Rat ACTB one-step RT-qPCR

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Abstract

RNA from matched FFPE, PFPE and cryo preserved rat tissues, stored for up to nine years at 22°C, 4°C, -20°C or -80°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 rat tissues, stored for up to nine years at 22°C, 4°C, -20°C or -80°C, examined for integrity and usability in quantitative RT-PCR		
			Provider/ manufacturer
Sample	Species	Rats (rattus norvegicus) raised to a weight of approximately 500 g	
		Sacrificed by CO2 asphyxiation	
		Organs removed within 5 min of sacrifice	
	Tissue types	Adjacent, equally sized tissues no larger than 15 × 15 × 4 mm grossed from Liver, Kidney, Spleen, Intestine, Lung	
	Fixation	Snap frozen in liquid nitrogen or placed into standard tissue cassettes and completely submerged in a container filled with fixative with fixation solutions in a ratio of at least 20 parts fixative to one part of tissue (v/v)	
	Fixative	NBF (neutral buffered formalin) for 24 hours at room temperature	Merck KGaA, Darmstadt, Germany
		PAXgene Tissue Fix for 2-4 hours at room temperature, afterwards transfer into PAXgene Tissue Stabilizer for 24-72 hours at room temperature	PreAnalytiX Gmbh, Hombrechtikon, CH
		Snap-frozen in liquid nitrogen, transported on dry ice, and stored at -80°C.	
	Processing and paraffine embedding	Samples fixed with formalin or PAXgene Tissue were processed in separate runs on an automated tissue processor TP1020	Leica-microsystems, Wetzlar, German
		Incubation at 80%, 90%, 99% ethanol (2x), followed by isopropanol (2x), xylene (2x) for no longer than 1h at each position	
		Low-melting point paraffin was used for infiltration and embedding	Surgipath Paraplast-XTRA, Carl Roth GmbH, Karlsruhe, Germany
		For infiltration of tissue with paraffin, samples were incubated (3 x 1 h) under vacuum at 56°C	
		Within 30 min after infiltration, samples were, embedded	
	Storage	PFPE and FFPE blocks stored in the dark at 22°C, 4°C, -20°C and -80°C	
		Cyro preserved tissue stored for up to one year at -80°C, RNA extracted and stored at -20°C	
RNA extraction	Cryo	10 mg of frozen tissue	
		RNeasy Mini kit	QIAGEN GmbH, Hilden, Germany
		Elution in 40µl RNAse free water, storage at -20°C	
	FFPE	Three sections, each 10 µm thick	
		miRNeasy FFPE kit	QIAGEN GmbH, Hilden, Germany
		Elution in 40µl RNAse free water, storage at -20°C	
	PFPE	Three sections, each 10 µm thick	
		PAXgene Tissue RNA kit	PreAnalytiX Gmbh, Hombrechtikon, CH
		Elution in 40µl Buffer TR4, storage at -20°C	
	Replicates	All preparations were done in triplicate	
	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	10 ng of total RNA	
	Assay	one-step RT-PCR assay, 25µl assay volume	
	Kit	QuantiTect® SYBR Green RT-PCR	QIAGEN GmbH, Hilden, Germany
	Intrument	Rotor-Gene Q series II	QIAGEN GmbH, Hilden, Germany

RT-qPCR target information	Gene	PCR primers specific to the rat beta-actin gene (NC_005111)	
	Amplicons	One common forward primer and six different reverse primers to amplify six different transcript sequences with lengths between 109 and 610 nucleotides	
RT-qPCR	Oligonucleotides	All oligonucleotides ordered lyophilized and HPLC-purified.	Metabion GmbH, Planegg/Steinkirchen, Germany
		Lyophilized oligonucleotides dissolved to 100 µM (stock solution)	
		Stored at −15°C to −30°C until use.	
		Name and amplicon length	Sequence 5´ - 3´
		Rn_actB for1	CCACACTGTGCCCATCTATGA
		Rn_actB rev109 - 109 bp	ACGCTCGGTCAGGATCTTCATG
		Rn_actB rev189 - 189 bp	AAGTCTAGGGCAACATAGCAC
		Rn_actB rev287 - 287 bp	GGAACCGCTCATTGCCGATAG
		Rn_actB rev331 - 331 bp	TTCCATACCCAGGAAGGAAGG
		Rn_actB rev438 - 438 bp	TACATGGTGGTGCCACCAGAC
		Rn_actB rev465 - 465 bp	TTCTGCATCCTGTCAGCAATG
	Reaction Mix	10ng RNA	
		12.5µl 2x Quantitect SYBR Green Master Mix	
		1.25µl forward primer (working solution 10µM) - 0.5µM	
		1.25µl revers primer (working solution 10µM) - 0.5µM	
		0.25µl RT Mix	
		Water ad 25µl	
	Cycle condition:		
	Stage	Time - Temperature	Cycle
	1 - Reverse Transcription Stage	30min - 50°C - 1x	1
	2 - Hold Stage	15min - 95°C - 1x	1
	3 - PCR Stage	15sec - 94°C - 40x	40
		30sec - 60°C - 40x	
		30sec - 70°C - 40x	
	4 - Melt Curve Stage	15sec - 95°C - 1x	1
		30sec - 60°C -1x	
		15sec - 95°C - 1x	
	Tubes and Caps	Rotorgene 72-Well Rotor Strip Tubes and Caps, 0.1mL	QIAGEN GmbH, Hilden, Germany
	Replicates	One amplification per triplicate RNA preparation	
	Instrument	Rotor-Gene Q series II	QIAGEN GmbH, Hilden, Germany
	Software	Rotor Gene Q Series Software 2.3.1	
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	