

# TaqMan® multiplex qPCR

Accurate, sensitive, and as efficient as traditional qPCR

In this application note, we show:

- A new multiplexing solution with probes having ABY® and JUN® reporter dyes and a QSY® nonfluorescent quencher
- An optimized TaqMan® Multiplex Master Mix that can be used for genotyping and gene expression analysis
- The new multiplexing solution is as accurate and sensitive as singleplex PCR on the same loci
- Multiplex qPCR assay results comparable to singleplex or duplex qPCR results can be obtained with less starting input material per assay

## Introduction

Current analyses of cell and tissue functionality require extracting as much information as possible from materials that are often limited. For example, samples such as tumor biopsies are difficult to collect and usually yield only a small amount of usable nucleic acid. Singleplex qPCR has been the gold standard for analyzing clinical research samples on the nucleic acid level, and has been invaluable in extending the limits of biological knowledge for more than a quarter century.

Genotyping	Gene expression
<ul style="list-style-type: none"> <li>• Obtain genomic DNA from:               <ul style="list-style-type: none"> <li>– SW620 colorectal adenocarcinoma cell line</li> <li>– Panc 03.27 pancreatic adenocarcinoma cell line</li> <li>– MDA-MB-468 breast adenocarcinoma cell line</li> <li>– 23 normal Coriell cell lines for reference</li> </ul> </li> <li>• Design multiplex genotyping assays:               <ul style="list-style-type: none"> <li>– <i>TP53</i> R273H</li> <li>– <i>KRAS</i> G12V</li> </ul> </li> <li>• Compare results from multiplex to singleplex SNP assays run on QuantStudio® 7 system</li> </ul>	<ul style="list-style-type: none"> <li>• Obtain total RNA from:               <ul style="list-style-type: none"> <li>– SW620 cells</li> <li>– Human total colon RNA</li> </ul> </li> <li>• Convert to cDNA using SuperScript® VILO™ cDNA Synthesis Kit</li> <li>• Design multiplex gene expression assays (gene selection was done following profiling of the samples using TaqMan® OpenArray® Human Signal Transduction Panel run on QuantStudio® 12K Flex Real-Time PCR System)</li> <li>• Compare results from multiplex to singleplex gene expression assays run on QuantStudio® 7 system</li> </ul>

Figure 1. Experimental approach for two different multiplexing applications.

However, the limited amount of nucleic acid obtained from clinical research specimens often forces choices to be made about how best to utilize these precious samples. Furthermore, if the sample is limited, the number of loci that can be analyzed is also limited, reducing the amount of information that can be extracted from the sample. Finally, the additional time and materials required to set up multiple single-assay reactions could increase the expense of a complex project significantly.

Multiplex qPCR analysis of nucleic acids, a strategy where more than one target is amplified and quantified from a single sample aliquot, is an attractive solution to these problems. In multiplex PCR, a sample aliquot is queried with multiple probes that contain fluorescent dyes in a single PCR reaction. This increases the amount of information that can be extracted from that sample. With multiplex qPCR, significant savings in sample and materials can be realized.

In this application note, we describe a multiplex qPCR solution for SNP genotyping and mRNA gene expression analysis. To demonstrate the versatility of the new multiplex solution we characterized oncogenic lesions in the colorectal adenocarcinoma cell line SW620, the pancreatic adenocarcinoma cell line Panc 03.27, and the breast adenocarcinoma cell line MDA-MB-468. These cells are highly tumorigenic when transplanted in nude mice, are known to harbor mutations in the *KRAS* and *TP53* genes, and thus are good models for tumor dynamics [1,2]. First, we verified that the cell lines have pathogenic alleles of the *KRAS* and *TP53* loci, but that 23 normal DNA samples (Coriell) do not. Next, we simultaneously profiled the expression levels of three sets of four different genes in different multiplex groups in SW620 cells and compared them to normal colon total RNA as a reference sample. The overall experimental approach is shown in Figure 1.

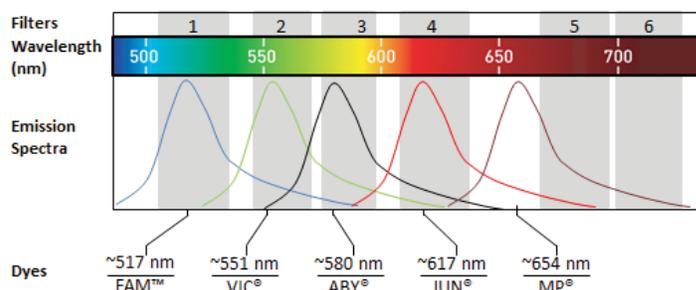


Figure 2. Fluorescence emission spectra of different dyes used for multiplex qPCR.

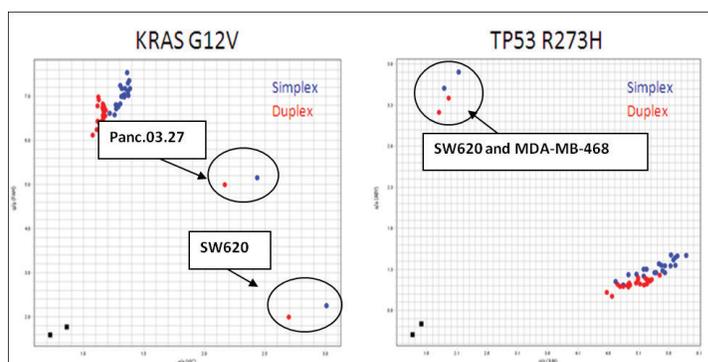


Figure 3. Comparing *KRAS* G12V and *TP53* R273H genotyping in singleplex (blue) and duplex (red) reactions across three cancer cell lines and 23 normal samples. (A) Panc 03.27 and SW620 are shown to be heterozygous and homozygous for *KRAS* G12V mutation (G>T), respectively. (B) MDA-MB-468 and SW620 are homozygous for a G>A mutation in *TP53*.

Multiplex qPCR requires introduction of new dyes. We have introduced a new multiplexing solution with four reporter dyes, a new quencher, and a new master mix. The ABY® and JUN® reporter dyes complement our existing offering of FAM™ and VIC® reporter dyes for multiplexing. These four reporter dyes with distinct spectra are optimized to work together with minimal spectral overlap (Figure 2). We have developed the new QSY® nonfluorescent quencher in order to facilitate multiplex qPCR with the four dyes. Finally, we have developed a new TaqMan® Multiplex Master Mix to be compatible with these four reporter dyes and that gives accurate and precise results. The master mix uses MUSTANG PURPLE® (MP) as a passive reference dye and AmpliTaq® UP polymerase, which only requires a 20-second activation time. This multiplexing solution is compatible with the QuantStudio® 6, 7, and 12K Flex systems, as well as the ViiA™ 7 and 7500/7500 Fast Real-Time PCR Systems.

### Application of multiplex qPCR to genotyping

To demonstrate the utility of multiplex PCR, we first examined the genotypes of different cell lines at two different loci. We used the predesigned TaqMan® SNP Genotyping Assay (C\_31385346\_10) that interrogates *KRAS* G12V with FAM™ dye- and VIC® dye-labeled NFQ-MGB probes and a custom-made SNP assay to *TP53* R273H that contains custom ABY®-QSY® and JUN®-QSY® probes. Each SNP assay was run individually and also run together in a multiplex reaction with the cell-line derived gDNAs. Reactions were performed on a QuantStudio® 7 instrument using identical thermocycling profiles (95°C 20 s, [95°C 5 s + 60°C 30 s] x 40 cycles). The following parameters served as the criteria for comparison between individual assay and multiplex assay reactions: call concordance and signal intensity ( $\Delta$ RN).

The resulting genotype calls are illustrated in Figure 3. The 100% call concordance between singleplex and duplex reactions shows that the new multiplex reagents provide similar efficiencies to the traditional singleplex reagents. Notably, the two single-assay reactions required a total of 20 ng of gDNA, whereas the multiplex assay reaction required only 10 ng. This illustrates how multiplex PCR is capable of extracting more information from a smaller amount of sample. Together, these results demonstrate that duplex assay genotyping using TaqMan® Multiplex Master Mix and the newly developed ABY® dye-labeled and JUN® dye-labeled QSY® probes is an accurate and attractive solution to analysis of limited or precious samples. In most cases, multiplex optimization is probably not necessary but refer to the TaqMan® Multiplex Master Mix User Guide for optimization recommendations if needed.

### Application of multiplex qPCR to gene expression analysis

We next evaluated the ability of multiplex qPCR to analyze mRNA levels. For these experiments, we took an approach similar to that used to identify genes that are differentially regulated in cancer. We obtained purified RNA from SW620 colon carcinoma cells and normal human colon. Both RNA samples were converted to cDNA using the SuperScript® VILO™ cDNA Synthesis Kit. To identify candidates that are differentially expressed in these samples, we

**Table 1. Gene expression assays for the multiplex gene expression application.** Assay IDs were submitted to our custom services for redesign with QSY® probes.

Gene symbol, assay ID	Reporter dye/quencher
<i>BAX</i> , Hs00180269_m1	JUN®
<i>EP3000</i> , Hs00914223_m1	VIC®
<i>EGFR</i> , Hs01076078_m1	FAM™
<i>CFLAR</i> , Hs01116280_m1	JUN®
<i>MYC</i> , Hs00905030_m1	VIC®
<i>AKT2</i> , Hs01086102_m1	FAM™ and JUN®
<i>MALT1</i> , Hs00198984_m1	VIC®
<i>EGR1</i> , Hs00152928_m1	FAM™
<i>B2M</i> , 4310886E	ABY®

performed an initial profiling experiment using the TaqMan® OpenArray® Human Signal Transduction Panel on the QuantStudio® 12K Flex Real-Time PCR System. This panel contains 573 unique assays related to signaling pathways and 24 reference assays. Genes that showed more than two-fold change in measured levels were selected for the multiplex gene expression study. Eight gene targets from the OpenArray® profiling results were chosen and gene expression assays were designed using a custom design process for QSY®, non-MGB probes. Assays for these targets plus *B2M* (used as the endogenous control) were synthesized; each had a different dye at the 5' end and the QSY® quencher on the 3' end (Table 1). Primer-probe mixes were formulated at 20x concentration for reaction concentrations of 300 nM forward primer, 300 nM reverse primer, and 250 nM probe.

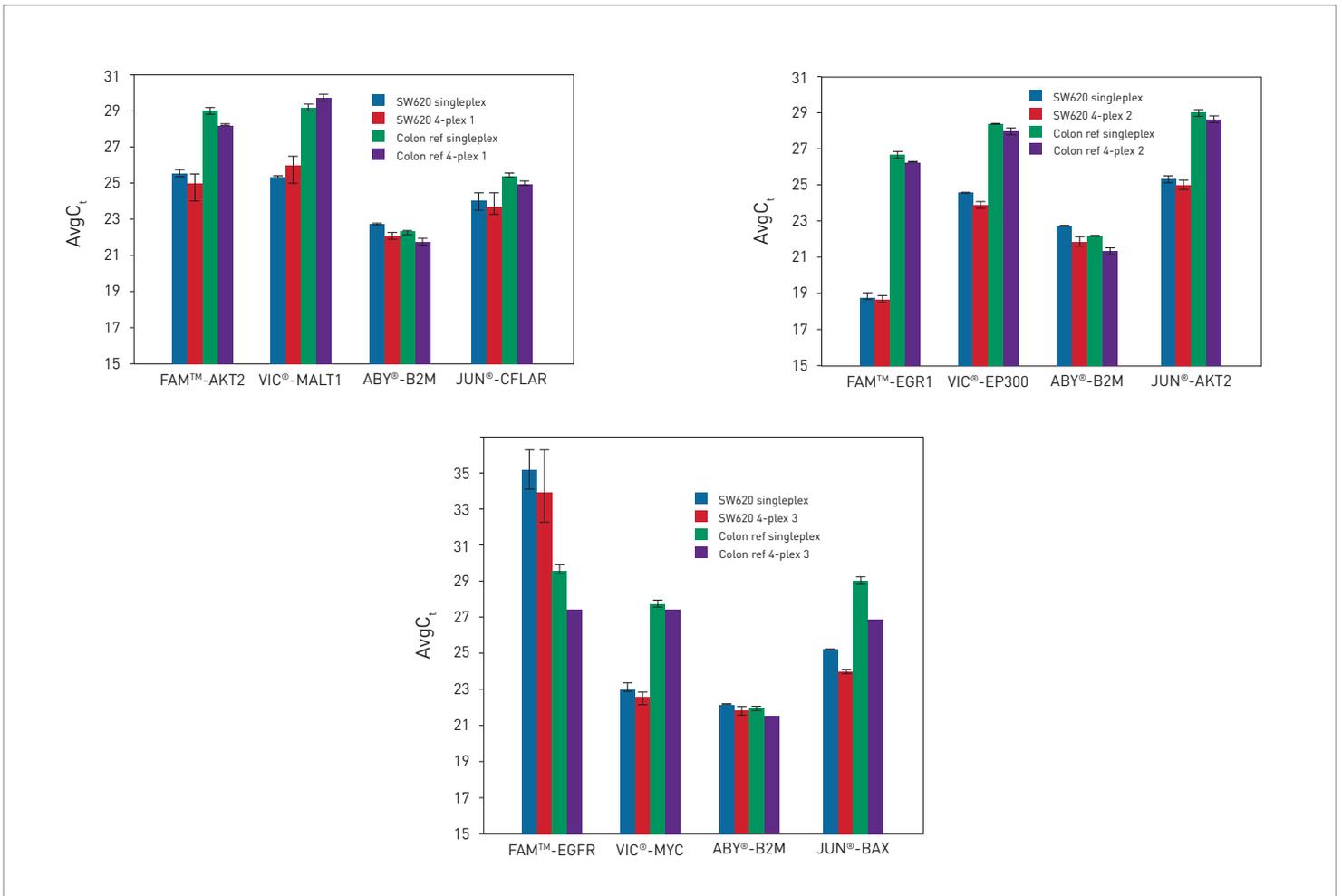


Figure 4. Comparison of average C<sub>t</sub> (n = 4) of nine loci and one reference gene from SW620 cell line RNA and normal colon RNA in singleplex and multiplex qPCR format.

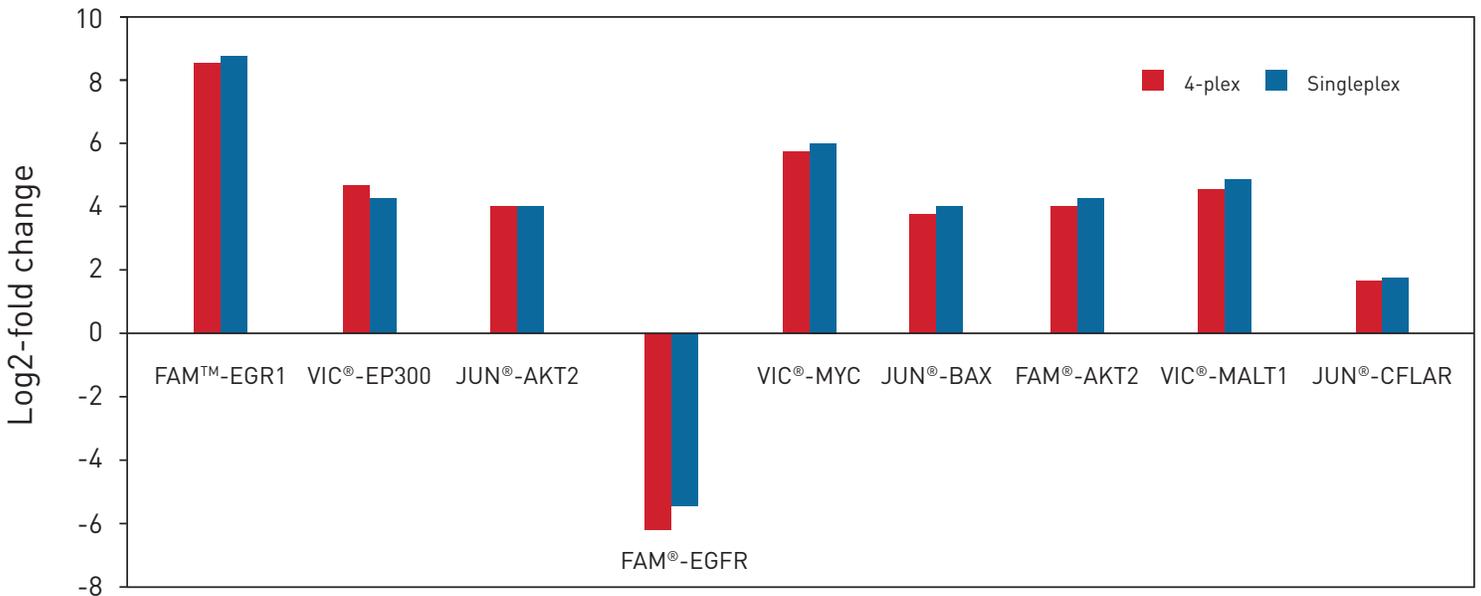
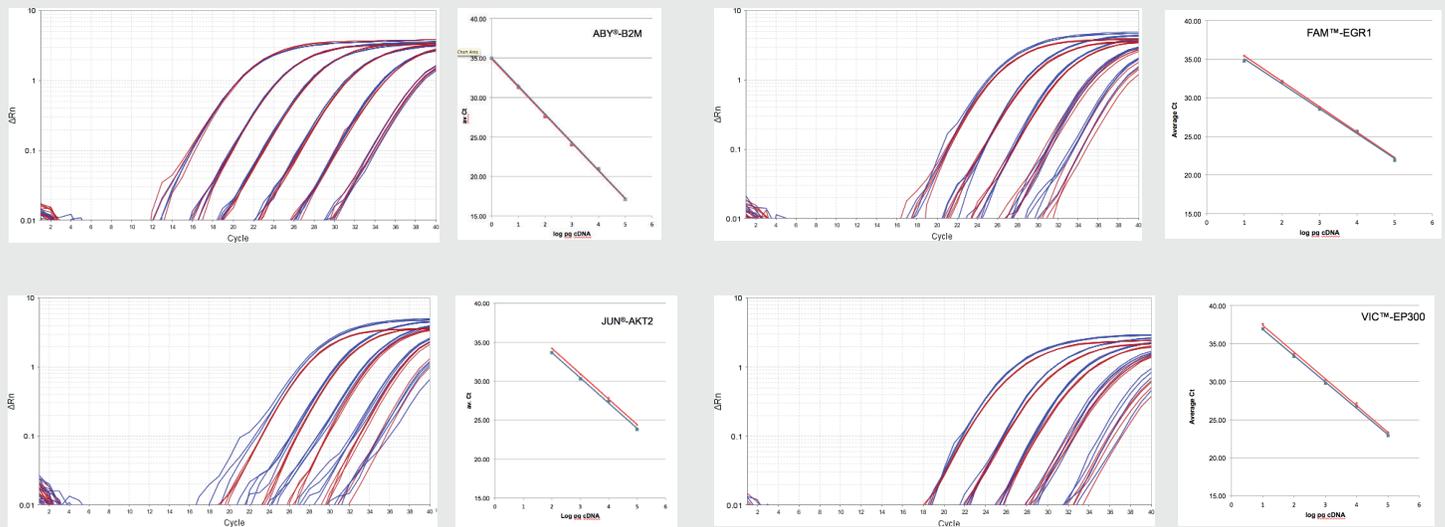


Figure 5. Fold change comparison of nine targets normalized to B2M in singleplex and multiplex qPCR format.

## Comparison of singleplex and multiplex gene expression experiment

It's important to validate that a multiplex gene expression experiment provides similar results to singleplex experiments before performing any sample comparisons. To do this, we performed serial dilutions of the colon reference cDNA. Reactions ( $n = 4$ ) were run over four logs

for each assay in singleplex and 4-plex format. The TaqMan® Multiplex PCR Optimization guide provides guidance on how to evaluate data such as  $C_t$  values, PCR efficiency, coefficient of correlation, and standard deviation.



	PCR efficiency		Coefficient of correlation ( $R^2$ )	
	Singleplex	4-plex	Singleplex	4-plex
<b>FAM™-EGR1</b>	101.21%	104.18%	0.997	0.996
<b>VIC®-EP300</b>	92.57%	94.54%	0.996	0.994
<b>ABY®-B2M</b>	91.04%	91.54%	0.999	0.999
<b>JUN®-AKT2</b>	102.35%	103.38%	0.993	0.995

**Figure 6.** EGR1 assay using FAM™ dye, EP300 assay using VIC® dye, B2M assay using ABY® dye, and AKT2 assay using JUN® assay were analyzed in singleplex and 4-plex reactions. Amplification was performed using a serial dilution of reference colon cDNA from 20,000 pg to 2 pg per 10  $\mu$ L reaction. In all four amplification plots, blue refers to 4-plex reactions and red represents singleplex reactions. The table summarizes the PCR efficiency and coefficient of correlation for each assay. Data for other multiplex experiments are not shown.

TaqMan® Multiplex Master Mix was used for amplification of both the single-target and multiple-target reactions. Following the TaqMan® Multiplex Master Mix User Guide, 15 ng of each cDNA was used in a 10  $\mu$ L reaction in a 384-well plate. Thermal cycling parameters were 95°C (20 s) followed by 40 cycles of 95°C (5 s) and 60°C (40 s). Real-time PCR data was collected using a QuantStudio® 7 Flex system. Three 4-plex reactions were performed: 4-plex 1 contained *AKT2* (FAM™ dye), *MALT1* (VIC® dye), *B2M* (ABY® dye), and *CFLAT* (JUN® dye) assays; 4-plex 2 contained *EGR1* (FAM™ dye), *EP300* (VIC® dye), *B2M* (ABY® dye), and *AKT2* (JUN® dye) assays; 4-plex 3 contained *EGFR* (FAM™ dye), *MYC* (VIC® dye), *B2M* (ABY® dye), and *BAX* (JUN® dye). *AKT2* gene assay was tested with two different dyes: FAM™ dye (4-plex 1) and ABY® dye (4-plex 2).

We calculated the average  $C_t$  values ( $n = 4$ ) for each assay in single reactions and 4-plex reactions in both samples (Figure 4). The correlation between the singleplex and multiplex measurements is evident; there was little or no significant difference between the two different measurement strategies. Note that a total of 135 ng of cDNA was needed for the nine singleplex reactions, while only 45 ng was required for the three multiplex reactions.

Next, the average  $C_t$  for each assay-sample combination was used to determine the  $\Delta C_t$  between the target gene and beta-2 microglobulin (*B2M*) control gene to normalize for RNA input amount. Finally, to measure the fold change between the SW620 cells and normal colon cells, the  $\Delta\Delta C_t$  was calculated from the  $\Delta C_t$  of the sample minus the  $\Delta C_t$  of the reference for each assay (Figure 5). One gene, *EGFR*, was down-regulated [71-fold] while seven genes were

up-regulated (*EGR1*: 362-fold, *EP300*: 21-fold, *AKT2*: 16-fold, *MYC*: 51-fold, *BAX*: 11-fold, *MALT1*: 21-fold, and *CFLAR*: 3-fold) and all were in concordance with singleplex results (see side bar, Figure 6). Interestingly, these eight genes are known to play roles in colon cancer [3–10].

Together, these results demonstrate that (1) multiplex PCR is as accurate at measuring differences in transcript levels as singleplex PCR, (2) sample *can* be preserved by performing a multiplex reaction, since the differences in transcript abundance measured by multiplex PCR were identical to traditional singleplex PCR, but with one quarter of the cDNA input amount, and (3) multiplex PCR is an efficient way to analyze mRNA levels from precious samples.

### Conclusion

Some of the challenges facing researchers are related to obtaining as much genetic information as possible from a small amount of sample. Here, we demonstrate that a new multiplex PCR solution can alleviate some of those challenges. We have shown that multiplex qPCR is as accurate as traditional singleplex or duplex qPCR in two different applications commonly used by research scientists. We have also shown that multiplex qPCR makes more efficient use of samples than singleplex PCR, because the same sample can be efficiently queried with more than one assay at a time. Finally, the same information can be obtained without setting up multiple single-assay reactions, saving time and materials that can reduce the cost associated with complex projects.

### References

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## Ordering information

Product	Cat. No.
Cell line SW620	ATCC®: CCL-227™
Cell line Panc 03.27	ATCC®: CRL-2549™
Cell line MDA-MB-468	ATCC®: HTB-132™
Human Colon Total RNA	AM7986
TaqMan® Control Genomic DNA®	4312660
TaqMan® Multiplex Master Mix (5 mL)	4461882
SuperScript® VILO™ cDNA Synthesis Kit (50 reactions)	11754050
TaqMan® OpenArray® Real-Time PCR Master Mix (5 mL)	4462164
TaqMan® Genotyping Master Mix (400 reactions)	4371355
TaqMan® OpenArray® Human Signal Transduction Panel, QuantStudio® 12K Flex	4475392
QuantStudio® 12K Flex System with OpenArray™ Block (with AccuFill™ System)	4471090
QuantStudio® 7 Flex Real-Time PCR System, 96-well, desktop	4485690
MicroAmp® Optical 96-well Reaction Plate with Barcode (20 plates)	4306737
MicroAmp® Optical Adhesive Film (100 covers)	4311971

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