

# Validation of Reference Genes for qPCR Analysis of Resistance Training and Androgenic Anabolic Steroids on Hypothalamus, Adrenal Gland and Fat Tissue

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## Abstract

**Background:** Real-time quantitative Polymerase Chain Reaction (qPCR) is a technique used for quantification of gene expression and the use of reference genes is very important to normalize the quantification results.

**Aim:** To validate the most suitable reference genes for resistance exercise training (REx) and use of nandrolone decanoate (DECA) in three different rat tissues.

**Methods:** A total of 40 adult male Wistar rats were distributed into four groups: exposed to vehicle three times per week (wk) (CT); eight wk of REx exposed to vehicle three times per wk (T); exposed to DECA three times per wk (D); eight wk of REx exposed to DECA three times per wk (TD). Stability of the following genes was evaluated: *beta actin (Actb)*, *alpha Tubulin (Tubulin)*, *Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)*, *Hypoxanthine phosphoribosyltransferase-1 (Hprt1)* and *18s Ribosomal RNA (18s)* in hypothalamus, adrenal gland and mesenteric fat tissue using GeNorm, NormFinder and BestKeeper software.

**Results:** In hypothalamus and adrenal, all genes were suitable and none was rejected by statistical analysis; however, in fat tissue, *Actb*, *Gapdh* and *Hprt1* genes were rejected by geNorm but not the others two software.

**Conclusion:** In hypothalamus and adrenal all selected genes analyzed were stable and can be used for qPCR gene expression analysis. However, in fat tissue we suggest the *Tubulin* gene as most stable gene.

**Keywords:** qPCR; Endogenous control gene; Resistance training; Androgenic anabolic steroids; Rats

**Abbreviations:** 18s: 18s Ribosomal RNA; AAS: Androgenic Anabolic Steroids; *Actb*: Beta Actin; Bp: Base Pair; Cq: Cycle Quantification; CT: Control Group; D: Nandrolone Group; DECA: Nandrolone Decanoate; *Gapdh*: Glyceraldehyde-3-phosphate Dehydrogenase; Hypoxanthine; *Hprt1*: Phosphoribosyltransferase-1; REx: Exercise Training; qPCR: Real-time Quantitative Polymerase Chain Reaction; T: Training group; TD: Training and Nandrolone Group; *Tubulin*: Alpha Tubulin; Wk: Week

## Introduction

Real-time quantitative chain reaction (qPCR) relative or absolute analysis requires appropriated endogenous gene as reference gene for data normalization, which are known by: housekeeping gene, normalization gene, endogenous control gene, internal reference gene and suitable reference genes [1]. The reference gene is used to normalize the target gene expression and, for this reason, the incorrect choice of reference genes can alter final results [1].

Our group has validated reference genes for rat models of sleep deprivation [2] and hypoxia [3]. To our knowledge, there are no studies concerning validation of reference genes to analyze the effects of resistance exercise (REx) and androgenic anabolic steroids (AAS) use on gene expression. A good reference gene must show minimum variation of expression in all experimental groups or, in other words, its expression should not be influenced by experimental conditions. Considering the increasing number of exercise-related and/or anabolic steroids articles, validation of the most stable reference genes for qPCR was considered of interest.

AAS are manipulated compounds derivatives of testosterone, whose main function is to isolate the anabolic effect. They are important for the treatment of growth-related diseases, osteoporosis and anemia; however, when used at supraphysiological doses, they may produce

side effects such as water retention, early closing of the bone epiphysis [4], aggressiveness [5,6], irritability, hostility, cognitive symptoms such as distractibility, forgetfulness and confusion, testicular atrophy, changes in the prostate and seminal vesicles, gynecomastia, growth changes [7], development of hepatic cysts [5], cardiovascular events such as myocardial infarction, cerebral infarction and pathological hypertrophy, increasing the likelihood of arrhythmias and stroke [8,9]. Thus, despite the abovementioned risks and being prohibited in many countries, athletes and amateur practitioners use supra physiological dosages of steroids to increase the performance and free fat mass. Resistance exercise, commonly called weight training, is a type of exercise that has as main objective muscle strength gaining. The authors suggest that REx training is a valid strategy to improve blood pressure, insulin resistance, muscle mass and reduce circulating levels of inflammatory markers [10]. Furthermore, physiological and psychological benefits of REx are considered important in physical rehabilitation and treatment programs [11]. Therefore, a number of gene expression and molecular biological studies has been conducted in REx training and steroids models, including ladder exercise models [12-15]. There is a methodological gap, in which there is a lack of studies that identify the best reference gene in these areas of knowledge.

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It is known that there is no universal reference gene although *Actb*, *Gapdh*, *Hprt1* and  $\beta 2M$ , among others are the most commonly used [16]. There are no studies regarding reference genes in this area; therefore, the aim of this study was to validate reference genes for REX and AAS use in rat hypothalamus, adrenal gland and mesenteric fat tissue.

## Materials and Methods

### Animals

The study was performed using 40 male Wistar rats (10-wk-old; 300-350 g) from CEDEME (Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia). Animals were maintained in the Department of Psychobiology facility (Universidade Federal de São Paulo). Room temperature was 22°C ( $\pm 1$ ) with 12:12 h light-dark cycle and access to food and water *ad libitum* was allowed. This study was conducted according to the Ethical of the use of Laboratory Animals Guidelines and its experimental protocol was approved by the Ethical Committee of Universidade Federal de São Paulo (#177700/2013).

### Groups

A total of 40 Wistar rats (10 wk old) was distributed into four groups: exposed to vehicle (peanut oil-subcutaneous administration 1 ml/kg) 3 times/wk during 8 wk (CT); resistance exercise during 8wk and exposed to vehicle (peanut oil-subcutaneous administration 1 mL/kg) 3x/wk during 8 wk (T); exposed to DECA (subcutaneous administration 5 mg/kg) 3 times/wk during 8 wk (D); submitted to resistance exercise during 8 wk and exposed to DECA (subcutaneous administration 5 mg/kg) 3 times/wk during 8 wk (TD).

### Exercise training and drug treatment

The training protocol consisted of progressive REX, 5 times/wk during 8 wk [adapted from 17-19]. A vertical ladder (110 cm high by 18 cm wide, inclined at 80° with 2 cm spacing between rungs) was used; at the top of the ladder there was a dark box (20 cm  $\times$  20 cm  $\times$  20 cm), where the animal could rest between sets (1 min). Every week, the maximum carrying loading (MCL) was tested, so that the periodization could be determined (Table 1).

Supraphysiological nandrolone decanoate doses (5 mg/kg) were injected subcutaneously to each animal 3 times/wk for 8 wk (15 mg/kg/wk). This dosage was chosen for being equivalent to that used by athletes in physical exercise [20,21]. The peanut oil was used as vehicle at the same volume of DECA (1 ml/kg).

### Gene selection

Exercise training and androgenic anabolic steroids affect several systems at cellular level; thus, candidate reference genes were selected

among the most common reference genes from animal models in the literature. Reference genes selected were *beta actin (Actb)*, *alpha Tubulin (Tubulin)*, *glyceraldehyde-3-phosphate dehydrogenase (Gapdh)*, *hypoxanthine phosphoribosyltransferase-1 (Hprt1)* and *18s ribosomal RNA (18s)*. Primers (Table 2) were designed and synthesized by IDT (Integrated DNA Technologies - www.idtdna.com) according to published Genbank sequences.

### RNA extraction, cDNA and qPCR

After 24 h of experimental issue, the animals were euthanatized by decapitation between 07:00 am to 10:00 am. Fasting for at least 2 hours was established.

The hypothalamus and adrenal gland were collected and RNA extraction was performed using TRizol<sup>®</sup> Plus RNA Purification Kit (CAT#12183-555 Ambion RNA, Life Technologies). Mesenteric fat tissue was also collected and total RNA was extracted using the RNeasy Plus Universal Mini Kit (CAT#73404, QIAGEN), according to manufacturer's specifications. RNA was pretreated with DNase I (2 U/ $\mu$ l), 10X DNase I Buffer (100 mM Tris- pH 7.5, 25 mM MgCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>) and incubated for 37°C for 30 sec (Invitrogen) according to manufacturer's specifications. The 28S and 18s integrity of RNA was evaluated using agarose gel electrophoresis. RNA quantification was performed using spectrophotometry (NanoDrop) and purity was evaluated using two optimal wavelengths: ratio of 260/280 for nucleic acids (1.8<sample>2.2) and ratio of 260/230 for organics contaminations (1.8<sample>2.2).

cDNAs were synthesized using 1  $\mu$ g of total RNA were placed in the presence of first mixture containing 0.5  $\mu$ g/ $\mu$ l of Random Primers (Promega) and 3 mM of MgCl<sub>2</sub> (Promega), after that incubated at 70°C for 5 minutes. Then were placed in second mixture using Reaction Buffer 5X (Promega), 25 mM of deoxyribonucleotide triphosphates (dNTP), 40 U/ $\mu$ l of RNase inhibitor (RNasin) (Promega) and enzyme Improm II (Reverse Transcriptase) (Promega). The final volume was 20  $\mu$ l. The conditions used for reverse transcription were as follows: 25°C for 5 min, 42°C for 60 min and 70°C for 15 min, according to manufacturer's specifications.

qPCR was performed using SYBR Green PCR Master Mix (AppliedBiosystem, Warrington, UK) and StepOnePlus Real-Time PCR (AppliedBiosystem, Warrington, UK). Each reaction was performed using 2  $\mu$ l of cDNA, 6  $\mu$ l of H<sub>2</sub>O, 2  $\mu$ l of primers (forward and reverse at 0.5  $\mu$ M each) and 10  $\mu$ l SYBR green PCR Master Mix to the final volume of 20  $\mu$ l. All samples were analyzed in duplicates and the average values were used. Design layout was: Holding stage: 3 min at 50°C and 10 min at 95°C; Cycling stage (no of cycles: 40): 15 sec at 95°C and 30 sec at 60°C. Melt curve stage: 15 sec at 95°C and 60° up 3°C each 15 sec to 95°C.

### Data analysis

**GeNorm, NormFinder and BestKeeper:** Three software were used to assess the stability of selected reference genes by different methods. All software are freely available to download permanently or as a demo for free for 14 days: geNorm (<https://www.biogazelle.com/qbaseplus/>); NormFinder (<http://moma.dk/normfinder-software/>); and

BestKeeper (<http://www.gene-quantification.de/bestkeeper.html#download>).

The software geNorm uses the M-value as a stability variable, directly assessing linear scale expression quantities by using the standard curve and absolute quantification. The gene with the lower value of M

Sessions/wk	1 wk	2 wk	3 wk	4 wk*	5 wk*	6 wk*	7 wk*#	8 wk**
1 <sup>st</sup> Session	50	50	50	50	50	50	50	75
2 <sup>nd</sup> Session	50	50	50	50	50	50	75	75
3 <sup>rd</sup> Session	50	75	75	75	75	75	75	75
4 <sup>th</sup> Session	75	75	75	75	75	75	75	75
5 <sup>th</sup> Session	75	75	75	75	90	90	90	90
6 <sup>th</sup> Session	75	75	90	90	90	90	90	90
7 <sup>th</sup> Session		90	90	90	100	100	100	100
8 <sup>th</sup> Session				100	100	100	100	100

3<sup>rd</sup> day of training, all session were done using 50% of MCL. # 4<sup>th</sup> day, day-off. \*\* 4<sup>th</sup> day, all session was done using 75% of MCL. MCL (Maximum Carrying Loading).

**Table 1:** Resistance training periodization in % of MCL.

Gene	ID GeneBank	Forward (5' – 3')	Reverse (5' – 3')	Bp	Efficiency*	T°C	Primer [ ]
<i>Beta Actin</i>	NM_031144.3	GTGTGGATTGGTGGCTCTATC	CAGTCCGCCTAGAAGCATT	122	Hypothalamus: 97.8% Adrenal Gland: 100.7% Fat Tissue: 97.4%	60°C	I: 10 µM F: 0.5 µM
<i>Alpha Tubulin</i>	NM_022298.1	GACCTGGAACCCACAGTTATT	ATCTTCCTGCCTGTGATGAG	90	Hypothalamus: 97.3% Adrenal Gland: 100.8% Fat Tissue: 98.8%	60°C	I: 10 µM F: 0.5 µM
<i>Gapdh</i>	NM_017008.4	CATGGCCTCCGTGTTCTTA	GCGGCATGTCAGATCCA	55	Hypothalamus: 103.6% Adrenal Gland: 102.6% Fat Tissue: 101.6%	60°C	I: 10 µM F: 0.5 µM
<i>Hprt1</i>	NM_012583	GCGAAAGTGAAAAGCCAAGT	GCCACATCAACAGGACTCTTGAG	76	Hypothalamus: 98.7% Adrenal Gland: 98.0% Fat Tissue: 99.0%	60°C*	I: 10 µM F: 0.5 µM
<i>18s</i>	NR_046237	CGGACAGGATTGACAGATTG	CAAATCGCTCCACCACTAA1	83	Hypothalamus: 99.5% Adrenal Gland: 94.0% Fat Tissue: 97.8%	60°C	I: 10 µM F: 0.5 µM

Bp: Base pair; T°C: Temperature; Primer [ ]: Primer concentration in initial and final volume; I: Initial; F: Final. \*In fat tissue, was the addition of stretch of 30 sec at 72°C for cycle. \*r<sup>2</sup>>99 was established. The amplification efficiency is calculated using the slope of the regression line in the standard curve.

Table 2: Reference genes ID and primers design.

is considered the “most stable”. NormFinder also uses the values from absolute quantification to calculate stability, which indicates as Stability value the best candidate by the lower value. BestKeeper uses the HKG index which calculates the geometric average of the “most stable” reference genes by Repeated Pair-wise Correlation Analysis and p-value (p<0.05). GeNorm and Normfinder use 2<sup>-ΔCt</sup> and the BestKeeper uses Cq values for analysis.

## Results

### Cycle quantification (Cq) distribution

The results related to fractional qPCR cycles are represented as follow: 1) Hypothalamus (Figure 1)-*Gapdh* showed the lowest standard deviation (± 0.69), followed by *Actb* (± 1.27), *Hprt1* (± 1.35), *Tubulin* (± 1.69) and *18s* (± 2.05); 2) Adrenal gland (Figure 2)-results showed that *Hprt1* had the lowest standard deviation (± 0.96) followed by *Gapdh* (± 1.25), *Actb* (± 1.33), *Tubulin* (± 1.87) and *18s* (± 1.92); 3) Fat tissue (Figure 3), *Gapdh* showed the lowest variation Cqs (± 1.52), followed by *Hprt1* (± 1.65), *Tubulin* (± 2.26), *18s* (± 2.48) and *Actb* (± 2.83).

### BestKeeper analysis

In hypothalamus, when comparing all experiments groups, CT vs. D, CT vs. D or CT vs. TD the most stable gene was *18s* gene followed by *Actb*, *Tubulin*, *Hprt1* and *Gapdh* genes.

For adrenal gland, *18s* gene was the most stable when analyzing all groups, CT vs. D, and CT vs. T vs. D groups and the less stable was *Hprt1*. Rank sequence are available in Table 3.

In fat tissue when all groups were compared, as well as CT vs. T, the most stable gene was *Tubulin* followed by *Actb*, *18s*, *Hprt1* and *Gapdh*. However, when CT vs. D and CT vs. TD were compared *Tubulin* remains the most stable gene and *Hprt1* was rejected (Table 3).

### Normfinder analysis

Analysis in Normfinder software showed that all genes in all groups were suitable, in other words, all genes showed stability values less than 0.15 [22], value considered by software. Moreover, this software showed best combination and stability value of two genes, e.g. in hypothalamus when compared all groups the best combination stability were *Actb* and *18s* with 0.02 M-value. The other values are presented in Table 4.

In hypothalamic tissue, the *Actb* gene was the most stable gene when compared all groups and CT vs. D groups. Moreover, when comparing

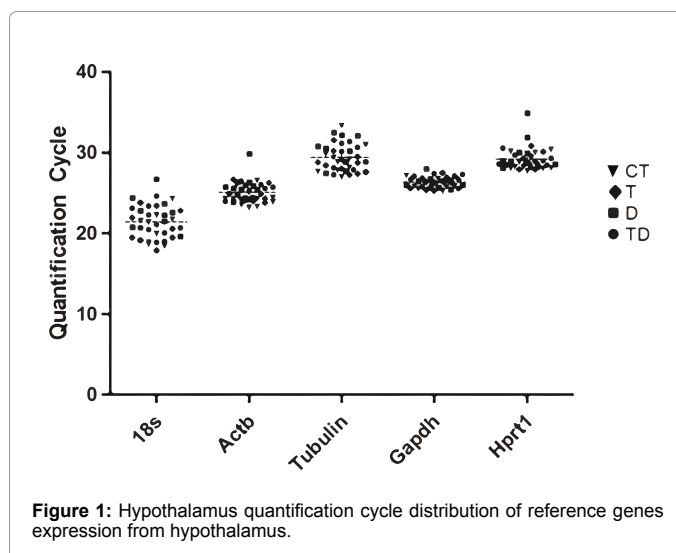


Figure 1: Hypothalamus quantification cycle distribution of reference genes expression from hypothalamus.

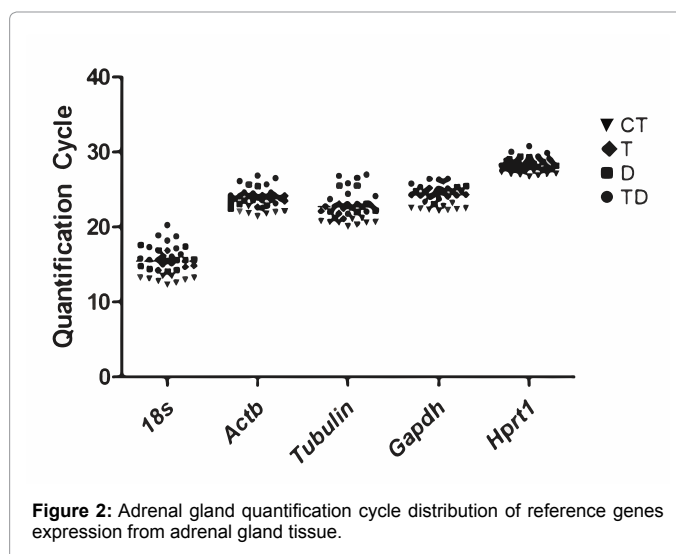


Figure 2: Adrenal gland quantification cycle distribution of reference genes expression from adrenal gland tissue.

CT vs. T and CT vs. TD groups, the *Hprt1* gene was considered the most stable gene. When all groups were analyzed the following rank was obtained: *Actb*, *18s*, *Hprt1*, *Tubulin* and *Gapdh* genes. When

comparing CT vs. D groups, *Actb*, followed by *18s*, *Tubulin*, *Hprt1* and *Gapdh* genes. At CT vs. T and CT vs. TD groups, *Hprt1*, followed by *18s*, *Tubulin*, *Gapdh* and *Actb* genes.

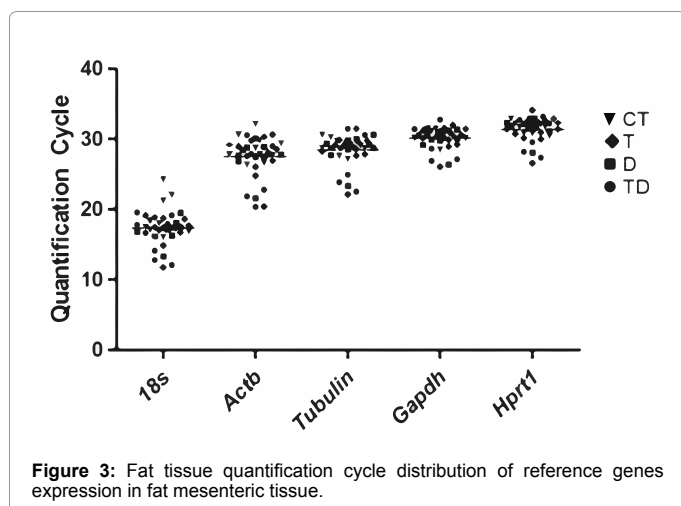
At adrenal gland, the ranks considering all groups and CT vs. TD groups were: *18s*, followed by *Tubulin*, *Gapdh*, *Actb* and *Hprt1* genes; for the CT vs. T groups: *Tubulin*, followed by *Actb*, *18s*, *Gapdh* and *Hprt1* genes; and for the CT vs. D groups: *Tubulin*, followed by *18s*, *Gapdh*, *Hprt1* and *Actb* genes.

The *Tubulin* gene was considered the most stable gene for fat tissue in all moments and *Gapdh* was the less stable gene, except in CT vs. D the *Hprt1* gene was the less suitable gene (Table 4).

### GeNorm analysis

Analysis made by software showed that in hypothalamus and adrenal gland all candidates are stable and could be used as reference genes but in mesenteric fat tissue, only *Tubulin* gene was stable in all analysis. The others genes (*Hprt1*, *Actb*, *18s* and *Gapdh* genes) had a 1.5 M-value and it, was not considered good reference genes at least one time.

In hypothalamus the most stable gene was *Actb* when comparing



all groups, CT vs. D and CT vs. TD groups, followed by *Hprt1*, *Tubulin*, *Gapdh* and *18s* genes; and for the CT vs. T groups: *Actb*, followed by *Hprt1*, *Gapdh*, *Tubulin* and *18s* genes.

In adrenal gland, it was observed that the *Hprt1* gene had the lower rank in all analysis. In all groups and CT vs. TD groups the *Actb* gene had the higher rank, following by *Gapdh*, *Tubulin*, *18s* and *Hprt1* genes. For the CT vs. T groups, *Gapdh* was the most stable gene, followed by *Actb*, *Tubulin*, *18s* and *Hprt1* genes; and for the CT vs. D, *Gapdh* was most suitable gene followed by *Actb*, *18s*, *Tubulin* and *Hprt1* genes.

Considering the mesenteric fat tissue the unique stable gene was the *Tubulin* in all analyzes. *Hprt1* was stable when all groups and CT vs. T were compared. The other genes exceeded the M-value allowed by the software (Table 5).

### Discussion

To determination of relative expression of target gene in any assay there is a necessity of use a reference gene. Accordingly, the reference gene is usually an endogenous gene in which expression is unchanged regardless of intervention [23]. The use of reference genes is appropriated only if they are tested, normalized and considered stable, some authors believe that it is wrong any gene as reference gene without validating their suitability before running the experiment [24]. In this study, this amount of genes gives us an overview of what can be used in the experiments using AAS and REX.

A simple way to find the most stable reference gene is to analyze the Cq variation and use the one with the lowest variation between experimental groups [25]. However, there are specific software for this type of analysis; the most used in the literature are BestKeeper, Normfinder and geNorm [24]. To our knowledge, there are no studies investigating the most stable genes regarding exercise and anabolic steroids use; therefore, our study will contribute to a more adequate choice of reference genes for those experiments. We separate the analysis group to group to have a more complete analysis, so analyze all groups and then each separate factor, so there is an interpretation according to each intervention.

The analysis of more than one reference gene has been shown to be useful to validate the data, as well as to confirm the results. The authors suggest the use of three reference genes; if the results with the first

Tissue	Hypothalamus					Adrenal gland					Mesenteric fat				
	CT, D, T and TD					CT, D, T and TD					CT, D, T and TD				
Gene	18s	Actb	Tubulin	Hprt1	Gapdh	18s	Tubulin	Actb	Gapdh	Hprt1	Tubulin	Actb	18s	Hprt1	Gapdh
<b>r</b>	0.96	0.93	0.91	0.89	0.77	0.98	0.97	0.96	0.95	0.86	0.98	0.95	0.89	0.86	0.82
<b>p-value</b>	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Groups	CT vs. T					CT vs. T					CT vs. T				
Gene	18s	Actb	Tubulin	Hprt1	Gapdh	18s	Gapdh	Tubulin	Actb	Hprt1	Tubulin	Actb	18s	Hprt1	Gapdh
<b>r</b>	0.94	0.91	0.88	0.88	0.68	0.98	0.98	0.96	0.95	0.87	0.96	0.92	0.85	0.78	0.64
<b>p-value</b>	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002
Groups	CT vs. D					CT vs. D					CT vs. D				
Gene	18s	Actb	Tubulin	Hprt1	Gapdh	18s	Tubulin	Gapdh	Actb	Hprt1	Tubulin	Actb	18s	Gapdh	Hprt1
<b>r</b>	0.98	0.96	0.92	0.90	0.85	0.98	0.96	0.95	0.95	0.81	0.92	0.88	0.87	0.57	0.234
<b>p-value</b>	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.008	0.321
Groups	CT vs. TD					CT vs. TD					CT vs. TD				
Gene	18s	Tubulin	Actb	Hprt1	Gapdh	18s	Tubulin	Actb	Gapdh	Hprt1	Tubulin	Actb	18s	Gapdh	Hprt1*
<b>r</b>	0.96	0.96	0.88	0.87	0.79	0.99	0.98	0.97	0.96	0.94	0.97	0.96	0.92	0.73	0.025
<b>p-value</b>	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.00	0.922

Data expressed as Pair-Wise Analysis Correlation (r). Higher r correlation indicates the most stable gene. p<0.05 was used in Pearson Correlation. CT: Control Group; D: DECA Group; T: Training Group; TD: Training and DECA Exposed Group. \*Result above the limit considered adequate by software analysis.

**Table 3:** Ranking of reference genes by Bestkeeper software analysis.



Tissue	Hypothalamus					Adrenal gland					Mesenteric fat				
Groups	CT, D, T and TD					CT, D, T and TD					CT, D, T and TD				
Genes	<i>Actb</i>	<i>18s</i>	<i>Hprt1</i>	<i>Tubulin</i>	<i>Gapdh</i>	<i>18s</i>	<i>Tubulin</i>	<i>Gapdh</i>	<i>Actb</i>	<i>Hprt1</i>	<i>Tubulin</i>	<i>18s</i>	<i>Hprt1</i>	<i>Actb</i>	<i>Gapdh</i>
Stability Value	0.04	0.04	0.05	0.05	0.06	0.03	0.03	0.05	0.05	0.06	0.00	0.02	0.02	0.02	0.02
Best Comb.	<i>Actb and 18s</i>					<i>Tubulin and 18s</i>					<i>18s and Tubulin</i>				
Stability value for best combination	0.02					0.03					0.01				
Groups	CT vs. T					CT vs. T					CT vs. T				
Genes	<i>Hprt1</i>	<i>18s</i>	<i>Tubulin</i>	<i>Gapdh</i>	<i>Actb</i>	<i>Tubulin</i>	<i>Actb</i>	<i>18s</i>	<i>Gapdh</i>	<i>Hprt1</i>	<i>Tubulin</i>	<i>Actb</i>	<i>Hprt1</i>	<i>18s</i>	<i>Gapdh</i>
Stability Value	0.05	0.07	0.07	0.08	0.08	0.04	0.05	0.05	0.06	0.08	0.00	0.00	0.00	0.01	0.01
Best Comb.	<i>Hprt1 and Gapdh</i>					<i>Actb and 18s</i>					<i>Actb and Tubulin</i>				
Stability value for best combination	0.05					0.03					0.00				
Groups	CT vs. D					CT vs. D					CT vs. D				
Genes	<i>Actb</i>	<i>18s</i>	<i>Tubulin</i>	<i>Hprt1</i>	<i>Gapdh</i>	<i>Tubulin</i>	<i>18s</i>	<i>Gapdh</i>	<i>Hprt1</i>	<i>Actb</i>	<i>Tubulin</i>	<i>Actb</i>	<i>Gapdh</i>	<i>18s</i>	<i>Hprt1</i>
Stability Value	0.01	0.03	0.04	0.05	0.05	0.03	0.03	0.05	0.05	0.06	0.00	0.01	0.01	0.01	0.01
Best Comb.	<i>Actb and 18s</i>					<i>Tubulin and 18s</i>					<i>Actb and Tubulin</i>				
Stability value for best combination	0.02					0.03					0.00				
Groups	CT vs. TD					CT vs. TD					CT vs. TD				
Genes	<i>Hprt1</i>	<i>18s</i>	<i>Tubulin</i>	<i>Gapdh</i>	<i>Actb</i>	<i>18s</i>	<i>Tubulin</i>	<i>Gapdh</i>	<i>Actb</i>	<i>Hprt1</i>	<i>Hprt1</i>	<i>18s</i>	<i>Tubulin</i>	<i>Actb</i>	<i>Gapdh</i>
Stability Value	0.06	0.06	0.07	0.07	0.07	0.03	0.04	0.06	0.06	0.06	0.00	0.00	0.01	0.01	0.03
Best Comb.	<i>Hprt1 and 18s</i>					<i>Tubulin and 18s</i>					<i>18s and Hprt1</i>				
Stability value for best combination	0.04					0.03					0.00				

Data expressed as Stability value. Lowest Stability value indicates most stable gene. Best Comb. (Best combination of two genes); CT: Control Group; D: DECA group; T: Training Group; TD: Training and DECA Exposed Group.

**Table 4:** Ranking of reference genes by the NormFinder software analysis.

Tissue	Hypothalamus					Adrenal gland					Mesenteric fat				
Groups	CT, D, T and TD					CT, D, T and TD					CT, D, T and TD				
Genes	<i>Actb</i>	<i>Hprt1</i>	<i>Tubulin</i>	<i>Gapdh</i>	<i>18s</i>	<i>Actb</i>	<i>Gapdh</i>	<i>Tubulin</i>	<i>18s</i>	<i>Hprt1</i>	<i>Tubulin</i>	<i>Hprt1</i>	<i>Gapdh</i>	<i>Actb</i>	<i>18s</i>
M-Value	0.95	1.02	1.14	1.23	1.26	0.75	0.77	0.87	0.88	0.99	1.21	1.39	1.50*	1.63*	1.79*
Groups	CT vs. T					CT vs. T					CT vs. T				
Genes	<i>Actb</i>	<i>Hprt1</i>	<i>Gapdh</i>	<i>Tubulin</i>	<i>18s</i>	<i>Gapdh</i>	<i>Tubulin</i>	<i>Actb</i>	<i>18s</i>	<i>Hprt1</i>	<i>Tubulin</i>	<i>Hprt1</i>	<i>Gapdh</i>	<i>Actb</i>	<i>18s</i>
M-Value	0.93	0.94	1.14	1.22	1.35	0.43	0.49	0.50	0.63	0.69	1.32	1.50*	1.69*	1.79*	2.01*
Groups	CT vs. D					CT vs. D					CT vs. D				
Genes	<i>Actb</i>	<i>Tubulin</i>	<i>Hprt1</i>	<i>18s</i>	<i>Gapdh</i>	<i>Gapdh</i>	<i>Actb</i>	<i>Tubulin</i>	<i>18s</i>	<i>Hprt1</i>	<i>Tubulin</i>	<i>Hprt1</i>	<i>Gapdh</i>	<i>Actb</i>	<i>18s</i>
M-Value	0.96	1.14	1.14	1.16	1.36	0.66	0.69	0.78	0.79	0.94	1.25	1.44	1.57*	1.63*	2.03*
Groups	CT vs. TD					CT vs. TD					CT vs. TD				
Genes	<i>Actb</i>	<i>Hprt1</i>	<i>Tubulin</i>	<i>Gapdh</i>	<i>18s</i>	<i>Actb</i>	<i>Gapdh</i>	<i>Tubulin</i>	<i>18s</i>	<i>Hprt1</i>	<i>Tubulin</i>	<i>Hprt1</i>	<i>Gapdh</i>	<i>Actb</i>	<i>18s</i>
M-Value	0.92	0.95	1.01	1.04	1.21	0.84	0.89	0.95	1.02	1.10	1.34	1.60*	1.72*	1.77*	2.07*

Data expressed as M-Value. Lowest M-value indicates most stable gene. CT: Control Group; D: DECA Group; T: Training Group; TD: Training and DECA Exposed Group. \*Result above the limit considered adequate by software analysis.

**Table 5:** Ranking of reference genes by GeNorm software analysis.

two are different, a third reference gene must be used, and, if they are similar, it is not necessary to evaluate a third gene [26]. Dheda et al. [27] demonstrated after three experiments with different reference genes that the results can be significantly different from those obtained when an invalidated reference gene is used. This incorrect choice, therefore, results may be erroneous. The same authors also suggest strongly supporting the argument for validation of reference genes prior to their use.

We conducted a search in PubMed and selected 20 articles that investigated the effects of physical exercise and/or androgenic anabolic steroids on gene expression, through qPCR (SYBR Green method), in order to verify if there were common reference genes in these studies. Two groups used only the *18s* gene [13,28], five used only *Actb* gene [29-33]. Other authors used the Cyclophilin gene [15,31] and eight authors used *Gapdh* as a reference gene in their studies [12,14,34-40]. Only three studies used more than one gene as internal control: *Gapdh* and *large ribosomal protein P0 (RPLP0)* genes [41]; *Gapdh*, *Actb*, *Hprt1* and *Cyclophilin C* gene [42]; the other authors used *Ubiquitin C* gene as reference gene [43]. Considering the abovementioned articles, the

most used reference genes were *Gapdh* and *Actb* in different species as human, monkey, rat, and mouse.

The results of the reference gene is controversial, some authors did not find stable results for *Actb* and *Gapdh* genes in injured muscle, the results were rejected by geNorm and BestKeeper [44]. In other study, also did not find stable results for *Actb* gene from hypothalamus of an obesity rat model [45]. *Actb* and *Gapdh* genes were rejected in muscle tissue by qBase, software that uses M-Value to analyze reference genes [26]. In our study, *Actb* and *Gapdh* genes were shown to be stable in all tissues analyzed by three different software; the only exception was for mesenteric fat analysis through geNorm, which rejected both genes, along with *Hprt1*.

In hypothalamus and adrenal gland, the three software used in our study showed similar results using different analyses (all groups together or separately, as in CT vs. T, CT vs. D or CT vs. TD). Many types of exercise and/or AAS could alter gene expression in different ways but, in most of our analyses, the values and ranking of genes were similar in all experimental groups. None of the genes were rejected by

any of the software; therefore, they are all suitable reference genes for qPCR analysis in rat hypothalamus and adrenal gland.

In mesenteric fat tissue, however, there were some discrepancies between results. All genes were stable and considered suitable as reference genes by Normfinder, but not by BestKeeper and geNorm analysis. GeNorm shows that the only stability gene was *Tubulin* (in all analysis) and when used Bestkeeper software, the only exception was *Hprt1* gene, showed above the limit considered adequate.

All software used to check the stability of genes are validated and considered replicated. Most often, results are repeatable despite using different calculations, as M-value and pair-wise correlation. Whereas two of the three software considered all stable candidates and only geNorm considered only *Tubulin* as stable, we suggest that the best gene to be used of adipose tissue is indeed *Tubulin*, however, could be used the other candidates for qPCR analysis if confirm with other reference gene.

It is also valid to emphasize that when we analyze different groups with different interventions, the software will also modify the results. When the NormFinder was used for each analysis groups, the software put genes in a different position and we showed the best combination.

In our study the three software utilized in these analyses produced similar results but the order of the results was not identical and in some cases was considerably different, which corroborate the findings reported by other study [46]. This difference may be attributed to different mathematical models used in each program [24]. On the other hand, there was found similar results for the software geNorm and Normfinder but not for BestKeeper, this can be justified by the fact that this software used the Pearson correlation method to classify the reference genes, a different method compared to the others software [3].

This study is the first to validate reference genes for the evaluation of REx and AAS use effects in different rat tissues. It is important to note that there is no ideal universal reference gene. This work can help you find good candidates, though, for each experiment, species, tissue and other conditions, it is necessary to perform and confirm a specific validation of reference genes, in order to analyze gene expression results more adequately.

## Conclusion

In conclusion, our results do not suggest a specific reference gene for hypothalamus and adrenal gland, since all genes analyzed (*Actb*, *18s*, *Hprt1*, *Tubulin* and *Gapdh*) were stable and suitable for gene expression normalization through qPCR. However, in mesenteric fat tissue, the only suitable reference gene accepted by three software was *Tubulin* gene.

## Competing Interests

None of the authors has any conflict of interest in submitting this manuscript.

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## Contribution

Design and experimental procedures: RP, LF, BFAC and VDA. Data analysis: RP, LF and VDA. Contribution with reagents, materials and analysis tools: RP and VDA. Article writer: RP, LF, BFAC and VD.

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