TaqMan® Gene Expression Assays for Quantitative Measurement of Mitochondrial DNA Transcription

Introduction

Mitochondria, in the form of ATP, provide most of the energy used in vital cellular processes. ATP is generated via the oxidative phosphorylation (OXPHOS) system; a series of four large enzymatic complexes. Assembling these complexes requires coordination of mitochondrial and nuclear gene expression.

Human mitochondrial DNA comprises 37 genes, 13 of which code for polypeptides and form part of the OXPHOS system, whereas the other 24 genes code for 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA) (Maechler and Wolheim, 2001). The remainder of the OXPHOS system components are encoded in the nuclear genome, synthesized in the cytosol and then imported into the mitochondria.

Defects in mitochondrial metabolism cause a wide range of neurological diseases including Parkinson's, Alzheimer's, and Friedreich Ataxia (Moreira et al., 2006; Schapira, A.H., 2006; and Kwong, J.Q. et al., 2006), among others, which have all been associated with mutations in mitochondria encoded genes. Moreover, mutations in the mitochondrial genome have also been associated with accelerated aging (Trifunovic, 2006), several cancers (Ohta, 2006), and type II diabetes (Moohta, 2003). The expression levels of COX1, a mitochondria encoded gene, have also been shown to be altered in patients with human nephrotic syndrome (Holthoffer, 1999). This points to a crucial role of the mitochondria in the incidence of major human diseases. In principle then, modulating mitochondrial gene expression might be an interesting therapeutic approach to help address these mitochondria based diseases. To address this, we have designed and validated TaqMan® Gene Expression Assays that target mitochondria encoded genes. These assays can be used in conjunction with our existing TaqMan® Assays that target nuclear encoded mitochondria genes. Both sets of assays can be used to better understand the role the OXPHOS system plays in mitochondria based disease.

Abstract

We have designed TaqMan Gene Expression Assays for 19 mitochondrial (mt) DNA encoded transcripts, including 13 mt mRNAs, two mt rRNAs, and one mt D-loop. Three additional assays targeting the mt inter-tRNA region are also available as Custom TaqMan® Gene Expression Assays. To validate these mitochondrial TaqMan Assays, gene expression was measured in both mitochondrial and nuclear transcripts that encode several subunits of the human OXPHOS system. Both HeLa cells and mtDNA depleted (~90%) HeLa cells were interrogated. Nineteen mitochondrial transcripts and 13 nuclear transcripts (ten OXPHOS transcripts and three endogenous controls) were quantified. The expression level of most of the mtDNA transcripts was reduced more than ten-fold in mtDNA depleted cells compared to the normal cells, while the majority of nuclear DNA encoded OXPHOS transcripts and endogenous controls showed similar expression levels in both cell types. Our results indicate that these TaqMan Assays targeting mtDNA transcripts are indeed sensitive and specific, and thus constitute useful tools for specific and accurate quantification of mtDNA transcription.

TABLE 1. List of TaqMan® Assays used in this study including the 19 newly designed TaqMan Assays for mtDNA encoded transcripts.

Assay ID	Gene ID	Symbol	Assay target	Transcript characteristics
TaqMan® Assays for mitochondrial encoded genes				
Hs02596873_s1	4535	MT-ND1	mt mRNA*	OXPHOS complex I
Hs02596874_g1	4536	MT-ND2	mt mRNA*	OXPHOS complex I
Hs02596875_s1	4537	MT-ND3	mt mRNA*	OXPHOS complex I
Hs02596876_g1	4538	MT-ND4	mt mRNA*	OXPHOS complex I
Hs02596877_g1	4539	MT-ND4L	mt mRNA*	OXPHOS complex I
Hs02596878_g1	4540	MT-ND5	mt mRNA*	OXPHOS complex I
Hs02596879_g1	4541	MT-ND6	mt mRNA*	OXPHOS complex I
Hs02596867_s1	4519	MT-CYB	mt mRNA*	OXPHOS complex III
Hs02596864_g1	4512	MT-CO1	mt mRNA*	OXPHOS complex IV
Hs02596865_g1	4513	MT-CO2	mt mRNA*	OXPHOS complex IV
Hs02596866_g1	4514	MT-CO3	mt mRNA*	OXPHOS complex IV
Hs02596862_g1	4508	MT-ATP6	mt mRNA*	OXPHOS complex V
Hs02596863_g1	4509	MT-ATP8	mt mRNA*	OXPHOS complex V
Hs02596859_g1	4549	MT-RNR1	mt rRNA*	
Hs02596860_s1	4550	MT-RNR2	mt rRNA*	
Hs02596861_s1	MT- D-loop	MT-7S	mt D-loop*	
AIN1HHE	MT- inter-tRNA1	MT- L_Ala_to_Gln	mt inter-tRNA region**	
AIPAFNM	MT- inter-tRNA2	MT- L_Pro_to_Glu	mt inter-tRNA region**	
AIQJDTU	MT- inter-tRNA3	MT- L_Ser1_to_Tyr	mt inter-tRNA region**	
TaqMan® Assays for nuclear encoded mitochondrial genes				
Hs00159575_m1	NM_002488.2	NDUFA2	Nuclear mRNA*	OXPHOS complex I
Hs00159583_m1	NM_182739.1	NDUFB6	Nuclear mRNA*	OXPHOS complex I
Hs00188166_m1	NM_004168.1	SDHA	Nuclear mRNA*	OXPHOS complex II
Hs00268117_m1	NM_003000.1	SDHB	Nuclear mRNA*	OXPHOS complex II
Hs00163415_m1	NM_003365.2	UQCRC1	Nuclear mRNA*	OXPHOS complex III
Hs00268685_m1	NM_003366.2	UQCRC2	Nuclear mRNA*	OXPHOS complex III
Hs00362067_m1	NM_004255.2	COX5A	Nuclear mRNA*	OXPHOS complex IV
Hs00269977_m1	NM_004374.2	COX6C	Nuclear mRNA*	OXPHOS complex IV
Hs00969567_g1	NM_001686.3	ATP5B	Nuclear mRNA*	OXPHOS complex V
Hs00426889_m1	NM_001697.2	ATP5O	Nuclear mRNA*	OXPHOS complex V
TaqMan® Assay controls				
Hs99999901_s1		18S	Nuclear mRNA*	Endogenous control
Hs99999903_m1	NM_001101.2	ACTB	Nuclear mRNA*	Endogenous control
Hs99999904_m1	NM_021130.2	PPIA	Nuclear mRNA*	Endogenous control

*Available as Inventoried TaqMan® Gene Expression Assays (P/N 4331182).

**Available as Custom TaqMan® Gene Expression Assays (P/Ns 4331348 [small], 4332078 [medium], 4332079 [large]).

Results

Design of TaqMan Assays for Mitochondrial DNA Encoded Genes

The target sequences of mtDNA encoded genes were derived from the annotation of a single RefSeq entry for the entire human mtDNA (NC_001807) from NCBI, in which individual tRNAs, rRNAs, protein coding regions and other features were annotated. Polymorphisms, sequence discrepancies, and repeating sequences were masked by a multiple alignment analysis of 1,774 human mitochondrial genomes across different ethnicities. TaqMan Assays (including gene-specific probes and primers) were designed for 21 mtDNA encoded transcripts, including 13 mt mRNAs, two rRNAs and four other transcribed regions mapping to D-loop or inter-tRNA regions on the light strand of mtDNA (Table 1). All assays were designed using the "single exon" version of our pipeline (as mitochondrial genes lack introns) and were BLASTed against all known human mRNAs, B35 human genome sequences, and NCBI reference mitochondrial DNA sequences. Analysis of the designed assays showed very little sequence similarity against human mRNAs (data not shown).

Generation of mtDNA Depleted HeLa Cells

Mitochondrial DNA depleted HeLa cells were obtained by growing cells in the presence of 50 ng/mL ethidium bromide (EtBr, King and Attardi, 1996). The treatment was carried out for five passages (~2 weeks). After each passage, genomic DNA was extracted using a Qiagen DNAeasy tissue kit, and the samples were analyzed for mtDNA copy number using a quantitative real-time PCR (qRT-PCR) assay. The mtDNA copy number was calculated as a ratio of mtDNA/nuclear (n) DNA. We used an ND2 gene specific probe and primers for the mtDNA quantification, and an NRF1 specific probe and primers for nDNA copy number. The qRT-PCR was carried out on an Applied Biosystems 7500 Real-Time PCR System. After five passages, mtDNA copy number was reduced by ~90% (Figure 1).

RNA Preparation

The total cellular RNA was extracted from these cells using an RNAeasy mini-kit (Qiagen). Again, because mitochondrial genes lack introns, the mitochondrial TaqMan Assays were designed using the "single exon" version of our pipeline. Therefore, residual DNA contamination may confound accuracy in quantification of mtDNA encoded transcripts. To monitor any residual DNA contamination in our RNA sample, we conducted PCR reactions using the following human mtDNA specific primers:

HVRIIF 5'CTCACGGGAGCTCTCCATGC 3'

HVRIIR 5'CTGTTAAAAGTGCATACCGCCA 3'

As shown in Figure 2, mtDNA contamination was evident in RNA samples (lanes 5–7). These samples were treated with RNase free DNase I (Qiagen). The absence of PCR product upon DNase I treatment (lanes 8–10) indicated that our RNA samples were free of DNA contamination.

Quantification of Transcription of mtDNA and Nuclear DNA Encoded OXPHOS Genes Using TaqMan Assays

To validate the specificity and accuracy of the 19 TaqMan Assays that target mtDNA encoded transcripts, we performed real-time RT-PCR analysis using these assays and ten TaqMan Assays designed to target nuclear DNA encoded subunits of the OXPHOS system (Table 1) in normal and mtDNA depleted HeLa cells. Three TaqMan Assays targeting common housekeeping genes, 18S, ACTB, and PPIA, were also included as potential endogenous controls. A total of six samples were analyzed with three biological replicates for each cell line. Next, cDNAs were generated from 2 µg of total RNA from each sample using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Each TaqMan Assay was run in quadruple (10 ng/µL cDNA, 10 µL reaction) using an Applied Biosystems 7900HT Fast Real-Time PCR System. PPIA was chosen as the endogenous control as it displayed the most constant expression level across different samples (data not shown).

Because the mtDNA copy number in depleted HeLa cells is significantly

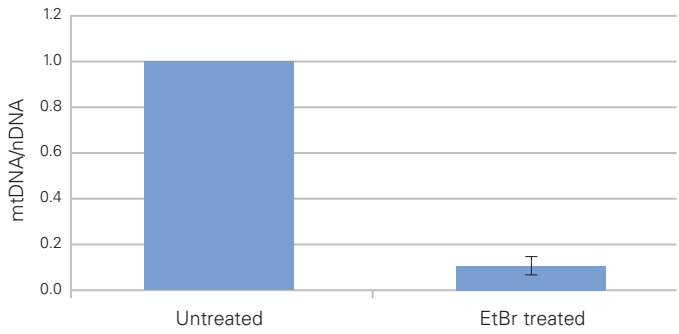

Figure 1. Quantification of mtDNA copy number using mtDNA-to-nuclear-DNA ratio assay. The mtDNA copy number is reduced by 90% upon ethidium bromide (EtBr) treatment.

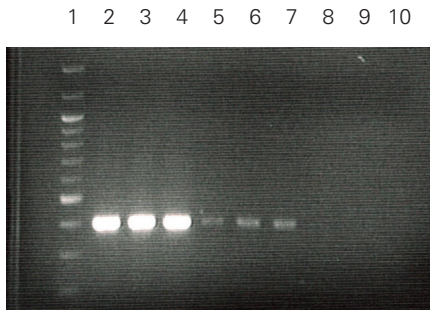

Figure 2. Detection of residual DNA in RNA samples by PCR. PCR products were run on 1.2% agarose gel. Lane 1: 100 bp DNA ladder; lanes 2–4: genomic DNA from HeLa cells; lanes 5–7: RNA from HeLa cells; and lanes 8–10: DNase I treated RNA samples.

reduced to ~10% relative to that of the normal cells (Figure 1), the mtDNA encoded transcripts were expected to have reduced expression levels in depleted cells while the levels of the nuclear DNA encoded transcripts should remain constant. The majority of the mtDNA encoded transcripts indeed showed reduced expression levels (<10%) compared to the normal cells, while most of the nuclear encoded OXPHOS genes and housekeeping genes exhibited similar expression level in both the depleted cells and normal cells (Figure 3 and Table 2). Two of the nuclear DNA encoded OXPHOS transcripts (NDUFB6 and ATP5B) showed significantly higher expression in mtDNA depleted HeLa cells (Table 2). This suggests that nuclear transcription may be programmed to closely coordinate with mitochondrial transcription.

Discussion

We have designed TaqMan Gene Expression Assays for 19 mitochondrial (mt) DNA encoded transcripts. These assays were experimentally validated to be highly sensitive and specific to mtDNA encoded transcripts.

The 19 mt TaqMan Assays cover all 13 protein coding transcripts in the mt genome and two mt rRNAs (Table 1). Although five assays also map to one or two nuclear encoded GenBank mRNAs other than the targeted mitochondrial transcript (Table 2), the expression level of these assays was highly correlated with the mitochondrial depletion level in our validation study. Therefore, they were experimentally validated to be very specific to interrogate the mitochondrial transcripts. It is possible that the mRNAs mapped by GenBank lack strong experimental evidence or result from mitochondrial contamination during sample preparation.

In addition to the assays designed to target mtDNA coding regions, we also designed and validated four assays that target the mt-D-loop or inter-tRNA regions. The D-loop region is a replication start site of the mtDNA. A transcript of that region is reportedly being generated to prime the replication process, and this assay may be able to measure the abundance of that transcript.

Three assays (available through the Custom TaqMan Gene Expression Assays product line) were designed to target the inter-tRNA regions on the light strand of mtDNA. The inter-tRNA regions are part of the polycistronic transcript of the light strand and are spliced out during processing of the transcript. Although they do not have any known function either as RNA or as coding proteins, measuring them may provide information regarding the mtDNA transcription process: the mtDNA light strand has three different transcript lengths (corresponding to three different transcription end sites), and the differences between the levels of these inter-tRNA regions may reflect the relative abundance of these three transcripts. The L prefix of these targets refers to the fact that they are on the mtDNA light strand, and the two amino acid names are the tRNA boundaries of the target (Ojala, 1981).

Conclusion

The TaqMan Assays designed and validated in this study provide a useful tool for the quantitative analysis of mtDNA transcription regulation in human genes. They also enable a better understanding of the coordination of expression between the nuclear and mitochondria encoded genes, which may be essential in furthering our knowledge of mitochondria based diseases, and lead to potential therapeutic approaches.

Contributors

This study was carried out through a collaboration between Applied Biosystems and Broad Institute at MIT. Key contributors include Applied Biosystems scientists Yulei Wang, Ph.D., Raymond Samaha, Ph.D., Yu Wang, Ph.D.; and Broad Institute scientists, Vishal Gohil, Ph.D., Oded Shaham, and Vamsi Mootha, Ph.D. We thank Pius Brzoska, Ph.D., Fangqi Hu, Ph.D., George Wang, Ph.D., and Regina Nagle, Ph.D. and Kathy Lee, Ph.D. (product management) for technical input and helpful discussions.

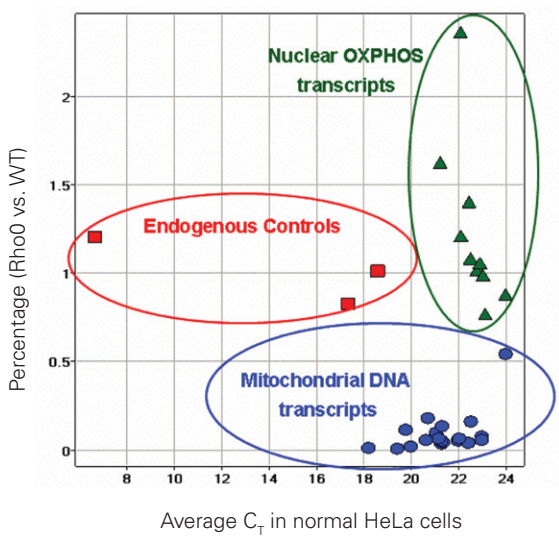

Figure 3. Comparison of gene expression level of mtDNA encoded transcripts and nuclear DNA encoded transcripts in mtDNA depleted HeLa cells and normal HeLa cells. X-axis: average C_T (normalized by PPIA endogenous control) for each assay in the normal HeLa cells derived from 12 measurements (three biological replicates, four technical replicates); Y-axis: ratio of expression level for each gene in mtDNA depleted cells vs. normal cells calculated from average C_T in mtDNA depleted cells and average C_T in normal cells.

TABLE 2. Gene expression comparison of mtDNA and nuclear DNA encoded genes in mtDNA depleted and normal HeLa cells.

Symbol	Assay target	Avg C_T (mtDNA depleted)	Avg C_T (normal)	Delta C_T	Delta C_T stdev	mtDNA depleted/normal ratio
MT-ND1	mt mRNA	20.7	18.2	2.5	0.1	0.18
MT-ND2	mt mRNA	21.3	18.4	2.9	0.2	0.14
MT-ND3	mt mRNA	23.0	19.3	3.7	0.1	0.08
MT-ND4	mt mRNA	21.2	17.0	4.2	0.6	0.05
MT-ND4L	mt mRNA	21.1	17.2	4.0	0.2	0.06
MT-ND5	mt mRNA	22.0	18.0	4.0	0.1	0.06
MT-ND6	mt mRNA	22.9	18.8	4.2	0.3	0.06
MT-CYB	mt mRNA	22.5	19.9	2.6	2.8	0.16
MT-CO1	mt mRNA	19.7	16.6	3.1	0.2	0.11
MT-CO2	mt mRNA	21.2	16.4	4.8	0.2	0.03
MT-CO3	mt mRNA	21.3	16.8	4.5	0.2	0.04
MT-ATP6	mt mRNA	21.9	17.7	4.3	0.2	0.05
MT-ATP8	mt mRNA	20.6	16.5	4.1	0.2	0.06
MT-RNR1	mt rRNA	18.2	11.7	6.4	0.2	0.01
MT-RNR2	mt rRNA	19.4	12.4	7.0	0.2	0.01
MT- 7S	mt D-loop	19.9	14.2	5.7	0.1	0.02
MT- L_Ala_to_Gln	mt inter-tRNA region	23.9	23.1	0.9	0.7	0.54
MT- L_Pro_to_Glu	mt inter-tRNA region	22.4	17.7	4.6	0.2	0.04
MT- L_Ser1_to_Tyr	mt inter-tRNA region	21.0	17.7	3.4	0.2	0.10
NDUFA2	Nuclear mRNA	23.9	23.8	0.2	0.5	0.88
NDUFB6	Nuclear mRNA	22.1	23.3	-1.2	0.8	2.37
SDHA	Nuclear mRNA	23.1	22.7	0.4	0.2	0.77
SDHB	Nuclear mRNA	22.4	22.9	-0.5	0.1	1.41
UQCRC1	Nuclear mRNA	22.1	22.4	-0.3	0.2	1.21
UQCRC2	Nuclear mRNA	23.0	23.0	0.0	0.2	0.99
COX5A	Nuclear mRNA	22.7	22.8	0.0	0.2	1.02
COX6C	Nuclear mRNA	22.9	22.9	-0.1	0.1	1.06
ATP5B	Nuclear mRNA	21.2	21.9	-0.7	1.0	1.63
ATP5O	Nuclear mRNA	22.5	22.6	-0.1	0.2	1.09
18S	Nuclear mRNA	6.6	6.9	-0.3	0.2	1.21
ACTB	Nuclear mRNA	17.3	17.0	0.3	0.1	0.83
PPIA	Nuclear mRNA	18.6	18.6	0.0	0.1	1.01

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