

1 The title is a declarative statement of key finding(s) and includes the species:
2

3 **Transcript Analysis of the Selenoproteome Indicates that Dietary Selenium**
4 **Requirements of Rats Based on Selenium-regulated Selenoprotein mRNA Levels**
5 **are Uniformly Less Than Those Based on Glutathione Peroxidase Activity¹⁻³**

6 Authors' first and last names are given and degrees are not included. List all authors' last names separately, and include names as they should appear in PubMed, if different.

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8 Author Last Names for PubMed indexing: Barnes Evenson, Raines, Sunde

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11 NUMBER OF TABLES: 1

This information is required for all submitted papers.

This is required if the paper includes online supplementary material.

12 SUPPLEMENTARY MATERIAL: Online Supporting Materials: 1 figure, 5 tables

13 A running head of < 48 characters and spaces is included.

14 RUNNING TITLE: Rat Selenoprotein Regulon

15 **FOOTNOTES**

Required footnotes are included concerning financial support, conflicts of interest, supplementary material, current addresses, contact information for the corresponding author, and an alphabetized footnote of all author-defined abbreviations.

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20 conflicts of interest.

This required wording is used for this footnote:

21 ³Supplemental Figure 1 and Supplemental Tables 1-4 are available as Online

22 Supporting Material with the online posting of this paper at <http://jn.nutrition.org>.

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All author-defined abbreviations are listed in alphabetical order.

26 ⁵Abbreviations used: Actb, β -actin; Dio, iodothyronine deiodinase; Gpx1, glutathione

27 peroxidase-1; Gpx3, plasma glutathione peroxidase; Gpx4, phospholipid

28 hydroperoxide glutathione peroxidase; Gapdh, glyceraldehyde-3-phosphate

29 dehydrogenase; qRT-PCR, quantitative real time polymerase chain reaction; Se,

30 selenium; selenophosphate synthetase-2, Sephs2; Selh, selenoprotein H;

31 Sepw1, selenoprotein W; Sepp1, selenoprotein P; Sepx1, methionine-R-sulfoxide

32 reductase; Txnrd, thioredoxin reductase.

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34

Abstract

Abbreviations are defined the first time used in the Abstract.

Dietary Selenium (Se) requirements in rats have been based largely upon glutathione peroxidase (Gpx1) enzyme activity, and Gpx1 mRNA levels can also be used to determine Se requirements. The identification of the complete selenoprotein proteome

suggests that we might identify additional useful molecular biomarkers for assessment

of Se status. To characterize Se regulation of the entire rat selenoproteome, weanling male rats were fed a Se-deficient diet (<0.01 µg Se/g) supplemented with graded levels of Se (0-0.8 µg/g diet) for 28 d, Se status was determined by tissue Se concentration

and selenoenzyme activity, and selenoprotein mRNA abundance in liver, kidney, and

muscle was determined by qRT-PCR. Tissue Se and selenoenzyme biomarkers

indicated that minimal Se requirements were at or below 0.1 µg Se/g diet for most

biomarkers. Liver Gpx1 mRNA also decreased to <10% of Se adequate levels, with a

minimum Se requirement at 0.07 µg/g diet. Five selenoprotein mRNAs in liver, 4 in

kidney, and 2 in muscle decreased to <41% of Se adequate levels, all with minimum Se

requirements at ≤0.07 µg/g diet; the majority of selenoprotein mRNAs in each tissue

were not significantly regulated by Se status, and one selenoprotein, Sephs2, was up-

regulated in Se-deficient kidney. Plateau breakpoints for all regulated selenoprotein

mRNAs were very similar, suggesting that one underlying mechanism is in play in Se

regulation of selenoprotein mRNAs. Lastly, we did not find any selenoprotein mRNAs

that could be used as biomarkers for super-nutritional/anticarcinogenic levels (up to 0.8

µg Se/g diet) of Se. The abstract is not longer than 250 words.

55

56

The reason for conducting the study is stated.

The experimental design is described briefly.

Standard JN units and abbreviations are used.

A concluding statement concerning the importance of the findings is included.

The paper is written in past tense; present tense is reserved for prevailing knowledge and overall conclusions.

Manuscript sections are labeled.

Literature citations are called out sequentially.

57 **Introduction**

Abbreviations are defined the first time used in the text.

Dietary selenium (Se^5) requirements for the rat were first reported to be 0.04 $\mu\text{g/g}$ diet based on prevention of liver necrosis (1) and 0.05 $\mu\text{g Se/g}$ diet based on maintenance of growth in weaning rats (2). The discovery that Se is an essential cofactor for glutathione peroxidase (Gpx) and that Gpx activity drops dramatically in Se deficiency (3) provided a biochemical marker that was used to establish a dietary requirement of 0.1 $\mu\text{g Se/g}$ diet in the rapidly growing rat (4,5). Discovery of additional selenoproteins, such as Gpx4, selenoprotein P (Sepp1), iodothyronine deiodinase (Dio), thioredoxin reductase-1 (Txnrd1), selenoprotein W (Sepw1), and Gpx3, provided further biomarkers of Se status that also indicate that the dietary Se requirements are 0.1 $\mu\text{g Se/g}$ diet or less (6-11). The current dietary Se requirements (12) now cite 0.15 $\mu\text{g Se/g}$ diet as the requirement for the growing rat; this level includes a safety factor and is based in part on prevention of cataract development in rats fed high sucrose diets.

Gpx1 mRNA level also drops dramatically in Se deficiency in rats (13), increases sigmoidally with increasing dietary Se, and reaches well-defined plateaus (6,14-16), providing a molecular biology-based biomarker for Se status. Dietary Se requirements based on hepatic Gpx1 mRNA levels are $\sim 0.05 \mu\text{g Se/g}$ diet for both male and female rats, even though female rats have twice the level of liver Gpx1 mRNA as well as Gpx1 activity as compared to male rats (14). In contrast, Gpx4 mRNA levels are not regulated by Se status in rats, eliminating use of Gpx4 mRNA as a molecular biomarker for assessing Se requirements (6). Effects of Se status on mRNA levels for several other selenoproteins have also been studied, including Sepp1, Txnrd1, and Dio1 (6,9,14-17), but these mRNAs do not fall as dramatically as does Gpx1 mRNA in liver and other

Lines are numbered sequentially throughout the paper.

The objectives of the study are stated but results are not given in the Introduction.

103 To characterize the complete selenoprotein regulon in the rat, we conducted two
104 experiments by feeding graded levels of dietary Se to young, rapidly growing rats. Our
105 objectives were to expand the molecular biology biomarkers for Se status to include the
106 full selenoproteome in the well-characterized rat model, to expand the selenoprotein
107 regulon analysis to include multiple tissues, and to use super-nutritional levels of dietary
108 Se in this characterization of the selenoprotein regulon.

The supplier of animals is included.

109 **Methods**

110 **Reagents.** Molecular biology reagents were purchased from Promega (Madison, WI),
111 Invitrogen (Carlsbad, CA) or Sigma (St. Louis, MO). All other chemicals were of
112 molecular biology or reagent grade.

A *Journal of Nutrition* (or *American Journal of Clinical Nutrition*) paper that gives the diet composition is cited or a diet composition table provided (see Sample Tables).

113 **Animals and Diets.** *Experiment 1.* Male w
114 from Holtzman (Madison, WI) and housed individually in hanging wire-mesh cages. The
115 basal diet was a Se-deficient torula-yeast diet (0.007 $\mu\text{g Se/g}$ by analysis),
116 supplemented with 100 mg/kg of all-rac- α -tocopherol acetate to ensure prevention
117 liver necrosis, and supplemented with 0.4% L-methionine to ensure adequate growth,
118 as described previously (14,16) . Rats were allocated randomly to dietary treatments
119 and fed the basal diet supplemented with graded levels of Se (0, 0.02, 0.05, 0.075, 0.1,
120 0.15, 0.2, or 0.3 $\mu\text{g Se/g}$ as Na_2SeO_3) for 28 d (4 rats/treatment). *Experiment 2.* Male
121 weanling rats (n=60; 21 d old) were obtained from Holtzman (Madison, WI) and treated
122 as in Expt. 1. Rats were fed the basal Se-deficient diet (0.005 $\mu\text{g Se/g}$ by analysis)
123 supplemented with graded levels of Se (0, 0.016, 0.04, 0.06, 0.08, 0.12, 0.16, 0.24, 0.4
124 or 0.8 $\mu\text{g Se/g}$ as Na_2SeO_3 for 28 d (6 rats/treatment). Body weight was measured bi-
125 weekly. Animals had free access to feed and water, and the care and treatment

Specific compounds are given using JN nomenclature.

The committee and institution that approved the study are specified.

126 protocols were approved by the Institutional Animal Care and Use Committee at the
127 University of Missouri (Expt. 1) and University of Wisconsin (Expt. 2).

128 **Tissue Analysis.** Rats were anesthetized with ether, and blood was drawn by cardiac
129 puncture using EDTA-containing syringes. Livers were perfused in situ with ice-cold
130 0.15 mol/L KCl and liver, kidney and muscle were removed and quick-frozen in liquid
131 nitrogen (16). Blood was centrifuged (1500 X g, 15 min, 4°C, Eppendorf 5415R, F-45-
132 24-11 rotor, Brinkmann, Westbury, NY) to separate plasma from red cells and the red
133 cells were reconstituted to original volume using saline phosphate buffer (76 mmol/L
134 NaCl, 50 mmol/L sodium phosphate, pH 7.4). Liver and kidney (only Expt. 2), and diet
135 Se concentrations (Expts. 1 and 2) were determined by neutron activation analysis (22).

136 **Enzyme Activity Assays.** Liver, kidney, and muscle were homogenized in 9 volumes
137 of sucrose buffer (20 mmol/L tris/HCl, pH 7.4, 0.25 mol/L sucrose, 1.1 mmol/L EDTA
138 and 0.1 % peroxide-free Triton X 100) and centrifuged (10,000 X g, 15 min, 4°C, model
139 J2-21M, JA-21 rotor, Beckman Instruments, Palo Alto, CA), as described previously
140 (6,16). Gpx1 activity in liver, kidney, muscle, and red blood cells (designated as Gpx1)
141 and Gpx3 activity in plasma (designated as Gpx3) were measured by the coupled assay
142 procedure (23) using 120 $\mu\text{mol/L}$ H_2O_2 . Gpx4 activity was measured by the coupled
143 assay procedure (6) using 78 $\mu\text{mol/L}$ phosphatidylcholine hydroperoxide, the specific
144 substrate. For both assays, 1 enzyme unit (EU) is the amount of enzyme that will
145 oxidize one μmole of GSH per min under these conditions. Thioredoxin reductase
146 (Txnrd) was measured using the gold-inhibition assay with DTNB as a substrate (24).
147 Protein concentration of each sample was determined by the method of Lowry et al.
148 (25).

Methods are concisely described and when possible, references cited.

SI units are used in Methods, Results, tables, and figures.

149 **RNA Isolation and Analysis.** Total RNA from liver and kidney (50-100 mg tissue,
150 n=3/diet group) was isolated using the guanidinium isothiocyanate method with TRIzol
151 Reagent (cat. #15596-026, Invitrogen, Carlsbad, CA), following the manufacturer's
152 protocol. The RNA pellet was dissolved in diethyl pyrocarbonate-treated water and
153 quantitated using a ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies,
154 Wilmington, DE).

155 Relative mRNA abundance was determined by quantitative RT-PCR. RNA (1 µg)
156 was reverse transcribed to cDNA using the RETROscript kit (AM1710, Ambion Inc.,
157 Austin, TX), following the manufacturer's instructions. Gene specific primers were
158 designed to span a splice-junction and amplify ~150 base segment (see **Supplemental**
159 **Table 1**). The final 25 µL real time reactions contained 10 ng reverse transcribed RNA,
160 0.2 mmol/L gene specific forward and reverse primers, and 1X SybrGreen PCR Master
161 Mix (#4309155, Applied Biosystems, Foster City, CA). Reactions were followed in an
162 ABI Prism 7000 (Applied Biosystems) with initial stages of 50°C for 2 min and 95°C for
163 10 min, followed by 50 cycles consisting of 95°C for 15 sec and 60°C for 2 min. A
164 dissociation curve was run for each plate to confirm the production of a single product.
165 The amplification efficiency for each gene was determined using the DART-PCR
166 program (26). The mRNA relative abundance was calculated according to Pfaffl (27),
167 accounting for gene-specific efficiencies, normalized to the mean of β-Actin (Actb) and
168 glyceraldehyde-3-phosphate dehydrogenase (Gapdh), and expressed as a percentage
169 of Se-adequate levels (0.2 µg Se/g in Expt. 1; 0.24 µg Se/g or plateau level in Expt. 2).
170 **Statistical Analysis.** Data are presented as means ± SEM. For Expt. 1 for all analyses,
171 n=3/treatment. For Expt. 2, for body weight and for all enzyme analyses except skeletal

All statistical tests conducted are completely described in Methods.

The identity of data presented in the text is clearly stated in Methods.

Alpha is stated in Methods.

172 muscle Gpx4, n = 5 or 6/treatment; for skeletal muscle Gpx4 activity, for Se
173 concentrations, and for liver and kidney mRNA analyses, n = 3/treatment; for skeletal
174 muscle mRNA analysis, n = 3 or 4/treatment. All data were analyzed by ANOVA using a
175 fixed model testing the main effect of diet (SAS Inst. Inc., Cary, NC). When the main
176 effect of diet was significant, differences between means were assessed by Duncan's
177 multiple range analysis ($P < 0.05$), with Kramer's modification for unequal class sizes
178 where necessary (28). When variance equality was significant, as tested by Bartlett's
179 test ($\alpha = 0.05$), significant differences between means were assessed instead by
180 Scheffé's F-test. For all tests, $P < 0.05$ was considered significant. The plateau
181 breakpoint for each Se response curve, defined as the intersection of the line tangent to
182 the point of steepest slope and the plateau, was calculated as described previously (14-
183 16) using sigmoidal or hyperbolic regression analysis (Sigma Plot, Jandel Scientific) to
184 estimate the minimum dietary Se necessary to obtain plateau responses.

185 **Results**

All OSM material is called out sequentially in the text.

186 **Animal Growth.** In both experiments, there were no significant differences in initial
187 body weights of the rats, and dietary Se supplementation did not alter the growth rate in
188 Expt. 1 (not shown) nor in Expt. 2 (**Supplemental Fig. 1**), where the rats grew at an
189 mean rate of 7.7 g/d. Thus in these studies neither Se deficiency nor Se
190 supplementation affected growth.

191 **Tissue Selenium Analysis.** Tissue Se levels were not determined in Expt. 1. In Expt.
192 2, liver Se concentrations in rats fed the basal diet were 3% of levels in Se-adequate
193 (0.24 μg Se/g diet) rats, showing that these rats were Se deficient (**Fig. 1**). Se
194 supplementation resulted in a sigmoidal response in liver Se concentration, with a

All tables and figures are called out sequentially in the text.

Bolding here can prevent references from being extracted from the text file for the Reference Link; normal type is preferred.

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Journal of Nutrition format is used:

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Issue numbers and months are not included.

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et al. is not used until 9 authors have been listed.

Figure legends are not on the same page as figures to be printed but are compiled on a separate page.

Titles concisely describe treatments and specify the animals studied.

FIGURE LEGENDS

FIGURE 1 Effect of graded dietary Se on liver and kidney Se concentration in rats. Male weanling rats were supplemented with Se at the indicated levels for 28 d (n = 5 or 6/diet). Values are means \pm SEM, and the legend indicates the level of significance. Detailed statistical analysis is provided in Supplemental Table 2.

FIGURE 2 Effect of dietary Se concentration on tissue selenoenzyme activities in rats.

Shown are enzyme specific activities for (A) Plasma Gpx3 activity, (B) RBC Gpx1 activity, (C) tissue Gpx1 activity, (D) tissue Gpx4 activity, and (E) liver Txnrd activity, expressed as EU/g protein. Values are means \pm SEM, n = 5 or 6/diet, except muscle Gpx4 activity where n = 3/diet; the legend in each panel indicates the level of significance. Detailed statistical analysis is provided in Supplemental Table 2.

Abbreviations in the manuscript's abbreviation footnote are used but not redefined in figure legends or table footnotes; nonstandard abbreviations used on the figure or table but not in the manuscript are defined in legends or footnotes.

The data are explained and independent *n* given.

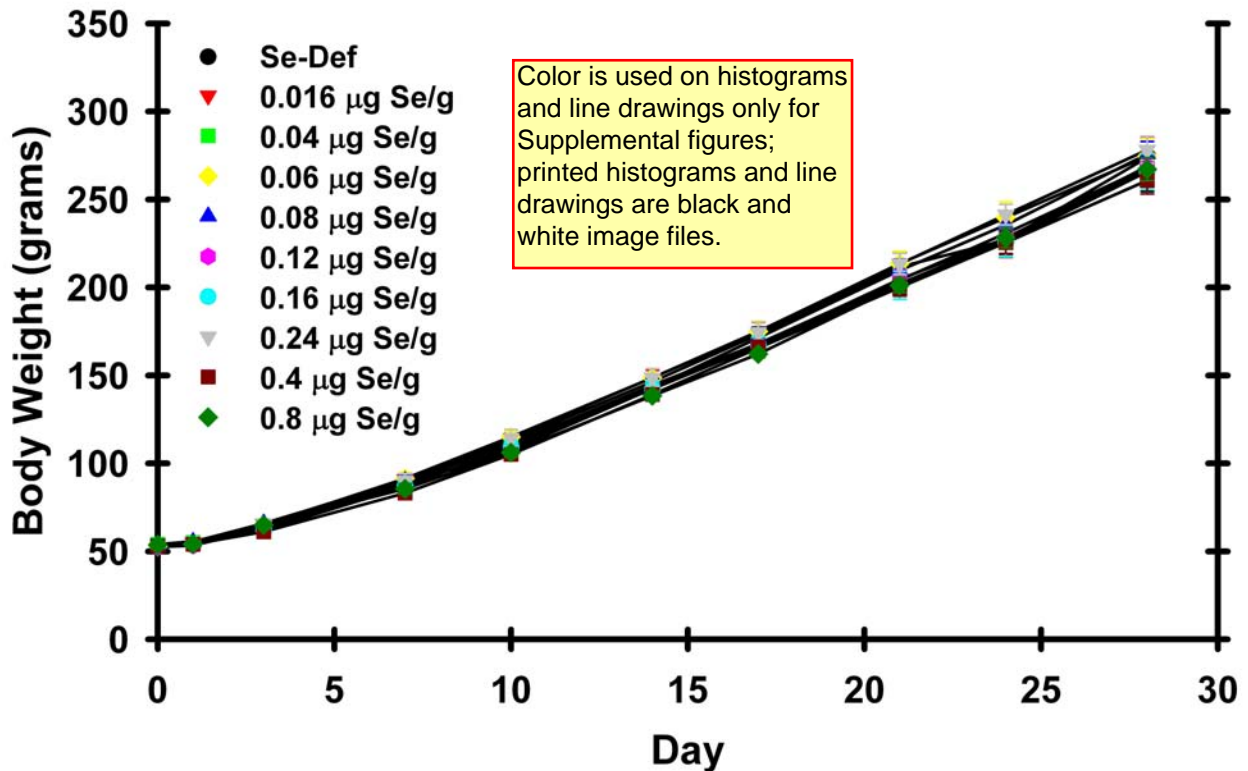
FIGURE 3 Effect of dietary Se concentration on tissue selenoprotein mRNA abundance in rats. Shown are relative mRNA levels for (A) Gpx1, (B) Sepw1, (C) Selh, (D) Gpx4, and (E) Sephs2, as determined by qRT-PCR on total RNA isolated from the indicated tissues. Values were determined in triplicate for each sample, normalized to the mean of Gapdh and Actb mRNA levels in each sample, expressed as a percentage of Se-adequate plateau levels, and plotted as mean \pm SEM, n = 3/diet. The legend in each panel indicates the level of significance. Detailed statistical analysis is provided in Supplemental Table 4.

The required label for all OSM pages is included.

Online Supporting Materials - Barnes et al

Barnes et al., Supplemental Figure 1

Body Weight



SUPPLEMENTAL FIGURE 1 Effect of dietary Se on rat body weight. Male weanling rats were supplemented with Se at the indicated levels for 28 d (n = 5 or 6/diet) and weighed biweekly. Values are the mean weights \pm SEM. There were no significant effects ($P > 0.05$) of dietary Se level at any timepoint.

The figure legend for supplemental figures is single-spaced on the same page.

Large primer tables are OSM, not printed. When 6 or fewer primer pairs are used, they are listed in the text of the Materials and Methods section.

Online Supporting Material - Barnes et al.

SUPPLEMENTAL TABLE 1 Selenoprotein genes and qRT-PCR primers for rat selenoproteins

Gene ¹	Accession ²	Gene Name	Forward Primer ³	Reverse Primer ³
Actb	NM_031144	Actin, beta	5'-cctgggatggaatcctgtg	5'-cttctgcatcctgtcagcaa
Dio1	NM_021653	Deiodinase, iodothyronine, type I	5'-gcctctcaggacagaagtgc	5'-gtcagctgtggaggcaaagt
Dio2	NM_031720	Deiodinase, iodothyronine, type II	5'-ggactacgctgtgtctggaa	5'-ctgcacaggcaaagtcaaga
Dio3	NM_017210	Deiodinase, iodothyronine, type III	5'-ctgtgctctggttctggaca	5'-cgcaactcagacacctggta
Gapdh	NM_017008	Glyceraldehyde-3-phosphate dehydrogenase	5'-ccatcaccatcttccaggag	5'-cggagatgatgacccttttg
Gpx1	NM_030826	Glutathione peroxidase 1	5'-gctgctcattgagaatgtcg	5'-gaatctctcattcttgccatt
Gpx2	NM_183403	Glutathione peroxidase 2	5'-cctagtggttctcggcttcc	5'-tgcccattgacatcacactt
Gpx3	NM_022525	Glutathione peroxidase 3	5'-cgagtagggagcccttacca	5'-aatgggccaagtctctctg
Gpx4	NM_017165	Glutathione peroxidase 4	5'-ccggctacaatgtcaggttt	5'-acgcagccgttctatcaat
Selh	NM_001114939	Selenoprotein H	5'-aactggaggccccagagata	5'-ggctcaggaaatttgagcttt
Seli	XM_343031	Selenoprotein I	5'-tcactgctgcctcactctg	5'-ggaccgatactcttcttcca
Selk	NM_207589	Selenoprotein K	5'-aaccggaggaaagatggttt	5'-ccccgtagcctctcttttc
Selm	XM_001115013	Selenoprotein M	5'-aaggagggtgaaggcctttgt	5'-tcatttgctgagtgaggatt
Sepp1	XM_342942	Selenoprotein N	5'-gttcaccggccatcatct	5'-catgttgctggtctactgg
Selo	NM_001085485	Selenoprotein O	5'-ctcattggcactcaagcaaa	5'-tcctgtccagacgctctct
Sepp1 (SelP)	NM_019192	Selenoprotein P	5'-tccttcctcactttcccgtg	5'-tctgagggtctgtggtttt
Sepp1 (MsrB1)	NM_001044285	Methionine-R-sulfoxide reductase	5'-aagtgcggctatgagctgtt	5'-acttgccacaggacaccttt
Sels	NM_173120	Selenoprotein S	5'-cttcagctgcgtccttctct	5'-ctgcattctcaaacgagcag
Selt	NM_001014253	Selenoprotein T	5'-cgtgcccagcaagagatta	5'-tcaatgaggatgtctggata
Selv	XR_009209	Selenoprotein V	5'-cccagcacagaacttcgttt	5'-tgatgctccagggtctttt
Sepw1	NM_013027	Selenoprotein W	5'-gccaagatctccagctca	5'-ttccggaactgctctctgt
Sep15	NM_133297	Selenoprotein 15	5'-ctgcatctccttgacagt	5'-ggaacctcccaattttcat
Sephs2 (SPS2)	NM_001079889	Selenophosphate synthetase 2	5'-actcagtgtagccagagca	5'-cccaccgatgataatccaag
Txnrd1	NM_031614	Thioredoxin reductase 1	5'-ggcctcagctcactgtaat	5'-tccctgctcaatctgttca
Txnrd2	NM_022584	Thioredoxin reductase 2	5'-cgctggagaagtacacaag	5'-cagtaggatccaggccagag
Txnrd3	NM_001106609	Thioredoxin reductase 3	5'-tgaagtcacacaggggttg	5'-gtaatgtccagccctgagga

¹Gene symbol with common alternative symbol given in parentheses.

²Accession number from NCBI Entrez Gene database.

³Forward and reverse primers used to amplify ~150 bp qRT-PCR product that span an intron.