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Enhanced Ratio of Signals Enables Digital Mutation Scanning for Rare Allele Detection

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The use of droplet digital PCR (ddPCR) for low-level DNA mutation detection in cancer, prenatal diagnosis, and infectious diseases is growing rapidly. However, although ddPCR has been implemented successfully for detection of rare mutations at pre-determined positions, no ddPCR adaptation for mutation scanning exists. Yet, frequently, clinically relevant mutations reside on multiple sequence positions in tumor suppressor genes or complex hotspot mutations in oncogenes. Here, we describe a combination of coamplification at lower denaturation temperature PCR (COLD-PCR) with ddPCR that enables digital mutation scanning within approximately 50-bp sections of a target amplicon. Two FAM/HEX-labeled hydrolysis probes matching the wild-type sequence are used during ddPCR. The ratio of FAM/HEX-positive droplets is constant when wild-type amplicons are amplified but deviates when mutations anywhere under the FAM or HEX probes are present. To enhance the change in FAM/HEX ratio, we employed COLD-PCR cycling conditions that enrich mutation-containing amplicons anywhere on the sequence. We validated COLD-ddPCR on multiple mutations in *TP53* and in *EGFR* using serial mutation dilutions and cell-free circulating DNA samples, and demonstrate detection down to approximately 0.2% to 1.2% mutation abundance. COLD-ddPCR enables a simple, rapid, and robust two-fluorophore detection method for the identification of multiple mutations during ddPCR and potentially can identify unknown DNA variants present in the target sequence. *(J Mol Diagn 2015, 17: 284—292; http://dx.doi.org/10.1016/j.jmoldx.2014.12.003)*

In the era of personalized medicine, mutation detection methods that target mutations known to influence therapy response or clinical outcome are of great interest. Although real-time PCR methodologies have been described and are widely used for detecting mutations in clinical samples,[1—4] interest in digital PCR[5] is rising in view of the unique aspects of the technology and the emergence of commercial droplet digital PCR (ddPCR) platforms.[5—8] ddPCR has been implemented in a variety of fields such as cancer biomarker and viral load detection, fetal screening, or library quantification for next-generation sequencing.[9] One of the most common ddPCR applications is in the detection of known DNA variants present within a large excess of wild-type DNA, for instance in DNA from heterogeneous samples that harbor subclonal populations of mutated tumor cells.[10]

In ddPCR, the amplification reaction is compartmentalized into microscopic emulsion-based droplets containing at most a few target molecules per droplet. By segregating the interrogated sample, the effect of in-droplet target competition is reduced, which translates into increased assay discrimination and facile determination of wild-type versus mutant status.[11] However, as currently applied, ddPCR can only be used to detect mutations at known sequence positions. ddPCR

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Table 1 Summary of Cell Lines Used

Target region	Cell line	Mutation (nt)	Mutation (aa)
EGFR exon 20	H1975	c.2369C>T	p.T790M
TP53 exon 8	SW480	c.818G>A	p.R273H
	DU-145	c.820G>T	p.V274F
	MDA-MB-231	c.839G>A	p.R280K
	HCC2218	c.847C>T	p.R283C

aa, amino acid; nt, nucleotide.

incorporates two reporter probes, one mutant-specific and one wild-type, because of the requirement to account for PCR-amplification variability among droplets. This approach, by design, allows only the detection of previously known mutations. In cancer, tumor suppressor genes such as *TP53* harbor mutations that are scattered throughout the gene as opposed to oncogenes that usually carry mutations located in specific hotspots.[12] Although mutation scanning methods based on amplicon fluorescent melting analysis following real-time PCR have been developed,[13–16] monitoring fluorescent melting within individual droplets during ddPCR is not available at this time. Accordingly, a ddPCR approach that could be implemented in a mutation scanning format is desirable.

Here, we demonstrate that incorporation of coamplification at lower denaturation temperature PCR (COLD-PCR),[17–21] within the ddPCR workflow in conjunction with two fluorescently labeled probes matching the wild-type amplicon, provides a simple and robust method for mutation scanning of target amplicons. COLD-PCR suppresses wild-type sequences and enables preferential amplification of mutation-containing droplets, for any mutation along the amplicon.[21,22] Detecting changes to the ratio of COLD-PCR–enhanced signals caused by mutations anywhere within the probed region enables mutation scanning with high selectivity. This novel enhanced ratio of signal-based mutation scanning COLD-ddPCR enables a rapid method for the detection of mutations during ddPCR without prior knowledge of the specific DNA variant present in the target sequence. We demonstrate in this paper the application of COLD-ddPCR to the detection of multiple mutations present in *TP53* exon 8, as well as for the T790M resistance mutation in *EGFR* exon 20 in DNA from mutated cell lines and cell-free circulating DNA (cfDNA) from clinical cancer samples.

Materials and Methods

Cell Lines and Clinical Samples

Missense mutations in several positions of the *TP53* exon 8 and mutation p.T790M in *EGFR* exon 20 were assessed in this study. Human genomic DNA from cancer cell lines DU-145 (ATCC HTB-81D), HCC2218 (ATCC CRL-2343), and MDA-MB-231 (ATCC HTB-26D) was purchased from ATCC (Manassas, VA). Genomic DNA from commercial cell lines SW480 (ATCC no. CCL-228) and H1975 (ATCC no. CRL-5908) was extracted using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol (Table 1). Human genomic DNA (Promega, Madison, WI) was used as wild-type control DNA and for creating dilutions of gradually decreasing mutation abundances. All experiments were replicated at least three independent times for assessing the reproducibility of the results.

To evaluate the efficacy of this assay in characterizing specimens from different origins, we analyzed a colorectal tumor sample, known to harbor a G>A (p.R273H) missense mutation present at a low frequency (approximately 1%). This mutation had been previously identified and validated using different methods [COLD-PCR sequencing, denaturing high-pressure liquid chromatography, restriction endonuclease-mediated selective PCR, and differential strand separation at critical temperature (T_c)[16,23]]. We also evaluated cfDNA isolated from patients with lung adenocarcinomas at different stages of disease progression and treatment, and a cfDNA sample from an esophageal cancer case. Mutations in these cfDNA samples had been previously identified using a ddPCR allele-specific approach with hydrolysis probes specific either to the wild-type or the mutant allele.[24] Samples were obtained from patients after informed consent and Dana Farber-Cancer Institute Institutional Review Board approval (Table 2).

Table 2 List of Clinical Samples Used for Validation Purposes

Target region	Sample name	Type	Tumor/origin	Mutation (nt)	Mutation (aa)	Estimated frequency*
TP53 exon 8	CT20	gDNA	Colorectal cancer	c.818G>A	p.R273H	~1%
	FC39–4	cfDNA	Esophageal cancer	c.847C>T	p.R283C	~5%
EGFR exon 20	PT-04–09	cfDNA	Lung adenocarcinoma	c.2369C>T	p.T790M	~0.1%
	PT-21	cfDNA	Lung adenocarcinoma	c.2369C>T	p.T790M	Wild type
	PT-04–11	cfDNA	Lung adenocarcinoma	c.2369C>T	p.T790M	~2.1%
	PT-10–06	cfDNA	Lung adenocarcinoma	c.2369C>T	p.T790M	~0.43%
	PT-07–16	cfDNA	Lung adenocarcinoma	c.2369C>T	p.T790M	~2.03%
	PT-10–33	cfDNA	Lung adenocarcinoma	c.2369C>T	p.T790M	~0.38%
	PT-04–17	cfDNA	Lung adenocarcinoma	c.2369C>T	p.T790M	~4.08%

Circulating cell-free tumor DNA samples (cfDNA).
*Mutation frequencies were estimated by previous analysis or conventional digital PCR using allele-specific probes.
aa, amino acid; DNA, genomic DNA; nt, nucleotide.