**Supplementary file 2**

**Long-term alcohol ingestion causes dysregulation of iron homeostasis, but not hepatic iron accumulation, in mice**

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**DESCRIPTION OF QA/QC/ASSAY PERFOMANCE**

1. **Table 1: MIQE checklist for qPCR**

|  |  |  |
| --- | --- | --- |
| **Item to check** | **Importance\*** | **Response** |
| **EXPERIMENTAL DESIGN** |  |  |
| Definition of experimental and control groups | E | Mice were pair-fed with the Lieber DeCarli liquid alcohol/control diet for various periods of time as detailed in the Methodology section. |
| Number within each group | E | Samples from 3 to 6 alcohol fed mice and pair-fed controls were analyzed in each group |
| Assay carried out by core lab or investigator's lab? | D | Assays were carried out in the investigators’ lab |
| **SAMPLE** |  |  |
| Description | E | Mouse liver |
| Volume/mass of sample processed | D | 100 mg of wet liver tissue was used for RNA isolation |
| Microdissection or macrodissection | E | Macrodissection |
| Processing procedure | E | Liver was quickly removed from the mouse, placed on an ice slab, cut into 8 equal pieces and transferred to pre-cooled micro-centrifuge tubes |
| If frozen - how and how quickly? | E | Samples were immediately frozen in liquid nitrogen |
| If fixed - with what, how quickly? | E | Not applicable |
| Sample storage conditions and duration (especially for FFPE samples) | E | Samples were stored at - 70°C until processed for RNA isolation |
| **NUCLEIC ACID EXTRACTION** |  |  |
| Procedure and/or instrumentation | E | Guanidinium thiocyanate-phenol-chloroform extraction method using Tri-reagent (Sigma) |
| Name of kit and details of any modifications | E | Not applicable |
| Source of additional reagents used | D | Chloroform and isopropanol used for RNA isolation were of molecular biology grade obtained from Sigma. |
| Details of DNase or RNAse treatment | E | Not done |
| Contamination assessment (DNA or RNA) | E | All samples were run on an 1% agarose gel to look for DNA contamination |
| Nucleic acid quantification | E | Done using a nanospectrophotometer |
| Instrument and method | E | NanoDrop2000c from ThermoFischer |
| Purity (A260/A280) | D | A260/A280 for all samples were > 1.80 |
| RNA integrity method/instrument | E | All samples were run on a 1% agarose gel. Only those samples that showed clear and distinct bands corresponding to 18s and 28s rRNA were used for cDNA construction |
| RIN/RQI or Cq of 3' and 5' transcripts | E | Not done |
| **REVERSE TRANSCRIPTION** |  |  |
| Complete reaction conditions | E | Reaction buffer containing 5mM MgCl2, 500µM dNTPs, 2.5µM random nonamers, 0.4U/µL RNAase inhibitor, 1.25U/µL reverse transcriptase (final concentration) |
| Amount of RNA and reaction volume | E | 500ng of total RNA was added to a total volume of 10µL |
| Priming oligonucleotide (if using GSP) and concentration | E | Random nonamers 2.5µM (final conc.) |
| Reverse transcriptase and concentration | E | Moloney Murine leukemia virus reverse transcriptase 1.25U/µL (final conc.) |
| Temperature and time | E | 25°C for 10 min, 48°C for 30 min, 95°C for 5 min |
| Manufacturer of reagents and catalogue numbers | D | Reverse Transcriptase Core kit from Eurogentec, Belgium (Catalogue no. RT-RTCK-05) |
| Storage conditions of cDNA | D | -20°C |
| **qPCR TARGET INFORMATION** |  |  |
| If multiplex, efficiency and LOD of each assay | E | Not applicable |
| Sequence accession number | D | Information provided in Supplementary Table 1 |
| Amplicon length | E | Information provided in Supplementary Table 1 |
| In silico specificity screen (BLAST, etc) | E | Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to check the specificity of each primer-pair used. |
| Pseudogenes, retropseudogenes or other homologs? | D | No |
| Location of each primer by exon or intron (if applicable) | E | Not applicable |
| What splice variants are targeted? | E | Primers were designed to amplify all splice variants of the target genes |
| **qPCR OLIGONUCLEOTIDES** |  |  |
| Primer sequences | E | Information provided in Supplementary Table 1 |
| RTPrimerDB Identification Number | D | Not applicable |
| Probe sequences | D | Not applicable |
| Location and identity of any modifications | E | Not applicable |
| Manufacturer of oligonucleotides | D | Eurogentec, Belgium |
| **qPCR PROTOCOL** |  |  |
| Reaction volume and amount of cDNA/DNA | E | 10 µL reaction volume containing 2 µL cDNA diluted 1:10 |
| Primer, (probe), Mg++ and dNTP concentrations | E | Final concentrations were:  Primer: 250nM  Mg2+ : 2.5mM  dNTPs: not specified by the kit manufacturer |
| Polymerase identity and concentration | E | TakyonTM DNA polymerase (concentration not specified by the kit manufacturer) |
| Buffer/kit identity and manufacturer | E | TakyonTM No Rox SYBR MasterMix dTTP Blue (Catalogue number: UF-NSMT-B0701) |
| Exact chemical constitution of the buffer | D | Information not provided by the kit manufacturer |
| Additives (SYBR Green I, DMSO, etc.) | E | Not applicable |
| Manufacturer of plates/tubes and catalog number | D | 96-well plates from Axygen Scientific (catalogue number: PCR-96-FS-C) |
| Complete thermocycling parameters | E | 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec) |
| Reaction setup (manual/robotic) | D | Manual |
| Manufacturer of qPCR instrument | E | BioRad Chromo4 real-time PCR machine |
| **qPCR VALIDATION** |  |  |
| Specificity (gel, sequence, melt, or digest) | E | Melt curve analysis was done for all PCR runs for all the genes. Single peaks were detected. No primer dimers were seen in any of reaction wells. |
| For SYBR Green I, Cq of the NTC | E | Information provided in the table below (Table 2) |
| Standard curves with slope and y-intercept | E | Information provided in the table below (Table 2) |
| PCR efficiency calculated from slope | E | Information provided in the table below (Table 2) |
| r2 of standard curve | E | Information provided in the table below (Table 2) |
| Linear dynamic range | E | Information provided in the table below (Table 2) |
| Cq variation at lower limit | E | Information provided in the table below (Table 2) |
| If multiplex, efficiency and LOD of each assay. | E | Not applicable |
| **DATA ANALYSIS** |  |  |
| qPCR analysis program (source, version) | E | MJ OpticonMonitor Analysis Software Version 3.1 (BioRad) |
| Cq method determination | E | Manual |
| Outlier identification and disposition | E | Not applicable |
| Results of NTCs | E | Information provided in the table below (Table 2) |
| Justification of number and choice of reference genes | E | The reference gene used was RPL19. The choice was based on previous publications which have used RPL19 as the reference gene. |
| Description of normalisation method | E | The delta delta Ct method was used for normalization |
| Number and stage (RT or qPCR) of technical replicates | E | All reactions were conducted in duplicate when qPCR was carried out. |
| Repeatability (intra-assay variation) | E | Average of Ct values for duplicates was taken for calculation. Runs were repeated in samples where the Ct SD exceeded 0.2 |
| Statistical methods for result significance | E | The Krushkal Wallis test was used to detect statistically significant changes occurring in the different groups of mice. Mann Whitney test was used for all pair-wise comparisons. |
| Software (source, version) | E | SPSS version 16.0 |

\* E – essential, D - desirable

**Table 2: qPCR validation data**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Sl. No** | **Gene** | **Standard curve slope** | **R2 of standard curve** | **Linear dynamic range (cDNA dilution)** | **Standard deviation of Ct at lower limit of dynamic range** | **Primer dimer (melting curve analysis)** | **Ct of amplification (if any) in the NTC** |
| 1 | Hepcidin1 | -3.332 | 0.996 | 1:5 to 1:625 | 0.16 | None | No amplification detected |
| 2 | Ferritin (L) | -3.631 | 0.997 | 1:5 to 1:625 | 0.05 | Primer dimer in NTC | 36 |
| 3 | Ferritin (H) | -3.529 | 0.999 | 1:5 to 1:625 | 0.07 | None | No amplification detected |
| 4 | Transferrin receptor 1 (TfR1) | -3.689 | 0.999 | 1:3 to 1: 243 | 0.06 | None | No amplification detected |
| 5 | Transferrin receptor 2 (TfR2) | -3.541 | 0.997 | 1.3 to 1:243 | 0.07 | None | No amplification detected |
| 6 | BMP6 | -3.49 | 0.999 | 1:5 to 1: 125 | 0.07 | Primer dimer in NTC | 36 |
| 7 | HO-1 | -2.929 | 0.999 | 1:5 to 1:625 | 0.34 | Primer dimer in NTC | 36 |

1. **Estimation of glutathione:**

Glutathione was estimated using the Cayman Chemicals Glutathione Assay Kit (#703002).

All assays were performed according to manufacturer’s instructions. The CV of the standard curve was within the acceptable range suggested in the kit insert. Samples were diluted so that the readings for all samples were within the range of the standard curve. All samples were processed in a single batch and assayed together.

1. **Estimation of alanine transaminase (ALT) activity**

ALT activity was estimated using the Cayman Chemicals Alanine Transaminase Colorimetric Activity Assay Kit (#700260). All assays were performed according to manufacturer’s instructions. Positive and negative controls were included and were within the acceptable range suggested in the kit insert. All samples were processed in a single batch and assayed together.

1. **Estimation of serum hepcidin**

Serum hepcidin was estimated using the Hepcidin-25 (mouse) EIA kit from Peninsula Laboratories International Inc. The kit has a range of 0 - 25 ng/dL with an IC50 of 0.5 ng/dL. Samples were therefore diluted 250-fold using the assay dilution buffer provided in the kit. Readings for all samples were within the range of the standard curve. All samples were processed in a single batch and assayed together.

1. **Estimation of thiobarbituric acid reactive substances (TBARS), cytochrome P450 2E1 enzyme activity and haem oxygenase enzyme activity**

These assays were performed based on previously published protocols (as described in the Methodology section of the manuscript) which were standardized in the PI’s laboratory.

1. **Liver and serum iron:**

Liver and serum iron levels were estimated by a colorimetric assay as described in the Methodology section of the manuscript. The validity of this assay has been established in our laboratory by estimating iron in 8 random liver samples by atomic absorption spectrophotometry (AAS) in the Clinical Biochemistry laboratory. Results obtained from these samples were compared to those obtained with the colorimetric assay in the same samples. The results were found to be strongly positively correlated (Pearson’s correlation coefficient = 0.985, p-value < 0.001).

1. **Light microscopy and Prussian Blue staining**

Tissue processing, H&E staining, Prussian Blue staining and interpretation of results were done by a trained pathologist. Positive and negative controls were included for Prussian Blue staining.

1. **Western blotting:**

The following measures were taken to ensure the validity of data obtained by western blotting.

1. Samples were lyzed in RIPA buffer containing protease inhibitors. All samples were kept in ice throughout the sample processing period.
2. Protein content was estimated using a BCA protein assay kit (Pierce) to ensure equal loading (50 µg protein)
3. A positive control was included in blots for TfR1, ferroportin and HO-1.
4. Bands for all blots were normalized to those obtained for beta actin which was used as the loading control.
5. Densitometric quantifications were done using the ImageJ v1.50d software.