

Table 4: Mean PCR amplification efficiencies (n = 4)

Muscle sample #	Amplification efficiencies and coefficient of correlation (E/R2)		
	AS1	AS2	HPRT
1	1.921/0.9993	1.941/0.9993	1.956/0.9987
2	2.068/0.9989	2.104/0.9995	1.999/0.9995
3	2.030/0.9989	2.045/0.9996	2.028/0.9988
4	2.011/0.9986	2.079/0.9993	1.976/0.9895

Dilutions series used for the determination of the PCR amplification efficiencies for each of the four MHS muscle tissues: 1; 1:5 dilution steps, 2; 1:3 dilution steps, 3; 1:3 dilution steps, 4; 1:3 dilution steps.

tions of 1:3 dilution steps instead 4 or 5 dilutions of 1:5 dilution steps.

Determining the relative allele frequencies

After assessing the linearity of the reverse transcription reactions and determining the PCR amplification efficiencies, the samples were screened to determine the relative quantities of the two *RYS1* alleles in each of the four samples. To confirm accuracy and reproducibility, the allele frequencies were determined by performing three independent experiments. Targets were amplified in triplicate within each experiment. Intra- and inter assay variabilities as obtained by screening sample #1 are shown in Table 5 and 6, respectively. As the results show, both intra- and inter assay variations are low.

Several mathematical models are available for relative quantification during real-time PCR. There are some small differences between the individual models. Nevertheless, all relative quantification analyses are based on the assumption that the concentration of the template (e.g. cDNA) at a sample's crossing point is the same for every sample containing the same target cDNA. The model used in this study, is a rearranged version of the

efficiency calibrated mathematical method (see Equation 2) [28]. In this model, calculations are based on E and the Ct values of an unknown "sample" versus a "calibrator". The "target" is the nucleic acid of interest, while the "reference" is a nucleic acid that is found at constant copy number in all samples and serves as an endogenous control. The "calibrator" is typically a sample with a stable ratio of target-to-reference and can be used to normalize all samples within a run, but in addition provides a constant calibration point between several runs.

$$\text{Normalized ratio} = \frac{(E_{\text{ref}})^{Ct_{\text{sample}}}}{(E_{\text{target}})^{Ct_{\text{sample}}}} + \frac{(E_{\text{ref}})^{Ct_{\text{calibrator}}}}{(E_{\text{target}})^{Ct_{\text{calibrator}}}} \quad (2)$$

In this study the relative expression levels of all targets were measured simultaneously in one target tissue. When the two *RYS1* alleles have identical mRNA expression levels it can be assumed that their HPRT:AS1 and HPRT:AS2 ratios will also be identical. Therefore, the two can be compared as long as the amplification efficiencies are corrected for. This eliminates the need for a calibrator, and if preferred also the reference. Here, the reference

Table 5: Intra-assay variability of real-time AS-PCR when screening #1 (n = 3)

Experimental run #	AS1			AS2			HPRT		
	Mean Ct	Stdev	CV%	Mean Ct	Stdev	CV%	Mean Ct	Stdev	CV%
1	23.66	0.13	0.54	24.08	0.10	0.40	27.21	0.12	0.42
2	23.65	0.06	0.06	24.13	0.02	0.09	27.16	0.05	0.19
3	23.53	0.27	1.14	24.08	0.11	0.11	27.09	0.12	0.43

Stdev. = Standard deviation, CV%= coefficient of variance.