Real-Time RT-PCR Protocol

Total RNA was purified from the GITC lysates using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Following extraction, the RNA preparation was precipitated with sodium acetate/ethanol using RNase-free glycogen as a non-specific carrier. RNA samples were treated with DNA-free™ (Ambion, Austin, TX, USA) to ensure that all DNA had been removed. The RNA preparations were quantified and purity assessed using spectrophotometry and electrophoresis.

Primers directed against the human sequence of the genes listed in Table 1 were designed using Primer Express® software v1.0 (Applied Biosystems, Foster City, CA, USA) to generate an amplicon of 300 to 350 bp from MARC-145 genomic DNA. The amplicons were purified and sequenced using capillary fluorescence based sequencing methods [10]. The identity of each gene was confirmed by sequence homology against the known human sequence and "nested" sequence-specific primers were designed using Primer Express® software v1.0 and synthesized (Integrated DNA Technologies, Inc., Coralville, IA, USA) to generate optimal sized (100 to 150 bp) amplicons for real-time reverse transcription-polymerase chain reaction (RT-PCR) (Table 1). Primers were designed that flanked splice sites to eliminate signal from potential genomic DNA contamination. An initial annealing temperature was selected for each primer pair based on their computed T_m (Table 1).

RT-PCR amplifications were performed using the MessageSensor™ (Ambion) real-time RT-PCR kit as per the manufacturer's instructions with the following modifications. Briefly, a master mixture was prepared for a one-step RT-PCR reaction containing RT-PCR grade water, RT-PCR buffer containing 3 mM $MgCl_2$, SYBR Green I (final concentration of 1:40,000, Molecular Probes, Inc.,

Eugene, OR, USA), 8% glycerol, dNTPs (2.5 mM each), RNase inhibitor (10 U/μl), ROX internal reference dye (250 μM, Ambion), Super*Taq* polymerase (5 U/μl), Moloney murine leukemia virus (M-MLV) reverse transcriptase, gene specific RT-PCR primer mix (1 mM each of the gene-specific forward and reverse primers), and 50 ng RNA template. Triplicate reactions for each gene were prepared along with a "no-template" negative control (NTC) and a "no-amplification" control (NAC). The real-time RT-PCR reaction was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) in either 8-well strips, or 96-well plates. The cycling conditions were as follows: RT for 15 min at 42°C, denaturation for 5 min at 95°C, amplification for 40 cycles, with denaturation for 15 sec at 95°C, annealing for 30 sec at 60°C, extension for 50 sec at 76°C, and detection after 2 sec at 76°C. A melting curve analysis was performed from 60°C to 95°C post-amplification. The temperature transition rate for all segments of the amplification cycles and the melting curve cycle were set at 20°C/sec, except for product melting curve analysis which was set at 0.2°C/sec.

To quantify RNA abundance, the comparative threshold cycle (C_T) method was used which compares the relative amount of target sequence to any of the reference values chosen and the result is presented as relative to the reference value (expression level of mock-infected MARC-145 cells). The comparative C_T method ($\Delta\Delta C_T$) is the most practical method if the target and reference have similar dynamic ranges [21]. Calculations for target transcript quantitation begin with the difference (ΔC_T) between the C_T values of the target and the reference: $\Delta C_T = C_T$ (target) - C_T (reference). This is followed by transforming these values to absolute values, using the formula: Comparative expression level = $2^{-\Delta\Delta C_T}$.

Table 1. Oligonucleotide sequences designed for real-time RT-PCR

Gene Name	Gene Symbol	GenBank Accession #	RT-PCR Product Expected size (bp)*	Ta	Tm	Forward primer	Reverse primer	Position in cDNA
Human Housekeeping gene family								
Beta Actin	ACTB	NM_001101	102	59		CCATCATGAAGTGTGACGTGG	TCTGCATCCTGTCGGCA AT	915-1016
Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH	NM_002046	106	60		GCAAATTCCATGGCACCGT	TCGCCCCACTTGATTTTGG	236-341
Hypoxanthine-Guanine Phosphoribosyltransferase	HPRT1	NM_000194	263	59		TCTTTGCTGACCTGCTGGATT	TGCGACCTTGACCATCTTTG	306-568
Cyclophilin A	PPIA	NM_021130	111	60		CCCAACACAAATGGTTCCCA	GGCCTCCACAATATTCATGCC	357-467
Transferrin Receptor	TFRC	NM_003234	138	60		AAAATCCGGTGTAGGCACAGC	GCACCAACCGATCCAAAGTCT	1514-1651
Human Type I Interferon pathway genes								
Interferon α_2	IFNA	V00549	121	58	79	ACCTTTGCTTTACTGGTGGCC	ATCTGTGCCAGGAGCATCAAG	70-191
Interferon β_1	IFNB	V00546	108	58	80	TAGGCGACACTGTTCGTGTTG	CCAAGCAAGTTGTAGCTCATGG	39-147
Activating transcription factor-2	ATF2	BE616960	117	58	79	GACAGATGACCCCCAATTGAA	GACTGCAACTCGGTTTTCCAG	218-335
v-jun avian sarcoma virus 17 oncogene homolog	JUN	AI417972	114	56	76	TCTCCGTCGCAACTTGTCAAG	GCTAACGCAGCAGTTGCAAAC	105-219
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	NFKB	M58603	100	58	79	ATGTGAAGGCCCATCCCAT	TTGCTGGTCCCACATAGTTGC	577-677
Interferon regulatory factor 3	IRF3	NM_001571	117	58	79	AGGATGCACAGCAGGAGGATT	GCAGAGCGGAAATTCCTCTTC	177-294
Human Caspase gene family								
Caspase 2 (ICH1)	CASP2	NM_032982	109	60		CATGATATGCGGCTATGCCTG	ACAAGCCCGCTCAGAAAACAC	1236-1344
Caspase 3 (cpp32)	CASP3	NM_004346	114	60		TGGTTTGAGCCTGAGCAGAGA	TCATCCACACATACCAGTGCG	2004-2117
Caspase 6 (mch2)	CASP6	NM_032992	134	60		TGGCGAAGGCAATCACATTT	TTCCCCGACATGCCTGAAT	174-307
Caspase 7 (mch3)	CASP7	NM_001227	113	61	83	TCTTCGCCTATTCCACGGTTC	TTCCAGGTCTTTTCCGTGCTC	971-1083
Caspase 8 (FLICE)	CASP8	NM_001228	108	57	78	AAGGAGCTGCTCTTCCGAATT	AATTTGAGCCCTGCCTGGT	443-550
Caspase 9 (MCH6)	CASP9	NM_001229	101	58	79	TCAATGCCAGTAACGCGTCTT	GGTTGTCAGGCGAGGAAAGTT	1738-1838
Human Bcl-2 gene family								
Bax	BAX	NM_004324	101	61	83	TGGAGCTGCAGAGGATGATTG	CCAGTTGAAGTTGCCGTCAGA	290-390
Bcl-2	BCL2	NM_000633	101	62	84	GTTTCAACACAGACCCACCCA	GCGTAAGCACCACTGCATTTC	3016-3116

Bcl-xL	BCL2L1	NM_138578	121	60	81	ATCATTTTCCCCCCACTCTCC	GCATCTTTATCCCAAGCAGCC	1500-1620
Human p53 Apoptosis Effectors								
Apaf-1	APAF1	NM_001160	196	58	78	TGCCTGTAATCCCAGCTCCTT	TTGACCGACTGCATGAACTGC	6581-1776
PRRSV isolate 16244b (Michelle)								
ORF7 positive strand	ORF7	AF046869	101	60	81	ACAACGGCAAGCAGCAGAA	TCTGGACTGGTTTTGCTGAGC	14889-15260
ORF7 negative strand (260-366)	ORF7neg	AF046869	106	59	81	CCTCTGGACTGGTTTTGCTGA	AATAACAACGGCAAGCAGCAG	14889-15260