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Selecting Suitable Reference Gene for RT-qPCR Normalization in FFPE Breast Cancer Tissues for Duhok/Iraqi Women

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Abstract

Nowadays, with the continuously increasing advances in understanding cancer system biology. It has led to the discovery of valuable disease-specific biomarkers. By targeting these markers using molecular-based techniques a highly specific and sensitive measurements can be obtained. Molecular diagnosis through PCR-based methods and next-generation sequencing (NGS) can detect many diseases caused by DNA and RNA changes that cannot be detected through antibody-based techniques. RT-qPCR is a valuable tool in measuring biomarker's transcriptional signature and will have a great potential in personalized medicine or molecular diagnostics. In related gene expression analysis, using a suitable reference gene is crucial for the accurate interpretation of the results. For this reason, ref. gene should be validated for particular tissue, cell and experimental conditions. This study was conducted to identify and validate the most suitable housekeeping gene (HKG) as a reference (ref.) gene to obtain accurate normalization for AXL receptor tyrosine kinase gene expression in Formalin-Fixed Paraffin-Embedded "FFPE" of breast cancer tissues. Based on the five different statistical algorithms used, ACTB was the least variable ref. gene and ∝-GLOBIN showed the highest variability. Norm finder, Best Keeper and the comparative ΔCt method suggested to use ACTB as a ref. gene in our experiment while geNorm suggested to use the combination of both ACTB and UBC since they had the same exact stability value. In conclusion, Reffinder suggested that ACTB had the least geomean of ranking values of the four used methods showing the least variance among the other genes and the three groups of our samples.

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1. Introduction

Very sensitive and specific techniques provided by molecular biology can assist in numerous fields such as disease diagnosis and prognosis, molecular medicine, and food safety[1]. The benefits of using nucleic acid detection exceed over protein detection for example, it is more cost-efficient and can detect many diseases caused by DNA changes such as DNA rearrangements and point mutations that can be detected using PCR-based techniques and next-generation sequencing (NGS) that are difficult to measure via antibody-based techniques[1]. Transcriptional biomarker studies often target and utilize messenger RNA since its genetic sequence is recognized for protein synthesis, also its expression levels correlate with the amount and type of protein that is directly linked to pathological disorder or disease[2]. In this context many techniques have been developed to measure messenger RNA, Reverse Transcription Quantitative Polymerase Chain Reaction RT-qPCR is a valuable tool in establishing disease-specific transcriptional biomarkers[1]. In relative gene expression analysis, a normalizer is necessary to correct differences in data caused by

differences in cellular input, quality of RNA, sample to sample variation and reverse transcriptase efficiency across samples in order to interpret gene expression measurements for clinical samples. In addition, the including of reference gene (ref. gene) improves the reliability of RT-qPCR experiments [3, 4]. The most common method in normalizing data is using a ref. gene as an internal control in gene expression experiments. Subsequently, selecting suitable ref. gene is not and should not be an arbitrary process, each ref. gene should be examined and validated based on the tissue or cell type and experimental conditions (ref. gene is exposed to the same experimental conditions as the target gene of interest and their expression profile should not be affected by them)[5]. Formalin-fixed paraffin-embedded (FFPE) is a valuable source of samples for retrospective analysis of clinical samples, gene expression analysis on FFPE tissues could be challenging due to degraded nature of mRNA, it might be caused by cross-linking and/or oxidative deamination that affects mRNA quality and integrity as a starting material for RT-qPCR leading to variation of gene expression between samples, thus validating and selecting suitable ref. genes or introducing two or more of them is crucial to reflect this variation and facilitate the reliable use of FFPE tissue for mRNA analysis and designing useful retrospective studies[6-8]. Computational tools that are based on different statistical algorithms allows for evaluating candidate ref. genes stability. Different statistics produce different stability rankings within the same experimental results of a ref. gene study, hence the use of Reffinder a web-based tool is beneficial for comparing and evaluating candidate ref. genes[9]. In this study, we attempted to identify the least variable ref. gene among five of the most used candidate ref. genes in normalizing RT-qPCR in respect to our gene of interest (AXL receptor tyrosine kinase) in breast cancer and non-neoplastic breast tissues in FFPE samples. These genes included (ACTB, GAPDH, □-GLOBIN, UBC, and TEGT).

2. Materials and Methods:

2.1 Patients and Study Samples:

This is a part of a study that was conducted to measure AXL receptor tyrosine kinase gene expression in breast tissues FFPE samples. The original study included a total number of 75 FFPE samples and was arranged in groups based on the grade (H. grade = 25 represented by high grade malignant tumors, L. grade = 25 represented by low and intermediate grade of malignant tumors and B, C group = 25 represented by fibroadenoma benign tumors for comparison and non-neoplastic cases represented mostly by inflammatory conditions as a control group). Five samples from each group were used (a total of 15 samples for each candidate ref. gene). The tissue blocks age ranged from two months to 6 years (from 2018 to 2024). Breast sample cases were collected from the central lab in Duhok governorate / Iraq. Ethical approval was obtained from the ethics committee of science research ethics committee under the number and date of (No.2 date: 23rd Dec. 2023). An experienced pathologist had reviewed the Hematoxylin and Eosin (H&E) slides to confirm the original diagnosis and to determine the malignant parts of interest for RNA extraction.

2.2 Candidate Genes:

Five candidate ref. genes were selected out of a set of significant genes previously reported in the literature (ACTB, GAPDH, □-GLOBIN, UBC, and TEGT)[7, 10-13] as shown in (Table.1). Their sequences [10, 14] are shown in (Table.2).

Table 1. Ref. genes of choice related information.

Gene name	Cellular function	Symbol (accession number)
Beta actin	cell motility, structure, integrity, and intercellular signaling	ACTB[a] (NM_001101.3)
Hemoglobin beta	Hemoglobin subunit	∝-GLOBIN (NM_004048.2)
Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis enzyme	GAPDH[a] (NM_002046.3)
Ubiquitin C	Polyubiquitin precursor	UBC[a] (NM_021009,4)
Testis enhanced gene transcript (BAX inhibitor 1)	suppressor of programmed cell death	TEGT (NM_003217.2)

Table 2. The sequence of ref. genes and their annealing temperature.

Ref. gene symbol	Primer sequences (5´→ 3´)	Ta °C	Product length (bp)
ACTB [a]	F: ATGTGGCCGAGGACTTTGATT	60	107
	R: AGTGGGGTGGCTTTTAGGATG		
∝-GLOBIN	F: ACA CAA CTG TGT TCA CTA GC	65	110
	R: CAA CTT CAT CCA CGT TCA CC		
GAPDH [a]	F: TCTCCTCTGACTTCAACAGCGAC	60	126
	R: CCCTGTTGCTGTAGCCAAATTC		
UBC [a]	F: ATTTGGGTCGCAGTTCTTG	60	91
	R: TGCCTTGACATTCTCGATGGT		
TEGT	F: TGCTGGATTTGCATTCCTTACA	60	151
	R: ACGGCGCCTGGCATAGA		

2.3 RNA Isolation

From each sample, a serial of 15 sections of 5µm were sliced. The first 3 sections were discarded to avoid contamination. Subsequently, sections were macro dissected before RNA extraction and purification. RNA was isolated from FFPE tissue using the "Amoy Dx FFPE DNA/RNA kit" (Amoy Dx, China). The detailed steps of extraction were performed according to the manufacturer's recommendations. RNA quantification was performed using "Quantifluor RNA System" (Promega, USA).

2.4 One-step RT-qPCR

GoTaq® One-Step RT-qPCR System was used (Promega, USA). The detailed steps, materials concentrations, and volumes were applied according to manufacturer's protocols, the final qPCR reaction was carried out in a total of 10µL of reaction volume (Table. 3). RT-qPCR was carried out using MIC-qPCR cycler (BMS, Australia). The cycling conditions at each step are shown in (Table.4). A no template controls were included for each candidate ref. gene run.

Table 3. Dye-based RT-qPCR reaction mix for each sample.

Total volume (µL)	10 (µL)
Master mix	5
Forward primer	0.5
Reverse primer	0.5
Nuclease free water	2.5
$MgCl_2$	0.25
RT mix	0.25
RNA template	1

Table 4. Dye-based One-step RT-qPCR cycling conditions.

Step	Temperature	Duration
Hold	37°C	15 min
Hold	95°C	5 min
40 cycles	95°C	20s
	60°C	20s acquiring on Green
	72°C	20s
Melt on Green	Melt from 72°C to 95°C at 0.3°C/s	

2.5 Stability analysis:

The stability analysis was performed by using Reffinder on raw Ct values as suggested by the website (bit.ly/3WUMpy3). Firstly, Reffinder runs statistical analysis based on the traditional four methods (geNorm, Norm finder, Best Keeper, and the comparative ΔCt method). Followed by utilizing ranks generated from each method and combining them to provide a comprehensive analysis for ranking each gene based on its variability (most stable to least stable order). The accepted thresholds for gene stability: the GeNorm M-value <1.5, NormFinder stability value <1.0 and Best Keeper Standard deviation <1.0 [15].

2.5.1 Comparative Delta Ct

Comparative delta Ct was employed by using Excel application and Reffinder calculations. The ranking was made possible by comparing the means of delta Ct of the housekeeping genes. Lower mean delta Ct values indicate higher expression levels of the housekeeping gene, which it may suggests a better choice as a ref. gene, also by taking the standard deviation of delta Ct values into consideration, a lower standard deviation indicates more stable expression across samples.

2.5.2 Best keeper

An Excel-based spreadsheet add-in software named Best Keeper was established and examined on biological material by Michael W. Pfaffl in 2004 [16]. BestKeeper (version 1) could be used through the Excel-based tool obtained from (https://www.gene-quantification.de/bestkeeper.html#download). The algorithm runs multiple statistics on Ct values as input data of which are denoting markers of variation in data and include: arithmetic mean, minimum and maximum Ct, Geo Mean, Ct std and coefficient of variation. After that, the algorithm performs repeated pair-wise correlation analyses and for every calculated correlation, the Pearson correlation coefficient (r) and the p value are calculated. The Best keeper index is the combination of highly correlated (HKGs), and then a comparison is made between candidate ref. gene and the index produces Pearson correlation coefficient(r), coefficient of determination (r2) and p-value [17]. Best keeper was performed via Reffinder. Lower index scores denote greater transcriptional stability and thus better suitability as a ref. gene[18].

2.5.3 Norm Finder

It is a model-based algorithm that estimates the variations of candidate ref. genes expression [19]. The stability value calculated by Norm Finder reflects the size of the systematic error for each ref. gene[20]. It can be performed using Excel Add-in and it's available in Reffinder.

2.5.4 GeNorm

Firstly, the algorithm calculates stability value (M) for each gene and. Then it compares the pair-wise variation (V) of this gene with the others. The Vn/Vn+1value indicates the pairwise variation between two serial normalization factors and determines a suitable number of ref. genes for accurate normalization[18].

2.5.5 RefFinder

It is a user-friendly website tool developed to provide a comprehensive analysis to screen for optimal ref. genes from provided experimental datasets. It integrates (geNorm, Norm finder, Best Keeper, and the comparative ΔCt method) to compare and rank candidate ref. genes. Based on the rankings from each program, a final rank is established by the Reffinder algorithm by calculating the geometric mean of ranking values from each program [21]. It is often used to select suitable ref. gene for normalization of qPCR final results [22].

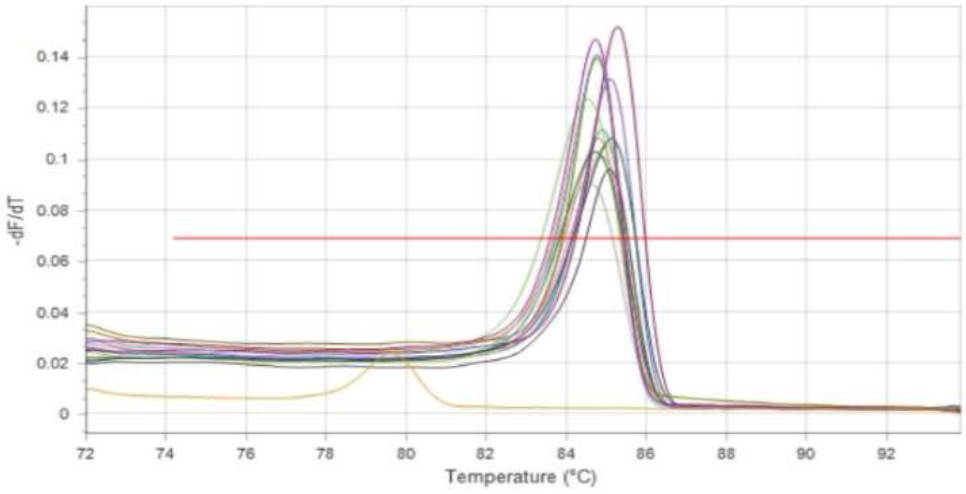
3. Results

3.1 RNA Isolation:

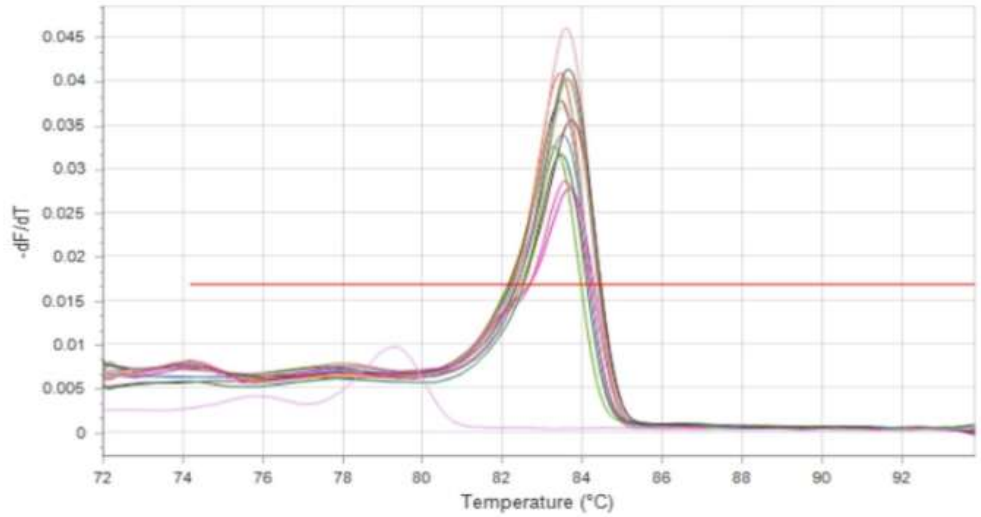
Total RNA was extracted, and the purity ratio mean of A260/A280 was (≥1.6) using nanodrop. Total RNA concentration ranged from 17 to 315 ng/µl measured by using Quantus Fluorometer (Promega, USA). Concentrations are shown in (Table.5).

3.2 One-step RT-qPCR:

Expression levels were determined as the number of cycles needed for amplification to reach a fixed threshold in the exponential phase of the qPCR reaction [23]. Raw Ct values and melting curves of ref. genes are shown in the (Table.5) and (Figuer.1).



(a)



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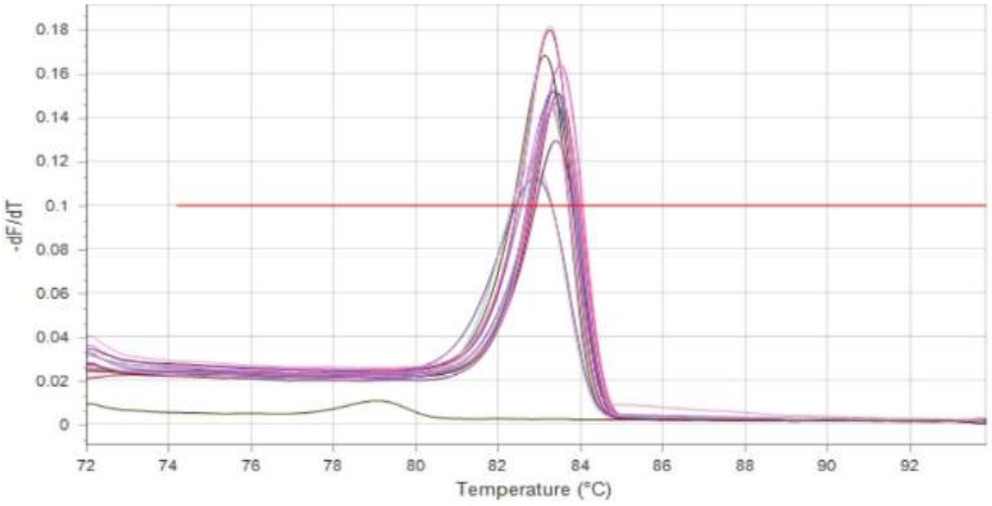

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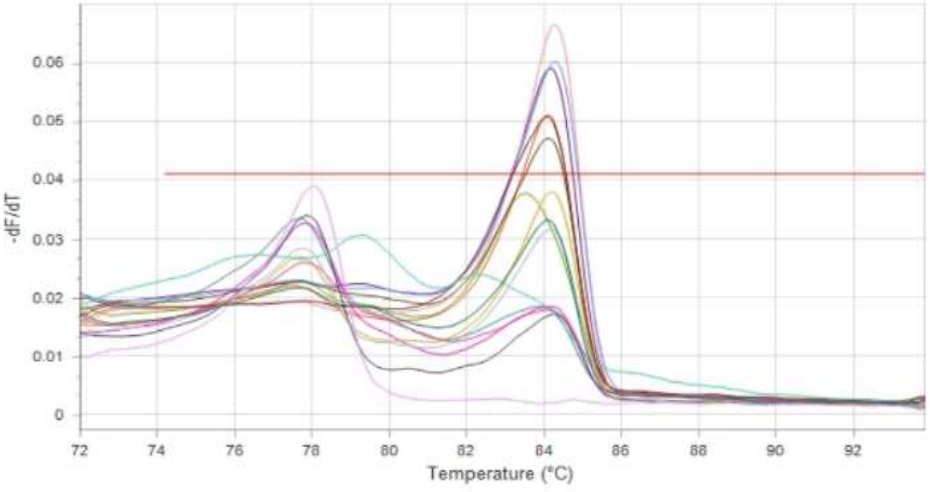

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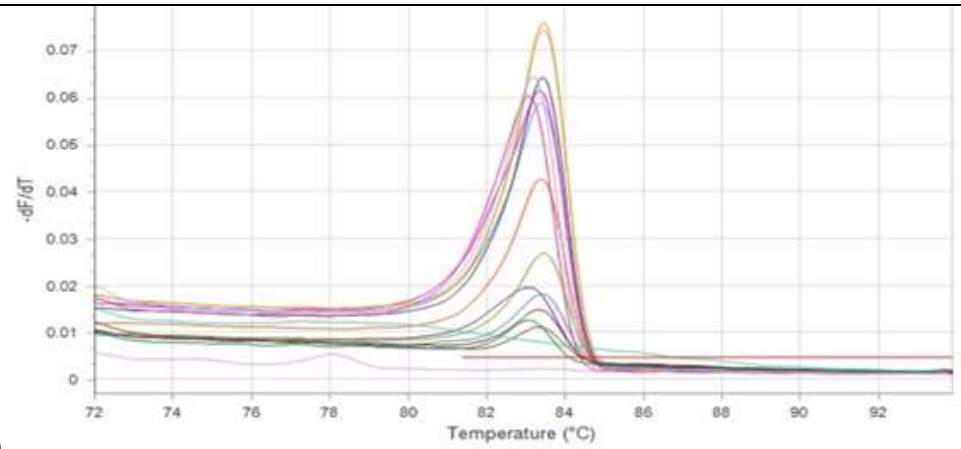

(e)

Figure 1. candidate reference genes melting curves: (a) TEGT melting curve with threshold of (0.063) starting at 74.18°C; (b) ACTB melting curve threshold (0.017) starting at starting at 74.18°C; (c) UBC's threshold is (0.100) starting at 74.18°C; (d) The GAPDH's curve threshold is (0.041) starting at 74.18°C; (e) □-Globin melting curve threshold is (0.005) starting at 81.38°C. All melting curves were generated using micPCR version 2.10.0 software, thresholds demonstrate the starting points for significant peaks where the concentration of amplicons starts to increase in the mixture and represented by red horizontal line.

Table 5. Ct values and concentrations of ACTB, GAPDH, □-GLOBIN, UBC and TEGT.

Sample	FFPE block year	Total RNA conc. (ng/µl)	GAPDH	TEGT	UBC	ACTB	Beta Globin
L1	2022	285	17.08	21.32	17.37	17.01	33.48
L2	2023	279	19.42	26.27	20.26	20.60	31.41
L3	2023	214	19.17	24.64	19.69	19.55	26.10
L4	2019	155	20.24	26.95	21.55	20.04	33.52
L5	2020	315	18.32	26.37	20.12	18.93	Excluded
H1	2023	36	17.18	23.24	18.22	18.90	21.81
H6	2020	164	16.84	22.66	17.64	18.31	24.57
H10	2019	77.5	20.55	27.16	20.40	21.50	23.60
H15	2018	224	17.61	24.11	18.94	18.98	Excluded
H24	2024	45.8	20.19	23.58	20.13	20.63	23.28
C1	2023	26.6	20.93	25.63	20.34	20.99	25.08
C2	2023	17	21.33	30.13	24.19	24.25	24.40
C3	2023	310	17.45	25.62	20.26	19.61	Excluded
C4	2023	44.3	19.96	23.35	19.41	20.22	24.28
C6	2023	84.6	18.39	22.32	18.30	19.12	36.74

The median CT value is 19.17 with a mean of 18.92 and an average of 18.98. The STD 1.47 for GAPDH indicates mild variability in its levels of expression across samples, showing fluctuation level in its expression. As for TEGT, it has a median of Ct value 24.64, an average of 24.89, and a mean of 24.79. The standard deviation was 2.22, indicating more variability compared to GAPDH in expression levels. This suggests that TEGT is less stable as a ref. gene. The median Ct value for UBC is 20.12, with an average of 19.79 and a mean of 19.72. The standard deviation is 1.63, indicating moderate variability.

UBC shows a similar level of stability to GAPDH, with slightly higher variability. ACTB has a median Ct value of 19.61, an average of 19.91, and a mean of 19.85. With a standard deviation of 1.60, ACTB demonstrates moderate variability and is comparable to GAPDH and UBC in terms of stability. On the other hand, Beta Globin shows a median of Ct values of 24.82, with a significantly higher average of 27.36 and a mean of 26.97. The high standard deviation of 4.78 indicates substantial variability, suggesting that Beta Globin is the least stable of the housekeeping genes evaluated. GAPDH, UBC, and ACTB have relatively consistent expression levels with moderate variability, while TEGT shows slightly more variability. Beta Globin, on the other hand, has the highest variability and a distribution with some high outliers, as indicated by the significant difference between the median and the average.

3.3 Delta Ct

Genes average of STDEV were calculated by Reffinder (Table.6). Demonstrated in (Figure.2-3).

Table 6. Comprehensive delta Ct.

Housekeeping gene	Average of STDEV
ACTB	3.87
UBC	3.89
GAPDH	3.96
TEGT	4.28
∝-GLOBIN	12.64

The lower values of ACTB, UBC, GAPDH, and TEGT show their lower variability and greater Stability. The significantly higher average for □-GLOBIN highlights its higher variability and less stability.

3.4 Best keeper:

Bestkeeper calculations were made using excel add-in and by Reffinder in (Table.7-9). Demonstrated in (Figure.2-3).

Table 7. Best Keeper vs. Coeff. Of corr. [r].

Best Keeper vs.	Coeff. Of corr. [r]		p-value
GAPDH	0.816		0.001
TEGT	0.859		0.001
UBC	0.893		0.001
ACTB	0.782		0.001
∝-GLOBIN	0.244		0.381

Table 6. Repeated pair-wise Pearson correlation coefficient **r** by BEST KEEPER between the HKG.

	GAPDH	TEGT	UBC	ACTB	□-globin
TEGT	0.662	-	-	-	-
p-value	0.007	-	-	-	-
UBC	0.777	0.934	-	-	-
p-value	0.001	0.001	-	-	-
ACTB	0.850	0.822	0.894	-	-
p-value	0.001	0.001	0.001	-	-
∝-GLOBIN	0.31	-0.16	-0.057	0.09	-
p-value	0.257	0.547	0.841	0.74	-

In correlation with Best keeper index of (p=0.001), the ACTB, TEGT, GAPDH and UBC ref. genes showed significance and the pairwise correlation between them resulted in high correlation coefficients signifying their consistent expression across samples. While ß-globin had an insignificant p value compared with best keeper index and low coefficients with the other ref. genes indicating its instability and unsuitability.

Table 9. Descriptive statistics performed by Best keeper.

	GAPDH	TEGT	UBC	ACTB	∝-globin
N	15	15	15	15	15
geo Mean [CP]	18.92	24.79	19.72	19.85	-
AR Mean [CP]	18.98	24.89	19.79	19.91	21.68
min [CP]	16.84	21.32	17.37	17.01	-1.00

max [CP]	21.33	30.13	24.19	24.25	36.74
std dev [+/- CP]	1.33	1.85	1.19	1.18	9.07
CV [% CP]	7.01	7.45	6.03	5.94	41.84
min [x-fold]	-4.23	-11.11	-5.11	-7.15	-2.00
max [x-fold]	5.31	40.41	22.11	21.15	114773609703.35
std dev [+/- x-fold]	2.51	3.61	2.29	2.27	538.90

3.5 Norm finder:

Based on Norm finder stability values listed in (Table.10), both ACTB and GAPDH were less than one which is suitable for normalization, as for UBC's stability value, it was slightly more than Genorm suggested ACTB/UBC, GAPDH and TEGT respectively in that order due to their M value were less than (1.5). Stability values are listed in (Table.11) and demonstrated in (Figure.2-3).

Table 10. HKG stability values based on Norm finder calculations

Gene name	Stability value
ACTB	0.385
GAPDH	0.439
UBC	1.073
TEGT	2.323
∝-GLOBIN	12.615

Table 11. HKG stability value based on GeNorm.

Gene name	Stability value
UBC | ACTB	0.770
GAPDH	0.910
TEGT	1.120
∝-GLOBIN	5.727

One showing moderate variability. TEGT and ∝-GLOBIN showed the highest variability in expression across samples demonstrated in (Figure.2-3).

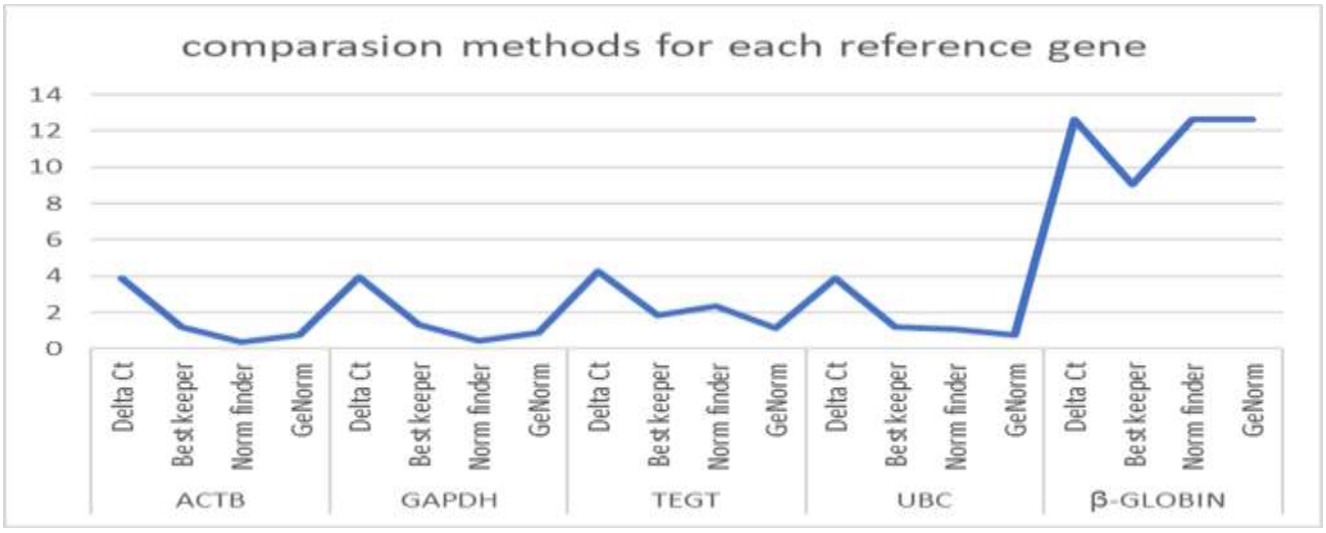

Figure.2 line graph showing stability values for each reference gene based on different statistical algorithms.

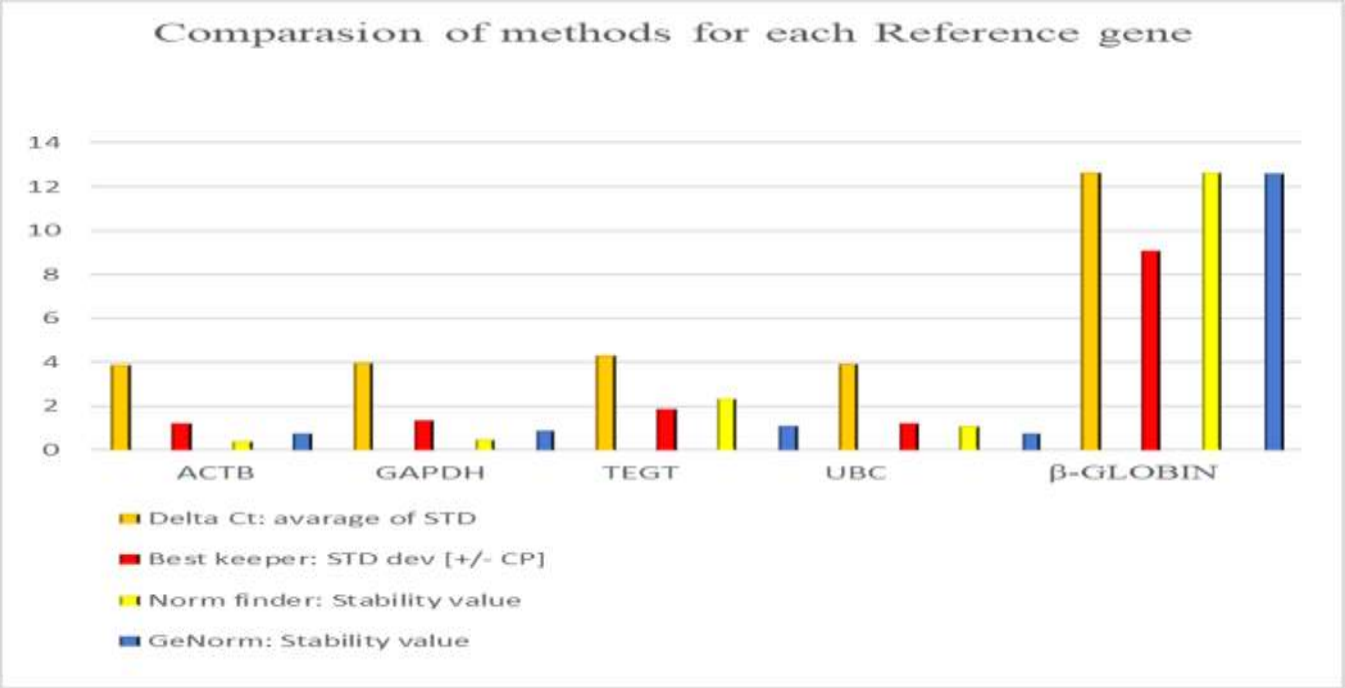

Figure.3 comprehensive Delta Ct ranking, the bar graph represents the average STDEV values for each reference gene. The shorter bars for ACTB, UBC, GAPDH, and TEGT indicate their lower variability and greater stability. The significantly taller bar for ∝-GLOBIN highlights its higher variability and less stability. Gene stability by Best keeper, was achieved using the standard deviation and coefficient of variance. □-GLOBIN has the highest bar indicating low stability in comparison to the other genes. While, ACTB has relatively lower bar signifying its good stability. UBC, GAPDH and TEGT bars gradually increase higher than ACTB but still stable. Gene stability by Norm finder. The provided stability values calculated by Norm finder indicate that ACTB have very high stability represented by the lowest bar. GAPDH, UBC, and TEGT values present very stable, stable and less stable respectively. While ∝-GLOBIN have the highest bar among the other genes, indicating the least stability. Gene stability by Genorm, the bars represent the average expression stability value (M) for each gene. ACTB/UBC showing similarity in their stability value indicating high stability, GAPDH bar slightly increases but remains stable, TEGT bar indicates lower stability in comparison with the first three genes and □-GLOBIN having the highest M-value showing poor stability.

3.7 Reffinder

The Reffinder has finalized the results as demonstrated below in (Table.12) and (Figure.4).

Table.12 HKG final ranks based on Genorm, Norm Finder, Best Keeper, The Comparative ΔCt and Reffinder.

Method	1	2	3	4	5
Delta CT	**ACTB**	**UBC**	**GAPDH**	**TEGT**	**∝-GLOBIN**
BestKeeper	**ACTB**	**UBC**	**GAPDH**	**TEGT**	
Normfinder	**ACTB**	**GAPDH**	**UBC**	**TEGT**	
Genorm	**UBC | ACTB**		**GAPDH**	**TEGT**	
Recommended comp. ranking (Geomean of ranks)	**ACTB (1)**	**UBC (1.86)**	**GAPDH (2.71)**	**TEGT (4)**	**∝-GLOBIN (5)**

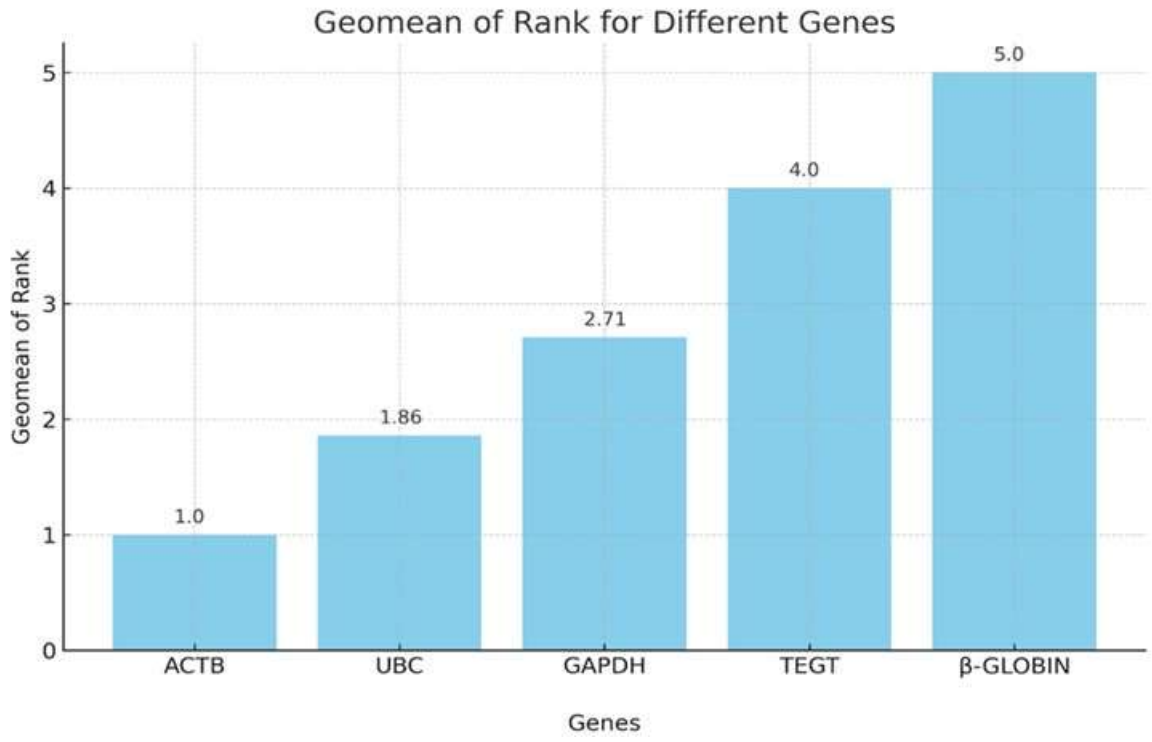

Figure.4 The finale recommended ranking by Reffinder by calculating the geomean of ranks derived from the four methods. This histogram was generated using python.

4. Discussion

FFPE tissue provides a valuable source of clinical samples that could be utilized to address key questions in the breast cancer field. In addition to its abundance and availability it is also connected to rich clinical data and patient's outcome[24]. For personalized medicine, RT-qPCR stands out as a promising tool for molecular diagnostics, potentially becoming routine in clinical testing. It has proven effectiveness in establishing disease-specific biomarkers[1]. RT-qPCR is simple, economical, fast, and yet highly sensitive, accurate, and reproducible [23]. However, studies have indicated the poor quality and quantity nature of RNA extracted from FFPE tissues due to formalin fixation and storage duration [25]. Our data suggested that RNA quality and concentrations were adequate for RT-qPCR. Also, by considering other variables such as differences in cellular content, Reverse Transcriptase efficiency between samples and experimental conditions[3] have major effects on the results of RT-qPCR. In order to correct the errors that arise from such conditions (variables), the need for suitable endogenous calibrator (ref. gene) is crucial. The past studies have shown that ref. genes commonly used behave differently based on the experimental design and cell type thus it is important to measure their stability before implementing them as internal controls [26]. For example, GAPDH is widely used as an internal control, but it has been reported to be dysregulated in many cancers and it was revealed that GAPDH had an implicated role in tumor development thus signifying doubts about its role as an internal ref. gene in cancer studies[27]. Another study suggested that commonly used ref. genes, ACTB, GAPDH, B2M, and 18S rRNA, were unsuitable for normalization in some ovarian tissue related studies [10]. In this present study, geNorm, Norm finder, Best Keeper and the comparative ΔCt method were used to determine the most suitable ref. gene for normalization. □-GLOBIN showed the highest variability across the samples based on these methods thus it was excluded. On the other hand, TEGT took the fourth rank in all of them. As for GAPDH, ranked third based on delta Ct and bestkeeper while it took the second rank according to Norm finder and geNorm.

For UBC, it had the second lowest variability according to delta Ct and bestkeeper. Norm finder ranked UBC in third place. Norm finder, Best Keeper, and the comparative ΔCt suggested that ACTB had the lowest variability among the five ref. gene while Genorm recommended including the combination of both UBC | ACTB since they both have the same stability value. By using these

different methods, different and variable results were generated thus we favored Reffinder due to its user-friendly accessibility and it is continuously updated. Reffinder has finalized the ranks in the order (ACTB>UBC>GAPDH>TEGT> ∝–GLOBIN) by calculating the geometric mean of each ref. gene ranks from the four methods.

5. Conclusions

ACTB was the least variable ref. gene and ∝-GLOBIN showed the highest variability. Norm finder, Best Keeper, and the comparative ΔCt method suggested using ACTB as a ref. gene in our experiment while geNorm suggested using the combination of both ACTB and UBC since they had the same stability value. In conclusion, Reffinder suggested that ACTB had the least geomean of ranking values of the four used methods showing the least variance among the other genes and the three groups of our samples.

Acknowledgments

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Conflicts of Interest

"The authors declare no conflict of interest."

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