Selection of internal references for transcriptomics and RT-qPCR assays in Neurofibromatosis type 1 (NF1) related Schwann cell lines

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Abstract

Transcriptomics has been widely applied in uncovering disease mechanisms and screening potential biomarkers. Internal reference selection determines the accuracy and reproducibility of data analyses. The aim of this study was to identify the most qualified reference genes for the relative quantitation analysis of transcriptomics and real-time quantitative reverse-transcription PCR in fourteen NF1 related cell lines, including non-tumor, benign and malignant Schwann cell lines. The expression characteristics of eleven candidate reference genes (RPS18, ACTB, B2M, GAPDH, PPIA, HPRT1, TBP, UBC, RPLP0, TFRC and RPL32) were screened and analyzed by four software programs: geNorm, NormFinder, BestKeeper and RefFinder. Results showed that GAPDH, the most used internal reference gene, was significantly unstable. The combinational use of two reference genes (PPIA and TBP) was optimal in malignant Schwann cell lines and the use of single reference genes (PPIA or PRLP0) alone or in combination was optimal in benign Schwann cell lines. Our recommended internal reference genes may improve the accuracy and reproducibility of the results of transcriptomics and real-time quantitative reverse-transcription PCR in further gene expression analyses of NF1 related tumors.

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominantly inherited tumor predisposition syndrome that affects multiple organ systems and has a wide range of variable clinical manifestations, such as pigmentary lesions, skeletal abnormalities[1-3]. The major defining features in NF1 patients are peripheral nerve sheath tumors, including plexiform neurofibromas (pNF) and malignant peripheral nerve sheath tumors (MPNST)[4]. Therefore, the majority of NF1 related tumor researches could be categorized into benign tumor studies or malignant tumor studies.

Currently, Omics studies play an increasingly important role in uncovering disease mechanisms and screening potential therapeutic biomarkers. Among different omics studies, transcriptomics is the bridge between genomics and proteomics. Key methods, including microarray and RNA-seq, could provide deep and precise measurements of levels of transcripts and different isoforms. Several studies are conducted to figure out the molecular mechanisms of NF1 and to advance the development of targeted drugs[5-7]. These high-throughput results need further validation by real-time quantitative reverse-transcription PCR (RT-qPCR), which is considered as the gold standard for gene expression studies. However, the accuracy and reproducibility of these results may be affected by several factors at multiple stages. In data analysis, the stability of reference genes is critical for appropriate standardization and obtaining accurate gene expression data. The Minimum Information for Publication of Quantitative Real-Time

PCR experiments (MIQE) guidelines highlight the importance of experimental validation of reference genes for particular tissues or cell types[8-11]. However, to date, there is no previous research on the validation of suitable reference genes for the relative quantification analysis of target gene expression in NF1 related cell lines.

In the present study, eleven candidate reference genes, which are most frequently used for relative quantification analysis in other neoplastic diseases, were selected, including 18S Ribosomal RNA (RPS18), β-actin (ACTB), β-2-microglobulin (B2M), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Peptidylprolyl isomerase A (PPIA), Hypoxanthine phosphoribosyltransferase 1 (HPRT1), TATA binding protein (TBP), Ubiquitin C (UBC), Ribosomal protein large P0 (RPLP0), Transferrin receptor (P90, CD71) (TFRC), and Ribosomal protein 32 (RPL32)[12-16].

We investigated the stability of these eleven reference genes in fourteen different NF1 related cell lines, including two non-tumor *NF1*$^{+/-}$ Schwann cell lines, five benign pNF cell lines and seven malignant MPNST cell lines. Based on study design, we analyzed the data obtained as two separate groups: (1) benign NF1 tumor study including Schwann cell (SC) + pNF cell lines and (2) malignant NF1 tumor study including SC + pNF + MPNST cell lines, aiming to identify the most qualified reference genes for gene expression analysis in benign and malignant NF1 tumor study respectively.

Materials and Methods

Neurofibromatosis type 1 (NF1) related cell lines

Five MPNST cell lines (ST-8814, STS26T, S462, S462TY, T265) were generous gifts from Dr.Vincent W Keng (Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong) and Jilong Yang (International Medical School, Tianjin Medical University, Tianjin). Two pNF cell lines (A68 and WZJ) are immortalized as described previously[17]. Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine approved this study. Informed consent was achieved from patients under institutional reviewer board protocols. hTERT NF1 ipNF05.5 (ATCC® CRL-3388™), hTERT NF1 ipNF95.6 (ATCC® CRL3389™), hTERT NF1 ipNF95.11b (ATCC® CRL3390™), hTERT NF1 ipnNF95.11c (ATCC® CRL3391™), hTERT NF1 ipn02.3 2λ (ATCC® CRL3392 ™), sNF96.2 (ATCC® CRL2884 ™) and sNF02.2 (ATCC® CRL2885 ™) were obtained from the American Type Culture Collection (ATCC). All the cell lines used in this study were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified incubator at 5% CO2 and were

confirmed negative for mycoplasma prior to use. Verification of cell lines was performed by Short Tandem Repeat (STR) DNA profiling (Applied Biological Materials Inc, Canada).

Total RNA extraction and cDNA synthesis

Total RNA was extracted from all NF1 related cell lines using AxyPrep Multisource RNA Miniprep Kit (Axygen, USA) according to the manufacturer's instructions. The quality and quantity of RNA were measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) through OD260/280 and OD260/230 ratios. 500 ng of total RNA was used for the cDNA synthesis reaction using PrimeScript™ RT Master Mix kit (TaKaRa RR036A) according to the manufacturer's instructions. After the reaction, the cDNA was diluted to 20 ng/ml. All RT-qPCR experiments were performed with the same batch of cDNA.

RT-qPCR

Reference gene primers were designed and synthesized by Sangon Biotech (Shanghai, China). The efficiency, dynamic range and specificity of all primers were tested by Sangon Biotech (Shanghai, China). RT-qPCR was performed using TB Green® Premix Ex Taq™ Kit (TaKaRa RR420A) on a Applied Biosystems 7500 Real-Time PCR System, as described previously[18]. Each sample was measured in three technical replicates. The relative quantification of RT-qPCR data were calculated using the $2^{-\Delta\Delta C}{}_{T}$ method, as described previously[8] .

Statistical analysis

The stability of the eleven candidate reference genes was examined by four frequently used software programs, geNorm[19], NormFinder[20] BestKeeper[21] and RefFinder[22], as described previously[23-26].

Results

The expression characteristics of eleven internal reference gene candidates

The threshold cycle (C_t) value was used to assess the expression characteristics of the internal reference gene candidates. Higher C_t values indicate lower expression levels. The distribution of the C_t values of eleven reference genes in fourteen samples, including two *NF1*$^{+/-}$ Schwann cell lines, five plexiform neurofibroma cell lines and seven MPNST cell lines was displayed in Fig 1. The C_t values represented in this research from all samples ranged from 18.09 to 43.65. It is worth noting that UBC showed significantly higher C_t values in MPNST samples compared to other samples, suggesting lower mRNA expression level.

Genorm analysis

In order to identify the best reference genes for benign and malignant NF1 tumor study, we applied four statistical approaches including geNorm, NormFinder, BestKeeper and ΔC_t method. The *M* value of eleven reference genes were calculated and ranked by genorm. The genorm system identifies those genes with lowest *M* values as the most stably expressed genes and an *M* value under 0.5 represents relatively stable gene expression. GeNorm analysis showed that PPIA and TBP, sharing an *M* value of 0.549, were the most stable reference genes in SC + pNF + MPNST cell lines (Fig 2). However, when only considering SC + pNF cell lines, the most stable genes were PPIA and PRLP, with an *M* value of 0.225, followed by TBP and S18 (*M* value, 0.295 and 0.454, respectively). The optimal number of reference genes to obtain a stable normalization index was demonstrated by V-values. When SC + pNF + MPNST cell lines were considered, the V8/9 value was 0.127, indicating that eight reference genes combination is optimal for RT-qPCR normalization. In SC + pNF cell lines, the V2/3 value was 0.104, suggesting that merely two reference genes were necessary. And the V8/9 value was lower than 0.110 in this group, of which the V values kept stable in different subsets.

NormFinder analysis

Eleven reference genes underwent normalization factor calculation and were ranked by NormFinder according to their minimal combined SC, pNF and MPNST gene expression variation. According to the result, TBP was the optimal reference gene (stability value, 0.394), followed by PPIA, ACTB and GAPDH (stability values, 0.403, 0.427 and 0559 respectively) (Fig 3). The best combination of two genes are GAPDH and S18 with a combination stability value of 0.150. For SC + pNF cell lines, PPIA, RPLP and TBP were the most stably expressed genes (stability values, 0.142, 0.185 and 0.186 respectively), while the rest ones were extremely unstable amongst those cell lines.

Best keeper analysis

In best keeper algorithm, the C_t values, SD (standard deviation) and CV (coefficient of variance) of each gene were calculated and analyzed to identify stable reference gene candidates. Under general conditions, genes with a SD greater than 1.0 are determined to be unstable. Among SC + pNF + MPNST cell lines, TBP turned out to be the best choice with a standard deviation [+/- CP] (which is the stability value in best keeper algorithm) of 0.48 (Fig 4). The stability value of TBP kept stable (0.44) when it came to SC + pNF cell lines. However, RPLP and PPIA were esteemed as more stable genes, sharing a stability value of 0.42.

ΔC_t analysis

In ΔC_t method, the differential expression of "gene pairs" were analyzed to determine the optimal reference genes. According to the results, PPIA (1.24) and TBP (1.26) were the most stably expressed reference genes in SC + pNF + MPNST cell lines (Fig 5). While RPLP (0.78) was far more stable in SC + pNF cell lines.

Comprehensive ranking order

Using RefFinder, we integrated four analysis approaches mentioned above to comprehensively evaluate the expression stability of these candidate genes. PPIA turned out to be the optimal reference gene in SC + pNF + MPNST cell lines with a ranking value of 1.19, followed by TBP and S18 (ranking value, 1.41 and 3.66, respectively) (Fig 6). And PRLP (1.19) was the most stably expressed in SC + pNF cell lines.

Discussion

High-throughput transcriptome sequencing identifies the key pathogenic genes that play crucial roles in the pathogenesis, development and malignant transformation of NF1 related neurofibroma. RT-qPCR has been considered as golden standard for gene expression analysis because of its accuracy and sensitivity[27]. It is a robust and specific method for the validation of the identity of these pathogenic genes that aberrantly expressed in neurofibroma.

The ease to generate RT-qPCR data is in sharp contrast with the challenges to guarantee that the results obtained are reliable. In the absence of proper reference genes, data obtained are possibly inaccurate and unreproducible, as it has been shown that the

use of a single reference gene without validation results in a significant bias (ranging from 3-fold in 25% of the results up to 6-fold in 10% of the results)[19].

Prior to this study, no validated reference gene has been identified for NF1 related cell lines. In previous studies, GAPDH and ACTB have been most frequently used in gene expression analysis of NF1 without solid validation[28-30]. However, the expression characteristics of reference genes vary remarkably under different experimental conditions and with different samples. The use of GAPDH and ACTB as an internal control has been proven to be unsuitable in other samples, such as lymphoblastoid cell lines and human mesenchymal stem cells[31-33]. Therefore, it is crucial to confirm reliable and qualified reference genes for particular tissues or cell types and specific experimental designs.

In our study, in order to find the internal reference genes stably expressed in different NF1 samples, including normal peripheral nerve, benign and malignant tumor tissues, we investigated fourteen different NF1 related cell lines, derived respectively from the tissues mentioned above, which include two non-tumor *NF1*$^{+/-}$ Schwann cell lines, five pNF cell lines and seven MPNST cell lines. The expression stability of eleven frequently used reference genes, including RPS18, ACTB, B2M, GAPDH, PPIA, HPRT1, TBP, UBC, RPLP0, TFRC, and RPL32 were investigated and analyzed separately in these cell lines.

Four different mathematical and statistical models were utilized to analyze the data obtained, including geNorm, NormFinder, BestKeeper and ΔC_t. Each model uses different algorithms to estimate both the intra- and the intergroup expression variations and rank candidate genes based on the instability score. GeNorm and NormFinder use the stability (actually instability) value, ΔC_t uses the average of S.D. and BestKeeper uses the S.D. of the crossing points[19-21]. In addition, A web-based tool RefFinder was finally used to evaluate the stability of candidate reference genes and identify the most stable gene by calculating the geometric mean of ranking values obtained from the above-mentioned four methods[22].

Due to the fact that the software applied are based on different mathematical models, the ranking orders of reference gene stabilities varied slightly between these tools. However, the top two positions of reference genes in SC + pNF group and SC + pNF + MPNST group determined by geNorm were identical to those determined by NormFinder, BestKeeper and ΔC_t. Unanimously, according to the results of RefFinder, the top two reference genes in two groups are also the same and they share similar ranking values which are significantly lower than others. Therefore, conclusion can be drawn that PPIA and PRLP0 are the best reference genes for normalization in benign NF1 tumor study, while PPIA and TBP are as well in malignant NF1 tumor study.

PPIA is a gene encoding for a cyclosporin binding-protein, TBP is a gene encoding for a TATA-binding protein, and PRLP0 encodes a ribosomal protein that is a component of the 60S subunit. In NF1 related tumors, they were stably expressed irrespective of tumor pathology and severity. However, the most frequently used reference gene GAPDH was proved to be an inappropriate option in two groups (*M* values > 0.5). As a multifunctional gene, the use of GAPDH as a reference gene has been also questioned and challenged in other cancers including lung cancer[34], breast cancer[35] and bladder cancer[36]. Accumulated evidences indicate that GAPDH is deregulated in various cancers under certain conditions and potentially participates in tumorigenesis and tumor progression[34-38].

In addition, considering the difference of opinion on the minimal number of reference genes required for RT-qPCR, we explored the necessity of selecting multiple reference genes for data normalization in our samples. In previous studies, some investigators showed that the combination of more than one reference gene improved the accuracy of results[14, 19, 39-41] while other investigators proved that normalization with a single gene is sufficient for most research applications[12, 36, 42, 43]. In present study, according to the analyses of geNorm, combination of eight reference genes is the most precise plan for normalization in SC + pNF + MPNST group. However, considering the stability and precision of using two reference genes in combination is sufficient for data normalization, we suggest using a normalization factor calculated by the geometrical mean of the most stable reference genes (PPIA and TBP) for normalization of target gene expression in SC, pNF and MPNST cells[44]. What's more, it is worth noting that the use of single reference gene, with a M-value over 0.5, should be avoided in these samples. In SC + pNF group, although the combination of two reference genes significantly improved precision over normalization with PPIA or PRLP0 alone, the use of single reference genes (PPIA or PRLP0), sharing an *M* value of 0.225, is acceptable for data normalization.

Conclusions

In this study, we systematically explored the suitability of fourteen candidate reference genes for normalization of gene expression in different NF1 related cell lines, including non-tumor *NF1*$^{+/-}$ Schwann cell lines, pNF cell lines and MPNST cell lines. According to the results, we recommend using two reference genes (PPIA and TBP) in combination for gene expression analyses in MPNST related researches and using single reference genes (PPIA or PRLP0) alone or in combination in pNF related studies.

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References

1. Gutmann DH, Ferner RE, Listernick RH, Korf BR, Wolters PL, Johnson KJ. Neurofibromatosis type 1. Nature Reviews Disease Primers. 2017;3(1). doi: 10.1038/nrdp.2017.4.
2. Stevenson DA, Zhou H, Ashrafi S, Messiaen LM, Carey JC, D'Astous JL, et al. Double inactivation of NF1 in tibial pseudarthrosis. Am J Hum Genet. 2006;79(1):143-8. Epub 2006/06/15. doi: 10.1086/504441. PubMed PMID: 16773574; PubMed Central PMCID: PMCPMC1474128.
3. De Schepper S, Maertens O, Callens T, Naeyaert JM, Lambert J, Messiaen L. Somatic mutation analysis in NF1 cafe au lait spots reveals two NF1 hits in the melanocytes. J Invest Dermatol. 2008;128(4):1050-3. Epub 2007/10/05. doi: 10.1038/sj.jid.5701095. PubMed PMID: 17914445.
4. Ariel IM. Tumors of the peripheral nervous system. Semin Surg Oncol. 1988;4(1):7-12. Epub 1988/01/01. doi: 10.1002/ssu.2980040104. PubMed PMID: 3127871.
5. Solga AC, Pong WW, Kim KY, Cimino PJ, Toonen JA, Walker J, et al. RNA Sequencing of Tumor-Associated Microglia Reveals Ccl5 as a Stromal Chemokine Critical for Neurofibromatosis-1 Glioma Growth. Neoplasia. 2015;17(10):776-88. Epub 2015/11/21. doi: 10.1016/j.neo.2015.10.002. PubMed PMID: 26585233; PubMed Central PMCID: PMCPMC4656811.
6. Kohlmeyer JL, Kaemmer CA, Pulliam C, Maharjan CK, Samayoa AM, Major HJ, et al. RABL6A Is an Essential Driver of MPNSTs that Negatively Regulates the RB1 Pathway and Sensitizes Tumor Cells to CDK4/6 Inhibitors. Clin Cancer Res. 2020;26(12):2997-3011. Epub 2020/02/23. doi: 10.1158/1078-0432.CCR-19-2706. PubMed PMID: 32086342; PubMed Central PMCID: PMCPMC7299809.
7. Cui Z, Shen Y, Chen KH, Mittal SK, Yang JY, Zhang G. KANK1 inhibits cell growth by inducing apoptosis through regulating CXXC5 in human malignant peripheral nerve sheath tumors. Sci Rep. 2017;7:40325. Epub 2017/01/10. doi: 10.1038/srep40325. PubMed PMID: 28067315; PubMed Central PMCID: PMCPMC5220314.
8. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8. Epub 2002/02/16. doi: 10.1006/meth.2001.1262. PubMed PMID: 11846609.
9. Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. 2005;6(4):279-84. Epub 2005/04/09. doi: 10.1038/sj.gene.6364190. PubMed PMID: 15815687.

10. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611-22. Epub 2009/02/28. doi: 10.1373/clinchem.2008.112797. PubMed PMID: 19246619.
11. Kozera B, Rapacz M. Reference genes in real-time PCR. J Appl Genet. 2013;54(4):391-406. Epub 2013/10/01. doi: 10.1007/s13353-013-0173-x. PubMed PMID: 24078518; PubMed Central PMCID: PMCPMC3825189.
12. Kriegova E, Arakelyan A, Fillerova R, Zatloukal J, Mrazek F, Navratilova Z, et al. PSMB2 and RPL32 are suitable denominators to normalize gene expression profiles in bronchoalveolar cells. BMC Mol Biol. 2008;9:69. Epub 2008/08/02. doi: 10.1186/1471-2199-9-69. PubMed PMID: 18671841; PubMed Central PMCID: PMCPMC2529339.
13. Liu DW, Chen ST, Liu HP. Choice of endogenous control for gene expression in nonsmall cell lung cancer. Eur Respir J. 2005;26(6):1002-8. Epub 2005/12/02. doi: 10.1183/09031936.05.00050205. PubMed PMID: 16319328.
14. Normann KR, Oystese KAB, Berg JP, Lekva T, Berg-Johnsen J, Bollerslev J, et al. Selection and validation of reliable reference genes for RT-qPCR analysis in a large cohort of pituitary adenomas. Mol Cell Endocrinol. 2016;437:183-9. Epub 2016/08/27. doi: 10.1016/j.mce.2016.08.030. PubMed PMID: 27561203.
15. Wang X, He J, Wang W, Ren M, Gao S, Zhao G, et al. Validation of internal reference genes for relative quantitation studies of gene expression in human laryngeal cancer. PeerJ. 2016;4:e2763. Epub 2016/12/14. doi: 10.7717/peerj.2763. PubMed PMID: 27957397; PubMed Central PMCID: PMCPMC5149058.
16. Rho HW, Lee BC, Choi ES, Choi IJ, Lee YS, Goh SH. Identification of valid reference genes for gene expression studies of human stomach cancer by reverse transcription-qPCR. BMC Cancer. 2010;10:240. Epub 2010/05/29. doi: 10.1186/1471-2407-10-240. PubMed PMID: 20507635; PubMed Central PMCID: PMCPMC2887403.
17. Li H, Chang LJ, Neubauer DR, Muir DF, Wallace MR. Immortalization of human normal and NF1 neurofibroma Schwann cells. Lab Invest. 2016;96(10):1105-15. Epub 2016/09/13. doi: 10.1038/labinvest.2016.88. PubMed PMID: 27617404.
18. Liu Y, Qin Z, Cai L, Zou L, Zhao J, Zhong F. Selection of internal references for qRT-PCR assays of human hepatocellular carcinoma cell lines. Biosci Rep. 2017;37(6). Epub 2017/11/29. doi: 10.1042/BSR20171281. PubMed PMID: 29180379; PubMed Central PMCID: PMCPMC5741833.
19. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3(7):RESEARCH0034. Epub 2002/08/20. doi: 10.1186/gb-2002-3-7-research0034. PubMed PMID: 12184808; PubMed Central PMCID: PMCPMC126239.

20. Andersen CL, Jensen JL, Orntoft TF. 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 Res. 2004;64(15):5245-50. Epub 2004/08/04. doi: 10.1158/0008-5472.CAN-04-0496. PubMed PMID: 15289330.

21. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol Lett. 2004;26(6):509-15. Epub 2004/05/07. doi: 10.1023/b:bile.0000019559.84305.47. PubMed PMID: 15127793.

22. Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol. 2012. Epub 2012/02/01. doi: 10.1007/s11103-012-9885-2. PubMed PMID: 22290409.

23. Hellemans J, Vandesompele J. Selection of reliable reference genes for RT-qPCR analysis. Methods Mol Biol. 2014;1160:19-26. Epub 2014/04/18. doi: 10.1007/978-1-4939-0733-5_3. PubMed PMID: 24740218.

24. Ali H, Du Z, Li X, Yang Q, Zhang YC, Wu M, et al. Identification of suitable reference genes for gene expression studies using quantitative polymerase chain reaction in lung cancer in vitro. Mol Med Rep. 2015;11(5):3767-73. Epub 2015/01/13. doi: 10.3892/mmr.2015.3159. PubMed PMID: 25573171.

25. Yu S, Yang Q, Yang JH, Du Z, Zhang G. Identification of suitable reference genes for investigating gene expression in human gallbladder carcinoma using reverse transcription quantitative polymerase chain reaction. Mol Med Rep. 2015;11(4):2967-74. Epub 2014/12/02. doi: 10.3892/mmr.2014.3008. PubMed PMID: 25434674.

26. Ma H, Yang Q, Li D, Liu J. Validation of suitable reference genes for quantitative polymerase chain reaction analysis in rabbit bone marrow mesenchymal stem cell differentiation. Mol Med Rep. 2015;12(2):2961-8. Epub 2015/05/16. doi: 10.3892/mmr.2015.3776. PubMed PMID: 25976103.

27. Derveaux S, Vandesompele J, Hellemans J. How to do successful gene expression analysis using real-time PCR. Methods. 2010;50(4):227-30. Epub 2009/12/09. doi: 10.1016/j.ymeth.2009.11.001. PubMed PMID: 19969088.

28. Hummel TR, Jessen WJ, Miller SJ, Kluwe L, Mautner VF, Wallace MR, et al. Gene expression analysis identifies potential biomarkers of neurofibromatosis type 1 including adrenomedullin. Clin Cancer Res. 2010;16(20):5048-57. Epub 2010/08/27. doi: 10.1158/1078-0432.CCR-10-0613. PubMed PMID: 20739432; PubMed Central PMCID: PMCPMC4837895.

29. Karaosmanoglu B, Kocaefe CY, Soylemezoglu F, Anlar B, Varan A, Vargel I, et al. Heightened CXCR4 and CXCL12 expression in NF1-associated neurofibromas. Childs Nerv Syst. 2018;34(5):877-82. Epub 2018/02/20. doi: 10.1007/s00381-018-3745-6. PubMed PMID: 29455242.

30. Longo JF, Brosius SN, Black L, Worley SH, Wilson RC, Roth KA, et al. ErbB4 promotes malignant peripheral nerve sheath tumor pathogenesis via Ras-independent

mechanisms. Cell Commun Signal. 2019;17(1):74. Epub 2019/07/12. doi: 10.1186/s12964-019-0388-5. PubMed PMID: 31291965; PubMed Central PMCID: PMCPMC6621970.

31. Li X, Yang Q, Bai J, Yang Y, Zhong L, Wang Y. Identification of optimal reference genes for quantitative PCR studies on human mesenchymal stem cells. Mol Med Rep. 2015;11(2):1304-11. Epub 2014/11/06. doi: 10.3892/mmr.2014.2841. PubMed PMID: 25369870.

32. Oturai DB, Sondergaard HB, Bornsen L, Sellebjerg F, Christensen JR. Identification of Suitable Reference Genes for Peripheral Blood Mononuclear Cell Subset Studies in Multiple Sclerosis. Scand J Immunol. 2016;83(1):72-80. Epub 2015/09/24. doi: 10.1111/sji.12391. PubMed PMID: 26395032.

33. Toritsuka M, Makinodan M, Yamauchi T, Yamashita Y, Ikawa D, Komori T, et al. Altered gene expression in lymphoblastoid cell lines after subculture. In Vitro Cell Dev Biol Anim. 2018;54(7):523-7. Epub 2018/06/28. doi: 10.1007/s11626-018-0267-1. PubMed PMID: 29948745.

34. Tokunaga K, Nakamura Y, Sakata K, Fujimori K, Ohkubo M, Sawada K, et al. Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res. 1987;47(21):5616-9. Epub 1987/11/01. PubMed PMID: 3664468.

35. Revillion F, Pawlowski V, Hornez L, Peyrat JP. Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer. Eur J Cancer. 2000;36(8):1038-42. Epub 2000/07/08. doi: 10.1016/s0959-8049(00)00051-4. PubMed PMID: 10885609.

36. Ohl F, Jung M, Radonic A, Sachs M, Loening SA, Jung K. Identification and validation of suitable endogenous reference genes for gene expression studies of human bladder cancer. J Urol. 2006;175(5):1915-20. Epub 2006/04/08. doi: 10.1016/S0022-5347(05)00919-5. PubMed PMID: 16600798.

37. Colell A, Green DR, Ricci JE. Novel roles for GAPDH in cell death and carcinogenesis. Cell Death Differ. 2009;16(12):1573-81. Epub 2009/09/26. doi: 10.1038/cdd.2009.137. PubMed PMID: 19779498.

38. Guo C, Liu S, Sun MZ. Novel insight into the role of GAPDH playing in tumor. Clin Transl Oncol. 2013;15(3):167-72. Epub 2012/08/23. doi: 10.1007/s12094-012-0924-x. PubMed PMID: 22911551.

39. Coulson DT, Brockbank S, Quinn JG, Murphy S, Ravid R, Irvine GB, et al. Identification of valid reference genes for the normalization of RT qPCR gene expression data in human brain tissue. BMC Mol Biol. 2008;9:46. Epub 2008/05/08. doi: 10.1186/1471-2199-9-46. PubMed PMID: 18460208; PubMed Central PMCID: PMCPMC2396658.

40. Pombo-Suarez M, Calaza M, Gomez-Reino JJ, Gonzalez A. Reference genes for normalization of gene expression studies in human osteoarthritic articular cartilage. BMC Mol Biol. 2008;9:17. Epub 2008/01/30. doi: 10.1186/1471-2199-9-17. PubMed PMID: 18226276; PubMed Central PMCID: PMCPMC2248200.

41. Abruzzo LV, Lee KY, Fuller A, Silverman A, Keating MJ, Medeiros LJ, et al. Validation of oligonucleotide microarray data using microfluidic low-density arrays: a new statistical method to normalize real-time RT-PCR data. Biotechniques. 2005;38(5):785-92. Epub 2005/06/11. doi: 10.2144/05385MT01. PubMed PMID: 15945375.

42. Dydensborg AB, Herring E, Auclair J, Tremblay E, Beaulieu JF. Normalizing genes for quantitative RT-PCR in differentiating human intestinal epithelial cells and adenocarcinomas of the colon. Am J Physiol Gastrointest Liver Physiol. 2006;290(5):G1067-74. Epub 2006/01/10. doi: 10.1152/ajpgi.00234.2005. PubMed PMID: 16399877.

43. Lyng MB, Laenkholm AV, Pallisgaard N, Ditzel HJ. Identification of genes for normalization of real-time RT-PCR data in breast carcinomas. BMC Cancer. 2008;8:20. Epub 2008/01/24. doi: 10.1186/1471-2407-8-20. PubMed PMID: 18211679; PubMed Central PMCID: PMCPMC2248196.

44. Urbatzka R, Galante-Oliveira S, Rocha E, Castro LF, Cunha I. Normalization strategies for gene expression studies by real-time PCR in a marine fish species, Scophthalmus maximus. Mar Genomics. 2013;10:17-25. Epub 2013/03/23. doi: 10.1016/j.margen.2013.02.001. PubMed PMID: 23517768.

Figure legend

Fig 1. C_t values of the candidate internal reference genes. Dots represent the mean C_t value; bars represent the mean ± standard deviation. (A) C_t values of each candidate internal reference gene in *NF1*$^{+/-}$ SC, *NF1*$^{-/-}$ pNF and MPNST cell lines (n=14). (B) C_t values of each candidate internal reference gene in SC and pNF cell lines (n = 7). SC, Schwann cell; pNF, plexiform neurofibroma; MPNST, malignant peripheral nerve sheath tumor.

Fig 2. Analysis results of geNorm program. The expression stability of eleven candidate genes in SC + pNF + MPNST subset (A) and SC + pNF subset (C) was calculated and ranked separately. The *x-axis* represents various candidate reference genes, and the *y-axis* represents stability value (*M* value). Lower *M* value suggests higher expression stability. B and D show the optimal number of reference genes in different subsets. The *x-axis* represents the number of genes selected for comprehensive analysis *V* (n/n+1), and the *y-axis* means the pairwise variation value (*V* value). When *V* value is under 0.15, the corresponding combination is esteemed stable and n is the best number of internal reference genes. SC, Schwann cell; pNF, plexiform neurofibroma; MPNST, malignant peripheral nerve sheath tumor.

Fig 3. Analysis results of NormFinder program. The *x-axis* represents various candidate reference genes, and the *y-axis* represents stability value. (A) The stability value of each candidate internal reference gene in SC + pNF + MPNST subset (*n*=14). (B) The stability value of each candidate internal reference gene in SC + pNF subset (*n*=7). SC, Schwann cell; pNF, plexiform neurofibroma; MPNST, malignant peripheral nerve sheath tumor.

Fig 4. Analysis results of BestKeeper program. The *x-axis* represents various candidate reference genes, and the *y-axis* represents stability value. (A) The stability value of each candidate internal reference gene in SC + pNF + MPNST subset (*n*=14). (B) The stability value of each candidate internal reference gene in SC + pNF subset (*n*=7). SC, Schwann cell; pNF, plexiform neurofibroma; MPNST, malignant peripheral nerve sheath tumor.

Fig 5. Analysis results of ΔC_t algorithm. The *x-axis* represents various candidate reference genes, and the *y-axis* represents stability value. (A) The stability value of each candidate internal reference gene in SC + pNF + MPNST subset (*n*=14). (B) The stability value of each candidate internal reference gene in SC + pNF subset (*n*=7). SC, Schwann cell; pNF, plexiform neurofibroma; MPNST, malignant peripheral nerve sheath tumor.

Fig 6. Analysis results of ReFinder program. The *x-axis* represents various candidate reference genes, and the *y-axis* represents stability value. (A) The stability value of each candidate internal reference gene in SC + pNF + MPNST subset (*n*=14). (B) The stability value of each candidate internal reference gene in SC + pNF subset (*n*=7). SC, Schwann cell; pNF, plexiform neurofibroma; MPNST, malignant peripheral nerve sheath tumor.

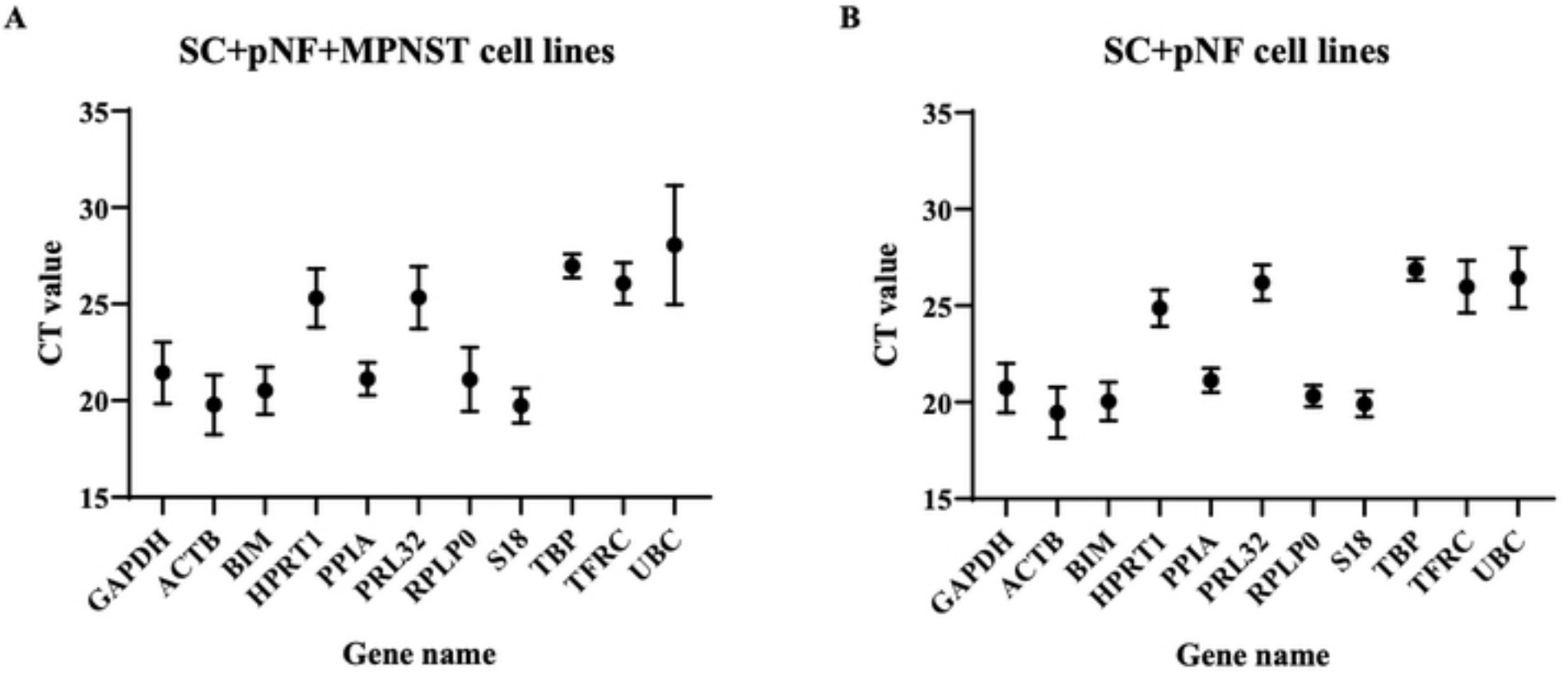
A
SC+pNF+MPNST cell lines
CT value
35
30
25
20
15
GAPDH
ACTB
BIM
HPRT1
PPIA
PRL32
RPLP0
S18
TBP
TFRC
UBC
Gene name
B
SC+pNF cell lines
CT value
35
30
25
20
15
GAPDH
ACTB
BIM
HPRT1
PPIA
PRL32
RPLP0
S18
TBP
TFRC
UBC
Gene name

Fig 1

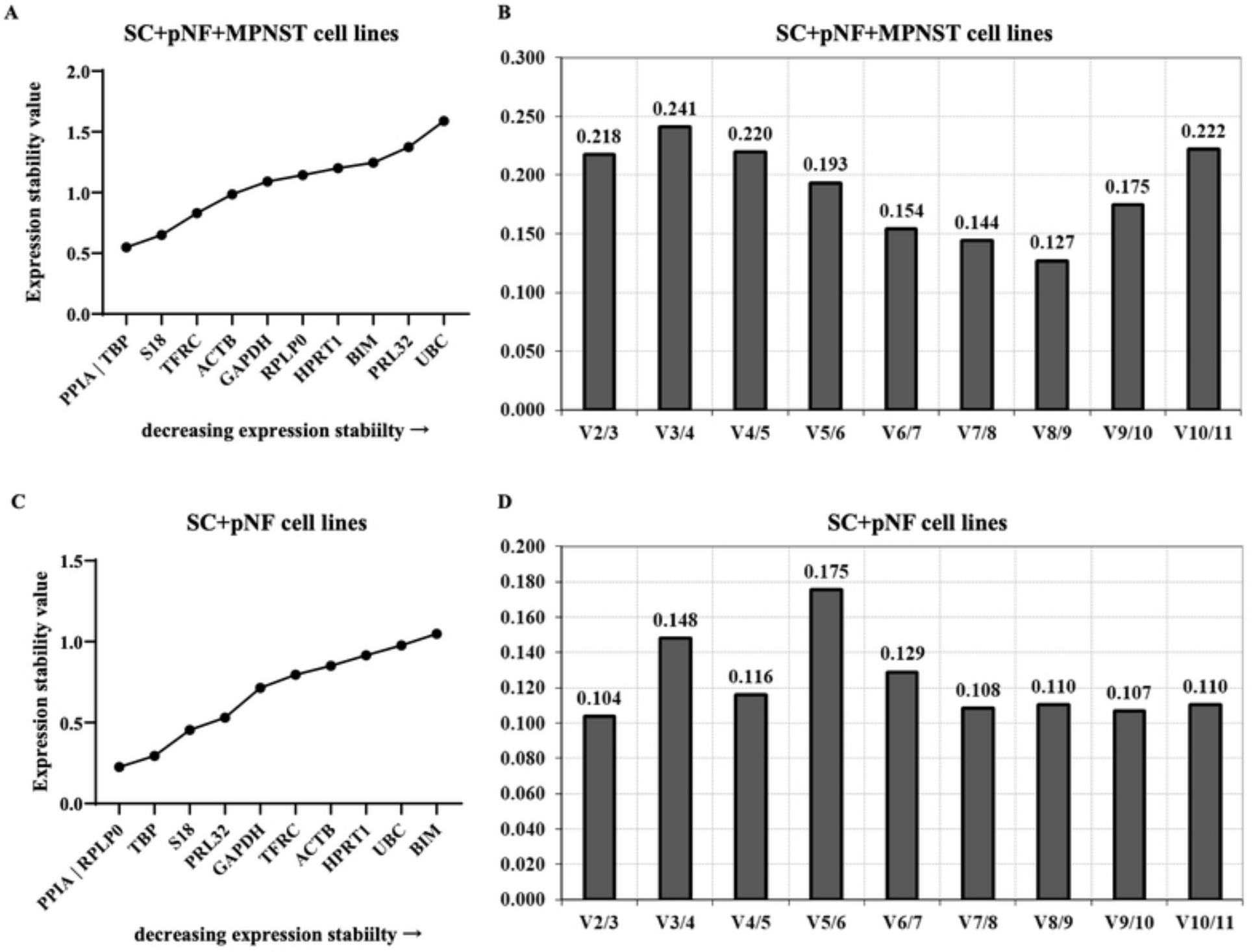
A
SC+pNF+MPNST cell lines
Expression stability value
2.0
1.5
1.0
0.5
0.0
PPIA | TBP
S18
TFRC
ACTB
GAPDH
RPLP0
HPRT1
BIM
PRL32
UBC
decreasing expression stabiilty →
B
SC+pNF+MPNST cell lines
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0.218
0.241
0.220
0.193
0.154
0.144
0.127
0.175
0.222
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
V8/9
V9/10
V10/11
C
SC+pNF cell lines
Expression stability value
1.5
1.0
0.5
0.0
PPIA | RPLP0
TBP
S18
PRL32
GAPDH
TFRC
ACTB
HPRT1
UBC
BIM
decreasing expression stabiilty →
D
SC+pNF cell lines
0.200
0.180
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
0.104
0.148
0.116
0.175
0.129
0.108
0.110
0.107
0.110
V2/3
V3/4
V4/5
V5/6
V6/7
V7/8
V8/9
V9/10
V10/11

Fig 2

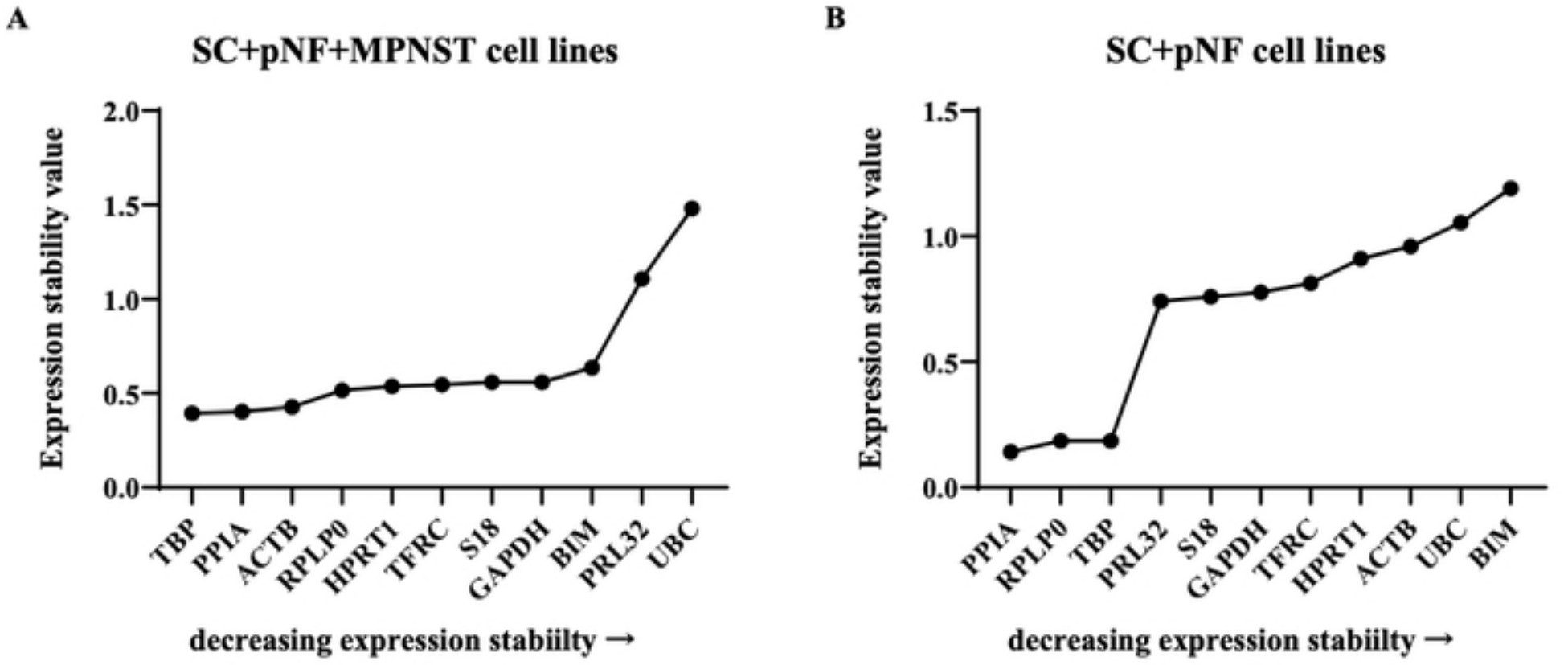
A
SC+pNF+MPNST cell lines
Expression stability value
2.0
1.5
1.0
0.5
0.0
TBP
PPIA
ACTB
RPLP0
HPRT1
TFRC
S18
GAPDH
BIM
PRL32
UBC
decreasing expression stabiilty →
B
SC+pNF cell lines
Expression stability value
1.5
1.0
0.5
0.0
PPIA
RPLP0
TBP
PRL32
S18
GAPDH
TFRC
HPRT1
ACTB
UBC
BIM
decreasing expression stabiilty →

Fig 3

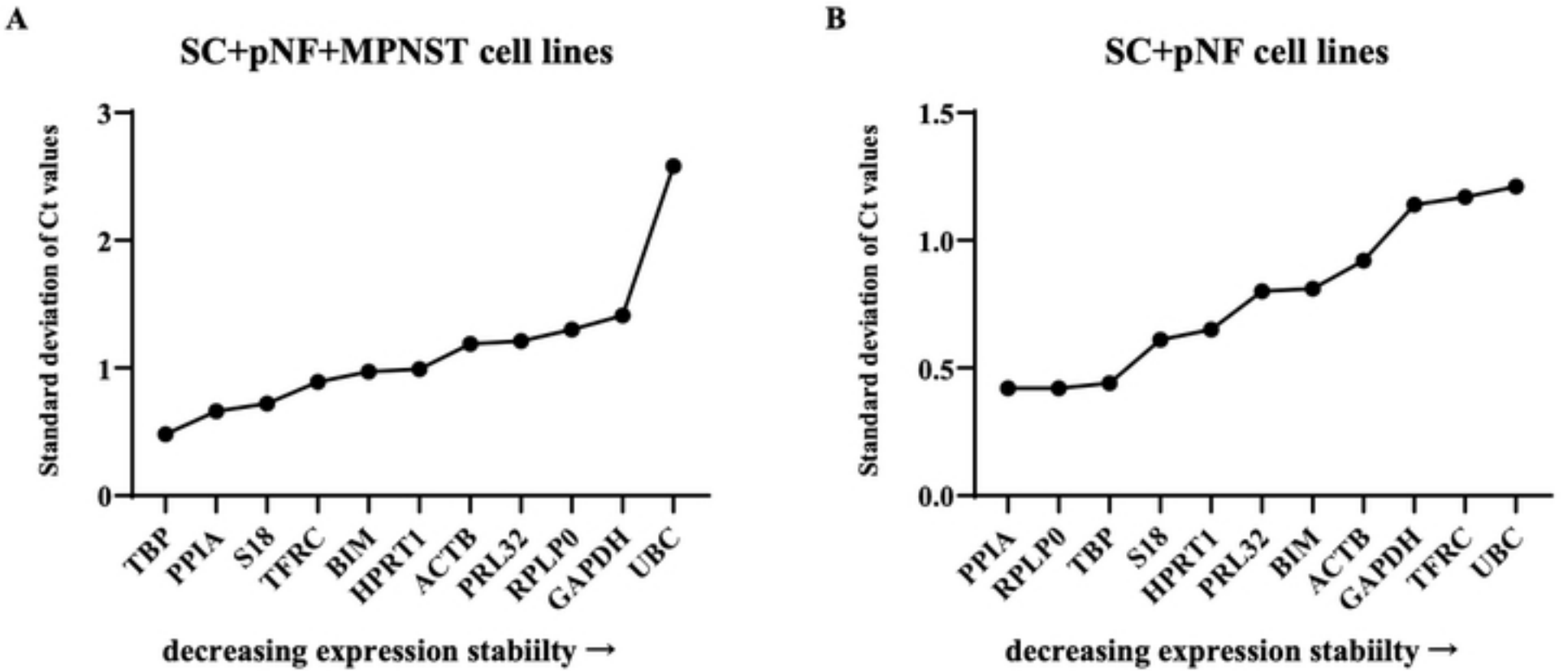
A
SC+pNF+MPNST cell lines
Standard deviation of Ct values
3
2
1
0
TBP
PPIA
S18
TFRC
BIM
HPRT1
ACTB
PRL32
RPLP0
GAPDH
UBC
decreasing expression stabiilty →
B
SC+pNF cell lines
Standard deviation of Ct values
1.5
1.0
0.5
0.0
PPIA
RPLP0
TBP
S18
HPRT1
PRL32
BIM
ACTB
GAPDH
TFRC
UBC
decreasing expression stabiilty →

Fig 4

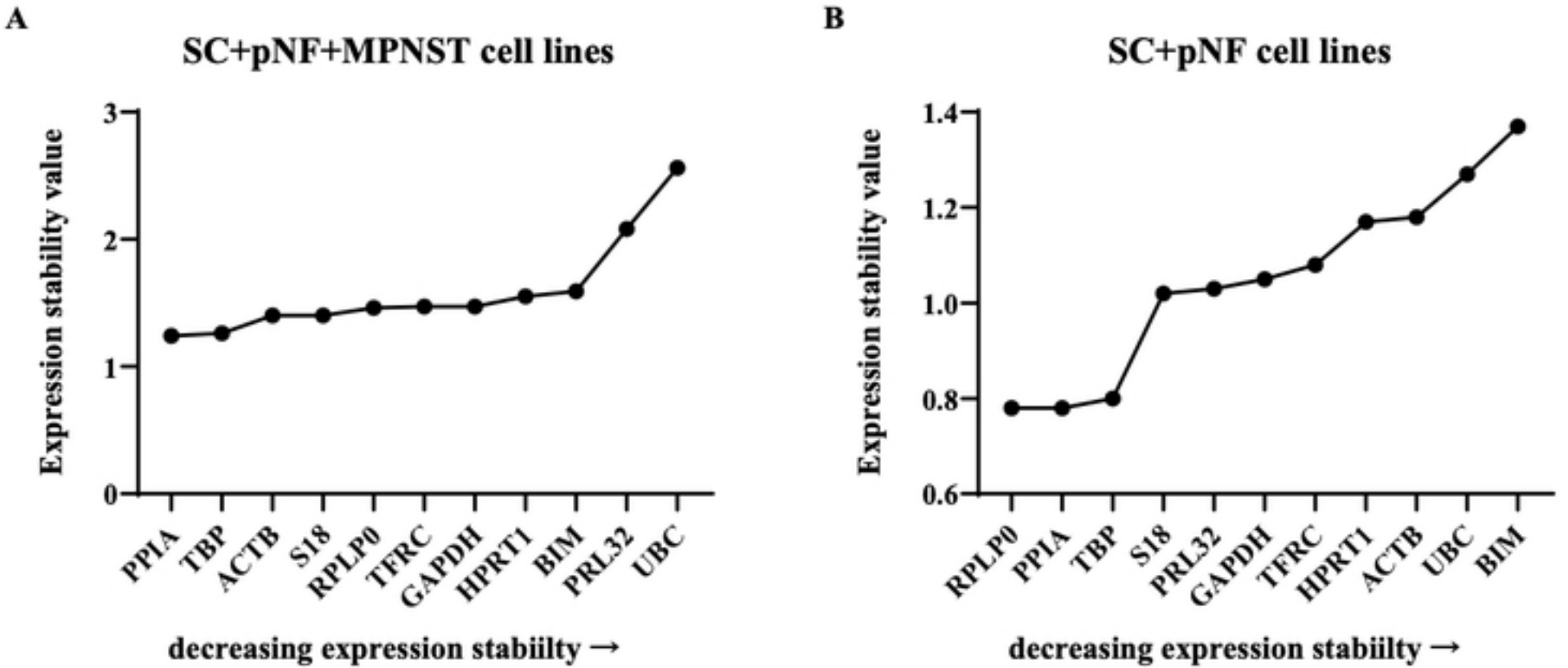
A
SC+pNF+MPNST cell lines
Expression stability value
3
2
1
0
PPIA
TBP
ACTB
S18
RPLP0
TFRC
GAPDH
HPRT1
BIM
PRL32
UBC
decreasing expression stabiilty →
B
SC+pNF cell lines
Expression stability value
1.4
1.2
1.0
0.8
0.6
RPLP0
PPIA
TBP
S18
PRL32
GAPDH
TFRC
HPRT1
ACTB
UBC
BIM
decreasing expression stabiilty →

Fig 5

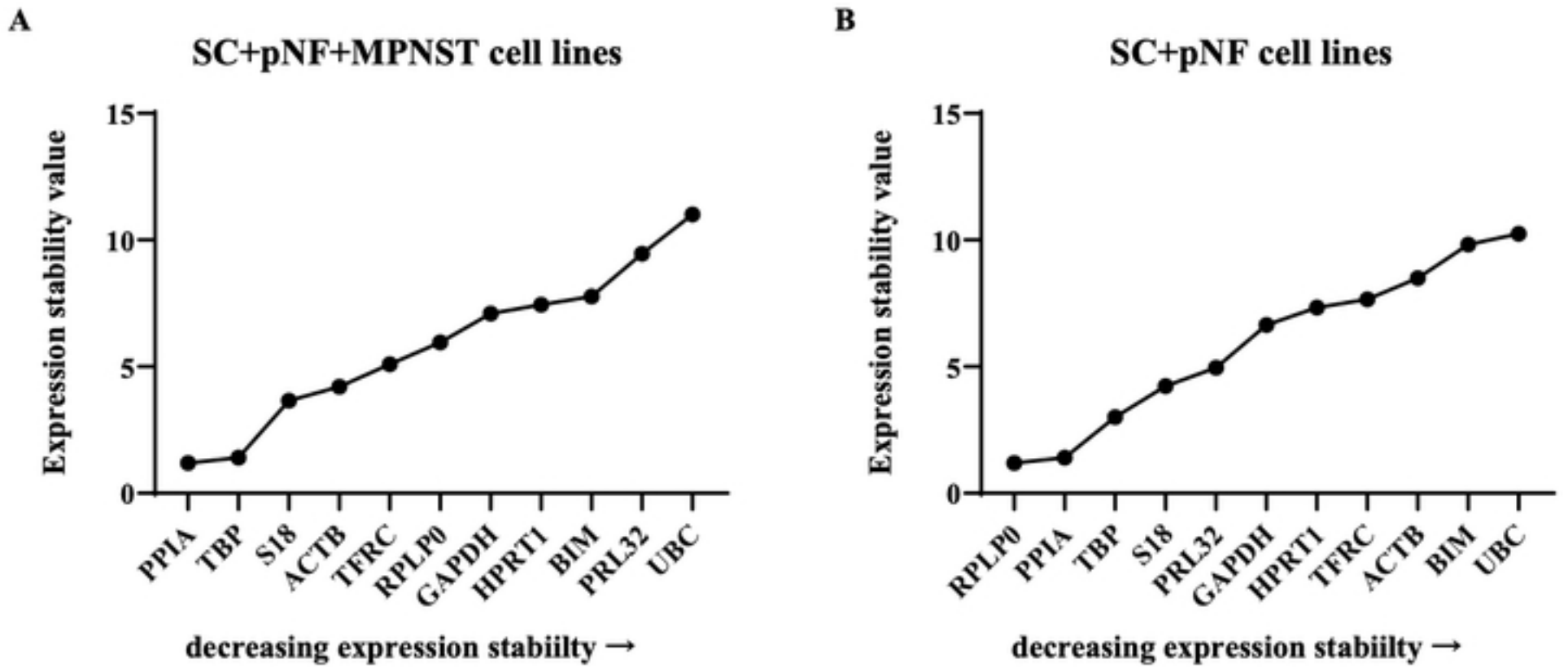
A
SC+pNF+MPNST cell lines
Expression stability value
15
10
5
0
PPIA
TBP
S18
ACTB
TFRC
RPLP0
GAPDH
HPRT1
BIM
PRL32
UBC
decreasing expression stabiilty →
B
SC+pNF cell lines
Expression stability value
15
10
5
0
RPLP0
PPIA
TBP
S18
PRL32
GAPDH
HPRT1
TFRC
ACTB
BIM
UBC
decreasing expression stabiilty →

Fig 6