TOWARDS A STANDARDISED METHOD TO ACQUIRE AND STORE LIVER SAMPLES AND GUIDELINES TO IMPROVE QUALITY CONTROL AND EXCHANGE OF RELATIVE EXPRESSION DATA

Frank M. Riemers, Jan Rothuizen, Louis C. Penning

Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht, the Netherlands.

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ABSTRACT

The current 'state-of-the-art' molecular techniques are extremely sensitive and consequently prone to false results. Even more so than in the past, today's hepatology research depends on high quality samples, especially for the molecular analyses. In all steps, starting with specimen sampling, fixation, storage, molecular processing and finally data calculation, variations in procedures between research laboratories may have a profound effect on the final conclusions. At the end of the day, this is an enormous drawback once data from different research institutes need to be reproduced, compared and/or combined. To improve standardisation, the so-called MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) were presented for quantitative PCR (qPCR) studies.[1,2] Furthermore, around the same time, recommendations were presented regarding human biospecimen collection, storage and processing, the so-called BRISQ-guidelines (Biospecimen Reporting for Improved Study Quality).[3] Finally, the editors of *The Journal of Pathology* as well as *Histopathology* required in the December 2012 issue of *The Journal of Pathology* that researchers needed to follow the BRISQ guidelines in their papers in order to improve the sample quality in biomedical research.[4]

These initiatives hold great promise to improve the comparison and independent reproduction of data acquired in different research centres. Pancreas, gall bladder and liver research will especially benefit from the standardisation protocols since these organ systems are highly vulnerable to post-biopsy autolytic degradation. This comment illustrates that standardisation in molecular liver research is not yet at the point where experiments can be easily replicated, and data can be compared and combined.

Keywords: Quantitative PCR, MIQE-precise, normalisation, reference genes.

INTRODUCTION

Molecular expression studies on biospecimen can gain insight into the etiology of a disease, and may lead to information on therapeutic effects and potentially facilitate biomarker studies. These samples need to be acquired, stored and processed in such a way that laboratory-to-laboratory comparison is possible and independent reproduction can be achieved. Standardisation of protocols in all three steps mentioned above is a way to come to meaningful comparisons. Cost-effective scientific progress can be achieved by different means, for instance, by combining data and data-comparisons of different research groups. High quality data is crucial in this respect. Space limitations often hamper detailed description of materials and methods, and consequently comparisons between laboratories, not to mention meta-analyses, are often flawed. For biopsies the BRISQ guidelines exist and there are guidelines to standardise quantitative PCR (qPCR) expression studies (MIQE-precise guidelines).[1-3] The MIQE guidelines are summarised in a checklist format and assist in experimental design, facilitate accurate data analysis, relieve the job of a manuscript reviewer, and make data interpretation easier for the readers of the scientific paper. Altogether they are beneficial in all steps from experimental design and biospecimen sampling

to acceptance and implementation in the scientific community. This chapter is an initiative to raise awareness of the cost-effective progress molecular liver research can make once data are calculated and presented in such a way that experiments can be easily repeated and data can be combined and compared.

Scientists prefer their biopsies, taken at surgery not under time pressure or other forms of stress, to be fixed specifically for their individual research questions which can be either at tissue, cellular or molecular level. However, these separate research questions require different fixation and storage methods. Such a complexity of tissue handling is clearly prone to the introduction of mistakes, leading to biospecimen of potentially lesser quality for a specific analysis. Although RNA is far less stable then DNA some studies indicate that the RNA integrity is not largely influenced even up to 48 hours on ice.[5,6] The last study included tonsil and liver samples. In contrast, two studies exemplified the effects of variations in liver tissue sampling on subsequent mRNA expression studies.[7,8] One study described the influence of the biopsy needle size in rat liver biopsies on the RNA quality in a subsequent micro-array expression study.[7] The second study assessed different sampling techniques, fixation methods, and storage procedures for canine liver tissue to optimise the use of a single liver biopsy for histological and molecular (qPCR) measurements.[8]

Not only can total RNA be subject to degradation (usually measured as a RNA Integrity Number (RIN) based on 18S and 28S) during the sampling, storage and processing, but mRNA (only 2-5% of total RNA, but most often the compound of interest) can also be degraded. One way to correct for mRNA degradation, and other steps in mRNA expression studies is the inclusion of so-called reference genes (previously erroneously called housekeeping genes) to normalise for mRNA input and PCR efficiency. The assumption here is that the expression of reference genes is always constant, irrespective of variations in samples, experimental conditions etc. In fact this assumption has been debated for about one decade now.[9] Obviously data comparison in molecular liver research faces an enormous hurdle if reference gene stability is either not evaluated nor are other parts of the sample and data processing are not described in detail. Whether this molecular deficit indeed exists in molecular liver research was not reported previously, and is investigated in this chapter. As it turned out, based on a PubMed search (http://www.ncbi.nlm.nih.gov/), the crucial step in expression studies, viz, evaluation in reference gene stability, was often omitted in molecular liver studies. This book chapter therefore is a clear advocacy to implement MIQE-precise guidelines as soon as possible.

Variation	Reference gene used	Ref.
Colorectal liver metastases	18S rRNA	21
HCC and HCA	18S rRNA	23
HCC	18S rRNA	25
Hepatocellular adenoma	18S rRNA	30
HepG2 cell line	18S rRNA	31
Nonalcoholic fatty liver disease	18S rRNA	32
Hepatocellular adenoma	18S rRNA	39
Gallstone disease	18S rRNA	41
HCC progression in mice	18S rRNA	25
Fibrogenesis in ABCb4 /- mice	18S rRNA	45
PHX in ob/ob mice	18S rRNA	58
BDL-induced fibrosis	Beta-2-Microglobulin	46
BDL- or TAA-induced fibrosis	Beta-2-Microglobulin	50
HCC patients	Beta-Actin	17
HBV-related HCC	Beta-Actin	18
HCC	Beta-Actin	19
HEV	Beta-Actin	28
HBV-related HCC	Beta-Actin	29
Freshly isolated hepatocytes	Beta-Actin	34
HCC	Beta-Actin	35
Hepatoma cell line	Beta-Actin	42
HBx-transgenic mice	Beta-Actin	18
Lipid accumulation in mice	Beta-Actin	49
Hepatosteatosis in mice	Beta-Actin	55
Iron-dextran overload	Beta-Actin	56
HCC and cell lines	Beta-Actin (HCC) GAPDH(cellline)	10
HCC	Beta-Globin	40
Rosiglitazone and LPS treatment	cyclophilin	52
LPS injections	cyclophilin	53
Rats	Cyclophilin A	47
Primary malignant liver tumor	GAPDH	33
NASH	GAPDH	36
HCC	GAPDH	37
HCC	GAPDH	38
Mouse model for NAFLD	GAPDH	43
DEN-treated cyclD -/- mice	GAPDH	44
Lithogenic diet	GAPDH	51
Doxorubicin mdr trangenenic mice	GAPDH	57
Alcohol liver disease	HPRT	26
HepG2 cell line	POLR2A	22
HCV + HIV	RPL0	27
HCV-induced dysplasia and HCC	RPL41 and SFRS4	11
CBS +/+, CBS +/-, CBS -/- mice	SOD-1	54
Mouse primary hepatocytes	TBP	22
Biliary atresia	Unspecified "housekeeping gene"	24
C57Bl6 mice	Unspecified commercial	48
Primary biliary cirrhosis	Villin	20

Table 1. Papers reporting on quantitative PCR in human and murine liver samples and cell lines, with emphasis on the reference gene included to normalise expression data.

MATERIAL AND METHODS

A PubMed search was performed via http://www.ncbi.nlm.nih.gov/ on Tuesday March 19th 11am CET on the terms 'human AND quantitative PCR AND expression AND hepatology'. The search was limited to *Hepatology* and the *Journal of Hepatology* only, the two highest top-ranked journals in the ISI-field of 'Gasteroenterology and Hepatology' specifically for hepatology. Moreover both are official journals of the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL) respectively. A similar search was performed on '(murine OR mouse) AND quantitative PCR AND expression AND hepatology'.

Finally, a PubMed search on papers evaluating reference expression stability in liver samples from human and other mammalian species was performed to reveal which reference genes were evaluated under what kind of research samples, and which freeware was used to indicate expression stability and consequently which were most reliable reference gene under that specific condition.

RESULTS

Approximately the first 50 hits on the combined terms 'human AND quantitative PCR AND expression AND hepatology' and '(mouse OR murine) AND quantitative PCR AND expression AND hepatology' were screened to establish which presumed stable reference gene was used **(Table 1)**. The preference for the classical reference genes, *viz*, beta-Actin, GAPDH or 18S rRNA, was obvious. Thirteen times was normalised against beta-actin, eleven times with 18S rRNA, and nine times with GAPDH. In one paper for the clinical samples normalisation was performed with beta-actin, whereas in cell lines GAPDH was used.[10]

None of these papers provided information on whether or not the indicated reference gene was expressed at a stable level. Most surprising was the observation that in all papers analysed, except for one, only one reference gene was used for normalisation. The exception included two independent reference genes: SFRS4 and RPL41.[11] Even worse, in view of data comparison, was the number of other reference genes used, including beta-2-microglobulin, beta-globin, cyclophilin A, villin, POLR2A, RPLP0, SOD-1, cyclophilin, TBP, or HPRT. There were no calculations on the expression stability of the reference genes included in any of the papers summarised in **Table 1**.

Six papers described the evaluation of reference gene expression stability in human samples as depicted in **Table 2**. GAPDH, beta-actin and HPRT, were included in five out of six studies, TBP was used three times, SFRS4, GUSB, 18S rRNA and B2M were include twice. RPL13A, HMBS, SDHA, RPL41, CYCC, RPS0, UBC, PMM1 and POLR2L were evaluated once. GeNorm analysis[12] and Normfinder[13] were used to evaluate expression levels and depending on the paper, either GUSB (twice), HPRT (twice) or TBP (twice) performed the best, exhibiting the highest stability of expression. SFRS4, HMBS, RPL41 and PMM1 turned out to be the best only once. The three most frequently used reference genes (beta-Actin, 18S rRNA or GAPDH) never ranked as most stably expressed reference genes **(Table 2)**. The GeNorm algorithm allows us to calculate the set of reference genes minimally required to normalise the expression of genes of interest. This analysis ('pairwise variation') has been included in as little as two of the six papers described above. Romanowski et al.[14] concluded that two reference genes, *viz* GUSB and PMM1, were sufficient to obtain a pairwise variation below 0.15, the recommended threshold to calculate the number

Variation	Reference genes	Software	Best reference gene(s)	Pairwise variation	Reference
HCV patients	18S rRNA, Beta-Actin, GAPDH, GUSB, HPRT, SFRS4	G, N, B	SFRS4 GUSB	Not tested	16
HBV-induced HCC	18S rRNA, Beta-Actin, GAPDH, HPRT, RPL13A, TBP	G, N	TBP HPRT	Not tested	59
HCC patients	B2M, GAPDH, HMBS, HPRT, SDHA, UBC	G, N	HMBS	Paired samples: V3/V4<0.15	15
HBV-induced HCC	B2M, Beta-Actin, GAPDH, HPRT, TBP	G, N	HPRT TBP	Not tested	60
HCV-induced HCC	Beta-Actin, GAPDH, RPL41, RPS20, SFRS4, TBP	G, N	RPL41 SFRS4	Not tested	61
HCV and HBV patients	Beta-Actin, CycC, GUSB, HPRT, PMM1, POLR2L	G, N	GUSB PMM1	V2/V3<0.15	14

Table 2. Papers reporting on the evaluation of expression stability of potential reference genes in human liver samples. Abbreviations in the software column: G=GeNorm, N=NormFinder, B=Bestkeeper.

Variation	Reference genes used	Software	Best reference gene(s)	Reference
Steatotic mice	B2M, Beta-Actin, GAPDH, HMBS, HPRT, RPL13A, RPLPO, TBP, TFRC, TuBP	G, N, B	HPRT GAPDH	62
Bos Taurus, cattle	Beta-Actin, GAPDH, HPRT, SDHA, TBP, YWHAZ	G	TBP Beta-Actin	63
Specific liver cells after Phx in rats	18S rRNA, B2M, Beta-Actin, GAPDH, HK1, UBC	G	Cell type dependent	64
90% PHx in rats	Alb, GAPDH, HPRT, UBC, YWHAZ		HPRT	65
Sus scrofa, pig	B2M, Beta-Actin, GAPDH, HPRT, HTPAP, RPL13A	G, N	GAPDH HPRT	66
Felis catus, cat	B2M, GAPDH, GUSB, HMBS, HPRT, RPL17, RPL30, RPS19, RPS5, YWHAZ	G	RPL17 HMBS	67
Canis lupus familiaris, dog	B2M, Beta-Actin, GAPDH, HMBS, HPRT, RPL13A, RPL32, RPS18, SDHA, TBP, YWHAZ	G	B2M Beta-Actin GAPDH	68
Canis lupus familiaris, dog	B2M, GAPDH, GUSB, hnRNPH, HPRT, RPL8, RPS19, RPS5	G	RPS5 HPRT B2M	69

Table 3. Papers reporting on the evaluation of expression stability of potential reference genes in mammalian non-human liver samples. Abbreviations in the software column: G=GeNorm, N=NormFinder.

of reference genes minimally required.[12] Combining tumourous and non-tumourous tissues revealed that at least four reference genes were needed.[15] The paper by Congiu et al.[16] clearly showed that a different set of reference genes were most stably expressed if the groups were arranged according to the levels of inflammation, or the levels of steatosis or fibrosis. Unfortunately, it was not indicated by pair-wise variation which number of reference genes was optimal for each specific condition. The situation is similarly disturbing once the expression stability is evaluated in liver samples from other mammalian species like mice, rats, pigs, cats, dogs and cattle **(Table 3)**. Again, a large list of potentially stably-expressed reference genes evaluated for their respective expression stability, including the favourable, but not necessarily the most stably expressed, human reference genes beta-actin, GAPDH and HPRT.

DISCUSSION

For relative expression levels of gene products, normalisation is needed. The expression of reference genes, of which the expression is to be stable amongst different conditions, is then used to standardise. The stability of their expression is tacitly presumed to be high. Analysis of the expression stability, by the inclusion of several independent reference genes, showed that this assumption does not always hold true. The few calculations on the minimal number of reference genes needed to properly normalise relative mRNA expression levels showed that, depending on the experimental comparison, at least two and sometimes more reference genes are needed. The plethora of various reference genes and the variable outcome in the papers evaluating reference gene expression stability, made one point clear: there are no standardised descriptions incorporated in the papers, nor are relevant details for data comparison, experimental repetition or data combination provided in most liver-related expression studies. Is this a purely academic fine-tuning issue? This is a rhetorical question. What are the cost-benefits for the inclusions of more reference genes? Imagine a simple *in vivo* experiment, two groups of six mice, six weeks of age, one group treated with a fibrotic agent and the other group as control. After six weeks (cost of animal housing around $500, 42 days 12 mice $1 per day per mouse), histology, slicing of slides, HE staining and one specific staining with an antibody (altogether costing $350). Molecular assays including one reference gene and three genes of interest ($200, qPCR for one gene around $50). So in total this imaginative experiment does cost around $1000, not taking into account the working hours. Histology and immunohistochemistry, once proper negative and positive controls are included, will be clear and open to comparisons and replications. Expression data can be replicated, however, they might not be comparable with other studies which use another reference gene to normalise expression. Even worse, the relative expression is potentially miscalculated, since it is unknown whether the reference gene was indeed expressed at a stable level throughout the two experimental conditions. For as little as $100 (two additional reference genes) the expression

data will be much more reliable, and since reference gene expression stability was evaluated and recorded a comparison of these expression data with other reports becomes feasible. The investment of just $100 will save a multitude of this amount once one can avoid a repetition of the experiment due to a lack of proper information on the stability of the included reference genes.

BRISQ-guided standardisation for histological research and biobanking is obligatory in leading pathological journals at present. Liver research can make great progress if an improved standardisation can be accomplished for molecular investigations. The proposed MQIE guidelines and MQIE-precise guidelines, including proper reference gene expression stability evaluation, offer an easy way to make the presented data easy to repeat, allow data comparison, and facilitate manuscript reviewing.[1,2]

ABBREVIATIONS

Alb, albumin
BDL, bile duct ligation
B2M, beta-2-microglobulin
CycC, cyclophillin C
GAPDH, glyceraldehyde-3 phosphate dehydrogenase
GUSB, beta-Glucoronidase
HBC, hepatitis B virus
HCC, hepatocellularcarcinoma
HCV, hepatitis C virus
HEV, hepatitis E virus
HIV, human immunedeficinecy virus
HMBS, hydroxymethyl-bilane synthase
HPRT, hypoxanthine phosphoribosyl-transferase
HTPAP, PPAP2 domain-containing protein 1B
LPS, lipopolysaccaride
NAFLD,nonalcoholic fatty liver disease
NASH, nonalcoholic steatohepatitis
PHx, partial hepatectomy
PMM1, Phosphomannomutase 1
POLR2, polymerase (RNA) II polypeptide L
RPL0, Ribosomal Protein Large0
RPL17, Ribosomal Protein Large17
RPL31A, Ribosomal Protein Large13A
RPL41, Ribosomal Protein Large41
RPS5, Ribosomal Protein Small
SDHA, succinate dehydrogenase complex, subunit A
SFRS4, splicing factor serine/arginine-rich 4
SOD1, Super Oxide Dismutase-1
TBP, TATA Box Binding Protein
TFRC, transferrin receptor
TuBP, tubulin alpha 4a
UBC, Ubiquitin C
YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

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Robust protection against recurrent episodes of hepatic encephalopathy[1]

Significant reductions in episodes† of hepatic encephalopathy and hospitalisation rates‡ have been demonstrated with XIFAXAN® 550 b.d. and concomitant lactulose*[1]. XIFAXAN® 550 b.d. provides a cost-effective treatment option[2] that enhances quality of life for patients.[3]

† p<0.001 ‡ p=0.01
* >90% were receiving concurrent lactulose in both treatment arms

NEW

Xifaxan®550
Targaxan®550▼
Rifaximin-α

INTERNATIONAL ABBREVIATED PRESCRIBING INFORMATION: XIFAXAN®/ TARGAXAN® 550 mg (rifaximin)
Presentation: Blister pack containing 14 film-coated, pink tablets of 550 mg rifaximin for oral administration. **Indication:** Reduction in recurrence of episodes of overt hepatic encephalopathy in patients ≥ 18 years of age. **Dosage and administration:** Recommended dose: 550 mg twice a day orally with a glass of water, with or without food. No specific dosing adjustment is necessary for patients with hepatic insufficiency or for the elderly. **Contraindications:** Hypersensitivity to rifaximin, any rifamycin antimicrobial agents or any of the excipients. **Warnings and precautions:** The safety and effectiveness of XIFAXAN® for the prevention of recurrence of hepatic encephalopathy have not been established in patients under 18 years of age. *Clostridium difficile*-associated diarrhoea (CDAD) has been reported with use of nearly all antibacterial agents, including rifaximin. The potential association of rifaximin treatment with CDAD and pseudomembranous colitis (PMC) cannot be ruled out. Caution is advised in patients with impaired renal function. Concomitant administration of rifaximin with other rifamycins is not recommended. Caution should be exercised when administering XIFAXAN® to patients with severe hepatic impairment (Child-Pugh C) and in patients with MELD (Model for End-Stage Liver Disease) score >25. **Interactions:** Due to the negligible gastrointestinal absorption of orally administered rifaximin, the systemic drug interaction potential is low. *In vitro* studies have shown that rifaximin did not inhibit cytochrome P450 isozymes 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and CYP3A4 at concentrations up to 200 ng/mL (at least 10 times the clinical C_{max}). Rifaximin is not expected to inhibit these enzymes in clinical use. The effectiveness of oral oestrogenic contraceptives could decrease after rifaximin administration. Additional contraceptive precautions are recommended, in particular if the oestrogen content is less than 50 μg. **Pregnancy and lactation:** Nonclinical studies of placental transfer of rifaximin/metabolites have not been conducted. There was no evidence of teratogenicity in pregnant rats or rabbits treated with rifaximin during the period of organogenesis. It is unknown whether rifaximin/metabolites are excreted in human milk. A risk to the child cannot be excluded. A decision must be made whether to discontinue breast-feeding or to discontinue/abstain from rifaximin therapy. Use of rifaximin during pregnancy is not recommended. **Undesirable effects:** The adverse effects identified from the pivotal clinical trial most likely to be associated with rifaximin treatment (incidence ≥10%) are: nausea, dizziness, ascites, oedema peripheral. The following adverse reactions have been identified during post approval use of rifaximin. Common (≥1/100 to <1/10): Depression, dizziness, headache, dyspnoea, abdominal pain upper, abdominal distension, diarrhoea, nausea, vomiting, ascites, rashes, pruritus, muscle spasms, arthralgia. Prescribers should consult country approved prescribing information for further information in relation to undesirable effects. **Overdose:** No case of overdose has been reported. In patients with normal bacterial flora, rifaximin in dosages of up to 2,400 mg/day for 7 days did not result in any relevant clinical symptoms related to the high dosage. In case of accidental overdosage, symptomatic treatments and supportive care are suggested. **Price and pack sizes:** PVC-PE-PVDC/Aluminium foil blisters in cartons of 28 or 56 tablets. Contact local distributor for price. **Legal category:** POM. **Prescribing information:** Medicinal product subject to medical prescription. **Marketing authorisation holder:** Norgine Pharmaceuticals Ltd. Norgine House, Widewater Place, Moorhall Road, Harefield, Middlesex UB9 6NS, UK. **Product licence number:** PL20011/0020. **ATC code:** A07AA11. **Date International Prescribing Information prepared:** 10 December 2012. **Company reference:** INT/XIF/1212/0160.

XIFAXAN® has varying availability and licensing internationally. Before prescribing, consult your country approved prescribing information, available from your local distributor or Norgine Ltd.

Adverse events should be reported to your regulatory agency. Adverse events should also be reported to your local distributor or Norgine Limited, Norgine House, Moorhall Road, Harefield, Uxbridge, Middlesex UB9 6NS, United Kingdom. Email: globalmedinfo@norgine.com

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XIFAXAN® 550 is indicated for the reduction in recurrence of episodes of overt hepatic encephalopathy in patients ≥ 18 years of age.[4]

Rifaximin-α is licensed under the Trade Names of XIFAXAN®, TARGAXAN®, and others. Please note that Trade Names and licensed indications may vary throughout Europe and between countries.

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