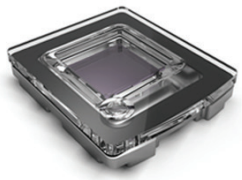
Detection of the TMPRSS2:ERG fusion transcript

Optimized workflow with TaqMan Assays and digital PCR

Current biomedical research aims at personalized treatments in the future for various cancers, for example, prostate cancer. Prostate-specific antigen (PSA) is a well-established biomarker for early detection of prostate cancer, but it has substantial false-positive and false-negative rates [1]. More recently, the *TMPRSS2:ERG* gene fusion was discovered in most prostate cancers. Due to the high prevalence of the *TMPRSS2:ERG* gene fusion, which occurs in more than 50% of prostate cancer cases [2], this gene fusion appears to be a suitable biomarker for monitoring prostate cancer. Furthermore, it can be detected in less-invasive sample material such as blood and urine. Research is presently focused on the development of benchtop methodologies that detect biomarkers quickly, simply, and sensitively in order to initiate therapeutic measures correctly and in a timely manner. Use of digital PCR is currently being used as a highly sensitive method for reproducible and robust measurements without the use of standard curves. The Applied Biosystems™ QuantStudio™ 3D Digital PCR System has a high point-of-care potential in the future due to its small footprint and easy workflow.

The aim of this project was to establish a system for the detection of the TMPRSS2:ERG fusion transcript on the QuantStudio 3D Digital PCR platform. A predesigned Applied Biosystems™ TaqMan® fusion assay and a standard TaqMan® Gene Expression Assay were used to detect mutant and wild-type alleles. Assay performance was



optimized by adjusting cycling conditions. RNA isolated from fusion-positive VCaP and fusion-negative LNCaP cell lines were used as reference material. In wet-lab experiments, it was possible to detect TMPRSS2:ERG transcripts with a detection limit of 0.05% fusion rate in a correspondingly high wild-type background. Spike-in experiments allowed the confirmation of an easy workflow. This is the first step in examining the potential of the assay as a noninvasive molecular test to monitor prostate cancer in the future.

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Several isoforms of the fusion transcript have been detected in prostate cancer (Figure 1). Different isoforms and their expression levels are suspected of having different effects on disease progression [3].

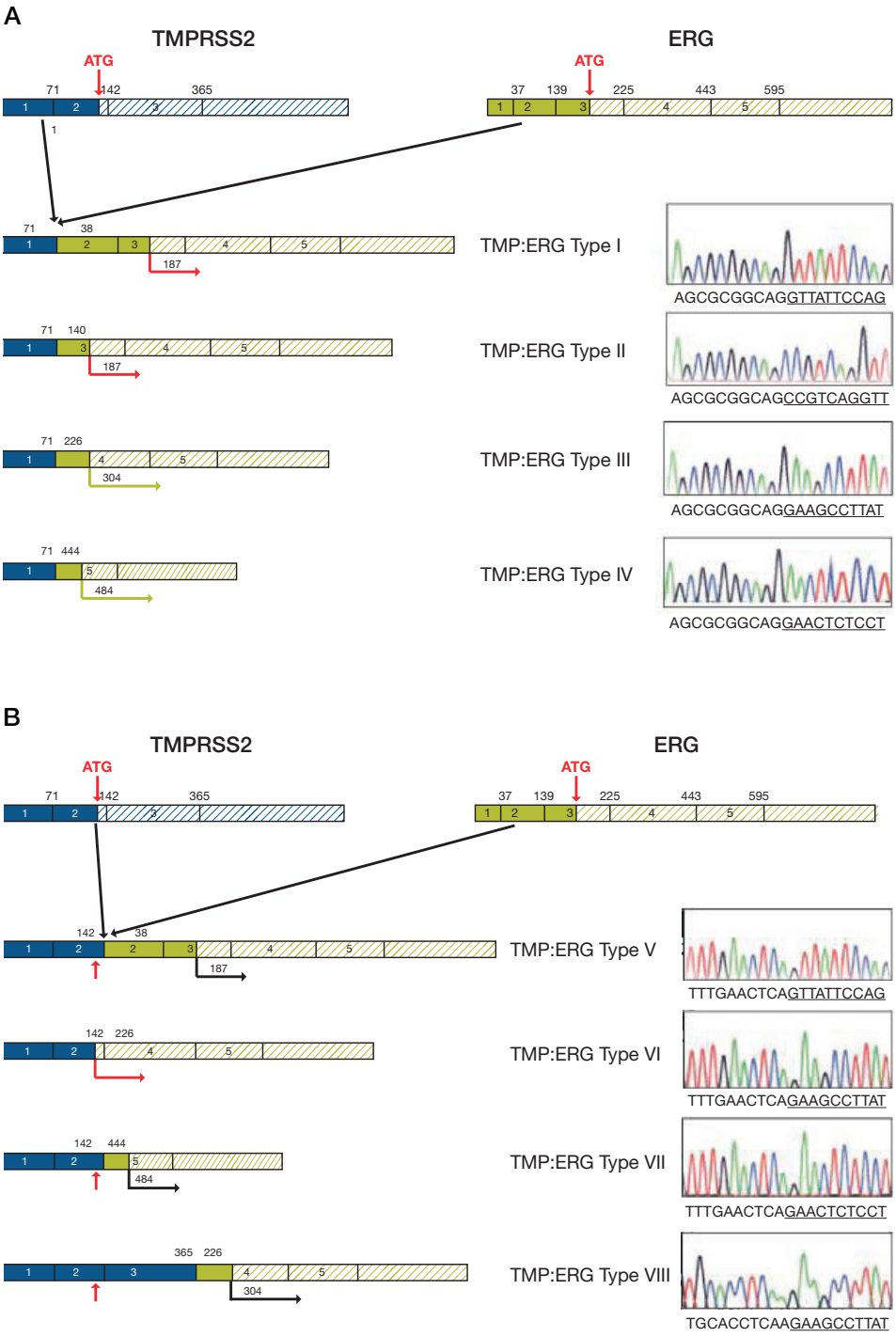

Figure 1. Fusion transcripts of the *TMPRSS2:ERG* gene. Chromosomal rearrangement leads to the *TMPRSS2:ERG* gene fusion that has a high known prevalence in prostate cancer research samples. Shown here are fusion transcripts detected in prostate cancer research samples. (**A**) Fusion involving only exon 1 of the *TMPRSS2* gene. (**B**) Fusion involving exons 1 and 2 of the *TMPRSS2* gene [3].

Materials and methods

Cell lines

Prostate carcinoma cell lines VCaP (heterozygous mutant for the *TMPRSS2:ERG* gene fusion) and LNCaP (homozygous wild-type) were used for the experiments. These cell lines have been previously used in biomarker research [5,6]. Mutation and wild-type status of these cell lines were verified by qPCR (data not shown). Cells were counted using an Invitrogen™ Countess™ Automated Cell Counter.

RNA isolation and transcription

The Qiagen™ RNeasy™ Mini Kit and QIAcube™ system were used for total RNA preparation. The protocol included an on-column DNase digest. RNA was quantitated on a Thermo Scientific™ NanoDrop™ 1000 spectrophotometer. RNA integrity was analyzed using an Agilent™ Bioanalyzer™ 2100 system and the Agilent™ RNA 6000 Nano Reagents Part I-kit according to the protocol. The Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit was used to generate cDNA.

Assay selection

For the real-time PCR experiments, a TaqMan fusion assay and standard TaqMan Gene Expression Assays for detection of the wild-type allele were ordered. The fusion assay carried an Applied Biosystems™ FAM™ dye label. The TaqMan Gene Expression Assays were used with an Applied Biosystems™ VIC™ dye label in order to facilitate dual detection of mutant and wild-type alleles. Details of the assay selection process are described in the results section.

Digital PCR

The QuantStudio 3D Digital PCR System was used for digital PCR. Each chip contained 0.1–6 ng of cDNA, dissolved in 14.5 µL reaction volume and cycled on an Applied Biosystems™ GeneAmp™ PCR System 9700. Cycling conditions are outlined in the results section.

Optimizing cycling conditions for digital PCR

Thermal cycling was performed on the GeneAmp PCR System 9700. Two cycling protocols were tested: the digital PCR protocol and the qPCR protocol.

Results

Steps in transcript detection

Evaluating the feasibility of fusion transcript detection in prostate cancer research samples involves three steps (Figure 2).

Figure 2. Overview of the steps for detecting the TMPRSS2:ERG fusion transcript in prostate cancer research samples with Applied Biosystems™ TaqMan® Assays on the QuantStudio 3D system. Step 1 is the selection of suitable assays to detect fusion and wild-type transcripts. Step 2 is optimizing experimental conditions to maximize VIC and FAM signals and achieve optimal cluster separation. Step 3 is determining the specificity and sensitivity of the assay.

Assay selection

Selection of suitable assays is critical for detecting the mutant and wild-type alleles. The *TMPRSS2:ERG* gene fusion is detected at the transcript level and calls for the use of a TaqMan fusion assay. The corresponding wild-type transcript is detected with a standard TaqMan Gene Expression Assay that was selected from a wide range of predesigned assays. Placement of the assay detecting the wild-type allele is crucial. The two options result in different outcomes for the signal (Figure 3).

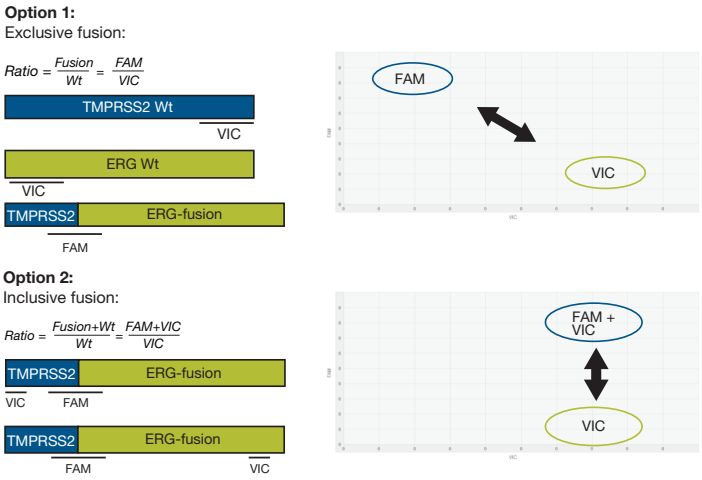

Figure 3. Considerations for assay selection. Two options were considered for the placement of the assay detecting the wild-type allele: exclusive fusion (top) and inclusive fusion (bottom).

In option 1 (exclusive fusion), the two alleles are detected on different transcripts. The FAM label detects the mutant allele only, and the VIC label detects the wild-type allele only. This option yields maximum separation of the FAM and VIC clusters. In this option, the VIC-labeled probe detects the wild-type allele in the 5′ region of the ERG transcript, which is not part of the fusion transcript. Alternatively, the VIC probe may detect the wild-type allele in the 3′ region of the TMPRSS2 transcript, which again is not part of the fusion transcript.

In option 2 (inclusive fusion), the VIC probe detects the 5′ region of the TMPRSS2 transcript or the 3′ region of the ERG transcript, that is also detected by the FAM probe if the fusion is present. This results in mixed VIC and FAM signals, with inferior cluster separation.

We selected an assay using option 1 for detecting the wild-type allele in the 3′ region of the TMPRSS2 transcript.

Optimizing the digital PCR thermal cycling

For the final experiment, the assays were run as duplexes as outlined in Table 1. In order to develop the optimal performance, two assay combinations were tested for the duplex assay detecting mutant and wild-type alleles. The fusion assay (FAM label) was tested in combination with two different gene expression assays (VIC label), one detecting exons 7–8 of the *TMPRSS2* gene and the other detecting exons 13–14 of the *TMPRSS2* gene.

PCR protocols

Two PCR protocols were tested to get optimal cluster separation of the duplex assay (Tables 2 and 3): digital and real-time PCR protocols. The real-time PCR profile generated the better assay performance compared to that obtained from the digital PCR profile (Figure 4).

Table 1. Assay selection for detection of the TMPRSS2:ERG fusion and TMPRSS2 wild-type transcripts.

	Assay ID*	Gene symbol	Label	Exon(s)	Amplicon size	RefSeq ID
A	Hs01120965_m1	*TMPRSS2*	VIC	13–14	66 bp	NM_005656.3
B	Hs01122332_m1	*TMPRSS2*	VIC	7–8	79 bp	NM_005656.3
C	Hs03063375_ft	*TMPRSS2:ERG*	FAM	Fusion	106 bp	DQ204772.1

* Two alternative assays for wild-type allele detection (A and B) were each mixed with the fusion assay (C) in the duplex PCR detecting both alleles. The two duplex assays (A and C, and B and C) were used for digital PCR experiments

Table 2. Digital PCR temperature profile.

Phase	Hold	Cycles (39)		Hold	Hold
Step	DNA polymerase activation	Anneal/extend	Denature	Final extension	Storage
Temperature	96.0°C	60.0°C	98.0°C	60.0°C	10.0°C
Time (min)	10:00	02:00	00:30	02:00	99:59

Table 3. Real-time PCR temperature profile.

Phase	Hold	Hold	Cycles (39)		Hold
Step	UDG activation	DNA polymerase activation	Denature	Anneal/extend	Storage
Temperature	50.0°C	95.0°C	95.0°C	60.0°C	10.0°C
Time (min)	2:00	10:00	00:15	01:00	99:59

Initial experiments were performed with standard digital PCR conditions and analyzed in Applied Biosystems™ QuantStudio™ 3D AnalysisSuite™ Cloud Software. The clusters were separated under these conditions, but better separation was desired. An experiment with standard qPCR cycling conditions improved the separation from the non-amplified cluster and increased signal strength. The fusion/wt 7–8 assay run under standard qPCR cycling conditions showed superior performance over the other duplex assay and run conditions with clear distinction of the mutant and wild-type clusters (Figure 4).

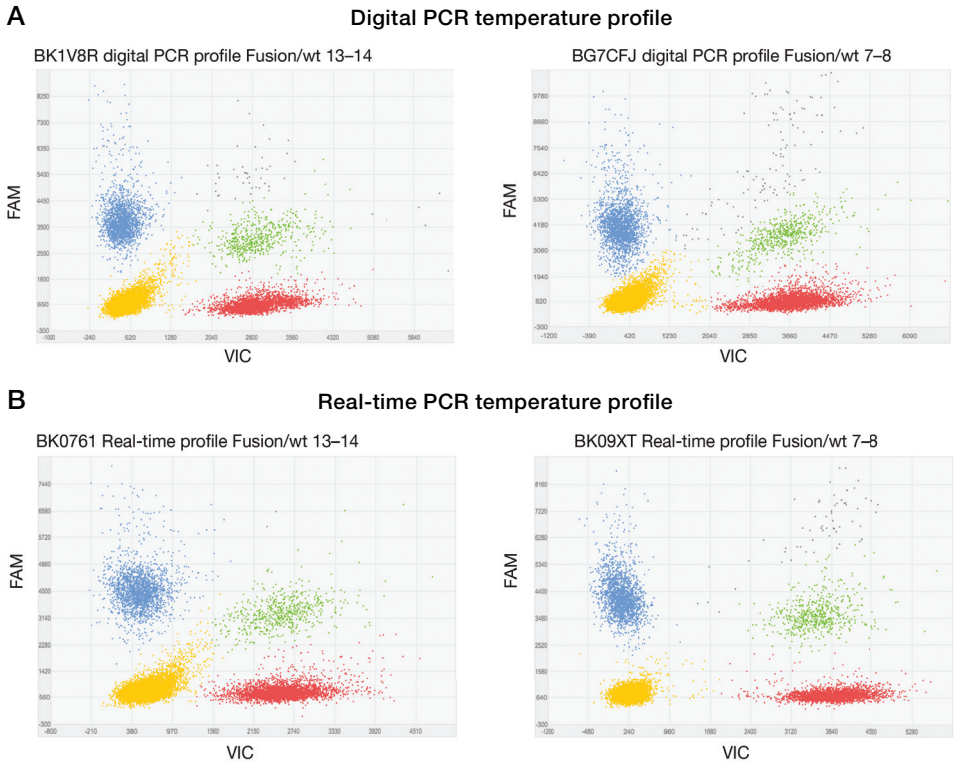

Figure 4. Two assay combinations tested with two thermal cycling protocols. Scatter plots comparing the temperature profiles of **(A)** digital PCR and **(B)** real-time PCR for VCaP cDNA (heterozygous for fusion and wild-type). The best cluster separation was achieved using the fusion/wild-type 7–8 assay combination and the real-time PCR temperature profile. Double-positive data points (mutant and wild-type) are shown in green. Plot scales are identical for easy comparison of signal intensities. 0.5 ng cDNA was loaded per chip.

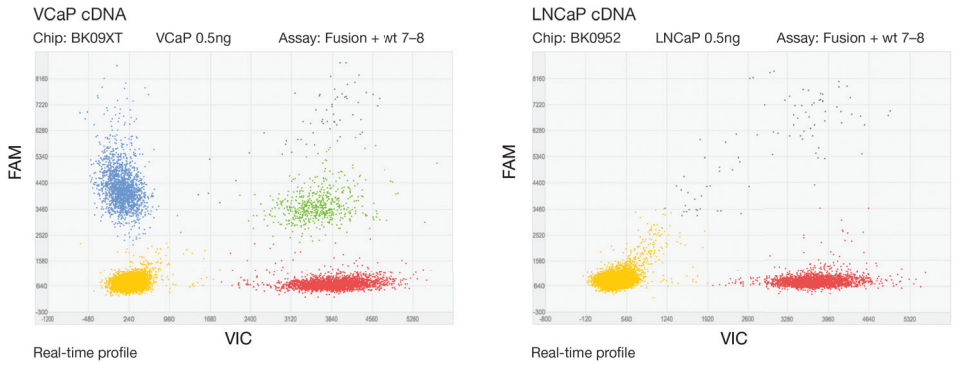

Figure 5. Comparison of fusion and wild-type signal in positive and negative controls. Scatter plots comparing signals of fusion-positive VCaP cDNA (left) and wild-type only LNCaP cDNA (right). The real-time PCR temperature profile was run using the fusion/wt 7–8 assay. Plot scales are identical.

Specificity and sensitivity

Assay specificity of individual and duplex assays was confirmed by qPCR (data not shown). The fusion assay did not amplify the LNCaP wild-type only cell line. The wild-type allele–specific assay showed a higher C_t for the heterozygous VCaP cell line than for the wild-type only LNCaP cell line, consistent with the assumption that the wild-type probe is specific. Specificity was confirmed for the duplex assay as the wild-type only LNCaP cell line was detected by the VIC-labeled probe only (Figure 5).

Assay sensitivity for the digital PCR experiment was assessed based on the guidelines of Armbruster and Pry [4]. VCaP cDNA containing the heterozygous fusion was spiked into wild-type LNCaP cDNA background. Wild-type TMPRSS2 and mutant TMPRSS2 fusion alleles were expected to have equal expression levels. 100% LNCaP cDNA served as a wild-type only control. All samples and controls were run in triplicate. FAM and VIC signal counts were obtained in QuantStudio 3D AnalysisSuite Cloud Software. The number of positive data points represents the number of transcripts (Figure 6).

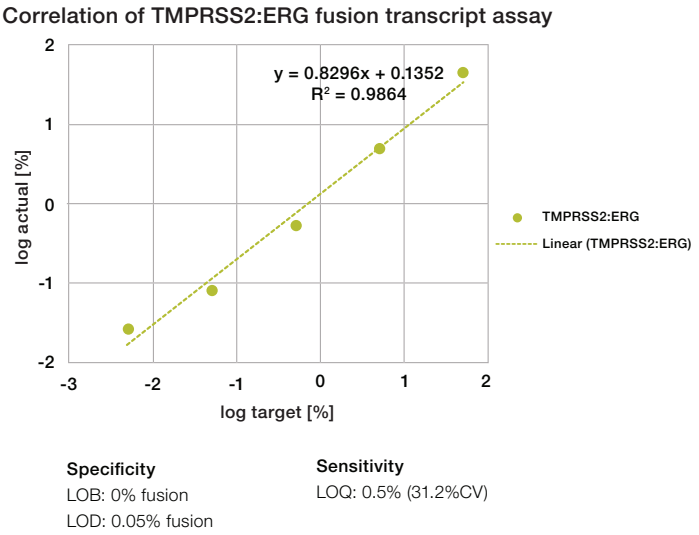

Figure 6. Determination of sensitivity and specificity. Serial dilutions were generated by mixing cDNA from LNCaP und VCaP cell lines to reflect a 0.005%–50% mutation rate. The values in the graph reflect the mean of triplicates. Limit of blank (LOB), limit of detection (LOD), and limit of quantitation (LOQ) were calculated according to the protocols by Armbruster and Pry [4]. Mean values and standard deviations of triplicates were used for calculations, and the coefficient of variation (CV) was calculated. Data points outside of the four clusters appeared on both wild-type and mutant-control chips and could be overlaid on the chips. They were considered nonspecific background and eliminated from further analysis by designating them as undefined. For the final duplex assay, we obtained a LOB = 0%, LOD = 0.05%, and an LOQ of 0.5% (target/total), set at 10 times above LOD, with a CV of 31.2% of the measured mutant allele (target quantification at LOD).

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Conclusions

This study demonstrates that off-the-shelf TaqMan gene expression and fusion assays may be run as a duplex assay to detect the TMPRSS2:ERG fusion transcript. The choice of digital PCR technology enabled detection of these transcripts at a LOD of 0.05% fusion in a correspondingly high wild-type background. These experiments demonstrated that the easy workflow can be used to quantify fusion transcripts and obtain rapid and reproducible results. Spike-in experiments allowed the confirmation of easy handling and the point-of-care potential of the method.

References:

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Ordering information

Product	Cat. No.
QuantStudio 3D Digital PCR System package—includes:**	**A25581†**
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