2010

Geno/Grinder®

Tissue
Homogenization
Cell Lysis

APPLICATION NOTE

Extraction of RNA/cDNA and Genomic DNA from Tissue with Real-Time PCR

With kind permission of Christian M. Leutenegger, Ph.D., University of California, Davis, cmluetenegger@ucdavis.edu, January 2003

Sample Collection, Preparation and Tissue Grinding

Fresh samples of animal tissue were collected, trimmed to approximately 50 – 100 mg of wet weight, snap-frozen in liquid nitrogen, and stored at –80°C. The animals were man, dog, cat, mouse, cow, and horse, as well as fish and clams; see Table 1. Before DNA and/or RNA extraction, the tissues were transferred frozen to a deep-well titer plate standing on a block of dry ice. Each well contained two 4-mm stainless steel balls (SPEX CertiPrep cat. no. 2150) and 500 microliters of buffer (Applied Biosystems nucleic acid purification lysis buffer). The plates were sealed with a plastic cover and subjected to grinding in the 2000 Geno/Grinder for 2 minutes at a setting of 1000 strokes per minute. After 30 minutes at 4°C, lysates were used for either gDNA extraction or total RNA extraction. Conditions were optimized for an Applied Biosystems 6700 automated nucleic acid (ANA) workstation, according to the manufacturer's instructions. The final amount of tissue subjected to RNA and/or DNA extractions was between 10 and 20 mg.

Quality Control of Extracted RNA/cDNA and Genomic DNA Using Real-Time Taqman® PCR

To assess the quality of the extracted RNA, complementary DNA (cDNA) was synthesized using Invitrogen products: 200 units of SuperScript III, 600 ng of random hexadeoxyribonucleotide ($pd(N)_6$) primer (random hexameter priner), 10 U RnaseOut (Rnase inhibitor), and 1 mM dNTPs in a final volume of 40 l. The reverse transcription reaction took place for 50 minutes at 50°C. After addition of 60 ⌈l of water, the reaction was terminated by heating for 5 minutes to 95°C and cooling on ice. The quality of the cDNA is judged according to the CT value obtained with a endogenous control TaqMan PCR system from a defined amount of tissue. Samples with values above a certain threshold indicate impaired sample quality and degradation of total RNA and warrant re-extraction of a back-up sample. To assess the quality of cDNA, we normally use TaqMan PCR systems targeting species specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or ribosomal genes (such as 18S rRNA or ITS-2).

To assess the quality of extracted genomic DNA, species specific TaqMan PCR systems were developed targeting single copy genes to allow the quantitation of genome equivalents and cell numbers. An overview of TaqMan systems used for gDNA quality control is given in Table 1. The gDNA quality from a defined amount of tissue is judged according to the CT value using a TaqMan PCR system targeting a single copy gene. Samples with values above a certain threshold indicate degraded DNA and warrant re-extraction of a back-up sample. GAPDH TaqMan systems can be

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used to target the single copy GAPDH pseudogene (Galland et al., 1990; Garcia-Meunier et al., 1993).

Each real-time TaqMan PCR reaction contained 400 nM of each primer, 80 nM of the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 ⌈l of the diluted cDNA sample or the gDNA in a final volume of 25 ⌈l. The samples were placed in 96 well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C.

References

GAPDH pseudogenes:

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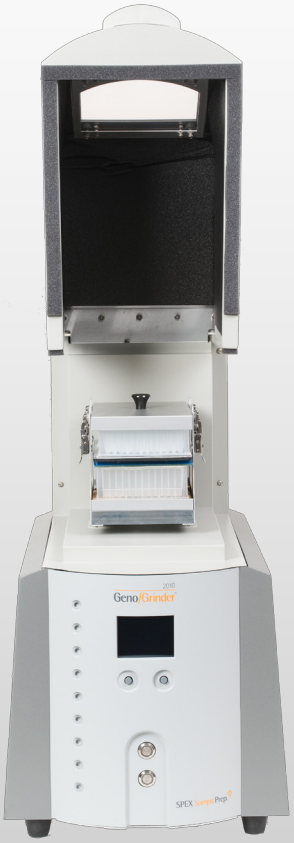

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Table 1

Overview of tissue samples, species and TaqMan systems used to assess quality of cDNA and gDNA

Tissue	Species	RNA/cDNA		Genomic DNA	
		Target Gene	CT Value	Target Gene	CT Value
Skin	Dog	GAPDH	<21	GAPDH	<24
Skeletal Muscle	Dog	GAPDH	<24	GAPDH	<25
Lung	Cow	GAPDH	<24	GAPDH	<25
Brain	Cat	GAPDH	<24	CCR5	<25
Lymph Node	Cat	GAPDH	<21	CCR5	<22
Spleen	Cat	GAPDH	<21	CCR5	<22
Thymus	Cat	GAPDH	<21	CCR5	<22
Tonsil	Cat	GAPDH	<21	CCR5	<22
Cartilage	Horse	GAPDH	<24	IGF-I	<26
Heart	Mouse	GAPDH	<24	IGF-I	<24
Tail	Mouse	GAPDH	<21	IGF-I	<21
Fin	Rainbow Trout	ITS-2	<14	IGF-I	<26
Gill	Rainbow Trout	ITS-2	<14	IGF-I	<24
Spine Tissue	Rainbow Trout	ITS-2	<14	IGF-I	<24
Mussel	Rainbow Trout	ITS-2	<14	IGF-I	<26
Liver	Rainbow Trout	ITS-2	<14	IGF-I	<21
Cranium	Rainbow Trout	ITS-2	<16	IGF-I	<24
Gill	Koi	ITS-2	<14	Glucokinase	<24
Liver	Koi	ITS-2	<14	Glucokinase	<21
Digestive Gland	Clams	18S rRNA	<11	Na	Na
Mussel	Clams	18S rRNA	<11	Na	Na
Liver	Human	GAPDH	<21	IL-2	<21
Skeletal Muscle	Human	GAPDH	<24	IL-2	<24
Kidney	Human	GAPDH	<24	IL-2	<24

Na: Not Available

CT Value: Cycle Threshold Value: PCR cycle at which the fluorescent intensity exceeds the threshold

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Table 2

Composition of real-time fluorogenic Taqman PCR assay

	GAPDH	IL4	IL10	IL 12 p35	IL 12 p40	IFN-g	Il16
2x Mastermix	12.5	12.5	12.5	12.5	12.5	12.5	12.5
10x Buffer A	1x	1x	1x	1x	1x	1x	1x
MgCl2 (mM)	5	5	5	5	5	5	5
dATP (mM)	300	300	300	300	300	300	300
dCTP (mM)	300	300	300	300	300	300	300
dGTP (mM)	300	300	300	300	300	300	300
dUTP (mM)	300	300	300	300	300	300	300
Forward primer (nM)	400	400	400	400	400	400	400
Reverse primer (nM)	400	400	400	400	400	400	400
TaqMan probe (nM)	80	80	80	80	80	80	80
AmpliTaq Gold (U)	0.75	0.75	0.75	0.75	0.75	0.75	0.75
AmpErase UNG (U)	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Template (cDNA)	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml
H2O	Ad 25 ml	Ad 25 ml	Ad 25 ml	Ad 25 ml	Ad 25 ml	Ad 25 ml	Ad 25 ml

10 x TaqMan Buffer A contains 500 mM KCl, 100 mM Tris-HCl, 100 mM EDTA, 600 nM, passive reference ROX, pH 8.3 at room temperature

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