History of Medicine, 2024, 10(2): 542-556
DOI: 10.17720/2409-5834.v10.2.2024.40

qRT-PCR data normalization by the identification of expression analysis of the most stable and the least stable housekeeping genes (HKGs) in Covid-19

Kanza Batool[1], Saira Jabeen[2], Hafiz Khawar[1], Muhammad Osama Malik[3], Muhammad Ijaz[4], Shahwana Tehreem[5], Aunza Nayab Ansar[6], Rehman Shahzad[7], Sumaira Ashraf[1*]

[1]Dr Ikram Ul Haq Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan
[2]Provincial Public Health Reference Laboratory, Lahore
[3]Collin College of Business, University of Tulsa, Oklahoma, USA
[4]Department of Entomology, University of Agriculture, Faisalabad
[5]Institute of Food Science and Nutrition, University of Sargodha
[6]Institute of Molecular Biology and Biotechnology, The University of Lahore, Pakistan
[7]Hi-Tech Biotechnology Laboratory Lahore, Pakistan

Abstract

Housekeeping genes (HKGs) are known as constitutive and metabolic genes which remain functionally active throughout the life span of an organism. The selection of appropriate housekeeping genes (HKGs) is the prime step for qRT-PCR data normalization. In present study, it was focused to compare the stability of ten HKGs systematically to identify the most stable endogenous internal control for our study groups i.e. control Covid-19 groups (healthy candidates), and Covid-19 effected group (mild, moderate severe). Expression levels of *GAPDH, β -actin, 18S rRNA, TBP, RPL 13a, EEF1G, UBE2D2, HPRT1* have determined by using three different biostatical based applets, geNorm, NormFinder, and BestKeeper. For comparative analysis, IL-10 gene was selected as target gene. The results suggested that B2M showed significant expression variation, and graded as the least stable housekeeping genes (HKGs). However, beta-Actin, and TBP were identified as the most stable housekeeping genes (HKGs) to carry out genetic based expression analysis of all samples associated with Covid-19. This study has applied the knowledge of molecular biology, immunological, pathological, and bioinformatics in an integrated manner to pave the ways for SARS-CoV-2 transcriptome analysis for the development of effective vaccines against Covid-19 in the future.

Keywords: Housekeeping genes (HKGs), Covid-19, gene expression, expression levels, vaccines

1.1. Introduction

Covid-19 was found to be a highly contagious, venereal, life-threatening, and human-to-human disease-causing virus. It has a pathogenic character due to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Ciotti, Ciccozzi et al. 2020; Huang and Pranata 2020; Nossmann 2020; Wrapp, Wang et al. 2020; Hu, Guo et al. 2021). This novel virus was first identified in Wuhan, Hubei province of China. Since then, it has spread throughout the world in a very short time and was declared a pandemic by World Health Organization (WHO) in 2019 (Velavan and Meyer 2020). It is referred to as zoonotic which means it possesses high transmittance power between humans and some animals only. The origin of this virus is still under debate because some scientists speculated that the virus is the product of laboratory-based genomic manipulations (Grein, Ohmagari et al. 2020; Guo, Cao et al. 2020). With the advancement in molecular life science technology, researchers can study mutation at a single gene level via high-throughput techniques such as real-time PCR (RT-PCR). RT-PCR has proved to be the unique, powerful (Vandesompele, De Preter et al. 2002), the most sensitive, robust, and the most reliable technique for the expression analysis of targeted genes with real-time detection to provide reproducibly, and accurate results (Gachon, Mingam et al. 2004; Bustin, Benes et al. 2005; Dheda, Huggett et al. 2005; Nestorov, Matić et al. 2013). To optimize the study of expression analysis of normal genes against viral infected genes, nowadays, RNA sequencing is the most common approach to capture the comprehensive transcriptome dynamics of disease models. RT-PCR require housekeeping genes which are also known as reference genes, internal control, endogenous control genes or constitutive genes (Moein, Javanmard et al. 2017) and account for the most active genes of the genomes, thus these genes are expressed throughout the life span of an individual (Thellin, Zorzi et al. 1999; Dheda, Huggett et al. 2004; Zhu, He et al. 2008; Eisenberg and Levanon 2014). Housekeeping genes are considered as the basic unit that unites different genomic and evolutionary aspects because these genes are assumed to have an absolute stable response regardless of the metabolic path regulation. HKGs have shorter introns and exons and have simple sequence repeats with very low potential for nucleosome formation at 5’ region (Curina, Termanini et al. 2017). However, in RT-PCR, the expression of commonly used HKGs for humans (Eisenberg and Levanon 2013) considerably vary under different developmental stages of different tissues, cell types, experimental and environmental conditions which can affect expression study of the GOI (gene of interest) (Thellin, Zorzi et al. 1999; Stürzenbaum and Kille 2001; Radonić, Thulke et al. 2004; Bustin, Benes et al. 2005; Dheda, Huggett et al. 2005; Guénin, Mauriat et al. 2009). In current study, expression levels of HKGs were measured by using ReFfinder which is a bio statistical Microsoft excel based online algorithm. ReFfinder includes pack of software like ΔCt analyses, GeNorm, NormFinder, BestKeeper, and comprehensive ranking

to verify the expression stability levels of each individual HKG. All software packages works on the manipulation of obtained raw Ct values of RT-PCR. This study included nine candidate housekeeping genes (*GAPDH, Beta-Actin, 18S rRNA, TBP, RPL 13a, EEF1G, UBE2D2,* and *HPRT1)* to identify the most reliable and the least stable HKGs. After the identification of the most stable HKG/ HKGs, the best possible approach to inspect the formal expression behavior of selected stable HKG/HKGs is to carry out comparative gene expression of all possible best combinations of HKGs against GOIs. Several studies showed that the expression of Interleukin 10 (IL-10) is significantly elevated in different disease spectrum (control, mild to moderate, severe) of Covid-19 (Islam, Chamberlain et al. 2021). Therefore, IL-10 was selected as GOI for this research study.

2.1.Materials and methods

2.1.1. Ethical statement and study design

The study was developed within the framework of the approved protocols by the Higher Education Commission (HEC), committee of research, which establishes the criteria for the execution of research projects for human health. Diagnostic and therapeutic maneuvers were carried out according to the Official Pakistan Standards (OPS). This study was analytical and comparative study, in which we compared the gene expression of 10 Housekeeping genes (HKGs) of healthy individuals with Covid-19 victims while considering IL-10 as targeted gene.

2.1.2. Study conducted

This study was conducted at Biomedical Sciences and Regenerative Medicine Laboratory at University of Health Sciences and Institute of Industrial Biotechnology, Government College University, Lahore.

2.1.3. Place of collection

Blood samples were collected from Covid-19 ward patients, and Covid-19 ICU patients of Mayo hospital and Jinnah Hospital (Lahore). The patients were identified by a positive Covid-19 test, and by the physician inspection team. Some other factors which were the appearance of symptoms in patients such as, headache, cough, breathing problem, diarrhea, fever, and prescribed treatments also considered from patient records.

2.1.4. Sampling technique

In this study, the sampling technique was non-probability purposive sampling technique. Whole blood was taken from the patients who voluntarily participated in this study.

2.1.5. Study duration

This study was conducted from October 2020 to November 2021.

2.1.6. Study subjects

The study group consisted of 50 participants (30 men and 20 women). These study subjects were divided into two categories: Inclusion group and exclusion group. Inclusion group was based on severity of the viral infection i.e. healthy candidates, mild to moderate, and severe ICU admitted covid-19 patients. Exclusion group included individual of less than 18 years old, patients with immune compromised or suppressed, bone marrow transplant, B cell lymphoma and who had active respiratory infections, HIV, syphilis, tuberculosis, flu infection, adenovirus infection, severe systemic diseases, malignancy or chronic diseases including hematological disorders, cachexia, active bleeding, malnutrition, cardiovascular, renal, lung, and liver dysfunction.

2.2.1. Total RNA isolation and cDNA synthesis

Total RNA was isolated from the blood samples using Trizol method according to the manufacturer's protocol (Invitrogen). The RNA quantity and quality parameters were ensured by Nanodrop ND-1000 (ThermoFisher Scientific) and Agilent 2100 Bioanalyzer. For the preservation of RNA from contamination, RNase free water (AMPD1-sigman RNAase free amplification grade RNase) was used. The first-strand cDNA synthesis was carried out with Thermo Scientific RevertAid First Strand cDNA Synthesis Kit #K1622 by following manufacturers' manuscript. 1 µg isolated RNA template, 1µL oligo dT primers and then added with nuclease free water to makeup total volume 12 µL. Followed by spinning the mixture incubated at 65 ºC for 5 min in thermo cycler, placed the mixture tube on ice, added with 4 µL addition of 5X Reaction Buffer (RiboLock RNase inhibitor 1 µl, 10mM dNTP mix 2 µl and Reverse Aid M-MuLV Reverse Transcriptase 1 µl) to make total volume up to 20 µl which was spined to get homogenous mixture. The mixture was then placed inside the thermo cycler for incubation at 42 ºC for 60 minutes (two steps of 30 min each) for the enzyme reaction, and

incubation at 70 ºC for 5 min was done to inactivate the enzyme reaction. After that mixture was incubated at 4 ºC to stop the reaction. Then this cDNA was used for real time PCR or placed at -80 ºC for storage.

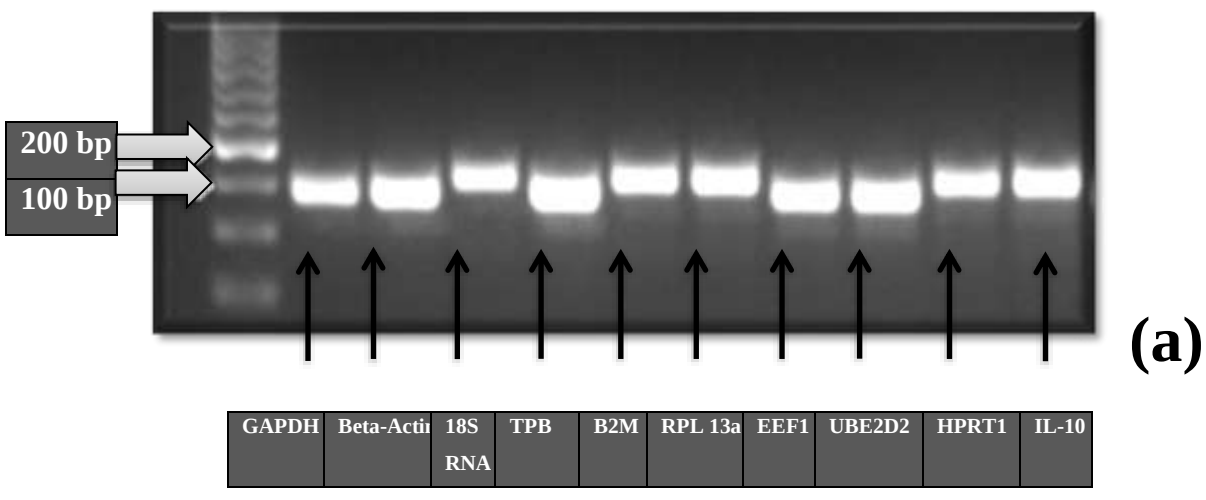
2.2.2. Selection of HKGs

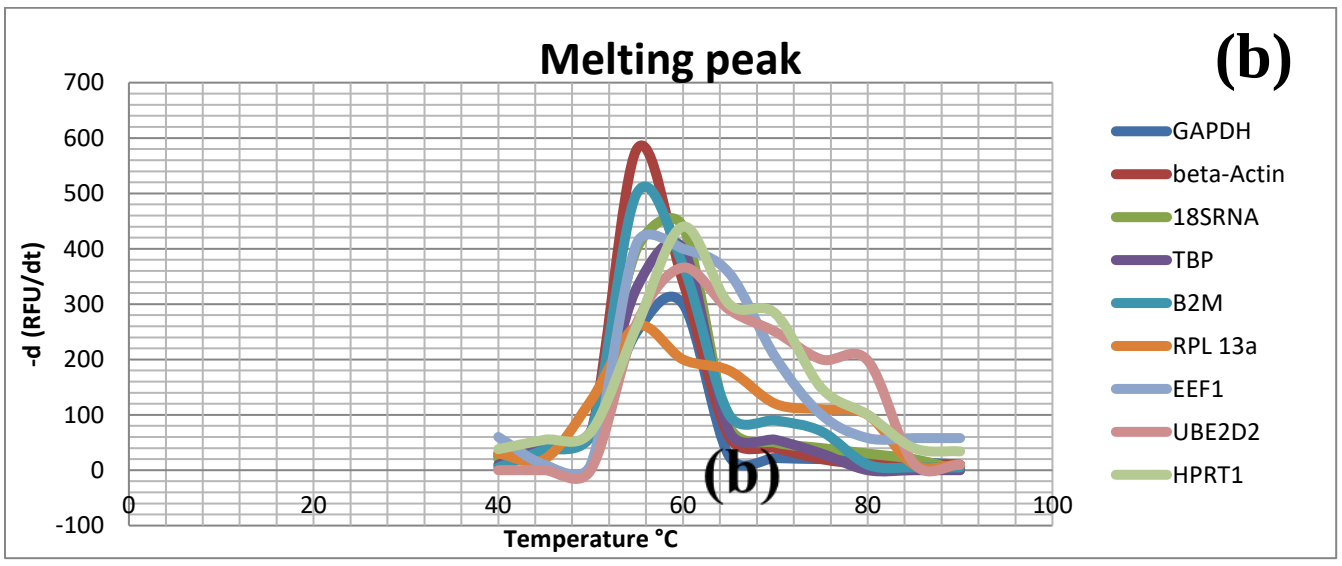
For this research study, HKGs were chosen through review of literature used stable HKG for the normalization of RT-PCR against RNA viral diseases (having characteristic properties similar to Covid-19). Based on stable expression profiles; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Beta actin (β -actin), 18S ribosomal RNA, TATA-binding protein (TBP), ribosomal protein L13A (RPL 13a), Homo sapiens eukaryotic translation elongation factor 1 gamma (EEF1G), mRNA, (cyclophilin A), ubiquitin conjugating enzyme E2 D2 (UBE2D2), hypoxanthine phosphoribosyl-transferase 1 (HPRT1) were selected. In addition to these, one target gene (IL-10) was contrasted to determine the stability patterns of the most stable HKGs.

2.2.3. Primers designing and optimization

Online database website https://www.ncbi.nlm.nih.gov/, https://blast.ncbi.nlm.nih.gov/Blast.cgi, online software such as CLC genomic workbench and Primer of Premier 5.0 was used to extract the sequence of primers specific to selected HKGs. Commercially synthesized primers specific to *Homo sapiens* were purchased from Origene Company (Table 1). Primers ranged 22-25 bp in length, their Tm was 57-60ºC and GC content was 42-55.

For the primers optimization, qPCR master mix (7µl volume of nuclease free water, 5µl SYBR Green qPCR Ready Mix, PCR grade water, template, e.g., 1µl forward primer and 1µl reverse primers) and 2 µL of cDNA template were added in PCR tubes.Run samples according to the two-step protocol. In steps 1, temperature 95 ºC for 30 seconds and 55 ºC for 60 seconds was provided to repeat the whole process through 40 cycles. In step 2, temperature 72 ºC was provided for 7 minutes. Followed by gel electrophoresis reaction was carried out through 1.5% agarose gel in Tris EDTA buffer at 80V to obtain single melting curves and peaks (Fig.1 b-d). qPCR Gel electrophoresis confirmed specificity of primers, the length of PCR-amplified specific product with no dimerization. [$E= (10^{[-1/slope]} - 1)$ formula was used to calculate individually tested primers amplification efficiency (E) by considering 10 fold dilutions (Fig 1a). Ct values were manually determined by using 0.2 as fixed value in SDS 2.1 software (Applied Biosystems). Ct values were used to calculate linear regression co-efficient values.





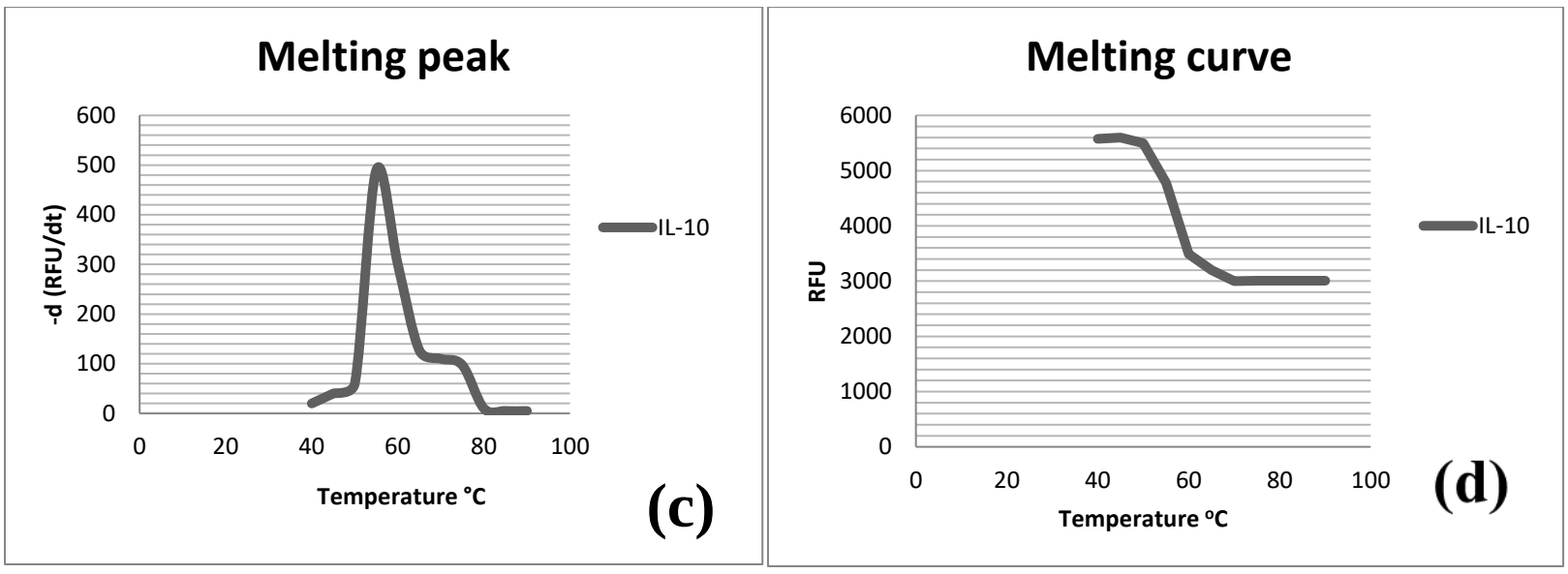


Figure 1: (a) PCR product with no primer dimerization (b) Melting peaks of candidate HKGs (c) Melting peak of IL-10 (d) Melting peak of IL-10

Table 1: Information of tested housekeeping genes, primers and amplicon for RT-PCR

Gene (*Homo sapiens*)	**Accession ID Number (NCBI GeneBank)**	**Sequence of commercially synthesized Origenes 5'-forward primers -3' (FP) and 3's-reverse primers-5' (RF)**	**Total base pairs (bp) for forward primers -3' (FP) and 3'-reverse primers-5' (RF)**	**Efficiency (E%), R^2 value**	**Gene Function**
(GAPDH) Glyceraldehyde-3-phosphate dehydrogenase	NM_002046.7	FP: GTCTCCTCTGACTTCAACAGCG RP: ACCACCCTGTTGCTGTAGCCAA	FP: 22 RP:22	98.9%, 0.9941	Glycolytic enzyme (Nicholls, Li et al. 2012)
(β –actin) Beta-actin	NM_001101.5	FP: CACCATTGGCAATGAGCGGTTC RP: AGGTCTTTGCGGATGTCCACGT	FP: 22 RP:22	92.6%, 0.9995	Cytoskeletal structural protein (Ruan and Lai 2007)
(18S rRNA) 18S ribosomal RNA	NR_003286.4	FP: ACCCGTTGAACCCCATTCGTGA RP: GCCTCACTAAACCATCCAATCGG	FP:22 RP:23	91.0%, 0.9919	Ribosomal RNA for protein synthesis (Ferreira-Cerca, Pöll et al. 2005)
(TBP) TATA-binding protein	NM_003194	FP: TGTATCCACAGTGAATCTTGGTTG RP: GGTTCGTGGCTCTCTTATCCTC	FP: 24 RP:22	101.6%, 0.997	Assist transcription by RNA polymerase (Lee and Young 1998)

(B2M) Beta 2-Microglobulin	NM_004048	FP: CCACTGAAAAAGATGAGTATGCCT RP: CAATCCAAATGCGGCATCTTCA	FP: 24 RP:22	92.9%, 0.991	Cytoskeletal protein involved in cell locomotion (Wang, Liu et al. 2021)
(RPL 13a) Ribosomal protein L13A	NM_012423.4	FP: CTCAAGGTGTTTGACGGCATCC RP: TACTTCCAGCCAACCTCGTGAG	FP: 22 RP:22	95%, 0.994	Inhibits the translation of certain transcripts in case of inflammation (Jia, Arif et al. 2012)
EEF1G *Homo sapiens* eukaryotic translation elongation factor 1 gamma (EEF1G), mRNA	NM_001404.5	FP: GTGGTACTCAGAGTATCGCTTCC RP: GGATGACACTGGCGAAGGCATT	FP: 23 RP:22	99.5%, 0.993	Deliver aminoacyl tRNAs to the ribosomes (Cristiano 2020)
(UBE2D2) Ubiquitin conjugating enzyme E2 D2	NM_181838.1	FP: CTACGATCACAGTGGTCTCCAG RP:CGAGCAATCTCAGGCACTAAAGG	FP: 22 RP:23	94.5%, 0.993	Nucleotide metabolism, degrade misfolded proteins (He, Sandford et al. 2008)
(HPRT1) Hypoxanthine phosphoribosyl-transferase 1	NM_000194	FP: CATTATGCTGAGGATTTGGAAAGG RP: CTTGAGCACACAGAGGGCTACA	FP: 24 RP:22	94.5%, 0.991	Metabolic salvage of purines (Duan, Nilsson et al. 2004)

Table 2: Primers used for IL-10

Targeted gene	**Primer sequence**	**Efficiency (%), R^2 value**	**Gene Function**
IL-10 forward	5'TCTCCGAGATGCCTTCAGCAGA3'	93.3%, 0.9978	Initiation of larger cascades of anti-inflammatory cytokines for immunosuppression and to combat the pathogenic agents causing tissue damage
IL-10 reverse	5'TCAGACAAGGCTTGGCAACCCA3'		

2.2.4. Quantitative qRT-PCR

qRT-PCR was performed with IQ^{TM} 5 Multicolor Real-Time PCR Systems (BioRad). The expression study of 9 selected HKGs was done in 384-well plates and amplified in automated florometer ABI Prism 7500HT sequence detection System (Applied Biosystems) 20µL diluted cDNA (15ng/µL), 0.4 µL of each primer from 10µM of stock solution, 5µL SYBR Premix Ex Taq II (TaKaRa) were added in PCR tubes along two step PCR conditions: temperature 95 ºC for 30 seconds and 55 ºC for 60 seconds was provided. The whole process was repeated upto 40 cycles. Followed by step 2 in which a temperature of 72 ºC was provided for 7 minutes. Each reaction was performed thrice. The signals of fluorescence were obtained during the annealing temperature and Ct values exported with a threshold of 0.1 and a baseline of 3-10.

3.1. Statistical analysis for the determination of the most stable and the least stable housekeeping genes (HKGs)

RefFinder is a web-based tool which includes pack of various bio statistical algorithms. This online software uses raw Ct values (obtained from qPCR) as an input to generate ranking positions for HKGs. It is a robust method to identify expression stability of reference genes by providing analysis values via ΔCt, GeNorm, NormFinder, and Bestkeeper programs. ΔCt method ranks HKGs by

comparing the mean Standard deviation. GeNorm program ranked HKGs based on the average expression stability (M) value. NormFinder applet employed inters and intra group analysis of HKGs and analysis was based on stability value (SV). BestKeeper work on the principle values of standard deviation (Std) of crossing point (CP) for reference genes, geometric mean (GM) value of crossing point (CP), and co-efficient of covariance (CV) value obtained in terms of % CP. In the end, the fold change in expression patterns of the most stable and the least stable genes against targeted gene (IL-10) were ascertained using p >0.05 Turkey multiple range tests).

4.1. Results

4.1.1. Primers specifications

Reproducibility values of RT-PCR (R^2) were obtained greater than 0.991 and amplification efficiency value was found within the range of 93% to 101%. These values were obtained on the basis of reaction calculated by the standard curve method for the commercial synthesized primers were used to perform qRT-PCR.

4.1.2. Quantification values of nine reference genes

RT-PCR (BioRad) system was operated to determine the stability of HKGs. Results were obtained in the form of untransformed Ct values. Lower Ct values indicate of presence of higher gene expression level and vice versa. Ct values were documented as reported by Sarwar et al. (Sarwar, Ahmad et al. 2020). Ct values obtained from blood samples of Covid-19 candidates were recounted within the range of 9 to 43. This wide fluctuation within the Ct values suggested that candidate HKGs has diverse response towards different molecular conditions (control, mild to moderate and severe) of blood samples. Ct value of GAPDH was expressed (C= 19-26 ±2.2) (M to M = 24-28 ±1.6) (S= 26-35 ±2.6) followed by Ct vales of beta-Actin (C= 19-25 ±1.6) (M to M = 19-24 ± 1.4) (S=10-16 ±1.6), 18S rRNA (C= 10-19 ±2.9) (M to M = 21-29 ±2.1) (S= 25-33 ±2.9), TBP (C= 30-36 ±1.7) (M to M= 26-35 ±2.5) (S = 22-35 ±4.1), B2M (C= 13-29 ±4.2) (M to M= 22-35 ±4.1) (S= 29-35 ±1.5), RPLA 13a (C= 14-25 ±2.8) (M to M = 23-43 ±4.4) (S= 11-19 ±2.2), EEF1 (C= 15-22 ±2) (M to M = 21-25 ±1.2) (S= 12-34 ±4.9), UBE2D2 (C= 13-29 ±3.6) (M to M = 12-19 ±2.5) (S= 20-29 ±3), and HPRT1 (C= 23-29 ±1.8) (M to M = 22-29±2.1) (S= 9-15 ±1.4). HKGs with the highest Std. represented the most diverse expression pattern (regarded as less stable ones) as compared to HKGs with the lowest Std. represented the least diverse expression pattern (regarded as more stable ones) (Sarwar, Ahmad et al. 2020) (Fig.2).

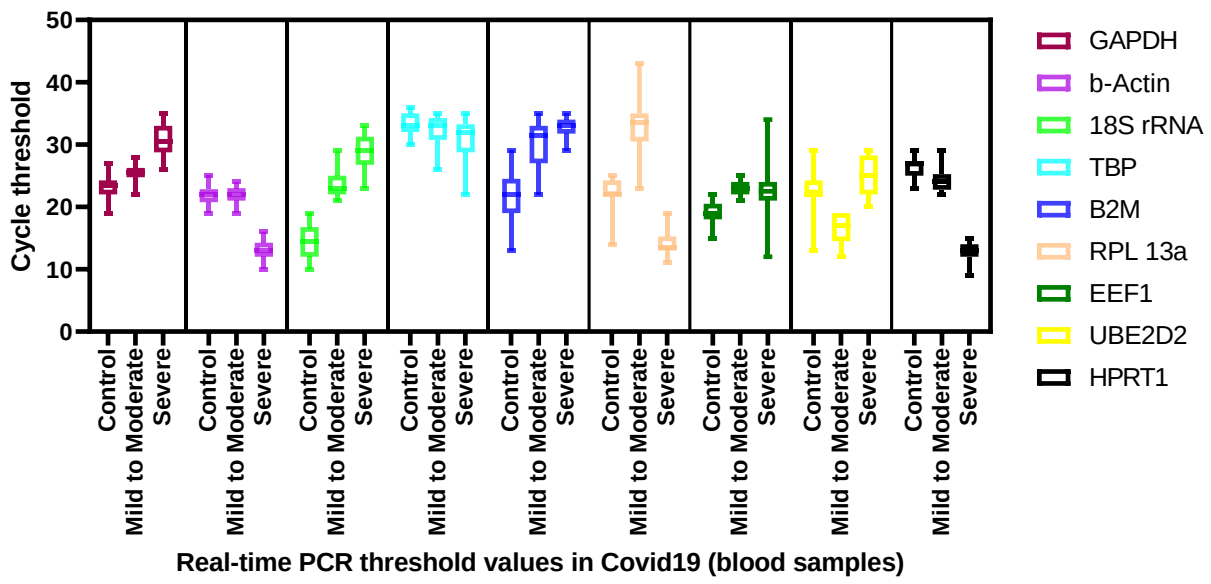


Figure 2: Analysis of threshold values in control, mild to moderate and severe Covid-19 samples. Bars across the square represented standard deviation, whereas whiskers indicate range of Ct values in each group (C, M to M and S). Data represented a total of 42 subjects (14 individuals of each experimental group)

4.1.3. Comparative ΔCt analysis of nine candidate HKGs

Expression profiles analysis reflects the expression level of selected reference genes. Higher the ΔCt value lower will be the stability factor of the gene (Chen, Han et al. 2017). ΔCt were obtained by using online ReFfinder software. For control group, ΔCt reported the lowest value for beta-Actin (M= 2.001) followed by the increasing ΔCt value for TBP (2.009), HPRT1 (M= 2.057), EEF1 (M= 2.438), GAPDH (M= 2.722), 18S rRNA (M= 3.018), RPL 13a (M= 3.247), UBE2D2 (M= 3.556), and B2M (M= 3.864). For mild to moderate group, ΔCt value was obtained lowest for GAPDH (M= 1.506), followed by the increase in the ΔCt value for EEF1 (M= 1.507), beta-Actin (M= 1.841), HPRT1 (M= 2.034), 18S rRNA (M= 2.192), UBE2D2 (M= 2.475), TBP (M= 2.710), B2M (M= 3.120), and RPL 13a (M= 3.470). For severe Covid-19 group, ΔCt value was obtained lowest for beta-Actin (M= 1.646), followed by the increase in the ΔCt value for B2M (M= 1.902), RPL 13a (M= 2.179), GAPDH (M= 2.392), 18S rRNA (M= 2.661), UBE2D2 (M=

2.998), TBP (M= 3.368), and EEF1 (M= 3.816). Results for pool samples convinced that beta-Actin found to be the most stable HKG (Fig.3).

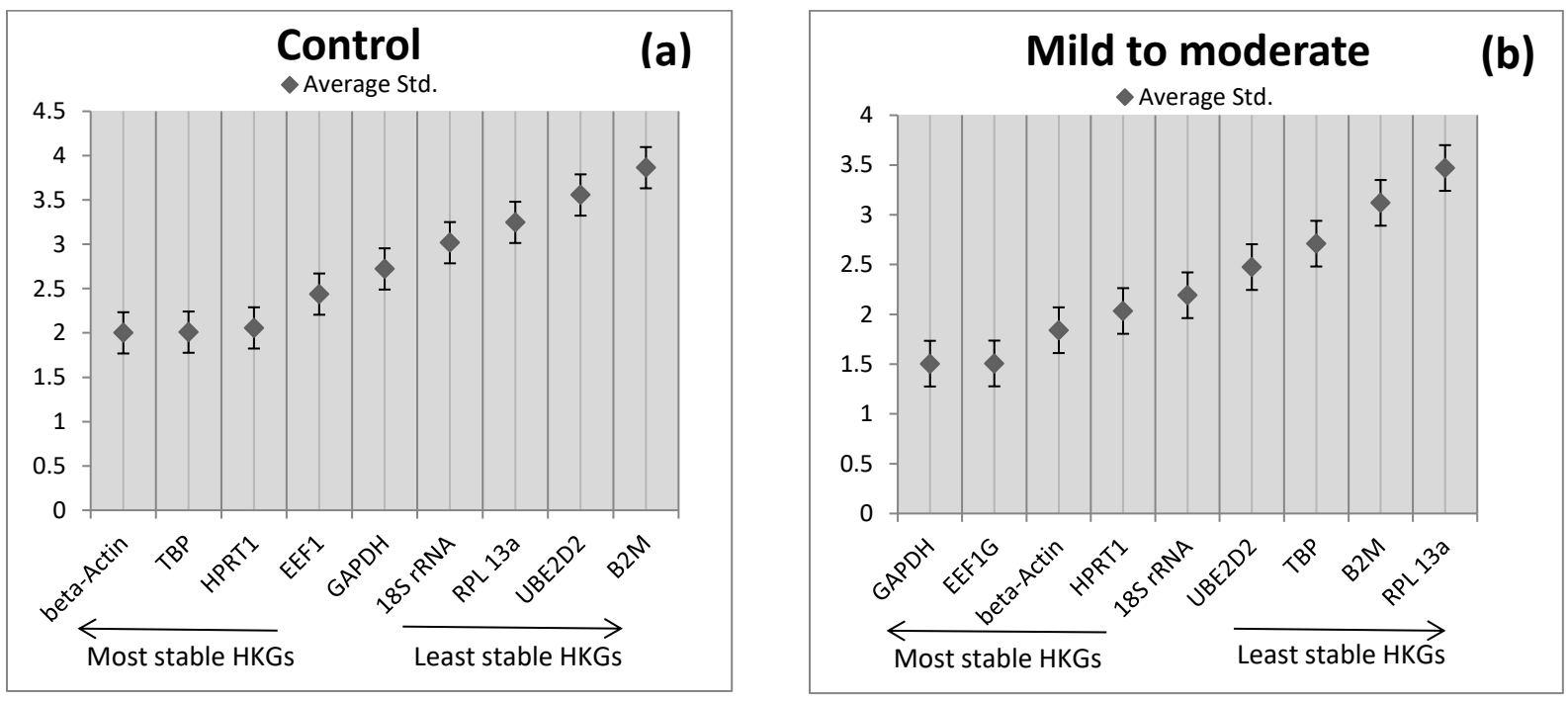


Fig. 3: ΔCt analysis of nine HKGs in Covid-19 blood samples (a) control group (b) mild to moderate group (c) severe group. Error bars with standard deviation are also indicated

4.1.4. Analysis of gene expression stability by GeNorm of online ReFfinder

All nine candidate HKGs were evaluated by using GeNorm software based on average stability (M) value. The lowest (M) values suggested highest stability character for respective HKG under all molecular conditions (control, and write full names for others) and vice versa (Sarwar, Ahmad et al. 2020). The M value for control group ranked beta-Actin (2.008) as the most stable HKG followed by TPB (2.009), HPRT1 (2.057), EEF1FG (2.438), GAPDH (2.722), 18S rRNA (3.018), RPL 13a (3.247), UBE2D2 (3.556) and B2M (3.864). Mild to Moderate group suggested M values for EEF1 (1.506), GAPDH (1.507), beta-Actin (1.841), HPRT1 (2.034), 18S rRNA (2.192), UBE2D2 (2.475), TBP (2.710), B2M (3.120), and RPL 13a (3.470). M values for beta-Actin (1.646), HPRT1 (1.647), B2M (1.902), RPL 13a (2.179), GAPDH (2.392), 18S rRNA (2.661), UBE2D2 (2.998), TBP (3.368), and EEF1 (3.816) were obtained for severe group. GeNorm for pooled samples analysis confirmed beta-Actin as the most stable HKG as shown in Fig. 4 (a-c).

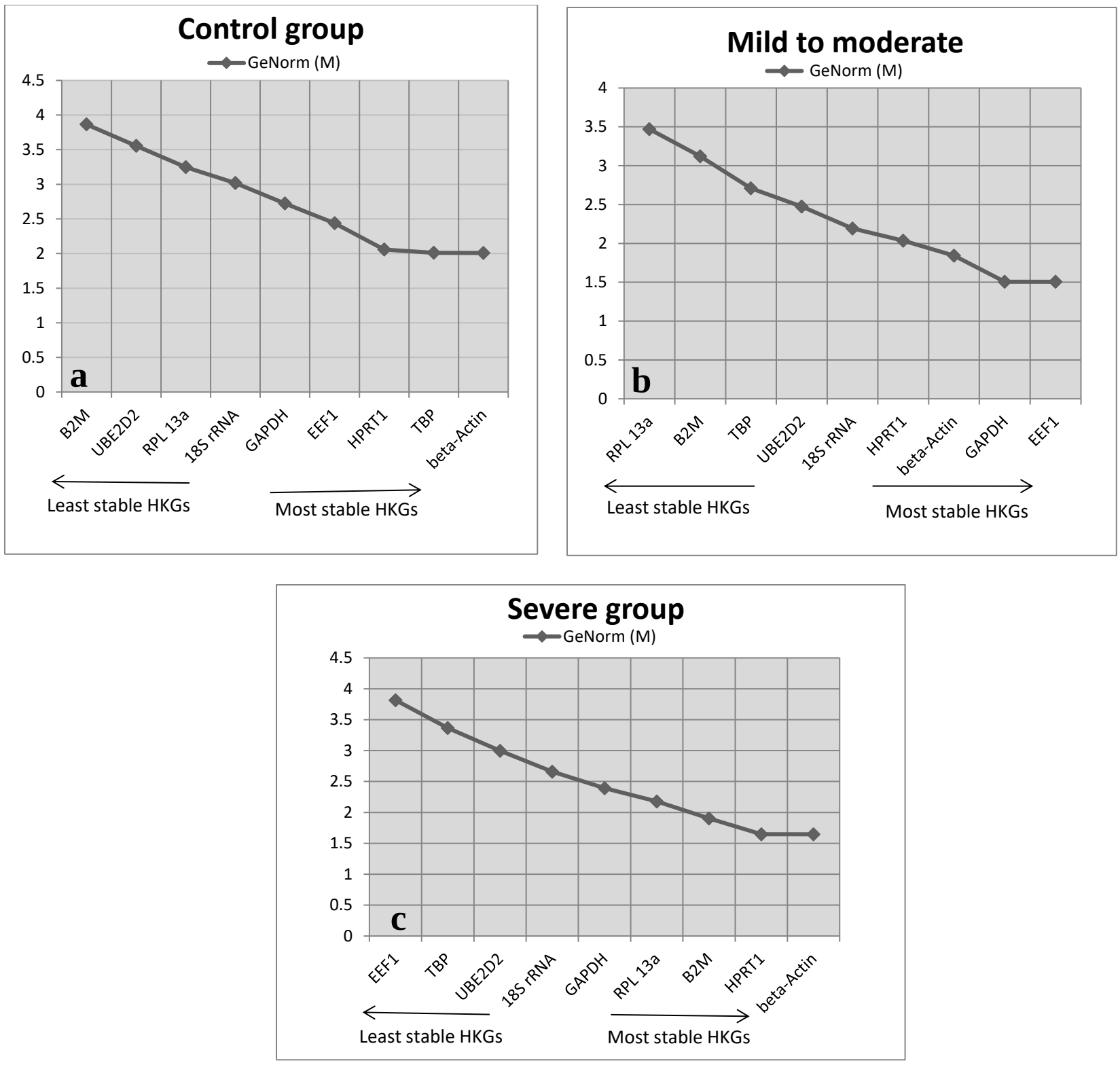


Fig. 4: Nine HKGs by GeNorm software based on (M) values (a) control group (b) mild to moderate group (c) severe group

4.1.5. Analysis of gene expression stability by NormFinder of online ReFfinder

NormFinder is statistical algorithm to determine the best possible candidate genes based on estimation of intra and inter group gene expression variations. Lower the SV value obtained higher will be the stability expression of that particular HKG and vice versa (Sarwar, Ahmad et al. 2020). SV values obtained for each group were comprehensively merged in Table 2. Results evinced that the order of SV values was the highest for beta-Actin (SV=1.143), HPRT1 (SV= 1.562), and TBP (SV=1.761) , thus found as the most stable genes in control group, whereas, EEF1 (SV= 1.571), GAPDH (SV= 1.600), and beta-Actin (SV= 1.636) emerged as the most stable genes in the mild to moderate group. For severe group, HPRT1 (SV= 1), beta-Actin (SV= 1.490), and B2M (1.564) revealed as the most stable expression profile. Results produced by comparative ranking of selected reference genes by NormFinder were different from those generated by GeNorm in the most of the Covid-19 samples. However, combine analysis of pooled samples by GeNorm and NormFinder identified beta-Actin (SV for C = 1.143, SV for M to M= 1.636, and SV for S= 1.490) as the most stable gene in all Covid-19 groups due to attaining the least SV values.

Table 2: Expression stability value (SV) of selected HKGs calculated by NormFinder under different molecular conditions (Control, Mild to Moderate, and Severe) of Covid-19 blood samples

NormFinder analysis	**Control**		**Mild to moderate**		**Severe**	
HKGs	**Stability values (SV)**	**Rank**	**Stability values (SV)**	**Rank**	**Stability values (SV)**	**Rank**

GAPDH	2.724	5	1.600	2	2.634	6
beta-Actin	1.143	1	1.636	3	1.490	2
18S rRNA	2.965	6	2.171	5	2.479	5
TBP	1.761	3	2.197	6	4.003	8
B2M	4.275	9	3.639	8	1.564	3
RPL 13a	2.987	7	4.137	9	2.205	4
EEF1F	2.464	4	1.571	1	4.820	9
UBE2D2	3.921	8	2.90	7	3.050	7
HPRT1	1.562	2	1.80	4	1	1

4.1.6. Analysis of gene expression stability by BestKeeper from online RefFinder

RefFinder tool also consisted of BestKeeper which ranked the candidate HKGs expression stability on the basis of (a) the co-efficient of covariance (CV), (b) standard deviation. Genes with lower Std. represented higher stability level and vice versa. The stability indicator values by Bestkeeper for individual genes are shown in Table 3. TBP (CV= 4.18, ±1), UBE2D2 (CV= 5.50, ±5.50), and beta-Actin (CV = 5.66, ±1.23) were ranked as the top three HKGs for control samples. EEF1G (CV=4.35, ±1), GAPDH (CV= 4.76, ±1.21), and beta-Actin (CV= 5.06, ±1.10) were ranked as the most stable genes for mild to moderate Covid-19 samples. TBP (CV= 3.70, ±3.70), GAPDH (CV= 7.23, ±2.21), and 18S RNA (CV= 7.60, ±2.18) were on the top of the list of ranking for severe Covid-19 patients. Under the pooled condition, three HKGs (B2M, GAPDH and TBP) got the least Std. values and ranked on the top of the list.

Table 3: Expression values of selected HKGs calculated by BestKeeper under different molecular conditions (Control, Mild to Moderate, and Severe) of Covid-19 affected blood samples

BestKeeper Analysis	**Control**			**Mild to moderate**			**Severe**		
HKGs	**Std.**	**CV (%CP)**	**Rank**	**Std.**	**CV**	**Rank**	**Std.**	**CV**	**Rank**
GAPDH	±1.71	7.41	4	±1.21	4.76	2	±2.21	7.23	2
Beta-Actin	±1.23	5.66	3	±1.10	5.06	3	±1.23	9.45	5
18S RNA	±2.53	17.80	9	±1.64	6.99	6	±2.18	7.60	3
TBP	±1	4.18	1	±1.98	6.16	4	±3.21	10.54	7
B2M	±3.08	13.96	8	±3.33	11.2	8	±1.20	3.70	1
RPL 13a	±1.71	7.79	5	±2.96	9.03	7	±1.70	11.99	8
EEF1FG	±2.24	9.88	7	±1	4.35	1	±2.93	13.27	9
UBE2D2	±1.45	5.50	2	±2.21	13.42	9	±2.37	9.50	6
HPRT1	±1.54	8.10	6	±1.55	6.35	5	±1.08	8.51	4

4.1.7. Comprehensive Ranking of HKGs by ReFfinder

Comparative stability analysis for candidate HKGs in the form of geometric mean values was obtained to rank all individual genes accordingly. HKGs with the lowest Geometric mean (GM) value reflected the highest stability characteristic in terms of gene expression and vice versa. By following this criterion, beta-Actin (GM= 1), TBP (GM= 2.06), HPRT1 (GM= 2.63), EEF1 (GM= 3.72), GAPDH (GM= 5.23), RPL 13a (GM= 6.44), 18S rRNA (GM= 6.45), UBE2D2 (GM=7.74), and B2M (GM= 9) were expressed in control group. EEF1 (GM= 1), GAPDH (GM= 1.86), beta-Actin (GM= 2.71), HPRT1 (GM=4), 18S rRNA (GM= 5), TBP (GM= 6.24), UBE2D2 (6.74), B2M (8.24), and RPL 13a (8.74) showed stability profiles in all mild to moderate samples. In severe Covid-19 experimental group, HPRT1 (GM=1), beta-Actin (GM=1.86), B2M (GM=2.71), RPL 13a (GM=4), 18S rRNA (GM= 5.23), GAPDH (GM= 5.73), UBE2D2 (GM=7), TBP (GM= 8.24), and EEF1 (GM= 8.47) were found to be the most stable HKGs (Fig.5 a-c).

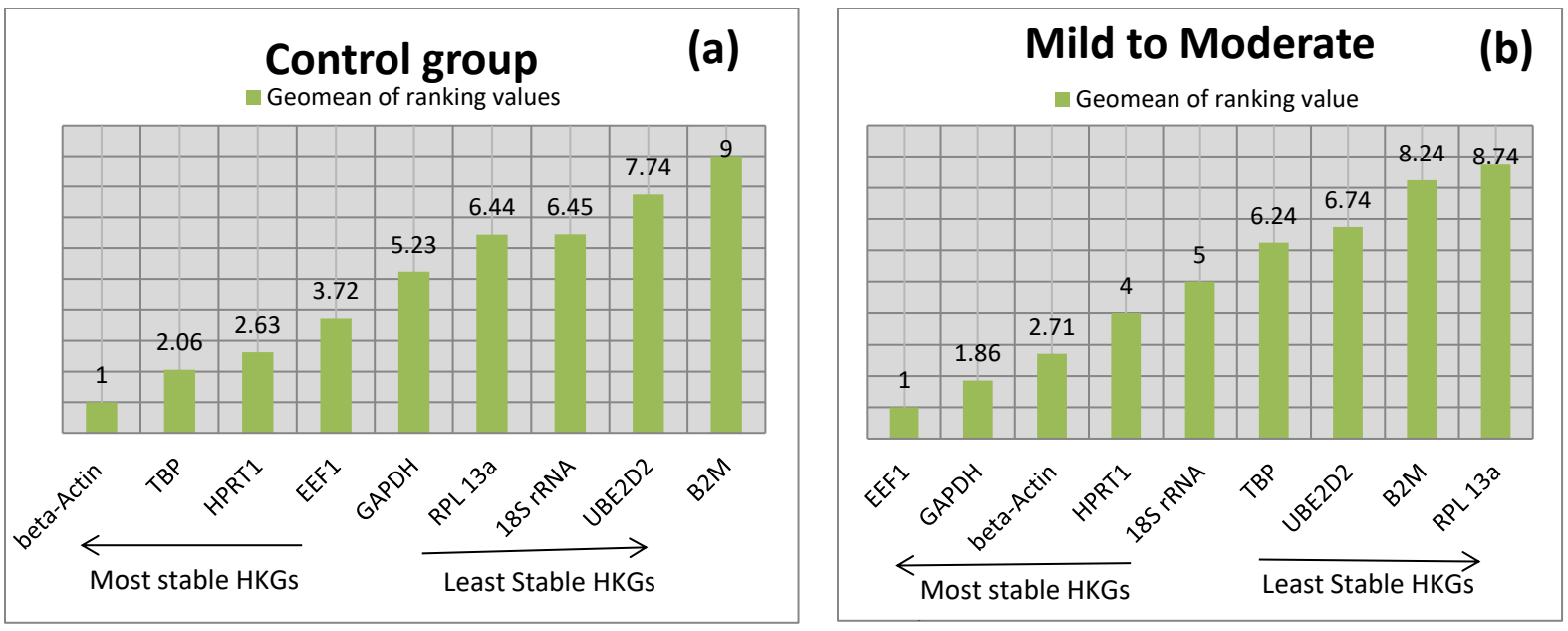


Fig.5: Graphical representation of comparative gene stability by ReFfinder based on Geomean ranking values in all Covid-19 (a-c) groups tested

4.1.8. The most stable and the least stable combinations of HKGs determined by RefFinder

Comprehensive ranking was carried out by ReFfinder. Recommended comprehensive ranking order (RO) for the control group samples was found as beta-Actin (RO= 1), TBP (RO= 2), HPRT1 (RO= 3), EEF1 (RO=4), GAPDH (RO=5), RPL 13a (RO=6), 18S rRNA (RO=7), UBE2D2 (RO=8), and B2M (RO=9). Recommended comprehensive ranking order (RO) for the mild to moderate group samples was determined as EEF1 (RO= 1), GAPDH (RO= 2), beta-Actin (RO= 3), HPRT1 (RO=4), 18S rRNA (RO=5), TBP (RO=6), UBE2D2 (RO=7), B2M (RO=8), and RPL 13a (RO=9). Recommended comprehensive ranking order (RO) for the severe Covid-19 group was determined as HPRT1 (RO= 1), beta-Actin (RO= 2), B2M (RO= 3), RPL 13a (RO=4), 18S rRNA (RO=5), GAPDH (RO=6), UBE2D2 (RO=7), TBP (RO=8), and EEF1 (RO=9) (Table 4).

Table 4: Comprehensive ranking of individual HKGs by ΔCt, BestKeeper, NormFinder and GeNorm methods from online ReFfinder

Control samples - Ranking Order (← Better------Good-------Average →)									
Methods	1	2	3	4	5	6	7	8	9
ΔCt	beta-Actin	HPRT1	TBP	EEF1	GAPDH	18S rRNA	RPL 13a	UBE2D2	B2M
BestKeeper	beta-Actin	TBP	EEF1	HPRT1	RPL 13a	GAPDH	UBE2D2	18S rRNA	B2M
NormFinder	beta-Actin	HPRT1	TBP	EEF1	GAPDH	18S rRNA	RPL 13a	UBE2D2	B2M
GeNorm	beta-Actin/ TBP		HPRT1	EEF1	GAPDH	18S rRNA	RPL 13a	UBE2D2	B2M
Recommended comprehensive ranking	**beta-Actin**	**TBP**	**HPRT1**	**EEF1**	**GAPDH**	**RPL 13a**	**18S rRNA**	**UBE2D2**	**B2M**

Mild to moderate samples - Ranking Order (← Better------Good-------Average →)									
ΔCT	EEF1	GAPDH	beta-Actin	HPRT1	18S rRNA	TBP	UBE2D2	B2M	RPL 13a
BestKeeper	EEF1	beta-Actin	GAPDH	HPRT1	18S rRNA	TBP	UBE2D2	RPL 13a	B2M
Normfinder	EEF1	GAPDH	beta-Actin	HPRT1	18S rRNA	TBP	UBE2D2	B2M	RPL 13a
GeNorm	GAPDH/ EEF1		beta-Actin	HPRT1	18S rRNA	UBE2D2	TBP	B2M	RPL 13a
Recommended comprehensive ranking	**EEF1**	**GAPDH**	**beta-Actin**	**HPRT1**	**18S rRNA**	**TBP**	**UBE2D2**	**B2M**	**RPL 13a**
Severe samples - Ranking Order (← Better------Good-------Average →)									
ΔCt	HPRT1	beta-Actin	B2M	RPL 13a	18S rRNA	GAPDH	UBE2D2	TBP	EEF1
BestKeeper	HPRT1	B2M	beta-Actin	RPL 13a	18S rRNA	GAPDH	UBE2D2	EEF1	TBP
Normfinder	HPRT1	beta-Actin	B2M	RPL 13a	18S rRNA	GAPDH	UBE2D2	TBP	EEF1
GeNorm	beta-Actin/ HPRT1		B2M	RPL 13a	GAPDH	18S rRNA	UBE2D2	TBP	EEF1
Recommended comprehensive ranking	**HPRT1**	**beta-Actin**	**B2M**	**RPL 13a**	**18S rRNA**	**GAPDH**	**UBE2D2**	**TBP**	**EEF1**

4.2. Evaluation of the target gene (IL-10) expression in Covid19 patients in relevance to the selected the most stable HKGs

Validation of the identified stable HKGs by comparing target gene (IL-10) was performed for normalization. It has been demonstrated that the use of inappropriate HKGs for the normalization of gene of interest (GOI) can result in significant wrong interpretation of expression data under specific molecular conditions (Control, Mild to Moderate, and Severe) of Covid-19 samples. Based on ReFfinder overall ranking, beta-Actin, and TBP were regarded as the most stable HKGs whereas, B2M was found as the least stable HKG. The trend of IL-10 was significantly similar to the most stable HKGs and differed when normalized with the least stable genes. To validate the usage efficiency of the identified superior HKGs, expression of a target gene (IL-10) was normalized in response to tested candidate HKGs. The relative fold expression of targeted gene (IL-10) was found to be uniform in the control samples when normalized with the most stable and the least stable genes. The results showed that the values of the relative fold change was reported the least for beta-Actin following the almost the similar trend followed by TBP. Hence, both ranked as the most stable HKGs, whereas, the significant relative fold change values were reported by the B2M thus ranked as inappropriate HKG for the process of normalization. Relative fold expression of GOI (IL-10) was up-regulated upto two relative folds change across Covid-19 spectrum (M to M and S) as compared to the control samples when normalized with the most stable and the least stable genes. For control group relative fold change with respect to time was reported by beta-Actin as 10 minutes= 0.05 fold changes, 20 minutes= 1.05 fold changes, 30 mints= 0.2, 40 minutes= 0.2, 50 minutes= 0.4, and 60 minutes=0.1 fold change. For mild to moderate group fold change reported by TBP was 10 minutes= 0.5 fold changes, 20 minutes= 1.09 fold changes, 30 mints= 0.4, 40 minutes= 0.5, 50 minutes= 0.7, and 60 minutes=0.9 fold change. B2M reported 10 minutes= 0 fold changes, 20 minutes= 1 fold changes, 30 mints= 2, 40 minutes= 2, 50 minutes= 4, and 60 minutes=1 fold change (Fig. 6A). For mild to moderate group relative fold change with respect to time was reported by beta-Actin as 10 minutes= 0.9 fold changes, 20 minutes= 0.5 fold changes, 30 mints= 0.5 fold changes, 40 minutes= 0.08 fold changes, 50 minutes= 0.06 fold changes, and 60 minutes=0.25 fold changes. Expression of TBP was found as 10 minutes= 1 fold change, 20 minutes= 2 fold changes, 30 mints= 2 fold changes, 40 minutes= 2 fold changes, 50 minutes= 0.93 fold changes, and 60 minutes=0.25 fold changes. B2M reported as 10 minutes= 1 fold change, 20 minutes= 0.75 fold changes, 30 mints= 12 fold changes, 40 minutes= 4 fold changes, 50 minutes= 5 fold changes, and 60 minutes=5 fold changes (Fig. 6B). Results demonstrated that the relative fold change value for severe group by beta-actin as 10 minutes= 0.01 fold changes, 20 minutes= 0.2 fold changes, 30 mints= 0.8 fold changes, 40 minutes= 1 fold change, 50 minutes= 2 fold changes, and 60 minutes= 2 fold changes. Expression of TBP was found as 10 minutes= 0.75 fold changes, 20 minutes= 1 fold change, 30 mints= 2 fold changes, 40 minutes= 4 fold changes, 50 minutes= 4 fold changes, and 60 minutes= 5 fold changes. B2M relative fold change reported as 10 minutes= 5 fold changes, 20 minutes= 15 fold changes, 30 mints= 16 fold changes, 40 minutes= 18 fold changes, 50 minutes= 20 fold changes, and 60 minutes= 25

fold changes (Fig 6C).However, the expression levels of B2M showed significant overestimation under least stable genes' normalization process ($p > 0.05$ Turkey multiple range tests).

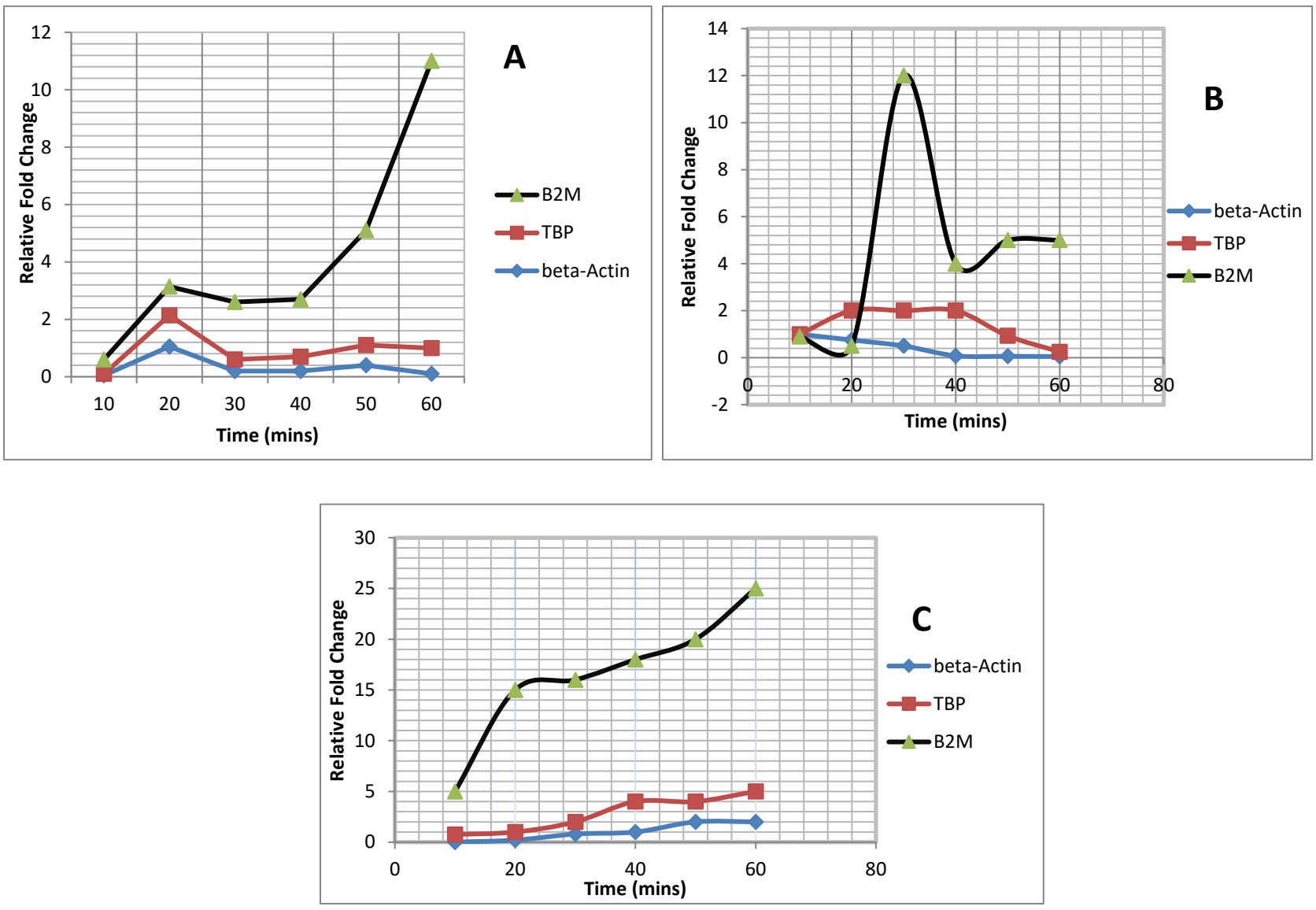


Fig. 6: Normalization of IL-10 gene expression for validation of selected reference genes under different molecular conditions of Covid-19 samples (A= Control group, B= mild to moderate, and C= severe group). The Error bars represent the standard deviation among the biological replicates. The solid blue and red lines represent the topmost stable reference while black solid lines with green triangles represent the least stable reference genes.

5.1. Discussion

An ideal HKG has the ability to maintain a stable RNA transcription level in all subjects regardless of disease status (control, mild, moderate or severe cases). Hence the purpose of the present study was to evaluate the variation in the expression among 9 potential HKGs from normal individuals to Covid-19 (Mild to Moderate and Severe group) affected victims. A literature search showed that no single gene can be declared as universal HKG which means for different viruses attacking the different body systems or even disrupting the functional ability of the same system, in all cases same HKGs cannot be used as normalizer in RT-PCR for the expression analysis of any particular gene. For example, Cyclophilin A (PPIA) gene is regarded as the best housekeeping gene in the case of RT-PCR in normal, and asthmatic human bronchial epithelial cells for the expression analysis purpose (He, Sandford et al. 2008). Similarly, for *Heliothis virescens ascovirus* 3h (HvAV-3H) expression analysis by RT-PCR, elongation factor 2 (EF2) is regarded as the most stable housekeeping gene (Chen, Han et al. 2017).

The RT-PCR is one of the most recognized technique to accurately measure the expression stability or instability levels of genes (Pfaffl 2001). The accuracy is compromised by numerous factors like quality of extracted RNA, usage of non-specific primers, providing non-optimal enzymatic conditions, selection of less stable housekeeping genes (HKGs) against the target genes, and inadequate application of statistical methods, etc. for the normalization process. These factors could lead to misinterpretation of the results (Nikalje, Srivastava et al. 2018). Thus, the use of appropriate HKGs for expression analysis studies becomes critical to correct sample to sample errors arising due to the said limitation.

In the current study commonly used HKGs were chosen from the literature which showed a diversity of function that enabled successful selection of reference genes for different cells, tissues, and disease status (Andersen, Jensen et al. 2004; Curtis, Gomez et al. 2010; Nicholls, Li et al. 2012; Wang, Bryant et al. 2017; Cristiano 2020). However, using the same HKGs for expression analysis

against specific genes showed that there could be great stability variation among them, which means for specific genes, some specific set of stable HKGs should be used as normalizers.

For this research study, from detailed literature review 9 selected HKGs; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Beta actin (β -actin), 18S ribosomal RNA, TATA-binding protein (TBP), ribosomal protein L13A (RPL 13a), Homo sapiens eukaryotic translation elongation factor 1 gamma (EEF1G), mRNA, Ubiquitin conjugating enzyme E2 D2 (UBE2D2), hypoxanthine phosphoribosyltransferase 1 (HPRT1) were selected. Statistical analysis for the determination of the most stable and the least stable HKGs was performed by RefFinder. RefFinder is a web-based tool which includes pack of various bio statistical algorithms. This online software uses raw Ct values (obtained from qPCR) as an input to generate ranking positions for HKGs. It is a robust method to identify expression stability of reference genes by providing analysis values via ΔCt, GeNorm, NormFinder, and Bestkeeper programs. ΔCt method ranks HKGs by comparing the mean Std. GeNorm program ranked HKGs based on the average expression stability (M) value. NormFinder applet employed inters and intra group analysis of HKGs and analysis was based on stability values (SV). BestKeeper work on the principle values of Std. of crossing point (CP) for reference genes, geometric mean (GM) values of crossing point (CP), and co-efficient of covariance (CV) values obtained in terms of % CP. In the end, the fold change in expression patterns of the most stable gene and the least stable gene against targeted gene (IL-10) were ascertained using $p > 0.05$ (Turkey multiple range tests). Results were obtained in the form of untransformed Ct values. Lower Ct value is the indication of presence of higher gene expression level and vice versa. Ct values were documented as reported by Sarwar *et al.* (Sarwar, Ahmad et al. 2020). Ct values obtained from blood samples of Covid-19 candidates were recounted within the range of 9 to 43. This wide fluctuation within the Ct values suggested that candidate HKGs have diverse response towards different molecular conditions (control, mild to moderate and severe) of blood samples.

Expression profiles analysis reflects the expression level of selected reference genes. Higher the ΔCt value lower will be the stability factor of the gene (Chen, Han et al. 2017). ΔCt were obtained by using online ReFfinder software. For control group, ΔCt reported the lowest value for beta-Actin (M= 2.001) followed by the increasing ΔCt value for TBP (M= 2.009), GeNorm software based on average stability (M) values. The lowest (M) values suggested highest stability character for respective HKG under all molecular conditions (Control, Mild to Moderate and Severe) and vice versa (Sarwar, Ahmad et al. 2020). The M value for control group ranked beta-Actin (M= 2.008) as the most stable HKG followed by TPB (M= 2.009). NormFinder is the statistical algorithm to determine the best possible candidate genes based on estimation of intra and inter group gene expression variations. Lower the SV values obtained higher will be the stability expression of that particular HKG and vice versa (Sarwar, Ahmad et al. 2020). SV values obtained for each group were comprehensively merged in Table 2. Results evinced that beta-Actin, HPRT1 and TBP were found the most stable genes in control group, whereas, EEF1, GAPDH and beta-Actin emerged as the most stable genes in the mild to moderate group. The stability indicator values analyzed by Bestkeeper for individual genes confirmed that TBP, UBE2D2 and beta-Actin were ranked as the top three HKGs for control samples. EEF1G, GAPDH and beta-Actin were ranked as the top first stable genes for mild to moderate Covid-19 samples. TBP, GAPDH and 18S RNA were on the top of the list of ranking for severe Covid-19 . Under the pooled condition, three HKGs (B2M, GAPDH and TBP) got the least std. values and ranked on the top of the list. Comparative stability analysis for candidate HKGs in the form of geometric mean values was obtained to rank all individual genes accordingly. HKGs with the lowest geometric mean (GM) value reflected the highest stability characteristic in terms of gene expression and vice versa. By following this criterion, beta-Actin (GM= 1), TBP (GM= 2.06) and HPRT1 (GM= 2.63) were found the most stable in the control group. EEF1 (GM= 1), GAPDH (GM= 1.86) and beta-Actin (GM= 2.71) showed highest stability profiles in all mild to moderate samples. In severe Covid-19 experimental group, HPRT1, beta-Actin and B2M were found to be the most stable HKGs. Recommended comprehensive ranking was carried out by ReFfinder. Based on the recommended comprehensive ranking order values (RO) for the control group samples confirmed beta-Actin (RO= 1), TBP (RO= 2), and HPRT1 (RO= 3) as the top three HKGs. Mild to moderate group samples confirmed the best HKGs as EEF1 (RO= 1), GAPDH (RO= 2), and beta-Actin (RO= 3), respectively. Recommended comprehensive ranking order of severe Covid-19 group determined HPRT1 (RO= 1), beta-Actin (RO= 2), and B2M (RO= 3) as the best HKGs due gene expression stability. Comparative profiling of the most and the least identified stable genes against GOI (IL-10) showed that there were two most stable genes i.e., beta-Actin and TBP as both followed the almost similar expression trends throughout the comparative profiling. However, the expression levels of the least stable gene i.e., B2M showed variable trends in all Covid-19 groups (C, M to M, and S). Therefore, fluctuation in their stability values suggested that the least stable HKGs should not be used as endogenous internal control in expression analysis process when carried out by RT-PCR.

The outcomes depicted that β –actin and TBP were the most stable HKGs with least variations, whereas, B2M was the least stable HKG with maximum fold change in expression profiles. To the best of our knowledge till now, it the first study of HKGs in Pakistan for the expression analysis study profile of Covid-19 which was not systematically evaluated before this study. Further research is also

recommended for better experience of normalization of genes by RT-PCR. This study is based on human immune system compromised due to Covid-19 to identify the most stable housekeeping genes by comparing their expression stability.

6.1. Conclusion and future prospective

Housekeeping genes play important role as gene analyzer in gene expression studies. These genes also act as internal endogenous control with respect to other target genes because the expression levels of stable housekeeping genes remain same in all molecular conditions i.e., if cells are in normal condition or infected with the virus. In contrast, the expression levels of other genes are altered (up-regulated or down-regulated) due to viral infection. These changes in expression levels are determined by RT-PCR. Similarly, the changes in expression levels of certain genes associated with compromised immune system due to SARS-CoV-2 is determined by selecting the most stable housekeeping genes against the altered genes. In this study, it was identified that for all the samples of Covid-19 (C, M to M and S), beta–Actin and TBP were identified as the most stable HKGs for RT-PCR normalization. It is always recommended for the accurate normalization i.e. a set of two housekeeping genes preferably be used against target gene.

The current pandemic era calls for the urgent need of generating plethora of biological data in the form of complete genome sequence of SARS-CoV-2. Advance molecular techniques, genetic information integrated with computational tools paved the ways to study the changes in the expression levels of certain defective genes due to viral infection. The identification of the most stable HKGs is the prime step in conduction of any expression analysis studies. By these identified HKGs which are showing invariability in all Covid-19 groups will assist in finding key lineage of fast mutating power of Covid-19 strains. Divergence in basal cellular transcriptome analysis for the Covid-19 vaccine development is the major future goal of this research study.

References

1. Andersen, C. L., J. L. Jensen, et al. (2004). "Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets." Cancer research 64(15): 5245-5250.
2. Bustin, S., V. Benes, et al. (2005). "Quantitative real-time RT-PCR–a perspective." Journal of molecular endocrinology 34(3): 597-601.
3. Chen, Z.-S., N.-N. Han, et al. (2017). "Selection of reference genes for expression analysis using RT-qPCR in the dissemination system of Heliothis virescens ascovirus 3 h (HvAV-3h)." Scientific reports 7(1): 1-10.
4. Ciotti, M., M. Ciccozzi, et al. (2020). "The COVID-19 pandemic." Critical reviews in clinical laboratory sciences 57(6): 365-388.
5. Cristiano, L. (2020). "EEF1G (Eukaryotic translation elongation factor 1 gamma)." Atlas of Genetics and Cytogenetics in Oncology and Haematology.
6. Curina, A., A. Termanini, et al. (2017). "High constitutive activity of a broad panel of housekeeping and tissue-specific cis-regulatory elements depends on a subset of ETS proteins." Genes & development 31(4): 399-412.
7. Curtis, K. M., L. A. Gomez, et al. (2010). "EF1α and RPL13a represent normalization genes suitable for RT-qPCR analysis of bone marrow derived mesenchymal stem cells." BMC molecular biology 11(1): 1-15.
8. Dheda, K., J. Huggett, et al. (2005). "The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization." Analytical biochemistry 344(1): 141-143.
9. Dheda, K., J. F. Huggett, et al. (2004). "Validation of housekeeping genes for normalizing RNA expression in real-time PCR." Biotechniques 37(1): 112-119.
10. Duan, J., L. Nilsson, et al. (2004). "Structural and functional analysis of mutations at the human hypoxanthine phosphoribosyl transferase (HPRT1) locus." Human mutation 23(6): 599-611.
11. Eisenberg, E. and E. Y. Levanon (2013). "Human housekeeping genes, revisited." TRENDS in Genetics 29(10): 569-574.
12. Eisenberg, E. and E. Y. Levanon (2014). "Human housekeeping genes, revisited (vol 29, pg 569, 2013)." Trends in Genetics 30(3): 119-120.
13. Ferreira-Cerca, S., G. Pöll, et al. (2005). "Roles of eukaryotic ribosomal proteins in maturation and transport of pre-18S rRNA and ribosome function." Molecular cell 20(2): 263-275.
14. Gachon, C., A. Mingam, et al. (2004). "Real-time PCR: what relevance to plant studies?" Journal of experimental botany 55(402): 1445-1454.
15. Grein, J., N. Ohmagari, et al. (2020). "Compassionate use of remdesivir for patients with severe Covid-19." New England Journal of Medicine 382(24): 2327-2336.

16. Guénin, S., M. Mauriat, et al. (2009). "Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references." Journal of experimental botany 60(2): 487-493.
17. Guo, Y.-R., Q.-D. Cao, et al. (2020). "The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak–an update on the status." Military medical research 7(1): 1-10.
18. He, J.-Q., A. J. Sandford, et al. (2008). "Selection of housekeeping genes for real-time PCR in atopic human bronchial epithelial cells." European Respiratory Journal 32(3): 755-762.
19. Hu, B., H. Guo, et al. (2021). "Characteristics of SARS-CoV-2 and COVID-19." Nature Reviews Microbiology 19(3): 141-154.
20. Huang, I. and R. Pranata (2020). "Lymphopenia in severe coronavirus disease-2019 (COVID-19): systematic review and meta-analysis." Journal of intensive care 8(1): 1-10.
21. Islam, H., T. C. Chamberlain, et al. (2021). "Elevated interleukin-10 levels in COVID-19: potentiation of pro-inflammatory responses or impaired anti-inflammatory action?" Frontiers in Immunology: 2485.
22. Jia, J., A. Arif, et al. (2012). "Protection of extraribosomal RPL13a by GAPDH and dysregulation by S-nitrosylation." Molecular cell 47(4): 656-663.
23. Lee, T. I. and R. A. Young (1998). "Regulation of gene expression by TBP-associated proteins." Genes & development 12(10): 1398-1408.
24. Moein, S., S. H. Javanmard, et al. (2017). "Identification of appropriate housekeeping genes for gene expression analysis in long-term hypoxia-treated kidney cells." Advanced biomedical research 6.
25. Nestorov, J., G. Matić, et al. (2013). "Gene expression studies: How to obtain accurate and reliable data by quantitative real-time RT PCR." Journal of Medical Biochemistry 32(4): 325-338.
26. Nicholls, C., H. Li, et al. (2012). "GAPDH: a common enzyme with uncommon functions." Clinical and Experimental Pharmacology and Physiology 39(8): 674-679.
27. Nikalje, G. C., A. K. Srivastava, et al. (2018). "Halophytes in biosaline agriculture: Mechanism, utilization, and value addition." Land Degradation & Development 29(4): 1081-1095.
28. Nossmann, M. (2020). "Optimization of a molecular diagnostic strategy to verify SARS-CoV-2 infections by RT-qPCR." Journal of Laboratory Medicine 44(6): 349-356.
29. Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT–PCR." Nucleic acids research 29(9): e45-e45.
30. Radonić, A., S. Thulke, et al. (2004). "Guideline to reference gene selection for quantitative real-time PCR." Biochemical and biophysical research communications 313(4): 856-862.
31. Ruan, W. and M. Lai (2007). "Actin, a reliable marker of internal control?" Clinica Chimica Acta 385(1-2): 1-5.
32. Sarwar, M. B., Z. Ahmad, et al. (2020). "Identification and validation of superior housekeeping gene (s) for qRT-PCR data normalization in Agave sisalana (a CAM-plant) under abiotic stresses." Physiology and Molecular Biology of Plants 26(3): 567-584.
33. Stürzenbaum, S. R. and P. Kille (2001). "Control genes in quantitative molecular biological techniques: the variability of invariance." Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 130(3): 281-289.
34. Thellin, O., W. Zorzi, et al. (1999). "Housekeeping genes as internal standards: use and limits." Journal of biotechnology 75(2-3): 291-295.
35. Vandesompele, J., K. De Preter, et al. (2002). "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes." Genome biology 3(7): 1-12.
36. Velavan, T. P. and C. G. Meyer (2020). "The COVID-19 epidemic." Tropical medicine & international health 25(3): 278.
37. Wang, H., B. Liu, et al. (2021). "Beta2-microglobulin (B2M) in cancer immunotherapies: Biological function, resistance and remedy." Cancer Letters.
38. Wang, Y., S. H. Bryant, et al. (2017). "Pubchem bioassay: 2017 update." Nucleic acids research 45(D1): D955-D963.
39. Wrapp, D., N. Wang, et al. (2020). "Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation." Science 367(6483): 1260-1263.
40. Zhu, J., F. He, et al. (2008). "On the nature of human housekeeping genes." Trends in genetics 24(10): 481-484.