APPLICATION NOTE
TaqMan® Mutation Detection Assays

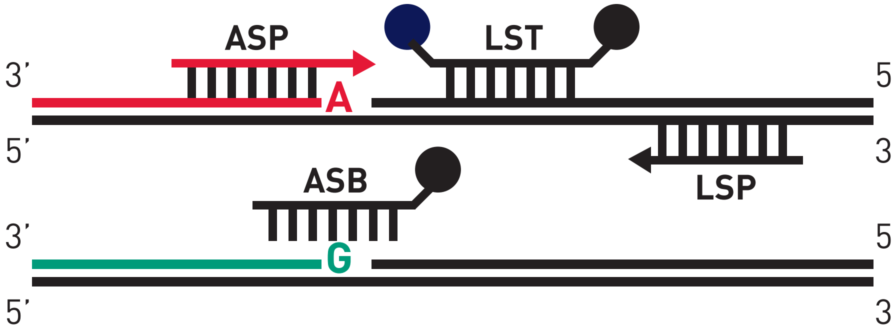
Accurate and sensitive mutation detection and quantitation using TaqMan® Mutation Detection Assays for disease research

In this research study, we addressed the feasibility of obtaining sensitive, accurate, and reproducible mutation detection results in synthetic templates, cell lines, and FFPE tissue samples using TaqMan® Mutation Detection Assays. Analytical performance assessed with synthetic templates and genomic DNA (gDNA) extracted from cell lines or model FFPE cell lines indicates that TaqMan® Mutation Detection Assays have a minimum of 0.1% sensitivity and can detect fewer than 10 copies of mutant DNA sequence in a background of wild type gDNA. Our data indicate that TaqMan® Mutation Detection Assays can accurately detect mutation status and quantify the percent mutation with a relative standard deviation (CV) <20%. Additionally, benchmarking to an established qPCR assay technology for mutation detection demonstrates that the TaqMan® Mutation Detection Assays have a wider detection range and superior limit of detection.

Introduction

Somatic mutations can be present in low abundance within a very high background of wild type sequences. Many mutation detection methods compatible with tumor specimens have been reported in the literature and/or are commercially available. Methods that are commonly used are gene sequencing (including pyrosequencing and traditional Sanger sequencing) and real-time PCR. Real-time PCR–based technologies include high-resolution melt curve analysis, peptide nucleic acid (PNA) clamping, and allele-specific amplification with bifunctional fluorescent primer/probe detection (ARMS/Scorpion). However, these commercially available kits have various limitations in terms of sensitivity, specificity, cost, workflow, and turnaround time. Also, there is a need for the technology to be able to accurately quantify the percent mutation within a sample.

We developed sensitive and quantitative TaqMan® Mutation Detection Assays to help accurately assess mutation status and percentage in research samples. TaqMan® Mutation Detection Assays were designed based on novel competitive allele–specific TaqMan® (castPCR™) technology, which combines allele-specific TaqMan® qPCR with allele-specific MGB blocker oligonucleotides to effectively suppress nonspecific amplification of the off-target allele (Figure 1).



ASP—Allele-specific primer
ASB—Allele-specific blocker (MGB)
LST—Locus-specific TaqMan® probe
LSP—Locus-specific primer

Figure 1. Schematic of a TaqMan® Mutation Detection Assay. A mutant allele assay or wild type allele assay is composed of an allele-specific primer (ASP), locus-specific primer (LSP), locus-specific TaqMan® probe (LST), and allele-specific blocker (ASB). The % mutation present in the sample is calculated based on the ΔC_t value between amplification reactions for a mutant allele assay and the corresponding wild type allele assay or a gene-specific reference assay.

Materials and methods

DNA samples

Twelve cell lines (from ATCC or other sources; Table 1) were cultured according to the suppliers' recommendations, and gDNA was isolated using the PureLink® Genomic DNA Mini Kit (Life Technologies) or the QIAamp® gDNA Mini Kit (Qiagen). gDNA from mutant cell lines was diluted into gDNA from wild type cell lines at various percentages. The total gDNA sample input in each reaction was 10 ng or 30 ng.

Eight model FFPE cell line specimens were prepared by AcroMetrix®, part of Life Technologies (Table 1). gDNA was extracted from 20 µm slices with the RecoverAll™ Nucleic Acid Extraction Kit (Ambion, part of Life Technologies) or QIAamp® DNA FFPE Kit (Qiagen). gDNA from mutant cell lines was diluted into gDNA from FFPE wild type cell lines at various percentages. The total FFPE sample input in each reaction was 30 ng.

FFPE or fresh frozen tumor specimens (colorectal and lung cancer) were collected and gDNA was extracted by an independent laboratory using their own validated method.

Synthetic plasmid constructs for seven KRAS mutant alleles and corresponding wild type alleles were each designed with 600 bp of genomic context sequence surrounding the mutant base. Gene synthesis, plasmid construction, and plasmid preparation were performed by GeneArt®, part of Life Technologies (Regensburg, Germany), and all sequences were verified by Sanger sequencing prior to use.

All DNA samples were quantified using a NanoDrop® ND1000 (NanoDrop Technologies) or TaqMan® RNase P Detection Reagents Kit. TaqMan® DNA Template Reagents Kit were used to create a standard curve.

TaqMan® Mutation Detection Assays

Mutation detection experiments were performed by running 10 µL or 20 µL reactions comprising DNA template, 1X Genotyping Master Mix, and 1X TaqMan® Mutation Detection Assay (containing allele-specific forward primer, locus-specific TaqMan® probe, locus-specific reverse primer, and allele-specific MGB blocker). qPCR was run on an Applied Biosystems® ViiA™ 7 or 7900HT Real-Time PCR System in 96-well or 384-well plates with the following conditions: 95°C for 10 min; 5 cycles: 92°C for 15 sec and 58°C for 1 min; 45 cycles: 92°C for 15 sec and 60°C for 1 min. All samples were run in quadruplicates or triplicates. C_t values were obtained from the instrument's real-time PCR data collection software using auto baseline and 0.2 manual threshold. The mutation status and percent mutation were analyzed using Mutation Detector™ software.

Table 1. Cell lines used in this study and their corresponding mutations.

Cell line	Sample type	Mutation description	COSMIC mutation ID
SW1463	Fresh/FFPE	KRAS G12C	516
A549	Fresh/FFPE	KRAS G12S	517
PSN-1	Fresh/FFPE	KRAS G12R	518
SW480	Fresh/FFPE	KRAS G12V	520
PANC-1	Fresh/FFPE	KRAS G12D	521
NCI-H2009	Fresh/FFPE	KRAS G12A	522
DLD-1	Fresh/FFPE	KRAS G13D	532
H460	Fresh	EGFR L858R	6224
H1975	Fresh	EGFR T790M	6240
H1650	Fresh	EGFR Exon19 Del	6223
Colo 201	Fresh	BRAF V600E	476
Jurkat	Fresh/FFPE	Wild type	

Table 2. KRAS gene mutations in the benchmarking study.

COSMIC mutation ID	Nucleotide change	Amino acid change
516	c.34G>T	p.G12C
517	c.34G>A	p.G12S
518	c.34G>C	p.G12R
520	c.35G>T	p.G12V
521	c.35G>A	p.G12D
522	c.35G>C	p.G12A
532	c.38G>A	p.G13D

Benchmarking of the TaqMan® Mutation Detection Assays to another commercially available qPCR technology for mutation detection was performed for seven KRAS gene mutation targets (Table 2). The other commercially available assays, or Vendor A assays, were purchased as a kit and the assay reactions were performed according to the protocol. The benchmarking experiments were performed on the Applied Biosystems® 7500 Real-Time PCR System in 96-well reaction plates. The TaqMan® Mutation Detection Assays were used with the following thermal cycling conditions: 95°C for 10 min; 5 cycles: 92°C for 15 sec and 58°C for 1 min; 40 cycles: 92°C for 15 sec and 60°C for 1 min; with the analysis settings described above. The Vendor A mutation detection assays were run in 25 µL reaction volume with the following thermal cycling conditions: 95°C for 4 min; 40 cycles: 95°C for 30 sec and 60°C for 1 min. Analysis of Vendor A assays was performed according to the vendor's protocol.

Results

Assay sensitivity, selectivity, and linearity

To evaluate assay sensitivity, selectivity, and linearity, gDNA from each of the mutant cell lines (fresh or FFPE cell line) was serially diluted into a background of wild type cell line gDNA of the same sample type. For fresh cell lines, the mutant allele percentage ranged from 100% to 0.1%. For FFPE cell lines, the mutant allele percentage ranged from 50% to 0.1%. The results show that assays can detect mutations at various percentages with excellent linearity (R^2>0.990) and PCR efficiency (100 ± 10%) (Figure 2A). In addition, assays can clearly detect 0.1% mutation (equivalent to 10 copies of mutant allele) in a background of 30 ng wild type gDNA (Figure 2B). Some of the assays have been shown to detect down to a single copy of the mutant allele in a background of 100 ng wild type gDNA (data not shown).

Assay accuracy and reproducibility

To assess the detection accuracy and reproducibility of the TaqMan® Mutation Detection Assays, three independent experiments were performed to measure the mutation percentage of model samples prepared in the above-mentioned cell line titration study. Both mutant allele assays and the corresponding wild type allele assays were run with each sample. The percent mutation present in the sample was calculated based on the ΔC_t value between amplification reactions for the mutant allele assay and corresponding wild type allele assay. Run-to-run variation was analyzed for detection precision. The data show that these research assays can precisely quantify the percent mutation with CV<20% when the target allele copy number is >30 (Table 3).

KRAS mutation detection in FFPE colorectal tumor research specimens

Seven KRAS TaqMan® Mutation Detection Assays were further tested with biological samples by an independent laboratory using a total of 31 gDNA samples from FFPE colon tissue research specimens. Among those 31 samples, 10 were non-tumor colon specimens and 21 were colorectal tumor specimens. The KRAS mutation status of these samples was validated by three different methods (TaqMan® allelic discrimination (AD) probe assay (custom TaqMan® SNP assay), TaqMan® AD probe assay + peptide nucleic acid (PNA) wild type allele blocker, and Sanger sequencing) (Table 4). Ten gDNA samples extracted from normal FFPE tissues were examined for KRAS mutation status using TaqMan® Mutation Detection Assays. No positive samples were found in the 10 non-tumor tissues. The results obtained by KRAS TaqMan® Mutation Detection Assays for those tumor samples were concordant with previously reported results (Table 4).

KRAS, BRAF, and EGFR mutation detection in cell line and tumor biopsy research samples

TaqMan® Mutation Detection Assays were evaluated using a total of 33 gDNA samples from cell lines and tumor biopsy research samples (fresh frozen and FFPE tissues) by an independent laboratory. Mutation status of these samples was initially assessed by two different methods (amplification refractory mutation system (ARMS®) technology and Sanger sequencing). ARMS® technology for mutation detection discriminates between the mutation and wild type DNA by selectively amplifying the target sequence. The results from the TaqMan® Mutation Detection Assays demonstrated complete concordance

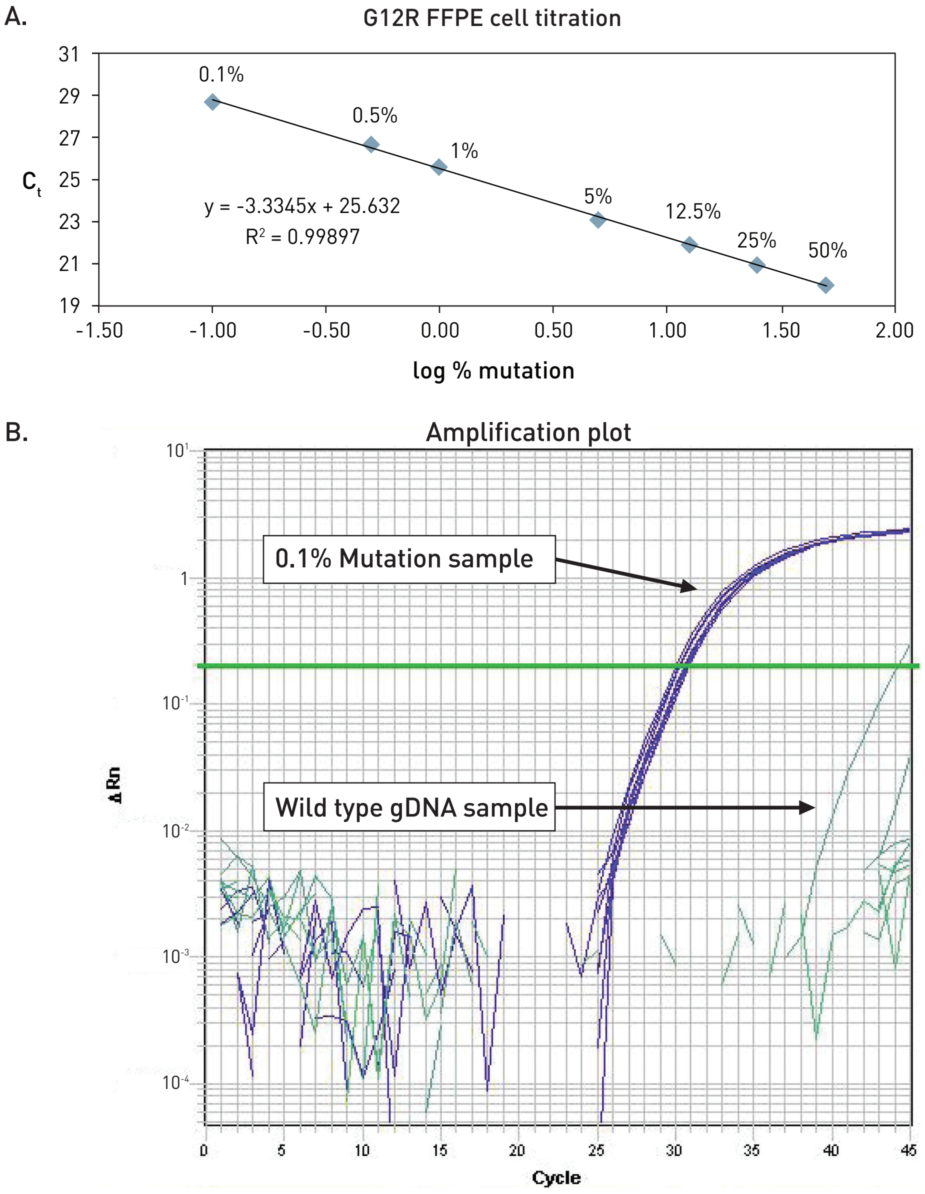


Figure 2. TaqMan® Mutation Detection Assays can detect mutations at various percentages with excellent linearity and PCR efficiency. The FFPE mutant cell line PSN-1 was serially titrated into the FFPE wild type Jurkat cell line from 50% to 0.1%. The total gDNA sample input was 30 ng. The KRAS_518_mu mutant allele assay was used to detect mutations in these model FFPE samples. **(A)** The KRAS_518_mu assay demonstrated excellent linearity (R^2 = 0.999) and PCR efficiency (99.5%). **(B)** The amplification plot shows the C_t difference between 0.1% mutation sample (10 copies mutant allele in 30 ng wild type gDNA and wild type gDNA sample (30 ng) for the KRAS_518_mu assay.

Table 3. KRAS G12C percent mutation measurement in cell line titration research samples from run to run. In this experiment, the total genomic DNA input was 10 ng.

	G12C mutant cell line gDNA titrated into wild type cell line gDNA								
Target mutant allele copy number	3000	1500	750	375	188	90	30	15	3
Expected (%)	100	50	25	12.5	6.3	3	1	0.5	0.1
Measured (%), average	100	48.9	23.3	11.2	5.7	2.6	0.8	0.4	0.1
CV (%) from run-to-run	0	2.2	3.8	7.8	7.5	9	17	26	23

Table 4. KRAS mutation detection in FFPE colorectal tumor research specimens using different mutation detection technologies.

	G12A	G12C	G12D	G12R	G12S	G12V	G13D	WT	
TaqMan® Mutation Detection Assays	2	1	4	0	0	4	8	2	n = 21
TaqMan® AD assay	2	1	4	0	0	4	8	2	
TaqMan® AD assay + PNA blocker	2	1	4	0	0	4	8	2	
Sequencing	2	1	4	0	0	4	8	2	

Table 5. Mutation detection in cell lines and tumor biopsy research samples. The same samples were tested independently at different sites.

	EGFR_L858R	EGFR_2235_2249del15	KRAS_G12C	KRAS_G12R	KRAS_G12V	KRAS_G12D
TaqMan® Mutation Detection Assay (data from an independent laboratory)	2	2	4	1	6	3
TaqMan® Mutation Detection Assay (internal testing data)	2	2	4	1	6	3
ARMS	2	2	4	1	6	3
Sanger sequencing	2	2	4	1	6	3

to those previously reported results. Eighteen of the 33 gDNA samples were then independently tested at Life Technologies to determine the robustness and reproducibility of the assays in biological samples. Results from this independent testing showed that TaqMan® Mutation Detection Assays are highly accurate and reproducible (Table 5).

Benchmarking to other commercially available mutation detection kits

Specificity and sensitivity of the TaqMan® Mutation Detection Assays compared to Vendor A's product for mutation detection were evaluated with synthetic plasmid DNA targets containing either the mutant allele or wild type allele. The seven KRAS mutant allele assays from each company were run with 10,000 copies (equivalent to 30 ng gDNA) of both target alleles. Three replicates were performed for each target, and the average C_t values were determined (Figures 3A and 3B).

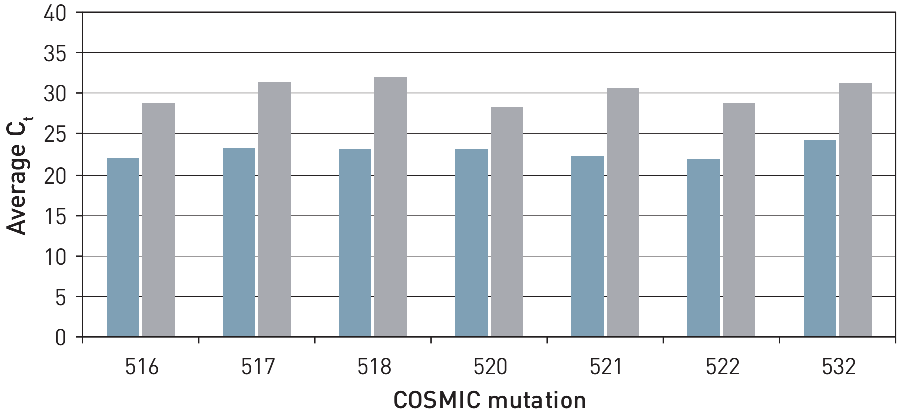
TaqMan® Mutation Detection Assays generated significantly lower C_t values compared to Vendor A's assays, indicating that the TaqMan® Mutation Detection Assays have greater sensitivity. In addition, the mutant allele assays did not amplify the wild type allele target, with the exception of one TaqMan® Mutation Detection Assay that resulted in minimal amplification for mutation target KRAS 521. The mutant allele assay on wild type allele target C_t value minus the mutant allele assay on mutant allele target C_t value provides a ΔC_t value for each target assessed. This ΔC_t correlates to the sensitivity capabilities of the assay. A larger ΔC_t value indicates the assay can detect a lower percent mutation in a larger quantity of sample. The TaqMan® Mutation Detection Assays provided significantly larger ΔC_t values than Vendor A's assays did, demonstrating the greater sensitivity of the TaqMan® Mutation Detection Assays.

The limit of detection for each of the seven assays for both technologies was evaluated with a dilution range of mutant allele targets, each combined with a constant 30 ng of wild type gDNA. Two replicates were performed for each mutant allele target input quantity from 10,000 copies down to 1–2 copies per reaction. The number of reactions that amplified and reached the cycle threshold value before cycle 40 was counted (Table 6). The TaqMan® Mutation Detection Assays detected down to 1 copy of mutant allele sequence in a background of 10,000 copies of wild type gDNA. Vendor A's mutation detection assays reproducibly detected 50 copies for all seven mutations; however, detection was inconsistent below this input quantity. These results demonstrate the superior limit of detection of TaqMan® Mutation Detection Assays compared to this current commercial qPCR technology.

Conclusions

This study demonstrates that TaqMan® Mutation Detection Assays can accurately detect mutation status and quantify percent mutation in research samples with a relative standard deviation (CV) <20% in fresh and FFPE cell line samples containing a high background of gDNA. The research assays demonstrate a minimum sensitivity of 0.1% mutation, and can detect down to a single copy of mutated DNA sequence in a background of wild type gDNA. Additionally, benchmarking to an established qPCR mutation detection technology shows that TaqMan® Mutation Detection Assays have a wider detection range and superior limit of detection.

A. Mutant allele DNA target



B. Wild type allele DNA target

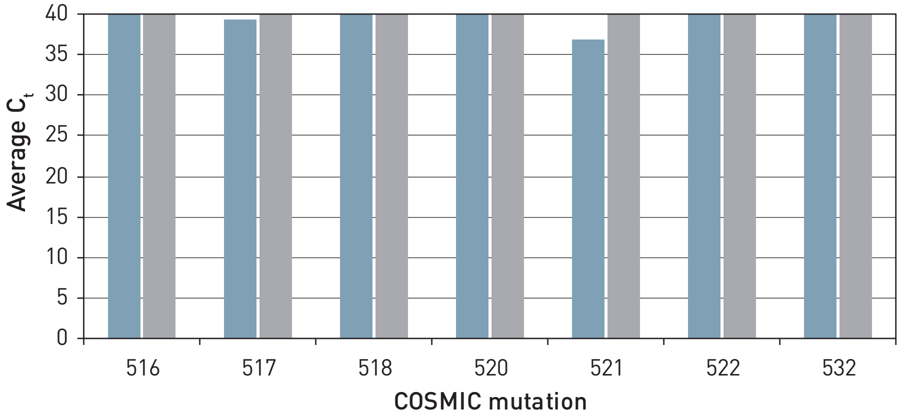


Figure 3. Comparison of TaqMan® Mutation Detection Assays with Vendor A's mutant allele assays. C_t values of TaqMan® Mutation Detection Assays (blue) versus the C_t values of Vendor A's mutant allele assays (grey) run on 10,000 copies of **(A)** mutant allele DNA target or **(B)** wild type allele DNA target. The data demonstrate that TaqMan® Mutation Detection Assays have higher sensitivity and equivalent specificity compared to Vendor A's assays.

Table 6. Limit of detection evaluation across a dynamic range of mutant allele DNA target inputs in a background of wild type allele DNA for TaqMan® and Vendor A's mutation detection assays (n = 2 per input quantity). Red indicates that less than two replicates were detected; gray indicates that no replicates were detected.

Mutant allele target input	COSMIC mutation 516		COSMIC mutation 517		COSMIC mutation 518		COSMIC mutation 520		COSMIC mutation 521		COSMIC mutation 522		COSMIC mutation 532	
	TaqMan®	Vendor A	TaqMan®	Vendor A	TaqMan®	Vendor A	TaqMan®	Vendor A	TaqMan®	Vendor A	TaqMan®	Vendor A	TaqMan®	Vendor A
10,000 copies	2	2	2	2	2	2	2	2	2	2	2	2	2	2
1,000 copies	2	2	2	2	2	2	2	2	2	2	2	2	2	2
100 copies	2	2	2	2	2	2	2	2	2	2	2	2	2	2
50 copies	2	2	2	2	2	2	2	2	2	2	2	2	2	2
25 copies	2	2	2	0	2	2	2	2	2	2	2	2	2	2
10 copies	2	1	2	0	2	1	2	2	2	1	2	2	2	0
5 copies	2	2	2	0	2	1	2	1	2	0	2	1	2	1
~1 to 2 copies	1	0	1	0	1	0	1	0	1	0	2	0	0	0

TaqMan® Mutation Detection Assays have the following features:

1. Assays can detect less than 0.1% mutation in a background of wild type gDNA. The limit of detection is down to 1 copy of mutant allele.
2. Assays have excellent linearity and PCR efficiency.
3. Assays are highly reproducible and can precisely quantify percent mutation.
4. Assays are highly accurate and highly concordant with other technologies.
5. Assays are compatible with multiple sample types including FFPE tissues, fresh frozen tissues, and cell lines.
6. Assays have superior detection range and sensitivity compared to current commercially available qPCR assays.

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