Human GAPDH RT-qPCR

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Abstract

RNA from matched FFPE, PFPE and cryo preserved human tissues, stored for up to seven years at 22°C and 4°C, examined for integrity and usability in quantitative RT-PCR

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Protocol

Step 1.

Experimental design	RNA from matched FFPE, PFPE and cryo preserved human tissues, stored for up to seven years at 22°C and 4°C, examined for integrity and usability in quantitative RT-PCR		
			Provider/ manufacturer
Sample	Species	Human, with written consent	
		Study approved by the Ethics Committee of the Medical University of Graz, Austria.	
	Tissue types	Nonmalignant cases - stomach, duodenum, liver	
		Malignant cases - liver, soft tissue	
	Fixation	Divided into equal aliquots for snap freezing, fixation with NBF or PAXgene Tissue to generate matched NBF-fixed, PAXgene Tissue-fixed and Cryo tissues per case	
	Fixative	Snap frozen in methyl butane, cooled by liquid nitorgen, storage at -80°C, or	
		NBF (neutral buffered formalin) for 3, 24 and up to 120 hours at room temperature or	Merck KGaA, Darmstadt, Germany
		PAXgene Tissue Fix for 3, 24 and up to 120 hours at room temperature, afterwards transfer into PAXgene Tissue Stabilizer for 24 and up to 120 hours at room temperature	PreAnalytiX Gmbh, Hombrechtikon, CH
	Processing and paraffine embedding	Samples fixed with formalin or PAXgene Tissue were processed on an automated tissue processor, Tissue-Tek VIP Vacuum Infiltration Processor	Sakura Finetek Europe
		Stepwise dehydration in 70%, 80%, 90%, 99% ethanol, followed by isopropanol, xylene for no longer than 1h at each position	
		Low-melting point paraffin was used for infiltration and embedding	
	Storage	PFPE and FFPE blocks stored in the dark at room temperature and 4°C	
		Cyro preserved tissue stored in liquid nitrogen	
RNA extraction	Cryo	20-70mg of frozen tissue	
		Invitrogen TRIzol procedure	Thermo Fisher Scientific, Germany
		Dissolved in 20-200µl RNase free water, storage at -70°C	
	FFPE	10-20 sections, each 5 µm thick	
		RNeasy FFPE kit	QIAGEN GmbH, Hilden, Germany
		Elution in 40µl RNase free water, storage at -70°C	
	PFPE	10-20 sections, each 5 µm thick	
		PAXgene Tissue RNA kit	PreAnalytiX Gmbh, Hombrechtikon, CH
		Elution in 40µl Buffer TR4, storage at -70°C	
	Replicates	Single preparations per sample	
	RNA analyses	RNA yield and purity was determined by spectrophotometric absorbance on Nanodrop ND-1000 spectrophotometer at 260nm	Nanodrop Technologies, Wilmington, USA
		RNA integrity was assessed by microcapillary electrophoresis on an Agilent 2100 Bioanalyzer and analyzed with the Agilent 2100 expert software	Agilent Technologies, Waldbronn, Germany
Reverse transcription	Template	1µg of total RNA in 20µl	
	Kit	High-Capacity cDNA Reverse Transcription Kit, according to manufacturers instructions	Thermo Fisher Scientific, Germany
		cDNA stored at -20°C until use	
RT-qPCR target information	Gene	PCR primers specific to the human GAPDH gene	
	Amplicons	One common forward primer and five different reverse primers to amplify five different transcript sequences with lengths between 71 and 323 nucleotides	

RT-qPCR	Oligonucleotides	All oligonucleotides ordered lyophilized and HPSF-purified.	
		Stored at -20°C until use.	
		Name and amplicon length	Sequence 5´ - 3´
		hu_GAPDH common fwd.	CCA CAT CGC TCA GAC ACC AT
		hu_GAPDH rev. 71bp	ACC AGG CGC CCA ATA CG
		hu_GAPDH rev. 153bp	GTA AAC CAT GTA GTT GAG GTC
		hu_GAPDH rev. 200bp	TTG ACG GTG CCA TGG AAT TT
		hu_GAPDH rev. 277bp	ACT TGA TTT TGG AGG GAT CT
		hu_GAPDH rev. 323bp	AAG ACG CCA GTG GAC TCC A
	Reaction Mix	4µl of 1:32 cDNA dilution (= 1,56ng/µl) served as template for PCR	
		5µl 2x Power SYBR Green PCR Master Mix	Thermo Fisher Scientific, Wilmington, DE
		0.05µl forward primer (working solution 100pmol/µl)	
		0.05µl revers primer (working solution 100pmol/µl)	
		Water ad 10µl	
	Cycle condition:		
	Stage	Time - Temperature	Cycle
	1 - Hold Stage	2min - 50°C	1
		10min - 95°C	
	2 - PCR Stage	15sec - 95°C	45
		1min - 60°C	
	3 - Melt Curve Stage	15sec - 95°C	1
		1min - 60°C	
		15sec - 95°C	
	Plates	MicroAmp Optical 384-Well Reaction Plate	
	Replicates	All samples and controls were analyzed in triplicates, mean Ct values used for further data analysis	
	Instrument	ABI 7900 Real-Time PCR System	Thermo Fisher Scientific, Wilmington, DE
	Software	ABI SDS software version 2.4	
qPCR validation	Specificity	Examination of amplicon length by agarose gel electrophoresis	
		Melting curve analysis	
	Run validity	No template controls no amplifiable, i.e. no Ct determined	
	Acceptance criteria for single reactions	Ct <40	
		Melting curves had to be free of extraneous peaks or peaks which indicated non-specific amplification to be accepted as valid. All data not meeting these acceptance criteria were excluded from data analysis	