the cfDNA concentration although the total amount of cfDNA molecules will naturally increase[62, 68].

In our view, separation and enrichment of ctDNA from non-neoplastic cfDNA molecules could contribute to overcome sensitivity limitations in the detection of somatic alterations. The

aforementioned shortening of ctDNA compared to wild type cfDNA in part 1 might be utilized as a selection factor favoring tumor-derived molecules. Indeed, Mouliere et al. have demonstrated that size selection for short plasma DNA molecules improves subsequent ctDNA detection rates[27]. Specifically, selection of short DNA fragments between 90 to 150 bp led to 11-fold enrichment of the mutant ctDNA fraction and enabled identification of somatic aberrations which were previously masked by wild type alleles in unselected cfDNA[27]. Enrichment of ctDNA alleles may therefore improve the detection of genetic alterations with higher sensitivity and potentially lower tumor stages.

3. Methods to accurately measure the quantity of cfDNA

Although elevated cfDNA levels have been reported for cancer patients compared to healthy individuals, the low abundance of cfDNA in the circulation constitutes yet another challenge for robust diagnosis. Low cfDNA quantities are mainly caused by fast DNA clearance via filtering by the liver and kidney[90, 91]. The average cfDNA half-lifetime of fetal DNA in the maternal circulation is only 16.3 min[31], whereas the half-lifetime of ctDNA is reportedly higher in blood (up to 2 h[43]) and urine (up to 5 h[92]). These fast turnover rates can -in theory- facilitate real-time assessment of genomic aberrations. On the other hand, these dynamics may also interfere with mutation detection, as reliable timing of blood sample draws in relation to the clinical course of disease is a critical factor. As the cfDNA amount is usually in a range of only 10-40 ng/ml plasma, sensitive methods are required for accurate quantification. Commonly used technologies include fluorescent dyes, spectrophotometrics, and quantitative PCR analysis[93]. Intercalating fluorophores that selectively bind to double stranded DNA, e.g. the PicoGreen or Qubit Fluorometer assays allow sensitive DNA quantification down to as little as 25 pg/ml or 50 pg/ml, respectively. However, background fluorescence of unbound dye molecules and interference with contaminants such as salts, detergents, solvents as well as free nucleotides and single-stranded nucleic acids might influence the accuracy of fluorescence-based DNA quantification. Real-time quantitative PCR (qPCR) with intercalating dyes (e.g. SYBR Green, Thermo Fisher) or 5' hydrolysis probes (e.g. Universal ProbeLibrary, Roche) are widely used for routine quantification of various nucleic acids, including low amounts of cfDNA[94]. This approach estimates concentrations by comparing the abundance of housekeeping genes in cfDNA to a standard curve of defined concentrations. Different housekeeping genes have been reported for the quantification of cfDNA, including Alu repeat sequences, endogenous retrovirus group 3 (*ERV-3*), telomerase reverse transcriptase[95], glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), or β-actin[94, 96, 97]. Furthermore, digital PCR (dPCR) approaches can be utilized to determine cfDNA concentrations[47, 98, 99]. While spectrophotometric and fluorescence measurements are insensitive to damage or shortening of DNA molecules, fragmentation of cfDNA molecules can affect PCR reactions. In particular, cfDNA quantification might be prone to underestimations, as the low abundance of intact cfDNA due to fragmentation considerably reduces PCR efficiencies. High-molecular weight genomic DNA as a reference standard might further provoke underestimation. Therefore, such reference DNA should be fragmented prior to the measurement in order to be comparable to cfDNA. In summary, methods for the determination of cfDNA or ctDNA concentration should be highly sensitive and preferably rely on amplification of small fragments to reliably assess both quantity and quality of the DNA.

4. Test parameters and key determinants of sensitivity in liquid biopsy diagnostics

For the implementation and validation of a diagnostic test, five key parameters should be kept in mind: i) accuracy, ii) precision, iii) robustness, iv) sensitivity, and v) specificity. While the precision of a test describes the reproducibility (variability) of measurement results of a given assay under normal assay conditions, accuracy provides information on the proximity of a value measured by an assay to the reference value. For example, fluorometric tests for cfDNA concentration have a high precision (the values do not differ much between measurements) but have lower accuracy if the cfDNA was extracted by methods using carrier RNA. This is because in this scenario the amount of cfDNA is overestimated as the tests measure both DNA and (partially) RNA. Robustness describes the degree of fluctuation in test performance and test results if the test is perturbed by non-optimal conditions. In other words, robustness measures the effect of changes in test conditions on test results and thus indicates the ability of an assay to perform under real-life test conditions. As discussed in this section and in section 2 of the review, changes in cfDNA yields, transport time or temperature may negatively affect the robustness of plasma-based testing and influence other test parameters as well. Sensitivity and specificity define the occurrence of true positive (sensitivity) and true negative (specificity) results. For example, a test would be highly specific if all patients with a mutation were correctly identified as mutated by liquid biopsy, but could have low specificity if the test is positive in mutation-negative patients. On the other hand, a test with high specificity may overlook patients which are positive for mutations.

Although considerable technological progress has been made in the past years, the detection of low ctDNA amounts and rare mutant alleles remains challenging. Highly sensitive and specific methods are required to detect small fractions of typically ≤1% ctDNA in limited amounts of cfDNA. A general recommendation is to use known characterized patient species or commercially available artificial cfDNA samples harboring low frequent mutations (e.g. Horizon Discovery) for establishing and validating methods. Thus, thresholds used for liquid biopsy analysis can be generated and the robustness of assays can be tested. Mutations that cannot be detected reliably in these samples in repeated analyses by the method of choice provide an estimate of the error rate. To get valid diagnostic analysis thresholds for both the number of positive reads (in case of targeted NGS) as well as allelic fraction, at least one standard deviation from this error rate should be added. Further increases of sensitivity are required for early detection of cancer and identification of minimal residual disease. While detection of ctDNA frequencies ranging from 1% to 0.001% have been reported for targeted approaches, including dPCR and BEAMing[11, 100, 101], a targeted NGS approach termed “cancer personalized profiling by deep sequencing” (CAPP-Seq) has demonstrated accurate detection of somatic alterations down to 0.00025%[56]. Despite all technical advances, the detection of mutations is evidently limited by the amount of available genome equivalents in a plasma sample. For example, at least 100,000 genome equivalents per locus are necessary to detect a heterozygous mutation with a sensitivity of 0.001% (as 0.001% is 1 in 100.000). Hence, since a diploid human genome weighs approx. 6.6 pg, in this theoretical scenario at least 660 ng cfDNA would be required to detect a single positive signal from a mutated DNA fragment. This scenario, however, becomes even more challenging when more than one signal (or read) is necessary to determine a true mutation, which is a *condicio sine qua non* in routine diagnostics. To come back to a theoretical example: More than three independent genomic signals should be detected for a positive test. If 4 genomic copies represent 0.1% allelic frequency, 4000 genomic copies in total would need to be included in the sample. If we calculate for 95% sensitivity which is the common routine diagnostic threshold, more than 4200 genomic copies or 13 ng of DNA are necessary for a validly positive test. If

the cfDNA harbors DNA derived from normal cells, the amount is even higher. To meet this benchmark, a common 10ml blood draw is usually not sufficient. As 1 ml of plasma typically comprises about 3000 genome copies[102, 103], the detection limit is strongly influenced and determined by the number of available DNA molecules per locus (Figure 2 A). In this context it is important to keep in mind that a healthy individual also harbors cfDNA concentrations of 2.3 ng/ml in the plasma[104] and tumor derived cfDNA is only a subfraction. In conclusion, a higher sequencing coverage does not automatically translate to higher sensitivity if the amount of reads per locus is not matched by the equivalent amount of DNA molecules per locus. Hence, assessment of mutation detection levels of a given assay should be performed at low (cf)DNA concentrations mirroring real life samples.

For all techniques and methods discussed here, different levels of detection apply for known tumor mutations and *de novo* mutation calling. Allelic thresholds for *de novo* calling must be higher to decrease the likelihood of finding false positives and to increase the specificity of the analysis. In agreement with these theoretical considerations, most studies report high specificity (80-99%; i.e. 80 to 99 patients (out of 100 mutation negative patients) are correctly identified as negative) while sensitivity is usually lower (65-98%; i.e. 65 to 98 patients (out of 100 who are mutation positive) are correctly identified as positive)[45, 58, 105-108]. In other words, a sensitivity of 65% implies that 35% of positive patients are incorrectly identified as negative, while a specificity of 80% would incorrectly identify 2 out of 10 patients as mutation positive although they are negative. Both scenarios highlight the limitations of current cfDNA analytics in a routine diagnostic scenario but may be overcome with advances in pre-analytics and analytics in the future. The threshold for mutation detection has to be low enough to identify as many patients as possible who will benefit from therapy. On the other hand, a low threshold for mutation detection increases the chances that patients without the mutation will be identified as (false) positive. Thus, lowering the threshold for mutation detection to the noise level inevitably leads to patients not responding to therapy and risking adverse events. Of note, this scenario also has economic implications such as increased cost for therapy of patients with low abundance of the targetable mutations which would currently be overlooked.

Usually, concurrent tissue biopsies are used to analyze sensitivity and specificity retrospectively. Retrospective comparison of liquid biopsy findings with FFPE results demonstrated an overall sensitivity of 65% and specificity of 98% for liquid biopsy[45, 58, 105-108]. Deviations and divergent results are currently explained by different models: In general, it is hypothesized that biopsies generally underestimate tumor heterogeneity which might be better assessed by cfDNA analysis, thus leading to mutation findings in liquid biopsies, which went undetected in the corresponding tissue. Following this explanation, it is important to keep in mind that tissue-based results usually serve as a benchmark and gold standard against which cfDNA based results are being compared. Negative tissue-based analyses are - in this scenario - declared false negative when compared to (positive) cfDNA-results, which now become the new (true positive) benchmark. While this benchmark swap results may still be valid, false positive results in cfDNA analysis need to be carefully controlled as they would obviously flaw interpretation of data and result in the detection of pseudo-heterogeneity that is due to artifacts and unrelated to tumor biology. Additionally, in our thought experiment it may well be that ctDNA is too low for valid analysis; hence liquid biopsy may be too insensitive to detect residual low frequency mutations of the tumor (Figure 2 C). In conclusion, one has to be careful when analyzing these data sets and premature conclusions should be avoided to leverage the full potential of liquid biopsies in the near future.

5. Different methods for mutation detection in routine diagnostics

5.1 Sanger Sequencing

Although NGS is applied in routine diagnostics, Sanger sequencing of tissue DNA is still the gold standard for mutation detection. For liquid biopsy, Sanger sequencing poses two problems: On the one hand, the low amount of cfDNA generated from the samples will prohibit the sequential analysis of several targets. On the other hand, Sanger sequencing is not sufficiently sensitive to detect mutated allele frequencies below 20% when the tumor cell content of the sample is low[109]. Both of these problems are common in liquid biopsy samples[110]. As the allele frequency thresholds for mutation detection in liquid biopsies range from to 1% to 0.1% and below, tests by Sanger sequencing are likely to be false negative and might cause inaccurate treatment decisions[74].

5.2 Quantitative PCR (qPCR)

As with Sanger sequencing, many qPCR kits such as Therascreen (Qiagen) or Cobas (Roche) are validated for "clinical use" in FFPE material. Thus, in theory they are suited for the analysis of liquid biopsies and have been used successfully in clinical studies[111-113]. By now, liquid biopsy testing by qPCR (Cobas and Therascreen) has been approved by the FDA only for EGFR exon 19 deletions, EGFR p.L858R and EGFR p.T790M in NSCLC patients, from whom tissue biopsies cannot be obtained. One limitation is that the kits were validated for allele frequencies of > 1%[83, 114]. As discussed above, liquid biopsy samples often display mutations at lower allele frequencies, and the sensitivity of commercially available allele specific PCR methods might not be sufficient to reliably identify the patients suitable for precision medicine treatment. In a head-to-head comparison of qPCR to dPCR and NGS-based mutation detection, qPCR with a TaqMan Assay was the only method not reaching the desired allele frequency threshold <1 %[115]. Consecutive testing of several (activating) variants in each gene also requires ample material which is clearly limited in liquid biopsies[115]. In addition, the commercial kits cover common hot spot mutations in each oncogene but are in no way comprehensive[46, 116]. With qPCR, 40-50 % positive patient samples may be missed because their (rare) responsive or oncogenic variants are simply not covered by the selected mutation panel[46].

5.3 Digital PCR (dPCR) and digital droplet PCR (ddPCR)

A variety of allele-specific assays have been introduced to improve the analytical sensitivity of conventional quantitative PCR methods. Digital PCR approaches utilize physical separation of template alleles followed by individual amplifications[12]. While the template molecules are separated in individual reaction chambers in digital approaches, the digital droplet PCR utilizes oil emulsion droplets for physical separation of individual molecules and reactions. The analysis enables absolute quantification of mutant and wild type molecules in cfDNA and allows for the detection of allele frequencies as low as 0.01%[117, 118]. Both dPCR and ddPCR share a lower sensitivity towards inhibitors in the sample that may make qPCR analysis less reliable[119]. The use of locked nucleic acid (LNA) probes further enhances the sensitivity for mutations as the amplification of wild type alleles is suppressed[120]. Physical partitioning of PCR reactions can be achieved by chip-based systems using microwell plates (Quantstudio 3D, Thermo Fischer Scientific)[47, 117] or microfluidic systems for parallel PCR reactions (Fluidigm)[121, 122] as well as by oil emulsion-based droplet systems, including droplet digital PCR (Bio-Rad)[123], picoliter droplet-based digital PCR (RainDance)[100], and BEAMing[124, 125]. Comparable to qPCR assays, the ability of dPCR as well as ddPCR to perform multiplex analysis is limited. Many publications showed the suitability of ddPCR in liquid biopsies[107, 126-129]. Reported sensitivities are 64-82 % in a routine diagnostic setting[130] and even higher in study settings, and detection of allele frequencies as low as 0.1% can be achieved. Here, sampling error may play a role

since mutated cfDNA molecules might not be present in each replicate[120]. Therefore, samples should always be analyzed in triplicates if low allele frequencies are expected[131-133].

Although dPCR and ddPCR are cheaper and have lower turnaround times than NGS-based approaches when testing for single mutations only, the main disadvantages are that mutations have to be tested sequentially and that detection of previously unknown mutations is impossible[118]. As all digital PCR technologies require only low quantities of template molecules, single molecule detection is advantageous for the analysis of limited amount of cfDNA alleles in plasma. The high analytical sensitivity of dPCR approaches down to single molecules[134] requires appropriate working standards as well as careful handling of samples and reagents to prevent (cross-) contaminations. Especially with highly sensitive methods like dPCR and ddPCR, careful upfront evaluation of known characterized patient species or commercially available artificial samples to determine limit of detection is needed as discussed above.

5.4 Next generation sequencing or massive parallel sequencing (NGS)

In contrast to the targeted approaches, NGS is theoretically applicable to all patients and scenarios, as it allows *de novo* identification of molecular alterations, including single nucleotide substitutions, structural rearrangements and copy number variations (CNVs). As NGS is currently becoming routine for tumor tissue profiling, different assays (e.g. AmpliSeq Cancer Panel, Thermo Fisher; RAS Panel, Illumina) are validated for clinical applications but have limited analytical sensitivities for ctDNA diagnostics. For example, in a proof-of-concept study, the FDA-approved Oncomine targeted sequencing kit demonstrated a sensitivity of only 77% for the detection of sensitizing *EGFR* mutations in cfDNA of NSCLC patients[135]. Similar results, with an overall concordance of 76% between tumor tissue and cfDNA, were reported for the AmpliSeq Cancer Panel[136]. To overcome these limitations, more sensitive targeted and genome-wide sequencing approaches with improved error suppression rates have been developed[56, 104, 137-140]. Targeted sequencing approaches utilize enrichment of recurrently altered loci by PCR amplification or hybrid capture and permits deep sequencing of up to several hundred kilobases. In contrast to amplicon sequencing, hybridization-based approaches require less amplification cycles and allow for the detection of rearrangements in ctDNA[141, 142]. However, disadvantageously larger amounts of cfDNA are required than for amplicon-based methods. Integration of digital error suppression (iDES) by molecular barcoding and reduction of sequencing background artifacts, further improved the sensitivity of the CAPP-Seq technology to a theoretical detection limit of 0.00025% mutant alleles (2.5 in 10^6 molecules)[56]. Hence, molecular barcodes can be utilized to suppress errors and thus increase the sensitivity in amplicon sequencing approaches[56, 143].

5.5 Whole exome sequencing (WES) or whole genome sequencing (WGS)

Besides focused panels for massively parallel sequencing, hybrid-capture allows to cover large fractions of the genome. With technological advances, studies have illustrated the utility of whole-exome sequencing (WES) to characterize the molecular tumor representation in cfDNA[98, 144, 145]. Murtaza et al. used WES for disease monitoring under therapy and identified resistance-associated mutations at tumor progression in several cancer entities[146]. Although WES facilitates comprehensive molecular profiling, its analytical sensitivity is limited. As the coverage is relatively low compared to targeted sequencing panels, WES does not allow for the detection of rare mutations with allele frequencies below 5%. Thus, WES of cfDNA is rather feasible for patients with advanced tumors and increased ctDNA fractions, but has less potential for longitudinal therapy monitoring where detection of low allelic variants plays a major role. Although WES can identify the same driver mutations as

targeted panels, additional insights from WES are primarily passenger mutations with currently unknown functional impact, which present only low information value for precision medicine approaches[147].

Whole-genome sequencing (WGS) approaches have been implemented to study chromosomal rearrangements and CNVs in plasma of cancer patients[139, 140, 148, 149]. Genome-wide copy number profiles could be established from cfDNA and identified somatic copy number gains and losses in patients with colorectal and breast cancer[140, 149]. Moreover, WGS of cfDNA was shown to detect alterations in 98 % of metastatic prostate cancer patients and to identify acquired focal amplifications as a driving force in tumor progression[150]. Mutational signatures or copy number signatures derived from WGS or WES may also help more accurately determine the localization of ctDNA origin in cancer of unknown primary patients[151]. Furthermore, whole genome bisulfite sequencing may detect methylation differences, which can help to identify the origin of DNA in the liquid biopsy and thus represent markers for distinguishing cf- from ctDNA[41].While WGS and WES are limited in their sensitivity to detect tumor-derived variants, they can potentially be excellent tools to obtain a 'mutational finger print' of the tumor at diagnosis. The detected genetic make-up can then provide the basis for different assays in which a large number of variants can be followed in the cfDNA during treatment.

6. Detection limits in cfDNA – how low can we go?

At present, only few commercial kits are available that allow for NGS analysis of ctDNA with high sensitivity in routine diagnostics, as discussed in detail in 5.4. In all these cases, molecular barcodes are used to reduce noise in the sequencing signal. Roche recently introduced the AVENIO ctDNA analysis kits that incorporate methods from CAPP-Seq and integrated digital error suppression[56, 141, 152]. Hybridization-based enrichment of recurrently mutated regions across various entities enables the detection of somatic alterations down to 0.1%. Furthermore, Thermo Fisher Scientific's Oncomine™ assays enable variant identification by amplicon-based sequencing with detections limits of 0.1% in 20 ng cfDNA. While hybridization-based methods can determine true genetic allele frequencies without amplification, amplicons-based NGS needs error suppression by e.g. molecular barcoding to correct for amplification artifacts. Importantly, the performances and sensitivities of both technologies need to be corroborated in comparative studies.

Using the CAPP-Seq technology, Newman et al. identified plasma ctDNA mutations in 100% of stage II–IV NSCLC patients, with 96% specificity for mutant allele frequencies down to approximately 0.02%. However, ctDNA was detected in only 50% of stage I patients due to the low abundances of ctDNA in early disease stages[141]. Even with individualized sequencing panels based on previous tumor tissue genotyping, only slightly higher ctDNA detection rates (ca. 60%) could be achieved for early stage NSCLC patients[46]. Similar ctDNA detection rates have been demonstrated for patients across 14 tumor types at the earliest disease stages[42]. Lowering the analytical sensitivity further with the techniques at hand will negatively influence signal to noise ratio. Especially considering the low abundance of ctDNA in most blood samples, detection of mutations with less than 0.1% allele frequency with the current techniques is random at best.

7. Liquid biopsy for mutation testing in different diagnostic settings

Liquid biopsies have advantages over tissue biopsies regarding resistance testing as they may be representative not only for tumor heterogeneity but also for multiple tumor sites within each patient[53, 54, 153]. Depending on the localization of the metastasis[154], attention has to be paid to the body fluids chosen: For example, brain metastases release ctDNA into the surroundings, but the blood brain barrier hinders ctDNA to enter the peripheral blood stream to a certain degree[14, 153]. Consequently, in those cases, cerebrospinal liquids present higher levels of ctDNA than blood. Urine may also present a useful liquid for ctDNA analyses as it is easy to collect high volumes, increasing the resulting cfDNA amounts. ctDNA in urine also represents tumors which are not directly connected to the urinary tract. For example, ctDNA of NSCLC patients has been detected in urine[155].

Noninvasive analysis of cfDNA facilitates various clinical applications in the diagnosis and treatment of cancer patients, including tumor diagnosis[156], evaluation of immediate treatment response[9, 47], longitudinal therapy monitoring[43, 157], detection of minimal residual disease[108, 158] or emerging resistance[159], and characterization of molecular tumor heterogeneity[53]. The choice of the appropriate technology is primarily dependent on the specific application and time point of cfDNA assessment.

7.1 Upfront testing for mutations – Can Liquid biopsy identify cancer patients *de novo*?

As in general the amount of cfDNA[104] as well as the presence of oncogenic driver mutations would indicate cancer in a patient, liquid biopsy could in theory be used to detect a tumor before the onset of clinical symptoms. Thus, liquid biopsy has the potential to complement current methods for early detection of cancer such as mammography or the fecal occult blood test with predictive blood sampling. In fact, publications showed that it is possible to detect mutations up to two years before cancer diagnosis[160, 161]. Although the authors claim high sensitivity for their plasma analysis, less than 50 % of the patients (16/33 patients) positive for oncogenic mutations also developed cancer[161]. No information on the occurrence rate of cancer in the patients that were negative in liquid biopsy was given, thus a comparison of the 50% incidence rate with plasma negative samples is not possible. For sputum, the test identified 53 % (8/15 patients) with a diagnosis of lung cancer correctly[160]. Other studies showed that liquid biopsy is not more sensitive than current screening methods. For example, for ovarian cancer of stage I and II, clinical sensitivity for cancer detection by ultrasound and biomarker CA-125 was 89 %[162] but only 68% in liquid biopsy[104]. For early stage colon cancer, a fecal immunochemical test (including testing for KRAS/NRAS mutations) in stool had a sensitivity of 92%[163], while blood testing had a sensitivity of 71%[104]. Even if the liquid biopsy is positive for a tumor driver mutation, if no information about malignant lesions of the patient is available, the whole body of the individual would have to be screened by e.g. a CT-scan. Considering the immense psychological and financial implications, roughly 50% false positive cases currently preclude cfDNA-based screening approaches in routine diagnostics. Assuming technical advances in the next 3-5 years, in any case a true positive finding in a screening scenario of non-symptomatic individuals, who are considered healthy, opens up a wide field of clinical, ethical, psychological, and financial challenges that require further discussion. For example, a patient with a driver mutation (e.g. in KRAS) but no radiological and clinical evidence of disease cannot be treated but may experience severe psychological burden that directly affects quality of live.

The predictive value of these upfront cancer tests in cfDNA is further influenced by several factors: i) clonal hematopoiesis[115], ii) benign tumors harboring mutations, and iii) deaminating mutations which mimic somatic alterations. First, during clonal hematopoiesis, stem cells or other early blood cell progenitors acquire specific genetic aberrations and form a genetically distinct subpopulation of blood cells sharing the same genetic profile. These changes can include oncogenic driver mutations

and thus increase blood cancer risk especially in older patients[164-168]. As circulating cell-free DNA is mainly derived from hematopoetic cells, individuals with oncogenic driver mutation positive blood may either have increased risk of blood cancer or increased necrosis/apoptosis of premalignant hematopoetic cells[169]. In a large WES datasets comprising 17,182 individuals in an analysis for risk factors for diabetes mellitus, such somatic mutations were present in 9.6% of age class 70 – 79 (n = 2299), 11.7% of age class 80 – 89 (n = 317), and 18.4% of age class ≥90 years (n = 103[168]). Second, benign tumor entities may harbor the same (putative) oncogenic driver mutations as malignant lesions. This has been described for example in benign adenomas of the breast[170], benign nevi[171], or anogenital papillary hidradenoma[172] among others. Thus, the detection of a classic oncogenic driver mutation without previous knowledge of cancer does not necessarily indicate a malignant tumor. Finally, DNA deamination of 5-methylcytosine leads to certain clock-like mutational signatures (Signature 1a and 1b) which are characterized by an increase of C>T substitutions at NpCpG trinucleotides in malignant as well as non-malignant tissues during aging[173]. These C>T exchanges can also affect oncogenic driver genes (e.g. leading to EGFR T790M or classic KRAS hotspot mutations). As with clonal hematopoiesis, mutations due to deamination accumulate and increase during aging[173]. Taken together, the detection of a mutation in an otherwise healthy individual may indicate an increased risk to develop cancer but the sensitivities and specificities are far too low for routine diagnostic use of liquid biopsy tests. Here, a combination of liquid biopsy with additional biomarkers may increase sensitivity and specificity of early detection of cancer in future. Moreover, the total amount of DNA molecules per locus is another crucial factor influencing the detection limits of such approaches (for details see part 4).

7.2 Primary testing for targeted therapies using liquid biopsy

For patients with histologically confirmed cancer diagnosis, a liquid biopsy might be used for mutational profiling if the tissue biopsy was of insufficient quantity or quality for molecular analysis. This situation is critically different from *de novo* testing. Here, one or several specific oncogenic driver mutations with known clinical utility are being investigated. In general, two different scenarios apply that should be considered when implementing and evaluating cfDNA-based testing concepts: In a tumor-at-baseline (non-resistance) scenario, the overwhelming majority of the current druggable genetic targets are truncal as they are founder mutations which occur early on in tumor development (e.g. BRAF mutations in melanoma, ALK gene fusions in lung cancer). In other words such events are not subclonal but present in all tumor cells. Consequently, inhibition of e.g. mutant BRAF by vemurafenib leads to shrinkage of all tumor lesions and not only one or a few[174]. During clinical resistance, resistance-mediating genetic variants may emerge and these may indeed be subclonal (e.g. T790M mutations in pretreated EGFR-mutated lung cancer; for details see part. 7.4). Evaluation of tumor tissue and corresponding plasma samples using targeted error correction sequencing (TEC-Seq) revealed an overall concordance of 72% for mutations in both specimens from early and late stage patients[104]. For alterations with mutant allele fractions >1% in plasma, the concordance was 93%. NGS analysis of cfDNA may be a non-invasive substitute to metastasis biopsy for tumor genotyping[57] and enables identification of molecular determinants for targeted therapies in the plasma of cancer patients[141]. Importantly, high analytical sensitivity is required for the accurate detection and quantification of mutations, but can lead to high false positive rates and over-diagnosis (as discussed in part 4). Despite the fact that somatic mutations might be undetectable in the plasma of patients at early tumor stages or during stable disease[42, 56, 104], the lack of ctDNA does not indicate the absence of disease in primary testing. Hence, the FDA and EMA recommend using liquid biopsy

as a complementary method in case tissue from patient cannot be obtained. Mutational testing using FFPE tissue currently is and remains the gold standard.

7.3 Longitudinal testing – Tracking tumors during therapy

The short half-life time of DNA in the circulation[31] facilitates serial analysis of ctDNA for real-time disease monitoring in response to therapy. Of note, the time points of sampling influence the fraction and abundance of ctDNA in the blood: Immediate release of mutant alleles by dying tumor cells as consequence of drug administration increases ctDNA levels and may thus inform about therapy response. Acute changes in plasma ctDNA levels within 26 hours of treatment onset were observed in response to *EGFR* tyrosine kinase inhibitor therapy in NSCLC patients[47]. Indications for early therapy response were also found by daily ctDNA quantification in urine samples from NSCLC patients[9]. Besides targeted therapies, an initial peak followed by a rapid decrease of ctDNA levels as an indicator for early response was reported for T-cell transfer immunotherapy in patients with metastatic melanoma[175]. This finding cannot be generalized, as such changes were not observed after chemotherapy initiation in CRC patients[108]. Several studies have illustrated the correlations between treatment responses and dynamic changes in ctDNA levels over long periods of therapy follow-up[43, 47, 157, 176]. As phases of stable disease are accompanied by low ctDNA levels, mutations might not be detected at each time point. Thus, serial (e.g. monthly) sampling might be necessary to constantly evaluate the tumor status in order to detect progressive disease. The ideal time points for serial sampling may differ between patients. As liquid biopsies represent a mere snapshot of the (tumoral) genetic information, ctDNA presence and composition may be different within hours or days. Rising ctDNA levels can indicate tumor progression even before there is clinical evidence[47]. Moreover, heterogeneous molecular manifestations of resistance mechanisms have been identified in plasma of patients treated with targeted agents[58, 61, 159, 177], illustrating the potential of ctDNA diagnostics for resistance testing.

7.4 Testing for resistance to treatment variants – second line therapies

Several studies[177-180] demonstrated the feasibility of liquid biopsies to monitor clonal evolution under therapy and identify the mechanisms underlying resistance to treatment. For example, positive selection of *KRAS* mutant clones in patients with colorectal cancer under anti-EGFR therapy was observed using serial ctDNA analyses[61, 181]. A similar effect was found in NSCLC patients under anti-EGFR therapy using antibodies[58, 182]. Mutation testing for BRAF p.V600E showed the same predictive effect on BRAF- and MEK inhibitor therapy response in plasma as in tissue biopsies[183]. The technique further allowed monitoring resistance development in melanoma patients under BRAF inhibitor therapy[184-186]. Liquid biopsy analysis could also identify ESR1 mutations conferring resistance to fulvestrant treatment in ER-positive breast cancer[107, 146, 178, 187].

Of note, analyzing tyrosine kinase inhibitor-resistance in NSCLC patients indicated by the EGFR p.T790M mutation is currently the only application for liquid biopsy in routine diagnostics. The general feasibility of this approach was demonstrated not only in clinical studies[46, 54, 83, 188-190], but also in first studies related to routine diagnostics[4, 45, 146, 191], and some reports showed that the resistance-conferring mutation was often detectable in ctDNA prior to clinical progression[192, 193]. Although a low amount of ctDNA copies may be close to noise, data in osimertinib treated, EGFR p.T790M-positive NSCLC patients indicated response even with less than 10 DNA copies per ml plasma[194]. A single study described tumor size reduction under osimertinib treatment to be associated with decreasing levels of EGFR p.T790M allelic fraction in ctDNA[195], and detection of a mutation independent of DNA source (plasma and/or tissue) indicated therapy response of the patient[196]. However, a recent large

retrospective analysis of the phase I AURA trial including cross-comparison of tissue vs. liquid biopsy (using BEAMing technology) genotyping reported a sensitivity and specificity of only 70% for cfDNA-based analysis and a corresponding Kaplan-Meier analysis of progression-free survival did not show a predictive value for cfDNA-based genotyping while tissue-based positive test results for T790M were indicative of improved survival[197]. Collectively, these data clearly demonstrate that cfDNA-based tests hold great potential for the detection of resistance variants but still require a better and more thorough understanding of tumor biology and technology as highlighted above. Like in primary testing, FDA and EMA recommends liquid biopsy testing for cases in which no tissue material is available for resistance testing. With further technological advances (i.e. increasing ctDNA yield, better noise filtering for variant calling) fields of applications and indications may broaden.

8. Liquid biopsy in diagnostic reporting - What information is important for the oncologist?

While the technical considerations and realization of liquid biopsy mutational analysis are usually in the hand of molecular pathologists or biologists, the reporting has to be adapted to the oncologist's needs and demands (Figure 3). According to the guidelines for reporting of diagnostic genetic testing[198], a report on liquid biopsy should include patient information and clinical information on the samples analyzed such as the material of the liquid biopsy (e.g. blood, cerebrospinal fluid, stool, etc.) and the condition of the sample (e.g. hemolysis, separation of liquids and solids).

The specifications of the genetic test used for the analysis are most important, given the different sensitivities and specificities of the methods described in detail above. Here, as much information as possible may help explain differences between results obtained in different settings or at different time points. Minimal required information includes testing method and the assay (i.e. the mutation spectrum covered by the test or the panel with complete information on screened genes and exons[198]). Additional information on the amount of cfDNA extracted and the resulting range of sensitivity to be expected with the mutation detection method that was employed is preferable (Figure 3).

The detected mutations should be stated clearly according to general guidelines[198]. Information about the biological and clinical implications of the detected mutations in the scientific literature, such as known response of mutation harboring patients to specific treatments, should be provided. Therapeutic recommendations should only be given by trained medical personal. In case of testing for resistance mutations, the presence or absence of both the initial mutation relevant for treatment and the potential resistance mutation should be reported. If no known cancer mutation was detected, the oncologist has to be informed that this may result from low abundance of ctDNA rather than from a truly negative sample. Here, re-testing should be recommended. In case several samples of the patient have been analyzed sequentially (longitudinal testing), information on previous tests should be provided and the current results should be interpreted in their context.

9. Future applications of liquid biopsies in individualized therapy

Although liquid biopsy is currently only used for resistance testing in routine diagnostics (specifically T790M in NSCLC), there are further fields of application. If the small size difference between healthy cfDNA and ctDNA[25, 28, 199] can be exploited to enrich samples for ctDNA, higher concentrations of ctDNA in the samples could improve mutation detection. In conjunction with larger volumes of blood subjected to analysis, yielding in turn more genomic equivalents per locus, this may eventually pave

the way towards reliable detection of relapse, minimal residual disease or early detection of disease. Recent data suggest that liquid biopsies also harbor the potential to infer tumor mutational load (or mutational burden) guiding checkpoint blockade[200, 201]. While these preliminary data are of obvious interest, the very same technical checks and balances influencing sensitivity and specificity that have been discussed herein apply and more data, particularly from prospective clinical trials, are warranted.

WGS or targeted hybrid capture approaches may be used for broad detection of oncogenic gene fusions across tumor entities. Indeed, single cases of *ALK*-fusion detection in liquid biopsies by DNA capture methods have already been described in the literature[56, 104, 202-206]. Data on RNA-based screening for gene fusions as routinely done in FFPE[207] are still preliminary and limited but maybe equally effective.

With improving technologies and increasing standards, liquid biopsies are likely to play increasingly important roles in molecular diagnostics and complement tissue-based genetic profiling. However, while the feasibility for mutation detection in general and clinical utility for cfDNA based T790M resistance analytics has been clearly demonstrated, further use in routine diagnostics requires proven clinical utility in controlled trials and – as discussed above- further improvement of pre-analytics and detection methods to push sensitivity and specificity to a level that reliably meets clinical-grade diagnostic standards. Since liquid biopsies potentially carry genetic information of all tumor sites in the body, cfDNA based profiling may provide complementary and additional insights into the complex field of tumor evolution. First studies on ctDNA guided decision-making regarding targeted therapies are already underway. In summary, liquid biopsies have demonstrated usability in a wide range of patient focused applications such as the detection of actionable mutations and resistance development. The ease of material collection will provide deeper insights into tumor development and response to different treatments. In future, cfDNA holds the potential to improve early diagnostics, treatment and monitoring of disease.

References

1. Mandel P, Métais P.Les acides nucléiques du plasma sanguin chez l'homme [The nucleic acids of blood plasma in humans]. C r hebd séances Acad sci. 1948;(142):241-243.
2. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ.Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res. 1977;(37):646-650.
3. Stroun M, Anker P, Maurice P, et al.Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology. 1989;(46):318-322.
4. Togneri FS, Ward DG, Foster JM, et al.Genomic complexity of urothelial bladder cancer revealed in urinary cfDNA. Eur J Hum Genet. 2016;(24):1167-1174.
5. Ward DG, Bryan RT.Liquid biopsies for bladder cancer. Transl Androl Urol. 2017;(6):331-335.
6. Millholland JM, Li S, Fernandez CA, Shuber AP.Detection of low frequency FGFR3 mutations in the urine of bladder cancer patients using next-generation deep sequencing. Res Rep Urol. 2012;(4):33-40.
7. Su YH, Wang M, Brenner DE, et al.Human urine contains small, 150 to 250 nucleotide-sized, soluble DNA derived from the circulation and may be useful in the detection of colorectal cancer. J Mol Diagn. 2004;(6):101-107.
8. Su YH, Wang M, Aiamkitsumrit B, Brenner DE, Block TM.Detection of a K-ras mutation in urine of patients with colorectal cancer. Cancer Biomark. 2005;(1):177-182.
9. Husain H, Melnikova VO, Kosco K, et al.Monitoring Daily Dynamics of Early Tumor Response to Targeted Therapy by Detecting Circulating Tumor DNA in Urine. Clin Cancer Res. 2017;(23):4716-4723.
10. Tchekmedyian N, Mudad R, Blanco FF, et al.Longitudinal monitoring of ctDNA EGFR mutation burden from urine correlates with patient response to EGFR TKIs: A case series. Lung cancer. 2017;(108):22-28.
11. Diehl F, Schmidt K, Durkee KH, et al.Analysis of mutations in DNA isolated from plasma and stool of colorectal cancer patients. Gastroenterology. 2008;(135):489-498.
12. Vogelstein B, Kinzler KW.Digital PCR. Proc Natl Acad Sci U S A. 1999;(96):9236-9241.
13. Pu D, Liang H, Wei F, et al.Evaluation of a novel saliva-based epidermal growth factor receptor mutation detection for lung cancer: A pilot study. Thorac Cancer. 2016;(7):428-436.
14. Pan W, Gu W, Nagpal S, Gephart MH, Quake SR.Brain tumor mutations detected in cerebral spinal fluid. Clin Chem. 2015;(61):514-522.
15. Soh J, Toyooka S, Ichihara S, et al.EGFR mutation status in pleural fluid predicts tumor responsiveness and resistance to gefitinib. Lung cancer. 2007;(56):445-448.
16. Jahr S, Hentze H, Englisch S, et al.DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res. 2001;(61):1659-1665.
17. Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P.About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. Clin Chim Acta. 2001;(313):139-142.
18. Stroun M, Maurice P, Vasioukhin V, et al.The origin and mechanism of circulating DNA. Ann N Y Acad Sci. 2000;(906):161-168.
19. Thierry AR, Mouliere F, Gongora C, et al.Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. Nucleic Acids Res. 2010;(38):6159-6175.
20. Gahan PB, Anker P, Stroun M.Metabolic DNA as the origin of spontaneously released DNA? Ann N Y Acad Sci. 2008;(1137):7-17.
21. Kahlert C, Melo SA, Protopopov A, et al.Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. J Biol Chem. 2014;(289):3869-3875.
22. Thakur BK, Zhang H, Becker A, et al.Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res. 2014;(24):766-769.
23. Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR.Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci U S A. 2008;(105):16266-16271.

24. Lo YM, Chan KC, Sun H, et al.Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. Sci Transl Med. 2010;(2):61ra91.
25. Mouliere F, Robert B, Arnau Peyrotte E, et al.High fragmentation characterizes tumour-derived circulating DNA. PLoS One. 2011;(6):e23418.
26. Jiang PY, Chan CWM, Chan KCA, et al.Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. Proc Natl Acad Sci U S A. 2015;(112):E1317-E1325.
27. Mouliere F, Piskorz AM, Chandrananda D, et al.Selecting Short DNA Fragments In Plasma Improves Detection Of Circulating Tumour DNA. bioRxiv. 2017;DOI: 10.1101/134437
28. Underhill HR, Kitzman JO, Hellwig S, et al.Fragment Length of Circulating Tumor DNA. PLoS Genet. 2016;(12):e1006162.
29. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J.Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. Cell. 2016;(164):57-68.
30. Peng M, Chen C, Hulbert A, Brock MV, Yu F.Non-blood circulating tumor DNA detection in cancer. Oncotarget. 2017;(8):69162-69173.
31. Lo YM, Corbetta N, Chamberlain PF, et al.Presence of fetal DNA in maternal plasma and serum. Lancet. 1997;(350):485-487.
32. Atamaniuk J, Vidotto C, Tschan H, et al.Increased concentrations of cell-free plasma DNA after exhaustive exercise. Clin Chem. 2004;(50):1668-1670.
33. Tug S, Helmig S, Deichmann ER, et al.Exercise-induced increases in cell free DNA in human plasma originate predominantly from cells of the haematopoietic lineage. Exerc Immunol Rev. 2015;(21):164-173.
34. Lo YM, Rainer TH, Chan LY, Hjelm NM, Cocks RA.Plasma DNA as a prognostic marker in trauma patients. Clin Chem. 2000;(46):319-323.
35. Rodrigues EM, Simon D, Ikuta N, et al.Elevated Cell-Free Plasma DNA Level as an Independent Predictor of Mortality in Patients with Severe Traumatic Brain Injury. J Neurotraum. 2014;(31):1639-1646.
36. Jiang N, Pisetsky DS.The effect of inflammation on the generation of plasma DNA from dead and dying cells in the peritoneum. J Leukoc Biol. 2005;(77):296-302.
37. Chang CP, Chia RH, Wu TL, et al.Elevated cell-free serum DNA detected in patients with myocardial infarction. Clin Chim Acta. 2003;(327):95-101.
38. Galeazzi M, Morozzi G, Piccini M, et al.Dosage and characterization of circulating DNA: present usage and possible applications in systemic autoimmune disorders. Autoimmun Rev. 2003;(2):50-55.
39. Rainer TH, Wong LKS, Lam W, et al.Prognostic use of circulating plasma nucleic acid concentrations in patients with acute stroke. Clin Chem. 2003;(49):562-569.
40. Tsai NW, Lin TK, Chen SD, et al.The value of serial plasma nuclear and mitochondrial DNA levels in patients with acute ischemic stroke. Clinica Chimica Acta. 2011;(412):476-479.
41. Sun K, Jiang PY, Chan KCA, et al.Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. Proc Natl Acad Sci U S A. 2015;(112):E5503-E5512.
42. Bettegowda C, Sausen M, Leary RJ, et al.Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med. 2014;(6):224ra224.
43. Diehl F, Schmidt K, Choti MA, et al.Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008;(14):985-990.
44. El Messaoudi S, Mouliere F, Du Manoir S, et al.Circulating DNA as a Strong Multimarker Prognostic Tool for Metastatic Colorectal Cancer Patient Management Care. Clinical Cancer Research. 2016;(22):3067-3077.
45. Thierry AR, Mouliere F, El Messaoudi S, et al.Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat Med. 2014;(20):430-435.
46. Abbosh C, Birkbak NJ, Wilson GA, et al.Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. Nature. 2017;(545):446-451.
47. Riediger AL, Dietz S, Schirmer U, et al.Mutation analysis of circulating plasma DNA to determine response to EGFR tyrosine kinase inhibitor therapy of lung adenocarcinoma patients. Sci Rep. 2016;(6):33505.

48. Jamal-Hanjani M, Wilson GA, McGranahan N, et al.Tracking the Evolution of Non-Small-Cell Lung Cancer. N Engl J Med. 2017;(376):2109-2121.
49. Yates LR, Gerstung M, Knappskog S, et al.Subclonal diversification of primary breast cancer revealed by multiregion sequencing. Nat Med. 2015;(21):751-759.
50. Zhang J, Fujimoto J, Zhang J, et al.Intratumor heterogeneity in localized lung adenocarcinomas delineated by multiregion sequencing. Science. 2014;(346):256-259.
51. Warth A, Muley T, Meister M, et al.The novel histologic International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification system of lung adenocarcinoma is a stage-independent predictor of survival. J Clin Oncol. 2012;(30):1438-1446.
52. Dietz S, Harms A, Endris V, et al.Spatial distribution of EGFR and KRAS mutation frequencies correlates with histological growth patterns of lung adenocarcinomas. Int J Cancer. 2017; 10.1002/ijc.30881
53. De Mattos-Arruda L, Weigelt B, Cortes J, et al.Capturing intra-tumor genetic heterogeneity by de novo mutation profiling of circulating cell-free tumor DNA: a proof-of-principle. Ann Oncol. 2014;(25):1729-1735.
54. Jamal-Hanjani M, Wilson GA, Horswell S, et al.Detection of ubiquitous and heterogeneous mutations in cell-free DNA from patients with early-stage non-small-cell lung cancer. Ann Oncol. 2016;(27):862-867.
55. Murtaza M, Dawson SJ, Pogrebniak K, et al.Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. Nat Commun. 2015;(6):
56. Newman AM, Lovejoy AF, Klass DM, et al.Integrated digital error suppression for improved detection of circulating tumor DNA. Nat Biotechnol. 2016;(34):547-555.
57. Lebofsky R, Decraene C, Bernard V, et al.Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. Mol Oncol. 2015;(9):783-790.
58. Chabon JJ, Simmons AD, Lovejoy AF, et al.Circulating tumour DNA profiling reveals heterogeneity of EGFR inhibitor resistance mechanisms in lung cancer patients. Nat Commun. 2016;(7):11815.
59. Pietrantonio F, Vernieri C, Siravegna G, et al.Heterogeneity of Acquired Resistance to Anti-EGFR Monoclonal Antibodies in Patients with Metastatic Colorectal Cancer. Clinical Cancer Research. 2017;(23):2414-2422.
60. Russo M, Siravegna G, Blaszkowsky LS, et al.Tumor Heterogeneity and Lesion-Specific Response to Targeted Therapy in Colorectal Cancer. Cancer Discov. 2016;(6):147-153.
61. Siravegna G, Mussolin B, Buscarino M, et al.Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. Nat Med. 2015;(21):795-801.
62. Page K, Guttery DS, Zahra N, et al.Influence of plasma processing on recovery and analysis of circulating nucleic acids. PLoS One. 2013;(8):e77963.
63. Mehrotra M, Singh RR, Chen W, et al.Study of Preanalytic and Analytic Variables for Clinical Next-Generation Sequencing of Circulating Cell-Free Nucleic Acid. J Mol Diagn. 2017;(19):514-524.
64. van Dessel LF, Beije N, Helmijr JC, et al.Application of circulating tumor DNA in prospective clinical oncology trials - standardization of preanalytical conditions. Mol Oncol. 2017;(11):295-304.
65. Rothwell DG, Smith N, Morris D, et al.Genetic profiling of tumours using both circulating free DNA and circulating tumour cells isolated from the same preserved whole blood sample. Mol Oncol. 2016;(10):566-574.
66. El Messaoudi S, Rolet F, Mouliere F, Thierry AR.Circulating cell free DNA: Preanalytical considerations. Clin Chim Acta. 2013;(424):222-230.
67. Parpart-Li S, Bartlett B, Popoli M, et al.The Effect of Preservative and Temperature on the Analysis of Circulating Tumor DNA. Clin Cancer Res. 2017;(23):2471-2477.
68. Sherwood JL, Corcoran C, Brown H, et al.Optimised Pre-Analytical Methods Improve KRAS Mutation Detection in Circulating Tumour DNA (ctDNA) from Patients with Non-Small Cell Lung Cancer (NSCLC). PLoS One. 2016;(11):e0150197.
69. Kang Q, Henry NL, Paoletti C, et al.Comparative analysis of circulating tumor DNA stability In K3EDTA, Streck, and CellSave blood collection tubes. Clin Biochem. 2016;(49):1354-1360.

70. Alidousty C, Brandes D, Heydt C, et al.Comparison of Blood Collection Tubes from Three Different Manufacturers for the Collection of Cell-Free DNA for Liquid Biopsy Mutation Testing. J Mol Diagn. 2017;10.1016/j.jmoldx.2017.06.004
71. Norton SE, Luna KK, Lechner JM, Qin J, Fernando MR.A new blood collection device minimizes cellular DNA release during sample storage and shipping when compared to a standard device. J Clin Lab Anal. 2013;(27):305-311.
72. Medina Diaz I, Nocon A, Mehnert DH, et al.Performance of Streck cfDNA Blood Collection Tubes for Liquid Biopsy Testing. PLoS One. 2016;(11):e0166354.
73. Sozzi G, Roz L, Conte D, et al.Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays. J Natl Cancer Inst. 2005;(97):1848-1850.
74. Fassunke J, Ihle MA, Lenze D, et al.EGFR T790M mutation testing of non-small cell lung cancer tissue and blood samples artificially spiked with circulating cell-free tumor DNA: results of a round robin trial. Virchows Arch. 2017;(10.1007/s00428-017-2226-8
75. Page K, Powles T, Slade MJ, et al.The importance of careful blood processing in isolation of cell-free DNA. Ann N Y Acad Sci. 2006;(1075):313-317.
76. Malentacchi F, Pizzamiglio S, Verderio P, et al.Influence of storage conditions and extraction methods on the quantity and quality of circulating cell-free DNA (ccfDNA): the SPIDIA-DNAplas External Quality Assessment experience. Clin Chem Lab Med. 2015;(53):1935-1942.
77. Stemmer C, Beau-Faller M, Pencreac'h E, et al.Use of magnetic beads for plasma cell-free DNA extraction: toward automation of plasma DNA analysis for molecular diagnostics. Clin Chem. 2003;(49):1953-1955.
78. Perez-Barrios C, Nieto-Alcolado I, Torrente M, et al.Comparison of methods for circulating cell-free DNA isolation using blood from cancer patients: impact on biomarker testing. Transl Lung Cancer Res. 2016;(5):665-672.
79. Sorber L, Zwaenepoel K, Deschoolmeester V, et al.A Comparison of Cell-Free DNA Isolation Kits: Isolation and Quantification of Cell-Free DNA in Plasma. J Mol Diagn. 2017;(19):162-168.
80. Spindler KL, Pallisgaard N, Vogelius I, Jakobsen A.Quantitative cell-free DNA, KRAS, and BRAF mutations in plasma from patients with metastatic colorectal cancer during treatment with cetuximab and irinotecan. Clin Cancer Res. 2012;(18):1177-1185.
81. Wolf A, Beller K, Groemminger S, et al.Purification of Circulating Cell-Free DNA from Plasma and Urine Using the Automated Large-Volume Extraction on the QIAsymphony(R) SP Instrument. Adv Exp Med Biol. 2016;(924):179-185.
82. Haselmann V, Ahmad-Nejad P, Geilenkeuser WJ, et al.Results of the first external quality assessment scheme (EQA) for isolation and analysis of circulating tumour DNA (ctDNA). Clin Chem Lab Med. 2017;10.1515/cclm-2017-0283
83. Wan JC, Massie C, Garcia-Corbacho J, et al.Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nat Rev Cancer. 2017;(17):223-238.
84. Breitbach S, Tug S, Helmig S, et al.Direct quantification of cell-free, circulating DNA from unpurified plasma. PLoS One. 2014;(9):e87838.
85. Beranek M, Sirak I, Vosmik M, et al.Carrier molecules and extraction of circulating tumor DNA for next generation sequencing in colorectal cancer. Acta medica. 2016;(59):54-58.
86. Lee TH, Montalvo L, Chrebtow V, Busch MP.Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. Transfusion. 2001;(41):276-282.
87. Jung M, Klotzek S, Lewandowski M, Fleischhacker M, Jung K.Changes in concentration of DNA in serum and plasma during storage of blood samples. Clin Chem. 2003;(49):1028-1029.
88. Board RE, Williams VS, Knight L, et al.Isolation and extraction of circulating tumor DNA from patients with small cell lung cancer. Ann N Y Acad Sci. 2008;(1137):98-107.
89. Breitbach S, Sterzing B, Magallanes C, Tug S, Simon P.Direct measurement of cell-free DNA from serially collected capillary plasma during incremental exercise. J Appl Physiol (1985). 2014;(117):119-130.

90. Botezatu I, Serdyuk O, Potapova G, et al.Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. Clin Chem. 2000;(46):1078-1084.
91. Gauthier VJ, Tyler LN, Mannik M.Blood clearance kinetics and liver uptake of mononucleosomes in mice. J Immunol. 1996;(156):1151-1156.
92. Cheng THT, Jiang P, Tam JCW, et al.Genomewide bisulfite sequencing reveals the origin and time-dependent fragmentation of urinary cfDNA. Clin Biochem. 2017;(50):496-501.
93. Fleischhacker M, Schmidt B.Circulating nucleic acids (CNAs) and cancer - A survey. Bba-Rev Cancer. 2007;(1775):181-232.
94. Devonshire AS, Whale AS, Gutteridge A, et al.Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. Anal Bioanal Chem. 2014;(406):6499-6512.
95. Alcaide M, Yu S, Davidson J, et al.Targeted error-suppressed quantification of circulating tumor DNA using semi-degenerate barcoded adapters and biotinylated baits. Sci Rep. 2017;(7):10574.
96. Kamat AA, Sood AK, Dang D, et al.Quantification of total plasma cell-free DNA in ovarian cancer using real-time PCR. Ann Ny Acad Sci. 2006;(1075):230-234.
97. Rago C, Huso DL, Diehl F, et al.Serial assessment of human tumor burdens in mice by the analysis of circulating DNA. Cancer Res. 2007;(67):9364-9370.
98. Dietz S, Schirmer U, Merce C, et al.Low Input Whole-Exome Sequencing to Determine the Representation of the Tumor Exome in Circulating DNA of Non-Small Cell Lung Cancer Patients. PLoS One. 2016;(11):e0161012.
99. Manokhina I, Singh TK, Penaherrera MS, Robinson WP.Quantification of Cell-Free DNA in Normal and Complicated Pregnancies: Overcoming Biological and Technical Issues. PLoS One. 2014;(9):e101500.
100. Taly V, Pekin D, Benhaim L, et al.Multiplex Picodroplet Digital PCR to Detect KRAS Mutations in Circulating DNA from the Plasma of Colorectal Cancer Patients. Clin Chem. 2013;(59):1722-1731.
101. Sanmamed MF, Fernandez-Landazuri S, Rodriguez C, et al.Quantitative Cell-Free Circulating BRAF(V600E) Mutation Analysis by Use of Droplet Digital PCR in the Follow-up of Patients with Melanoma Being Treated with BRAF Inhibitors. Clin Chem. 2015;(61):297-304.
102. Perkins G, Yap TA, Pope L, et al.Multi-purpose utility of circulating plasma DNA testing in patients with advanced cancers. PLoS One. 2012;(7):e47020.
103. Leung F, Kulasingam V, Diamandis EP, et al.Circulating Tumor DNA as a Cancer Biomarker: Fact or Fiction? Clin Chem. 2016;(62):1054-1060.
104. Phallen J, Sausen M, Adleff V, et al.Direct detection of early-stage cancers using circulating tumor DNA. Sci Transl Med. 2017;(9):
105. Karachaliou N, Mayo-de las Casas C, Queralt C, et al.Association of EGFR L858R Mutation in Circulating Free DNA With Survival in the EURTAC Trial. JAMA Oncol. 2015;(1):149-157.
106. Kuo YB, Chen JS, Fan CW, Li YS, Chan EC.Comparison of KRAS mutation analysis of primary tumors and matched circulating cell-free DNA in plasmas of patients with colorectal cancer. Clin Chim Acta. 2014;(433):284-289.
107. Schiavon G, Hrebien S, Garcia-Murillas I, et al.Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med. 2015;(7):313ra182.
108. Tie J, Kinde I, Wang Y, et al.Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Ann Oncol. 2015;(26):1715-1722.
109. Endris V, Penzel R, Warth A, et al.Molecular diagnostic profiling of lung cancer specimens with a semiconductor-based massive parallel sequencing approach: feasibility, costs, and performance compared with conventional sequencing. J Mol Diagn. 2013;(15):765-775.
110. Diaz LA, Jr., Bardelli A.Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol. 2014;(32):579-586.
111. Jenkins S, Yang JC, Ramalingam SS, et al.Plasma ctDNA Analysis for Detection of the EGFR T790M Mutation in Patients with Advanced Non-Small Cell Lung Cancer. J Thorac Oncol. 2017;(12):1061-1070.

112. Dearden S, Brown H, Jenkins S, et al.EGFR T790M mutation testing within the osimertinib AURA Phase I study. Lung cancer. 2017;(109):9-13.
113. Reck M, Hagiwara K, Han B, et al.ctDNA Determination of EGFR Mutation Status in European and Japanese Patients with Advanced NSCLC: The ASSESS Study. J Thorac Oncol. 2016;(11):1682-1689.
114. Wu YL, Zhou C, Liam CK, et al.First-line erlotinib versus gemcitabine/cisplatin in patients with advanced EGFR mutation-positive non-small-cell lung cancer: analyses from the phase III, randomized, open-label, ENSURE study. Ann Oncol. 2015;(26):1883-1889.
115. Bartels S, Persing S, Hasemeier B, et al.Molecular Analysis of Circulating Free DNA from Lung Cancer Patients in Routine Laboratory Practice: A Cross-Platform Comparison of Three Different Molecular Methods for Mutation Detection. J Mol Diagn. 2017;10.1016/j.jmoldx.2017.05.008
116. Xu T, Kang X, You X, et al.Cross-Platform Comparison of Four Leading Technologies for Detecting EGFR Mutations in Circulating Tumor DNA from Non-Small Cell Lung Carcinoma Patient Plasma. Theranostics. 2017;(7):1437-1446.
117. Oshiro C, Kagara N, Naoi Y, et al.PIK3CA mutations in serum DNA are predictive of recurrence in primary breast cancer patients. Breast Cancer Res Tr. 2015;(150):299-307.
118. Busser B, Lupo J, Sancey L, et al.Plasma Circulating Tumor DNA Levels for the Monitoring of Melanoma Patients: Landscape of Available Technologies and Clinical Applications. Biomed Res Int. 2017;(2017):5986129.
119. Dingle TC, Sedlak RH, Cook L, Jerome KR.Tolerance of droplet-digital PCR vs real-time quantitative PCR to inhibitory substances. Clin Chem. 2013;(59):1670-1672.
120. Arnason T, Hart RD, Taylor SM, et al.Diagnostic accuracy and safety of fine-needle aspiration biopsy of the parapharyngeal space. Diagn Cytopathol. 2012;(40):118-123.
121. Yung TKF, Chan KCA, Mok TSK, et al.Single-Molecule Detection of Epidermal Growth Factor Receptor Mutations in Plasma by Microfluidics Digital PCR in Non-Small Cell Lung Cancer Patients. Clinical Cancer Research. 2009;(15):2076-2084.
122. Madic J, Kiialainen A, Bidard FC, et al.Circulating tumor DNA and circulating tumor cells in metastatic triple negative breast cancer patients. Int J Cancer. 2015;(136):2158-2165.
123. Hindson BJ, Ness KD, Masquelier DA, et al.High-Throughput Droplet Digital PCR System for Absolute Quantitation of DNA Copy Number. Anal Chem. 2011;(83):8604-8610.
124. Diehl F, Li M, He YP, et al.BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. Nat Methods. 2006;(3):551-559.
125. Dressman D, Yan H, Traverso G, Kinzler KW, Vogelstein B.Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proc Natl Acad Sci U S A. 2003;(100):8817-8822.
126. Diehl F, Li M, Dressman D, et al.Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci U S A. 2005;(102):16368-16373.
127. Christensen E, Birkenkamp-Demtroder K, Nordentoft I, et al.Liquid Biopsy Analysis of FGFR3 and PIK3CA Hotspot Mutations for Disease Surveillance in Bladder Cancer. Eur Urol. 2017;(71):961-969.
128. Wan R, Wang Z, Lee JJ, et al.Comprehensive analysis of the discordance of EGFR mutation status between tumor tissues and matched circulating tumor DNA in advanced non-small-cell lung cancer. J Thorac Oncol. 2017;10.1016/j.jtho.2017.05.011
129. Guttery DS, Page K, Hills A, et al.Noninvasive detection of activating estrogen receptor 1 (ESR1) mutations in estrogen receptor-positive metastatic breast cancer. Clin Chem. 2015;(61):974-982.
130. Sacher AG, Paweletz C, Dahlberg SE, et al.Prospective Validation of Rapid Plasma Genotyping for the Detection of EGFR and KRAS Mutations in Advanced Lung Cancer. JAMA Oncol. 2016;(2):1014-1022.
131. Strain MC, Lada SM, Luong T, et al.Highly precise measurement of HIV DNA by droplet digital PCR. PLoS One. 2013;(8):e55943.
132. van Ginkel JH, Huibers MMH, van Es RJJ, de Bree R, Willems SM.Droplet digital PCR for detection and quantification of circulating tumor DNA in plasma of head and neck cancer patients. BMC cancer. 2017;(17):428.

133. Taylor SC, Laperriere G, Germain H.Droplet Digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable nonsense to publication quality data. Sci Rep. 2017;(7):2409.
134. Day E, Dear PH, McCaughan F.Digital PCR strategies in the development and analysis of molecular biomarkers for personalized medicine. Methods. 2013;(59):101-107.
135. Rachiglio AM, Abate RE, Sacco A, et al.Limits and potential of targeted sequencing analysis of liquid biopsy in patients with lung and colon carcinoma. Oncotarget. 2016;(7):66595-66605.
136. Xu S, Lou F, Wu Y, et al.Circulating tumor DNA identified by targeted sequencing in advanced-stage non-small cell lung cancer patients. Cancer Lett. 2016;(370):324-331.
137. Belic J, Koch M, Ulz P, et al.Rapid Identification of Plasma DNA Samples with Increased ctDNA Levels by a Modified FAST-SeqS Approach. Clin Chem. 2015;(61):838-849.
138. Forshew T, Murtaza M, Parkinson C, et al.Noninvasive Identification and Monitoring of Cancer Mutations by Targeted Deep Sequencing of Plasma DNA. Sci Transl Med. 2012;(4):136ra68.
139. Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B.Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A. 2011;(108):9530-9535.
140. Leary RJ, Sausen M, Kinde I, et al.Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med. 2012;(4):162ra154.
141. Newman AM, Bratman SV, To J, et al.An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med. 2014;(20):548-554.
142. Siravegna G, Sartore-Bianchi A, Mussolin B, et al.Tracking a CAD-ALK gene rearrangement in urine and blood of a colorectal cancer patient treated with an ALK inhibitor. Ann Oncol. 2017;(28):1302-1308.
143. Volik S, Alcaide M, Morin RD, Collins C.Cell-free DNA (cfDNA): Clinical Significance and Utility in Cancer Shaped By Emerging Technologies. Mol Cancer Res. 2016;(14):898-908.
144. Butler TM, Johnson-Camacho K, Peto M, et al.Exome Sequencing of Cell-Free DNA from Metastatic Cancer Patients Identifies Clinically Actionable Mutations Distinct from Primary Disease. PLoS One. 2015;(10):e0136407.
145. Klevebring D, Neiman M, Sundling S, et al.Evaluation of Exome Sequencing to Estimate Tumor Burden in Plasma. PLoS One. 2014;(9):e104417.
146. Murtaza M, Dawson SJ, Tsui DW, et al.Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature. 2013;(497):108-112.
147. Leary RJ, Kinde I, Diehl F, et al.Development of personalized tumor biomarkers using massively parallel sequencing. Sci Transl Med. 2010;(2):20ra14.
148. Chan KCA, Jiang PY, Zheng YWL, et al.Cancer Genome Scanning in Plasma: Detection of Tumor-Associated Copy Number Aberrations, Single-Nucleotide Variants, and Tumoral Heterogeneity by Massively Parallel Sequencing. Clin Chem. 2013;(59):211-224.
149. Heitzer E, Auer M, Hoffmann EM, et al.Establishment of tumor-specific copy number alterations from plasma DNA of patients with cancer. Int J Cancer. 2013;(133):346-356.
150. Ulz P, Belic J, Graf R, et al.Whole-genome plasma sequencing reveals focal amplifications as a driving force in metastatic prostate cancer. Nat Commun. 2016;(7):12008.
151. Molparia B, Nichani E, Torkamani A.Assessment of circulating copy number variant detection for cancer screening. PLoS One. 2017;(12):e0180647.
152. Tsao SC, Weiss J, Hudson C, et al.Monitoring response to therapy in melanoma by quantifying circulating tumour DNA with droplet digital PCR for BRAF and NRAS mutations. Sci Rep. 2015;(5):11198.
153. De Mattos-Arruda L, Mayor R, Ng CK, et al.Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. Nat Commun. 2015;(6):8839.
154. Budczies J, von Winterfeld M, Klauschen F, et al.The landscape of metastatic progression patterns across major human cancers. Oncotarget. 2015;(6):570-583.
155. Sands J, Li Q, Hornberger J.Urine circulating-tumor DNA (ctDNA) detection of acquired EGFR T790M mutation in non-small-cell lung cancer: An outcomes and total cost-of-care analysis. Lung Cancer. 2017;(110):19-25.

156. Sozzi G, Conte D, Mariani L, et al.Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients. Cancer Res. 2001;(61):4675-4678.
157. Dawson SJ, Tsui DW, Murtaza M, et al.Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013;(368):1199-1209.
158. Garcia-Murillas I, Schiavon G, Weigelt B, et al.Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med. 2015;(7):302ra133.
159. Diaz LA, Williams RT, Wu J, et al.The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012;(486):537-540.
160. Mao L, Hruban RH, Boyle JO, Tockman M, Sidransky D.Detection of oncogene mutations in sputum precedes diagnosis of lung cancer. Cancer Res. 1994;(54):1634-1637.
161. Gormally E, Vineis P, Matullo G, et al.TP53 and KRAS2 mutations in plasma DNA of healthy subjects and subsequent cancer occurrence: a prospective study. Cancer Res. 2006;(66):6871-6876.
162. Menon U, Gentry-Maharaj A, Hallett R, et al.Sensitivity and specificity of multimodal and ultrasound screening for ovarian cancer, and stage distribution of detected cancers: results of the prevalence screen of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS). Lancet Oncol. 2009;(10):327-340.
163. Imperiale TF, Ransohoff DF, Itzkowitz SH, et al.Multitarget stool DNA testing for colorectal-cancer screening. N Engl J Med. 2014;(370):1287-1297.
164. Steensma DP, Bejar R, Jaiswal S, et al.Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. Blood. 2015;(126):9-16.
165. Busque L, Patel JP, Figueroa ME, et al.Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. Nat Genet. 2012;(44):1179-1181.
166. Xie M, Lu C, Wang J, et al.Age-related mutations associated with clonal hematopoietic expansion and malignancies. Nat Med. 2014;(20):1472-1478.
167. Genovese G, Kahler AK, Handsaker RE, et al.Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med. 2014;(371):2477-2487.
168. Jaiswal S, Fontanillas P, Flannick J, et al.Age-related clonal hematopoiesis associated with adverse outcomes. N Engl J Med. 2014;(371):2488-2498.
169. Yeh P, Dickinson M, Ftouni S, et al.Molecular disease monitoring using circulating tumor DNA in myelodysplastic syndromes. Blood. 2017;(129):1685-1690.
170. Volckmar AL, Leichsenring J, Flechtenmacher C, et al.Tubular, lactating, and ductal adenomas are devoid of MED12 Exon2 mutations, and ductal adenomas show recurrent mutations in GNAS and the PI3K-AKT pathway. Genes Chromosomes Cancer. 2017;(56):11-17.
171. Marino-Enriquez A, Fletcher CD.Shouldn't we care about the biology of benign tumours? Nat Rev Cancer. 2014;(14):701-702.
172. Pfarr N, Sinn HP, Klauschen F, et al.Mutations in genes encoding PI3K-AKT and MAPK signaling define anogenital papillary hidradenoma. Genes Chromosomes Cancer. 2016;(55):113-119.
173. Alexandrov LB, Nik-Zainal S, Wedge DC, et al.Signatures of mutational processes in human cancer. Nature. 2013;(500):415-421.
174. Wagle N, Emery C, Berger MF, et al.Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. J Clin Oncol. 2011;(29):3085-3096.
175. Xi LQ, Pham THT, Payabyab EC, et al.Circulating Tumor DNA as an Early Indicator of Response to T-cell Transfer Immunotherapy in Metastatic Melanoma. Clinical Cancer Research. 2016;(22):5480-5486.
176. Maier J, Lange T, Kerle I, et al.Detection of mutant free circulating tumor DNA in the plasma of patients with gastrointestinal stromal tumor harboring activating mutations of CKIT or PDGFRA. Clin Cancer Res. 2013;(19):4854-4867.
177. Thress KS, Paweletz CP, Felip E, et al.Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M. Nat Med. 2015;(21):560-562.
178. Frenel JS, Carreira S, Goodall J, et al.Serial Next-Generation Sequencing of Circulating Cell-Free DNA Evaluating Tumor Clone Response To Molecularly Targeted Drug Administration. Clin Cancer Res. 2015;(21):4586-4596.

179. Russo M, Misale S, Wei G, et al.Acquired Resistance to the TRK Inhibitor Entrectinib in Colorectal Cancer. Cancer Discov. 2016;(6):36-44.
180. Thress KS, Brant R, Carr TH, et al.EGFR mutation detection in ctDNA from NSCLC patient plasma: A cross-platform comparison of leading technologies to support the clinical development of AZD9291. Lung cancer. 2015;(90):509-515.
181. Morelli MP, Overman MJ, Dasari A, et al.Characterizing the patterns of clonal selection in circulating tumor DNA from patients with colorectal cancer refractory to anti-EGFR treatment. Ann Oncol. 2015;(26):731-736.
182. Diaz LA, Jr., Sausen M, Fisher GA, Velculescu VE.Insights into therapeutic resistance from whole-genome analyses of circulating tumor DNA. Oncotarget. 2013;(4):1856-1857.
183. Santiago-Walker A, Gagnon R, Mazumdar J, et al.Correlation of BRAF Mutation Status in Circulating-Free DNA and Tumor and Association with Clinical Outcome across Four BRAFi and MEKi Clinical Trials. Clin Cancer Res. 2016;(22):567-574.
184. Gray ES, Rizos H, Reid AL, et al.Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. Oncotarget. 2015;(6):42008-42018.
185. Lipson EJ, Velculescu VE, Pritchard TS, et al.Circulating tumor DNA analysis as a real-time method for monitoring tumor burden in melanoma patients undergoing treatment with immune checkpoint blockade. J Immunother Cancer. 2014;(2):42.
186. Schreuer M, Meersseman G, Van Den Herrewegen S, et al.Quantitative assessment of BRAF V600 mutant circulating cell-free tumor DNA as a tool for therapeutic monitoring in metastatic melanoma patients treated with BRAF/MEK inhibitors. J Transl Med. 2016;(14):95.
187. De Mattos-Arruda L, Caldas C.Cell-free circulating tumour DNA as a liquid biopsy in breast cancer. Mol Oncol. 2016;(10):464-474.
188. Bernabe R, Hickson N, Wallace A, Blackhall FH.What do we need to make circulating tumour DNA (ctDNA) a routine diagnostic test in lung cancer? Eur J Cancer. 2017;(81):66-73.
189. Weerts MJA, van Marion R, Helmijr JCA, et al.Somatic Tumor Mutations Detected by Targeted Next Generation Sequencing in Minute Amounts of Serum-Derived Cell-Free DNA. Sci Rep. 2017;(7):2136.
190. Remon J, Menis J, Hasan B, et al.The APPLE Trial: Feasibility and Activity of AZD9291 (Osimertinib) Treatment on Positive PLasma T790M in EGFR-mutant NSCLC Patients. EORTC 1613. Clinical lung cancer. 2017;10.1016/j.cllc.2017.02.005
191. Mazloom AR, Dzakula Z, Oeth P, et al.Noninvasive prenatal detection of sex chromosomal aneuploidies by sequencing circulating cell-free DNA from maternal plasma. Prenat Diagn. 2013;(33):591-597.
192. Oxnard GR, Paweletz CP, Kuang Y, et al.Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. Clin Cancer Res. 2014;(20):1698-1705.
193. Sorensen BS, Wu L, Wei W, et al.Monitoring of epidermal growth factor receptor tyrosine kinase inhibitor-sensitizing and resistance mutations in the plasma DNA of patients with advanced non-small cell lung cancer during treatment with erlotinib. Cancer. 2014;(120):3896-3901.
194. Buder A, Hochmair M, Holzer S, et al.P3.02b-032 Association between EGFR T790M Mutation Copy Numbers in Cell-Free Plasma DNA and Response to Osimertinib in Advanced NSCLC. J Thorac Oncol. 2017;(12):S1205–S1206.
195. Piotrowska Z, Niederst MJ, Karlovich CA, et al.Heterogeneity Underlies the Emergence of EGFRT790 Wild-Type Clones Following Treatment of T790M-Positive Cancers with a Third-Generation EGFR Inhibitor. Cancer Discov. 2015;(5):713-722.
196. Remon J, Caramella C, Jovelet C, et al.Osimertinib benefit in EGFR-mutant NSCLC patients with T790M-mutation detected by circulating tumour DNA. Ann Oncol. 2017;(28):784-790.
197. Oxnard GR, Thress KS, Alden RS, et al.Association Between Plasma Genotyping and Outcomes of Treatment With Osimertinib (AZD9291) in Advanced Non-Small-Cell Lung Cancer. J Clin Oncol. 2016;(34):3375-3382.

198. Claustres M, Kozich V, Dequeker E, et al.Recommendations for reporting results of diagnostic genetic testing (biochemical, cytogenetic and molecular genetic). Eur J Hum Genet. 2014;(22):160-170.
199. Mouliere F, El Messaoudi S, Gongora C, et al.Circulating Cell-Free DNA from Colorectal Cancer Patients May Reveal High KRAS or BRAF Mutation Load. Transl Oncol. 2013;(6):319-328.
200. Khagi Y, Goodman AM, Daniels GA, et al.Hypermutated Circulating Tumor DNA: Correlation with Response to Checkpoint Inhibitor-Based Immunotherapy. Clin Cancer Res. 2017;(23):5729-5736.
201. Gandara DR, Kowanetz M, Mok TSK, et al.Blood-based biomarkers for cancer immunotherapy: Tumor mutational burden in blood (bTMB) is associated with improved atezolizumab (atezo) efficacy in 2L+ NSCLC (POPLAR and OAK). Ann Oncol. 2017;(28):v460-v496.
202. Lai AZ, Schrock AB, Erlich RL, et al.Detection of an ALK Fusion in Colorectal Carcinoma by Hybrid Capture-Based Assay of Circulating Tumor DNA. Oncologist. 2017;(22):774-779.
203. Liang W, He Q, Chen Y, et al.Metastatic EML4-ALK fusion detected by circulating DNA genotyping in an EGFR-mutated NSCLC patient and successful management by adding ALK inhibitors: a case report. BMC cancer. 2016;(16):62.
204. Muller JN, Falk M, Talwar J, et al.Concordance between Comprehensive Cancer Genome Profiling in Plasma and Tumor Specimens. J Thorac Oncol. 2017;10.1016/j.jtho.2017.07.014
205. Siravegna G, Marsoni S, Siena S, Bardelli A.Integrating liquid biopsies into the management of cancer. Nat Rev Clin Oncol. 2017;(14):531-548.
206. Wang Y, Tian PW, Wang WY, et al.Noninvasive genotyping and monitoring of anaplastic lymphoma kinase (ALK) rearranged non-small cell lung cancer by capture-based next-generation sequencing. Oncotarget. 2016;(7):65208-65217.
207. Pfarr N, Stenzinger A, Penzel R, et al.High-throughput diagnostic profiling of clinically actionable gene fusions in lung cancer. Genes Chromosomes Cancer. 2016;(55):30-44.

Figures

Figure 1

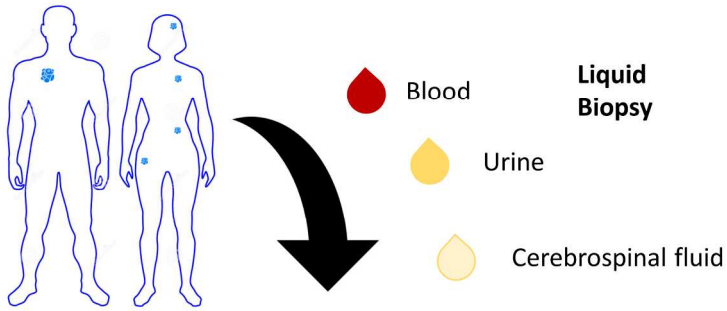
Workflow of the ctDNA from patient over extraction to the different extraction and mutation detection methods. Liquid biopsy (in from of e.g. blood, urine, cerebrospinal fluid) is taken from the cancer patient and can be extracted by various manual or automatic methods. Subsequently, the cfDNA sample is subjected to different mutation detection methods (Sanger sequencing, Whole exome/genome sequencing, quantitative PCR, digital PCR, digital droplet PCR, next generation sequencing, NGS with CAPP sequencing or molecular barcodes).

Figure 2

A) Genome equivalents and cfDNA input material calculations for several expected mutation allele frequencies. Of note: The amount of cfDNA does not necessarily rely solely on ctDNA derived from tumor. B) Tumor volume correlates strongly with the amount of ctDNA in the blood measured by oncogenic mutations (adapted from [48]). C) Maximum expected mutation allele frequency per cfDNA input. As cfDNA is not completely tumor derived, expected allele frequencies should be adapted.

Figure 3

Exemplary report for cfDNA-based mutation analysis in routine diagnostics.



cfDNA extraction

Method		cfDNA quality	cfDNA quantity	Usability
Non-extracted		high	medium	low
Trizol	manual	high	medium	low
Polymer	manual	medium	high	medium
Silicabased colums	manual	medium	high	high
Magnetic beads	manual	medium	high	high
Silicabased colums	automatic	high	high	high
Magnetic beads	automatic	high	high	high

Mutation detection

Sensitivity	Methods	Information	Cost	Time
10%	Sanger	medium	low	medium
10%	WES/WGS	high	high	high
1%	qPCR	low	low	Low
0.1%	dPCR	low	low	low
0.1%	ddPCR	low	low	low
0.1%	NGS	high	medium	medium
0.01%	NGS (Capp Seq or mol. BC)	high	high	medium

Figure 1

190x275mm (300 x 300 DPI)

A

Variant allele frequency	10%	1%	0.1%	0.01%	0.001%
Genome equivalents	10	100	1,000	10,000	100,000
Required cfDNA Input	66 pg	660 pg	6600 pg	66,000 pg	660,000 pg

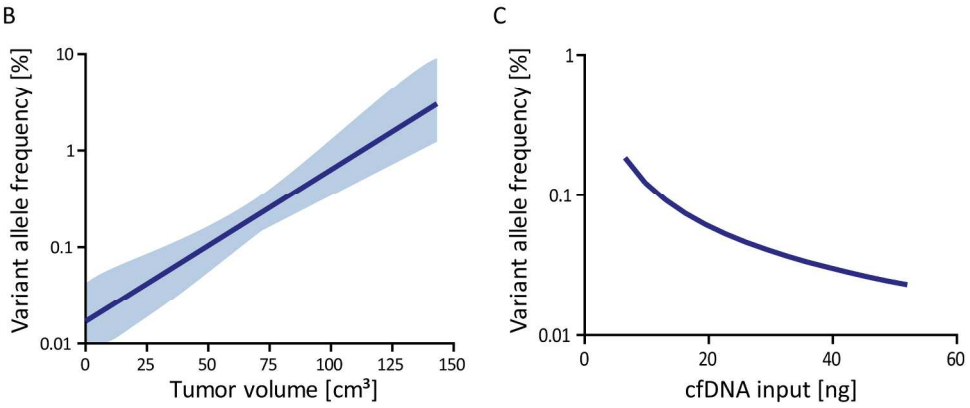


Figure 2

206x131mm (300 x 300 DPI)

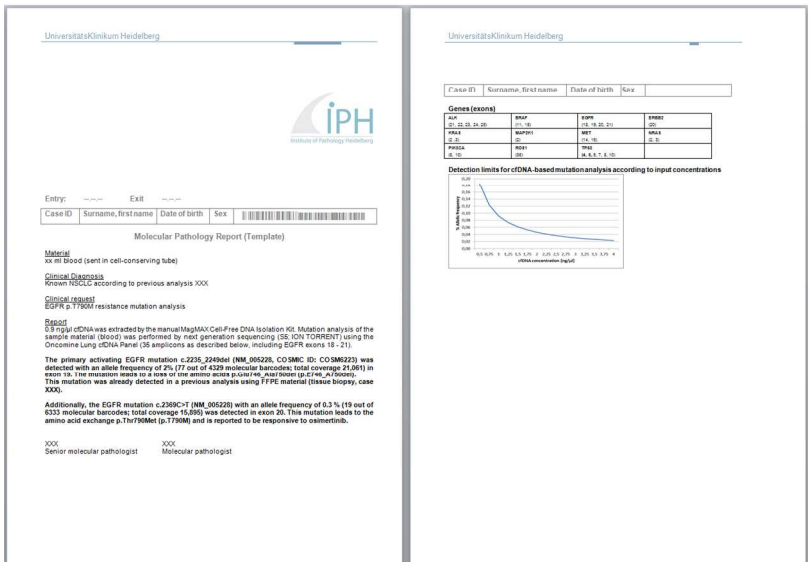


Figure 3

338x190mm (300 x 300 DPI)