Journal of Virological Methods 248 (2017) 100–106

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Determination of suitable reference genes for RT-qPCR analysis of murine Cytomegalovirus *in vivo* and *in vitro*

Marion Griessl, Michael Gutknecht, Charles H. Cook*

Department of Surgery, Division of Acute Care Surgery, Trauma and Surgical Critical Care, Beth Israel Deaconess Medical Center – Harvard Medical School, Boston, MA, United States

ARTICLE INFO

Keywords:
Murine Cytomegalovirus
RT-qPCR
Reference gene

ABSTRACT

Reverse transcription quantitative PCR (RT-qPCR) is the most commonly used method to evaluate gene expression. Reliable qPCR results are highly dependent on accurate normalization using suitable reference genes. We investigated expression of commonly used reference genes during murine Cytomegalovirus (mCMV) infection and latency to determine those genes least perturbed by infection. Following mCMV infection in BALB/c mice, lung, salivary gland, liver, spleen and kidney were evaluated. Liver sinusoidal endothelial cells and NIH-3T3 cells were also evaluated. RT-qPCR was performed during acute and latent mCMV infection for 11 commonly used reference genes with comparisons made to uninfected samples. Normfinder, BestKeeper, GeNorm and the comparative delta CT method produced comparable analyses that were combined in RefFinder to generate an overall ranking. Ppia, B2m and Gapdh are the most stable reference genes for *in vitro* infection studies. For *in vivo* studies the most suitable reference genes were highly tissue and cell type dependent. Comparing infected and uninfected groups revealed viral influence on transcription of some genes. We provide reference gene guidelines for investigations of gene expression for mCMV Smith strain infection of Balb/cJ mice or NIH-3T3 cells. These results also suggest careful consideration of reference genes for different host tissues evaluated.

1. Introduction

Reverse transcription quantitative PCR (RT-qPCR) is still the most commonly used method to measure levels of gene expression in various biological samples, not only in basic research but also in diagnostic laboratories. The technique's advantages are high sensitivity, reproducibility, cost effectiveness as well as speed and simplicity of performance.

One major obstacle to RT-qPCR is reproducibility of results. Consideration and disclosure of experimental design, such as nucleic acid extraction and sample information, details of reverse transcription and qPCR performance are all essential to prevent assay variation and ensure result reproducibility (Bustin et al., 2009; Derveaux et al., 2010). Reliable qPCR experiments depend highly on selection of appropriate reference genes (Hellemans and Vandesompele, 2014), but the importance of accurate normalization of results is often underestimated. Depending on experimental conditions, commonly used reference genes may not always represent the best fit (Glare et al., 2002). Several computer based tools are available to help choosing the most suitable control genes, including Normfinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), GeNorm (Vandesompele et al., 2002) and the comparative delta CT method (Livak and Schmittgen, 2001; Silver et al., 2006).

The term housekeeping gene has been used to describe genes whose expression is not altered by changes in experimental circumstance. As any gene's transcription might be influenced depending on experimental circumstances, the idea of universal housekeeping genes is probably erroneous (Glare et al., 2002; Selvey et al., 2001). Host gene expression can also vary depending on the tissue and cell type that is analyzed sometimes making it necessary to use different genes (Barber et al., 2005; Chapman and Waldenstrom, 2015; Suzuki et al., 2000). Different experimental conditions, in particular investigation of cellular transcription after virus infections, may significantly alter expression of commonly used control genes. It is therefore widely accepted that different experimental conditions require specific evaluations to determine the most suitable reference gene. Accurate normalization is a fundamental requirement when studying the significance of gene expression differences. Several studies have been conducted describing the ideal reference gene for many virus infections, including HIV, HSV, VZV, SARS and human CMV to name a few (Neerukonda et al., 2016;

* Corresponding author.
E-mail address: chcook@bidmc.harvard.edu (C.H. Cook).

http://dx.doi.org/10.1016/j.jviromet.2017.06.012
Received 23 January 2017; Received in revised form 21 June 2017; Accepted 23 June 2017
Available online 26 June 2017

Table 1
Reference gene candidates with corresponding primer information.

#[a]	Ref[b]- gene	Ref-Seq[c] #	Exon location	Sequence primer-for[d]	Sequence primer-rev[e]	Size RNA [bp]	Size DNA [bp]	IDT[f] catalog #
1	Gapdh	NM_008084	2–3	5′-GTG GAG TCA TAC TGG AAC ATG TAG-3′	5′-AAT GGT GAA GGT CGG TGT G-3′	150	1984	MmPT 39a1
2	Hsp90ab1	NM_008302	1–2	5′-CAT GAG CTG GGC AAT TTC TG-3′	5′-ACT CGG CTT TCC CGT CA-3′	94	1349	MmPT5841290500
3	B2m	NM_009735	1–2	5′-ACG TAG CAG TTC AGT ATG TTC G-3′	5′-GGT CTT TCT GGT GCT TGT CT-3′	120	3188	MmPT5810497647
4	Gusb	NM_010368	10–11	5′-GAA CAG CCT TCT GGT ACT CC-3′	5′-GAG AAC TGG TAT AAG ACG CAT CA-3′	121	3386	MmPT58138436l
5	Tbp	NM_013684	3–4	5′-CAA GTT TAC AGC CAA GAT TCA CG-3′	5′-TTC ACC AAT GAC TCC TAT GAC C-3′	116	2811	MmPT5810867035
6	Pgk1	NM_008828	4–5	5′-CTA GTT TGG ACA GTG AGG CT-3′	5′-AAC CTC CGC TTT CAT GTA GAG-3′	112	2178	MmPT5813087431
7	Ppia	NM_008907	4–5	5′-TTC ACC TTC CCA AAG ACC AC-3′	5′-CAA ACA CAA ACG GTT CCC AG-3′	85	277	MmPT39a2gs
8	Actb	NM_007393	1–2	5′-ATG CCG GAG CCG TTG TC-3′	5′-GCG AGC ACA GCT TCT TTG-3′	106	1065	MmPT5833540333
9	Hprt	NM_013556	2–3	5′-AGC AGG TCA GCA AAG AAC T-3′	5′-CCT CAT GGA CTG ATT ATG GAC A-3′	125	3015	MmPT5832192191
10	Tubb4a	NM_009451	1–2	5′-GTC GAT GCC GTG CTC AT-3′	5′-GAC ACC CGT CCA TCA GAC-3′	132	1249	MmPT56a9905332
11	Pthlh	NM_008970.3	3	5′-CAA GGG CAA GTC CAT CCA AG-3′	5′-GGG ACA CCT CCG AGG TAG CT-3′	101	101	custom made

[a] #-number.
[b] Ref,Reference.
[c] Seq, Sequence.
[d] for, forward.
[e] rev, reverse.
[f] IDT, Integrated DNA Technologies.

Radonic et al., 2005; Watson et al., 2007).

Surprisingly, for the most commonly used animal model of cytomegalovirus infection − the mouse, no general recommendations about reference genes of choice have been published. We describe results from 11 of the most frequently used reference genes in the context of mCMV infection, both in cell culture and in different murine tissues after infection.

2. Materials and methods

2.1. Animals

Female BALB/cJ mice 6–8 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were euthanized by cervical dislocation under isoflurane inhalation anesthesia. Mouse tissues were dissected aseptically, snap frozen in liquid nitrogen, and stored at −80 °C. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines of Beth Israel Deaconess Medical Center.

2.2. Viral infections

For *in vitro* experiments 70% confluent NIH-3T3 cells (ATCC®, CRL-1658™) were infected with murine CMV strain Smith (ATCC®, VR-194/1981™) at MOI of 0.4. Cells were harvested at 0 h, 6 h, 24 h, 48 h and 72 h after infection.

For *in vivo* experiments female Balb/cJ mice were infected intra peritoneal (i.p.) with 1×10^6 plaque forming units (pfu) of murine CMV strain Smith. All virus stocks were stored at −80° C and before use diluted in Dulbecco's phosphate buffered saline (DPBS) to reach an injection volume of 100 µl. Mock animals were injected with 100 µl of sterile DPBS. As previously published, mice were allowed to become latent over the course of at least 4 months (Cook et al., 2002). It has been previously shown that susceptible mice have replicating virus detectable in salivary gland, lungs and liver 2 weeks after infection so this time point was chosen for the acute tissue infection experiments. (Matsuzawa et al., 1995; Selgrade et al., 1984; Shanley and Pesanti, 1985; Yuhasz et al., 1994)

2.3. Isolation of liver sinusoidal endothelial cells (LSEC)

Non-parenchymal liver cells were isolated as described previously (Seckert et al., 2009). LSEC were isolated from non-parenchymal liver cells by magnetic cell separation using CD146 (LSEC) MicroBeads (Milteny Biotec, cat. no. 130-092-007). Positive selection of CD146 expressing cells was done according to manufacturer's instructions using LS columns. After cell enumeration, RNA were directly isolated from cell pellets.

2.4. RNA extraction and cDNA synthesis

RNA were isolated via TRIzol reagent (Ambion, cat. no. 15596-018) following manufacturer's instructions. Briefly, tissues were homogenized in 1 ml TRIzol using tissue lyser II (Qiagen, cat. no. 85300) according to manufacturer protocol 'purification of RNA from animal tissues'. RNA pellets were resuspended in 35 µl of RNase free water and incubated at 55 °C for less than 5 min. RNA were column purified with RNeasy Mini Kits (Qiagen, cat. no. 74104) with DNase treatment on-column (Qiagen, cat. no. 79254), then eluted in 35 µl RNase free water and stored at −80° C until cDNA synthesis was performed. RNA quantifications were done with Nanodrop 1000 (Thermo Scientific). cDNA were produced using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad laboratories, cat. no. 170–8841) according to manufacturer recommendations using RNA input amounts of 800 ng, or 200 ng when 800 ng was not available.