Rat ACTB one-step qPCR

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Abstract

Quantitative PCR for amplification of genomic DNA from FFPE (formalin-fixed and paraffin-embedded), PFPE (PAXgene Tissue-fixed and paraffin-embedded), and snap-frozen fixed tissues.

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Protocol

Step 1.

Experimental design	DNA 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 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, DNA extracted and stored at -20°C	
DNA extraction	Cryo	20 mg of frozen tissue	
		DNeasy Tissue kit	QIAGEN GmbH, Hilden, Germany
		Elution in 100µl Buffer AE	
	FFPE	Three sections, each 10 µm thick	
		QIAamp FFPE kit	QIAGEN GmbH, Hilden, Germany
		Elution in 40µl Buffer AE	
	PFPE	Three sections, each 10 µm thick	
		PAXgene Tissue DNA kit	PreAnalytiX Gmbh, Hombrechtikon, CH
		Elution in 40µl Buffer TD5	
	Replicates	All preparations were done in triplicate	
	DNA analyses	DNA purity and yield was determined by spectrophotometric absorbance on Nanodrop ND-1000 spectrophotometer at 260nm	Thermo Fisher Scientific Inc.
		DNA yield confirmed with Qubit® 2.0 Fluorometer with Qubit® dsDNA Assay	Thermo Fisher Scientific Inc.
		DNA integrity was assessed on Agilent 4200 TapeStation system with genomic DNA Analysis ScreenTape assay	Agilent Technologies, Waldbronn, Germany
qPCR target information	Gene	PCR primers specific to the rat beta-actin gene (NC_005111)	
		Three different forward primer and two different reverse primers to amplify four different sequences with lengths of 271, 523, 650 and 747 bp	
		Rn_ACTB DNA-for1/rev1 (271bp)	
		Rn_ACTB DNA-for2/rev1 (523bp)	

		Rn_ACTB DNA-for2/rev2 (650bp)	
		Rn_ACTB DNA-for3/rev2 (747bp)	
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	Sequence 5´- 3´
		Rn_ACTB DNA-for1	CTTGTGGCTTTAGGAGCTTGAC
		Rn_ACTB DNA-for2	TCGATCGCCTTTCTGACTAGG
		Rn_ACTB DNA-for3	CTTCTGCCATTCTCCCATAGG
		Rn_ACTB DNA-rev1	CATCGGAACCGCTCATTGCCGATAG
		Rn_ACTB DNA-rev2	TCTTCTCCAGGGAGGAAGAGGATG
	Reaction Mix	10ng DNA	
		10µl 2x Quantitect SYBR Green Master Mix	
		1µl forward primer (working solution 10µM) - 0.5µM	
		1µl revers primer (working solution 10µM) - 0.5µM	
		Water ad 20µl	
	Cycle condition:		
	Stage	Time - Temperature	Cycle
	1 - Hold Stage	15min - 95°C	1
	2 - PCR Stage	15sec - 94°C	40
		30sec - 60°C	
		30sec - 70°C	
	3 - Melt Curve Stage	15sec - 95°C	1
		30sec - 60°C	
		15sec - 95°C	
	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	