

Supplementary Materials: Selection of Suitable Reference Genes for Analysis of Salivary Transcriptome in Non-Syndromic Autistic Male Children

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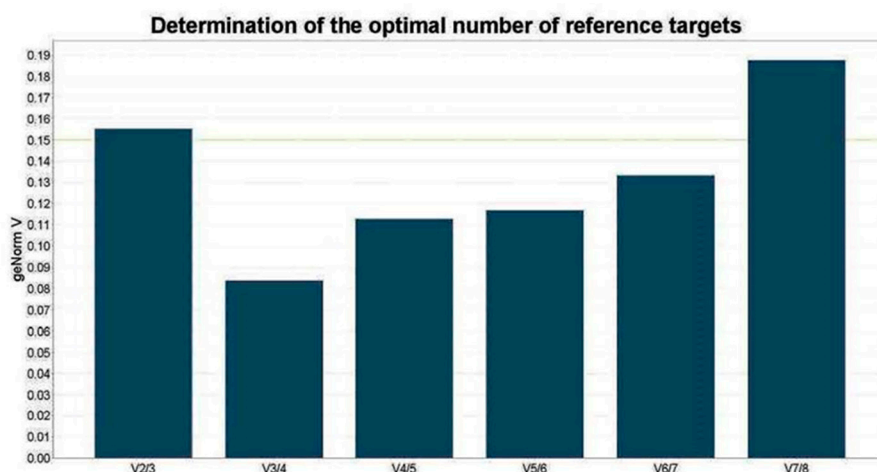


Figure S1. The optimal number of reference genes required for accurate normalization in phase I according to geNorm algorithm. Based on pairwise variation (V) by geNorm, the most stable three genes are required for normalization.

Table S1. Comparison of *ACTB* C_q values between adults and children (preliminary phase).

| Samples | Age (Year) | $C_q \pm SD$ | Average | S_{tot} | 2^{-C_q} | CV% |
|-----------------|------------|--------------|--------------|-----------|-------------------------|-------|
| Ad ₁ | 40 | 19.48 ± 0.31 | | | 1.36 × 10 ⁻⁶ | |
| Ad ₂ | 40 | 19.46 ± 0.86 | | | 1.38 × 10 ⁻⁶ | |
| Ad ₃ | 32 | 20.25 ± 0.38 | 19.54 ± 0.44 | 0.99 | 8.01 × 10 ⁻⁷ | 28.5 |
| Ad ₄ | 45 | 19.01 ± 0.07 | | | 1.89 × 10 ⁻⁶ | |
| Ad ₅ | 40 | 19.52 ± 0.03 | | | 1.33 × 10 ⁻⁶ | |
| Ch ₁ | 5 | 24.72 ± 0.31 | | | 3.61 × 10 ⁻⁸ | |
| Ch ₂ | 1 | 20.96 ± 0.61 | | | 4.90 × 10 ⁻⁷ | |
| Ch ₃ | 9 | 27.48 ± 0.04 | 25.12 ± 2.60 | 0.69 | 5.34 × 10 ⁻⁹ | 187.4 |
| Ch ₄ | 6 | 27.28 ± 0.01 | | | 6.13 × 10 ⁻⁹ | |
| Ch ₅ | 3 | 25.19 ± 0.10 | | | 2.61 × 10 ⁻⁸ | |

Ad, Adult; Ch, Children (Ch₁, represents an autistic patient, and Ch₂₋₅ healthy controls); SD, Standard deviation; S_{tot} , represents the sum of SD from replicate reactions calculated as $\sqrt{SD_1^2 + SD_2^2 + \dots + SD_N^2}$; CV, coefficient of variation for each group was calculated after linearization of the C_q values with 2^{-C_q} .

Table S2. The *C_q* values measured for samples studied in Phase 1.

| Samples | <i>18s rRNA</i> | | <i>ACTB</i> | | <i>GAPDH</i> | | <i>RPL13A</i> | |
|----------------|----------------------|------|----------------------|------|----------------------|------|----------------------|------|
| | <i>C_q</i> | SD | <i>C_q</i> | SD | <i>C_q</i> | SD | <i>C_q</i> | SD |
| A ₁ | 15.15 | 0.01 | 27.67 | 0.30 | 27.35 | 0.04 | 32.72 | 0.61 |
| A ₂ | 14.02 | 0.01 | 30.69 | 0.35 | 26.67 | 0.33 | ND | NA |
| C ₁ | 14.64 | 0.35 | 25.93 | 0.56 | 26.09 | 0.27 | 30.35 | 0.03 |
| C ₂ | 17.48 | 0.29 | 25.80 | 0.01 | 26.45 | 0.21 | 31.84 | 0.36 |

| Samples | <i>SDHA</i> | | <i>TFRC</i> | | <i>UBC</i> | | <i>YWHAZ</i> | |
|----------------|----------------------|------|----------------------|------|----------------------|------|----------------------|------|
| | <i>C_q</i> | SD | <i>C_q</i> | SD | <i>C_q</i> | SD | <i>C_q</i> | SD |
| A ₁ | 28.75 | 0.20 | 31.91 | 0.36 | 31.77 | 0.34 | 27.11 | 0.12 |
| A ₂ | 29.11 | 0.36 | 34.54 | 0.17 | 32.70 | 0.24 | 29.03 | 0.04 |
| C ₁ | 33.73 | 0.22 | 29.83 | 0.40 | 29.70 | 0.14 | 27.03 | 0.02 |
| C ₂ | ND | NA | 32.46 | 0.36 | 30.75 | 0.09 | 28.05 | 0.26 |

A, Autistic; C, Control; SD, standard deviation; ND, not detected; NA, not applicable. *C_q* values for each sample represent averages for respective triplicate reactions.

Table S3. The *C_q* values measured for all samples studied in Phase 2.

| Samples | <i>ACTB</i> | | <i>GAPDH</i> | | <i>UBC</i> | | <i>YWHAZ</i> | |
|----------------|----------------------|------|----------------------|------|----------------------|------|----------------------|------|
| | <i>C_q</i> | SD | <i>C_q</i> | SD | <i>C_q</i> | SD | <i>C_q</i> | SD |
| A ₁ | 30.29 | 0.07 | 31.36 | 0.56 | 38.28 | 0.03 | 34.28 | 1.34 |
| A ₂ | 26.28 | 0.30 | 25.28 | 0.11 | 28.50 | 0.23 | 26.02 | 0.10 |
| A ₃ | 32.65 | 0.23 | 29.48 | 0.12 | 33.67 | 0.46 | 28.76 | 0.11 |
| A ₄ | 35.47 | 0.57 | 31.20 | 0.54 | 37.04 | 0.24 | 32.41 | 0.01 |
| A ₅ | 29.90 | 0.01 | 30.41 | 0.10 | 33.89 | 0.32 | 33.50 | 0.39 |
| A ₆ | 24.06 | 0.08 | 22.24 | 0.04 | 24.82 | 0.22 | 23.17 | 0.01 |
| A ₇ | 22.29 | 0.13 | 20.84 | 0.06 | 24.92 | 0.53 | 21.50 | 0.08 |
| A ₈ | 24.65 | 0.34 | 25.03 | 0.02 | 26.35 | 0.28 | 24.52 | 0.31 |
| A ₉ | 21.26 | 0.27 | 21.98 | 0.33 | 24.33 | 0.32 | 23.77 | 0.13 |
| Average | 27.43 ± 4.89 | 0.83 | 26.42 ± 4.24 | 0.87 | 30.20 ± 5.55 | 0.97 | 27.55 ± 4.84 | 1.45 |
| C ₁ | 22.56 | 0.16 | 21.33 | 0.02 | 23.41 | 0.04 | 22.41 | 0.12 |
| C ₂ | 27.52 | 0.35 | 28.00 | 0.01 | 31.66 | 0.42 | 29.32 | 0.04 |
| C ₃ | 30.18 | 0.07 | 30.41 | 0.14 | 34.30 | 0.22 | 30.63 | 0.07 |
| C ₄ | 26.38 | 0.25 | 27.40 | 0.02 | 30.88 | 0.17 | 28.65 | 0.04 |
| C ₅ | 23.34 | 0.21 | 23.68 | 0.28 | 28.95 | 0.43 | 24.58 | 0.23 |
| C ₆ | 24.01 | 0.49 | 24.63 | 0.31 | 28.94 | 0.13 | 24.67 | 0.28 |
| C ₇ | 27.48 | 0.38 | 28.62 | 0.21 | 39.28* | 0.03 | 28.19 | 0.04 |
| C ₈ | 22.40 | 0.09 | 21.69 | 0.30 | 26.73 | 0.18 | 23.67 | 0.30 |
| C ₉ | 21.03 | 0.26 | 20.95 | 0.12 | 23.27 | 0.46 | 21.75 | 0.28 |
| Average | 24.99 ± 3.03 | 0.85 | 25.19 ± 3.53 | 0.59 | 28.52 ± 3.89 | 0.84 | 25.99 ± 4.89 | 0.57 |

A, Autistic; C, Control; SD, standard deviation. *C_q* values for each sample represent averages for respective triplicate reactions. The star in the *UBC* column indicates an undetected *C_q* value, which was replaced with an assigned value based on the maximum *C_q* detected for that assay plus 1 (Max *C_q* + 1). The rows labeled as Average show the mean *C_q* values (±SD, *n* = 9) for the corresponding gene in each group.

Table S4. Expression stability of the candidate HKGs in saliva samples evaluated by BestKeeper.

| Phase I: Eight Candidate HKGs; Four Samples | | | | | | | | |
|---|-----------|----------|----------|--------------------|----------|--------|----------------------------------|--------------|
| Gene | Geo. Mean | Ar. Mean | Min (Cq) | Max (Cq) | SD | CV (%) | Coeff. of Corr. (r) ¹ | Power of HKG |
| 18s rRNA | 15.27 | 15.32 | 14.02 | 17.48 | 1.08 (4) | 7.04 | 0.424 | 1.92 |
| ACTB | 27.45 | 27.52 | 25.80 | 30.69 | 1.66 (7) | 6.02 | 0.404 | 2.55 |
| GAPDH | 26.64 | 26.64 | 26.09 | 27.35 | 0.37 (1) | 1.39 | 0.365 | 1.22 |
| RPL13A | 32.56 | 32.61 | 30.35 | 35.54 ² | 1.52 (6) | 4.65 | 0.661 | 4.34 |
| SDHA | 31.65 | 31.78 | 28.75 | 35.54 ² | 2.85 (8) | 8.98 | -0.107 | 0.69 |
| TFRC | 32.14 | 32.19 | 29.83 | 34.54 | 1.32 (5) | 4.09 | 0.858 | 5.42 |
| UBC | 31.21 | 31.23 | 29.70 | 32.70 | 1.01 (3) | 3.22 | 0.673 | 2.43 |
| YWHAZ | 27.79 | 27.81 | 27.03 | 29.03 | 0.74 (2) | 2.64 | 0.272 | 1.30 |
| Phase II: Four Candidate Genes; 18 Samples | | | | | | | | |
| Gene | Geo. Mean | Ar. Mean | Min (Cq) | Max (Cq) | SD | CV (%) | Coeff. of Corr. (r) ³ | Power of HKG |
| ACTB | 25.91 | 26.21 | 21.03 | 35.47 | 3.36 (1) | 12.84 | 0.948 (4) | 1.91 (2/3) |
| GAPDH | 25.54 | 25.81 | 20.84 | 31.36 | 3.38 (2) | 13.10 | 0.991 (1) | 1.90 (1) |
| UBC | 29.54 | 29.96 | 23.27 | 39.28 | 4.37 (4) | 14.59 | 0.952 (3) | 2.08 (4) |
| YWHAZ | 26.48 | 26.77 | 21.50 | 34.28 | 3.51 (3) | 13.12 | 0.968 (2) | 1.91 (2/3) |

Geo. mean, Geometric mean of minimum and maximum Cq values; Ar. mean, Arithmetic mean of minimum and maximum Cq values; Min Cq, minimum Cq value; Max Cq, maximum Cq value; SD, standard deviation; CV, coefficient of variation; Coeff. of Corr., coefficient of correlation. The numbers in parentheses indicate the ranking of the HKGs in the respective columns. ¹ p-values for pair-wise correlations are not provided by BestKeeper algorithm for Phase I, which makes the ranking in the (r) column not applicable; ² Indicates an undetected Cq value, which was replaced with an assigned value based on the maximum Cq detected plus 1 (Max Cq + 1); ³ Phase II pair-wise correlation p-values = 0.001 for all combinations.