The role of CYP3A4 mRNA transcript with shortened 3’-UTR in hepatocyte differentiation, liver development, and response to drug induction

Dan Li, Roger Gaedigk, Steven N. Hart, J Steven Leeder, and Xiao-bo Zhong

Molecular Pharmacology

Supplemental Table 6. MIQE checklist

ITEM TO CHECK	IMPORTANCE #	CHECKLIST	
EXPERIMENTAL DESIGN			
Definition of experimental and control groups	E	X	Human liver tissues of fetal, neonatal/pediatric, and adult ages were compared with each other. HepaRG cells and primary human hepatocytes incubated with 0.1% DMSO were sovlent controls, and HepaRG cells and primary human hepatocytes incubated with rifampicin (10 µM) or phenobarbital (750 µM) were experimental groups, respectively.
Number within each group	E	X	HepaRG cells and primary human hepatocytes were incubated with sovlent control, rifampicin, or phenobarbital (n=3 for each). Human liver tissues were from fetal, neonatal/pediatric, and adult (n=4 for each).

Assay carried out by core lab or investigator's lab?	D	X	Investigator's lab
Acknowledgement of authors' contributions	D		
SAMPLE			
Description	E	X	Human liver tissues were collected from four fetal livers (estimated gestational ages: 170 to 227 days), four neonatal/pediatric livers (postnatal ages: 0 to 7 years), and four adult livers (ages: 17 to 36 years).
Volume/mass of sample processed	D		
Microdissection or macrodissection	E	X	Macrodissected
Processing procedure	E	X	The post mortem interval was < 6 hrs.
If frozen - how and how quickly?	E	N/A	
If fixed - with what, how	E	N/A	

quickly?			
Sample storage conditions and duration (especially for FFPE samples)	E	X	All the liver tissues were maintained at -80 ºC prior to preparation of subcellular fractions.
NUCLEIC ACID EXTRACTION			
Procedure and/or instrumentation	E	X	Total RNAs were extracted from HepaRG cells by TRIzol reagent or from human liver tissues by a Qiagen RNeasy kit according to the manufacturer's protocols.
Name of kit and details of any modifications	E	X	TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and Qiagen RNeasy kit (Valencia, CA, USA). We exactly followed the manufacturer's protocols
Source of additional reagents used	D	X	Absolute ethanol, isopropanol, and choroloform were from Sigma-Aldrich (St. Louis, MO, USA)
Details of DNase or RNAse treatment	E	X	No DNase treatment.
Contamination assessment (DNA or	E	X	No-reverse transcription controls was used to assess DNA contamination of RNA samples. The Cq values were more than 35.

RNA)			
Nucleic acid quantification	E	X	RNA quantification was assessed by Nanodrop 1000 spectrophotometer.
Instrument and method	E	X	Nanodrop 1000 spectrophotometer (Thermo Scientific)
Purity (A260/A280)	D	X	> 1.8
Yield	D		
RNA integrity method/instrument	E	X	RNA were assessed by Experion Bioanalyzer (Bio-Rad Laboratories, Hercules, CA, USA).
RIN/RQI or Cq of 3' and 5' transcripts	E	X	The 95% confidence interval of RQI values were between 7.7 and 9.3 for human liver RNA.
Electrophoresis traces	D	X	Not performed
Inhibition testing (Cq dilutions, spike or other)	E	X	Not performed.
REVERSE TRANSCRIPTION			
Complete reaction conditions	E	X	The total RNA was reverse transcribed by M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers

			(Invitrogen, Carlsbad, CA, USA) .
Amount of RNA and reaction volume	E	X	RNA is 1 μg ; Reaction volume is 50 μl.
Priming oligonucleotide (if using GSP) and concentration	E	N/A	
Reverse transcriptase and concentration	E	X	M-MLV reverse transcriptase (200 u/ μl) .
Temperature and time	E	X	The reverse transcription mixture was maintained at 45 ℃ for 30 min and heated at 95 ℃ for 5 min before storage at -20 ℃.
Manufacturer of reagents and catalogue numbers	D	X	M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA, Cat No. 28025-013) and random primers (Invitrogen, Carlsbad, CA, USA, P/N 58875), 5×first strand buffer (Invitrogen, Carlsbad, CA, USA, P/N y02321), RnaseOut TM recombinant ribonuclease inhibitor (Invitrogen, Carlsbad, CA, USA, P/N 100000840)
Cqs with and without RT	D*	X	Cq without RT >35. Cq with RT for canonical transcript < 24 , for canocial and shorter transcripts < 27 , for GAPDH < 22, and for ACTB <23 .

Storage conditions of cDNA	D	X	-20 ℃
qPCR TARGET INFORMATION			
If multiplex, efficiency and LOD of each assay.	E	X	PCR efficiency is between 0.97 to 1.03; LOD (Cq), for canonical transcript: 24 , for canocial and shorter transcripts: 27 , for GAPDH: 22, for ACTB: 23.
Sequence accession number	E	X	cytochrome P450 3A4 (CYP3A4): NM_017460.3; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): NM_002046.3; actin beta (ACTB): NM_001101.3.
Location of amplicon	D	X	For CYP3A4: two primer sets in 3'-UTR. For GAPDH: one primer set in exon 4 and exon 6. For ACTB: one primer set in exon 3 and exon 4.
Amplicon length	E	X	canonical 3’UTR transcript: 142 bp; canonical and shorter 3’UTR transcripts: 156 bp; GAPDH: 199 bp; ACTB: 192bp.
In silico specificity screen (BLAST, etc)	E	X	The amplicons are specifically matched to the targets by UCSC and BLAST.
Pseudogenes, retropseudogenes or other	D		

homologs?			
Sequence alignment	D		
Secondary structure analysis of amplicon	D		
Location of each primer by exon or intron (if applicable)	E	X	For CYP3A4: two primer sets in 3'-UTR. For GAPDH: one primer set in exon 4 and exon 6. For ACTB: one primer set in exon 3 and exon 4.
What splice variants are targeted?	E	X	For CYP3A4: Canonical and the shorter 3'-UTR transcripts.
qPCR OLIGONUCLEOTIDES			
Primer sequences	E	X	Please check Table 1 in the manuscript.
RTPrimerDB Identification Number	D	N/A	
Probe sequences	D**	N/A	
Location and identity of any modifications	E	X	No modification.

Manufacturer of oligonucleotides	D	X	Integrated DNA Technologies (IA, USA)
Purification method	D		
qPCR PROTOCOL			
Complete reaction conditions	E	X	cDNAs obtained from reverse transcriptions were diluted 5 fold and 1 μL were added with 10 μL of SYBR Green Master Mix (Fermentas, EU) containing 250 nM of each primer with the final volume of 20 μL. Real time PCR reactions were run on the 7900 HT Fast Real time PCR system (Applied Biosystem) under the following conditions: heating at 95 ℃ for 10 min, 40 cycles of denaturation at 95 ℃ for 15 sec, followed by an annealing/extension at 60 ℃ for 1 min. A negative control without cDNA template was run in every assays and all the measures were performed in triplicates for each sample.
Reaction volume and amount of cDNA/DNA	E	X	Reaction Volume: 20 mL; Amount of cDNA: 50 ng
Primer, (probe), Mg++ and dNTP concentrations	E	X	Primer concentration: 250 nM. SYBR green master mix (Fermentas, EU) includes Maxima® Hot Start Taq DNA polymerase and dNTPs in an optimized PCR buffer, containing

			both KCl and $(NH4)2SO4$ to provide high specificity of primer annealing. The buffer composition allows for PCR at a wide range of $MgCl2$ concentrations.
Polymerase identity and concentration	E	X	Maxima Hot Start Taq DNA polymerase in SYBR Green/ROX qPCR Master Mix (Fermentas, EU).
Buffer/kit identity and manufacturer	E	X	SYBR Green/ROX qPCR Master Mix (Fermentas, EU)
Exact chemical constitution of the buffer	D		
Additives (SYBR Green I, DMSO, etc.)	E	X	SYBR® Green I, ROX passive reference dye, dUTP
Manufacturer of plates/tubes and catalog number	D	X	384 well PCR Plate, Natural (Genemate by ISC BioExpress)
Complete thermocycling parameters	E	X	Real time PCR reactions were run under the following conditions: heating at 95 ℃ for 10 min, 40 cycles of denaturation at 95 ℃ for 15 sec, followed by an annealing/extension at 60 ℃ for 1 min.
Reaction setup	D		

(manual/robotic)			
Manufacturer of qPCR instrument	E	X	7900 HT Fast Real-Time PCR system (Applied Biosystems)
qPCR VALIDATION			
Evidence of optimisation (from gradients)	D		
Specificity (gel, sequence, melt, or digest)	E	X	Gel image was provided in Supplemental Figure 1. The melting curve analysis was performed with single peak for each primer set.
For SYBR Green I, Cq of the NTC	E	X	Cq > 38
Standard curves with slope and y-intercept	E	X	Slope: -3.22 to -3.42; y-intercept: 35 to 38 cycles
PCR efficiency calculated from slope	E	X	0.97 to 1.03
Confidence interval for PCR efficiency or	D	X	Please check the Supplementary Table 1.

standard error			
r2 of standard curve	E	X	0.9959 to 0.9996
Linear dynamic range	E	X	Cq, canonical 3’-UTR transcript: 18 to 30; canonical and shorter 3’-UTR transcripts: 15 to 31; GAPDH: 18 to 35; ACTB: 19 to 35.
Cq variation at lower limit	E	X	S.D. < 0.4
Confidence intervals throughout range	D		
Evidence for limit of detection	E	X	Cq, canonical transcript: 30; canonical and shorter 3'-UTR transcript: 31; GAPDH: 35; ACTB:35.
If multiplex, efficiency and LOD of each assay.	E	X	Efficiency and LOD (Cq) for canonical 3’-UTR transcript are 1.00 and 24; for canonical and shorter 3'-UTR transcript are 1.03 and 27; for GAPDH are 0.97 and 22; for ACTB are 0.99 and 23.
DATA ANALYSIS			
qPCR analysis program (source, version)	E	X	SDS Software v2.4 (Applied Biosystems)

Cq method determination	E	X	The threshold is used to specify Cq values of samples. The threshold is determined so that it is more than 10 times the standard deviation above the noise of the baseline.
Outlier identification and disposition	E	X	Runs were performed in triplicate. Single run was excluded when the melting curve analysis revealed unintended amplification products: melting curves with more than one peak, or one single peak but with a melt temperature different from the expected one.
Results of NTCs	E	X	Cq > 38
Justification of number and choice of reference genes	E	X	GAPDH and ACTB have been used as reference genes based on the literature.
Description of normalisation method	E	X	Data normalization has been carried out against the mean of GAPDH and ACTB as two endogenous reference genes. For each cDNA, the triplicate Cq values were averaged, and the normalized Cq was calculated by subtracting the mean Cq value for GAPDH and ACTB from each mean Cq value.
Number and concordance of biological replicates	D		

Number and stage (RT or qPCR) of technical replicates	E	X	qPCR reactions were performed in triplicate.
Repeatability (intra-assay variation)	E	X	Standard deviation (SD) for the Cq variance between triplicates <0.4.
Reproducibility (inter-assay variation, %CV)	D		
Power analysis	D		
Statistical methods for result significance	E	N/A	
Software (source, version)	E	X	GraphPad Prism 4.00
Cq or raw data submission using RDML	D	X	Supplementary Table 2, 3, 4, and 5.

[#]: All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If using primers obtained from RTPrimerDB, information on qPCR target, oligonucleotides, protocols and validation is available from that source.

*: Assessing the absence of DNA using a no RT assay is essential when first extracting RNA. Once the sample has been validated as RDNA-free, inclusion of a no-RT control is desirable, but no longer essential.

**: Disclosure of the probe sequence is highly desirable and strongly encouraged. However, since not all commercial pre-designed assay vendors provide this information, it cannot be an essential requirement. Use of such assays is advised against.