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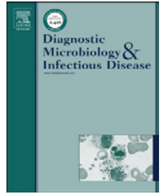

Review

Viral diagnostics in the era of digital polymerase chain reaction ☆

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Unlike quantitative polymerase chain reaction (qPCR), digital PCR (dPCR) achieves sensitive and accurate absolute quantitation of a DNA sample without the need for a standard curve. A single PCR reaction is divided into many separate reactions that each have a positive or negative signal. By applying Poisson statistics, the number of DNA molecules in the original sample is directly calculated from the number of positive and negative reactions. The recent availability of multiple commercial dPCR platforms has led to increased interest in clinical diagnostic applications, such as low viral load detection and low abundance mutant detection, where dPCR could be superior to traditional qPCR. Here we review current literature that demonstrates dPCR's potential utility in viral diagnostics, particularly through absolute quantification of target DNA sequences and rare mutant allele detection.

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Clinical viral diagnostic approaches rely heavily on quantitative polymerase chain reaction (qPCR) as a method to detect and quantify viral load in patient samples. For the past 20 years, fluorescence-based qPCR chemistries have revolutionized nucleic acid diagnostics and become the gold standard for viral load quantification (Mackay et al., 2002) and detection of bacterial pathogens, among myriad other applications. During qPCR, DNA is amplified until it produces a certain level of signal which is supplied through a DNA intercalating dye or sequence-specific fluorescent probe. The cycle threshold, defined as the number of amplification cycles required to reach that signal level, is used to calculate the number of DNA molecules originally present based on a standard curve (Bustin, 2004).

Although qPCR has driven major advances in disease diagnosis, this technology has notable limitations. Quantification is based on a standard curve, which requires careful calibration and consistent source material. Additionally, the choice of signal threshold can be made by the operator, introducing subjectivity into the analysis. Due to differences in standard curve construction and potential subjectivity in analysis, interlaboratory variation can be substantial even when using commercial kits and standardized protocols. Moreover, even within a highly trained laboratory the coefficient of variation for any single assay can be 20–30% or higher at lower template copy number (Cook et al., 2009; Lai et al., 2003). For example, the interassay variability for a cytomegalovirus (CMV) quantitation assay is considered low with a viral load coefficient of variation of 28% (Boeckh et al., 2004).

Digital PCR (dPCR) promises to remedy some of the shortcomings of qPCR by transforming the analog, exponential nature of PCR into a digital, linear signal (Vogelstein and Kinzler, 1999). Here we discuss the theoretical basis for dPCR and the currently available commercial dPCR systems. We also review current literature that demonstrates dPCR's potential utility in viral and microbial diagnostics, particularly through absolute quantification of target DNA sequences and rare mutant allele detection.

1. Digital PCR

First described in the 1990s (Sidransky et al., 1992; Vogelstein and Kinzler, 1999), dPCR uses the same primers and probes as qPCR, but touts increased sensitivity and precision. These improvements are achieved by diluting the sample and partitioning it into individual reactions so that ideally each reaction contains 1 or no copies of the DNA of interest (Fig. 1). The number of positive versus negative reactions is counted to directly calculate the number of DNA molecules in the original sample based on Poisson statistics. If the sample is not dilute, many of the individual reactions will be positive and will have contained 2, 3, or more target molecules. In this case, simply counting the positive reactions would underestimate the true number of molecules. This underestimation can be corrected using the Poisson equation [copies per reaction $= -\ln(1 - p)$, where p is the fraction of positive reactions], which calculates the average number of molecules per reaction from the observed proportion of positive reactions (Sykes et al., 1992). Using Poisson statistics, digital PCR provides absolute quantification of nucleic acids, reducing subjectivity in analysis by abrogating the need for signal threshold determination and standard curves.

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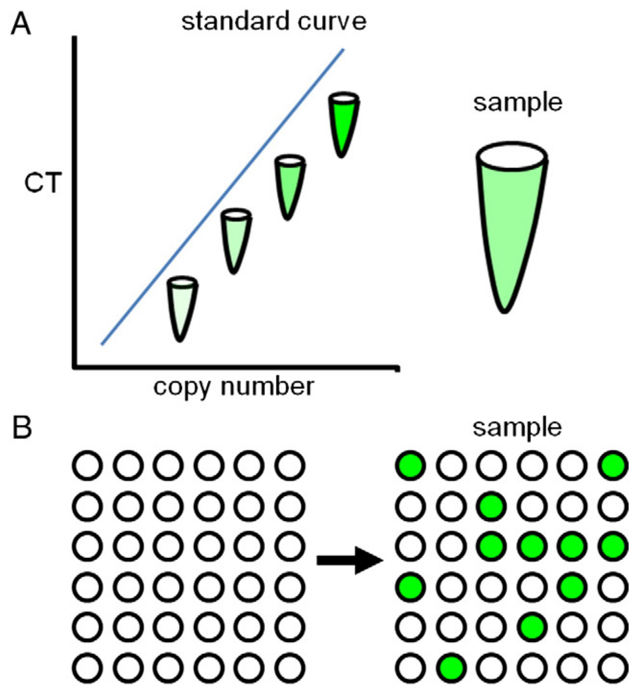

Fig. 1. Comparison of standard quantitative PCR and digital PCR. (A) Quantitative PCR. Quantitation of DNA in a sample is based on comparing the cycle threshold (CT) values for known DNA concentrations to the CT values of the measured sample. (B) Digital PCR. Absolute quantitation of DNA in a sample is achieved by compartmentalizing a sample into hundreds or thousands of separate reactions that are cycled to endpoint, each containing one (green) or no (white) target DNA particles.

Additionally, when amplification is carried out in bulk reactions, it is difficult to quantify poorly represented target sequences in a background of more abundant species. Digital PCR increases sensitivity by isolating rare target species so they are not competing with extraneous DNA targets for primers or other reagents (Fig. 2). Although the concept of dPCR is a powerful one for nucleic acid analysis, the technique has been limited by technical roadblocks associated with the sheer number of reactions required for statistically significant results. The advent of multiple commercially available platforms capable of running reactions on the nano- to picoliter scale has made dPCR a practical tool with great potential in research and clinical settings.

2. Digital PCR platforms

Four different dPCR platforms are currently marketed and differ mainly in their method of individual reaction partitioning (Table 1).

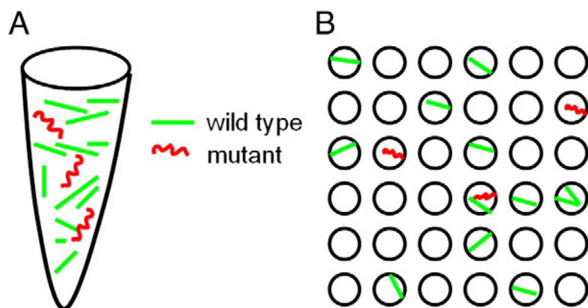

Fig. 2. Detection of rare mutant alleles in a background of wild-type DNA by digital PCR. (A) In conventional qPCR, mutant and wild-type alleles are mixed together in one bulk reaction where rare mutants compete for reagents with more abundant wild-type DNA. (B) Digital PCR increases sensitivity by compartmentalizing wild-type and mutant sequences, giving a less abundant mutant sequence equal access to reagents.

Fluidigm Corporation (San Francisco, CA) and Life Technologies (Carlsbad, CA) offer microfluidics-based systems that partition sample using sophisticated chips designed with microfluidics channels that deliver nanoliter volumes of sample into individual reaction wells. These systems are limited only by the number of reactions that fit onto a single microfluidics chip (hundreds to thousands) and the cost of the consumable chips (in the hundreds of dollars) (Baker, 2012). Bio-Rad Laboratories (Hercules, CA) and RainDance (Lexington, MA) have developed systems that divide diluted sample among many water-in-oil droplets (Hindson et al., 2011; Kiss et al., 2008). Each droplet represents a single reaction allowing simultaneous analysis of thousands (Bio-Rad) to millions (RainDance) of separate reactions (Baker, 2012).

In addition to commercially available dPCR systems, several laboratories are developing simpler dPCR systems with the goal of making this technology practical in resource-limited settings. For example, the SlipChip platform relies simply on the movement or “slipping” of 2 plates to reproducibly and precisely deposit discreet volumes suitable for parallel compartmentalization of nucleic acids (Shen et al., 2010). Any system capable of dividing 1 bulk PCR reaction into many discrete reactions is suitable for dPCR, whose utility derives simply from the ability to identify the amplification of a single nucleic acid template in many separate reactions.

3. Applications of dPCR

Although dPCR promises more sensitive and accurate nucleic acid detection, its use has been mainly limited to research applications. For example, Tadmor et al. (2011) used digital PCR instead of classical phage enrichment to identify virus–bacteria interactions in uncultured bacteria. The group targeted phage-like elements with degenerate primers and targeted bacterial small subunit ribosomal RNA genes with universal “all bacterial” primers in a microfluidics dPCR platform to identify previously unknown, uncultured bacteria in the termite hindgut (Tadmor et al., 2011).

Digital PCR has significantly advanced research capabilities, but its potential for clinical application has been investigated only to a limited degree, partly because devices that are practical, in both cost and dynamic range of detection, are just now becoming commercially available. As commercial systems gain wider use, dPCR could become a standard diagnostic approach for nucleic acid quantitation. Two areas where dPCR has shown potential clinical diagnostic utility are absolute quantification of target DNA sequences and rare mutant allele detection.

3.1. Absolute quantification

Digital PCR provides a sensitive method for the direct measure of viral nucleic acid, providing the absolute number of copies/mL without the need for a standard curve. White et al. (2012) utilized the Fluidigm dPCR system to quantify GB Virus Type C (GBV-C), an occult RNA virus associated with HIV-1 infection. Co-infection of HIV-1 patients with GBV-C has been suggested to lead to a decrease in the temporal progression to AIDS (Bhattarai and Stapleton, 2012; Gretch, 2012). Therefore, tracking the presence of GBV-C early in infection could provide the information needed for a more comprehensive patient prognosis. White et al. compared quantification of GBV-C isolated from transfected cells lines using standard qPCR and dPCR; they found that dPCR had an average coefficient of variation (CV, measure of precision) of 11.7 ± 2.2% for viral load testing, while standard qPCR had an average CV of 25.8 ± 4.9%. Using dPCR, they could detect between 3 and 10 DNA molecules/μL, a level that could not be detected by traditional qPCR in parallel experiments.

The second comparison of viral qPCR and dPCR was carried out by Henrich et al. (2012) on HIV-1 quantitation. They found that serial dilutions of HIV-1 or human CCR5 DNA amplicon standards

Table 1
Overview of commercial digital PCR systems.

Reaction partitioning format	Vendor	Instrument and list price	Consumables and list price	Number (and volume) of partitions	Multiplexing capabilities
Plate format	Fluidigm Corporation	Biomark HD $200–250K	$400/chip	9180 (6 nL) partitions/chip	5 colors
	Life Technologies	OpenArray Real-Time PCR System $140K and QuantStudio 12K Flex instrument $90–190K	$150/plate	3072 (33 nL) partitions/plate	2 colors
Droplet format	Bio-Rad Laboratories	QX100 ddPCR system $89K	$3/sample8samples/chip	20,000 (1 nL) droplets/sample	2 colors
	RainDance	RainDrop Digital PCR $100K	$10-30 sample8samples/chip	1 million (5 pL) droplets/sample	2 colors

Adapted from Baker (2012).

quantitated by droplet digital PCR (ddCPR, Bio-Rad) matched expected nominal copy numbers. When they ran the same assay on patient samples, they found that both ddPCR and qPCR had similar sensitivity, but ddPCR enumerated 10–40% fewer DNA copies compared with qPCR. Although the reason for this discrepancy is speculative, the authors offer that errors could be introduced into the qPCR assay by the spectrophotometric determination of the DNA concentration of the standards (Henrich et al., 2012).

A study by Kiss et al. (2008) validated the RainDance dPCR platform for sensitive absolute quantification. This system utilizes millions of picoliter droplets to divide a PCR reaction into millions of separate negative or positive outputs. They detected adenovirus at starting template concentrations as low as 1 template molecule/167 droplets or 92 molecules/μL (Kiss et al., 2008).

The move towards absolute quantification of viral load is driven not only by commercially available dPCR systems but also by simple systems suitable for point-of-care and resource-limited settings. Shen et al. (2011) used a SlipChip system to show that absolute quantification of HIV and hepatitis C virus (HCV) could be achieved within a large dynamic range. They utilized a rotational SlipChip, a microfluidic platform that manipulates liquid samples from microliter to picoliter scales through the relative movement of different plates without requiring complex control systems. They validated their assay with viral RNA from 2 HIV patients. Their measurements had good agreement with measurements from the standard clinical assay and achieved a dynamic range of 3-fold (0.5 $\log_{10}$) resolution from 1.7×10^2 to 2.0×10^7 molecules/mL, with a lower detection limit of 40 molecules/mL. They also validated a multiplex SlipChip with a 5-plex panel to simultaneously detect HIV and HCV along with a negative and positive control with a dynamic range of 1.8×10^3 to 1.2×10^7 molecules/mL.

Validation of dPCR for clinical viral diagnostics is still in its infancy. However, these initial studies demonstrate the potential clinical utility of dPCR for rapid, sensitive, and accurate quantification of viral load in patient samples.

3.2. Rare mutant detection

A second important application of dPCR in viral diagnostics, which has been the most well studied thus far in the oncology field, is the detection of rare point mutants in a background of wild-type sequences. Pekin et al. used a RainDance dPCR platform for the sensitive and quantitative detection of mutations in the KRAS oncogene, one of the most common oncogenic alterations in a range of human cancers (Pekin et al., 2011). In oncology diagnostics, somatic mutations in tumor DNA are used as highly specific biomarkers to distinguish cancer cells from their normal counterparts. Current qPCR TaqMan assays and pyrosequencing cannot detect less than 1–10% mutant genes in a nonmutated DNA background (Pekin et al., 2011). Digital PCR (on the RainDance platform) improves mutant detection by compartmentalizing genomic DNA (gDNA) into millions of picoliter droplets at a concentration of less than 1 genome equivalent per droplet with 2 TaqMan probes, 1 for mutant (green) and 1 for wild type (red). The ratio of green to red fluorescent reactions determines the ratio of mutant to wild-type genes. Pekin et al. quantified mutations in codons 12 and 13 of the KRAS oncogene in gDNA from several different human cell lines and were able to detect 1 mutant in a background of 200,000 wild-type KRAS genes (0.005% mutant) by analyzing 10^6 droplets (Pekin et al., 2011).

Another example of rare mutant detection by dPCR is the detection of low abundance epidermal growth factor receptor (EGFR) mutations in tumor tissue and plasma (Wang et al., 2010; Yung et al., 2009). EGFR tyrosine kinase inhibitors retard the progression of some lung cancers. Responsiveness to these inhibitors is associated with the presence of activating mutations in the EGFR kinase domain. Therefore, Yung et al. (2009) investigated dPCR analysis (Fluidigm platform) for detection of the 2 most common EGFR mutations in tumor tissues and plasma of lung cancer patients. Direct sequencing was commonly used in early studies, but this technique only detected mutant sequences greater than 30% of the total genetic content (Yung et al., 2009). Using dPCR, they were able to identify mutant sequences that were not detected by traditional sequencing methods. In these samples, mutant sequence constituted 2–14% of the total DNA. Similarly, Wang et al. utilized dPCR (Fluidigm platform) to detect and quantitate rare (0.02–9.26% abundance) drug-sensitizing EGFR mutations in tumor DNA.

These studies distinguish dPCR as a powerful tool for identifying low abundance mutant alleles in a background of high abundance wild-type sequence. While these data focus on oncology diagnostic applications, the principles demonstrated here translate to virology diagnostic applications where detection of low abundance mutant sequences, such as those mediating antiviral resistance, can significantly impact treatment outcome.

4. Potential applications and limitations of dPCR

Digital PCR's potential for sensitive and accurate quantitation of nucleic acids could offer significant improvements over current viral diagnostic procedures, particularly in detecting very low viral loads. The clinical significance of low-level viremia has not been well established, partly because the typical lower limit of 95% detection is around 40–60 copies/mL for typical viral assays (Waggoner et al., 2012; Widdrington et al., 2011). At this level, viral load is detectable but not reliably quantitated, resulting in a large number of patients with ongoing but unquantifiable or undetectable levels of viremia. One CMV study suggests that increases in viral load even at very low levels were clinically meaningful (Waggoner et al., 2012). Other studies on very low level viremia in HIV-infected patients suggest that low-level detection of HIV-1 viral load could be useful in predicting subsequent suboptimal viral control in patients on retroviral therapy (Doyle and Geretti, 2012; Doyle et al., 2012; Widdrington et al., 2011). Therefore, if future work indicates that dPCR assays have greater sensitivity and precision than qPCR assays at low viral loads, clinical treatment and outcome could be improved in situations where patient management relies on low-level viral load detection.

Moreover, just as dPCR has been utilized to identify low abundance oncogenic mutations, it could be adapted to identify low frequency virus variants, e.g., emerging drug-resistant mutants of CMV, HIV, or HBV in patients on antiviral therapy. As mentioned above, sequencing techniques, which are often employed for drug resistance mutant detection, cannot detect less than 1–10% mutant genes in a wild-type DNA background. Allele-specific digital PCR has the potential to detect very low abundance, emerging drug resistance mutations for applications where only a few key mutations need to be monitored.

Another application of dPCR could be the detection of chromosomally integrated viral genomes. Human herpes virus 6 (HHV-6) can integrate into human chromosome telomere regions, causing complications in the interpretation of HHV-6 PCR testing because normal PCR assays detect HHV-6 infections and integrated DNA. One study estimated that about half of all HHV-6–positive cerebrospinal fluid samples were due to detection of integrated HHV-6 rather than to actual infection (Ward et al., 2007). A current assay for integration involves detection of the ratio between cellular DNA and HHV-6 DNA. However, with the variation inherent in current qPCR assays, the ratio of cell DNA to viral DNA can range from 0.5 to 2.0 or worse. The precision and reproducibility of dPCR, which does not require a standard curve for quantitation, may improve such ratio-based chromosomal integration assays.

Despite dPCR's potential, there may be limitations to utilizing this technology in a clinical diagnostic setting. Some of the commercial dPCR platforms have a relatively small number of partitions that can only be scaled up using multiple, costly, 1-time-use, microfluidics chips. Also, studies need to investigate whether the sensitivity of dPCR assays exceeds current clinical qPCR assay sensitivity. Theoretically, dPCR should be more sensitive and more precise at low virus levels using the same qPCR TaqMan primers and probes, but practical issues such as limits on template input volume and master mix compatibility on the dPCR platform need to be considered. The digital platforms also add another layer of complexity to any assay, potentially slowing workflow and introducing error during sample pipetting and transfer, depending on the dPCR system used.

Digital PCR is unlikely to supplant qPCR in the short-term but, instead, will be a complementary approach in certain applications. Digital PCR has the potential to improve inter- and intralaboratory variation. Currently, qPCR assays base quantitation on a standard curve, which can vary from laboratory to laboratory or even from run to run. Digital PCR requires no standard curve to quantitate nucleic acid molecules, so it is a more direct and accurate method of quantitation. The sensitivity of dPCR is limited only by the number of individual reactions that are run simultaneously on a sample, so dPCR should provide the ability to detect below 1 viral copy per milliliter of sample using already established quantitative PCR protocols for many viruses, including CMV, HIV, and HCV. The absolute quantitation provided by dPCR could also improve specialty assays such as those for viral chromosomal integration. Digital PCR's sensitive and reproducible nucleic acid detection demonstrated thus far in research settings could translate well to a diagnostics setting as commercially available, high-throughput dPCR systems become more accessible.

References

Baker M. Digital PCR hits its stride. Nat Meth 2012;9:541–4.

Bhattarai N, Stapleton JT. GB virus C: the good boy virus? Trends Microbiol 2012;20:124–30.

Boeckh M, Huang M, Ferrenberg J, Stevens-Ayers T, Stensland L, Nichols WG, et al. Optimization of quantitative detection of cytomegalovirus DNA in plasma by real-time PCR. J Clin Microbiol 2004;42:1142–8.

Bustin SA. A-Z of Quantitative PCR. La Jolla, CA: International University Line; 2004.

Cook L, Atienza EE, Bagabag A, Obrigewitch RM, Jerome KR. Comparison of methods for extraction of viral DNA from cellular specimens. Diagn Microbiol Infect Dis 2009;64:37–42.

Doyle T, Geretti AM. Low-level viraemia on HAART: significance and management. Curr Opin Infect Dis 2012;25:17–25.

Doyle T, Smith C, Vitiello P, Cambiano V, Johnson M, Owen A, et al. Plasma HIV-1 RNA detection below 50 copies/ml and risk of virologic rebound in patients receiving highly active antiretroviral therapy. Clin Infect Dis 2012;54:724–32.

Gretch D. Advocating the concept of GB virus C biotherapy against AIDS. Clin Infect Dis; 2012.

Henrich TJ, Gallien S, Li JZ, Pereyra F, Kuritzkes DR. Low-level detection and quantitation of cellular HIV-1 DNA and 2-LTR circles using droplet digital PCR. J Virol Methods 2012;186:68–72.

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. Anal Chem 2011;83:8604–10.

Kiss MM, Ortoleva-Donnelly L, Beer NR, Warner J, Bailey CG, Colston BW, et al. High-throughput quantitative polymerase chain reaction in picoliter droplets. Anal Chem 2008;80:8975–81.

Lai KK, Cook L, Wendt S, Corey L, Jerome KR. Evaluation of real-time PCR versus PCR with liquid-phase hybridization for detection of enterovirus RNA in cerebrospinal fluid. J Clin Microbiol 2003;41:3133–41.

Mackay IM, Arden KE, Nitsche A. Real-time PCR in virology. Nucleic Acids Res 2002;30:1292–305.

Pekin D, Skhiri Y, Baret JC, Le Corre D, Mazutis L, Salem CB, et al. Quantitative and sensitive detection of rare mutations using droplet-based microfluidics. Lab Chip 2011;11:2156–66.

Shen F, Du W, Kreutz JE, Fok A, Ismagilov RF. Digital PCR on a SlipChip. Lab Chip 2010;10:2666–72.

Shen F, Sun B, Kreutz JE, Davydova EK, Du W, Reddy PL, et al. Multiplexed quantification of nucleic acids with large dynamic range using multivolume digital RT-PCR on a rotational SlipChip tested with HIV and hepatitis C viral load. J Am Chem Soc 2011;133:17705–12.

Sidransky D, Tokino T, Hamilton SR, Kinzler KW, Levin B, Frost P, et al. Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. Science 1992;256:102–5.

Sykes PJ, Neoh SH, Brisco MJ, Hughes E, Condon J, Morley AA. Quantitation of targets for PCR by use of limiting dilution. Biotechniques 1992;13:444–9.

Tadmor AD, Ottesen EA, Leadbetter JR, Phillips R. Probing individual environmental bacteria for viruses by using microfluidic digital PCR. Science 2011;333:58–62.

Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A 1999;96:9236–41.

Waggoner J, Ho DY, Libiran P, Pinsky BA. Clinical significance of low cytomegalovirus DNA levels in human plasma. J Clin Microbiol 2012;50:2378–83.

Wang J, Ramakrishnan R, Tang Z, Fan W, Kluge A, Dowlati A, et al. Quantifying EGFR alterations in the lung cancer genome with nanofluidic digital PCR arrays. Clin Chem 2010;56:623–32.

Ward KN, Leong HN, Thiruchelvam AD, Atkinson CE, Clark DA. Human herpesvirus 6 DNA levels in cerebrospinal fluid due to primary infection differ from those due to chromosomal viral integration and have implications for diagnosis of encephalitis. J Clin Microbiol 2007;45:1298–304.

White RA, Quake SR, Curr K. Digital PCR provides absolute quantitation of viral load for an occult RNA virus. J Virol Methods 2012;179:45–50.

Widdrington J, Payne B, Medhi M, Valappil M, Schmid ML. The significance of very low-level viraemia detected by sensitive viral load assays in HIV infected patients on HAART. J Infect 2011;62:87–92.

Yung TK, Chan KC, Mok TS, Tong J, To KF, Lo YM. Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. Clin Cancer Res 2009;15:2076–84.