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Determining lower limits of detection of digital PCR assays for cancer-related gene mutations

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ABSTRACT

Digital PCR offers very high sensitivity compared to many other technologies for processing molecular detection assays. Herein, a process is outlined for determining the lower limit of detection (LoD) of two droplet-based digital PCR assays for point mutations of the epidermal growth factor receptor (*EGFR*) gene. Hydrolysis probe mutation-detection assays for *EGFR* p.L858R and p.T790M mutations were characterized in detail. Furthermore, sixteen additional cancer-related mutation assays were explored by the same approach. For the *EGFR* L8585R assay, the assay sensitivity is extremely good, and thus, the LoD is limited by the amount of amplifiable DNA that is analyzed. With 95% confidence limits, the LoD is one mutant in 180,000 wild-type molecules for the evaluation of 3.3 μg of genomic DNA, and detection of one mutant molecule in over 4 million wild-type molecules was achieved when 70 million copies of DNA were processed. The measured false-positive rate for the *EGFR* L8585R assay is one in 14 million, which indicates the theoretical LoD if an unlimited amount of DNA is evaluated. For the *EFGR* T790M assay, the LoD is one mutant in 13,000 for analysis of a 3.3 μg sample of genomic DNA, and the dPCR assay limit sensitivity approaches one mutant in 22,000 wild-type molecules.

1. Introduction

1.1. Digital PCR

Invention of the Polymerase Chain Reaction (PCR) [1] changed life science research and molecular diagnostics. Now, digital PCR (dPCR) is changing the field of PCR and re-defining expectations for mutation detection. The power of digital PCR arises from evaluating individual molecules as positive or negative for a particular parameter, such as mutation status. For many assays, dPCR sensitivity is significantly higher than traditional PCR analysis, and the accuracy and precision of the assay improves by counting larger numbers of molecules individually. The sensitivity of digital PCR will facilitate detection limits that redefine our understanding of disease onset, progression, and recurrence.

One particularly attractive application of digital PCR is the quantitative detection of a small number of mutated DNA molecules among a large number of wild-type molecules, which is relevant to cancer research, and especially for the detection of minor alleles. Cancerous tissue is often highly heterogeneous and cancer biomarkers vary across types of disease and stages of disease progression which complicates cancer detection and identification at early stages. Subclonal populations of cells within a tumor may contain a mutation that differs from the primary mutation, but the subclonal mutation could be correlated to a prognosis and/or a response to specific therapy regimens. Similarly, detecting mutations in circulating tumor DNA (i.e., a Fluid Biopsy™ sample) is a relatively simple and non-invasive approach to monitoring disease recurrence, which requires a high sensitivity of mutation detection to provide effective therapies at the earliest stage of progression [2–4]. These examples reflect the need for mutation detection tools that are qualitatively more sensitive than existing tools, enabling sensitivity better than 1 in 10,000 and maximized to enable transformational advances in cancer research.

Digital PCR technologies can be deeply sensitive and highly precise for detection of low abundance minority alleles, such as those observed in progressive cancer and metastatic samples. The

Abbreviations: PCR, Polymerase Chain Reaction; *EGFR*, epidermal growth factor receptor; LoB, limit of blank; LoD, limit of detection; N, total number of droplet events counted; N_{WT}, number of droplets with only wild-type DNA; N_{Mut}, number of droplets with only mutated DNA; λ, average number of targets "loaded" per droplet; p, fraction of PCR-positive droplets; R, ratio of mutant to wild-type molecules; Λ_{FP}, average number of false-positive events; R_{FP}, average false positive rate (Λ_{FP}/#WT).

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Table 1
EGFR assay details.

Protein Variant	p.T790M	p.L858R
Nucleotide Variant	c.2369C>T	c.2573T>G
Amplicon Location	chr7: 55249045-55249095	chr7:55259486-55259563
Amplicon Length	51 base pairs	78 base pairs
Forward Primer	5′-CCTCACCTCCACCGTGCA-3′	5′-GCAGCATGTCAAGATCACAGATT-3′
Reverse Primer	5′-AGGCAGCCGAAGGGCA-3′	5′-CCTCCTTCTGCATGGTATTCTTTCT-3′
Wild-type Probe	/5TET/T+CATC+A+C+GC/ZEN/A+GCTC/3IABkFQ/	/VIC/-AGTTTGGCCAGCCCAA-MGBNFQ
Mutant Probe	/6FAM/T+CATC+A+T+GC/ZEN/A+GC+TC/3IABkFQ/	/6FAM/-AGTTTGGCCCGCCCAA-MGBNFQ
Probe Type (Vendor)	PrimeTime® LNA-ZEN (Integrated DNA Technologies)	TaqMan® MGB (Life Technologies)

epidermal growth factor receptor (*EGFR*) is frequently impacted by mutations that arise in cancer. Mutations in the *EGFR* gene (chromosomal locus 7p12.3-p12.1) are common across several cancer types, and the mutations often result in altered expression and activity. Upregulation and overexpression of *EGFR* can lead to uncontrolled cellular division, resulting in rampant tissue growth and lead to cancer. A common example is a point mutation (c.2573T>G) in exon 21 of *EGFR*, commonly known as variant L858R. This type of activating mutation occurs frequently in epithelial cancers, particularly lung cancer and glioblastomas. Non-small cell lung cancers (NSCLC) are some of the most common cancers in the world and are frequently associated with mutations in *EGFR*. Two tyrosine kinase inhibitors (TKIs), Gefitinib (Iressa®) and Erlotinib (Tarceva®), are common treatments for NSCLC. Nearly all responders to treatment possess somatic alterations in exons 18–21 of the *EGFR* gene. Approximately 90% of *EGFR* mutations in NSCLC are highly variable deletions located in exon 19 or point mutations in exon 21[5]. The L858R and T790M alterations are two of the most frequent *EGFR* mutations. The *EGFR* T790M mutation is particularly important because it is linked to known drug resistance, reinforcing the value of early detection [6].

Herein, the process of determining lower limits of detection (LoD) for assays performed using the RainDance RainDrop® digital PCR system for the detection of mutations in *EGFR* and other common cancer-related mutations is reported. The data demonstrate that digital PCR supports the potential for detecting mutated DNA in highly heterogeneous tumor samples or body fluids, which provides a broad view of all biomarkers arising from heterogeneous and highly localized tumor(s).

2. Methods

2.1. EGFR assays for digital PCR analysis

Assays comprised of primers and hydrolysis probes were developed to screen for *EGFR* T790M and L858R point mutations via digital PCR. The digital PCR system utilized for this evaluation was the RainDrop® Digital PCR System (RainDance Technologies). Table 1 presents the assay details for the *EGFR* T790M and L858R assays. Probes were labeled with 6-carboxyfluorescein (FAM, ex 494 nm/em 522 nm), VIC (from ABI, ex 528 nm/em 554 nm), or TET (from Integrated DNA Technologies, ex 522 nm/em 539 nm). The *EGFR* L858R TaqMan® MGB probes were designed using the Life Technologies online TaqMan design tool, whereas the PrimeTime® LNA-ZEN probes were designed with the assistance of Integrated DNA Technologies, as these are custom probes (non-cataloged item). The *EGFR* T790M probe sequence contains LNA nucleotides that are denoted with "+".

Table 2
Samples evaluated to measure and verify the sensitivity of each *EGFR* assay.

Sample (Mut/WT)	*EGFR* T790M	*EGFR* L858R
0% (wild-type only)	$N = 58$	$N = 71$
~0.5 to 1.0%	$N = 4$	$N = 4$
~0.05 to 0.1%	$N = 4$	$N = 4$
~0.005 to 0.01%	$N = 4$	$N = 4$
~0.0005 to 0.001%	$N = 4$	$N = 4$

2.2. Samples and reagent components

A series of experimental samples were processed to determine the LoD for each *EGFR* assay. Table 2 summarizes the replicate samples that were analyzed in this study. A two-part design was executed; one subset of samples is composed of wild-type genomic DNA samples to assess the false positive rate of the assay, and the second subset included a mutation titration series to verify the linearity and sensitivity of the assay.

The *EGFR* T790M and L858R mutation templates were synthetic GeneArt® plasmid templates (Life Technologies Inc.). Each plasmid was linearized using restriction enzyme digestion. Wild-type genomic DNA (G3041, Promega Inc.) was nebulized to approximately 3 kb in length according to manufacturer's protocols (K7025-05, Life Technologies). DNA was quantified and qualified using a NanoDrop 2000 spectrophotometer. Fragmentation length was confirmed via gel electrophoresis.

Following DNA preparation, PCR reagent components were prepared as presented in Table 3 in a pre-PCR room to limit the risk of reagent contamination. All sample PCR reactions were prepared to 50 μl volumes, containing approximately ~20,000 copies/μl of genomic DNA (~3.3 μg DNA per each 50 μl reaction). The mutant template was only incorporated into the second subset of samples, following a titration of the DNA sample, according to Table 2.

Outside of the samples defined within this evaluation, preliminary assessment, verification, and optimization of both assays was performed. The false positive mutant count is zero for both

Table 3
Digital PCR reagent components.

Reagent	Vendor	Item #	Final concentration
TaqMan® Genotyping Master Mix	Life Technologies	4371355	1×
TaqMan® MGB Probes	Life Technologies	Custom	0.2 μM
PrimeTime® LNA-ZEN qPCR Probes	Integrated DNA Technologies	Custom	0.2 μM
Oligonucleotide primers	Integrated DNA Technologies	Custom	0.9 μM
Droplet Stabilizer	RainDance Technologies	20-00803	1×
WT genomic DNA control	Promega	G3041	~20,000 copies/μl
Mutant plasmid DNA control (GeneArt® Gene Synthesis)	Life Technologies	Custom	Variable by sample type
DNase/RNase-free water	Sigma–Aldrich	W4502-1L	To volume