Rapid Identification of Plasma DNA Samples with Increased ctDNA Levels by a Modified FAST-SeqS Approach

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BACKGROUND: Recent progress in the analysis of cell-free DNA fragments [cell-free circulating tumor DNA (ctDNA)] now allows monitoring of tumor genomes by noninvasive means. However, previous studies with plasma DNA from patients with cancer demonstrated highly variable allele frequencies of ctDNA. The comprehensive analysis of tumor genomes is greatly facilitated when plasma DNA has increased amounts of ctDNA. Therefore, a fast and cost-effective prescreening method to identify such plasma samples without previous knowledge about alterations in the respective tumor genome could assist in the selection of samples suitable for further extensive qualitative analysis.

METHODS: We adapted the recently described Fast Aneuploidy Screening Test-Sequencing System (FAST-SeqS) method, which was originally established as a simple, effective, noninvasive screening method for fetal aneuploidy from maternal blood.

RESULTS: We show that our modified FAST-SeqS method (mFAST-SeqS) can be used as a prescreening tool for an estimation of ctDNA percentage. With a combined evaluation of genome-wide and chromosome arm-specific z-scores from dilution series with cell line DNA and by comparisons of plasma-Seq profiles with data from mFAST-SeqS, we established a detection limit of ≥10% mutant alleles. Plasma samples with an mFAST-SeqS z-score ≥5 showed results that were highly concordant with those of copy number profiles obtained from our previously described plasma-Seq approach.

CONCLUSIONS: Advantages of this approach include the speed and cost-effectiveness of the assay and that no prior knowledge about the genetic composition of tumor samples is necessary to identify plasma DNA samples with >10% ctDNA content.

Recent progress in the analysis of cell-free circulating tumor DNA (ctDNA) now allows monitoring of tumor genomes by noninvasive means (1–7). Basically, 2 strategies to monitor ctDNA have emerged. First, targeted approaches, i.e., single or few tumor-specific somatic mutations present in the primary tumor, are used to monitor residual disease in the peripheral blood (6, 8, 9) (Fig. 1). Disadvantages of targeted approaches include the need for a detailed characterization of the primary tumor genome to identify suitable targets, which is not always possible (e.g., if only a biopsy was obtained). Furthermore, the analysis of only a single mutation or a few mutations limits the options to monitor the clonal evolution of a tumor genome. However, with highly sensitive approaches, mutations can be detected even if they are present at low allele frequencies (1, 10–13), which is important, as several recent publications demonstrated a high variability of ctDNA allele frequency even in advanced-stage metastatic disease (1, 6, 14–16).

Second, untargeted approaches establish genome-wide patterns of copy number aberrations (CNAs) from ctDNA (2, 4, 15, 17) or assess the mutation spectrum by exome sequencing from plasma DNA (7) (Fig. 1). Advantages of untargeted strategies include independence of prior knowledge about characteristics of the primary tumor and easier identification of novel changes occurring during tumor evolution. However, a disadvantage is that a certain percentage of ctDNA in plasma DNA is required for the reliable reconstruction of tumor-specific copy number changes. According to our experience, this
percentage is in the range of ≥10% (4, 14, 15, 18). Owing to the high variability of ctDNA (from 0.01% to >90% (1, 6, 15, 16)), not all blood samples will therefore be suitable for genome-wide analyses (Fig. 1). We sought an untargeted strategy to identify plasma DNA samples that are suitable for a further workup with complex whole-genome sequencing approaches.

To this end, we used a modified Fast Aneuploidy Screening Test-Sequencing System (FAST-SeqS) method described by Kinde et al. (19) that was developed as a prenatal screening method to establish fetal chromosome status. We adapted this method to prescreen plasma DNA samples from cancer patients to estimate the fraction of ctDNA and its suitability for genome-wide analyses, such as plasma-Seq (4). We show that our modified FAST-SeqS (mFAST-SeqS) can be used to estimate the amount of ctDNA in plasma in a cost-effective and rapid manner without any prior knowledge of specific aberrations of the primary tumor (Fig. 1).

Methods

PATIENT SAMPLES AND CANCER CELL LINES
We analyzed 35 control samples (18 women, 17 men) without malignant disease and a set of samples from cancer patients with different tumor entities at different
stages, which were available from previous studies (4, 14). We obtained 24 blood samples from 21 patients with advanced breast cancer (mean age 61 years; range 37–84), including hormone receptor–positive/negative and/or human epidermal growth factor receptor (HER2)–positive/negative tumors, from the Department of Obstetrics and Gynecology, Medical University of Graz. Follow-up samples taken at 13, 12, and 11 months were available from 3 patients (B1, B4, and B5). Prostate cancer patients (n = 56) (mean age 70.4 years; range 51–87) were recruited at the Department of Urology or the Division of Clinical Oncology, Department of Internal Medicine, Medical University of Graz. Four patients were under surveillance, 10 patients had a prostatectomy with localized cancer, and 42 patients had metastatic disease. Follow-up samples were available at 3, 10, 11, 9, and 6 months from 5 patients (P39, P40, P102, P111, and P147). All patients were of Caucasian origin. The study was approved by the ethics committee of the Medical University of Graz (approval numbers 21–227 ex 09/10, breast cancer; 21–228 ex 09/10, prostate cancer) and conducted according to the Declaration of Helsinki, and written informed consent was obtained from all patients. Additionally, this study includes cancer cell lines (HepG2, HT29, and MCF7) that were purchased from American Type Culture Collection and cultured according to the supplier’s recommendations.

SERIAL DILUTIONS OF CELL LINES
To validate the reliability and repeatability, i.e., inter- and intraassay variability, we used a dilution series of HT29 DNA (100%, 50%, 10% 5%, 0%). The sensitivity of mFAST-SeqS was determined with serial dilutions of DNA from cancer cell lines (HepG2, HT29, MCF7) diluted in noncancerous DNA (Promega) in the following ratios: 50%, 25% 10%, 5%, and 1%.

EXTRACTION OF GENOMIC DNA AND PLASMA DNA
Genomic DNA was extracted with the QIAamp DNA Midi Kit (Qiagen) according to the manufacturer’s protocol. Plasma DNA was prepared with the QIAamp DNA Blood Mini Kit (Qiagen) as previously described (15). For quantification of plasma DNA, we used the Qubit dsDNA HS Assay Kit (Life Technologies).

MODIFIED FAST-SeqS
Line-1 (L1) amplicon libraries were prepared on the basis of the protocol by Kinde et al. (19) with the following exceptions. We excluded the unique identifier in the primer sequences and adapted the amount of input DNA. For analyses of cell lines, we used 20 ng DNA. Depending on the concentration of plasma DNA, we used 5–10 μL DNA corresponding to 0.1–5 ng total DNA. For samples with very high concentrations (>100 ng/mL plasma), a maximum of 5 ng was used for the assay.

Phusion HF Buffer (5×), 2 U Phusion Hot Start II Polymerase, 0.25 μmol/L target-specific L1 primer, and 200 μmol/L dNTPs were mixed and amplified with the following cycling conditions: denaturation at 98 °C for 2 min, followed by 5 cycles of 98 °C for 10 s, 57 °C for 120 s, and 72 °C for 120 s. PCR products were purified by incubation with 70 μL AMPure Beads (Beckman Coulter) for 10 min. The bead pellet was washed twice with freshly prepared 70% ethanol. After drying, the pellet was resuspended in 12 μL 1× Tris-EDTA-buffer. Purified PCR products (10 μL) were directly used for a second PCR, in which sequencing adaptors and sample-specific indexes were added (for primer sequences, see Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue6). Reaction setup and cycling conditions remained the same but with increased numbers of cycles. For plasma DNA samples, we used 18 cycles; all other samples were amplified with 15 cycles. PCR products were quality checked and quantified on an Agilent Bioanalyzer with a 7500 DNA kit (Agilent).

SEQUENCING AND DATA ANALYSIS
L1 amplicon libraries were pooled in equimolar amounts and sequenced on a MiSeq (Illumina) generating 150-bp single reads. Because mFAST-SeqS libraries represent low-diversity libraries, we spiked either 10%–20% of a PhiX control library or mFAST-SeqS libraries into plasma-Seq libraries in different ratios depending on the number of samples to be sequenced. Sequence reads were aligned to the hg19 genome with Burrows-Wheeler alignment, version 0.7.4. Reads with a mapping quality >15 were counted with an in-house script. To correct for different sequencing yields, read counts were normalized to the total read count per sample. To test for over- and under-representation of each chromosome arm, we calculated z-scores by subtracting the mean and dividing by the SD of normalized read-counts for the respective chromosome arm from 35 controls. Because no reads aligned to the short arms of the acrocentric chromosomes 13p, 14p, 15p, 21p, 22p, and Y, these were excluded from the analysis. To get a general overview of aneuploidy, we introduced a genome-wide z-score. Therefore, normalized read counts per chromosome arm were squared and summed.

PLASMA-SEQ
The plasma-Seq method was described in detail previously (4). Briefly, shotgun libraries were prepared with the TrueSeq DNA Nano Sample Preparation Kit (Illumina) with the following changes. We used 5–10 ng input DNA. On the basis of previous data, where we observed an enrichment of fragments in the range of 160–340
bp with the Agilent Bioanalyzer (14, 15), we omitted the fragmentation step. For selective amplification of the library fragments, we used 20–25 PCR cycles. The libraries were sequenced on a MiSeq (Illumina).

TARGETED RESEQUENCING
With Sure Select Custom DNA Kit (Agilent), we enriched 1.3 Mbp including exonic sequences of 55 cancer genes and 38 introns of 18 genes, where fusion breakpoints have been described. Analysis criteria have been previously described (4). Identified somatic mutations were validated with deep sequencing (18) to determine the mutant allele frequencies more accurately. We set the threshold for reliable detection of a sequence variation at 1%. Allelic fractions of <1% were considered sequencing errors.

ACCESSION NUMBERS
All sequencing raw data were deposited at the European Genome-Phenome Archive (http://www.ebi.ac.uk/ega/), which is hosted by the European Bioinformatics Institute, under the study accession number EGAS00001001133.

STATISTICS
To compare mFAST-SeqS with plasma-Seq, we used Pearson correlation. Comparisons were performed with genome-wide and chromosome arm–specific z-scores. Correlations were visualized with linear regression. ROC analysis was performed in R (20, 21).

Results
SEQUENCING OF CONTROL SAMPLES TO ESTABLISH REFERENCE VALUES
We modified the original FAST-SeqS protocol in 2 important aspects. First, whereas Kinde et al. performed read count and z-score analysis for whole chromosomes (19), we expanded the analysis to chromosome arms. Second, as our goal was the development of a prescreening tool that should perform fast at low cost, we used a lower amount of total reads than Kinde et al. (19). We analyzed plasma samples from controls to assess the consequences of these modifications (n = 35). After filtering for reads with a Phred-scaled mapping quality >15, we obtained a mean amount of 242,190 reads (range 106,205–925,843) (see online Supplemental Table 2) that aligned to a mean of 22,297 positions (see online Supplemental Table 2). Although the number of reads varied about 8.7-fold, the number of uniquely aligned positions varied only 2-fold (see online Supplemental Table 2). We applied read count analysis by counting the aligned reads to each chromosome arm and calculated a relative ratio for each chromosome arm. Although these ratios varied by a mean of 1.53-fold for smaller chromosomes (chromosomes 15–22), there was only a slight variability of 1.02-fold for larger chromosomes (chromosomes 1–14) (see online Supplemental Table 2). We calculated a z-score for each chromosome arm, with the mean ratios of control samples of the same sex. Altogether, we calculated 41 chromosome arm–specific z-scores for each control, accounting for a total of 1435 z-scores (range 3.35 to 3.85). Ten z-scores were >3 (0.07%) and 7 were less than 3 (0.05%) (see online Supplemental Table 3). Compared with data from Kinde et al., we observed a slightly higher variability, which was not unexpected since we used lower amounts of total reads. Moreover, we calculated a genome-wide z-score as a general measure for aneuploidy consisting of the squared sum of all chromosome-specific z-scores. This genome-wide z-score ranged from 1.50 to 2.51 for all healthy controls (n = 35) (see online Supplemental Table 3).

INTRA- AND INTERASSAY VARIABILITY OF mFAST-SeqS
We validated the reliability and repeatability of mFAST-SeqS by analyzing different dilutions of HT29 DNA (50%, 10% 5%, 0%) with 3–5 duplicates of each dilution in 5 independent runs. We obtained a good repeatability of the genome-wide z-score, with only small inter- and intraassay variations (Table 1) (see online Supplemental Fig. 1), although the number of reads varied 2-fold from run to run. Chromosome arm–specific z-scores were highly correlated within (r = 0.992) and between (r = 0.998) runs (see online Supplemental Fig. 1, A and B). Linear regression analysis revealed a good
correlation between genome-wide z-scores and the dilution of HT29 cell lines (mean $r = 0.974$) (see online Supplemental Fig. 1C).

**SPECIFICITY AND SENSITIVITY OF mFAST-SeqS**

Next, we evaluated our mFAST-SeqS assay by analyzing 3 cancer cell lines, HepG2, HT29, and MCF7 (Fig. 2, A–C) (see online Supplemental Table 4). When we used raw data of our plasma-Seq to establish comparable chromosome arm–specific z-scores, we obtained correlation coefficients of 0.895, 0.883, and 0.857 for HepG2, HT29, and MCF7, respectively, indicating that copy number changes obtained with the mFAST-SeqS assay were highly concordant with those obtained from plasma-Seq (Table 2). Not surprisingly, high-level amplifications of specific parts of chromosomes resulted in high overall chromosome arm–specific z-scores. For instance, the high-level am-
plications of chromosome 8q in both HT29 and MCF7 were reflected in 8q-specific \(z\)-scores of 42.2 and 15.0, respectively (Fig. 2D, first and third panel). We observed a close correlation between plasma-Seq and mFAST-SeqS if the entire chromosome arm was lost or gained, as exemplified by the loss of the short arm of chromosome 3 in HT29 (Fig. 2D, second panel) or gain of the entire chromosome 2 in HepG2 (Fig. 2D, fourth panel). In contrast, some chromosome arm–specific \(z\)-scores were below the threshold of 5, although CNAs were detected with plasma-Seq, which was the case when gains and losses co-occurred at the same chromosome arm (Fig. 2D, fifth panel).

To evaluate the sensitivity of mFAST-SeqS, we performed serial dilutions of 3 cancer cell lines. As expected, the genome-wide \(z\)-scores decreased with increasing dilution. Depending on the total amount and extent of aberrations, we observed a stronger decrease of genome-wide \(z\)-scores in the different dilutions (for details, see online Supplemental Fig. 2). Nevertheless, characteristic high-level amplifications such as the distal amplification of chromosome 8q in HT29 (see online Supplemental Fig. 2B) or the gain of chromosome 2q in HepG2 (see online Supplemental Fig. 2A) were detected when only 10% of cell line DNA was present. This suggests that a combined evaluation of genome-wide \(z\)-scores and chromosome arm–specific \(z\)-scores will detect the majority of plasma samples with a percentage of 10% ctDNA.

### Table 2. Comparison of mFAST-SeqS and plasma-Seq data.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Correlation of chromosome arm-specific (z)-scores</th>
<th>Genome-wide (z)-score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mFAST-SeqS</td>
<td>plasma-Seq</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.895</td>
<td>84.2</td>
</tr>
<tr>
<td>HT29</td>
<td>0.883</td>
<td>128.5</td>
</tr>
<tr>
<td>MCF7</td>
<td>0.857</td>
<td>93.8</td>
</tr>
<tr>
<td>B4_1</td>
<td>0.907</td>
<td>213.4</td>
</tr>
<tr>
<td>B40_1</td>
<td>0.870</td>
<td>54.1</td>
</tr>
<tr>
<td>B41_1</td>
<td>0.885</td>
<td>131.0</td>
</tr>
<tr>
<td>B4_2</td>
<td>0.906</td>
<td>259.9</td>
</tr>
<tr>
<td>B1_2</td>
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<td>94.9</td>
</tr>
<tr>
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<tr>
<td>P111_1</td>
<td>0.956</td>
<td>41.9</td>
</tr>
<tr>
<td>P111_4</td>
<td>0.962</td>
<td>94.1</td>
</tr>
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</table>

mFAST-SeqS FOR NONINVASIVE ANEUPLOIDY SCREENING IN CANCER PATIENTS

To evaluate whether mFAST-SeqS can be used as a prescreening tool for the presence of increased ctDNA levels, we analyzed 90 plasma samples from cancer patients. To determine \(z\)-score cutoffs for the identification of plasma samples where subsequent plasma-Seq would detect CNAs with a high probability, we used 24 plasma samples from 21 metastatic breast cancer patients for which copy number profiles established with plasma-Seq were available (mean plasma DNA concentration 64.4 ng/mL, range 5.1–440.8). The mean genome-wide \(z\)-score was 86.9 (range 2.2–349.6) (see online Supplemental Table 5). Genome-wide \(z\)-scores were only weakly correlated with plasma DNA concentrations (\(r = 0.419\)). All samples with a genome-wide \(z\)-score >5 (\(n = 20\)) demonstrated CNAs with plasma-Seq, whereas samples with genome-wide \(z\)-score <5 (\(n = 4\)) showed balanced plasma-Seq profiles. Despite this relatively low sample size, we performed ROC analysis of genome-wide \(z\)-scores, which revealed a cutoff of 6 for the detection of CNAs with plasma-Seq. To not miss samples with potential CNAs, we chose a cutoff of 5, which revealed a sensitivity of 100% and specificity of 80% for the detection of copy number changes with plasma-Seq.

Example heat maps showing chromosome arm–specific \(z\)-scores for various values of genome-wide \(z\)-scores and highly concordant mFAST-SeqS and plasma-Seq copy number profiles are displayed in Fig. 3. Chromosome-specific \(z\)-scores established from plasma-Seq data (\(n = 6\)) showed a strong correlation with those from mFAST-SeqS (mean \(r = 0.90\) (Table 2). When we compared genome-wide \(z\)-scores from mFAST-SeqS with those of available plasma-Seq data (\(n = 18\)), we again observed a good concordance (\(r = 0.677\) (Table 2). However, when we analyzed chromosome arm–specific \(z\)-scores, we noted 2 samples from the same patient (B5_3 and B5_4, genome-wide \(z\)-scores 4.0 and 3.8, respectively) with increased chromosome arm–specific \(z\)-scores for 8q of 6.4 and 7.0, respectively. In these 2 cases, plasma-Seq did not confirm the 8q CNAs (data not shown).

When we analyzed follow-up samples from the same patients collected 13 (B1) and 12 (B4) months after the first sample, mFAST-SeqS profiles were highly consistent (\(r = 0.97\) for B1 and \(r = 0.99\) for B4) (see online Supplemental Fig. 3, A and B), indicating that mFAST-SeqS delivers highly concordant results even when using biological replicates.

To validate these results, we analyzed a set of 66 samples from 56 prostate cancer patients at various disease stages (Fig. 4A) (see online Supplemental Table 6). Samples from patients with metastatic disease (\(n = 52\)) had significantly higher plasma DNA concentrations (mean 36.5 ng/mL; range 0.36–390) than patients in earlier stages (\(n = 14\)) (mean 2.4 ng/mL; range 0.5–5.5).
Fig. 3. mFAST-SeqS of plasma samples from patients with metastatic breast cancer.

(A), Heat map of selected plasma samples with different values of genome-wide z-scores. Blue bars indicate chromosome-specific z-scores less than −5, and red bars indicate chromosome-specific z-scores >5. (B), Comparison of mFAST-SeqS profiles and copy number profiles established with plasma-Seq of selected samples. Chr, chromosome.
Twenty-eight samples (42.4%) had a genome-wide $z$-score $<5$. Not surprisingly, these patients included all patients under surveillance and 9 of 10 patients with localized cancer after prostatectomy, for which a low fraction of tumor-specific DNA was expected. Also, 15 samples from metastatic patients had a $z$-score $<5$, indicating low amounts of tumor-specific DNA, which was confirmed by a balanced profile after plasma-Seq of 3 samples (see online Supplemental Fig. 4). In 38 patients (57.6%), a genome-wide $z$-score $>$5 was observed, including 8 patients (12.1%) with a genome-wide score $>$100. Again, we did not observe a strong correlation between plasma DNA concentrations and $z$-scores ($r = 0.304$). However, when we correlated mutant allele frequencies (mAFs) of 11 samples identified by targeted resequencing of known cancer driver genes with the mFAST-SeqS $z$-score, we observed a strong correlation ($r = 0.902$) (see online Supplemental Fig. 5). On the basis of linear regression, a $z$-score of 5 would predict an mAF of 10.5%.

Furthermore, we observed a good concordance between mFAST-SeqS and plasma-Seq results as exemplified in Fig. 4B and Table 2 ($r = 0.777$ for 38 genome-wide $z$-score comparisons, and $r = 0.945$ for chromosome arm-specific $z$-scores). Analysis of follow-up samples of 3 patients (P40, P147, and P111) revealed strong correlations of $r = 0.940$, 0.992, and 0.988, respectively (see online Supplemental Fig. 6, A–C).
Fig. 4. mFAST-SeqS of plasma samples from patients with prostate cancer.

(A), Heat map of selected plasma samples with different values of genome-wide z-scores. Blue bars indicate chromosome-specific z-scores less than −5, and red bars indicate chromosome-specific z-scores >5. (B), Comparison of mFAST-SeqS profiles and copy number profiles established with plasma-Seq of selected samples. Chr, chromosome.
Discussion

Previous studies with plasma DNA from patients with cancer demonstrated highly variable allele frequencies of ctDNA (1, 16). In cases with low ctDNA allele frequency, targeted approaches allow high-sensitivity monitoring of ctDNA dynamics (Fig. 1) (10, 12, 22, 23). However, in cases with high ctDNA allele frequency, analyses can be extended to untargeted, genome-wide approaches to uncover novel changes occurring during tumor evolution. Hence, an important question is how to distinguish between plasma samples with low and high ctDNA content to decide on the optimal strategy for further processing of a plasma sample (Fig. 1). To address this, we adapted the FAST-SeqS screening method for fetal aneuploidy (19). Because our aim was to use mFAST-SeqS as a prescreening tool for an estimation of the ctDNA percentage, we used a lower total number of reads for FAST-SeqS than used by Kinde et al. (19). With cell line DNA and a set of plasma from breast cancer patients, we established a cutoff z-score of 5 for a subsequent detection of CNAs with our established plasma-Seq approach. We confirmed this cutoff with 11 plasma samples in which comparisons of mAF in established cancer driver genes such as BRCA1 (breast cancer 1, early onset), TP53 (tumor protein p53), and CTNNB1 (catenin (cadherin-associating protein), beta 1, 88 kDa).

Fig. 4. Continued.
DNA in patients with prostate cancer with a sensitivity of focal amplifications, may result in aberrant other hand, few changes with high amplitudes, such as focal amplifications, may result in aberrant z-scores even if the ctDNA is <10%. Therefore, we suggest a combined evaluation of genome-wide and chromosome arm-specific z-scores. Furthermore, our assay may be influenced by very low plasma DNA concentrations, which would result in low input templates. However, these are likely to be cases unsuited for untargeted approaches to which highly-sensitive targeted techniques should be applied.

Advantages of this approach include that no prior knowledge about the genetic composition of tumor samples is necessary to estimate the amount of ctDNA, the speed of analysis (<1 day: hands-on time 1 h, bioinformatic and statistical analysis <1h), and the low cost per analysis (approximately €10 for consumables). The loss of resolution is, in our opinion, acceptable, as mFAST-SeqS merely serves as a decision support tool, to select the most appropriate in-depth/high-resolution strategy (Fig. 1).

It has previously been reported that ctDNA fragments might be smaller than 100 bp (24). However, this is in contrast to our own observations (14, 15) and reports by others (2). Indeed, the Qiagen Mini Kit for DNA extraction limits the isolation to fragment sizes of ≥100 bp, and furthermore, the FAST-SeqS assay amplifies PCR products in the range of 124–142 bp. Smaller DNA fragments are omitted from our assay and mFAST-SeqS and plasma-Seq copy number profiles are closely correlated, which further suggests that a substantial number of tumor DNA fragments in the circulation must have a size of >100 bp.

With our plasma-Seq method that applies low-coverage whole-genome sequencing to establish copy number profiles, tumor-specific changes can be detected in the circulation at levels ≥10% of circulating tumor DNA in patients with prostate cancer with a sensitivity of >80% and specificity of >80% (4). With plasma-Seq, we were able to identify CNAs in plasma samples of many metastasis patients (4, 14, 18); however, in some cases, we would not have performed plasma-Seq if we had had prior knowledge about the ctDNA fraction. Similar data about ctDNA variability were obtained by a recent study, which showed that approximately 80% of patients with metastatic disease had detectable levels of ctDNA, albeit with a high variability of mutant fragments (1). In another comprehensive study with exome sequencing, results could be achieved only in patients with a high systemic mutation burden in plasma >5%–10% (7). Chan et al. showed that the fractional concentration of tumor DNA in plasma and the class of CNA strongly influence the detectability of such alterations in plasma (2).

Although sequencing costs have dropped rapidly in recent years, genome-wide, in-depth, high-coverage analysis is still expensive and time consuming, which hampers the introduction of ctDNA diagnostics into the clinic. mFAST-SeqS may contribute to a significant reduction of cost and increase of speed and could therefore serve as a valuable, untargeted prescreening tool to identify plasma DNA samples with high ctDNA content for decisions on further diagnostic steps (Fig. 1).

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