Supplementary Information – Laurell et al.

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GOI Symbol | Forward | Reverse | Amplicon length | UCSC Gene Reference
--- | --- | --- | --- | ---
CcI2 | TTCAACGATCTGAGGCGGCTGTC | TGTGTTGAGTGGTTGCTGTA | 80 | uc007km.1_CcI2:418+497
Ccbp | AGGACCTTAGGGTATGCTGCTG | GTTTCATGAGGCTGCTGCTG | 87 | uc009gj.1_Ccbp:1712+1798
Ch25h | TGTAATTTACCTGGTGTGTCG | AAAGGCGGACAGTTGTCAGTGA | 78 | uc008gh.1_Ch25h:622+699
Fdx1 | CCAAGATCGGGGATGACAAAGA | CATTTTCCCCGTCGCTGTT | 79 | uc009pl.1_Fdx1:1256+334
Gadph,Tataa | Included in the mouse reference gene panel (Tataa biocenter) | | | |
Plat | TATATTGCAGGATCCAGTATGCTG | GCATGGGGACAGTTGACAGTAT | 93 | uc009dx.2_Plat:900+992
Ppia | GAAGCTTCTGGACAAATATCTGG | CCCCAGGACATTTGCTGGTG | 125 | uc007hy.1_Ppia:108+232
Ppia,Tataa | Included in the mouse reference gene panel (Tataa biocenter) | | | |
Ptg1s | CCAGAACGAGGGTGCTGCTG | GTAGCCGGTGCGAGTACAATC | 70 | uc008jll.1_Ptg1s:216+335
Rtnla | CCAATCCGCTAATCTATCTCCC | GGGAGCGCATCTGTCATAGTCC | 80 | uc007jy.1_Rtnla:221+300
Rhoa | TGCCATCTGAGAAGTCTCAGGTACT | ACCACACCGCCATCTACACCCA | 125 | uc009pe.2_Rhoa:353+477
Serpine1 | ACAAGCTTGTGCTACTCGACC | CGGAAACCACAGAGAGGAGAA | 75 | uc009abu.2_Serpine1:1229+1303
Vcam1 | CATGGAGCCGCTTGACTTTTGTAG | GGGGAAAGATAGTAGTGGCAG | 106 | uc008bx.1_Vcam1:564+669

High confidence GOI assays used in the Biomek framework

GOI | Forward | Reverse | Amplicon length | UCSC Gene Reference
--- | --- | --- | --- | ---
Cebpb | CAACACGTCGGCAGGCCGGTGT | TACTCGAGGCGCGCCCTGCA | 72 | uc008oaf.1_Cebpb:1210+1281
Ctsf | TGGCCCGGCAGGAAGGAGGCAGG | CTGGCTCTCCGAGCTGCTG | 122 | uc008gb.1_Ctsf:883+994
Cyr61 | ACGGCTGCTGCTGCTGCTAG | GGTGGACGGCTGGCGAGGAAAT | 113 | uc008rq.2_Cyr61:336+448
Id1 | ACAGCCGCGGAGGTGGTACT | TGTCGCTCTGCAAGGACCG | 80 | uc008ngf.1_Id1:134+228
Ier2 | TGCGTGGCGACGTGAGTTGCT | TGGGCGGCGGTGCTGCTTCT | 90 | uc009mmr.2_Ier2:723+828
Igfbp5 | AGAAGCGAGTGAAGCCCTCCC | CCGGAGCTCTCAGTCTCAGTACT | 79 | uc007bk.1_Igfbp5:1402+1480
Ipo5 | CTCCTTCTCTTCTCAGCCTAGC | AGCAGGTTTCCAGGAGGGAGGTA | 84 | uc007zz.1_Ipo5:33+116
Junb | GAAGCGGAGGTGTGACCGGCA | GCTCCGCCGTGAGACCCGCGA | 101 | uc012ghn.1_Junb:1138+1238
Mad2l1 | AAGGACCGGGAGGGAGGACTT | CTCCTTCTCAGGACCACCAAAA | 120 | uc009cen.1_Mad2l1:1096+1215
Nfkbia | CTGTGAGACGGCTGCTGCTG | GGTGGCCGCTAGGGGAGGTA | 84 | uc007nor.2_Nfkbia:574+657
Pik3r2 | TCAATAGCGCCATCTACTCCCA | AGCTCGTGCTGCTGCTGCTTC | 90 | uc009mbn.2_Pik3r2:796+900
Rasd1 | AGACCCCTAGCGGTGGTCCG | TGCCATGCGACGAGGACCGC | 90 | uc007fh.1_Rasd1:634+723
Sox4 | TGCACATACCCAGAACACCAAA | CGAACGGAGACGAGGCTC | 84 | uc009pk.1_Sox4:1407+1480
Sprr2f | CAAAGCACATCCGGCCGCTCCT | AGGCTGAGGCGACACCGGGA | 74 | uc008qdu.1_Sprr2f:86+167
Ubb | TGCCGAGCACCTCTTCTGTAG | GCATCGCCACCTCAGGGC | 77 | uc007ijj.1_Ubb:254+330

High confidence GOI assays used in both qPCR systems

GOI | Forward | Reverse | Amplicon length | UCSC Gene Reference
--- | --- | --- | --- | ---
Gapdh | AGTGTCGTTGGAAGGAGGATTG | TGTAAGACCTACCTAGGTCAG | 122 | uc009dts.1_Gapdh:58+180
Mapk3 | TCAGAGGCACCCACATTGGA | TGAAGGCCCTGGAGGAATGCT | 144 | uc009jsm.1_Mapk3:343+486
Odc1 | TCGAGAGGAGGAGCTGCTG | GGAATGGCAGCCAACTCGCTC | 108 | uc007ncv.1_Odc1:343+450
Tgfb3 | GCAAGATCTGGACCAAGG | CACTGCCGAGAGTGTAAGT | 69 | uc007ohn.2_Tgfb3:1672+1741

GOI assays used in the Biomark for correction of endogenous DNA

GOI Symbol | Forward | Reverse
--- | ---
II1b gDNA A+ | CAACCAACAGTATATTCTTACTACG | GATCACCACCTCTCCAGCTGCA | 152 | uc008mht.1_II1b:527+678
II1b gDNA | TGAAGAAGGAGGCCCTGCTG | GCAGGAGCGCGCAGAGTCT | 50 | uc008mht.1_II1b:359+488
Serpine1 A+ | ACAAGCTTCTGCGACCAAGCAG | TGCTTATCCTGTGCTGCTG | 71 | uc009abu.2_Serpine1:1133+1203
Serpine1 | ACAAGCTTCTGCGACCAAGCAG | TGCTTATCCTGTGCTGCTG | 71 | uc009abu.2_Serpine1:1133+1203
Chi3L3 A+ | GAAGCCCTCTCAGCAGAAACA | GCCATTGGAATGCTTCTCAGC | 96 | uc008qww.2_Chi3L3:480+575
Chi3L3 | AGAAGGAGGTTCAAACCTG | GTTCGCTCAGTGGTGTAAGTGA | 109 | uc008qww.2_Chi3L3:154+262

VPAs used in the study

GOI | Forward | Reverse | UCSC Gene Reference
--- | --- | --- | ---
mVPA1 | GAAGCGCAGCTGAGGAGCGA | AGGCCAGCAACCTACCTGTA | 87 | chr1:41857082+41857168
mVPA5 | ACAAGGAGCCAGCTGTATC | ACTCCGCTTCTGGACGCT | 111 | chr5:117408738+117408848

Supplementary Table S1.

**Primer sequence information for GOI assays and VPAs.** A+ indicates pri mers that do not amplify gDNA. UCSC references refer to sequence coordinates in the genome browser at UCSC [http://genome.ucsc.edu](http://genome.ucsc.edu) according to the NCBI37/mm9 assembly.
### Supplementary Table S2.

**Presence of cDNA or RNA does not affect the quantification of gDNA with VPA.**

Addition of 20 ng of cDNA (DNase treated), RNA (DNase treated) or tRNA to different amounts of gDNA (0.15, 0.6, 3.0 ng corresponding to 50, 200, 1000 haploid genome copies) did not affect the amplification efficiency (expressed in %) of mVPA1 (n=4/condition). A one-way ANOVA analysis (Tukey-Kramer, GenEx v.5.3) did not reveal any significant difference between the groups with same gDNA concentration. Indeed, the coefficient of variation (CV), calculated on relative quantities (), of all 24 data points (=6*4) for each gDNA concentration was less than 20%. Furthermore; the absence of signal (ND) in all samples with no gDNA demonstrates that there is no transcriptional activity at the VPA locus.

| Spiking Source | Description       | gDNA copy# | Mean Cq (VPA) | SD (n=4) | slope       | VPA Efficiency (%) | R²   |
|----------------|------------------|------------|---------------|----------|-------------|---------------------|------|
| Adipose Tissue | DNase treated cDNA | 0          | ND            |          |             |                     |      |
|                |                  | 50         | 30.46         | 0.02     | -3.267      | 102.3               | 0.9997 |
|                |                  | 200        | 28.55         | 0.18     |             |                     |      |
|                |                  | 1000       | 26.21         | 0.06     |             |                     |      |
| Adipose Tissue | DNase treated RNA | 0          | ND            |          |             |                     |      |
|                |                  | 50         | 30.42         | 0.36     | -3.365      | 98.2                | 0.9999 |
|                |                  | 200        | 28.43         | 0.25     |             |                     |      |
|                |                  | 1000       | 26.05         | 0.08     |             |                     |      |
| Liver          | DNase treated cDNA | 0          | ND            |          |             |                     |      |
|                |                  | 50         | 30.33         | 0.06     | -3.378      | 97.7                | 0.9994 |
|                |                  | 200        | 28.39         | 0.07     |             |                     |      |
|                |                  | 1000       | 25.94         | 0.09     |             |                     |      |
| Liver          | DNase treated RNA | 0          | ND            |          |             |                     |      |
|                |                  | 50         | 30.51         | 0.23     | -3.397      | 96.9                | 0.9993 |
|                |                  | 200        | 28.57         | 0.12     |             |                     |      |
|                |                  | 1000       | 26.1          | 0.07     |             |                     |      |
| Yeast          | tRNA             | 0          | ND            |          |             |                     |      |
|                |                  | 50         | 30.26         | 0.34     | -3.303      | 100.8               | 0.9982 |
|                |                  | 200        | 28.1          | 0.09     |             |                     |      |
|                |                  | 1000       | 25.95         | 0.05     |             |                     |      |
| H₂O            | H₂O              | 0          | ND            |          |             |                     |      |
|                |                  | 50         | 30.26         | 0.28     | -3.359      | 98.5                | 0.9978 |
|                |                  | 200        | 28.42         | 0.18     |             |                     |      |
|                |                  | 1000       | 25.9          | 0.06     |             |                     |      |

| gDNA copy# | CV (%) (n=24) |
|------------|---------------|
| 50         | 16.26         |
| 200        | 14.52         |
| 1000       | 8.51          |
Supplementary Figure S1A

\[N_{Cq_{DNA}} = N^0_{DNA} 2^{Cq_{DNA}}\]

\[N_{Cq_{RNA}} = N^0_{RNA} 2^{Cq_{RNA}}\]

\[N_{NA} = N_{DNA} + N_{RNA}\]

\[N_{Cq_{NA}} = N^0_{DNA} 2^{Cq_{NA}} + N^0_{RNA} 2^{Cq_{NA}-1}\]

\[N_{Cq_{NA}} = N_{Cq_{DNA}} = N_{Cq_{RNA}}\]

\[N^0_{DNA} 2^{Cq_{DNA}} = N^0_{DNA} 2^{Cq_{NA}} + N^0_{RNA} 2^{Cq_{RNA}-1}\]

\[N^0_{RNA} 2^{Cq_{RNA}-1} = N^0_{DNA} 2^{Cq_{DNA}} - 2^{Cq_{RNA}}\]

\[N^0_{RNA} 2^{Cq_{RNA}-1} - 2^{Cq_{RNA}-1} = N^0_{DNA} 2^{Cq_{DNA}} - 2^{Cq_{RNA}}\]

\[\frac{2^{Cq_{RNA}-1} - 2^{Cq_{RNA}-1}}{2^{Cq_{RNA}-1} - 2^{Cq_{RNA}}} = \frac{2^{Cq_{DNA}}}{2^{Cq_{DNA}} - 2^{Cq_{RNA}}}\]

\[2^{Cq_{RNA}-1} = \frac{2^{2Cq_{DNA}-1}}{2^{Cq_{DNA}} - 2^{Cq_{RNA}}} + 2^{Cq_{RNA}-1}\]

\[Cq_{RNA} = 1 + \log_2 \left(\frac{2^{2Cq_{DNA}-1}}{2^{Cq_{DNA}} - 2^{Cq_{RNA}}} + 2^{Cq_{RNA}-1}\right)\]

\[Cq_{RNA} = 1 + \log_2 \left(\frac{2^{Cq_{DNA} + Cq_{RNA}-1}}{2^{Cq_{DNA}} - 2^{Cq_{RNA}}}\right)\]

\[Cq_{RNA} = Cq_{DNA} + Cq_{NA} - \log_2 \left(2^{Cq_{DNA}} - 2^{Cq_{RNA}}\right)\]

\[Cq_{RNA} = -\log_2 \left(2^{-Cq_{RNA}} - 2^{-Cq_{DNA}}\right)\]

Derivation of CqRNA

Double stranded DNA provides twice the number of template molecules for PCR amplification compared to single stranded cDNA produced from RNA (S1, S2). We define the number of nucleic acid (NA) molecules in a sample as the sum of the number of dsDNA and single stranded cDNAs produced from RNA (S3, S4). At threshold the number of molecules in the test tube is the same regardless of whether or not the template was double or single stranded (S5). This gives a relation between \(Cq_{DNA}\) (measured in a PCR sensing only the DNA component), \(Cq_{RNA}\) (measured in a PCR sensing only the RNA component) and \(Cq_{NA}\) (measured in a PCR sensing both nucleic acids) (S6, S7). After rearrangement (S8, S9) the two equations are combined to eliminate starting amounts (S10). After further arrangements (S11-S15) CqRNA is expressed as function of the measured \(Cq_{NA}\) and \(Cq_{DNA}\) (measured either directly by a RT(-)qPCR or indirectly using the more sensitive ValidPrime assay), which is used in equation 4 in Figure 1 of the main text. The derivation above assumes 100 % PCR efficiency.
Supplementary Figure S1B

(S16) \[ (C_q^{\text{GOI}_{\text{Sample}}} - C_q^{\text{RG}_{\text{Sample}}}) - (C_q^{\text{GOI}_{\text{Calibrator}}} - C_q^{\text{RG}_{\text{Calibrator}}}) = \Delta \Delta C_t \]

(5) \[ C_q^{\text{DNA}} = C_q^{\text{VPA}_{\text{Sample}}} + C_q^{\text{GOI}_{\text{gDNA}}} - C_q^{\text{VPA}_{\text{gDNA}}} \]

(S17) \[ C_q^{\text{DNA}} - C_q^{\text{VPA}_{\text{Sample}}} = C_q^{\text{GOI}_{\text{gDNA}}} - C_q^{\text{VPA}_{\text{gDNA}}} \]

(S18) \[ (C_q^{\text{DNA}} - C_q^{\text{VPA}_{\text{Sample}}}) - (C_q^{\text{GOI}_{\text{gDNA}}} - C_q^{\text{VPA}_{\text{gDNA}}}) = \Delta \Delta C_q = 0 \]

Comparison between ValidPrime \( C_q^{\text{DNA}} \) determination and the \( \Delta \Delta C_t \) method.

Being based on the addition and subtraction of Cq values, the calculation of \( C_q^{\text{DNA}} \) by ValidPrime has a certain resemblance with the \( \Delta \Delta Ct \) equation (Equation S16) proposed by Livak and Schmittgen (10). The \( \Delta \Delta Ct \) method was the first to propose a mean for normalization of qPCR data of a GOI (or target) against a reference gene (RG) and a calibrator (control) sample. It was later further developed to include the use of multiple reference genes (22-24). The resemblance with the \( C_q^{\text{DNA}} \) determination in ValidPrime is more clearly appreciated in the rearrangement of Equation 5 into Equations S17 and S18. In ValidPrime, the gDNA can thus be considered as the calibrator sample and the ValidPrime assay (VPA) as the reference gene. As in the \( \Delta \Delta Ct \) method, in which similar amplification efficiencies for the GOI and RG assays are presumed, ValidPrime assumes similar efficiencies for the VPA and the GOI assays in order to correctly estimate \( C_q^{\text{DNA}} \) and consequently \( C_q^{\text{RNA}} \). However, in contrast to the \( \Delta \Delta Ct \) method, in which all four terms are experimentally determined, the result of equation S18 always equals zero\(^{(*)}\), since the \( C_q^{\text{DNA}} \) is unknown and defined by the other 3 Cq values in the equation. Furthermore, the aim of the “normalization” is clearly different. In ValidPrime the inclusion of gDNA serves as reference to evaluate gDNA-sensitivity of GOI assays compared to the VPA. Thus, despite the resemblance, the two methods are distinct and not interchangeable.

\(^{(*)}\) Implementation of equation S18 on the data shown in Figure 2 with the replacement of \( C_q^{\text{DNA}} \) with the RT(-) data, generated indeed a result close to zero: 0.14+/-0.35 (n=24) (mean+/-SD).

10. Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(\(\Delta\Delta C_t\)) Method. Methods, 25, 402-408.
22. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol, 3, RESEARCH0034.
23. Andersen, C.L., Jensen, J.L. and Orntoft, T.F. (2004) 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.
24. Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. and Vandesompele, J. (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol, 8, R19.
**Supplementary Figure S2**

**Limit of detection (LOD) and Limit of quantification (LOQ) of ValidPrime assay mVPA1**

| Quantities (genome copies) | Mean Cq | SD (Cq) | Number of calls (of 6) |
|----------------------------|---------|---------|------------------------|
| 0                          | NA      | NA      | 0                      |
| 1                          | 35.26   | 1.148   | 5 (5)                  |
| 2                          | 34.36   | 0.687   | 5 (5)                  |
| 4                          | 33.51   | 0.346   | 6 (5)                  |
| 8                          | 32.82   | 0.343   | 6 (5)                  |
| 16                         | 31.73   | 0.366   | 6 (6)                  |
| 32                         | 30.56   | 0.179   | 6 (5)                  |
| 64                         | 29.79   | 0.200   | 6 (6)                  |

| Quantities (#copies) | slope | efficiency (%) | R²  |
|----------------------|-------|----------------|-----|
| 1-64                 | -3.080| 111.2          | 0.994|
| 2-64                 | -3.159| 107.3          | 0.993|
| 4-64                 | -3.336| 99.4           | 0.996|
| 8-64                 | -3.416| 96.2           | 0.993|

Genex LOD | Copy number  
Cut-Off Cq: 36 | 3.69  
Cut-Off Cq: 37 | 3.89  
(95%CI)

**Capacity of mVPA1 to amplify low copy number templates. Determination of LOD and LOQ.**

Different concentrations of gDNA (from 0 to 64 haploid genome copies) were used as template in qPCR reactions with the mVPA1 assay. The melting curve analysis includes all positive calls (n=40). Limit of detection (LOD) and PCR efficiency were determined with GenEx software and the limit of quantification (LOQ) was defined using SD<0.45 as the upper limit of precision. Taking the concentrations above LOD into account the PCR efficiency (E) was estimated to: 1.00+/-0.16 (95% CI). Similar results were obtained with the mVPAS assay as well as with higher gDNA concentrations (see Supplementary Table 2 and Material and Methods section).
**Supplementary Figure S3**

**A.**

![Graph A](image1.png)

**B.**

![Graph B](image2.png)

**ValidPrime correction is more reliable after GOI assay validation using gDNA dilution series.** For certain target genes it is impossible to design gDNA-insensitive assays. This could be due to the presence of well-conserved pseudogenes (eg. *Gapdh* (A)) or the absence of introns (eg. *Ier2* (B)). When validated according to the MIQE guidelines (17) gDNA-sensitive assays are normally perfectly compatible with ValidPrime. Nonetheless, we recommend using a dilution series of four or more reference gDNA concentrations in the initial validation of a GOI assay with ValidPrime. This does not replace the established criteria for assay validation, but specifically aims to ensure that assays do not behave unexpectedly when purified gDNA is used as template. To obtain accurate estimates of gDNA background contributions, the GOI and the VPA amplification efficiencies should be similar, which is the case if the slopes of the standard curves are the same. Consequently, the distance between the curves (∆Cq in panel a) is similar at all gDNA concentrations. Based on the training data sets obtained with two different qPCR technologies, we found 0.3 as maximal standard deviation (SD) of the ∆Cq values between GOI and VPA appropriate to assign a high confidence level to the GOI assay used for ValidPrime correction. The data shown were generated on the Biomark instrument.

17. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer C.T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.*, 55, 611-622.
Supplementary Figure S4

A. 

Fold over control

|                | Without ValidPrime | With ValidPrime |
|----------------|--------------------|-----------------|
| No spike       |                    |                 |
| 3-25%          |                    |                 |
| 25-60%         |                    |                 |
| 60-90%         |                    |                 |

B. 

Fold over control

|                | Without ValidPrime | With ValidPrime |
|----------------|--------------------|-----------------|
| No spike       |                    |                 |
| 3-25%          |                    |                 |
| 25-60%         |                    |                 |
| 60-90%         |                    |                 |

ValidPrime applied on targets with one or multiple genomic loci.
cDNA samples were spiked with gDNA and analyzed with or without Validprime. Uncorrected ratios of relative quantities (left) and ValidPrime corrected ratios (right) are shown as mean +/- SD relative to the unspiked control. gDNA background contribution is indicated under the bars. (A) Data were generated with assays for Ch25h, Serpine1 and Tgfβ3, which all target a single locus. 88 samples were analyzed. (B) Using an assay targeting Gapdh, which has more than 50 well-conserved intronless pseudogenes in vertebrates and more than 300 in rodents (25), only 1 out of 25 samples spiked with up to 3000 genome copies had more than 60% DNA contribution as consequence of high Gapdh expression. Experiments were performed on the StepOnePlus.

25. Liu, Y.J., Zheng, D., Balasubramanian, S., Carriero, N., Khurana, E., Robilotto, R. and Gerstein, M.B. (2009) Comprehensive analysis of the pseudogenes of glycolytic enzymes in vertebrates: the anomalously high number of GAPDH pseudogenes highlights a recent burst of retrotrans-positional activity. BMC Genomics, 10, 480.
Supplementary Figure S5

The effect of ValidPrime correction is modest when the contribution of endogenous gDNA on the total signal is low.

Similar experiment as described in Figure 4, with gDNA sensitive and insensitive assays targeting Il1b. Data are from cDNAs obtained from mouse peritoneal macrophages, treated or not with LPS, and measured by qPCR on the Biomark. The gDNA sensitive assay passed the high confidence criteria for gDNA amplification (Supplementary Figure S3). Data are from 81 independent RNA preparations (mean of duplicates). (A) Scatter plot showing the correlation between %DNA (ie the gDNA derived signal as fraction of the total signal expressed in relative quantities (Equation 6)) and CqNA measured with gDNA sensitive assay. Mean and median values refer to %DNA levels. (B) Cq data obtained with the 2 assays, either as CqNA shown in dark blue or as ValidPrime corrected CqRNA in light blue. (C) Table summarizing the effect of ValidPrime correction on the slope and the coefficient of determination (R²) of logarithmic (Cq) data. (D) The impact of ValidPrime correction becomes more evident in the linear scale. Same data as in (B) but presented as inversed relative quantities. (E) Summary of the linear regression analyses on data in (D), illustrating more clearly the positive effect of ValidPrime on linearity.
Absence of correlation between gDNA levels and GOI expression levels.

Data from the experiment described in Figure 4 and Supplementary Figure 5, but with gDNA levels (as measured by VPA qPCR) plotted against the raw signal (CqNA) obtained with gDNA sensitive assays targeting Il1b, Serpine1 or Chi3l3. Data are from 81 cDNAs obtained from mouse peritoneal macrophages, treated or not with LPS ex vivo, and measured by qPCR on the Biomark. The relatively large dispersion of gDNA levels between samples (up to 4.5 Cq difference) is coherent with previous findings in the literature (1).

1. Bustin, S.A. (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol., 29, 23-39.
Supplementary Figure S7

A. **ValidPrime GOI assay validation : ≥4 gDNA conc**

qPCR data (including ValidPrime controls)

GOI assays not amplifying gDNA

- A+ assays

GOI assays amplifying gDNA

- confidence evaluation

High confidence assays

| Grade | %DNA | ValidPrime correction |
|-------|------|-----------------------|
| A     | <3 % | NOT NECESSARY         |
| B     | 3-25 %| YES                   |
| C     | 25-60 %| YES                 |
| D     | >60 %| NO                    |

B. **ValidPrime-validated GOI assays : 1 - 3 gDNA conc**

| Grade | %DNA | VP correction | OutPut Data/Flags |
|-------|------|---------------|-------------------|
| A+    | ND   | -             | CqNA              |
| A     | <3 % | NO            | -                 |
| B     | 3-25 %| YES          | -                 |
| C     | 25-60 %| YES        | -                 |
| F     | >60 %| NO            | -                 |

*ValidPrime GOI assay validation and grading.*

(a) An initial screening with a gDNA dilution series serves two purposes: 1) A robust identification of gDNA insensitive (A+) assays; 2) The classification of GOI assays amplifying gDNA. High confidence assays should amplify gDNA over the entire range of gDNA concentrations, with an estimated efficiency close to that of the VPA (Supplementary Figure S3). The gh-validprime software ([https://code.google.com/p/gh-validprime](https://code.google.com/p/gh-validprime)) attributes grades A,B,C and F to the gDNA sensitive assays depending on the DNA contribution in each qPCR reaction according to the table. (b) Once an assay has been appropriately tested in ValidPrime, High confidence assays and A+ assays can be used with a reduced number of gDNA samples. The table summaries the nature of the default output from the gh-validprime software, which can be used for further pre-analytic data processing.