Systematic identification of human housekeeping genes possibly useful as references in gene expression studies

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Received November 15, 2016; Accepted March 16, 2017

DOI: 10.3892/mmr.2017.6944

Abstract. The ideal reference, or control, gene for the study of gene expression in a given organism should be expressed at a medium-high level for easy detection, should be expressed at a constant/stable level throughout different cell types and within the same cell type undergoing different treatments, and should maintain these features through as many different tissues of the organism. From a biological point of view, these theoretical requirements of an ideal reference gene appear to be best suited to housekeeping (HK) genes. Recent advancements in the quality and completeness of human expression microarray data and in their statistical analysis may provide new clues toward the quantitative standardization of human gene expression studies in biology and medicine, both cross- and within-tissue. The systematic approach used by the present study is based on the Transcriptome Mapper tool and exploits the automated reassignment of probes to corresponding genes, intra- and inter-sample normalization, elaboration and representation of gene expression values in linear form within an indexed and searchable database with a graphical interface recording quantitative levels of expression, expression variability and cross-tissue width of expression for more than 31,000 transcripts. The present study conducted a meta-analysis of a pool of 646 expression profile data sets from 54 different human tissues and identified actin γ 1 as the HK gene that best fits the combination of all the traditional criteria to be used as a reference gene for general use; two ribosomal protein genes, RPS18 and RPS27, and one aquaporin gene, POM121 transmembrane nucleoporin C, were also identified. The present study provided a list of tissue- and organ-specific genes that may be most suited for the following individual tissues/organs: Adipose tissue, bone marrow, brain, heart, kidney, liver, lung, ovary, skeletal muscle and testis; and also provides in these cases a representative, quantitative portrait of the relative, typical gene-expression profile in the form of searchable database tables.

Introduction

The quantitative study of gene expression in terms of the amount of RNA produced by a certain gene in a given biological condition is fundamental to our understanding of gene structure and function. Molecular laboratory techniques used to quantitatively measure RNA expression levels include northern blot analysis, reverse transcription-polymerase chain reaction (RT-PCR), expression microarrays and, recently, RNA sequencing (RNA-Seq). These techniques typically require a form of normalization of the measured RNA expression level of a gene to account for the potentially different RNA input quantities used in the assay. The best way to do this is to relate the transcripts to the number of templates (DNA strands) creating them. Owing to difficulties in obtaining these parameters from the same samples, several methods have been proposed over the years and are commonly used to relate the RNA amount of a given molecular species to one or more reference RNAs that are assumed to be expressed at a constant level in the cell type under consideration (1). More specifically, the ideal reference (or control) gene for the study of gene expression in a given organism should: i) Be expressed at a medium-high level so it can be easily detected; ii) be expressed at a constant/stable level in different cell types and within the same cell type undergoing different treatments; and iii) maintain these features through as many different tissues as possible within the organism (that is, ubiquitously expressed). These features would maximize the usefulness of the genes in the expression studies (2).

From a biological point of view, the theoretical requirements for an ideal reference gene appear to be best suited to the housekeeping (HK) genes, a large class of genes that are constitutively expressed, subjected to low levels of regulation in different conditions and perform biological actions that are fundamental for the basic functions of the cell (1). Their fundamental roles also mean that they tend to be expressed in high levels, confirming their suitability as reference genes.
Since the 1980s, several human genes have been widely used as ‘classic’ reference genes based on their fulfilling of the aforementioned requirements, as assessed typically by northern blot analysis (3), and this set of genes was seamlessly transferred for use in RT-PCR analyses in the 1990s (4). However, in a situation in which there was only preliminary knowledge of the human genome, the choice of these genes could be only anecdotal, among the limited pool of the genes known at the time. Following the widespread use of expression microarray techniques in the early 2000s (5), along with the initial sequencing and characterization of the human genome, it became theoretically possible to study and select HK genes by the systematic analysis of transcriptomes. This possibility was readily exploited in certain initial studies (6,7), which demonstrated that common control genes used in human studies, including the most popular glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin β (ACTB) and β2–microglobulin (B2M) (3,8,9), actually exhibited considerable variability in expression within and across microarray data sets, and in certain cases this was confirmed by quantitative RT-PCR (RT-qPCR) analysis (10). The main conclusion was that the choice of a reference gene should be suited to the specific investigated tissue. In the following years, the problem of selecting a human HK gene by exploiting the availability of transcriptome-scale data was addressed by several studies and remains under debate (11).

The present study considered whether recent advancements in the quality and completeness of human expression microarray data, along with developments in statistical analysis, may be able to provide new clues toward the quantitative standardization of human tissue gene expression studies, cross- and within-tissue. A general framework is presented for choosing reference genes that may be useful in gene expression studies on normal human tissues and organs; the present study also addresses certain previous assumptions and provides an approach that is based on the Transcriptome Mapper (TRAM; http://apollo11.isto.unibo.it/software/TRAM) tool, which can overcome a number of problems associated with cross-platform analysis (such as, probe assignment to locus, intra- and inter-sample normalization and scaled quantile statistics) (12,13). TRAM can integrate data from hundreds or thousands of complete microarray data sets and provide unique combinations of features that are particularly suited to allow the choice of reference genes based on the three properties aforementioned. TRAM calculates a quantitative measurement for a consensus mean-expression value for tens of thousands of human transcripts expressed in a specific tissue or organ, thus allowing for a precise estimation of the intensity of its expression in terms of a percentage of the mean expression value in the pool of analyzed transcriptomes and the choice of a reference gene expressed at medium-high or high level (12,14). In addition, TRAM provides the standard deviation (SD) from the mean (normalized as the percentage of the mean value) for the mean expression value of a given locus, thus allowing for the selection of genes that may have more stable expression values in a variety of different samples and/or experimental platforms that have been investigated for a given tissue/organ. Finally, TRAM is able to integrate data from numerous sources, allowing verification of the consistency of the first two features through a wide range of different tissues within the organism studied (12).

The present study conducted a meta-analysis of 646 data sets that were obtained from different studies associated with 54 different normal human tissues and organs, using various experimental platforms. This meta-analysis produced results for 35,131 individual loci, including known genes and expressed sequence tag (EST) clusters, in the form of a database that may be extensively queried by freely combining a number of criteria, thus identifying the best intersection of moderate-high level of expression, low expression-value variability and expression in a large number of tissues. Results from the present study demonstrated that the human actin γ 1 (ACTG1) gene may potentially be used as a general reference gene for human cross-tissue studies and that specific genes are most suited for individual within-tissue studies. An enrichment analysis in functional classes for the identified HK genes is also presented.

Materials and Methods

Database search. To retrieve data sets that have been derived from normal adult human tissues, a systematic search of the gene expression data repository Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo) (15) was performed for any available sample series associated with pools of normal human tissues/organs, choosing Homo sapiens as the organism. The query used was: ‘Homo sapiens [ORGANISM] AND tissue* [TI] OR organ* [TI]’. These selection criteria led to the generation of a pool of samples that included all of the main human organs and tissues, which served as a reference set already partially used by the authors of the present study (14). The searches were performed up to May 2013, and search results were then filtered using inclusion and exclusion criteria as explained in the Data set selection subsection below.

Data set selection. Inclusion criteria of data sets used in the present analysis were: i) Experiments were carried out on whole organs or tissues; ii) organs/tissues were obtained from individuals exhibiting normal phenotypes; iii) samples used in the studies were obtained from adults; and iv) the availability of the raw or pre-processed data. Exclusion criteria included: i) Studies using exon arrays (which hamper data elaboration by TRAM, owing to an exceedingly high number of data rows) or platforms using probes that are split into several different arrays for each sample, which hampers intra-sample normalization; ii) platforms that examine an atypical number of genes (that is, <5,000 or >60,000); and iii) data that is derived from cell lines, pathological or treated tissue, or children or fetal tissues.

A quantitative transcriptome map was obtained by linearizing values from each data set that were provided as logarithms. If only raw files (such as CEL files) were available in the GEO database, they were converted into pre-processed data using the AltAnalyze version 2.0 program (http://www.altanalyze.org) (16). Tables I and II summarize the data sets used to build the integrated transcriptome map from different human tissues/organs. The mean number of retrieved data sets was 22.2±6.6 for the group of the main tissues/organs (Table I) and 6.5±5.2 for the group of the minor tissues/organs.
was performed. When a very low condition is the mean of all available values for that locus. The quantile. The value for each locus within each biological mean value and then to inter-sample normalization by scaled subject into the TRAM 1.2.1 software. Briefly, gene expression values were assigned to individual loci using UniGene, data were subjected to intra-sample normalization as a percentage of the mean value, followed by ascending order of SD% and then to inter-sample normalization by scaled quantile. The value for each locus within each biological condition is the mean of all available values for that locus. The median value of whole genome gene expression was used to determine the percentiles of expression for each gene. Only mapped genes or EST clusters with an assigned gene name or UniGene code, respectively, that have start and end genomic coordinates, and have a non-empty raw intensity value were selected for the analysis.

Further improvements were made for the present study by adding the total number of biological samples (microarrays) from which each gene expression value is derived, in addition to the number of data points, as certain experimental platforms used to assess expression levels for a sample may contain a variable number of microarray spots, each with a different probe, which generate multiple expression data points for the same gene. These improvements are available (upon request) as a dedicated script and are to be fully integrated into the next version of TRAM (TRAM 1.3), which is due to be released in 2017. Since a different number of microarray spots may be available on a platform for a given gene, these new features offer the possibility to normalize the quantity of information that is available for a gene based on the actual number of distinct biological samples that provide measured expression values for that gene.

To create transcriptome maps, TRAM does not consider probes for which expression values are not available, assuming that an expression level has not been measured. Furthermore, the software gives 95% of the minimum positive value present in a sample to those expression values ≤0 to obtain meaningful numbers when it is required to obtain a ratio between values in pool A and pool B. If it is assumed that, in these cases, the expression level is too low to be detected under the experimental conditions used, then this transformation may be useful to highlight differential gene expression.

HK gene search. The predicted genes that behave as HK genes were determined, as they are mainly involved in fundamental cellular functions and are ubiquitously and constitutively expressed in all tissues (19-21). A search for HK genes in the transcriptome maps was performed using the following parameters: i) An expression value ≥300, which in TRAM is given as a percentage of the mean expression value in a sample in order to select genes that are expressed at least threefold above the mean value and are therefore expressed at an easily appreciable level; ii) a SD ≤30, expressed as a percentage of the mean value to identify genes with a low expression variation among different samples; and iii) a sample number ≥80% of the total number of samples for each analyzed pool to select commonly expressed HK genes (for example, ≥80% indicates 16 out of 20 samples for adipose tissue). When a very low number of suitable HK genes were identified by these criteria, the parameters were relaxed to ≥150 mean expression value, ≤45% SD and ≥65% of samples in the pool with a measured value for the gene (Table I).

To select the HK genes with the best overall features to be proposed as reference genes, the genes identified as fitting the described criteria were first arranged in descending order of expression value, followed by ascending order of SD% and finally by descending order of sample number. An ascending rank number was assigned for each sorting criterion, the mean among these three ranks was calculated and the lowest mean rank was considered to correspond to the gene with the.
Table I. Summary of the samples used in the meta-analysis for 10 specific tissues/organs.

| Sample         | ID   | n     | Data points | Genes | HK | Value | Samples % (n) | SD% |
|----------------|------|-------|-------------|-------|----|-------|---------------|-----|
| Whole pool     | 1-646| 646   | 21,840,330  | 35,131| 27 | ≥150  | ≥65 (420)     | ≤45 |
| Adipose tissue | 1-20 | 20    | 943,480     | 25,722| 363| ≥300  | ≥80 (16)      | ≤30 |
| Bone marrow    | 46-60| 15    | 548,322     | 26,097| 222| ≥300  | ≥80 (12)      | ≤30 |
| Brain          | 61-84| 24    | 507,799     | 21,603| 174| ≥150  | ≥65 (16)      | ≤45 |
| Heart          | 150-166| 17   | 452,683     | 34,486| 56 | ≥150  | ≥65 (11)      | ≤45 |
| Kidney         | 167-183| 17   | 405,285     | 33,816| 65 | ≥150  | ≥65 (11)      | ≤45 |
| Liver          | 184-216| 33   | 971,110     | 35,090| 49 | ≥150  | ≥65 (21)      | ≤45 |
| Lung           | 217-249| 33   | 817,145     | 34,872| 49 | ≥150  | ≥65 (21)      | ≤45 |
| Ovary          | 281-304| 24   | 788,447     | 34,853| 157| ≥150  | ≥65 (16)      | ≤45 |
| Skeletal muscle| 403-418| 16   | 324,382     | 27,160| 77 | ≥300  | ≥80 (13)      | ≤30 |
| Testis         | 495-517| 23   | 745,430     | 34,853| 227| ≥150  | ≥65 (15)      | ≤45 |

4Please refer to the text for a description of the selection criteria used to identify HK genes according to expression values, number of samples and SD. Whole pool contains all sample data sets related to the 54 different human tissues/organs listed in Tables I and II. ID, identifier used in the present study; HK, number of housekeeping genes retrieved; value, expression value; Samples % (n), the percentage of samples in each dataset in which the HK genes fulfilled the selection criteria; n, total sample number; SD%, standard deviation from the mean expression value of a given locus expressed as a percentage.

Discussion

Following the diffusion of expression microarray technology, a number of attempts have been made to use microarray data to perform a systematic analysis of the features of gene expression to identify HK genes that may be best suited as reference genes. Early attempts suffered from the limited number of samples available for analysis in addition to a lack of choice of computational biology techniques to analyze them, in
Table II. Samples used in the meta-analysis for the whole pool in addition to all samples listed in Table I.

| Sample                      | ID     | n  |
|-----------------------------|--------|----|
| Adrenal gland               | 21-30  | 10 |
| Aorta                       | 31-34  | 4  |
| Appendix                    | 35-38  | 4  |
| Bladder                     | 39-41  | 3  |
| Blood                       | 42-45  | 4  |
| Breast                      | 85-89  | 5  |
| Bronchus                    | 90-95  | 6  |
| Ciliary ganglion            | 96-99  | 4  |
| Colon                       | 100-105| 6  |
| Connective                  | 106    | 1  |
| Dental pulp                 | 107-108| 2  |
| Dorsal root ganglia         | 109-130| 22 |
| Esophagus                   | 131-141| 11 |
| Fallopian tube              | 142-148| 7  |
| Gall bladder                | 149    | 1  |
| Lymph node                  | 250-266| 17 |
| Mammary gland               | 267-272| 6  |
| Oral mucosa                 | 273-280| 8  |
| Pancreas                    | 305-315| 11 |
| Parathyroid                 | 316-318| 3  |
| Penis                       | 319-324| 6  |
| Pericardium                 | 325-326| 2  |
| Pharyngeal mucosa           | 327-334| 8  |
| Pituitary gland             | 335-351| 17 |
| Prostate                    | 352-382| 31 |
| Salivary gland              | 383-402| 20 |
| Skin                        | 419-431| 13 |
| Small intestine             | 432-437| 6  |
| Smooth muscle               | 438-441| 4  |
| Spinal cord                 | 442-465| 24 |
| Spleen                      | 466-483| 18 |
| Stomach                     | 484-488| 5  |
| Synovial membrane           | 489-494| 6  |
| Thymus                      | 518-532| 15 |
| Thyroid                     | 533-554| 22 |
| Tongue                      | 555-564| 10 |
| Tonsil                      | 565-578| 14 |
| Trachea                     | 579-592| 14 |
| Trigeminal ganglion         | 593-612| 20 |
| Urethra                     | 613-620| 8  |
| Uterus                      | 621-628| 8  |
| Vagina                      | 629-637| 9  |
| Vena cava                   | 638    | 1  |
| Vulva                       | 639-646| 8  |

ID, identifier used in the present study; n, total sample number.

particular for cross-platform analysis (6). Similar investigations were also conducted using the EST database (23) and, in recent years, RNA-Seq data (24,25). However, the generation of ESTs is subject to certain biases depending on the level of ability for an mRNA to be cloned during the generation of the cDNA EST libraries; therefore, although these data are useful to identify expressed sequences, they are less useful for quantitative analysis. RNA microarrays and RNA-Seq are the two main types of high-throughput technologies used to assess gene expression (26). Although RNA-Seq is considered to be more sensitive and has a broader dynamic range than RNA microarrays (27), in large comparative studies these two methods have produced comparable results in terms of gene expression profiling (27-29). Microarrays remain an accurate tool for measuring the levels of gene expression (29) and continue to provide useful data-mining resources. The approach of the present study takes advantage of the large number of previous transcriptomic studies that were performed with microarray technology and stored in publicly available databases, and also of the results provided in the form of a list of genes and the corresponding expression values.

The diverse origin of the data, in terms of different investigated individuals, different experimenters and different experimental platforms in the field of microarray analysis, provided a richness in the context of an analysis such as in the present study. That is, following data integration, the final results were not affected by systematic biases that may be linked to the particular samples or experimenters/platforms involved in the generation of the data, and they are likely to best represent the actual ‘mean’ status for a gene (12), compared with works based only on the original data obtained through a single platform (6). In addition, the approach of the present study exploited the combination of: i) Automated reassignment of probes to the corresponding genes by the updated UniGene data embedded in TRAM; ii) intra- and inter-sample normalization, including the scaled-quantile method that allows for comparison among platforms with a highly different number of probes; and iii) elaboration and representation of gene expression values in linear form within an indexed, searchable database, with a graphical interface recording quantitative levels of expression (mean expression values), expression variability (SD) and cross-tissue expression of more than 31,000 transcripts. These features represent a clear advancement in comparison with other meta-analyses that were based on published microarray data and were also aimed at identifying human reference genes (30,31), particularly considering that several studies on the subject were conducted in years when there was a reduced availability of samples and/or the experimental platforms were less complete (32).

The meta-analysis in the present study was performed on a pool of 646 data sets from 54 different human whole tissues/organs, and excluded analyses of individual cell types as the whole organ/tissue includes a vast number of cell types in its structure [as discussed by Fagerberg et al (33)], and also due to the requirement of selecting a representative set of samples as a result of the very long elaboration time for each analysis. The ACTG1 gene was identified as the HK gene that best fit the selection criteria for use as a general reference gene in the study of human gene expression. This gene proved to be statistically significantly over-expressed in the transcriptome map, according to the described criteria (34), with the following features: A very high mean expression value (3,453.7, indicating an expression level of ~35-fold
Table III. A list of the eight genes that best fulfill the criteria proposed for use as a reference in gene expression studies across all 646 pool B samples of human tissues and organs that were examined.

| Mean rank | Gene\(^a\) | Chromosome | Value | n   | SD% |
|-----------|------------|------------|-------|-----|-----|
| 2.8       | ACTG1\(^b\) | 17         | 3453.7| 513 | 37.8|
| 5.5       | RPS18      | 6          | 4933.5| 472 | 41.4|
| 5.8       | POM121C    | 7          | 349.7 | 425 | 28.0|
| 6.5       | MRPL18     | 6          | 226.6 | 546 | 39.4|
| 6.5       | TOMM5      | 9          | 273.6 | 426 | 38.2|
| 7.0       | YTHDF1     | 20         | 213.0 | 546 | 39.1|
| 7.3       | TPT1       | 13         | 5941.7| 508 | 43.1|
| 8.0       | RPS27      | 1          | 4355.6| 513 | 44.1|

\(^a\)Please see Table VIII for a complete list of gene definitions. \(^b\)ACTG1 was significantly overexpressed in the transcriptome map (q=0.03; where q is the P-value corrected for false discovery rate). Value, expression value; SD%, standard deviation expressed as percentage of the mean expression value for the locus.

Table IV. A list of the eight genes that best fulfill the selection criteria proposed for a reference in gene expression studies of human adipose tissue and bone marrow.

A, Adipose tissue

| Mean rank | Gene\(^a\) | Chromosome | Value A | Value B | A/B | Sample count A | Sample count B | SD% A | SD% B |
|-----------|------------|------------|---------|---------|-----|----------------|----------------|-------|-------|
| 25.0      | RPL6       | 12         | 2207.0  | 2069.0  | 1.1 | 20             | 592            | 11.6  | 89.5  |
| 27.5      | RPS25      | 11         | 2189.7  | 2113.3  | 1.0 | 20             | 568            | 14.2  | 75.2  |
| 27.5      | SOD1       | 21         | 1273.0  | 1231.8  | 1.0 | 20             | 592            | 12.1  | 67.9  |
| 33.5      | RNASEK     | 17         | 912.6   | 881.1   | 1.0 | 20             | 335            | 11.3  | 41.7  |
| 35.8      | GABARAP    | 17         | 1112.7  | 1185.6  | 0.9 | 20             | 580            | 12.7  | 72.6  |
| 41.3      | ACTG1      | 17         | 3821.3  | 3453.7  | 1.1 | 20             | 513            | 15.2  | 37.8  |
| 43.8      | GABARAPL2  | 16         | 591.6   | 591.9   | 1.0 | 20             | 592            | 12.7  | 69.8  |
| 45.0      | MRFAP1     | 4          | 1583.2  | 1499.0  | 1.1 | 20             | 480            | 17.6  | 64.2  |

B, Bone marrow

| Mean rank | Gene\(^a\) | Chromosome | Value A | Value B | A/B | Sample count A | Sample count B | SD% A | SD% B |
|-----------|------------|------------|---------|---------|-----|----------------|----------------|-------|-------|
| 13.0      | RPL41      | 12         | 5739.4  | 5838.5  | 1.0 | 13             | 568            | 18.5  | 45.1  |
| 17.8      | RPLP0      | 12         | 3659.6  | 3341.5  | 1.1 | 13             | 508            | 16.5  | 44.5  |
| 17.8      | RPS27      | 1          | 4494.8  | 4355.6  | 1.0 | 13             | 513            | 19.5  | 44.1  |
| 23.3      | TUBAI1B    | 12         | 2893.1  | 2478.6  | 1.2 | 13             | 508            | 16.4  | 81.1  |
| 24.3      | RPSA       | 3          | 2016.1  | 2036.8  | 1.0 | 13             | 575            | 20.6  | 80.1  |
| 25.5      | SLC25A3    | 12         | 918.5   | 956.7   | 1.0 | 13             | 592            | 17.6  | 73.5  |
| 26.5      | ACTG1      | 17         | 3728.3  | 3453.7  | 1.1 | 13             | 513            | 20.3  | 37.8  |
| 30.0      | EEF1G      | 11         | 2713.6  | 2686.9  | 1.0 | 13             | 587            | 22.6  | 48.3  |

\(^a\)Please see Table VIII for a complete list of gene definitions. Value A, mean expression values of the gene in the pool A, including the sample related to the specific tissue; value B, mean expression values of the gene in the whole pool B consisting of all the 646 samples; A/B, ratio between values A and B; SD%, standard deviation expressed as percentage of the mean expression value (A or B) for the locus.

In comparison with the mean expression value of all the genes in each sample, set as equal to 100) and an SD% of 37.8%; the number of samples in which a measure for this gene was available was 513 out of 646 (79.4%). Notably, this well-characterized gene, whose coding sequence appears to be completely characterized (35), encodes for a cytoplasmic form of actin that is known to be ubiquitously expressed in human cells, but is different from the actin β (ACTB) that is routinely used as a reference gene. According to the data of the present study, the commonly used reference genes ACTB and GAPDH had excellent features in terms of high expression value and diffuse expression in human cells. However,
they have an SD% almost double that of ACTG1. Owing to the high similarity between ACTB and ACTG1 (91% identity with no gaps between their coding sequences, as determined by standard BLASTN analysis; data not shown), probes and primers need to be accurately selected to specifically identify the desired form of RNA.

Among the HK genes identified to be best suited as general reference genes for human studies, two ribosomal protein genes, RPS18 and RPS27, and one aquaporin gene, POM121 transmembrane nucleoporin C (POM121C), were identified. ACTG1 and RPS27 were also included in the top 20 HK human genes across 42 human tissues in a previous study based on a single platform (36), however, the data sets from the present study were not included in the present meta-analysis since it was not possible to derive the expression values as linear numbers for each microarray channel from the deposited data. Notably, the eight genes listed in Table III were also classified at the transcript and protein level as ‘expressed in all tissues’ in the Human Protein Atlas (33), further supporting the results of the present study regarding them as the most generally suitable reference genes.

In accordance with the relevance of the ACTG1 gene in cross-tissue analysis, this gene is also present in the greatest number of lists (n=5) of the 10 tissue-specific genes best fulfilling criteria to be used as reference genes (Tables IV-VII). Ribosomal proteins were another notable example of known classes of general HK genes that are well represented in several human tissues.

### Table V. A list of the eight genes that best fulfill the selection criteria proposed for a reference in gene expression studies of human brain, heart and skeletal muscle.

#### A, Brain

| Mean rank | Gene<sup>a</sup> | Chromosome | Value A | Value B | A/B | Sample count A | Sample count B | SD% A | SD% B |
|-----------|----------------|------------|---------|---------|-----|----------------|----------------|-------|-------|
| 9.3       | NDUFB4         | 3          | 590.9   | 545.7   | 1.1 | 20             | 551            | 20.6  | 110.0 |
| 9.8       | NDUFB1         | 14         | 546.7   | 586.0   | 0.9 | 22             | 592            | 22.7  | 59.9  |
| 17.5      | GSTO1          | 10         | 345.9   | 370.6   | 0.9 | 22             | 592            | 22.0  | 75.5  |
| 28.0      | AM22           | 17         | 299.5   | 276.1   | 1.1 | 20             | 568            | 29.3  | 99.5  |
| 28.8      | POLR21         | 19         | 302.8   | 254.7   | 1.2 | 22             | 568            | 23.0  | 79.2  |
| 29.5      | NDUFA3         | 19         | 365.0   | 280.9   | 1.3 | 20             | 551            | 19.7  | 59.0  |
| 30.0      | RRAGA          | 9          | 463.0   | 350.9   | 1.3 | 22             | 592            | 26.5  | 45.1  |
| 30.5      | RPL41          | 12         | 4,995.8 | 5,838.5 | 0.9 | 16             | 568            | 17.3  | 45.1  |
| 8.3       | PRDX1          | 1          | 921.2   | 1,096.5 | 0.8 | 16             | 592            | 18.5  | 71.7  |
| 10.8      | RPL8           | 8          | 2,065.6 | 2,069.0 | 1.0 | 16             | 513            | 25.2  | 44.1  |
| 11.3      | C14orf166      | 14         | 576.0   | 505.7   | 1.1 | 16             | 546            | 19.1  | 57.0  |
| 11.8      | JTB            | 1          | 714.0   | 606.9   | 1.2 | 16             | 592            | 20.6  | 61.6  |
| 11.8      | RPS29          | 14         | 2,751.4 | 2,551.2 | 1.1 | 16             | 592            | 25.4  | 57.9  |
| 13.0      | SNRPD2         | 19         | 516.4   | 524.7   | 1.0 | 16             | 592            | 21.9  | 57.5  |
| 14.5      | NOP10          | 15         | 497.5   | 426.5   | 1.2 | 16             | 546            | 19.2  | 48.0  |

<sup>a</sup>Please see Table VIII for a complete list of gene definitions. Value A, mean expression values of the gene in the pool A, including the sample related to the specific tissue; value B, mean expression values of the gene in the whole pool B consisting of all the 646 samples; A/B, ratio between these value A and value B; SD%, standard deviation expressed as percentage of the mean expression value (A or B) for the locus.

### B, Heart

| Mean rank | Gene<sup>a</sup> | Chromosome | Value A | Value B | A/B | Sample count A | Sample count B | SD% A | SD% B |
|-----------|----------------|------------|---------|---------|-----|----------------|----------------|-------|-------|
| 10.5      | MIF            | 22         | 896.7   | 845.6   | 1.1 | 11             | 527            | 42.1  | 66.5  |
| 12.5      | ECHS1          | 10         | 567.8   | 571.0   | 1.0 | 15             | 592            | 43.2  | 100.3 |
| 13.0      | FAM96A         | 15         | 338.6   | 289.7   | 1.2 | 11             | 480            | 35.0  | 63.8  |
| 13.5      | NOP10          | 15         | 474.8   | 428.5   | 1.1 | 13             | 546            | 41.4  | 48.0  |
| 13.5      | TBCB           | 19         | 319.8   | 290.6   | 1.1 | 15             | 592            | 38.8  | 67.9  |
| 14.8      | RRAGA          | 9          | 292.4   | 350.9   | 0.8 | 15             | 592            | 37.0  | 45.1  |
| 15.0      | IFI27          | 14         | 510.4   | 441.9   | 1.2 | 15             | 592            | 42.2  | 103.1 |
| 15.5      | MB             | 22         | 6,845.8 | 745.2   | 9.2 | 15             | 592            | 30.6  | 260.2 |

### C, Skeletal muscle

| Mean rank | Gene<sup>a</sup> | Chromosome | Value A | Value B | A/B | Sample count A | Sample count B | SD% A | SD% B |
|-----------|----------------|------------|---------|---------|-----|----------------|----------------|-------|-------|
| 4.3       | RPL41          | 12         | 4,995.8 | 5,838.5 | 0.9 | 16             | 568            | 17.3  | 45.1  |
| 8.3       | PRDX1          | 1          | 921.2   | 1,096.5 | 0.8 | 16             | 592            | 18.5  | 71.7  |
| 10.8      | RPL8           | 8          | 2,065.6 | 2,069.0 | 1.0 | 16             | 513            | 25.2  | 44.1  |
| 11.3      | C14orf166      | 14         | 576.0   | 505.7   | 1.1 | 16             | 546            | 19.1  | 57.0  |
| 11.8      | JTB            | 1          | 714.0   | 606.9   | 1.2 | 16             | 592            | 20.6  | 61.6  |
| 11.8      | RPS29          | 14         | 2,751.4 | 2,551.2 | 1.1 | 16             | 592            | 25.4  | 57.9  |
| 13.0      | SNRPD2         | 19         | 516.4   | 524.7   | 1.0 | 16             | 592            | 21.9  | 57.5  |
| 14.5      | NOP10          | 15         | 497.5   | 426.5   | 1.2 | 16             | 546            | 19.2  | 48.0  |
expected prevalence among the identified loci of genes encoding for basic cell structure (such as genes encoding for cytoskeletal components) and function (such as genes encoding for transcription and translation, reduction-oxidation metabolism and signaling proteins), it is worth noting that specific members of the same gene family involved in these processes may be identified in one particular tissue/organ and not in the others. The vast majority of genes that may be more suitable as reference genes for individual tissues (Tables IV-VII) are still typical HK genes, with the clear exception of the tissue-specific myoglobin gene in the heart (Table V).

The approach used in the present study allows for the systematic search for ideal reference genes and, at the same time, made available a ‘consensus’ reference gene-expression profile for 10 human tissues/organs. From this particular point of view, the presented results are less systematic than other previous attempts conducted in the case of the brain (34) and heart (14).

Only the samples belonging to experiments in which a series of normal human tissues were analyzed have been included here, without searching for any single samples recorded for a given tissue in any type of available experiment (for example, comparisons between normal and pathological samples). However, the present data have been obtained through an improvement of the search algorithm for HK genes and may still offer interesting hints to their biological specificity in the transcriptome of these tissues. The same approach may be applied to data sets deriving from cell lines or pathological samples.

Table VI. A list of the eight genes that best fulfill the selection criteria proposed for a reference in gene expression studies of human kidney, liver and lung.

A, Kidney

| Mean rank | Gene | Chromosome | Value A | Value B | A/B | Sample count A | Sample count B | SD% A | SD% B |
|-----------|------|------------|---------|---------|-----|----------------|----------------|-------|-------|
| 6.5       | PGAM1| 10         | 688.8   | 741.8   | 0.9 | 14             | 556            | 29.6  | 64.0  |
| 7.5       | NOP10| 15         | 413.9   | 428.5   | 1.0 | 12             | 546            | 31.9  | 48.0  |
| 8.0       | FIS1 | 7          | 340.7   | 329.0   | 1.0 | 12             | 546            | 29.6  | 52.9  |
| 9.0       | GPX1 | 3          | 459.9   | 496.7   | 0.9 | 15             | 592            | 34.2  | 66.9  |
| 10.3      | GANAB| 11         | 266.7   | 260.0   | 1.0 | 15             | 587            | 35.1  | 49.5  |
| 11.0      | NDUFB11| X         | 343.8   | 294.9   | 1.2 | 12             | 551            | 25.0  | 67.7  |
| 12.8      | HEBP1| 12         | 288.6   | 249.2   | 1.2 | 12             | 551            | 27.7  | 53.9  |
| 13.0      | HDGF | 1          | 444.3   | 432.2   | 1.0 | 14             | 568            | 40.5  | 58.4  |

B, Liver

| Mean rank | Gene | Chromosome | Value A | Value B | A/B | Sample count A | Sample count B | SD% A | SD% B |
|-----------|------|------------|---------|---------|-----|----------------|----------------|-------|-------|
| 8.5       | HEBP2| 6          | 335.1   | 362.5   | 0.9 | 31             | 592            | 35.8  | 79.2  |
| 11.8      | NDUFS3| 11         | 289.3   | 325.3   | 0.9 | 31             | 592            | 36.1  | 60.8  |
| 12.3      | POLR2H| 3          | 162.0   | 160.1   | 1.0 | 33             | 641            | 32.4  | 45.2  |
| 12.5      | MRPS24| 7          | 438.5   | 445.0   | 1.0 | 23             | 426            | 42.0  | 57.9  |
| 13.3      | FAM96B| 16         | 287.1   | 325.2   | 0.9 | 29             | 546            | 37.0  | 47.9  |
| 13.3      | GTF3A | 13         | 349.5   | 310.3   | 1.1 | 22             | 501            | 38.6  | 62.0  |
| 13.3      | HIST1H2BK| 6       | 216.3   | 186.6   | 1.2 | 28             | 568            | 30.3  | 72.7  |
| 13.8      | CRELD2| 22         | 297.3   | 279.9   | 1.1 | 24             | 467            | 39.5  | 52.7  |

C, Lung

| Mean rank | Gene | Chromosome | Value A | Value B | A/B | Sample count A | Sample count B | SD% A | SD% B |
|-----------|------|------------|---------|---------|-----|----------------|----------------|-------|-------|
| 7.3       | RBX1 | 22         | 283.7   | 315.4   | 0.9 | 29             | 551            | 36.6  | 57.8  |
| 7.5       | RRAGA| 9          | 289.7   | 350.9   | 0.8 | 31             | 592            | 34.9  | 45.1  |
| 11.5      | LAMTOR5| 1         | 253.1   | 344.6   | 0.7 | 33             | 646            | 34.1  | 55.7  |
| 11.8      | CNIH1| 14         | 207.4   | 250.4   | 0.8 | 31             | 592            | 32.3  | 63.4  |
| 12.0      | EPCAM| 2          | 252.8   | 256.7   | 1.0 | 31             | 592            | 39.0  | 218.7 |
| 12.8      | EIF4A3| 17         | 271.2   | 340.0   | 0.8 | 25             | 563            | 37.8  | 47.1  |
| 13.5      | ACTG1| 17         | 3,067.3 | 3,453.7 | 0.9 | 27             | 513            | 43.9  | 37.8  |
| 14.0      | FAM96B| 16        | 277.0   | 325.2   | 0.9 | 24             | 546            | 39.2  | 47.9  |

aPlease see Table VIII for a complete list of gene definitions. Value A, mean expression values of the gene in the pool A, including the sample related to the specific tissue; value B, mean expression values of the gene in the whole pool B consisting of all the 646 samples; A/B, ratio between these value A and value B; SD%, standard deviation expressed as percentage of the mean expression value (A or B) for the locus.
Table VII. A list of the eight genes that best fulfill the selection criteria proposed for a reference in gene expression studies of human ovary and testis.

A, Ovary

| Mean rank | Gene 4 | Chromosome | Value A | Value B | A/B | Sample count A | Sample count B | SD% A | SD% B |
|-----------|--------|------------|---------|---------|-----|----------------|----------------|-------|-------|
| 10.0      | ACTG1  | 17         | 3,547.3 | 3,453.7 | 1.0 | 17             | 513            | 30.8  | 37.8  |
| 20.0      | AP2M1  | 3          | 387.9   | 375.5   | 1.0 | 17             | 513            | 32.0  | 51.8  |
| 22.5      | MIF    | 22         | 885.0   | 845.6   | 1.0 | 17             | 527            | 36.5  | 66.5  |
| 24.3      | NELFCD | 20         | 240.2   | 239.5   | 1.0 | 17             | 522            | 31.3  | 118.6 |
| 24.5      | RNF181I| 2          | 331.3   | 356.5   | 0.9 | 16             | 426            | 24.8  | 52.5  |
| 28.0      | PSMC1  | 14         | 367.9   | 421.4   | 0.9 | 17             | 513            | 29.3  | 48.8  |
| 30.0      | TMEM147 | 19        | 295.9   | 282.8   | 1.0 | 22             | 592            | 35.3  | 47.2  |
| 30.3      | TERF21P| 16         | 323.5   | 344.6   | 0.9 | 22             | 592            | 34.7  | 69.9  |

B, Testis

| Mean rank | Gene 4  | Chromosome | Value A | Value B | A/B | Sample count A | Sample count B | SD% A | SD% B |
|-----------|---------|------------|---------|---------|-----|----------------|----------------|-------|-------|
| 18.5      | TUBA1B  | 12         | 2,774.3 | 2,478.6 | 1.1 | 18             | 508            | 30.9  | 81.1  |
| 21.0      | FAM96B  | 16         | 339.6   | 325.2   | 1.0 | 19             | 546            | 28.0  | 47.9  |
| 22.8      | RPL8    | 8          | 1,637.9 | 2,069.0 | 0.8 | 18             | 513            | 27.9  | 44.1  |
| 23.0      | RPS18   | 6          | 4,434.5 | 4,933.5 | 0.9 | 16             | 472            | 34.9  | 41.4  |
| 24.5      | ACTG1   | 17         | 4,320.0 | 3,453.7 | 1.3 | 18             | 513            | 27.7  | 37.8  |
| 29.3      | RPS27   | 1          | 5,444.6 | 4,355.6 | 1.3 | 18             | 513            | 32.5  | 44.1  |
| 31.5      | TBCB    | 19         | 343.1   | 290.6   | 1.2 | 21             | 592            | 29.1  | 67.9  |
| 32.3      | RPL41   | 12         | 4,676.1 | 5,838.5 | 0.8 | 20             | 568            | 36.1  | 45.1  |

*Please see Table VIII for a complete list of gene definitions. Value A, mean expression values of the gene in the pool A, including the sample related to the specific tissue; value B, mean expression values of the gene in the whole pool B consisting of all the 646 samples; A/B, ratio between these value A and value B; SD%, standard deviation expressed as percentage of the mean expression value (A or B) for the locus.*

Systematic analysis aimed to evaluate if the genes identified as possible reference genes (Tables IV-VII) were significantly enriched in a particular class of genes confirmed to be involved in the most basic biological processes; in particular, in the metabolism of the informational macromolecules (nucleic acids and proteins). This therefore justifies their tendency to constitutive, stable and almost universal expression, which was also observed in a previous analysis that ranked genes by combining the average expression level and its SD in a single score (37). The biological peculiarity of HK genes was also highlighted by a significant difference in complexity between HK and tissue-specific gene promoters, as revealed by DNA entropy analysis (38).

While the present study was in progress, an article on the topic was published that suggested that a ‘universal’ human HK gene does not exist and provided a list of suitable reference genes for individual tissues/organs (11). However, the method employed by that study was different from the approach of the present study in a number of relevant aspects. In particular, the results of the previous study were originally obtained by combining the lists of genes retrieved from studies performed using heterogeneous techniques (such as microarray, EST or RNA-Seq analysis), were analyzed using the logic of classification, previous judgments concerning the suitability of certain genes as HK/reference genes were accepted and then these lists were combined. This approach of combining the lists of results was also used by Chang et al (39) and, following ranking, by Shaw et al (40). By contrast, the present study re-elaborated and normalized original raw data, and generated a fully quantitative analysis of human gene expression, which may explain certain differences in the results obtained by the algorithm used in the current study. Conversely, certain shared general conclusions were highlighted, including the necessity to calibrate the search criteria for HK genes according to cell/tissue type; however, the general analysis of the present study can still identify certain general-purpose genes with acceptable criteria that may be proposed as reference genes. Several previous studies have demonstrated that the results provided by the TRAM tool were highly reliable, having been confirmed by RT-qPCR experiments for several diverse human tissues, demonstrating a correlation coefficient (r) between TRAM and RT-qPCR data of r=0.98 for brain (34), r=0.99 for hippocampus (41) and r=0.98 for heart (14). However, it is commonly accepted that additional experimental studies may be required to verify that the identified candidate reference gene is suitable for the actual biological condition investigated (42). Additional studies are in progress to verify if the HK profile identified in normal tissues may be applicable to aneuploid cells, in particular for systematic analysis of trisomy 21 cells (43), in light of the fact that all of the best reference genes identified by that study are not
Table VIII. Gene symbols, corresponding descriptions and the number of recurrences (n) in the Tables for the genes listed in Tables III-VII.

| Gene symbol | n | Gene description |
|-------------|---|------------------|
| ACTG1       | 5 | Actin $\gamma$ 1 |
| AMZ2        | 1 | Archaelysin family metallopeptidase 2 |
| AP2M1       | 1 | Adaptor-related protein complex 2, $\mu$1 subunit |
| C14orf166   | 1 | Chromosome 14 open reading frame 166 |
| CNHI1       | 1 | Cornichon family AMPA receptor auxiliary protein 1 |
| CRELD2      | 1 | Cysteine rich with EGF like domains 2 |
| ECHS1       | 1 | Enoyl-CoA hydratase, short chain 1 |
| EEF1G       | 1 | Eukaryotic translation elongation factor 1$\gamma$ |
| EIF4A3      | 1 | Eukaryotic translation initiation factor 4A3 |
| EPCAM       | 1 | Epithelial cell adhesion molecule |
| FAM96A      | 1 | Family with sequence similarity 96 member A |
| FAM96B      | 3 | Family with sequence similarity 96 member B |
| FIS1        | 1 | Fission, mitochondrial |
| GABARAP     | 1 | GABA type A receptor-associated protein |
| GABARAPL2   | 1 | GABA type A receptor associated protein like 2 |
| GANAB       | 1 | Glucosidase II $\alpha$ subunit |
| GPX1        | 1 | Glutathione peroxidase 1 |
| GSTO1       | 1 | Glutathione S-transferase 01 |
| GTF3A       | 1 | General transcription factor IIIA |
| HDGF        | 1 | Heparin binding growth factor |
| HEBP1       | 1 | Heme binding protein 1 |
| HEBP2       | 1 | Heme binding protein 2 |
| HIST1H2BK   | 1 | Histone cluster 1 H2B family member k |
| IFI27       | 1 | Interferon $\alpha$-inducible protein 27 |
| JTB         | 1 | Jumping translocation breakpoint |
| LAMTOR5     | 1 | Late endosomal/lysosomal adaptor, MAPK and MTOR activator 5 |
| MB          | 1 | Myoglobin |
| MIF         | 2 | Macrophage migration inhibitory factor (glycosylation-inhibiting factor) |
| MRFAP1      | 1 | Morf4 family associated protein 1 |
| MRPL18      | 1 | Mitochondrial ribosomal protein L18 |
| MRPS24      | 1 | Mitochondrial ribosomal protein S24 |
| NDUFA3      | 1 | NADH:ubiquinone oxidoreductase subunit A3 |
| NDUFB1      | 1 | NADH:ubiquinone oxidoreductase subunit B1 |
| NDUFB4      | 1 | NADH:ubiquinone oxidoreductase subunit B4 |
| NDUFB11     | 1 | NADH:ubiquinone oxidoreductase subunit B11 |
| NDUFS3      | 1 | NADH:ubiquinone oxidoreductase core subunit S3 |
| NELFCD      | 1 | Negative elongation factor complex member C/D |
| NOP10       | 3 | NOP10 ribonucleoprotein |
| PGAM1       | 1 | Phosphoglycerate mutase 1 |
| POLR2H      | 1 | RNA polymerase II subunit H |
| POLR2I      | 1 | RNA polymerase II subunit I |
| POM121C     | 1 | POM121 transmembrane nucleoporin C |
| POMP        | 1 | Proteasome maturation protein |
| PRDX1       | 1 | Peroxiredoxin 1 |
| PSMC1       | 1 | Proteasome 26S subunit, ATPase 1 |
| RBX1        | 1 | Ring-box 1 |
| RNASEK      | 1 | Ribonuclease K |
| RNF18I      | 1 | Ring finger protein 181 |
| RPL6        | 1 | Ribosomal protein L6 |
| RPL8        | 2 | Ribosomal protein L8 |
located on chromosome 21 and that a very small portion of this chromosome appears to be associated with the basic features of Down syndrome (DS) (44). In this regard, consulting the published differential transcriptome map comparing acute megakaryoblastic leukemia (AMKL) cells from children with DS (DS AMKL) and euploid megakaryocyte cells (euploid MK) (45) reveals promising values for \( \text{ACTG1} \) (DS AMKL/euploid MK gene expression ratio=1.08) and \( \text{POM121C} \) (DS AMKL/euploid MK gene expression ratio=1.01); the ratios range between 0.47 and 3.55 (45) for the other genes listed in Table III.

A previous study using RNA-Seq data to identify reference genes across multiple human tissues focused mainly on low SD and so proposed a list of 11 genes with exceptionally low variability (1). The corresponding values have been checked by TRAM analysis in the present study, which confirmed their expression across multiple tissues, with a generally low SD (although not exceptional in the data of the current study). However, these identified genes had low expression values, all in the range of 150-400 in terms of a percentage of the mean value. This was recognized by Kwon et al (46), who selected low variability as the leading parameter, as have other studies, whereas the approach of the present study was aimed at finding genes with the best combination of criteria, considering that a high expression value may be advantageous in practice for the usability of a reference gene. Finally, it should be noted that other studies have frequently used very different and original approaches to the problem, using computational classifiers (47), the controlled vocabulary of Medical Subject Headings (48) and Gene Ontology classifications (29).

It may finally be noted that several of the available analysis tools are aimed at determining the best reference genes for normalization of gene expression data, and are largely based on the evaluation of the minimal variation of the expression level of a given gene among different conditions. BestKeeper (49) and GeNorm (50) are commonly used for screening the reference genes to perform an accurate normalization of RT-qPCR data, whereas NormFinder (51) may be used to evaluate reference genes for normalization of RT-qPCR and microarray experiments. Regarding NormFinder, a direct comparison with the tool employed in the present study is not possible, since NormFinder requires the same number of measured values for each gene and the TRAM algorithm does not have this limitation. In addition, NormFinder evaluation is based only on the analysis of variation among different samples, whereas the TRAM approach integrates this parameter with the level of expression (at least medium-high) and with the highest possible number of samples in which each gene is measured; all are essential parameters by which to search for a suitable reference gene, thus allowing the identification of genes with the overall best fit to the three criteria. Finally, although NormFinder has the ability to identify the ideal combination of biologically independent genes for each tissue, this analysis requires the creation of a matrix with two groups of data deriving from two different conditions, which in the case of TRAM can be either one of the ten tissues analyzed or the whole pool. In the whole pool sample, the difference in the number of measures for each gene increases, so the exclusion of a large number of measures to create a matrix of data does not make the

| Gene symbol | n  | Gene description                                      |
|-------------|----|-------------------------------------------------------|
| **RPL41**   | 3  | Ribosomal protein L41                                  |
| **RPLP0**   | 1  | Ribosomal protein lateral stalk subunit P0             |
| **RPS18**   | 2  | Ribosomal protein S18                                  |
| **RPS25**   | 1  | Ribosomal protein S25                                  |
| **RPS27**   | 3  | Ribosomal protein S27                                  |
| **RPS29**   | 1  | Ribosomal protein S29                                  |
| **RPSA**    | 1  | Ribosomal protein SA                                   |
| **RRAGA**   | 3  | Ras related GTP binding A                              |
| **SLC25A3** | 1  | Solute carrier family 25 member 3                      |
| **SNRPD2**  | 1  | Small nuclear ribonucleoprotein D2 polypeptide         |
| **SOD1**    | 1  | Superoxide dismutase 1                                 |
| **TBCB**    | 2  | Tubulin folding cofactor B                              |
| **TERF2ZIP**| 1  | TERF2 interacting protein                               |
| **TMEM147** | 1  | Transmembrane protein 147                              |
| **TOMM5**   | 1  | Translocase of outer mitochondrial membrane 5           |
| **TPT1**    | 1  | Tumor protein, translationally-controlled 1             |
| **TUBA1B**  | 2  | Tubulin α1b                                            |
| **YTHDF1**  | 1  | YTH N6-methyladenosine RNA binding protein 1            |

The genes with a number of recurrences (n) >1 are shown in bold. AMPA, \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CoA, coenzyme A; EGF, epidermal growth factor; GTP, guanosine 5'-triphosphate; MAPK, mitogen-activated protein kinase; Morf4, mortality factor 4 (pseudogene); MTOR, mechanistic target of rapamycin; TERF2, telomeric repeat binding protein 2.
In conclusion, the present study provided, to the best of our knowledge, the first systematic analysis to quantitatively combine all of the traditional criteria aimed at identifying the HK genes that are best suited to be reference genes for the study of human gene expression. Several genes were identified and proposed to be suitable in cross-tissue studies, and certain genes were proposed as references for tissue/organ-specific studies. The wealth of data generated by this approach may also provide a representative portrait of typical gene-expression profiles for several human tissues and organs in the form of searchable database tables and suggested that currently uncharacterized transcripts, even EST clusters, may be worthy of further investigation as strong candidates to represent HK genes, or tissue-specific genes, expressed in high levels in human cells.

Acknowledgements

The authors wish to thank sincerely the Fondazione Umano Progresso, Milano, Italy for its fundamental support to our research on trisomy 21 and to the present study. The authors are pleased to thank Mrs. Vittoria Aiello and Mr. Massimiliano Albanese (Washington, DC, USA) for having undertaken an international initiative in support of our research in addition to all the donors contributing to this initiative listed at http://www.massimilianoalbanese.net/ds-research/?lang=en. The authors thank all the other people that have kindly contributed through individual donations to support part of the fellowships, in addition to computer hardware, that allowed the present study to be performed. In particular, we are grateful to Matteo and Elisa Mele (Bologna, Italy), to the Costa family, ‘Gruppo Arzdore’, ‘Parrocchia di Dozza’ the community of Dozza and ‘Associazione Turistica Pro Loco di Dozza’ (Dozza, Bologna, Italy), in addition to Mrs. Rina Bini (Fermo, Italy). The authors are grateful to Mrs. Kirsten Welter for her kind and expert revision of the manuscript. M.C.’s fellowship was funded by a donation from Fondazione Umano Progresso and by a grant from Fondazione Del Monte di Bologna e Ravenna (Bologna, Italy).

Table IX. A list of all 63 genes identified for the 10 specific tissues and organs presented in Tables IV-VII.

| Number | Gene |
|--------|------|
| 1      | ACTG1 |
| 2      | AMZ2  |
| 3      | AP2M1 |
| 4      | C1orf166 |
| 5      | CNH1  |
| 6      | CRELD2 |
| 7      | ECHS1 |
| 8      | EEF1G |
| 9      | EIF4A3 |
| 10     | EPCAM |
| 11     | FM96A |
| 12     | FM96B |
| 13     | FIS1  |
| 14     | GABARAP |
| 15     | GABARAPL2 |
| 16     | GANAB |
| 17     | GPX1  |
| 18     | GTO1  |
| 19     | GTF3A |
| 20     | HDGF  |
| 21     | HEBP1 |
| 22     | HEBP2 |
| 23     | HIST1H2BK |
| 24     | IF27  |
| 25     | JTB   |
| 26     | LAMTOR5 |
| 27     | MB    |
| 28     | MIF   |
| 29     | MRFAP1 |
| 30     | MRPS24 |
| 31     | NDUF1  |
| 32     | NDUF1  |
| 33     | NDUF11 |
| 34     | NDUF4  |
| 35     | NDUF5  |
| 36     | NELFCD |
| 37     | NOP10 |
| 38     | PGAM1 |
| 39     | POLR2H |
| 40     | POLR2I |
| 41     | POMP  |
| 42     | PRDX1 |
| 43     | PSMC1 |
| 44     | RBX1  |
| 45     | RNASEK |
| 46     | RNF181 |
| 47     | RPL41 |
| 48     | RPL6  |
| 49     | RPL8  |
| 50     | RPLP0 |
| 51     | RPS18 |

Table IX. Continued.

| Number | Gene |
|--------|------|
| 52     | RPS25 |
| 53     | RPS27 |
| 54     | RPS29 |
| 55     | RPSA  |
| 56     | RRAGA |
| 57     | SLC25A3 |
| 58     | SNRPD2 |
| 59     | SOD1  |
| 60     | TBCB  |
| 61     | TERF2IP |
| 62     | TMEM147 |
| 63     | TUBA1B |

direct comparison between NormFinder and TRAM results possible.

The wealth of data generated by this approach may also provide a representative portrait of typical gene-expression profiles for several human tissues and organs in the form of searchable database tables and suggested that currently uncharacterized transcripts, even EST clusters, may be worthy of further investigation as strong candidates to represent HK genes, or tissue-specific genes, expressed in high levels in human cells.
References

1. Eisenberg E and Levanon EY: Human housekeeping genes, revisited. Trends Genet 29: 569-574, 2013.
2. Casadei R, Pelleri MC, Vitale L, Facchin F, Lenzi L, Canaider S, Strippoli P and Frabetti F: Identification of housekeeping genes suitable for gene expression analysis in the zebrafish. Gene Expr Patterns 11: 271-276, 2011.
3. Finnegan MC, Goepel JR, Hancock BW and Goyns MH: Investigation of the expression of housekeeping genes in non-Hodgkin’s lymphoma. Leuk Lymphoma 10: 387-393, 1993.
4. Lion T: Current recommendations for positive controls in RT-PCR assays. Leukemia 15: 1033-1037, 2001.
5. Yue H, Eastman PS, Wang BB, Minor J, Doctolero MH, Nuttall RL, Stack R, Becker JW, Montgomery JR, Vainer M and Johnston LA: An evaluation of the performance of cDNA microarrays for detecting changes in global mRNA expression. Nucleic Acids Res 29: E41-E41, 2001.
6. Warrington JA, Nair A, Mahadevappa M and Tsyganskaya M: Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. Physiol Genomics 2: 143-147, 2000.
7. Lee PD, Sladek R, Greenwood CM and Hudson TJ: Control genes and variability: Absence of ubiquitous reference transcripts in diverse mammalian expression studies. Genome Res 12: 292-297, 2002.
8. Perfetti V, Manenti G and Dragani TA: Expression of housekeeping genes in Hodgkin’s disease lymph nodes. Leukemia 5: 1110-1112, 1991.
9. Bhatia P, Taylor WR, Greenberg AH and Wright JA: Comparison of glyceraldehyde-3-phosphate dehydrogenase and 28S-ribosomal RNA gene expression as RNA loading controls for northern blot analysis of cell lines of varying malignant potential. Anal Biochem 216: 223-226, 1994.
10. Barber RD, Harmer DW, Coleman RA and Clark BJ: GAPDH as a housekeeping gene: Analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol Genomics 21: 389-395, 2005.
11. Zhang Y, Li D and Sun B: Do housekeeping genes exist? PLoS One 10: e0123691, 2015.
12. Lenzi L, Facchin F, Piva F, Giuliani M, Pelleri MC, Frabetti F, Vitale L, Casadei R, Canaider S, Bortoluzzi S, et al: TRAM (Transcriptome Mapper): Database-driven creation and analysis of transcriptome maps from multiple sources. BMC Genomics 12: 121, 2011.
13. Piovesan A, Vitale L, Pelleri MC and Strippoli P: Universal tight correlation of codon bias and pool of RNA codons (codonome): The genome is optimized to allow any distribution of gene expression values in the transcriptome from bacteria to humans. Genomics 101: 282-289, 2013.
14. Carcausa M, Piovesan A, Vitale L and Pelleri MC: Integrated transcriptome map highlights structural and functional aspects of the normal human heart. J Cell Physiol 232: 759-770, 2017.
15. Barrett T and Edgar R: Gene expression omnibus: Microarray data storage, submission, retrieval and analysis. Methods Enzymol 411: 352-369, 2006.
16. Emig D, Salomoni N, Baumbach J, Lengauer T, Conklin BR and Albrecht M: AltAnalyze and DomainGraph: Analyzing and visualizing expression data. Nucleic Acids Res 38 (Web Server issue): W755-W762, 2010.
17. Lenzi L, Frabetti F, Facchin F, Casadei R, Vitale L, Canaider S, Carinci P, Zannotti M and Strippoli P: UniGene Tabulator: A full parser for the UniGene format. Bioinformatics 22: 2570-2571, 2006.
18. Piovesan A, Carcausa M, Ricci M, Strippoli P, Vitale L and Pelleri MC: Identification of minimal eukaryotic introns through GeneBase, a user-friendly tool for parsing the NCBI Gene database. DNA Res 22: 495-503, 2015.
19. Butte AJ, Dzau VJ and Glueck SB: Further defining housekeeping, or ‘maintenance,’ genes Focus on ‘A compendium of gene expression in normal human tissues.’ Physiol Genomics 7: 31-34, 2001.
20. Tu Z, Wang L, Xu M, Zhou X, Chen T and Sun F: Further understanding human disease genes by comparing with housekeeping genes and other genes. BMC Genomics 7: 31, 2006.
21. Pilbrow AP, Ellmers LJ, Black MA, Moravec CS, Sweet WE, Troughton RW, Richards AM, Frampton CM and Cameron VA: Genomic selection of minimal reference genes for real-time PCR in human myocardium. BMC Med Genomics 1: 64, 2008.
22. Berriz GF, King OD, Bryant B, Sander C and Roth FP: Characterizing gene sets with FuncAssociate. Bioinformatics 19: 2502-2504, 2003.
23. Li B, Yu J, Xu J, Chen J and Lai M: A novel approach to detect differentially expressed genes from count-based digital databases by normalizing with housekeeping genes. Genomics 94: 211-216, 2009.
24. Krupp M, Marquardt JU, Sahin U, Gaile PR, Castle J and Teufel A: RNA-Seq Atlas—a reference database for gene expression profiling in normal tissue by next-generation sequencing. Bioinformatics 28: 1184-1185, 2012.
25. Chen M, Xiao J, Zhang Z, Liu J, Wu J and Yu J: Identification of human HK genes and gene expression regulation study in cancer from transcriptomics data analysis. PLoS One 8: e54082, 2013.
26. Costa Ade F and Franco NC: Insights into RNA transcriptome profiling of cardiac tissue in obesity and hypertension conditions. J Cell Physiol 230: 959-968, 2015.
27. Zhao S, Fung-Leng WP, Bittner A, Ngo K and Liu X: Comparison of RNA-Seq and microarray in transcriptome profiling of activated T cells. PLoS One 9: e87644, 2014.
28. Guo Y, Sheng Q, Li J, Ye F, Samuels DC and Shyr Y: Large scale comparison of gene expression levels by microarrays and RNAseq using TCGA data. PLoS One 8: e71462, 2013.
29. Zhang Y, Akintola OS, Liu KJ and Sun B: Membrane gene ontology bias in sequencing and microarray obtained by housekeeping-gene analysis. Gene 575: 559-566, 2016.
30. Lee S, Jo M, Lee J, Koh SS and Kim S: Identification of novel universal housekeeping genes by statistical analysis of microarray data. J Biochem Mol Biol 40: 226-231, 2007.
31. Zhu J, He F, Song S, Wang J and Yu J: How many human genes can be defined as housekeeping with current expression data? BMC Genomics 9: 172, 2008.
32. de Jonge HJ, Fehrmann RS, de Bont ES, Hofstra RM, Gerbens F, Kamps WA, de Vries EG, van der Zee AG, te Meerman GJ and ter Elst A: Evidence based selection of housekeeping genes. PLoS One 2: e9098, 2007.
33. Fagerberg L, Hallström BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, Habuka M, Tahmasebpoor S, Danielsson A, Edlund K, et al: Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. Mol Cell Proteomics 13: 397-406, 2014.
34. Carcausa M, Vitale L, Pelleri MC, Piovesan A, Bruno S and Strippoli P: A quantitative transcriptome reference map of the normal human brain. Neurogenetics 15: 267-287, 2014.
35. Casadei R, Piovesan A, Vitale L, Facchin F, Pelleri MC, Canaider S, Bianconi E, Frabetti F and Strippoli P: Genome-scale analysis of human mRNA 5’ coding sequences based on expressed sequence tag (EST) database. Genomics 100: 125-130, 2012.
36. She X, Rohl CA, Castle JC, Kulkarni AV, Johnson JM and Chen R: Definition, conservation and epigenetics of housekeeping and tissue-enriched genes. BMC Genomics 10: 269, 2009.
37. Thomas D, Finan C, Newport MJ and Jones S: DNA entropy reveals a significant difference in complexity between housekeeping-gene analysis. Gene 575: 559-566, 2016.
38. Thomas D, Finan C, Newport MJ and Jones S: DNA entropy reveals a significant difference in complexity between housekeeping and tissue specific gene promoters. Comput Biol Chem 58: 19-24, 2015.
39. Chang CW, Cheng WC, Chen CR, Shu WY, Tsai ML, Huang CL and Hsu IC: Identification of human housekeeping genes and tissue specific genes by microarray meta-analysis. PLoS One 6: e22859, 2011.
40. Shaw GT, Shih ES, Chen CH and Hwang MJ: Preservation of ranking order in the expression of human Housekeeping genes. PLoS One 6: e29314, 2011.
41. Carcausa M, Rigon V, Piovesan A, Strippoli P, Vitale L and Pelleri MC: A quantitative transcriptome reference map of the normal human hippocampus. Hippocampus 26: 13-26, 2016.
42. Cheng WC, Chang CW, Chen CR, Tsai ML, Shu WY, Li CY and Hsu IC: Identification of reference genes across physiological states for qRT-PCR through microarray meta-analysis. PLoS One 6: e17347, 2011.

43. Strippoli P, Pelleri MC, Caracausi M, Vitale L, Piovesan A, Locatelli C, Mimmi MC, Berardi AC, Ricotta D, Radeghieri A, et al: An integrated route to identifying new pathogenesis-based therapeutic approaches for trisomy 21 (Down Syndrome) following the thought of Jérôme Lejeune. Sci Postprint 1: e00010, 2013.

44. Pelleri MC, Cicchini E, Locatelli C, Vitale L, Caracausi M, Piovesan A, Rocca A, Poletti G, Seri M, Strippoli P and Cocchi G: Systematic reanalysis of partial trisomy 21 cases with or without Down syndrome suggests a small region on 21q22.13 as critical to the phenotype. Hum Mol Genet 25: 2525-2538, 2016.

45. Pelleri MC, Piovesan A, Caracausi M, Berardi AC, Vitale L and Strippoli P: Integrated differential transcriptome maps of acute megakaryoblastic leukemia (AMKL) in children with or without down syndrome (DS). BMC Med Genomics 7: 63, 2014.

46. Kwon MJ, Oh E, Lee S, Roh MR, Kim SE, Lee Y, Choi YL, In YH, Park T, Koh SS and Shin YK: Identification of novel reference genes using multiplatform expression data and their validation for quantitative gene expression analysis. PLoS One 4: e6162, 2009.

47. Chiang AW, Shaw GT and Hwang MJ: Partitioning the human transcriptome using HKera, a novel classifier of housekeeping and tissue-specific genes. PLoS One 8: e63040, 2013.

48. Ersahin T, Carkacioglu L, Can T, Konu O, Atalay V and Cetin-Atalay R: Identification of novel reference genes based on MeSH categories. PLoS One 9: e93341, 2014.

49. Pfaffl MW, Tichopad A, Prgomet C and Neuvians TP: Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol Lett 26: 509-515, 2004.

50. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH0034, 2002.

51. Andersen CL, Jensen JL and Ørntoft 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 64: 5245-5250, 2004.