# Comparison of BEAMing and droplet digital PCR for circulating tumor DNA analysis

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**Abstract (250 word limit)**

*Background*

Circulating tumor DNA (ctDNA) assays are increasingly utilized for clinical decision-making, but it is unknown how well different assays agree. We aimed to assess the agreement in ctDNA mutation calling between BEAMing and droplet digital PCR (ddPCR), two of the most commonly used digital PCR techniques for detecting mutations in ctDNA.

*Methods*

Baseline plasma samples from patients with advanced breast cancer enrolled in the phase III PALOMA-3 trial were assessed for *ESR1* and *PIK3CA* mutations in ctDNA using both BEAMing and ddPCR. Concordance between the two approaches was assessed, with exploratory analyses to estimate the importance of sampling effects.

*Results*

Of the 521 patients enrolled, 363 had paired baseline ctDNA analysis. *ESR1* mutation detection was 24.2% (88/363) for BEAMing and 25.3% (92/363) for ddPCR, with good agreement between the two techniques ($\kappa$ = 0.9, 95%CI 0.85 – 0.95). *PIK3CA* mutations detection rates were 26.2% (95/363) for BEAMing and 22.9% (83/363) for ddPCR, with good agreement ($\kappa$ = 0.87, 95%CI 0.81 – 0.93). Discordancy was observed for 3.9% patients with *ESR1* mutations and 5.0% with *PIK3CA* mutations. Assessment of individual mutations suggested higher rates of discordancy for less common mutations ($P$ = 0.019). The majority of discordant calls occurred at allele frequency <1%, predominantly resulting from stochastic sampling effects.

*Conclusions*

This large, clinically-relevant comparison showed good agreement between BEAMing and ddPCR, suggesting sufficient reproducibility for clinical use. Much of the observed discordancy may be related to sampling effects, potentially explaining many of the differences in the currently available ctDNA literature.

**Manuscript text (3500 word limit)**

## Introduction

Circulating tumor DNA (ctDNA) has been identified in the cell-free DNA of patients with many different cancers, enabling investigation of the genomic aberrations that define an individual's cancer*(1)*. Work over the last decade has shown ctDNA in certain settings to be an adequate surrogate for tumor biopsy*(2)*, also offering an insight into response to treatment*(3-5)* and clonal evolution*(6-10)*, as such showing great promise as a potential clinical tool to advance personalized medicine. These data have led in the case of *EGFR* mutations in lung cancer to FDA approval for a ctDNA companion diagnostic to direct therapy with tyrosine kinase inhibitors*(11)*, the first of its kind.

Despite the growing evidence for circulating tumor DNA (ctDNA) in the research setting*(12)*, only a small minority of assays have demonstrated the analytical validity and clinical utility to qualify as 'liquid biopsies'*(13)*, steps necessary for effective translation into routine practice. There is a considerable body of work that has established the reproducibility of different digital PCR techniques*(14-16)*, but fewer data are available for comparison between different methods in clinical samples from patients with cancer. There are few datasets directly comparing different approaches, and no large ones, but a recent report of 40 patients with prostate cancer tested with two commercially available ctDNA sequencing assays demonstrated substantial discordance - 40% of patients showing no agreement in positive mutation calls*(17)*, an observation that provoked considerable debate*(18)*. Confidence that different ctDNA assays are comparable is critical for their meaningful integration into care, and for assessment of clinical studies using different techniques for ctDNA analysis. Where aberrations identified in ctDNA are used to select therapy*(2)*, or to monitor response to treatment*(6, 7, 19-21)*, a good understanding of the factors that may lead to discordant results between analyses is important and yet to be addressed in the current literature.

We aimed to investigate these challenges to effective ctDNA assessment with contemporaneous ctDNA data derived from a clinical trial. Here we report the largest comparison to date between two different ctDNA approaches - BEAMing*(22)* and droplet digital PCR*(23)* (ddPCR, including multiplex ddPCR*(24, 25)*), two of the most prevalent digital PCR based methods for assessment of ctDNA*(26)*. There are few datasets directly comparing testing of clinical plasma samples using these two methods, the largest comprising 27 patients*(27, 28)*. Our testing cohort was derived from PALOMA-3, a randomized phase III trial of palbociclib plus fulvestrant versus placebo plus fulvestrant in patients with advanced, estrogen receptor positive breast cancer. Baseline plasma samples from this study were analyzed separately with BEAMing*(29)* and ddPCR*(3, 30)*. This data set permits the unique opportunity of a

detailed assessment of assay results in contemporaneous plasma collected under strict protocol in a homogeneous patient group at the same stage of treatment.

## Materials and Methods

*Plasma collection*

The PALOMA-3 trial enrolled patients with advanced estrogen receptor positive, HER2 negative breast cancer which had previously progressed on endocrine therapy. Blood samples were collected in two 10ml K2 EDTA tubes from study participants on day 1 of treatment. Blood was centrifuged within 30 minutes at 1500-2000g for 10 minutes. The plasma supernatant was then separated and stored at -80$^{o}$C until DNA extraction. Separate aliquots of plasma were sent to two independent laboratories for separate testing of mutations by BEAMing and droplet digital PCR. No matched tumor data were available.

*Droplet digital PCR (ddPCR)*

Plasma was thawed and centrifuged at 3000g for 10 minutes with the supernatant separated and the DNA extracted using the Circulating Nucleic Acid kit from Qiagen with a vacuum manifold. DNA was eluted into 50µl of AVE buffer and stored at -20 $^{o}$C. Cell free DNA content was estimated using a VIC fluorophore Taqman assay against *RPPH1*, the gene encoding RNAseP (Life Technologies) using 1µl of eluate on the Bio-Rad QX-200. DNA concentration per microliter was estimated using 3.3pg as the mass of a haploid human genome. Samples were screened initially with 3 multiplexes of custom Taqman ddPCR assays with FAM fluorophore mutant probes and HEX fluorophore wild type probes (online **Supplemental Figure 1**). These comprised a *PIK3CA* multiplex testing for p.E542K (c.1624G>A), p.E545K (c.1633G>A), p.H1047R (c.3140A>G) and p.H1047L (c.3140A>T) (online **Supplemental Table 1**) and two *ESR1* multiplexes testing for p.E380Q (c.1138G>C), p.L536R (c.1607T>G), p.Y537C (c.1610A>G), p.D538G (c.1613A>G) (Bio-Rad, Cat Number: dHsaMDXE91450042) and p.S463P (c.1387T>C), p.Y537N (c.1609T>A) and p.Y537S (c.1610A>C) (Bio-Rad, Cat Number: dHsaMDXE65719815) respectively. The *PIK3CA* assays were run at 95 $^{o}$C for 10 minutes, 95 $^{o}$C for 15 seconds, 54 $^{o}$C for 1 minute, 98 $^{o}$C for 10 minutes, all ramp increments 2.5 $^{o}$C/sec. The *ESR1* assays were run at 95 $^{o}$C for 10 minutes, 95 $^{o}$C for 15 seconds, 52 $^{o}$C for 1 minute, 98 $^{o}$C for 10 minutes, all ramp increments 2.5 $^{o}$C/sec. Sample input into each assay was 0.25ml equivalent or 1.3ng, whichever was the greater. At least 300 wild type droplets were required for the assay to be considered assessable. Positive results required at least 2 droplets on the mutant channel. Non-template controls were run with every assay, gating was performed manually using an oligonucleotide positive control run for each batch of assay. Mutations were then subsequently validated with single assays with allele fraction estimated from these (online **Supplemental Figure 1**). All ddPCR was performed on the Bio-Rad QX-200 with data analysis using the QuantaSoft package version1.7.4.0917. No false positive mutant partitions were observed. Reproducibility

data for the ddPCR assays are given in online **Supplemental Figure 2** and **Supplemental Figure 3**. Example ddPCR plots are shown in online **Supplemental Figure 4**, **Supplemental Figure 5**, and **Supplemental Figure 6**.

*BEAMing*

DNA was extracted using the Circulating Nucleic Acid kit from Qiagen, with 2ml of plasma used for the analysis. BEAMing was performed by Sysmex Inostics, using the OncoBEAM assay, for which reproducibility has been previously published*(31)*. DNA was quantified using a *LINE1* real-time qPCR assay with DNA then subjected to PCR pre-amplification before the reaction products were split into variant-specific emulsion PCR and hybridization reactions. For *PIK3CA* these variants included p.E542K (*c.1624G>A*), p.E545K (*c.1633G>A*), p.H1047R (*c.3140A>G*) and p.H1047L (*c.3140A>T*), and for *ESR1* p.E380Q (*c.1138G>C*), p.S463P (*c.1387T>C*), p.V534E (*c.1601T>A*), p.P535H (*c.1604C>A*), p.L536H (*c.1607T>A*), p.L536P (*c.1607T>C*), p.L536Q (*c.1607-1608TC>A*), p.L536R (*c.1607T>G*), p.Y537C (*c.1610A>G*), p.Y537N (*c.1609T>A*), p.Y537S (*c.1610A>C*) and p.D538G (*c.1613A>G*). Positive calls were only made where allele fraction was consistent with >1 mutant copy being present in the initial reaction based on the results of the *LINE1* qPCR, with a threshold of 0.02% allele fraction from a negative control.

*Comparison cohort*

Patients were considered eligible for comparison if they had available data for all the mutations tested with both platforms, namely E380Q, S463P, L536R, Y537C, Y537N, Y537S and D538G for *ESR1* and E542K, E545K, H1047R and H1047L for *PIK3CA*. A pre-specified cutoff threshold of a mutant allele fraction of 0.1% was used for comparison, with either assay considered negative if estimated allele fractions were below this value. For the primary outcome, comparisons were made on mutation status for both genes, that is, whether a patient's samples had any mutation in *ESR1* or *PIK3CA*. The secondary endpoint was agreement between specific mutations in each of the genes.

*Statistical analyses*

All statistical calculations were performed using R version 3.4.3. Agreement between the two approaches was calculated using the Cohen kappa κ. Allele fraction correlation was calculated both for composite allele fraction and individual mutation allele fraction using the Spearman r. The difference between assay input DNA was assessed using the Mann Whitney test. For the sampling error model the BEAMing allele fraction was taken as the probability of success with replacement in a binomial model. A cumulative probability was then calculated with the number of trials, n, being the number of alleles assessed by the ddPCR assay. The comparison was considered to be at risk of under-sampling if the probability of there being at least 1 mutant copy in the ddPCR assay was estimated at <95%.

*Sliding window analysis*

All paired samples with a positive call from either BEAMing or ddPCR were included and ranked from high to low on the basis of the highest observed allele fraction between the two platforms. A sliding window of 20 patients was started with the 20 highest allele fraction sample pairs. The percentage of sample pairs within the window that were concordant was calculated and this was plotted against the median allele fraction of the window. The next window was formed by removal of the sample pair with the highest allele fraction and inclusion of the sample pair with the next highest allele fraction below the existing window, with the process repeated until the window contained the 20 sample pairs with the lowest allele fractions.

## Results

*Concordance of mutation status for ESR1 and PIK3CA*

Of the 521 patients in the PALOMA-3 trial, 396 patients underwent baseline plasma assessment with BEAMing and 459 patients with ddPCR. Comparative analyses were conducted in the 363 patients with data available from both methods (**Figure 1A**). First, mutation status defined as detection of any mutation was compared between the two techniques. BEAMing detected an *ESR1* mutation in 24.2% (88/363) of patients while ddPCR detected an *ESR1* mutation in 25.3% (92/363). Comparing these directly there was close agreement between classification of *ESR1* mutation status by BEAMing and ddPCR, with a Cohen kappa $\kappa = 0.9$ (95%CI 0.85 – 0.95), with 3.6% (13/363) discordant cases (**Figure 1B**). Composite allele fraction of *ESR1* between the two techniques was tightly correlated (Spearman r = 0.84, $P < 0.0001$, **Figure 1B**). For *PIK3CA* mutations, BEAMing detected 26.2% (95/363) patients as having a mutation compared to 22.9% (83/363) with ddPCR (**Figure 1C**), with agreement similar to *ESR1* ($\kappa = 0.87$, 95%CI 0.81 – 0.93) with a comparable proportion of discordant cases (5%, 18/363). Composite allele fraction for *PIK3CA* mutation, as with *ESR1* mutation, was tightly correlated (Spearman r = 0.83, $P < 0.0001$, **Figure 1C**). Focusing specifically on discordant cases for mutations in the two genes revealed that the composite allele fractions in these cases were often close to the calling threshold of 0.1% allele fraction (online **Supplemental figure 7**).

*Agreement at a specific mutation level*

Next, agreement between specific mutations within *ESR1* and *PIK3CA* was assessed. Concordance at this level was lower, with $\kappa$ ranging from 0.40-0.93 for the 7 individual *ESR1* mutations (**Figure 2A**, online **Supplemental Figure 8**) and 0.44-0.90 for the 4 individual *PIK3CA* mutations (**Figure 2B**, online **Supplemental Figure 9**). Allele fraction of discordant calls did not appear to associate with any specific mutation for either *ESR1* or *PIK3CA* (**Figure 2C, Figure 2D**), but better agreement was associated with a greater overall number of positive calls (Pearson r = 0.69, 95%CI 0.15 - 0.91, $P = 0.0189$, **Figure 2E**, **Figure 2F**, online **Supplemental Figure 10**), suggesting disagreement was higher with mutations occurring at a lower prevalence.

*Contribution of sampling effects to observed results*

In light of the above we next assessed more closely the possible role of sampling effects in the data - for a mutation present at very low levels the assay-independent chance of false negative due to sampling effects increases with decreasing prevalence. Varying the cut off for assigning a positive call resulted in deterioration in kappa between the two techniques for thresholds below 0.1% for both *ESR1* and *PIK3CA* (**Figure 3A, B**). However, analyzing all patients with a positive result from at least one platform using a sliding window of 20 patients from high to low allele fraction revealed a sharp deterioration in concordance for windows with median

allele fractions below 1% (**Figure 3C, D**). This suggested that although overall concordance between BEAMing and ddPCR remained good when including all the data down to 0.1%, the proportion of discordancy actually began to increase rapidly below 1%.

With higher levels of discordancy associating with fewer positive calls overall, and lower calling thresholds consistent with stochastic error as a key factor, we next reviewed the potential role of input DNA for the two respective techniques and related this to discordance. There was considerably greater DNA input into the BEAMing assay (mean input 27,467 haploid genome equivalents where each allele detected is assumed to represent half a diploid genome, IQR 10,895), as, unlike ddPCR (1,008 haploid genome equivalents, IQR 583), all variants are called from the PCR product of an initial reaction (online **Supplemental Figure 11**). As the calling threshold was reduced, discordancy for both *ESR1* and *PIK3CA* was driven primarily by additional BEAMing calls not detected by ddPCR (**Figure 3E**, **F**, online **Supplemental Figure 12**). However, using a binomial sampling model accounting for the number of genomes assessed in the ddPCR assay (Methods, online **Supplemental Figure 13**), most of the discordant positive calls were found to be at an allele fraction too low to be sufficiently confident that mutant DNA was present in the ddPCR assay input (**Figure 3E**, **Figure 3F**).

## Discussion

In this analysis of plasma DNA from the PALOMA-3 trial there was close agreement between BEAMing and ddPCR for *ESR1* and *PIK3CA* mutation status. Concordance was slightly lower for individual mutations, with agreement better for specific mutations in both genes that occurred at a greater prevalence. Further analyses involving varying the calling threshold, analyzing concordance between patients with similar allele fractions and accounting for the input DNA in each assay implicated stochastic sampling error as a critical factor in discordance. These findings could potentially explain much of the disagreement observed in the published literature, and provide a framework for future comparisons between trials.

Circulating tumor DNA analysis is the most developed of the emerging liquid biopsy technologies, but the variety of ctDNA assays offers a challenge to meaningful comparisons between studies. The available data comparing different platforms is limited to small sets, in which it is difficult to robustly assess stochastic effects. One comparison of ddPCR versus BEAMing for 27 lung cancer patients with *EGFR* mutations from the phase I AURA trial showed concordance of 70-90%, the investigators noting higher discordance at lower allele fraction*(28)*. Another study of 40 patients with prostate cancer suggested considerable discordance between two different multi-gene ctDNA sequencing assays and found only 7.5% of patients had complete congruence*(17)*. However, the distribution of allele fractions in this study

was not initially provided and other groups who have repeated analyses of the source data have claimed that the initial presentation was overly negative*(17, 32, 33)*. Neither of these studies was able to explicitly examine the effect of input quantity of DNA, allele fraction or the effect of varying the threshold for positive calls. Reasons for discordance between different techniques are not clear and are difficult to delineate definitively. Inter-laboratory studies have shown impressively low intra-assay variance between the same experiments performed in different laboratories*(15, 16)*. However, in comparisons of mutation detection in the clinical setting there is no gold standard available for comparison of tumor heterogeneity, meaning even tumor biopsy cannot be relied upon for definitive comparison*(34)*. One approach to address some of these challenges is to use large datasets from randomized controlled trials, where much of the clinical variability is controlled, and samples are collected under strict protocol.

Our cohort of 363 patients from a phase III clinical trial with paired BEAMing and ddPCR data enabled analyses for 11 individual variants across two genes commonly mutated in advanced breast cancer. We found close agreement between the two techniques with regards to any *ESR1* or any *PIK3CA* mutation ($\kappa$ = 0.9 and 0.87 respectively, **Figure 1**) with a relatively low proportion of discordant cases (<5%). We suggest that this is an acceptable level for real-world applications and for meaningful comparisons across clinical cohorts, although we acknowledge that any discordance observed in tests that may be used to select a clinical treatment is potentially a cause for concern. Our findings that discordant results were associated with both low allele fraction and calling threshold suggest sampling error as a principal cause, a hypothesis further supported by integrating the effect of input DNA into the analysis (**Figure 3**). This highlights that it is crucial to account for sampling effects when considering the result of any ctDNA assay, particularly as the tumor fraction of cell-free DNA is often very low, and further reduced by treatment*(3-5)*. With concordance approaching 100% only as allele fractions increased over 1%, it is important to note that the often sub clonal *ESR1* mutations allele fractions were <1% for a substantial proportion of mutations (41/124 mutations, 33.1%, ddPCR set). Considerable amounts of material would need to be tested to avoid sampling error in these patients.

Strengths of our study include its large size in comparison to previously published cohorts and the blinding of the analyzing laboratories. Additionally, the plasma samples were collected under a strict protocol within in a multicenter phase III trial, with all patients at the same clinical treatment stage, that is, just prior to a new therapy having recently progressed on endocrine treatment.

An important limitation for precise comparison to consider is the fundamental difference between the workflow for BEAMing and ddPCR (online **Supplemental Figure 14**). BEAMing (beads, emulsion, amplification and magnetics) involves a pre-amplification step of starting DNA template with specific primers targeting the genomic loci of interest. The products are then input into a limiting dilution with

primer-coated beads and undergo a further PCR reaction before the beads are purified and attached to allele-specific fluorophore probes to delineate mutant from wild type DNA. In contrast, in droplet digital PCR the initial template is subjected to a limiting dilution before any PCR, which then takes place in an oil/water emulsion with a sequence-specific Taqman fluorophore assay releasing allele-specific probes according to the sequence of the initial template present in the droplet. Thus for BEAMing, the entire starting template for all tested mutations is present in the initial reaction, although a threshold must be applied to exclude PCR error. With the ddPCR the total DNA template is split for the different *PIK3CA* and *ESR1* multiplexes, the results of which are then confirmed in singleplex assays, such that these results effectively involve two tests, not one. To mitigate this challenge and achieve as fair a comparison as possible we used a pre-specified, though arbitrary, allele fraction cut off for both approaches, subsequently being able to demonstrate close agreement between the two techniques. Though our results suggest an important role for stochastic effects in discordance, it is not possible in this analysis to definitively demonstrate the reasons for observed discordance. Lastly, due to the commercial nature of the assays used for this comparison, we are unable to provide technical details of the assays that may be relevant to interpretation.

A further limitation is that the samples were taken contemporaneously, so our analysis cannot address potential further variation that could occur with samples taken on different days. This is an important consideration in real-world practice as pre-analytical factors are critical for interrogating cell free DNA, and can be expected to vary to a degree over time. Finally, although we can compare a larger number of variants than the currently available digital PCR studies, the 11 mutations examined here reflect a much lower number than would be possible with a typical NGS assay. However, the principal of sampling a particular genomic locus in ctDNA remains analogous between digital PCR and NGS.

## Conclusions

These data demonstrate that digital PCR, with BEAMing and ddPCR, for *ESR1* and *PIK3CA*, offers sufficiently high levels of agreement for clinical diagnostic testing. Discordance between the two techniques was observed, but was low, with much of the discordancy likely accounted for by sampling effects due to a combination of low mutant fraction or low DNA input. This is reassuring for clinical development of these assays, although it highlights the important factors that must be considered when making meaningful comparisons between ctDNA data derived from different platforms.

## Figure legends

### Figure 1. Agreement between BEAMing and droplet digital PCR in the PALOMA-3 study.

A - CONSORT diagram for study showing patients included from the PALOMA-3 trial. B – Contingency table for *ESR1* mutation status by patient from BEAMing and ddPCR, with agreement of composite allele fraction shown in right panel. C - Contingency table for *PIK3CA* mutation status by patient from BEAMing and ddPCR, with agreement of composite allele fraction shown in right panel. Cohen's kappa statistic with 95% confidence intervals.

### Figure 2. Agreement for individual mutation calling in *PIK3CA* and *ESR1* by digital PCR.

A – Cohen's kappa for individual *ESR1* mutations. B - Cohen's kappa for individual *PIK3CA* mutations. C – Correlation of mutant allele fraction for individual *ESR1* mutations. D - Correlation of mutant allele fraction for individual *PIK3CA* mutations. E – Number of positive calls for individual *ESR1* mutations. F - Number of positive calls for individual *PIK3CA* mutations.

### Figure 3. Sampling issues are the major factor in discordance at low allele fractions.

A – Cohen's kappa between BEAMing and ddPCR for *ESR1* varying the cut off threshold used to call positive variants. Results are shown both for any mutation and for each specific mutation. B – As panel A but for *PIK3CA*. C – Sliding window analysis of patients with a positive *ESR1* call in at least one platform showing how percentage of concordant cases varies with median allele fraction of window (see Supplementary methods). D – As panel C but for *PIK3CA*. E – Number of patients with discordant results varying the threshold for positive calling for *ESR1* with both BEAMing and ddPCR. Adjusted bar removes those patients in which the alternative assay does not have enough input DNA to confidently exclude sampling error (see online **Supplemental methods**). F – As panel E but for *PIK3CA*.

## References


1. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Science Translational Medicine 2014;6:224ra24.
2. Douillard JY, Ostoros G, Cobo M, Ciuleanu T, McCormack R, Webster A, Milenkova T. First-line gefitinib in caucasian egfr mutation-positive nsclc patients: A phase-iv, open-label, single-arm study. Br J Cancer 2014;110:55-62.
3. O'Leary B, Hrebien S, Morden JP, Beaney M, Fribbens C, Huang X, et al. Early circulating tumor DNA dynamics and clonal selection with palbociclib and fulvestrant for breast cancer. Nature communications 2018;9:896.
4. Mok T, Wu Y-L, Lee JS, Yu C-J, Sriuranpong V, Sandoval-Tan J, et al. Detection and dynamic changes of egfr mutations from circulating tumor DNA as a predictor of survival outcomes in nsclc patients treated with first-line intercalated erlotinib and chemotherapy. Clinical Cancer Research 2015;21:3196-203.
5. Parkinson CA, Gale D, Piskorz AM, Biggs H, Hodgkin C, Addley H, et al. Exploratory analysis of tp53 mutations in circulating tumour DNA as biomarkers of treatment response for patients with relapsed high-grade serous ovarian carcinoma: A retrospective study. PLOS Medicine 2016;13:e1002198.
6. Murtaza M, Dawson S-J, Tsui DWY, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497:108-12.
7. Forshew T, Murtaza M, Parkinson C, Gale D, Tsui DWY, Kaper F, et al. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Science Translational Medicine 2012;4:136ra68.
8. Murtaza M, Dawson SJ, Pogrebniak K, Rueda OM, Provenzano E, Grant J, et al. Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. Nature communications 2015;6:8760.
9. Diaz Jr LA, Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, et al. The molecular evolution of acquired resistance to targeted egfr blockade in colorectal cancers. Nature 2012;486:537-40.
10. Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, et al. Emergence of kras mutations and acquired resistance to anti-egfr therapy in colorectal cancer. Nature 2012;486:532-6.
11. FDA. Premarket approval p150044 — cobas egfr mutation test v2. Vol., 2016.
12. Wan JCM, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, et al. Liquid biopsies come of age: Towards implementation of circulating tumour DNA. Nat Rev Cancer 2017;advance online publication.
13. Merker JD, Oxnard GR, Compton C, Diehn M, Hurley P, Lazar AJ, et al. Circulating tumor DNA analysis in patients with cancer: American society of clinical oncology and college of american pathologists joint review. Journal of Clinical Oncology 2018;36:1631-41.
14. Pavšič J, Devonshire A, Blejec A, Foy CA, Van Heuverswyn F, Jones GM, et al. Inter-laboratory assessment of different digital pcr platforms for quantification

of human cytomegalovirus DNA. Analytical and bioanalytical chemistry 2017;409:2601-14.
15. Yoo H-B, Park S-R, Dong L, Wang J, Sui Z, Pavšič J, et al. International comparison of enumeration-based quantification of DNA copy-concentration using flow cytometric counting and digital polymerase chain reaction. Analytical Chemistry 2016;88:12169-76.
16. Whale AS, Devonshire AS, Karlin-Neumann G, Regan J, Javier L, Cowen S, et al. International interlaboratory digital pcr study demonstrating high reproducibility for the measurement of a rare sequence variant. Analytical Chemistry 2017;89:1724-33.
17. Torga G, Pienta KJ. Patient-paired sample congruence between 2 commercial liquid biopsy tests. JAMA Oncology 2018;4:868-70.
18. Hayes DF. Precision medicine and testing for tumor biomarkers—are all tests born equal? JAMA Oncology 2018;4:773-4.
19. Dawson S-J, Tsui DWY, Murtaza M, Biggs H, Rueda OM, Chin S-F, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. New England Journal of Medicine 2013;368:1199-209.
20. Akamatsu H, Koh Y, Okamoto I, Fujimoto D, Bessho A, Azuma K, et al. Clinical significance of monitoring egfr mutation in plasma using multiplexed digital pcr in egfr mutated patients treated with afatinib (west japan oncology group 8114ltr study). Lung Cancer 2019;131:128-33.
21. Taus Á, Camacho L, Rocha P, Hardy-Werbin M, Pijuan L, Piquer G, et al. Dynamics of egfr mutation load in plasma for prediction of treatment response and disease progression in patients with egfr-mutant lung adenocarcinoma. Clinical Lung Cancer 2018;19:387-94.e2.
22. Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. Beaming: Single-molecule pcr on microparticles in water-in-oil emulsions. Nat Meth 2006;3:551-9.
23. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital pcr system for absolute quantitation of DNA copy number. Analytical chemistry 2011;83:8604-10.
24. Chen W, Zheng J, Wu C, Liu S, Chen Y, Liu X, et al. Breast cancer subtype classification using 4-plex droplet digital pcr. Clinical Chemistry 2019:clinchem.2019.302315.
25. Hrebien S, O'Leary B, Beaney M, Schiavon G, Fribbens C, Bhambra A, et al. Reproducibility of digital pcr assays for circulating tumor DNA analysis in advanced breast cancer. PLoS ONE 2016;11:e0165023.
26. Diaz LA, Bardelli A. Liquid biopsies: Genotyping circulating tumor DNA. Journal of Clinical Oncology 2014.
27. Garcia J, Forestier J, Dusserre E, Wozny A-S, Geiguer F, Merle P, et al. Cross-platform comparison for the detection of ras mutations in cfdna (ddpcr biorad detection assay, beaming assay, and ngs strategy). Oncotarget 2018;9:21122-31.
28. Thress KS, Brant R, Carr TH, Dearden S, Jenkins S, Brown H, 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;90:509-15.
29. Cristofanilli M, Turner NC, Bondarenko I, Ro J, Im S-A, Masuda N, et al. Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, her2-negative metastatic breast cancer that

progressed on previous endocrine therapy (paloma-3): Final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. The Lancet Oncology 2016.
30. Fribbens C, O'Leary B, Kilburn L, Hrebien S, Garcia-Murillas I, Beaney M, et al. Plasma esr1 mutations and the treatment of estrogen receptor–positive advanced breast cancer. Journal of Clinical Oncology 2016.
31. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14:985-90.
32. Agarwal N, Lanman RB, Pal SK. Regarding the congruence between 2 circulating tumor DNA sequencing assays. JAMA Oncol 2018;4:1429-30.
33. Angiuoli SV, White JR, Jones S. Regarding the congruence between 2 circulating tumor dna sequencing assays. JAMA Oncology 2018.
34. Abbosh C, Birkbak NJ, Wilson GA, Jamal-Hanjani M, Constantin T, Salari R, et al. Phylogenetic ctdna analysis depicts early stage lung cancer evolution. Nature 2017;advance online publication.