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Research Paper

Exploring transcriptomic databases to identify and experimentally validate tissue-specific consensus reference gene for gene expression normalization in BALB/c mice acutely exposed to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a toxic compound affecting organs like the liver, kidney, lung, and reproductive systems in mammals. This study outlines a strategy for choosing appropriate HKGs for tissue-specific gene expression analysis in TCDD toxicity, including four steps: i) identifying candidate HKGs from literature and databases; ii) defining primers from literature or designing new ones; iii) validating primer efficiency and specificity; iv) experimentally assessing candidate HKGs' stability in various tissues of TCDD-exposed mice.

Based on this strategy, a total of 40 potential HKGs was selected, further filtered based on their database sources and ranked according to their frequency of use or expression stability. Ultimately, we identified a final set of 15 HKGs (*Rps18*, *Calr*, *Polr2b*, *Brms1l*, *P4hb*, *Esd*, *Hdgf*, *Gapdh*, *Mlec*, *Tbp*, *Rn18s*, *Sdha*, *B2m*, *Actr3* and *Actb*) with typical efficiencies for further evaluation. Then, the stability of the selected HKGs was determined in the liver, kidney, lung, ovary and testis of TCDD-exposed mouse compared to the control group using the [log ($2^{\Delta Ct}$)] and statistically analyzed using Pearson correlation coefficient (*r*) by BestKeeper algorithm. Our data analysis revealed that *Actb*, *Rps18*, and *Polr2b* were the most stable HKGs for normalizing gene expression in the liver, while *Sdha*, *Actb*, and *Gapdh* were suitable for kidney tissue. In the lung, *Tbp*, *Sdha*, and *Rps18* showed stability, while *Tbp*, *B2m*, and *Actb* were most stable in ovary. Lastly, *Actb*, *B2m*, and *Tbp* were accurately stable in the testis of TCDD-exposed mice. Our study identifies stable HKGs, improving TCDD toxicity research accuracy and reliability.

1. Introduction

Dioxins represent an exceptionally potent class of environmental organic contaminants, establishing their standing as one of the most formidable pollutants known to date. The International Agency for Research on Cancer (IARC) classifies dioxins as a "human carcinogen" based on strong evidence from studies in both humans and animals (IARC, 1997; Stockholm, 2001; WHO, 2016). Chemically, dioxins comprise two aromatic rings connected by either one or two oxygen atoms, giving rise to polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzodioxins (PCDDs), respectively. These chemical configurations allow for chlorination at 1 to 8 positions, making dioxins remarkably stable and extremely hydrophobic (Caruso et al., 2003; Pollitt, 1999). Among the dioxin congeners, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) stands out as the most potent, characterized by a half-life of 3–9 years in adult humans (Pirkle et al., 1989), and consequently serving as a prototype for dioxin-related toxicological investigations (Van den Berg et al., 2006; WHO, 2002).

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Dioxins cause permanent harm to humans, animals, plants, and microorganisms, leading to serious ecological, environmental, and economic impacts. (Gao et al., 2023; Hanano et al., 2018a; Hanano et al., 2016; Hanano et al., 2019b; Mahfouz et al., 2020; Mahfouz et al., 2022; Mathew et al., 2025). In mammals, the major toxicological effects of dioxins are mediated by the aryl hydrocarbon receptor (AhR), a member of the cytoplasmic receptor family that functions as a transcription factor (Sorg, 2014). The biological roles of AhR in mammals has been exhaustively explored in the liver (Larigot et al., 2018). Within this context, the canonical genomic AhR pathway leads to the activation of AhR target genes, encompassing those involved in xenobiotic and drug metabolism, inflammatory responses (Hirakawa et al., 2007; Ibrahim et al., 2020; Larigot et al., 2018), oxidative stress (Veith and Moorthy, 2018), reproductive systems (Jablonska et al., 2011), development and differentiation (Stanford et al., 2016), and even more in aging process (Salminen, 2022; Salminen, 2023). The extent of dioxin toxicity is closely related to its affinity for AhR, with 2,3,7,8-TCDD being the most potent AhR agonist (Larigot et al., 2018). Within this context, the toxicity of dioxins varies depending on the specific tissues of the exposed animals. This variability is due to differences in lipid content and the expression levels of AhR in each tissue (Frericks et al., 2007). Consequently, the reported tissue-related toxicities of dioxins showed a wide array of adverse effects, including developmental toxicity (Choi et al., 2017; Herlin et al., 2021; Hoyeck et al., 2020b; Liu et al., 2016a; Liu et al., 2016b), metabolic toxicity (Hammoudeh et al., 2023b; Hoyeck et al., 2020a; Matteo et al., 2021; Nunes et al., 2022), male and female reproductive toxicity (Aldeli et al., 2023; Faiad et al., 2022; Foster et al., 2010; Jin et al., 2010; Johnson et al., 2020), neurotoxicity (Pelclova et al., 2018), and cellular apoptosis (Zhao et al., 2016).

The assessment of tissue-specific dioxin toxicity can be carried out through various methods, such as microscopic examination, histological analysis, and physiological and biochemical measurements (Faiad et al., 2023). However, the effectiveness and precision of these methods often rely on the specific characteristics of the target tissue. Molecular methods at the genomic and transcriptomic levels remain the most effective tools for assessing tissue-specific dioxin toxicity. Relevantly, reverse transcription qPCR (RT-qPCR) is the method of choice for evaluating the expression levels of specific genes. Its popularity comes from being easy to use, highly sensitive, precise, and reliable (Bustin et al., 2013). Despite the emergence of high-throughput RNA sequencing techniques in monitoring of dioxin toxicity (Boutros et al., 2008; Yao et al., 2012), RT-qPCR continues to be widely utilized as a fundamental confirmatory technique (Fischer et al., 2016).

Accurate quantification of transcript level in RT-qPCR depends on several critical factors, encompassing the variability in RNA extraction, differences in reverse transcription, and the efficiency of PCR amplification (Bustin et al., 2009; Miranda and Steward, 2017; Redshaw et al., 2013). Utilizing high-quality reagents and components during RNA extraction and cDNA synthesis can substantially reduce the variability inherent in these steps. Nevertheless, it is essential to underscore that the precision of the relative quantification method for transcripts is fundamentally linked to the choice of endogenous "reference" genes employed to normalize the expression of the target genes, presumably maintaining a consistent level of mRNA abundance across various tissue-types, regardless of environmental conditions (Bustin et al., 2013; Bustin et al., 2009; Huggett et al., 2005; Miranda and Steward, 2017). In toxicological and pathological studies, the commonly used reference genes, often referred to as "normalizing genes" or "housekeeping genes," for the normalization of RT-qPCR expression data encompass beta-actin (*Actb*), glyeraldehyde-3-phosphate dehydrogenase (*Gapdh*) and ribosomal *Rn18S* (Dijkstra et al., 2014; Suzuki et al., 2000). However, several studies have shown that these reference genes do not maintain stable mRNA abundance levels in different tissues as well as under many experimental conditions (Goidin et al., 2001; Liu et al., 2020; Riveroll et al., 2023; Vandesompele et al., 2002). Using unstable housekeeping genes (HKGs) for qPCR normalization can lead to inaccurate gene expression quantification, false interpretations, and irreproducible results. If an HKG fluctuates across conditions, it can artificially alter target gene expression. For example, *Gapdh*, often used in inflammatory studies, is upregulated in immune responses, potentially masking cytokine gene upregulation (*IL-6*, *TNF-α*) (Millet et al., 2016). Similarly, *Actb* is highly variable in cancer tissues, making it unsuitable for normalizing oncogene expression (*HER2* in breast cancer) (Carlsson et al., 2004). Additionally, 18S rRNA (*Rn18S*), frequently used in drug response studies, is influenced by drug treatment, leading to misinterpretation of true gene expression changes (Moreno-Igoa et al., 2010). Thus, proper selection of stable HKGs is crucial for accurate, reproducible qPCR results in diverse experimental conditions.

Dioxin toxicity in mammalian models, particularly in mice, are routinely conducted in specific tissues, including the liver (Jones and Butler, 1973; Mejia-Garcia et al., 2013; Wang et al., 2011), lung (Zhang et al., 2020), kidney (Van Quang et al., 2024), ovary (Aldeli et al., 2023) and testis (Faiad et al., 2023). Hence, it is of utmost importance to meticulously choose the most precise reference genes for normalizing transcript levels in different types of exposed tissues, a crucial step in RT-qPCR quantification (Sanders et al., 2018). To the best of our knowledge, no prior information on this matter has been documented. In light of this, we hypothesized that an in silico analysis of candidate reference gene expression within relevant transcriptomic databases could offer an effective means to identify a subset of genes exhibiting highly consistent expression patterns. These selected genes could then be integrated into subsequent RT-qPCR assay design and validation. For that purpose, we conducted experimental evaluations to determine the stability of the chosen reference genes in various tissues, encompassing the liver, kidney, lung, ovary, and testis of adult BALB/c mice following acute exposure to 25 μg TCDD kg^{-1} for a week. These conditions mirror the most common scenarios encountered in toxicological studies of TCDD on mice.

2. Materials and methods

2.1. Exploring housekeeping genes (HGs) for mouse RT-qPCR in public databases

The published literature on the analysis of candidate HGs for the normalization of qPCR data in the context of mouse studies was retrieved through a search in PubMed (https://pubmed.ncbi.nlm.nih.gov/) performed on 1st November 2023 using the entry "Gene expression RT-qPCR mouse TCDD". The following PubMed filters were applied: species—mouse; search field—full text. This search resulted in a list of 102 unique publications published between 1996–2023. The studies were analyzed to extrapolate the following information: candidate reference genes tested, the normalizing approach used, and best-performing reference gene identified and the experimental set regarding the mouse strain, TCDD dose and the time of treatment (Fig. 1).

Additionally, housekeeping genes were extracted for Housekeeping and Reference Transcript Atlas (HRT Atlas) (https://housekeeping.unicamp.br/). HRT is an online publicly available transcriptomic tool, developed by Hounkpe his co-workers (Hounkpe et al., 2021), with user-friendly interface dedicated to mainly search human and mouse housekeeping and candidate reference genes/transcripts. Interestingly this tool offers tissue and cell selective candidate reference transcripts and some primers for qPCR normalization. The search of candidate reference genes at HRT was focused on the mouse database which included the analysis of 507 high quality RNA-seq datasets manually curated from Gene Expression Omnibus (GEO) portal (https://www.ncbi.nlm.nih.gov/geo/). In this portal, 22 tissues and cells types from wild type healthy control mice were included in the workflow. For each tissue, liver, kidney, lung, ovary, and testis, candidate reference genes, specific primers as well as the validation method were obtained using the filter criteria by default with a logarithmic a scale of the RPKM

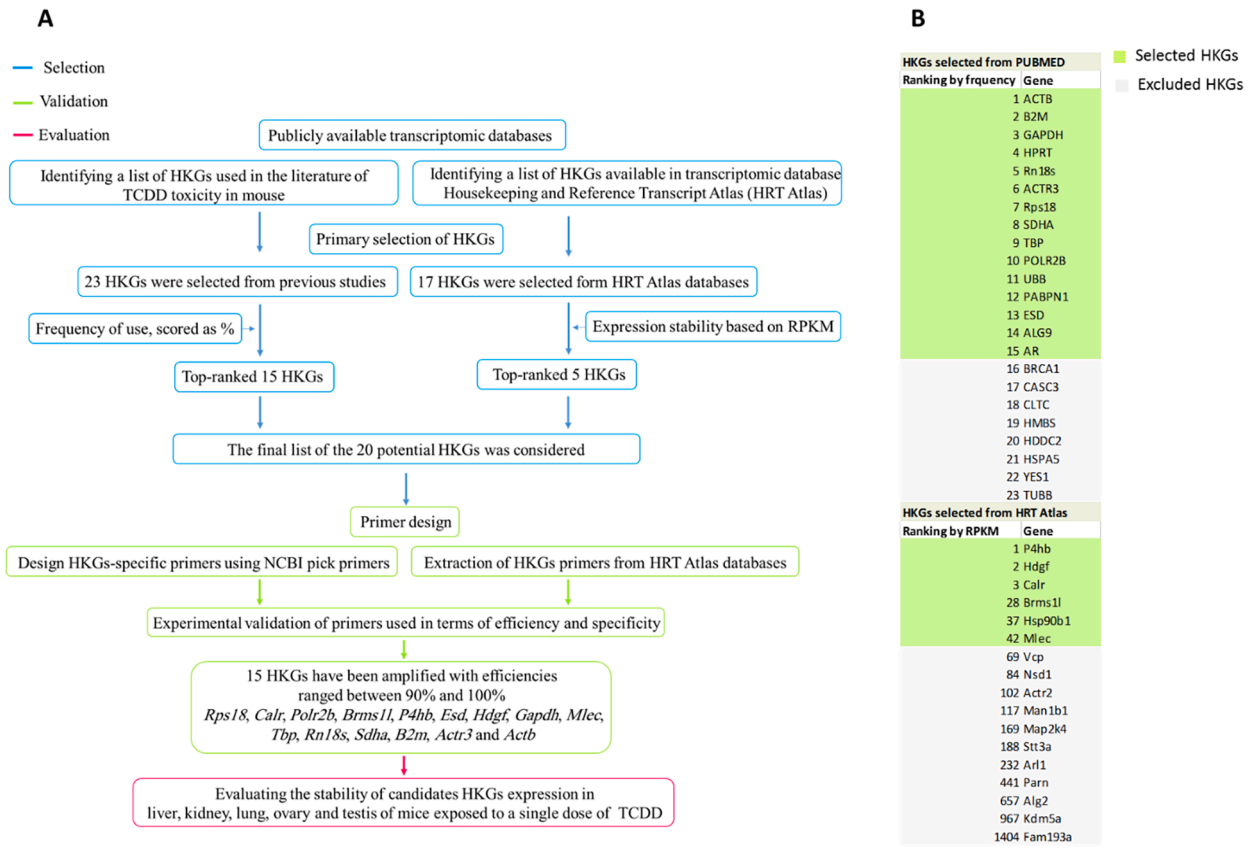

Fig. 1. A. A schematic representation of workflow. The workflow was structured into three main steps: first, the primary selection of housekeeping genes (HKGs) from transcriptomic databases (PUBMED literature and HRT Atlas) (indicated by blue boxes); second, the validation of primers and amplification conditions for the selected HKGs (indicated by green boxes); and finally, the assessment of the stability of the chosen 15 HKGs in the liver, kidney, lung, ovary, and testis of mice exposed to TCDD. **B.** Initially, a total of 40 HKGs were identified as potential candidates for normalizing gene expression; 23 from PUBMED and 17 from the HRT Atlas database. Among the 23 HKGs sourced from toxicological studies in mice, their frequency of use was ranked, represented as a percentage. Subsequently, the top-ranked 15 HKGs were selected for further assessment. As for the 17 HKGs retrieved from the HRT Atlas databases, they were evaluated for expression stability based on mean RPKM (Reads Per Kilobase Million), standard deviation of log2 RPKM, and MFC (Median Fold Change) value. The top 5 HKGs were then designated as potential candidates for further evaluation.

(Reads Per Kilo base of transcript per Million mapped reads) and a mean value equal of higher than 30. For each tissues, only reference genes with an experimental validation available on HRT were selected.

2.2. Primers design

The primers of the selected reference genes used in our study were prepared using different sources; i) the primers of candidate reference genes selected from the RHT database were accordingly extracted. The entries of each candidate reference genes compromised specific sequences of forward and reverse primers, melting temperature and amplicon size. The validation parameters for each couple of primers was checked and only those having a qPCR effeminacy more than 98 % and with R^2 ranged between 0.98 and 0.99 with highly melting curve specificity were included in our collection; ii) primers of reference genes available in the published literature in PubMed (https://pubmed.ncbi.nlm.nih.gov/) performed on 1st November 2023 using the entry "Gene expression RT-qPCR mouse TCDD"; ii) primers of certain reference was designed using the pick primers tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi). Gens-specific forward and reverse primers sequences, amplicon size, and references are presented in Table S3.

2.3. Chemicals, animals and TCDD-treatment

2,3,7,8-TCDD (Supleco, USA, 10 μg/ml in toluene) was evaporated under nitrogen flow until complete dryness, then dissolved in dimethyl sulfoxide (DMSO) and diluted with corn oil. The study was conducted on 24 albino BALB/c mouse (12 females and 12 males) aged 12 weeks with weights ranged between 23–24 g. These animals were obtained from the unit of Breeding Animal at the Atomic Energy Commission of Syria (AECS).

Mice were kept in plastic cages with a solid bottom and a metal grid lid. The surface of the cage was covered with fine sawdust. Ad libitum feeding and watering systems were used in the animal's cages. Animals were fed with dry pellets food with a standard composition of carbohydrates, proteins, fats, minerals and vitamins. Usually the water is made available in a water bottle with a sipper tube. Mice cages were kept under standard experimental conditions (20–22 °C, 55–65 % humidity and 12-hour light/dark cycle). The Fig. S1 represents the workflow of our study that aims to select the most accurate housekeeping genes in liver, kidney, lung, ovary, and testis isolated from adult BALB/c mice following acute exposure to 25 μg TCDD kg^{-1} for 7 days. Our choice for the TCDD-dose and the time of exposure is effectively based on previous studies that demonstrated critical toxicity of TCDD in these parameters (Aida-Yasuoka et al., 2014; Bankoti et al., 2010; Stedtfeld et al., 2017).

24 adult mice were divided into 2 groups; an experimental group (TCDD-G) consists of 12 animals (6 females and 6 males) and a control group (Ct-G) consists of the same number of animals with similar female/males ratio. The TCDD-G was orally treated with a single dose of 25 μg of TCDD/kg dissolved into corn oil as vehicle, while the Ct-G was

fed with the same volume of corn oil only. Seven days later, mice of both groups were sacrificed and the organs of liver, kidney, lung, ovary, and testis were isolated and immediately frozen in liquid nitrogen for further analyses. The TCDD-induced toxicological effects in these tissues were verified at molecular, biochemical, and histological levels ((Aldeli and Hanano, 2025; Aldeli et al., 2023; Faiad et al., 2023; Hammoudeh et al., 2023a)), and the same samples were used in the current study. All animals' experiments were carried out in accordance with the guidelines of AECS Committee of animal Ethics for animal experiments which is adopted from the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments

2.4. RNA extraction and cDNA synthesis

0.5 mg of frozen tissue was used to extract the total RNA using TRIzo solution according using the RNeasy Mini Kit (Qiagen). Starting from 1 mL of frozen TRIzol samples, 200 μL of chloroform was added per 1 mL of TRIzol used. After the gradient centrifugation, the aqueous phase was transferred to a new tube and one volume of 100 % EtOH was added. Then, 700 μL was loaded in an RNeasy Mini spin column and processed according to the manufacturer's instructions. Genomic DNA traces were removed using the RNase-Free DNase Set (Qiagen, Germany), providing efficient on-column digestion of DNA during RNA purification using RNeasy Kit. The concentration and quality of RNA were determined with a NanoDrop Spectrophotometer ND1000 (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. For the cDNA synthesis, aliquots containing 1 μg total RNA were used for first-strand cDNA synthesis using M−MLV Reverse Transcriptase (Sigma-Aldrich, USA) as previously described (Hammoudeh et al., 2020). After a qualitative and quantitative standardization, the prepared cDNA samples were kept at at −20 °C until their use.

2.5. Real time-quantitative PCR

To select the most appropriate reference gene, the expression of candidate reference genes was analysed in term of their stability in different mouse tissues (liver, kidney, lung, ovary, and testis) as a function of TCDD treatment by reverse-transcription quantitative PCR (RT-qPCR) as described (Hanano et al., 2019a; Hanano et al., 2016). PCR efficiencies for each couple of primers were determined using serial dilutions of cDNA (10^{-1} to 10^{-5}). Then, the slope of the regression between the Log values of cDNA dilutions and the average Ct values was established. PCR efficiencies, expressed as a percentage, were calculated using the following equation; efficiency (%) = $[10^{(-1/\text{Slope})}\text{-}1]$ X 100. PCR efficiencies for all couples of primer were ranged between 96 and 98 %. Real-time PCR was performed in 96-well plates using an AriaMx Real-time PCR System from Agilent technologies, USA. In brief, 25 μL reaction mixtures contained 0.5 μM of each specific oligonucleotide primer of reference genes, 12.5 μL of SYBR Green PCR mix (Bio-Rad, USA) and 100 ng cDNA. qPCR conditions including melting curves analysis were performed as described before (Hanano et al., 2018b; Perez-Matas et al., 2022). Each point was replicated in triplicate and the average of C_T was taken for calculation of the $^{(-\Delta\Delta CT)}$.

2.6. Statistics

The C_t (cycle threshold) values were collected for amplification of each candidate HGs for each tissues (liver, kidney, lung, ovary, and testis) isolated from TCDD-G and C_t-G were loaded in the GraphPad Prism software v. 9 for all analyses.

These values were then used to determine the magnitude (M) of the differences in the abundance of gene transcripts between TCDD-G and Ct-G was evaluated using the [log ($2^{\Delta Ct}$)], where $\Delta C_t = C_{t(TCDD)} - C_{t\ (control)}$. Results were visualized as [log ($2^{\Delta Ct}$)] ± standard-deviation across experimental condition. Statistical analysis was performed using GraphPad Prism software, version 9 (https://www.graphpad.com/scientific-software/prism/). The stability ranking of candidate reference genes in each tissues isolated from TCDD-treated mice and control mice was performed using the RefFinder algorithm (Xie et al., 2012), a user-friendly web-based comprehensive tool developed for evaluating and screening reference genes from extensive experimental datasets. It integrates the currently available major computational programs, including geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and the comparative ΔC_t method (Silver et al., 2006) to compare and rank the tested candidate reference genes (http://blooge.cn/RefFinder/) accessed on 26 November 2023.

3. Results

3.1. Selection of candidate housekeeping genes used in the toxicological studies of TCDD in mouse

We devised a strategy to select candidates housekeeping gens for RT-qPCR based on publicly available transcriptomic databases and a pipeline to design and validate RT-qPCR assays. Our strategy consists of i) identifying a list of candidate reference genes explored as potential normalizing genes in the literature of TCDD toxicity in mouse, based on a systematic revision of public literature on this topic, as well as based on their expression profile available in transcriptomic database Housekeeping and Reference Transcript Atlas (HRT Atlas); ii) defining primer pairs for the candidate reference genes, primers were taken from the previous studies or from HRT Atlas databases, if not available, gene specific primers were designed using NCBI pick primers tool; v) performing an experimental validation of primers used in terms of efficiency and specificity; iv) finally, evaluating the stability of the candidates housekeeping genes expression in different tissues of mice acutely exposed to TCDD. A schematic representation of the proposed strategy is represented in Fig. 1A. Basing on this strategy, a total of 40 HKGs were selected as potential candidates for normalizing of genes expression. Of them, 23 HKGs were selected from previous studies and 17 HKGs were selected form HRT Atlas databases. Table S1 summarized all the information of the selected HKGs including gene NCBI-accession number or transcript ID in HRT Atlas databases, gene full name, gene name abbreviation and the related reference.

Then, the 40 HKGs selected in the first round were filtered in two ways according their databases sources. The 23 HKGs extracted from previous toxicological studies in mouse were ranked according to their frequency of use, scored as %. Subsequently, the 23 selected HKGs was ranked from 1 to 23 according to their frequency % that ranged between 90 % for the *Actb* and 2 % for *Tubb*, respectively. Accordingly, the top-ranked 15 HKGs were considered for further evaluation. For the 17 HKGs selected form HRT Atlas databases, these genes were ranked in terms of expression stability based on mean RPKM, standard deviation of log2 RPKM and MFC value. The ranking of these genes was largely varied and ranged between 1 for *P4hb* and 1404 for *Fam193a*, respectively (Table S2). The top 5 HKGs were considered as potential HKGs candidate for further evaluation. The final list of the 20 potential HKGs that considered in our study is presented in Fig. 1B.

3.2. Primers setup and validation in control mouse tissues

The 20 couples of primers defined for the selected HKGs were experimentally tested using the cDNA prepared from mouse liver of control group mice (Ct-G), as reference tissue in term of toxicological response to TCDD exposure. For that, the PCR specificity of each couple of primer was evaluated in three liver cDNA samples using both agarose gel electrophoresis and melting curve analysis. While the PCR efficiency was determined using a serial dilutions of each cDNA sample over five orders of magnitudes (from 10^{-1} to 10^{-5}). The Table 1 shows the values of R2, slope and corresponding PCR efficiency % for the selected HKGs. The PCR efficiency % was ranged between 80.5 % for *Ar* and 128.9 % for

Table 1
Shows the values of r^2, slope and corresponding PCR efficiency % for the selected HKGs.

Ranking	Gene abbreviation	R^2	Slope	PCR Efficiency %
15	*AR*	0.9923	−3.91	80.2
14	*ALG9*	0.9964	−3.85	81.8
11	*UBB*	0.9944	−3.6	89.5
7	*Rps18*	0.9968	−3.4	96.8
18	*Calr*	0.9984	−3.34	99.2
10	*Polr2b*	0.9993	−3.33	99.6
19	*Brms1l*	0.9974	−3.33	99.6
16	*P4hb*	0.9995	−3.31	100.5
13	*Esd*	0.999	−3.3	100.9
17	*Hdgf*	0.9994	−3.29	101.3
3	*Gapdh*	0.9906	−3.28	101.7
21	*Mlec*	0.9989	−3.27	102.2
9	*Tbp*	0.9993	−3.26	102.6
5	*Rn18s*	0.9987	−3.23	103.9
8	*Sdha*	0.998	−3.23	103.9
2	*B2m*	0.9825	−3.21	104.8
6	*Actr3*	0.9965	−3.16	107.2
1	*Actb*	0.9987	−3.12	109.1
20	*Hsp90b1*	0.9969	−2.93	119.4
4	*HPRT*	0.9964	−2.84	124.9
12	*PABPN1*	0.995	−2.78	128.9

Pabpn1. Three HKGs (*Ar*, *Alg9* and *Ubb*) had PCR efficiencies less than 90 % and three others HKGs (*Hsp90b1*, *Hprt* and *Pabpn1*) had PCR efficiencies over than 110 %, accordingly they were excluded from the study. Potentially, 15 HKGs (*Rps18*, *Calr*, *Polr2b*, *Brms1l*, *P4hb*, *Esd*, *Hdgf*, *Gapdh*, *Mlec*, *Tbp*, *Rn18s*, *Sdha*, *B2m*, *Actr3* and *Actb*) have been amplified with acceptable efficiencies typically ranged between 90 % and 100 %, therefore they have been chosen as a final set of candidate HKGs. To assess intra-assay variation of Ct for each gene amplification, three technical replicates were performed in one run for each cDNA preparation. The coefficient for intra-assay variation ranged from 0.75 % for *Sdha* to 1.5 % for *Rps18* (Fig. 2A). Next, the inter-assay variation was assessed for each gene by performing three separated runs. The coefficient for inter-assay variation was 0.5 % (for *Rps18*, *Brms1l*, *Ph4d*, *Hgdf*, *Gapdh*, *Tbp*, *Rn18s* and *B2m*) and 1 % (for *Calr*, *Polr2b*, *Esd*, *Mlec*, *Sdha*, *Actr3* and *Actb*) (Fig. 2B). These data indicate that all selected HKGs are amplified with high efficiencies and reproducibility, suggesting their appropriateness for further evaluation in term of their tissular stability as a function of TCDD treatment.

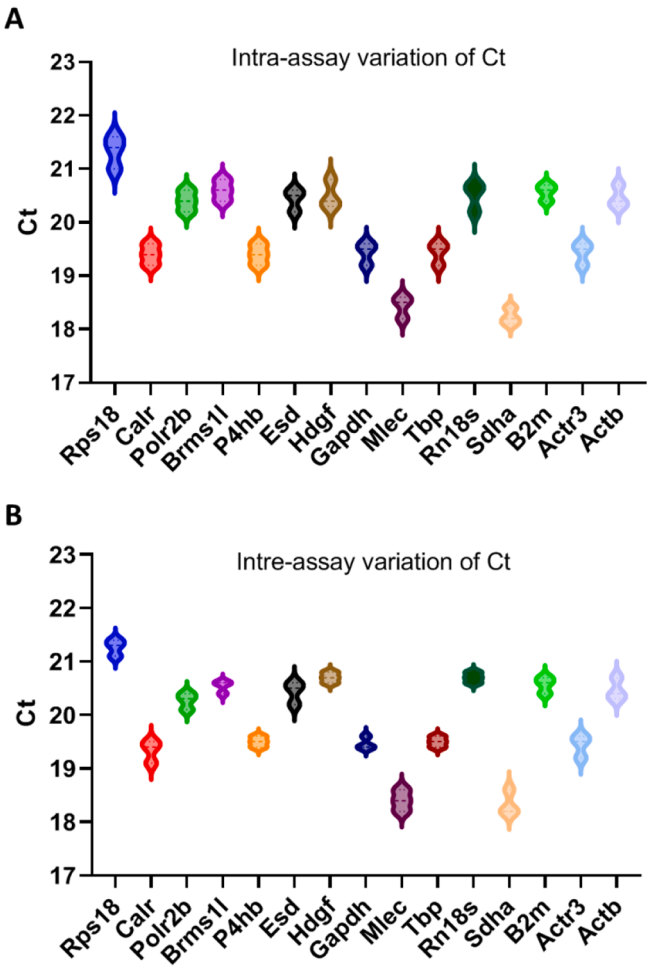

Fig. 2. Coefficient of intra-assay and inter-assay variation for the amplification of selected HKGs. **A.** Mean Ct values for six technical replicates, indicated by dots, for each candidate reference gene obtained from a single run (intra-assay variation). Horizontal lines depict the mean value for each group. **B.** Mean Ct values for six technical replicates for each candidate reference gene obtained from three different runs on separate days (inter-assay variation).

3.3. Stability of HKGs in mice liver as a function of TCDD exposure

Due to its richness in lipids, liver is considered the most affected organ by TCDD (Hammoudeh et al., 2023a). Our data showed that the values of [log ($2^{\Delta Ct}$)] varied between −0.21 and + 0.33 and the closest values to zero were those for *Rps18*, *Polr2b*, *Gapdh*, *Mlec*, *Rn18s*, *Sdha* and *Actb*. Inversely, the furthest [log ($2^{\Delta Ct}$)] values to zero were those for *Tbp*, *Esd*, *B2m*, *Hgdf* and *Calr* (Fig. 3A, Table S4). Subsequently, the obtained [log ($2^{\Delta Ct}$)] values of investigated HKGs were statistically analyzed by using BestKeeper algorithm, a web-based comprehensive tool developed for evaluating and ranking the HKGs. The statistics and Pearson correlation coefficient (*r*) by BestKeeper are presented in Table S5, accordingly the HKGs were ranked in the respect to the stability of their expression in mouse liver as a function of TCDD exposure. As the Fig. 3B shows that *Actb*, *Rps18*, *Polr2b*, *Gapdh* and *Sdha* were ranked in the top five places from 1 to 5, respectively, in the respect to their expression stability. Whilst the least stable HKGs in the mouse liver were *Tbp*, *Esd*, *B2m*, *Hgdf* and *Calr*, ranked from 11 to 15 positions, respectively. Consequently, the five most stable HKGs in the mouse liver as a function of TCDD exposure are *Actb*, *Rps18*, *Polr2b*, *Gapdh* and *Sdha* (Fig. 3C), suggesting therefore the use of *Actb*, *Rps18* and *Polr2b* as accurately stable HKGs for normalizing the expression of target genes in the TCDD-induced hepatotoxicity in mouse.

3.4. Stability of HKGs in mice kidney as a function of TCDD exposure

Beyond the liver, kidney is considered one of the most affected organs by the toxicity of TCDD (Boutros et al., 2009), thus it is quite worthy to determine the most appropriate HKGs for normalizing the gene expression in the kidney tissue following the exposure to TCDD. To this end, the expression stability of selected HKGs were assessed in a similar approach used in the liver. The expression of investigated HKGs was quantified in the kidney of mouse exposed and non-exposed to TCDD. As shown in the Fig. 4A and Table S6, [log ($2^{\Delta Ct}$)] varied between 0.02 and 0.22 and the closest values to zero were those for *Sdha*, *Actb*, *Gapdh*, *B2m* and *Rn18s*, respectively. On the other hand, the furthest [log ($2^{\Delta Ct}$)] values to zero were those for *Actr3*, *Hgdf*, *Esd*, *Melc* and *Brms1l*. The statistics and Pearson correlation coefficient (*r*) by BestKeeper are presented in Table S7, accordingly the HKGs were ranked in the respect to the stability of their expression in mouse kidney as a function of TCDD exposure. As the Fig. 4B shows that *Sdha*, *Actb*, *Gapdh*, *B2m* and *Rn18s*, were ranked in the top five places from 1 to 5, respectively, in the respect to their expression stability. Whilst *Actr3*, *Hgdf*, *Esd*, *Melc* and *Brms1l* genes were ranked as the least stable HKGs in the mouse kidney and positioned from 11 to 15, respectively. In the light of our data, the five most stable HKGs in the mouse kidney as a function of TCDD exposure are *Sdha*, *Actb*, *Gapdh*, *B2m* and *Rn18s* (Fig. 4C), suggesting therefore the use of *Sdha*, *Actb* and *Gapdh* as accurately stable HKGs for normalizing

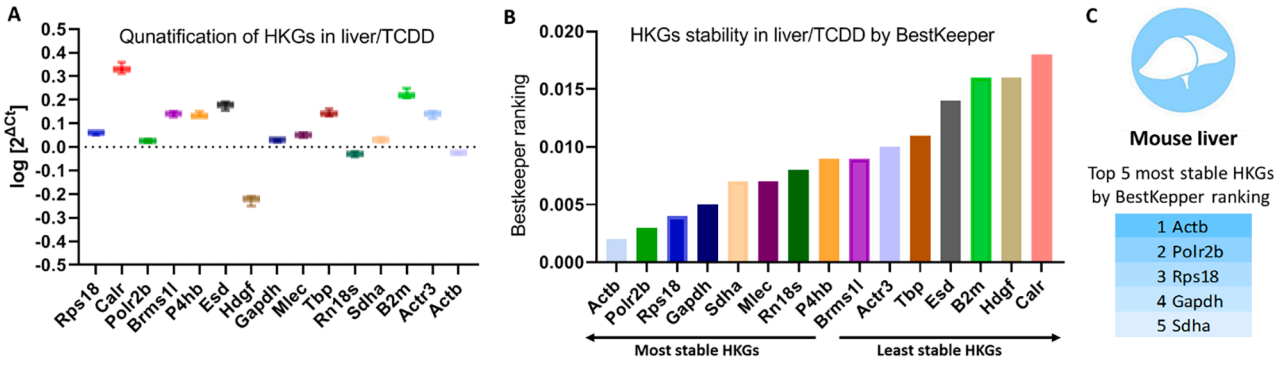

Fig. 3. Stability of HKGs Expression in Mouse Liver under TCDD Exposure. **A.** The logarithmic values of ΔCt ($\log 2^{\Delta Ct}$), where ΔCt = Ct (TCDD) − Ct (control), are depicted for the 15 HKGs in the livers of mice exposed to TCDD compared to the control group. The horizontal line represents the median value. **B.** The stability ranking of HKGs determined by RefFinder across all study subjects. The stability of HKG expression was assessed using the BestKeeper computational tool. The most stable HKGs are indicated by an arrow pointing to the left, while the least stable HKGs are indicated by an arrow pointing to the right. Measurements were performed in triplicate for three cDNA preparations ($n = 9$).

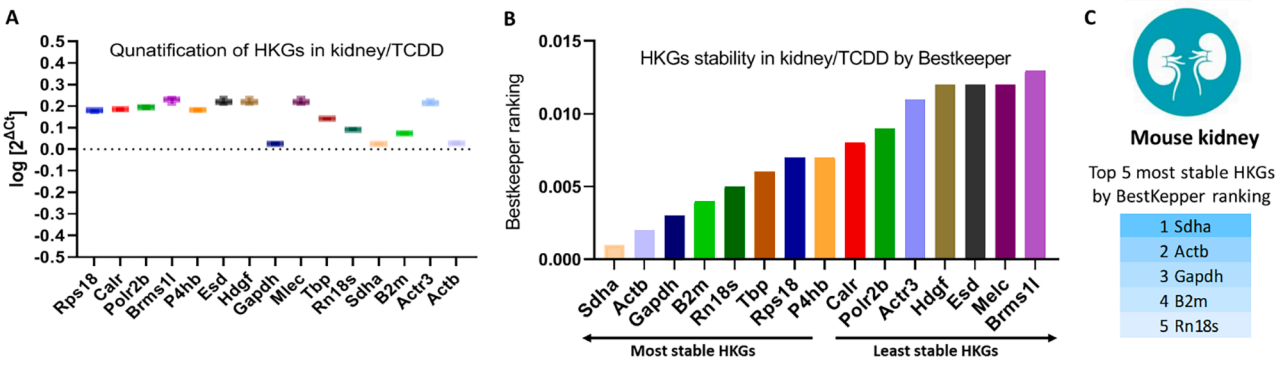

Fig. 4. Stability of HKGs expression in mouse kidney as a function of TCDD Exposure **A**. Logarithmic values of $2^{\Delta Ct}$, where ΔCt = Ct (TCDD) − Ct (control), are illustrated for the 15 HKGs in the kidneys of mice exposed to TCDD compared to the control group. The horizontal line denotes the median value. **B.** The stability ranking of HKGs determined by RefFinder across all study subjects. HKG expression stability was evaluated using the BestKeeper computational tool. The most stable HKGs are denoted by an arrow pointing to the left, while the least stable HKGs are indicated by an arrow pointing to the right. Measurements were conducted in triplicate for three cDNA preparations ($n = 9$).

the expression of target genes in the TCDD-induced toxicity in mouse kidney.

3.5. Stability of HKGs in mice lung as a function of TCDD exposure

Several lines of evidence demonstrated that the polluted air is one of the major exposure pathway of human to dioxins (Ok et al., 2002; Wei et al., 2021), thus lung is considered one of the organs that can be seriously affected by the toxicity of TCDD. As response to TCDD exposure, the expression of all investigated HKGs were assessed and [log ($2^{\Delta Ct}$)] values for each gene were determined (Table S8). Fig. 5A shows that the closest [log ($2^{\Delta Ct}$)] values to zero were those for *Tbp*, *Sdha*, *Rps18*, *B2m* and *Rn18s*, respectively. While, the furthest [log ($2^{\Delta Ct}$)] values to zero were those for *Brms1l*, *P4hd*, *Actr3*, *Esd* and *Hgdf*. The

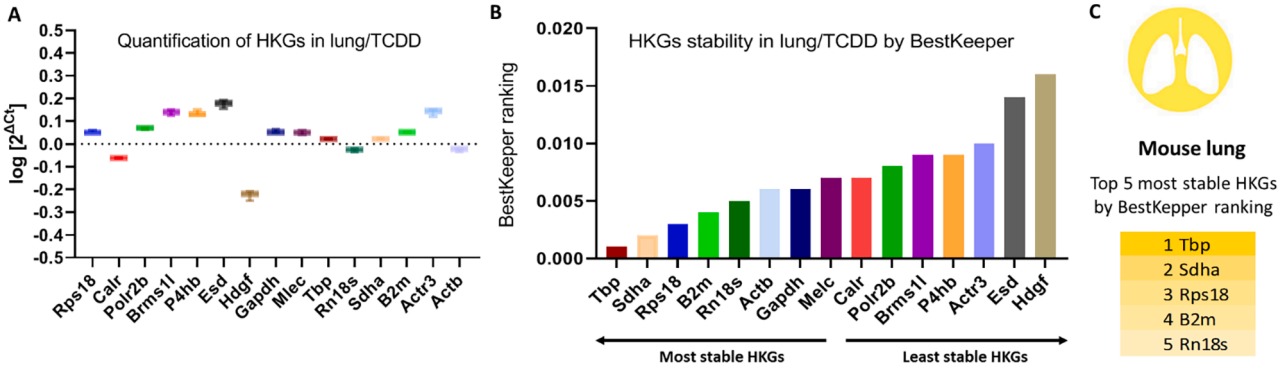

Fig. 5. Stability of HKGs in mice lung as a function of TCDD exposure. **A.** The $\log 2^{\Delta Ct}$ values, where ΔCt = Ct (TCDD) − Ct (control), for the 15 HKGs in the lungs of mice exposed to TCDD compared to the control group are presented. The horizontal line represents the median value. **B.** The stability ranking of HKGs determined by RefFinder across all study subjects. HKG expression stability was assessed using the BestKeeper computational tool. The most stable HKGs are indicated by an arrow pointing to the left, while the least stable HKGs are indicated by an arrow pointing to the right. Measurements were conducted in triplicate for three cDNA preparations ($n = 9$).

statistics and Pearson correlation coefficient (*r*) by BestKeeper are presented in Table S9, accordingly the HKGs were ranked in the respect to the stability of their expression in mouse lung as a function of TCDD exposure. As the Fig. 5B shows that *Tbp*, *Sdha*, *Rps18*, *B2m* and *Rn18s* were ranked in the top five positions from 1 to 5, respectively, in the respect to their expression stability. Whilst *Brms1l*, *P4hd*, *Actr3*, *Esd* and *Hgdf* genes were ranked as the least stable HKGs in the mouse lung and positioned from 11 to 15, respectively. In the light of our data, the five most stable HKGs in the mouse lung as a function of TCDD exposure are *Tbp*, *Sdha*, *Rps18*, *B2m* and *Rn18s* (Fig. 5C), suggesting therefore the use of *Tbp*, *Sdha* and *Rps18*, as accurately stable HKGs for normalizing the expression of target genes in the TCDD-induced toxicity in mouse lung.

3.6. Stability of HKGs in mice ovary as a function of TCDD exposure

Mammalian female reproductive system is seriously affected by the toxicity of dioxin by dysregulation the expression of key genes involved in the biosynthesis of female reproductive hormones (Aldeli et al., 2023). For that, it is worth to identify the most appropriate HKGs for normalizing the genes expression in ovary tissue in response to TCDD exposure. Fig. 6A and Table S10 show that the closest [log ($2^{\Delta Ct}$)] values to zero were those for *Tbp*, *B2m*, *Actb, Rn18s* and *Gapdh*, respectively. While, the furthest [log ($2^{\Delta Ct}$)] values to zero were those for *P4hd, Brms1l*, *Calr*, *Esd* and *Hgdf*. The statistics and Pearson correlation coefficient (*r*) by BestKeeper are presented in Table S11, accordingly the HKGs were ranked in the respect to the stability of their expression in mouse ovary as a function of TCDD exposure. As the Fig. 6B shows that *Tbp*, *B2m*, *Actb, Rn18s* and *Gapdh* were ranked in the top five positions from 1 to 5, respectively, in the respect to their expression stability. Whilst *P4hd, Brms1l*, *Calr*, *Esd* and *Hgdf* genes were ranked as the least stable HKGs in the mouse ovary and positioned from 11 to 15, respectively. Subsequently, the five most stable HKGs in the mouse ovary as a function of TCDD exposure were *Tbp*, *B2m*, *Actb, Rn18s* and *Gapdh* (Fig. 6C), suggesting therefore the use of *Tbp*, *B2m* and *Actb* as accurately stable HKGs for normalizing the expression of target genes in the TCDD-induced female reproductive toxicity in mouse.

3.7. Stability of HKGs expression in the mice testis as a function of TCDD exposure

Male reproductive system is seriously affected by the toxicity of dioxin by dysregulation the expression of key genes involved in the biosynthesis of female reproductive hormones (Faiad et al., 2023). For that, it is worth to identify the most appropriate HKGs for normalizing the genes expression in testis tissue in response to TCDD exposure. Fig. 7A and Table S12 show that the closest [log ($2^{\Delta Ct}$)] values to zero were those for *Actb*, *B2m*, *Tbp*, *Gapdh*, *Rp18s*, and *Rn18s*, respectively. While, the furthest [log ($2^{\Delta Ct}$)] values to zero were those for *Brms1l*, *Calr*, *Esd*, *Hgdf* and *P4hd*. The statistics and Pearson correlation coefficient (*r*) by BestKeeper are presented in Table S13, accordingly the HKGs were ranked in the respect to the stability of their expression in mouse testis as a function of TCDD exposure. As the Fig. 7B shows that *Actb*, *B2m*, *Tbp*, *Gapdh* and *Rp18s*, were ranked in the top five positions from 1 to 5, respectively, in the respect to their expression stability. Whilst *Brms1l*, *Calr*, *Esd*, *Hgdf* and *P4hd* genes were ranked as the least stable HKGs in the mouse testis and positioned from 11 to 15, respectively. In the light of our data, the five most stable HKGs in the mouse testis as a function of TCDD exposure are *Actb*, *B2m*, *Tbp*, *Gapdh* and *Rp18s* (Fig. 7C), suggesting therefore the use of *Actb*, *B2m* and *Tbp*, as accurately stable HKGs for normalizing the expression of target genes in the TCDD-induced male reproductive toxicity in mouse.

3.8. Comprehensive ranking of HKGs across all mouse organs in response to TCDD exposure

In the objective to identify the most stable trio of HKGs across all studied organs, the stability of the top five housekeeping genes (HKGs) in the liver, kidney, lung, ovary, and testis of TCDD-exposed mice was assessed using BestKeeper, NormFinder, and GeNorm algorithms. Table 2 presents a comprehensive ranking of HKGs, highlighting their stability in different organs under TCDD exposure. The results demonstrated that *B2m*, Gapdh, and *Actb* consistently ranked among the top three most stable genes in all examined tissues, suggesting their suitability as reference genes for normalizing gene expression in various organs of TCDD-exposed mice.

4. Discussion

Relevantly, reverse transcription qPCR (RT-qPCR) stands out as the preferred method for assessing the expression levels of specific genes. Its widespread adoption is owed to its ease of use, remarkable sensitivity, precision, and reliability (Bustin et al., 2013). RT-qPCR serves as a pivotal tool for generating precise quantitative data regarding gene expression levels, which complements conventional toxicological parameters in assessing the toxic effects of dioxins in mammals (Aldeli et al., 2023; Faiad et al., 2023; Van Quang et al., 2024). Nonetheless, it is crucial to emphasize that the accuracy of the relative quantification method for transcripts is intrinsically tied to the selection of endogenous housekeeping genes (HKGs) used for normalizing the expression of target genes. These HKGs are presumed to uphold a consistent level of

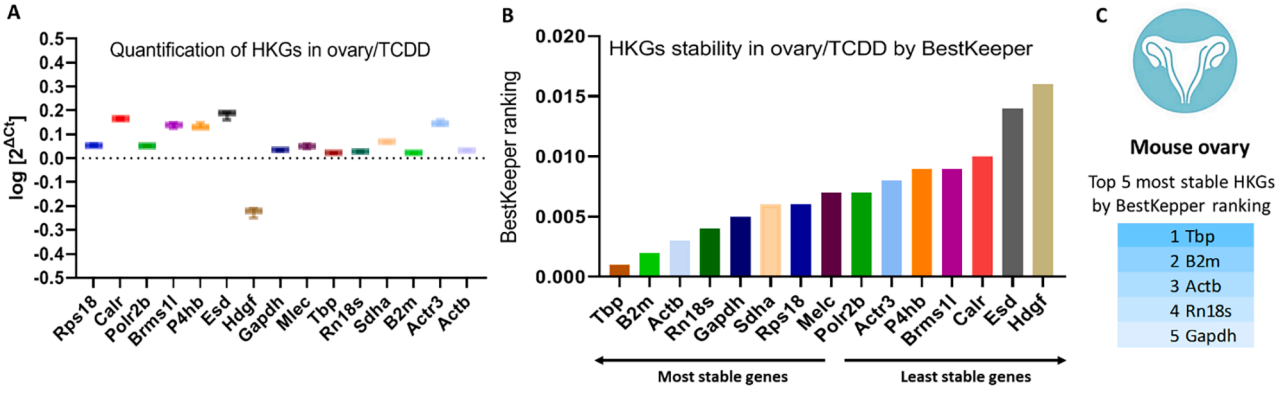

Fig. 6. Stability of Housekeeping Genes (HKGs) in Mouse Ovary under TCDD Exposure. **A.** Logarithmic values of $2^{\Delta Ct}$, where $\Delta Ct = Ct\ (TCDD) - Ct\ (control)$, for the 15 HKGs in the ovaries of mice exposed to TCDD compared to the control group are shown. The horizontal line indicates the median value. **B.** The stability ranking of HKGs determined by RefFinder across all study subjects. The stability of HKG expression was assessed using the BestKeeper computational tool. The most stable HKGs are denoted by an arrow pointing to the left, while the least stable HKGs are indicated by an arrow pointing to the right. Measurements were performed in triplicate for three cDNA preparations ($n = 9$).

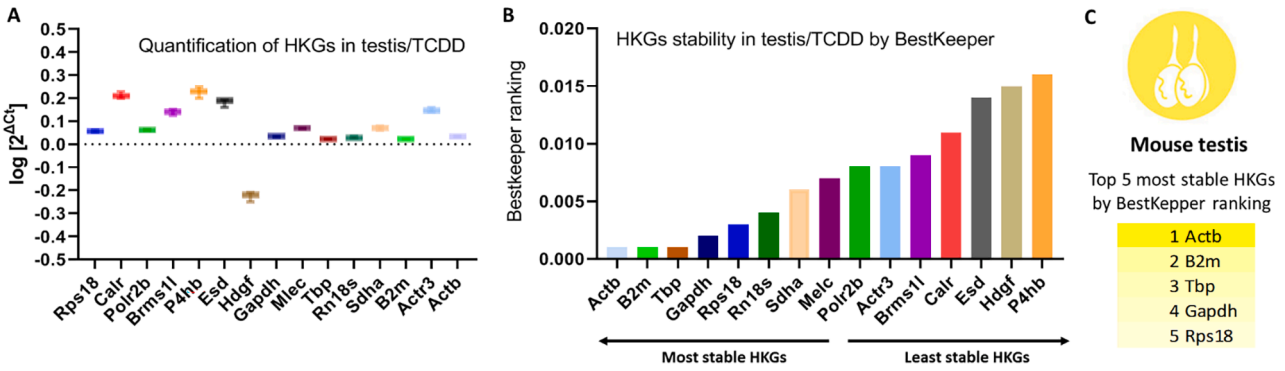

Fig. 7. Stability of Housekeeping Genes (HKGs) in Mouse Testis under TCDD Exposure. **A.** The $\log 2^{\Delta Ct}$ values for the 15 HKGs in the testes of mice exposed to TCDD compared to the control group are presented. The horizontal line represents the median value. **B.** The stability ranking of HKGs determined by RefFinder across all study subjects. The stability of HKG expression was evaluated using the BestKeeper computational tool. The most stable HKGs are depicted by an arrow pointing to the left, while the least stable HKGs are indicated by an arrow pointing to the right. Measurements were conducted in triplicate for three cDNA preparations ($n = 9$).

Table 2
Recommended comprehensive ranking order for the most stable HKGs in all studied organs in the TCDD-exposed mouse.

	Ranking Order							
Method	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Delta CT	B2m	Gapdh	Actb	Sdha	Polr2b	Rn18s	Tbp	Rps18
BestKeeper	Gapdh	B2m	Actb	Tbp	Sdha	Polr2b	Rps18	Rn18s
Normfinder	B2m	Actb	Gapdh	Sdha	Polr2b	Rn18s	Tbp	Rps18
Genorm	Actb	Gapdh	B2m	Polr2b	Sdha	Rn18s	Tbp	Rps18
Recommended comprehensive ranking	**B2m**	**Gapdh**	**Actb**	**Sdha**	**Polr2b**	**Tbp**	**Rn18s**	**Rps18**

mRNA abundance across diverse tissue types, irrespective of environmental conditions. (Bustin et al., 2013; Bustin et al., 2009; Huggett et al., 2005; Miranda and Steward, 2017). Several factors can greatly influence HKG stability, particularly the developmental stage, with notable differences between fetal and postnatal life due to changes in gene regulation. During fetal development, rapid cell proliferation, metabolic shifts, and differentiation may alter the expression of commonly used HKGs like *Gapdh*, *Actb*, and *Rn18S*. Postnatally, physiological homeostasis and tissue specialization further influence HKG stability. For instance, *Hprt1* and *Tbp* are more stable across developmental stages, whereas *Gapdh* fluctuates in response to metabolic demands (Uddin et al., 2011).

Therefore, selecting the most accurate housekeeping genes (HKGs) to normalize transcript levels across various exposed tissues is paramount in RT-qPCR quantification (Sanders et al., 2018). We proposed that conducting an in silico analysis of candidate HKGs expression within relevant transcriptomic databases could provide an effective method for identifying a subset of genes with highly consistent expression patterns. These selected genes could then be incorporated into subsequent RT-qPCR assay design and validation. To achieve this goal, we implemented a bioinformatics workflow utilizing publicly-available transcriptome resources to identify the most commonly used HKGs and assess their stability in expression across different tissues of mice acutely exposed to TCDD. Our approach in its main sequential steps comes in line with recent methodologies utilizing publicly available transcriptomic datasets to select and validate the most reliable HKGs for gene expression studies in pathology and toxicology (Nevone et al., 2023; Padhi et al., 2020). However, one important feature of our proposed strategy is the use of Housekeeping and Reference Transcript Atlas (HRT Atlas) to primarily identify the more accurate HKGs in a specific-tissue pattern. The HRT dataset, created by Hounkpe and colleagues (Hounkpe et al., 2021), is a publicly accessible online transcriptomic resource featuring a user-friendly interface. It is primarily designed for searching human and mouse housekeeping genes and candidate reference transcripts. Notably, this tool provides tissue- and cell-specific candidate reference transcripts along with some qPCR normalization primers. The exploration of candidate reference genes on the HRT platform was centered on the mouse database, comprising an analysis of 507 meticulously curated high-quality RNA-seq datasets sourced from the Gene Expression Omnibus (GEO) portal (https://www.ncbi.nlm.nih.gov/geo/). In this portal, 22 tissues and cells types from wild type healthy control mice were included in the workflow (Hounkpe et al., 2021).

Our method enabled the identification and selection of a final roster of 20 potential housekeeping genes (HKGs), which were subsequently subjected to experimental scrutiny in response to TCDD exposure, displaying a tissue-specific pattern. This quantity of HKGs is sufficiently ample to choose at least three with the most consistent expression patterns across experimental and control samples, as routinely recommended by the MIQE guidelines (Minimum Information for publication of Quantitative real-time PCR Experiments). Experts in the field advocate for the systematic validation of reference genes in a given experimental context and endorse the use of multiple pre-validated reference genes, alongside appropriate normalizing algorithms, for the normalization of RT-qPCR data (Bustin et al., 2009; Bustin and Wittwer, 2017; Nevone et al., 2023; Sanders et al., 2018). Furthermore, the number of housekeeping genes (HKGs) examined in our study far surpasses those explored in other investigations conducted on mice exposed to TCDD or in rat tissues under toxicological circumstances. (Prokopec et al., 2013; Svingen et al., 2015). Our data potentially indicate that out of the initially chosen housekeeping genes (HKGs), 15 genes (*Rps18*, *Calr*, *Polr2b*, *Brms1l*, *P4hb*, *Esd*, *Hdgf*, *Gapdh*, *Mlec*, *Tbp*, *Rn18s*, *Sdha*, *B2m*, *Actr3* and *Actb*) exhibited typical amplification efficiencies and notably low values in both intra- and inter-assay variations of Ct for each gene amplification. This suggests a precise selection process compared to other methodologies (Nevone et al., 2023).

Our findings indicate that the expression levels of *Actb*, *Rps18*, *Polr2b*, *Gapdh*, and *Sdha* remain consistently stable in the liver of mice following exposure to TCDD. Moreover, previous reports have highlighted the common usage of *Actb*, *Gapdh*, and *Rps18* in hepatotoxicological and pathological studies, affirming their reliability as reference genes in such contexts (Dijkstra et al., 2014; Suzuki et al., 2000). More

specifically, our findings are corroborated by numerous pieces of evidence demonstrating the stable gene expression of *Actb*, *Gapdh*, and *Rps18* in the liver of mice exposed to TCDD (Besteman et al., 2007; Fling et al., 2020). Moreover, *Gapdh* and *Sdha* was used as accurate HKGs to normalize the expression of genes exhibited TCDD-dependent responses including *Cyp1a1*, *Cyp1b1*, *Aldh1a3* and *Slc7a5* in human liver cell line (HL1-1) (Kim et al., 2009), and in rat liver (Watson et al., 2014).

In the kidney, our findings reveal that the five most stable HKGs in mice exposed to TCDD are *Sdha*, *Actb*, *Gapdh*, *B2m*, and *Rn18s*. Generally, toxicological studies conducted on mouse kidneys have consistently identified *Sdha* as one of the most stable genes when the expression of 15 HKGs was experimentally assessed in various tissues and statistically analyzed using BestKeeper. However, *B2m* was observed to be among the less stable genes in these studies (Svingen et al., 2015). Despite the scarcity of transcriptional studies in mouse kidneys exposed to TCDD, our findings receive support from a comprehensive transcriptional analysis that focused on 297 genes in the liver and 17 genes in the kidney of mice following exposure to TCDD (Boutros et al., 2009).

In relation to lung tissue, our investigation revealed that the five most stable housekeeping genes (HKGs) in the mouse lung following exposure to TCDD were *Tbp*, *Sdha*, *Rps18*, *B2m*, and *Rn18s*. Consistently, it has been reported that *Tbp* serves as a reliable reference gene in the mouse lung, taking into account various biological, technical, and experimental factors (Shin et al., 2022). Also, the stability of *Sdha* was reported in various mouse tissues (Svingen et al., 2015). Our findings in mouse ovaries indicate that *Tbp*, *B2m*, *Actb*, *Rn18s*, and *Gapdh* were the most stable housekeeping genes (HKGs) in response to TCDD exposure. Notably, the stability of *Actb* has been specifically noted in TCDD-exposed mouse ovaries within this context (Aldeli and Hanano, 2025; Aldeli et al., 2023). While, other studies suggested *Actb*, *Gapdh*, *Sdha* and *Cyc1* as the most stable HKGs in the ovary under normal conditions (Berruien et al., 2021; Cadenas et al., 2022; Kouadjo et al., 2007).

Moreover, our findings indicate that *Actb*, *B2m*, *Tbp*, *Gapdh*, and *Rp18s* are the most stable housekeeping genes for normalizing the expression of target genes in TCDD-induced male reproductive toxicity in mice. These results align with a recent report highlighting the accurate stability of *Actb* in the testis of mice exposed to TCDD (Faiad et al., 2023). Furthermore, *Actb* and *Gapdh* have been noted to exhibit stable expression patterns in various mouse tissues, including the testis (Kouadjo et al., 2007). In developmental studies, BestKeeper analysis identified *Gapdh*, *Actb*, and *Ppia* as the most stable genes in mouse testes during both embryonic and postnatal development (Gong et al., 2014). Similarly, *Actb* and *Gapdh* have been consistently expressed in the human testis (Cavalcanti et al., 2011). Finally, our study presents a robust and innovative strategy for identifying the most stably expressed control genes across various mouse tissues exposed to TCDD, as well as for determining the minimum number of genes required to calculate a reliable normalization factor. Our findings indicate that *B2m*, *Gapdh*, and *Actb* consistently ranked among the top three most stable genes in all tissues examined, supporting their suitability as reference genes for normalizing gene expression in TCDD-exposed mice. Importantly, it was demonstrated that relying on a single housekeeping gene for normalization can introduce substantial errors in a significant portion of the analyzed samples. In contrast, the geometric mean of multiple carefully selected reference genes provides a more accurate normalization approach, as previously validated using publicly available microarray data (Vandesompele et al., 2002). Based on our results, we recommend the use of at least two genes—*Gapdh* and *Actb*—from the proposed trio for reliable normalization in future gene expression studies involving TCDD-exposed mouse tissues. (Aldeli and Hanano, 2025; Aldeli et al., 2023; Faiad et al., 2023).

5. Conclusions

It is well recognized that TCDD provokes significant toxicity in mammals in a very pronounced tissue-specific manner, leading to dysregulation of numerous genes across various tissues. Therefore, precise quantification of the expression of TCDD-affected genes relies heavily on accurate normalization using suitable HKGs. In conclusion, we have devised a strategy to select appropriate HKGs for the normalization of RT-qPCR experiments based on publicly available and easily accessible transcriptomic datasets, along with a pipeline for designing and validating RT-qPCR assays. As a proof-of-concept, we applied this strategy to identify stable HKGs in the most affected tissues of mice exposed to TCDD, demonstrating that genes identified through this approach typically outperform commonly used normalizing genes for gene normalization purposes. Overall, our study offers valuable insights into the selection of stable HKGs for tissue-specific gene expression analysis in response to TCDD toxicity, thereby facilitating more accurate and reliable research in this field.

Authors' contributions

NH, RH, MD, ZR, OA, RA, MY, AK, RA, WF, NA performed the experimental work equally. AH supervised the work and wrote the manuscript. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2025.100234.

Data availability

No data was used for the research described in the article.

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