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Identification of Suitable Reference Gene for Normalization of microRNAs in Forensically Revenant Body Fluids by Reverse Transcription-Quantitative Polymerase Chain Reaction

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ABSTRACT
Body fluids are well known to be a very useful evidence type in criminal investigations.MicroRNAs are small non-coding RNAs that have previously been identified as possible markers for forensically related body fluid detection.Adequate data normalization is essential to reduce nonbiological technical changes to perform reliable miRNA expression analyzes using a quantitative PCR.The best reference genes are those that are biologically stable and whose expression does not vary across samples. The current study investigated ten selected candidate reference genes that could be used to normalize miRNA expression using an RT qPCR assay, including: SNORD-48; SNORD-44; SNORD-47; U6-2; hsa-miR-26b-5p; hsa-miR-92a-3p; hsa-miR-93-3p; hsa-miR-191-5p; hsa-miR-21; hsa-miR-484.The present results revealed that the cycle threshold (Ct) values of most genes ranged from 3.12 to 27.03, and identify SNORD-47 as a suitable reference gene for all sample types included venous blood, semen, and vaginal secretion.
Keywords: MicroRNAs; Body fluids; Reference genes; RT qPCR.

Introduction
In forensic casework, body fluid detection is important for reconstructing criminal activity.Many studies have shown that some tissue-specific mRNAs can confirm specific body fluids even under controlled conditions after long periods of time [1, 2].However, the analyzes of mRNA are disadvantaged, such as their susceptibility to degradation and the lack of specificity for determining or discriminating certain body fluids, particularly vaginal secretions [3,4]. As a result, several groups have assessed the feasibility and practicability of forensic miRNA analysis using quantitative PCR (qPCR). MiRNAs are non-coding RNA molecule and regulates gene expression at the post-transcriptional level [5],environmentally resistant, because it is stored in exosomes [6,7].The fundamentally short miRNAs have the potential to be ideal markers for forensic applications, including forensic body fluid recognition in the case of substantial differential expression.The number of miRNAs that have been detected is constantly increasing, with hundreds of species now being identified for humans.According to a number of reports that several microRNAs in forensically relevant body fluids are highly expressed [8,9,10].Reference genes are constitutive genes which perform basic cell metabolic functions,they were expected to be expressed in a consistent manner [11].They're suitable for calibration and normalization studies because of their distinct characteristics.The internal control genes are the most common for normalizing the mRNA,microRNAs fractions. This internal control often called a housekeeping gene-should not be different in or in response to the tissues or cells being tested. Therefore, the aim of this study is to investigate ten candidate reference genes in order to determine which ones are best for normalizing and calibrating qPCR data in forensically revenant body fluids.

Material and Methods
Ethical statement
Every volunteer has informed written consent. The ethics committee of the MOH and MOHSER in Iraq's ethical approval for scientific research has accepted this research.
Collection and preparation of samples
Five samples of venous blood, semen, and vaginal secretion were taken from healthy volunteers in the Iraqi province of Babylon. Venous blood was collected into EDTA vials using venipuncture and semen supplied by donors in specimen containers. On a sterile cotton tipped swab, vaginal secretions were collected.
Total RNA Extraction
Extraction of total RNA by RNAzol RT reagent (Sigma-Aldrich/USA), according to manufacturer'sinstructions. RNA yield and integrity measurements were performed.
Estimation of Concentration and purity of Total RNA
The concentration and purity of samples weremeasuredat 260 nm and 280 nm by Nanodrop (Biodrop / UK) instrument. A260/280 ratios of pure RNA would usually range from 1.8 to 2.2.
Candidate reference genes selection

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The following genes were obtained from the miRBase database: SNORD-48, SNORD-44, SNORD-47, U6-2, hsa-miR-26b-5p, hsa-miR-92a-3p, hsa-miR-93-3p, hsa-miR-191-5p, hsa-miR-21, and hsa-miR-484. (http://www.mirbase.org/index.shtml/).

Synthesis of cDNA from microRNA

Total RNA was extracted from three body fluid samples and used for synthesis cDNA synthesis by MiRNA All-In-One cDNA Synthesis Kit from abm/ USA.

RT-qPCR

The reverse transcription and RT-qPCR reactions were carried out according to the manufacturer's instructions for Bright Green miRNA qPCR Master Mix. In all samples and the negative control, the expression levels of ten reference genes were determined in duplicate. RT-PCR mixture was in 20 µl including 10 µl (1x) BrightGreen miRNA qPCR MasterMix, 300 nM of each primer, 3 µlcDNA product. The qPCR reactions were performed using the following 3-step cycling program as table 1:

Table 1. qPCR program

Step	Temperature	Duration	Cycles
Enzyme activation	95°C	10 min	1
Denaturation	95°C	10 sec	40
Annealing and data collection	63°C	15 sec	
Extension	72°C	30 sec	

Biostatistical Analysis

The SPSS statistical package for the Social Sciences was used to analyze the results (version 20.0 for windows, SPSS, Chicago, IL, USA), [12].

Results and Discussion

Body fluids are well known to be a reliable source of proof in criminal investigations. Furthermore, the ability to co-extract both miRNA and DNA is a welcome benefit to the forensic community because it allows for simultaneous body fluid and individual recognition with just a small sample size [13,14].

Collection Sample

Quantity of total RNA differ among samples of different bodyfluid included; venous blood, semen, and vaginal secretion, table (2).

Table 2. Total RNA concentration (ng/µl) for venous blood, semen, and vaginal secretion

Blood Sample	Age	Sex	Total RNA Concentration (ng/µl)	Semen Sample	Age	Total RNA Concentration (ng/µl)	V.S. Sample	Age	Total RNA Concentration (ng/µl)
1B	20	Female	332	1S	41	169	1V	17	323
2B	41	Female	229	2S	32	179	2V	41	174
3B	18	Female	273	3S	30	317	3V	26	170
4B	42	Female	362	4S	52	310	4V	32	188

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5B	19	Female	291	5S	55	438	5V	25	242
Mean± SD	28±1 2.34				42±1 1.33			28.2±8 .92	

Selection of most suitable reference genes

A suitable and robust normalizer is necessary to eliminate and reduce non-biological sample-by-sample variations during the experimental procedure to perform accurate miRNA expression analysis using qPCR. To find the best reference genes, a gene whose expression does not vary across different samples. Stable expression is characterized as small variations between samples of 0.0-0.5 Cts.The present results revealed that the cycle threshold (Ct) values of most genes ranged from 3.12 to 27.03. In this study, identify SNORD-47 as a suitable reference gene for all sample types, as table (3) and fig (1).

Table 3. Mean±SD for Reference gene

HKG ct	SNORD-48	SNORD-44	SNORD-47	U6-2	hsa-miR-26b-5p	hsa-miR-92a-3p	hsa-miR-93-3p	hsa-miR-191-5p	hsa-miR-21	hsa-miR-484
Blood	19.94±0.176	19.06±1.477	26.95±2.651	8.34±1.025	11.54±0.056	4.37±1.308	0±0	0±0	9.11±0.190	0±0
Semen	20.95±0.565	26.34±8.895	26.52±4.214	16.96±2.418	5.72±0.601	3.95±0.579	9.12±1.590	7.63±3.351	10.78±0.657	5.98±1.088
Vaginal Secretion	7.93±2.920	26.38±4.780	27.03±0.749	22.72±0.395	3.12±2.658	6.1±0.678	6.35±1.265	5.77±0.586	0±0	11.86±0.898

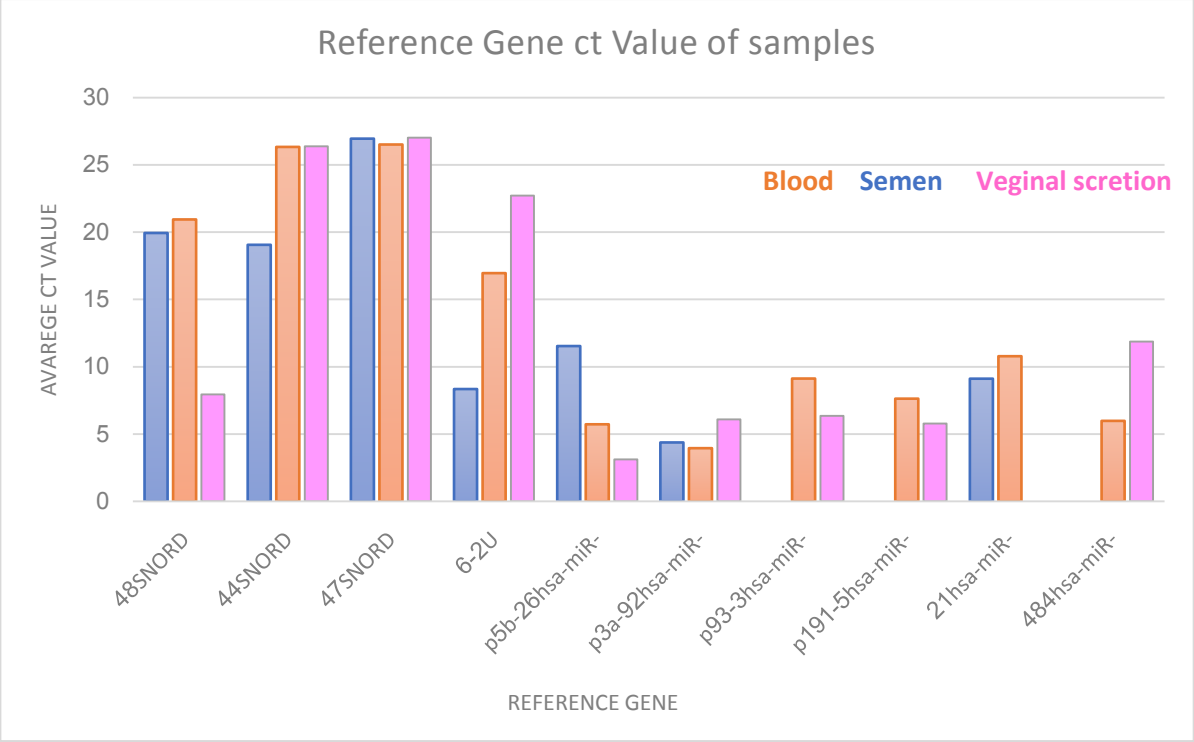

Figure 1: Reference genect values for each samples type

Reference genes are essential for the quantification of the target gene expression level in RT-qPCR analysis. Even a small change in miRNA expression may influence cleavage of mRNAs or translation repression [15].Previous research has found promising results in recognizing differentially expressed miRNAs that have the potential to be used as new biomarkers for forensic body fluid recognition [16,17].Despite the fact that real-time RT-PCR is commonly used to quantify biologically significant changes in mRNA levels, it still has a number of drawbacks. These include RNA inherent variability, extraction protocol variability that can copurify inhibitors, and various reverse transcription and PCR efficacy [18].

Conclusion

The current study investigated ten selected candidate reference genes that could be used to normalize miRNA expression using an RT qPCR assay, and identify SNORD-47 as a suitable reference gene for forensically revenant body fluids, included venous blood, semen, and vaginal secretion.

Conflict of Interests

The authors have declared no conflict of interests.

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