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Volume - Related Inhibitors Standardization For Reverse Transcription Quantitative Polymerase Chain Reaction Experiments

Pascal PUGNIÈRE, Sébastien BANZET, Thomas CHAILLOU, Catherine MOURET and André PEINNEQUIN

Genomic Core Facility, Institut de Recherche Biomédicale des Armées (IRBA) - La Tronche, 38702 La Tronche, FRANCE.

pascal.pugniere@neuf.fr

Introduction

A large part of the reliability of reverse transcription quantitative polymerase chain reaction (RT-qPCR) data depends on technical variations. Such variations are mainly attributable to the RT step. Standardization is a key factor in decreasing the inter sample variability. However, an ideal standardization is not always possible, and compromises must be found. Due to technical requirements, the current consensus is that a constant amount of total RNA should be used for the RT step (CA-RT). Because RNA isolation yields are variable, variable volumes of nucleic acid extracts are used in RT reaction. To overcome such drawbacks, we proposed to carry out the RT reaction with a constant volume of RNA extract by preserving a constant RNA amount through the supplementation of yeast transfer RNA[1] (CV-RT). The aim of this study was to determine whether CV-RT could improve the reliability of RT-qPCR assessment compared with the classical use of a constant amount of total RNA (CA-RT)

Methods

- RT inhibition assessment: previously described 5'-3' integrity assay[2] was used to assess RT efficiency. The RT reaction is primed using oligo-dT and separate PCR assays are used to quantify the levels of two target amplicons that are spatially separated, with one toward the 5' end and the second toward the 3' end of a single mRNA sequence. The ratio assesses the RT efficiency.

- RT inhibition assessment: we used three universal inhibition assays based on alien DNA template amplification[3] according to MIQE Guidelines[4].

- Gene quantification accuracy: we used a multigenic index to assess the reliability of the quantification, provided by the normalization software geNorm[5].

Results

Volume-related inhibitors are able to affect the efficiency of reverse transcription when the isolation method is not appropriate (Figure 1A, 1B and 1C). A significant inhibition of qPCR amplification was observed with CA-RT which is analysis and gene-dependent (Figure 2). CV-RT allows both a reduction of intersample variability (Figure 3A) and an improved geNorm normalization multiparametric index (Figure 3B). CV-RT shows better agreement than CA-RT using Bland-Altman ratio comparison. It promotes lower intersample variability, even when different RT reactions were compared (Figure 4).

Agreements between Methods

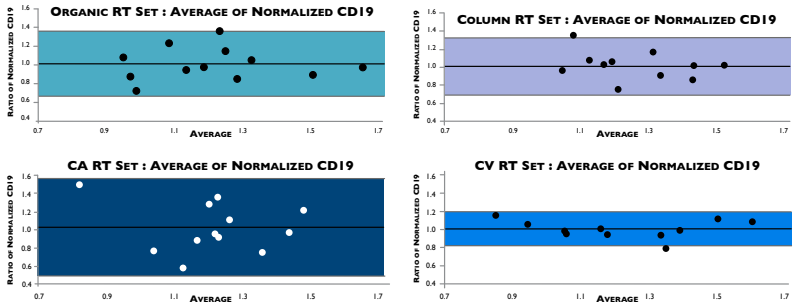

Figure 4: Quantification of CD19 mRNA levels in spleen: agreement between CD19 measurements using Bland–Altman ratio comparison among organic RT sets, column RT sets, CA-RT sets and CV-RT sets. Black line: bias; gray lines: 95% limits of agreement.

Conclusion

Due to the variability of RNA isolation yields, the achievement of RT using constant amounts of total RNA increases intersample RT-qPCR variability. Such drawbacks are caused by the presence of volume-related RT inhibitors in the RNA extracts. However, we show in this study that standardization is possible with the use of a constant volume of RNA extracts as RT template, allowing to decrease the intersample differences in volume -related inhibitors. Such a decrease is a prerequisite for the reliability of RT-qPCR assays.

References

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4. S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, **The MIQE guidelines: minimum information for publication of quantitative realtime PCR experiments,** Clin. Chem. 55 (2009) 609–620
5. J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, **Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes,** Genome Biol. 3 (2002) RESEARCH0034.

RT Inhibition Assessment

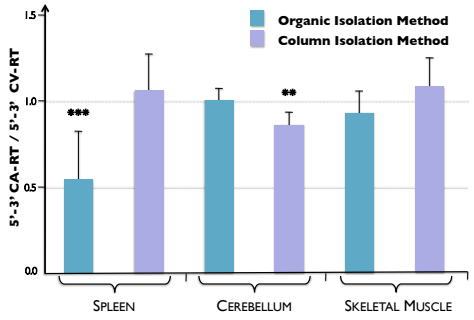

Figure 1A: 5'-3' CA/5'-3'CV: average of the ratios of RT efficiencies between CA-RT and CV-RT of each sample using 5'-3' qPCR assay for the whole tissues

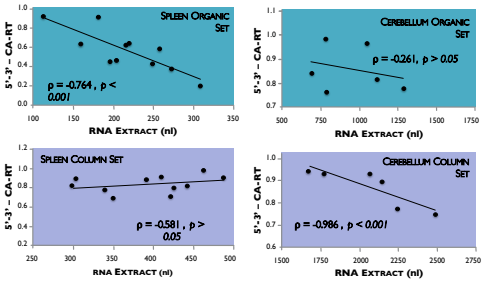

Figure 1B: Spleen extracts
Linear correlations between RNA extract volume and the 5'-3' ratio for CA-RT

Figure 1C: Cerebellum extracts
Linear correlations between RNA extract volume and the 5'-3' ratio for CA-RT

qPCR Inhibition Assessment

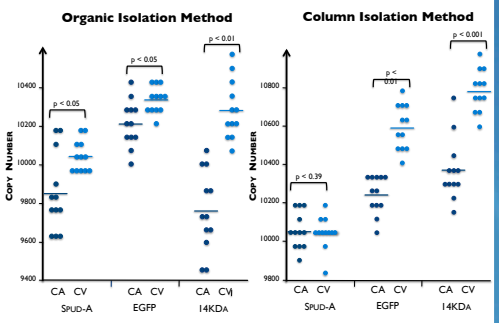

Figure 2: Spud-A, EGFP, and 14 kDa alien inhibition assays for each spleen sample RT set. Statistical comparisons were made employing the Wilcoxon test.

Gene Quantification Accuracy

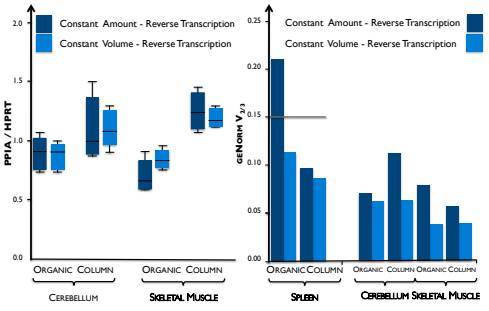

Figure 3A: Variability of the relative expression of PPIA *versus* HPRT in the cerebellum and muscle tissue.

Figure 3B: geNorm pairwise variation ($V_{2/3}$): multiparametric normalization index calculated using ARBP, HPRT1 and PPIA as reference genes for each RT set.

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