New Fusion Transcript Assay for the BCR-ABL Translocation

Applied Biosystems® TaqMan® Gene Expression Assays for Fusion Transcripts

HEMATOPATHOLOGY UNIT, HOSPITAL CLINIC, BARCELONA

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APPLICATION

Fusion transcript expression

TECHNOLOGIES

TaqMan® Gene Expression Assays for Fusion Transcripts

7900HT Fast Real-Time PCR System

Detecting Fusion Transcripts Caused by Chromosome Translocations

Chromosome translocations are abnormalities caused by rearrangement of chromosome sections between nonhomologous chromosomes. These rearrangements may result in chimeric genes that express fusion transcripts. Some of these transcripts can be translated into fusion proteins that affect normal regulatory pathways and stimulate abnormal cell growth. A well-known example is the *bcr-abl* chimeric mRNA (Philadelphia translocation), which is the result of a translocation of the abl gene on chromosome 9 to the *bcr* gene breakpoint cluster on chromosome 22.

In this study, new quantitative real-time PCR assays—Applied Biosystems® TaqMan® Gene Expression Assays for Fusion Transcripts, for detection of different bcr-abl transcripts (targeting p210 and p190 isoforms)—were compared to primers and probes for the same targets recommended and standardized by the Europe Against Cancer (EAC) Program and currently in widespread use [1–3]. The data presented here indicate that TaqMan® Gene Expression Assays for Fusion Transcripts have greater sensitivity and use an easier, ready-to-go workflow. The standardized assay format and protocol with an optimized master mix results in less variability in assay setup and allows laboratories to generate more reproducible data.

The Philadelphia Translocation (BCR-ABL Fusion Proteins)

BCR-ABL fusion proteins are associated with the formation of the Philadelphia translocation (Ph) and are one of the most common genetic abnormalities studied in blood cancer research. At the molecular level, the Ph chromosome, or t(9;22) (q34;q11) translocation, results from the fusion of the *bcr* gene (chromosome 22), which forms the 5' end of the fusion transcript, to the abl gene (chromosome 9), which forms the 3' end. In the vast majority of cases, the breakpoints in the *bcr* gene are found within three well-defined regions: the major breakpoint (M-bcr), minor breakpoint (m-bcr), and micro breakpoint (µ-bcr). Depending on which breakpoints are used, three main chimeric proteins of different sizes are generated (Table 1, Figure 1). These BCR-ABL chimeric proteins (p190, p210, p230) show increased, deregulated tyrosine kinase activity, which appears to deregulate normal cytokine-dependent signal transduction leading to inhibition of apoptosis, independent of growth factors.

Real-Time PCR Detects Translocations and Quantifies Expression

Current methods for identifying translocations include FISH and karyotyping, neither of which can be used to quantify the expression level of the fused gene as real-time PCR does. Real-time quantitative PCR can provide an appropriate monitoring strategy for analyzing BCR-ABL expression levels in the samples under study [5].

Table 1. *bcr-abl* Fusion Transcripts and Resulting Fusion Proteins.

Breakpoint Designation	Chrm 22 (*bcr* Gene) Break Location	Chrm 9 (abl Gene) Break Location	Variant Transcript Designation	Chimeric Protein Size (Name)
M-*bcr* (exons 12-16)	Intron 13	Intron 1	b2-a2 (e13-a2)	210 kDa (p210)
	Intron 14	Intron 1	b3-a2 (e14-a2)	" "
	Intron 13	Intron 2	b2-a3 (rare) (e13-a3)	" "
	Intron 14	Intron 2	b3-a3 (rare) (e14-a3)	" "
m-*bcr*	Intron 1	Intron 1	e1-a2	190 kDa (p190)
µ-*bcr* (rare)	Intron 19	Intron 1	e19-a2	230 kDa (p230)

Improved *bcr-abl* PCR Assays

Real-time PCR is the gold standard for quantitative measurement of nucleic acid. In collaboration with Applied Biosystems, EAC researchers developed primers and probes to detect specific *bcr*-abl fusion transcripts [1,2]. Recently Applied Biosystems has improved on these primer and probe designs, creating new TaqMan® Gene Expression Assays for all of the *bcr*-abl fusion transcripts (Table 3). Selected transcripts were annotated, and bases located at the fusion transcript breakpoint, known SNPs, and repetitive sequences were masked. TaqMan® minor groove binder (MGB) assays were then designed using the Applied Biosystems® bioinformatics design pipeline. The assays were designed such that the primers and probes bound on either side of the fusion transcript breakpoint (Figure 2), and each assay design was checked by in silico quality control.

TaqMan® Gene Expression Assays vs. EAC Assays

As proof of principle, the TaqMan® Gene Expression Assay designs were first tested using plasmids containing the translocation variant and human samples containing the translocation event. Amplification only occurred in samples containing the fusion transcript, confirming assay specificity (data not shown). Subsequently, researchers in the Hematopathology Unit of the Hospital Clinic in Barcelona used human samples to compare the Applied Biosystems® TaqMan® Gene Expression Assays for *bcr-abl* fusion transcripts and the EAC primer and probe designs [1,2]. (Note: The *bcr-abl* TaqMan® Gene Expression Assays include assays for several fusion transcripts for which there were no corresponding EAC designs.) The experimental procedure is provided in the sidebar, *Technical Details for Fusion Assays*. Results follow.

Detection of *bcr-abl* Fusion Transcript

Although it has been recommended to use a fixed threshold value using EAC designs (threshold 0.1), better results have been obtained using TaqMan® Gene Expression Assays with Automatic Analysis provided by the 7900HT Fast Real-Time PCR System. Standard curves generated by amplification of dilutions of each of the fusion transcripts (cloned into Ipsogen and pCR2.1 plasmid vectors) using both TaqMan® Gene Expression Assays and the ABL endogenous control TaqMan® Assay were very reproducible. PCR efficiencies were close to 100% with R2>0.99 (Figure 3).

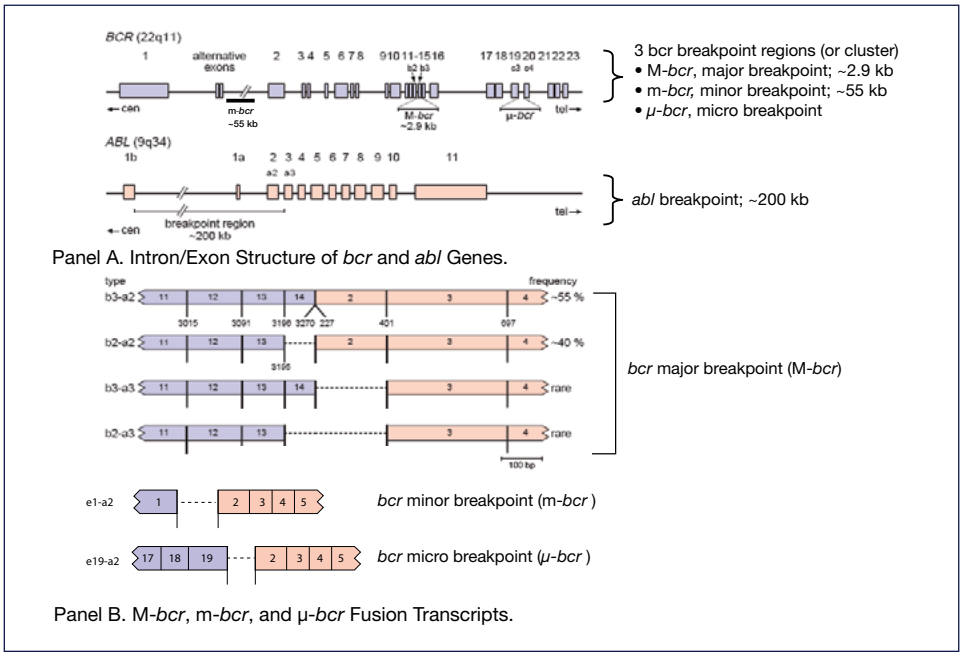


Figure 1. *bcr-abl* Chromosomal Breakpoints and Fusion Gene Transcripts. (A) Schematic diagram of the exon/intron structure of the *bcr* and *abl* genes involved in t(9;22) (q34;q11). The centromere (cen) and telomere (tel) orientation, exon numbering, and relevant breakpoint regions are indicated, including the micro breakpoint cluster region (µ-*bcr*). **(B)** Schematic diagram of *bcr-abl* major (M), minor (m), and micro (µ) transcripts. For M-*bcr*, the b3-a2 and b2-a2 transcripts are found most frequently, but sporadic cases with b3-a3 and b2-a3 transcripts have been reported. (Parts of this figure are used with permission from *Leukemia*.)

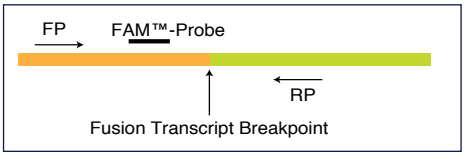


Figure 2. Design for TaqMan® Gene Expression Assays for Fusion Transcripts. Design of primer and probe binding locations [2]. Approximately 10 bp surrounding the breakpoint were masked to avoid designing the probe across this region, since the precise sequence around the breakpoint can be ambiguous. Thus, probes did not span transcript breakpoints. FP = forward primer; RP = reverse primer.

M-bcr and *m-bcr* Analysis

M-*bcr* and m-*bcr* fusion transcript quantification results were generated using the Ipsogen plasmid vector and the pCR2.1 plasmid vector (this second vector has only been used for M-bcr). A statistical study demonstrated that quantitative results obtained for M-*bcr* and m-*bcr* fusion transcripts with TaqMan® Gene Expression Assays for Fusion Transcripts were identical to those obtained with EAC assays for all 20 samples analyzed, independent of the plasmid vector used (Ipsogen or pCR2.1 plasmid vector) (Figure 4 and Figure 5).

To check the sensitivity of TaqMan® Gene Expression Assays compared with the EAC assays, samples amplified with TaqMan® Gene Expression Assays were reanalyzed using a fixed threshold value of 0.1. For M-*bcr*, the Ct values obtained with TaqMan® Gene Expression Assays were 0.49 to 1.97 lower (mean: 0.85) than Ct values obtained using EAC assays (Figure 6, Panel A). For m-*bcr*, the Ct values obtained with TaqMan® Gene Expression Assays were 1.76 to 2.53 (mean: 2.22) lower than Ct values obtained using the EAC assays (Figure 6, Panel B). This analysis demonstrates the greater sensitivity of TaqMan® Gene Expression Assays versus the EAC assays. Besides, quantitative results obtained with the fixed threshold value for both M-*bcr* and m-*bcr* translocations were almost identical to those obtained with the 7900HT Fast Real-Time PCR System's Automatic Analysis. The greater sensitivity achieved with TaqMan® Gene Expression Assays has also made possible the detection of the m-*bcr* fusion transcript in a sample that previously could not be amplified using the EAC probe and primers (data not shown).

Analysis of *abl* Expression

The expression levels of the abl endogenous control obtained with TaqMan® Assay Hs99999002_mH were higher than those obtained using the EAC designs in all 20 samples (on average 1.7 times higher). Samples were analyzed independently with the Ipsogen and the pCR2.1 plasmid vector (Figure 7).

Technical Details for Fusion Assays

Samples. Peripheral blood samples were a subset of routine samples collected by the Hematopathology Unit of the hospital, and were kept anonymous. Informed consent was obtained in accordance with the Institutional Ethics Committee of the Hospital Clinic (Barcelona, Spain) and the Helsinki declaration.

RNA and cDNA Preparation. Dra Colomer and colleagues used 10 human samples each for analysis of M-*bcr* and m-*bcr* expression. Leukocytes from BCR-ABL–positive peripheral blood samples were isolated by 2% dextran sedimentation. Total RNA was extracted from the leukocytes using TRIzol® Reagent (Invitrogen) following the manufacturer's instructions. Total RNA (1 µg; quantified by Nanodrop technology) was reverse transcribed into cDNA (50 µL reactions) using random primers and M-MLV reverse transcriptase (Invitrogen), following the protocol published by the EAC Consortium [2] (Table 2).

Fusion Transcript Assays. M-*brc* (b2-a2, b3-a2; TaqMan® Gene Expression Assay Hs030024541_ft) and m-*bcr* (e1-a2; TaqMan® Gene Expression Assay Hs03024844_ft) fusion transcript quantification was performed in two different reactions using both TaqMan® Gene Expression Assays and the EAC fusion transcript primers and probes. The Abelson (ABL) TaqMan® Gene Expression Assay (Hs99999002_mH) and EAC *abl* primers and probes were used together to amplify the endogenous control.

Quantitative real-time PCR (qPCR) for *bcr-abl* and *abl* control transcripts was performed in duplicate on the Applied Biosystems® 7900HT Fast Real-Time PCR System using standard run conditions. Reactions (25 µL) included TaqMan® Gene Expression Master Mix (Applied Biosystems) and cDNA (2 µL). A known positive and negative control were amplified for each assay.

Table 2. EAC Reverse Transcription Protocol.

Incubate 1 µg total RNA in 10 µL H_2O at 70°C for 10 min
Cool on ice and add other reagents to a final volume of 20 µL
Reverse transcriptase (either M-MLV or SuperScript® I or II): 100 U
RT buffer (according to the RTase used)
dNTP: 1 mM
DTT: 10 mM
Random hexamers: 25 µM
RNase inhibitor: 20 U
Incubate subsequently at:
Room temperature for 10 min
42ºC for 45 min
99ºC for 3 min
Place the sample at 4ºC
Dilute the final cDNA with 30 µL of H_2O

Data Analysis. For analysis of both the M-*bcr* breakpoint and *abl* endogenous control gene data, standard curves were created using the Ipsogen plasmid vector (Ipsogen) and/or the pCR2.1 T0P0® + *bcr-abl* plasmid vector [3]. For analysis of the m-*bcr* breakpoint data, standard curves were created using the Ipsogen plasmid vector. Detection of the rare M-*bcr* b3-a3 transcript was tested with a TaqMan® Gene Expression Assay (Hs 03043652_ft) on M-*bcr* b3-a3 positive samples using the protocol described above. No plasmid was available for this last fusion transcript.

Analysis of Rare Fusion Transcript Forms

A TaqMan® Gene Expression Assay (Hs03024652_ft) was able to detect the rare M-*bcr* b3-a3 transcript in human M-*bcr* b3-a3 positive samples. Figure 8 shows a time course of b3-a3 M-*bcr* transcript expression in the positive samples taken at time points out to one year, when the rare transcript was not longer detected. EAC designs were not available for detection of this transcript.

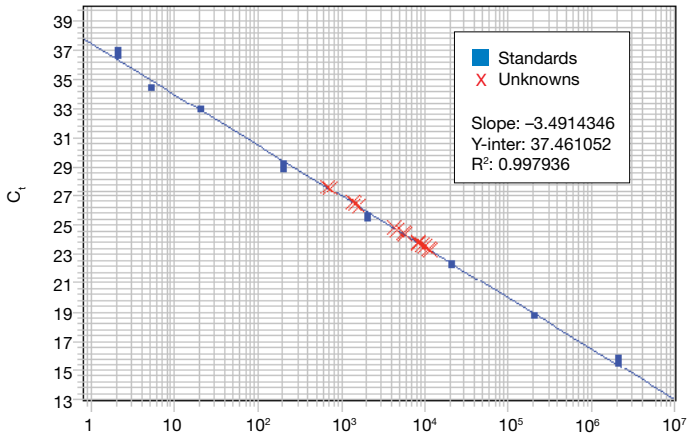
Ready-to-Use TaqMan® Assays Provide Many Advantages for Detecting Fusion Transcripts

Applied Biosystems has recently released a novel set of 165 TaqMan® Gene Expression Assays for quantitation of human fusion

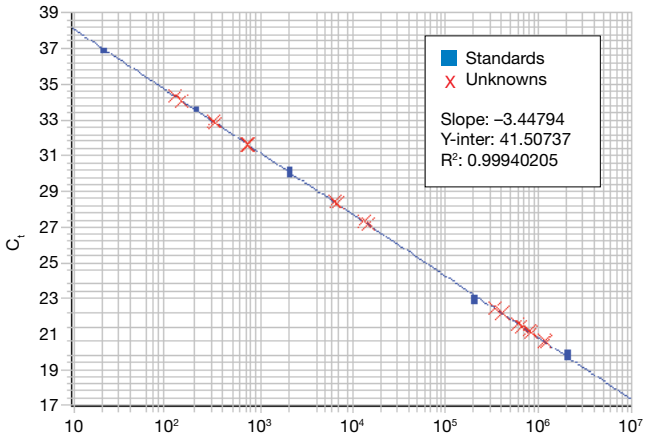
transcripts. These assays were developed with Applied Biosystems' validated bioinformatics pipeline used to design the 1.1 million TaqMan® Gene Expression Assays currently available. As with other TaqMan® Gene Expression Assays, the fusion transcript assays undergo a synthesis quality control test using mass spectrometry to verify primer and probe sequence and concentration.

In this study, Dra Colomer and colleagues use TaqMan® Gene Expression Assays for M-*bcr* (Hs030024541_ft) and m-*bcr* (Hs03024844_ft) to detect and quantify bcr-abl fusion transcripts. The TaqMan® Assays were able to detect M-*bcr* and m-*bcr* transcripts in the same samples as EAC probe and primers designed to these targets. However, the TaqMan® Gene Expression Assays provided more sensitivity, yielding transcript amplification in samples not amplifiable with the EAC designs. In addition, the single-tube 20X format of TaqMan® Gene Expression Assays and associated Applied Biosystems® reagents made the assays easier to process, and saved valuable time. "When used with the EAC program's standardized protocol, these TaqMan® Gene Expression Assays would help eliminate much of the variability seen across different laboratories due to individual primer and probe preparation protocols. Having standardized reverse transcription quantitative PCR (RT-qPCR) assays would harmonize the current technology for detecting *bcr-abl* transcripts, saving time and providing more reproducibility in results," notes Dra Colomer.

Slightly better results have been achieved using the automatic threshold software on the 7900HT Fast Real-Time PCR System to analyze results obtained with TaqMan® Gene Expression Assays. However, as it is recommended to use a fixed threshold value with the EAC primer and probe designs (threshold = 0.1), the data for all samples was reanalyzed using this fixed threshold. Under these conditions, TaqMan® Gene Expression Assays were shown to be more sensitive, allowing the amplification of a sample that was negative using EAC primer and probe designs.

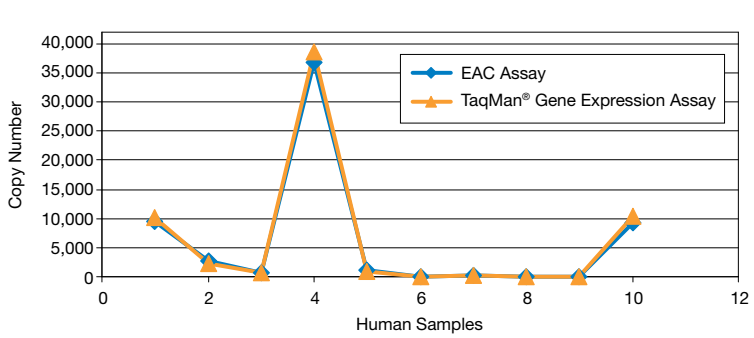


Panel A. M-*bcr* (Hs 030024541_ft) Standard Curve With pCR2.1 Plasmid Vector.

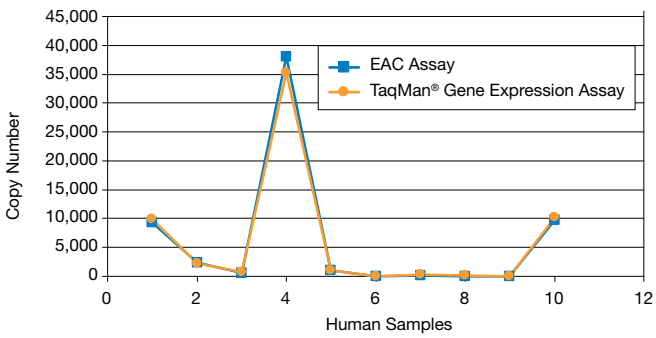


Panel B. m-*bcr* (Hs 03024844_ft) Standard Curve With Ipsogen Plasmid Vector.

Figure 3. Standard Curves Obtained Using TaqMan® Gene Expression Assays. (A) Two replicates of each of eight dilutions (2×10^6, 2×10^5, 2×10^4, 2×10^3, 2×10^2, 2×10, 5, and 2 copies/µL) of the M-*bcr* breakpoint cloned into the pCR2.1 plasmid vector were amplified, using 2 µL of each dilution. **(B)** Two replicates of each of five dilutions (2×10^6, 2×10^5, 2×10^4, 2×10^3, and 2×10 copies/µL) of the m-*bcr* breakpoint cloned into the Ipsogen plasmid vector were amplified using 2 µL of each dilution.



Panel A. M-*bcr* samples analyzed with pCR2.1 plasmid vector.



Panel B. M-*bcr* samples analyzed with Ipsogen plasmid vector.

Figure 4. M-*bcr* Copy Number Using TaqMan® Gene Expression Assays vs. EAC Assay. Copy number analysis was performed using TaqMan® Gene Expression Assay (Hs 030024541_ft) and the comparable EAC assay with either **(A)** pCR2.1 plasmid vector or **(B)** Ipsogen plasmid vector. The TaqMan® Gene Expression Assay gave the same results as the EAC assay in both cases.

The TaqMan® Assays were also able to successfully detect the rare M-*bcr* transcript b3-a3 and the µ-*bcr* transcript e19-a2 in human samples (data not shown).

It is especially important to note that these particular assays provide new tools to researchers, since there are no comparable EAC probe and primer designs for these specific translocations.

Applied Biosystems provides researchers a standardized, easy-to-use workflow to quantify the different bcr-abl fusion transcripts. This workflow makes it possible to obtain rapid and reproducible results within and between laboratories.

Acknowledgments

We acknowledge Beatriz Cabot, Applied Biosystems Senior Field Application Specialist, Molecular Biology, and Marisa Checa, Applied Biosystems Field Application Specialist, Molecular Biology, for their generous contribution in this collaboration.

References

1. p190 primers and probe: ENF401, ENR561, ENF541; p210 primers and probe: ENF501, ENR561, ENP541.

2. Gabert J, Beillard E, van der Velden VH et al. (2003) Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia—A Europe Against Cancer Program. *Leukemia* 17:2318–2357.

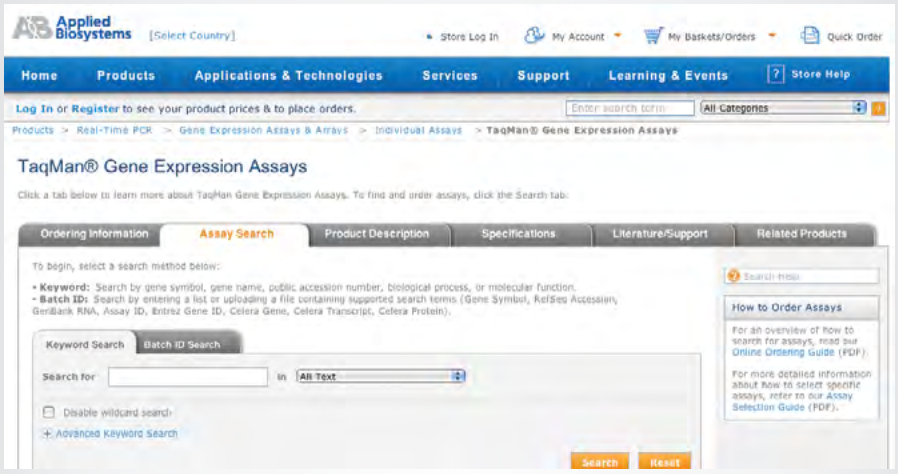
3. Beillard E, Pallisgaard N, Bi W et al. (2003) Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR)—A Europe Against Cancer Program. *Leukemia* 17:2474–2486.

4. Hughes T, Deininger M, Hochhaus A , et. al. (2006) *Blood* (ASH Annual Meeting Abstracts),108:28–371.

5. Melo JV (1996) The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype. *Blood* 88:2375–2384.

Simplifying Fusion Transcript Detection and Quantitation With Better Tools TaqMan® Gene Expression Assays for Fusion Transcripts

TaqMan® Gene Expression Assay products enable researchers to conduct fusion studies quickly and easily by eliminating the time-consuming processes involved in assay development. As all of the assays are ready to use (primer and probes formulated in single-tube, 20X mix), it is easier to set up the reaction and to compare results with other researchers and labs directly and accurately (see Table 3). See Tables 3 and 4 and page 8 for ordering information.



You can search online for the assay you need at **info.appliedbiosystems.com/gexassays.**

Table 3. *bcr-abl* and Endogenous Control TaqMan® Gene Expression Assays.

bcr-abl TaqMan® Gene Expression Assays		
Assay ID	**Transcript**	**Assay Accession No.**
Hs03024541_ft	b2-a2	AJ131467.1
Hs03024541_ft	b3-a2	AJ131466.1
Hs03024844_ft	e1-a2	AF113911.1
Hs03205538_ft	e19-a2	AM491363.1
Hs03043652_ft	b3-a3	AM491360.1
Hs03043652_ft	b3-a2	AJ131466.1
Hs03024646_ft	b2-a3	AY043457.1
Hs03024646_ft	b3-a3	AM491360.1
Hs03024646_ft	b2-a2	AJ131467.1
Endogenous Control TaqMan® Gene Expression Assay		
Assay ID	Assay Accession No.	
Abelson (ABL)	Hs 99999002_mH	

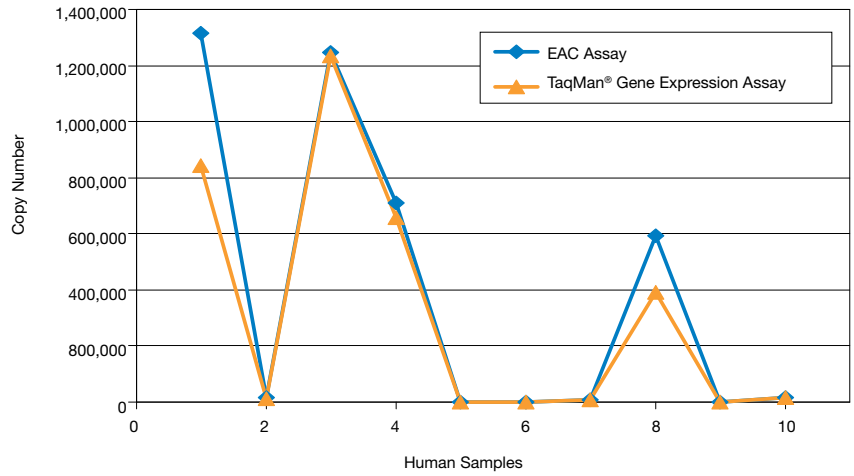
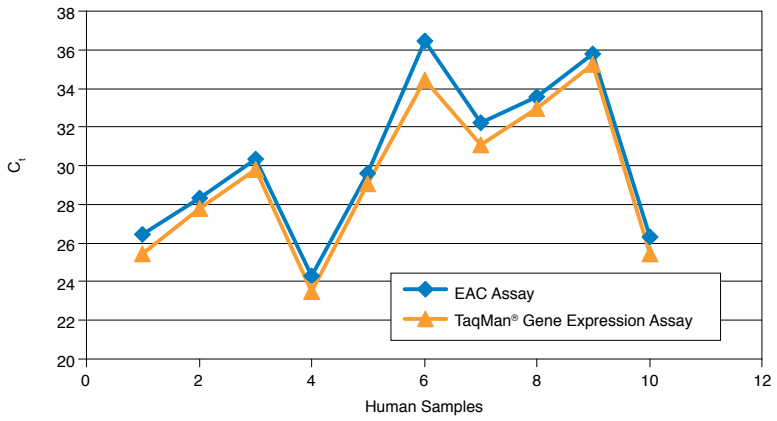
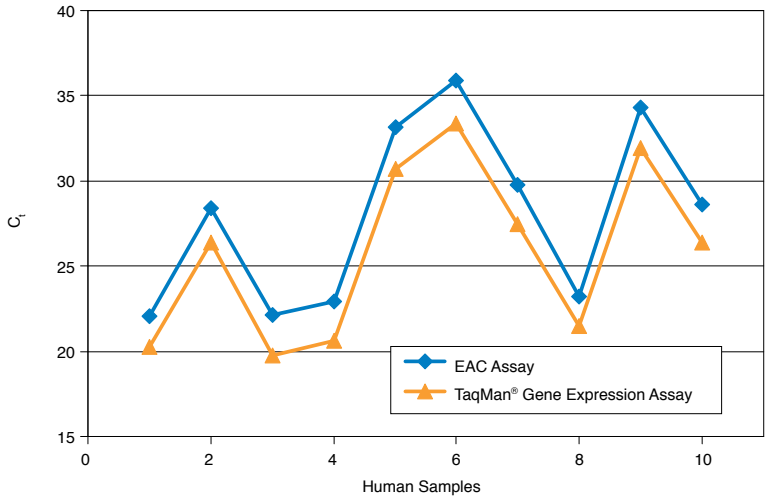


Figure 5. m-*bcr* Copy Number Using TaqMan® Gene Expression Assay vs. EAC Assay. Copy number analysis was performed using TaqMan® Gene Expression Assay Hs03024844_ft and the comparable EAC assay with the Ipsogen plasmid vector. The TaqMan® Gene Expression Assay gave the same results as the EAC assay.



Panel A. M-*bcr* Samples.



Panel B. m-*bcr* Samples.

Figure 6. C_t Values for M-*bcr* Using TaqMan® Gene Expression Assays vs. EAC Assays. Reanalysis of M-*bcr* samples (**A:** TaqMan® Gene Expression Assay Hs 030024541_ft and comparable EAC assay using a fixed 0.1 threshold value) and of m-*bcr* samples (**B:** TaqMan® Gene Expression Assay Hs03024844_ft and comparable EAC assay). The TaqMan® Gene Expression Assays were consistently more sensitive than the EAC assays.

Applied Biosystems® TaqMan® Gene Expression Master Mix

The TaqMan® Gene Expression Assay workflow can be further simplified by incorporating Applied Biosystems® TaqMan® Gene Expression Master Mix. The Gene Expression Master Mix comes concentrated with all needed reagents premixed, decreasing hands-on time for dilution, mixing, and pipetting. TaqMan® Gene Expression Master Mix delivers sensitive and specific detection across a broad range of template quantities, down to a single copy of target. For ease of use, TaqMan® Gene Expression Master Mix uses universal thermal cycling conditions and users can set up the reaction at room temperature. See page 8 for ordering information.

LeukoLOCK™ Total RNA Isolation System

The LeukoLOCK™ Total RNA Isolation System is an innovative method for cellular fractionation of whole blood, and total RNA stabilization and extraction from the leukocyte population. It has been optimized for use with human blood. Blood is a storehouse of cellular information; however, the presence of globin mRNA in RNA prepared from whole blood can interfere with downstream expression profiling applications. The LeukoLOCK™ System employs filter-based leukocyte-depletion technology to isolate leukocytes from whole blood, and Ambion® RNA*later*® to stabilize the cells on the filter. By excluding red blood cells, the RNA that is purified from captured leukocytes is inherently depleted of globin mRNA, which improves sample utility for expression profiling and other applications. See page 8 for ordering information.

High Capacity cDNA Reverse Transcription Kit

The High Capacity cDNA Reverse Transcription Kit delivers extremely high-quality, single-stranded cDNA from total RNA. It contains all components necessary for the quantitative conversion of 0.02 to 2 µg total RNA in a single 20 µL reaction to single-stranded cDNA. Downstream applications include real-time PCR, standard PCR, and microarrays. See page 8 for ordering information.

Custom Plating Service

The TaqMan® Custom Plating Service offers the convenience of pre-plated TaqMan® Gene Expression Assays, Custom Assays, and Custom Probe/Primer Sets in 96- or 384-well plates. Set up custom configurations using TaqMan® Gene Expression Assays (Inventoried, Made-to-Order, and Custom) and Custom TaqMan® Probes and Primers. You can select from a variety of reaction volumes and receive assays in dried or liquid formulation.

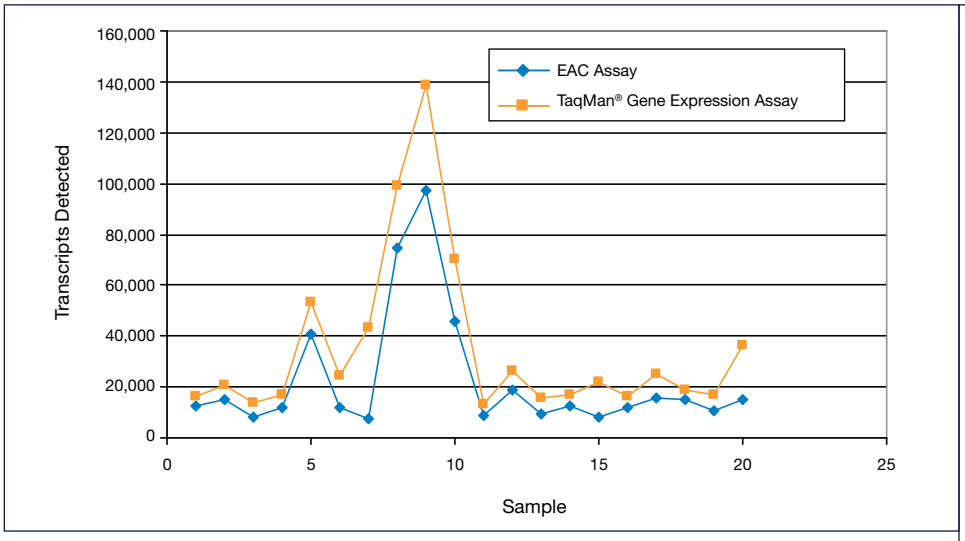


Figure 7. Better Detection of *abl* Expression Levels Using TaqMan® Gene Expression Assays. Samples (20) were amplified using probe and primer designs for the abl endogenous control transcript. Higher *abl* expression levels were detected with the TaqMan® Gene Expression Assay Hs99999002_mH than with the EAC designs (pCR2.1 plasmid vector). The same data was obtained using the Ipsogen plasmid (data not shown).

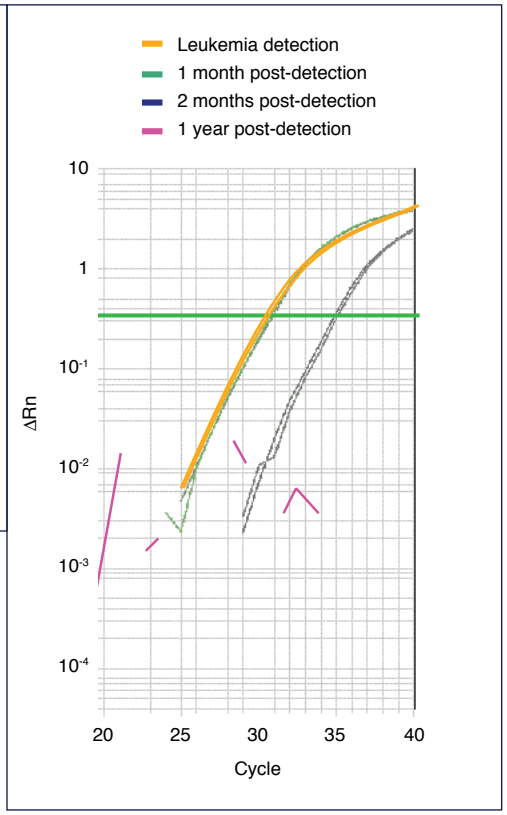


Figure 8. TaqMan® Assays Make Possible Detection of Rare M-*bcr* Transcript. Detection of the rare b3-a3 M-*bcr* translocation in a positive sample, over a time course out to 1 year. Amplifications were performed using the TaqMan® Gene Expression Assay Hs03024652_ft.

Ordering Information

Description	Size	Part Number
LeukoLOCK™ Total RNA Isolation System	20 rxn*	AM1923
High Capacity cDNA Reverse Transcription Kit	200 rxn*	4368814
TaqMan® Gene Expression Assays for Fusion Transcripts		
Inventoried	250 rxn	4331182†
Made-to-Order	360 rxn	4351372
TaqMan® Gene Expression Master Mix, 1 Mini-Pack (1 x 1 mL)	40 rxn*	4370048
TaqMan® Universal PCR Master Mix, 1-Pack (1 x 5 mL)	200 rxn*	4304437
7900HT Fast Real-Time PCR System with Standard 96-well Block Module	1 instrument	4329003

*Available in other sizes or in bundles.

†See list below for specific assays.

TaqMan® Gene Expression Assays for *bcr-abl* Fusion Transcripts

Assay ID	Transcript	Assay Accession No.
Hs03024541_ft	b2-a2	AJ131467.1
Hs03024541_ft	b3-a2	AJ131466.1
Hs03024844_ft	e1-a2	AF113911.1
Hs03205538_ft	e19-a2	AM491363.1
Hs03043652_ft	b3-a3	AM491360.1
Hs03043652_ft	b3-a2	AJ131466.1

Endogenous Control TaqMan® Gene Expression Assay

Assay ID	Assay Accession No.
Abelson (ABL)	Hs 99999002_mH

ThermoFisher SCIENTIFIC