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Suitable reference genes determination for real time PCR using induced sputum samples

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Take home message

The best reference genes to use for normalization when performing RT-qPCR from sputum cells were assessed.

ABSTRACT

Induced sputum is a non-invasive method, which allows collecting cells from airways. Gene expression analysis from sputum cells has been used to understand the underlying mechanisms of airway diseases such as asthma or Chronic Obstructive Pulmonary Disease (COPD). Suitable reference genes for normalization of target mRNA levels between sputum samples have not been defined so far.

The current study assessed the expression stability of 9 common reference genes in sputum samples from 14 healthy volunteers, 12 asthmatics and 12 COPD patients. Using three different algorithms (geNorm, NormFinder and BestKeeper), we identified HPRT1, GNB2L1 as the most optimal reference genes to use for normalization of RT-qPCR data from sputum cells. The higher expression stability of HPRT1 and GNB2L1 were confirmed in a validation set of patients including 9 healthy controls, 5 COPD and 5 asthmatic patients. In this group, the RNA extraction and RT-PCR methods differed which attested that these genes remained the most reliable whatever the method used to extract the RNA, generate complementary DNA or amplify it. Finally, an example of relative quantification of gene expression linked to eosinophils or neutrophils provided more accurate results after normalization with the reference genes identified as the most stable compared to the least stable and confirmed our findings.

Key words: sputum, qPCR, gene expression, reference genes

Introduction

Induced sputum (IS) is a non-invasive method to collect cells from airways which allows many applications such as measurement of mediators in the supernatant or detailed investigation using the sputum cells [1]. IS has been used in research to analyse gene expression profile and to better understand the pathophysiology of lung diseases. It has been used specifically to help revealing molecular mechanisms of common lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) [2–6]. The development of reverse transcription-polymerase chain reaction (RT-PCR) and microarray techniques allowed to detect various RNA-containing infectious agents in induced sputum with high sensitivity [7–9] and to investigate inflammatory mediators [10–12] and microRNA expression [13,14].

The use of reference genes for normalization of quantitative RT-PCR (RT-qPCR) data is now the method of choice. In the literature, the most frequently found reference genes when performing RT-qPCR analyses using sputum cells are β-actin [15,16], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [17,18] and ribosomal RNA 18S [19,20]. However, optimal reference genes for sputum gene expression analysis have not been explored so far. The choice of reference genes is indeed crucial for RT-qPCR data normalization and should be assessed in each specific experiment or biological sample [21] and it is therefore of prime importance to fill this lack.

For this purpose, we screened 9 commonly used reference genes in sputum cells. As their expression levels can vary according to the airway disease or cell type, we assessed their stability in samples obtained from healthy controls, asthmatic patients and patients suffering from COPD. They exhibit different sputum cellular profiles and there is therefore a need for invariant expression of the chosen reference gene(s). Three different algorithms for identifying the best reference genes among a set of candidates were applied (geNorm, NormFinder and BestKeeper). The experiment was also performed in a new set of patients using different RNA extraction and RT-qPCR protocols. Finally, we used an example of relative quantification of target genes (IL-5 and CXCL8) known to be linked to eosinophils [22] and neutrophils recruitment [23] respectively, to attest that the choice of stable endogenous reference genes is crucial to obtain unbiased results from RT-qPCR using sputum cells.

Material and methods

Subjects

The characteristics of the patients are given in Table 1. Asthmatic and COPD patients were recruited through the outpatient clinic and pulmonary rehabilitation centre (CHU, Sart-Tilman, Liege). Asthma was diagnosed as described in the GINA guidelines (http://ginasthma.org/). Mild to moderate asthma were defined as patients without maintenance treatment or with a low to moderate dose of inhaled corticosteroids (ICS; <1000 µg beclomethasone/day) and had $FEV_1 \geq 80\%$ predicted. Severe and refractory asthma were defined according to ATS criteria [24]. Diagnosis of COPD was made according to GOLD criteria obtained from the Global Initiative for Chronic Obstructive Lung Disease (http://goldcopd.org/). All asthmatics and COPD patients were recruited during stable state of the disease. Healthy volunteers were enrolled by advertisement among the hospital and staff. This study was approved by the local ethics Committee of CHU Liège and all subjects gave written informed consent for participation.

Study design

The objective of the study was to determine the most reliable reference gene(s) to use in RT-qPCR experiments using induced sputum samples. As recommended in the manual of geNorm, NormFinder and BestKeeper, we performed comparisons of more than 8 commonly used reference genes in groups of patients greater than 10 subjects including asthmatics, COPD and healthy subjects to obtain confident results. The 9 chosen reference genes were β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2-Microglobulin (B2M), β-Glucuronidase (GUSB), Hypoxanthine ribosyltransferase 1 (HPRT1), Guanine nucleotide-binding protein, b-peptide 2-like 1 (GNB2L1), TATA-box binding protein (TBP), Ribosomal protein L13A (RPL13A) and ribosomal RNA 18S (RNA18S) and were selected because they belong to different biological pathways and are then presumably not co-regulated.

Sputum induction and processing

The sputum was induced and processed as previously described [4,25]. Cell viability was assessed by trypan blue exclusion and the differential leukocyte count was performed on cytospins stained with Rapi Diff II Stain Kit (Atom Scientific, Manchester, UK) on 500 cells.

All samples were selected according to following selection criteria: < 30% of squamous cells and viability > 50%. These criteria has been determined in our lab as the optimal threshold to obtain reliable expression results. The cell pellet (median number of cells ((25-75%)): 1.8 (1.2-2.2) 10^6) was mixed with 5 volumes of RNAprotect cell reagent (Qiagen, Hilden, Germany) and kept at -80 °C until RNA extraction.

RNA extraction and RT-qPCR methods

These steps were performed according to the description of da Silva et al. [5] except that the Taqman PCR step was achieved in 96-well plates allowing sample maximization approach. All these procedure information were given according to the MIQE guidelines for the minimum information required for a qPCR experiment [26] (All the experimental procedure is included in the checklist in the online supplementary material).

Validation experiment

A new experiment was performed to analyze the stability of 7 genes out of the 9 previously assessed (ACTB, GAPDH, B2M, HPRT1, GNB2L1, RPL13A and RNA18S) in sputum collected from 9 healthy controls, 5 COPD and 5 asthmatic patients. Ribosomal Protein L32 (RPL32) was added in the panel as it was previously shown to be the most stable reference gene to use for bronchoalveolar lavage (BAL) cells [27]. The characteristics of the patients are given in Table 1 in the online supplementary material.

In contrast to the first experiment where the RNAs were isolated using trizol and phenol-chloroform extraction method followed by washing and elution on RNA binding column, the RNAs were directly extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All the materials cited afterwards are from Thermo Fisher Scientific, Wilmington, USA. Genomic DNA contamination was eliminated by a treatment with TURBO DNA free^{TM} kit from Ambion. The reverse transcription was performed starting from 500 ng of RNA with High Capacity cDNA Reverse Transcription kit according to the manufacturer's protocol. The qPCR was achieved with the Taqman Universal master mix II. The cDNA was loaded on custom TaqMan low density array (384 wells plate) as provided by the manufacturer's instructions. The plates were read using 7900 HT Fast Real-Time PCR System. The primers and probes and the efficiencies and specificities are included in the MIQE checklist file in the online supplementary material. The 3 algorithms were applied as for the primary experiment.

Immune cells correlation

In order to validate the findings in a real application, we proceeded to a relative quantification of genes correlated with eosinophils and neutrophils (IL5 and CXCL8 respectively). This experiment was performed with samples from 6 healthy controls, 6 asthmatics and 6 COPD patients (characteristics of the patients in Table 2 in the online supplementary material). The COPD patients exhibited an intense neutrophilic inflammation and the asthmatic cohort showed a high sputum eosinophil and neutrophil percentage. The RNA extraction and RT-qPCR procedures were performed as described for the first cohort. Relative quantification in gene expression compared to healthy controls was determined using qbase^{+} qPCR analysis software (Biogazelle, Zwijnaarde, Belgium) in accordance with the target specific amplification efficiency values.

Statistics

Three different algorithms were used to assess the stability of the 9 reference genes. GeNorm (implementation in qbase+) uses a normalization strategy which provides a ranking of the candidate genes according to an average stability value (M) of remaining reference genes calculated during stepwise exclusion of the least stable reference gene [28]. NormFinder, available as an excel add-in, is based on the analysis of overall gene expression variation and the variation between sample subgroups [29]. BestKeeper software, available as an excel based tool, determines the optimal reference gene with pairwise correlation analysis of all pairs of candidate genes and calculates the geometric mean of the best ones [30].

The demographic and functional characteristics of the patients were expressed as mean ± SD and comparisons between groups were performed by one-way ANOVA followed by Tukey's multiple comparisons test for continuous variables. Chi-square test was applied for categorical analyses. Sputum cell counts were expressed as median (25%-75%). Comparisons between groups were performed by Kruskal-Wallis test followed by Dunn's multiple comparisons test. In the application example, the relative expression between groups of subjects was analyzed in the same manner and with Mann-Whitney test when 2 groups were compared. Correlations were tested with Spearman's rank correlation analysis. Statistical analyses were carried out with Graphpad Prism 7.0 (Graphpad Software San Diego, CA, USA). Differences were considered statistically significant when a two-sided p-value was < 0.05.

Results

Baseline characteristics of the patients

The 3 groups of patients were well matched according to gender and age but not for tobacco habits and treatments. Regarding sputum cell counts, the COPD patients exhibited lower percentage of macrophages but higher proportion of neutrophils than controls. COPD had also a higher proportion of eosinophils than controls but to a lesser extent than asthmatic patients.

Raw quantification cycle (Cq) distribution of the candidate reference genes

The raw Cq distribution of candidate reference genes are presented in Figure 1. They displayed a wide range level, from 15.7 cycles (15.0-16.6) for RNA18S to 33.0 (32.0-35.2) for HPRT1.

Reference gene expression stability evaluation

The ranking obtained with the 3 algorithms are combined in Table 2. The M values obtained in the geNorm pilot experiment was low for the best reference gene and high for most unstable genes. In addition, the NormFinder algorithm gave a stability value for each candidate gene, the lowest being considered as the best. Finally, BestKeeper software combined all the candidate normalization genes into an index and analyzed the correlation of this index with each individual gene. The most appropriate genes had the greatest correlation coefficient values.

The final ranking was computed by the addition of each individual rank obtained with the 3 algorithms. It appeared that GNB2L1 and HPRT1 were the reference genes identified as the most suitable. These 3 analyses showed slight differences only and the 2 genes exhibiting the greater variation were the same for all (namely GAPDH and RNA18S) .

Validation experiment

The 3 algorithms were applied on the data obtained with a new set of patients and the results were combined in Table 3 in the online supplement. Even if GAPDH was ranked as the candidate gene with the highest stability, HPRT1 and GNB2L1 still occupied the next top positions. Ribosomal 18S and RPL32 were classified as the least stable genes.

Immune cells correlation

The relative quantification of IL5 and CXCL8 gene expression using either HPRT1 (shown as the most suitable) or RNA18S (shown as the most unstable) as reference genes in a new patients set was performed. As shown in Figure 2, the relative expression changed drastically depending on whether the normalization was made with RNA18S or HPRT1. Indeed, we

observed that, even if the quantification using RNA18S did not show any difference between groups (Kruskal-Wallis test: $p=0.82$), the relative quantification based on HPRT1 gave significant differences (Kruskal-Wallis test <0.05). When the data obtained from controls and asthmatics patients were compared, the results appeared significant (Mann-Whitney test: $p<0.05$). The correlation between the IL-5 expression and eosinophil percentage was non-significant when RNA18S was used and became significant when HPRT1 was applied ($r=0.63$, $p<0.05$).

As for CXCL8 gene expression normalized with RNA18S, the Kruskal-Wallis test gave a $p<0.001$ and the Dunn's multiple comparison tests were significant for controls vs COPD ($p<0.01$) and asthmatics vs COPD ($p<0.05$). In contrast, when the normalization was done with HPRT1, the Kruskal-Wallis test gave a $p<0.0001$ and the Dunn's multiple comparison tests were significant for controls vs COPD only ($p<0.001$). When the data obtained from controls and asthmatics patients were compared by Mann-Whitney test, the p value was significant too ($p<0.01$) as well as when the asthmatics and COPD patients were compared ($p<0.01$). The correlation between CXCL8 expression and the neutrophil percentage was more pronounced once the normalization was made with HPRT1 ($r= 0.9$, $p<0.0001$) instead of RNA18S ($r=0.77$, $p<0.001$). We observed a trend for a positive correlation between CXCL8 expression and the eosinophil percentage only when the data were quantified with HPRT1 ($r=0.37$, $p=0.13$).

When the normalization was done with both HPRT1 and GNB2L1 compared to RNA18S and GAPDH (Figure 3), the Kruskal-Wallis test was significant for IL5 and the Dunn's test gave a results < 0.05 for the comparison between controls and asthmatic patients.

Regarding CXCL8, the results were similar when normalized with HPRT1 and GNB2L1 compared to RNA18S and GAPDH and did not differ from those obtained with HPRT1 alone.

Discussion

Comparisons of gene expressions from sputum samples of controls, asthmatic and COPD patients are frequent. However, until now, information about the most suitable reference genes to normalize this kind of data are missing. This study is, to the best of our knowledge, the first to investigate the most appropriate reference genes to use when performing RT-qPCR analysis using sputum cells. For this purpose, 9 common reference genes known to be involved in distinct functions were chosen. Using the 3 algorithms, we found that GNB2L1 and HPRT1 were the most suitable reference genes to use in this context. Both were validated in another independent group of patients where the RNA extraction and RT-qPCR methods

differed. They were already shown as the most stably expressed reference genes in alveolar macrophages of COPD patients whatever the disease severity [31] and in isolated human neutrophils [32]. HPRT1 was already shown to be the most stably expressed reference gene in other systems but data regarding GNB2L1 appeared limited (see Table 4 in the online supplementary material). Based on the expression level, HPRT1 would be more suitable for low abundance transcripts in IS, GNB2L1 being more appropriate in case of higher abundance transcripts.

Even if commonly used in the context of sputum cells, GAPDH, β-actin and RNA18S did not appear as good candidate reference genes. In a previous study, GAPDH and β-actin were shown as unstable in BAL and bronchial biopsies from asthmatic patients due to different cellular profiles and activation status [33]. It is interesting to note that GAPDH is classified as one of the most variable reference gene in the first cohort and as the most stable in the validation cohort. This discrepancies may be explained by different reasons. Indeed, GAPDH is implicated in many cellular process and has many functions in addition to its glycolytic activity. Furthermore, the use of inhaled corticosteroids appeared to influence the expression level of GAPDH [33] and the proportions of patients treated with inhaled corticosteroids is different inside our 2 cohorts. Finally, the fact that the primer sequences are different between the 2 experiments could also explain the variability of our results.

In the same manner, even if the ribosomal RNA level variation is supposed to be low compared to mRNA, it is also regulated according to the cell type, the functional state and it varies between different individuals. Its really high abundance and nature limit also its use for mRNA normalization. Moreover, ribosomal RNA is thought to be less affected by RNA degradation compared to other genes and may not serve as a good endogenous control in this regard [34].

Finally, a practical example of relative quantification using RNA18S (classified as the most unstable gene by the 3 algorithms) compared with HPRT1 (identified as the most suitable) showed contrasting results and highlighted the importance of the reference gene choice. Indeed, some differences can be hidden and the interpretation of the results may be mistaken when the normalization is done using genes with variable expression. The expression of IL-5 is known to be linked with eosinophil recruitment and highly expressed in sputum of asthmatic patients [12,35] compared to healthy volunteers. CXCL8, for its part, was found to be increased in COPD [36,37] but also in patients with asthma where it participates to the neutrophil [23,38] and eosinophil chemotaxis [39] as shown previously and attested by our positive correlations. Although normalization against a single reference gene is acceptable

when there is a validation of its stability under the experimental conditions [26], the use of two reference genes is recommended to limits errors and increase the results accuracy [40]. In our experiment, even if the use of two reference genes gave the same results for CXCL8 when the normalization was performed with the two most stable genes compared to the two most unsuitable, the results for IL5 were still remarkably different.

These results could be confirmed using other technologies such as Droplet Digital PCR assay, NanoString, microarray or massive parallel RNA sequencing. Indeed, the use of reference genes is not mandatory as they provide additional normalization strategies. However, the applicability of these emerging technologies is restrained as not all research centers are equipped. The other issue is the extensive bioinformatic analyses linked to these technics. Nevertheless, It is important to note that qPCR remains the gold standard for expression analysis and is used to confirm results from high-throughput analyses.

The limitation of our study is the low number of patients which did not allow sub-group analyses. For this reason, the authors would recommend the validation of the reference genes prior to their use in an experimental protocol comparing patients with different treatments, tobacco habits or disease severity.

In conclusion, GNB2L1 and HPRT1 are the most ideal reference genes to use for RT-qPCR data normalization when working with induced sputum and are not affected by airway diseases, sputum cellular composition, RNA extraction and RT-qPCR methods.

Competing interest

Authors have no conflicts to disclose.

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Table 1: Demographic and functional characteristics

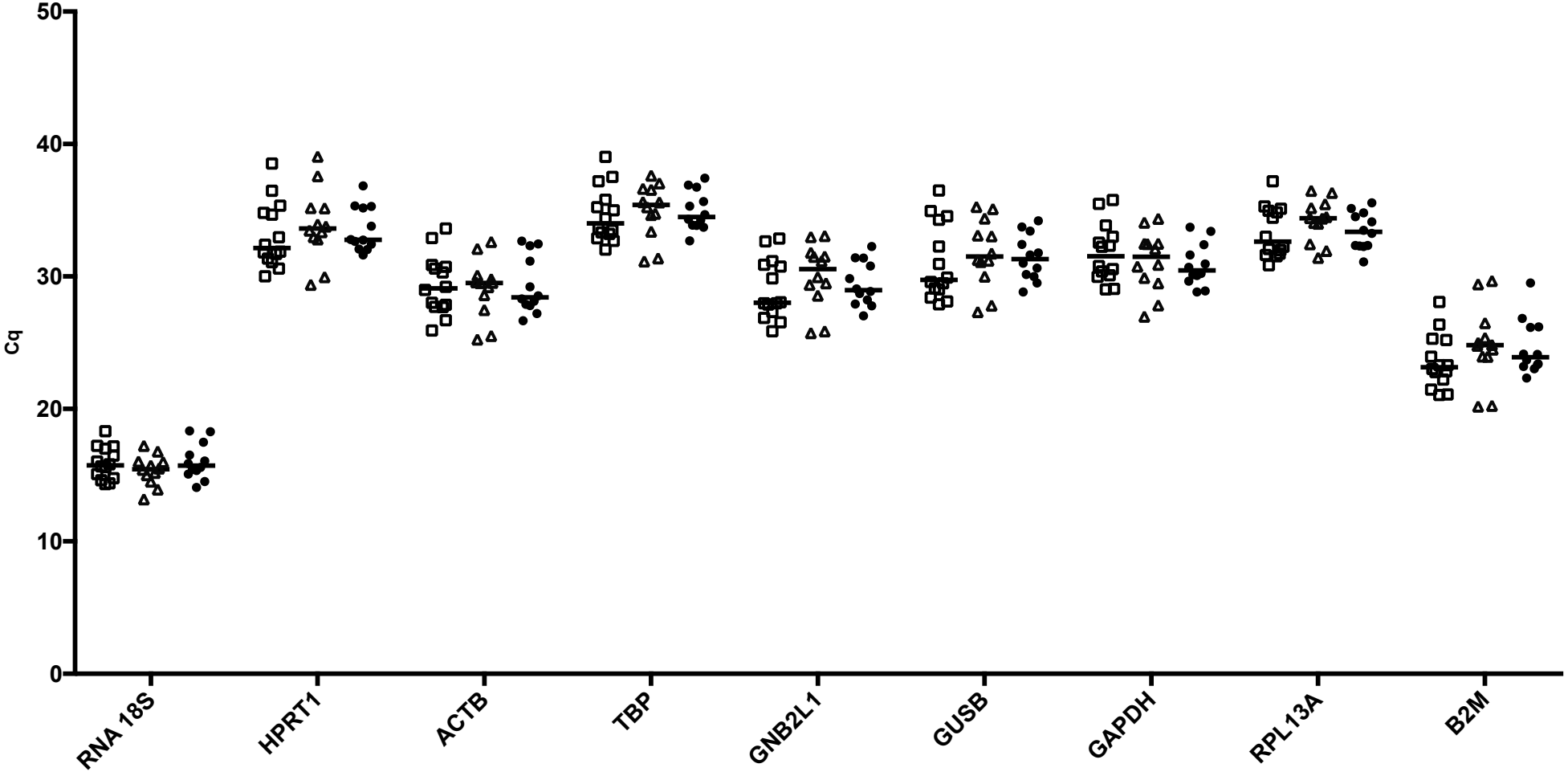
	Control	**COPD**	**Asthma**	P value
Subjects (n)	14	12	12	
Age (years)	48 ± 15	58 ± 13	55 ± 11	0.15
Sex (m/f)	6/8	6/6	4/8	0.71
Tobacco status (ns/es/cs)	9/5/0	0/5/7	5/6/1	<0.001
Pack years	6 ± 10	61 ± 27	14 ± 11	<0.0001
Treatment				
ICS (yes/no)	0/14	7/5	11/1	<0.0001
LABA (yes/no)	0/14	9/3	6/6	<0.001
LAMA (yes/no)	0/14	6/6	0/12	<0.001
SABA (yes/no)	0/14	0/12	3/9	<0.05
LTRA (yes/no)	0/14	1/11	4/8	<0.05
Severity status		3 GOLD 1 8 GOLD 2 1 GOLD 3	7 mild-moderate 5 severe-refractory	
FEV1(% predicted)	108 ± 11	63 ± 14***$	80 ± 18***	<0.0001
FEV1 post BD(% predicted)	111 ± 17	70 ± 15***	82 ± 19**	<0.0001
FVC (% predicted)	114 ± 13	89 ± 13***	92 ± 15***	<0.0001
FEV1/FVC (%)	80 ± 6	57 ± 9***$$	71 ± 8*	<0.0001
Sputum				
Squamous cells (%)	21.0 (4.7-27.5)	2.5 (0.0-10.5) *	4.0 (1.2-13.5)	<0.01
Total non-squamous (10^6/g)	1.0 (0.3-1.9)	3.3 (1.2-8.1)	4.6 (2.2-9.8) **	<0.01
Viability (%)	77.0 (68.5-81.8)	75.0 (64.5-86.5)	75.0 (62.0-81.0)	0.68
Macrophages (%)	37.6 (23.9-64.7)	18.3 (4.5-27.2) *	20.4 (11.2-25.8)	<0.01
Neutrophils (%)	53.7 (28.1-68.7)	69.6 (54.6-86.1)	39.0 (24.3-81.0)	<0.05
Lymphocytes (%)	1.9 (0.9-2.4)	2.4 (0.5-5.0)	0.5 (0.4-3.3)	0.50
Eosinophils (%)	0.0 (0.0-0.4)	2.3 (0.3-4.4) *	21.4 (1.8-44.2) ***	<0.001
Epithelial cells (%)	2.8 (2.3-6.2)	1.1 (0.4-4.8)	5.8 (3.6-7.7)	0.15

COPD: Chronic Obstructive lung disease, ns: non-smoker, es: ex-smoker, cs: current smoker, ICS: inhaled corticosteroids, LABA: long acting beta agonist, LAMA: long acting muscarinic antagonist. SABA: short acting beta agonist, LTRA: leukotriene receptor antagonist. FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity, post BD: post bronchodilation. Results are expressed as mean ± SD or median (IQR). * <0.05, ** <0.01, *** < 0.001 vs healthy subjects. $ <0.05, $$ <0.01, $$$ < 0.001 vs asthmatic patients.

Table 2: Reference gene expression stability obtained with the 3 algorithms.

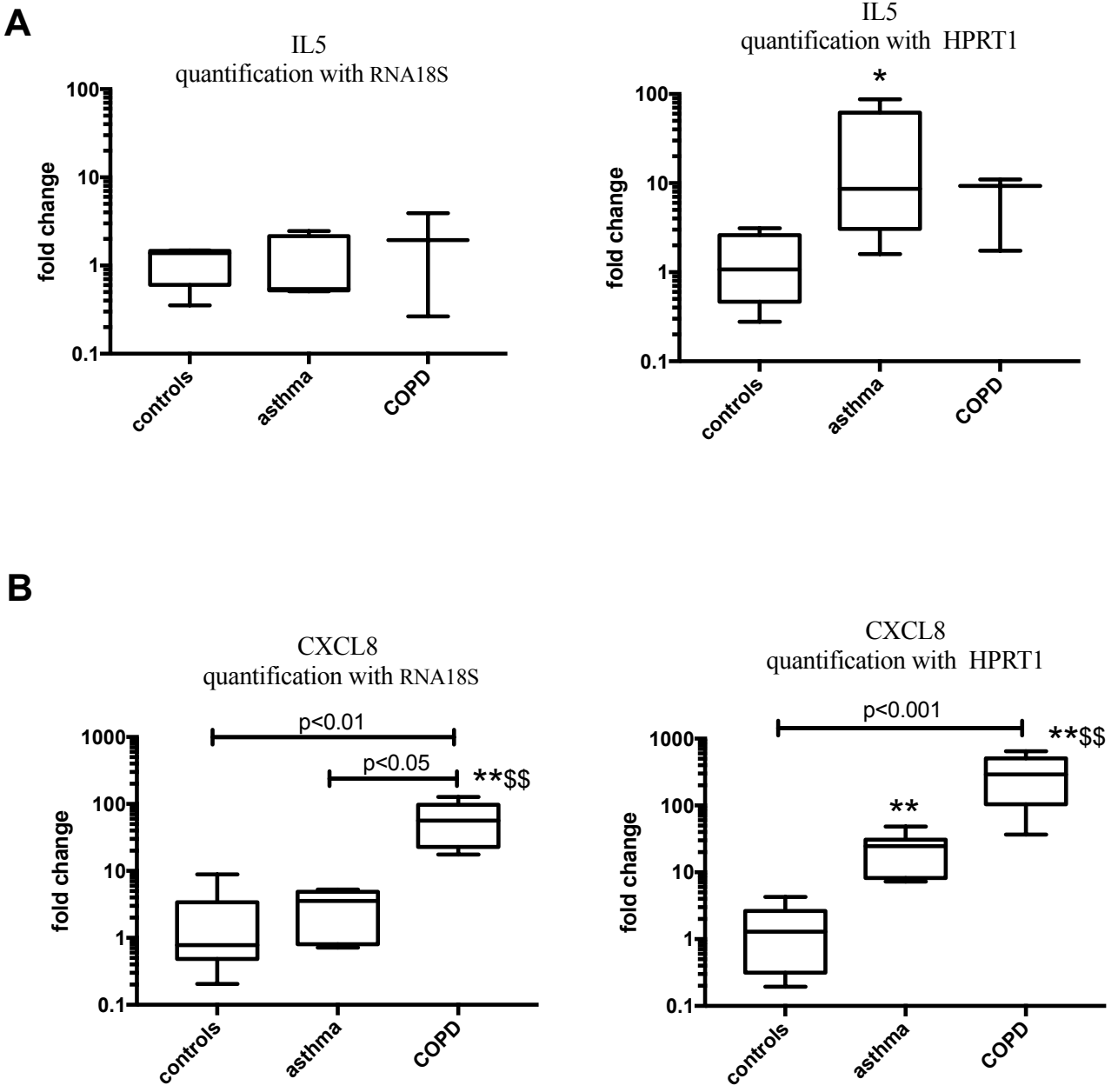
Gene	geNorm (M)	rank	NormFinder	rank	BestKeeper (r^+)	rank	Final rank
GNB2L1	0.717	1	0.275	4	0.95	1	1
GUSB	0.725	2	0.260	3	0.91	6	3
HPRT1	0.739	3	0.246	2	0.94	2	2
RPL13A	0.887	4	0.279	5	0.93	4	4
B2M	1.01	5	0.405	7	0.92	5	7
ACTB	1.119	6	0.309	6	0.94	3	5
TBP	1.175	7	0.230	1	0.91	7	6
GAPDH	1.227	8	0.411	8	0.89	8	8
RNA18S	1.336	9	0.423	9	0.73	9	9

Figure 1: Cq values of the 9 candidate reference genes in the 38 sputum samples.



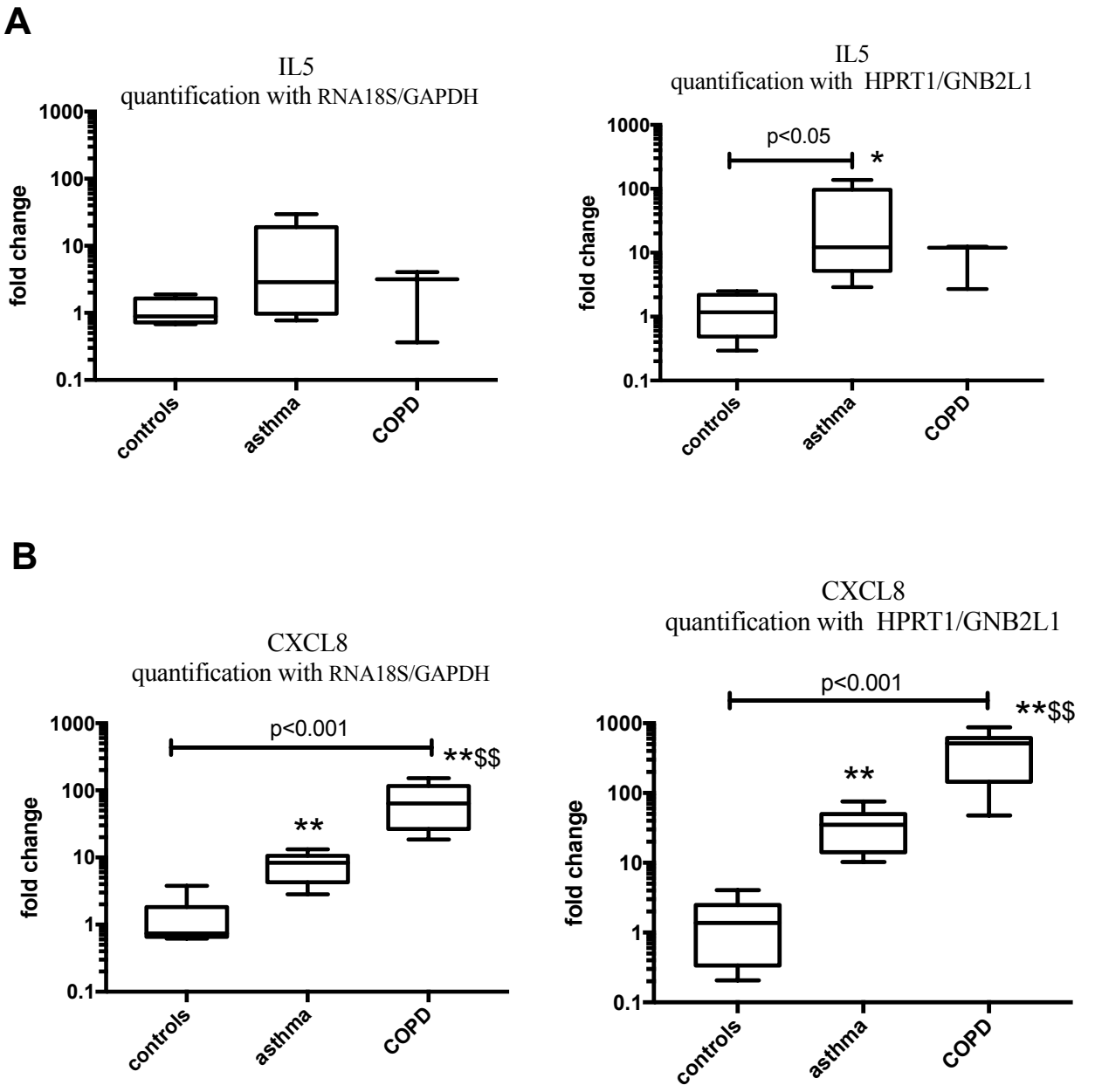
□ Healthy controls, ● COPD and Δ asthmatic patients. The median is indicated by a bar. RNA 18S: 18s ribosomal RNA; HPRT1: Hypoxanthine ribosyltransferase 1; ACTB: β-Actin; TBP: TATA-box binding protein; GNB2L1: Guanine nucleotide-binding protein, b-peptide 2-like 1; GUSB: β-Glucuronidase ; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RPL13A: Ribosomal protein L13A; B2M: β2 Microglobulin

Figure 2: Comparison of HPRT1 and RNA18S for normalizing IL5 (A) or CXCL8 (B) gene expression.



The comparisons were made with by Kruskal-Wallis test followed by Dunn's multiple comparisons test (bars) and with Mann-Whitney test (* $p<0.05$; ** $p<0.01$ vs healthy subjects. $ <0.05, $$ <0.01 vs asthmatic patients.) when 2 groups were compared. Control patients n=4, asthmatic patients n=5 and COPD patients n=3.

Figure 3: Comparison of HPRT1/GNB2L1 and RNA18S/GAPDH for normalizing IL5 (A) or CXCL8 (B) gene expression.



The comparisons were made with by Kruskal-Wallis test followed by Dunn's multiple comparisons test (bars) and with Mann-Whitney test (* $p<0.05$; ** $p<0.01$ vs healthy subjects. $ <0.05, $$ <0.01 vs asthmatic patients.) when 2 groups were compared. Control patients n=4, asthmatic patients n=5 and COPD patients n=3.

Online supplementary material: MIQE checklist (essential information)

Experimental design

-Definition of experimental and control groups: Controls, asthmatic and COPD patients are defined in the main body text.
- Number within each group : 14 healthy volunteers, 12 asthmatics and 12 COPD patients.

Sample

-Description : Sputum cells obtained after sputum processing in the laboratory which was made within 3 hours after induction. The sputum was kept at 4°C before being processed. The cell pellet (median number of cells ((25-75%)): 1.8 (1.2-2.2) 10^6) was mixed with 5 volumes of RNAprotect cell reagent (Qiagen, Hilden, Germany) and kept at -80 °C until RNA extraction. Applied quality criteria were: < 30% of squamous cells and viability > 50%.

-Microdissection or macrodissection: /

-Processing procedure: The whole sputum was collected in a plastic container, weighed, and homogenized with three volumes of phosphate-buffered saline (PBS), vortexed for 30 s, and centrifuged at 800g for 10 min at 4° C. The supernatant was separated from the cell pellet by filtration through 2 layers of sterile gauze. A mucolysis was performed by adding an equal volume of 6.5 mM dithiothreitol and the suspension was rocked during 20 min. After a centrifugation of 10 min at 550g, the squamous cells and total cell counts as well as the cell viability were checked by trypan blue exclusion with a manual hemocytometer. The differential leukocyte count was performed on cytospins stained with May–Grünwald–Giemsa on 500 cells.

-If frozen, how and how quickly? The cell pellets in RNAprotect cell reagent were frozen at -80°C right after the sputum processing.

-If fixed, with what and how quickly? /

-Sample storage conditions and duration : The samples remained at -80°C until RNA extraction (mean of storage: 4 years).

Nucleic acid extraction

-Procedure and/or instrumentation: The cell pellet was resuspended in Tripure isolation reagent (Roche, Pleasanton, CA, USA), and was homogenized using a bead (Stainless Steel Bead, 5mm, Qiagen) and a tissue Lyser II (Qiagen) for 2 min at 25 Hz. The RNA was then separated by phenol-chloroform extraction. The upper phase (300 µl) was diluted in an equal volume of ethanol and transferred to a RNA binding column of the NucleoSpin RNA Clean Up kit (Macherey Nagel, Düren, Germany). The washing and elution steps were performed according to the manufacturer's protocol.

-Name of kit and details of any modifications: NucleoSpin RNA Clean Up kit (Macherey Nagel, Düren, Germany).

-Details of DNase or RNase treatment: The genomic DNA was eliminated using the TURBO DNA free kit of Ambion (Thermo Fisher Scientific, Wilmington, USA) according to the manufacturer's protocol.

-Contamination assessment (DNA or RNA): To test for potential genomic DNA contamination, we performed for each sample a supplementary PCR from RNA for all genes. We did not get any amplification signal for any gene except for RN18S. For this gene, Cq values obtained from RNA samples (mean ± SD: 35.06 ±0.05) differed by more than 20 cycles from Cq values obtained from cDNA samples (mean ± SD: 14.81 ± 0.01). This corresponds to a gDNA contamination of 0.00001% if we consider a E of 2 (=100%). We then considered that this contamination was negligible. Finally we used primers located on exon-exon junction, which theoretically exclude genomic DNA as template.

-Nucleic acid quantification: 32 (20-67) ng/µl

-Instrument and method: The RNA concentration and purity were assessed by Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

-RNA integrity: method/instrument: The RNA integrity was assessed using an Agilent RNA 6000 Pico kit on an Agilent 2100 Bioanalyzer instrument (Agilent, Santa Clara, USA). RNAs were first diluted at approximately 1ng/µl to fulfill Pico Kit concentration range requirements.

-RIN/RQI or Cq of 3' and 5' transcripts: mean RIN ± SD: 6 ± 2

-Inhibition testing (Cq dilutions, spike, or other): No inhibition was noted concerning the results of PCR after samples dilution (see efficiency section).

Reverse transcription

-Complete reaction conditions: This reaction was performed using the Quantitect Reverse Transcription kit of Qiagen. The first step is another step of genomic DNA elimination. It was performed as followed: 2 µl of gDNA Wipeout Buffer 7X, 8.5 µl of RNA, 3.5 µl of RNAse-free water for a total reaction volume of 14 µl. After an incubation of 2 min at 42°C, the tubes were directly placed on ice.

-Amount of RNA and reaction volume, priming oligonucleotide and reverse transcriptase: The cDNA was prepared from maximum 1µg of RNA. The 14 µl of template RNA was mixed with 1µl of Quantiscript Reverse Transcriptase, 4 µl of Quantiscript RT Buffer 5X and 1 µl of RT primer mix (oligo-dT and random) for a total volume reaction of 20 µl.

-Temperature and time: Incubation of 15 min at 42°C and 3 min at 95°C to inactivate the Quantiscript Reverse Transcriptase.

-Cqs with and without reverse transcription: see genomic DNA contamination estimation and efficiency evaluation section.

qPCR target information

-Gene symbol: see table below.
-Sequence accession number: see table below

-Amplicon length:

RNA18S: 67 bp
ACTB: 110 bp
GAPDH: 143 bp
B2M: 143 bp
GUSB: 128 bp
HPRT1: 128 bp
GNB2L1: 147 bp
TBP: 104 bp
RPL13A: 90 bp

-In silico specificity screen (BLAST, and so on): Using UCSC genome browser, in silico PCR,
Max product size: 20,000
Min perfect match: 15
Min good match: 15
Genome: Human
Assembly: GRCh38/hg38
Target: genome assembly

we found:

RNA18S: 4 matches when using Gencode Genes as target.
ENST00000627981.1__FP236383.3:663-729 67bp F : CGCCGCTAGAGGTGAAATTCT R: CATTCTTGGCAAATGCTTTCG
ENST00000625598.1__FP236383.2:663-729 67bp F : CGCCGCTAGAGGTGAAATTCT R: CATTCTTGGCAAATGCTTTCG
ENST00000629969.1__FP671120.4:663-729 67bp F : CGCCGCTAGAGGTGAAATTCT R: CATTCTTGGCAAATGCTTTCG
ENST00000631211.1__FP671120.3:663-729 67bp F : CGCCGCTAGAGGTGAAATTCT R: CATTCTTGGCAAATGCTTTCG

ACTB: one match
chr7:5529603+5530572 970bp F : CCTTGCACATGCCGGAG R: ACAGAGCCTCGCCTTTG

GAPDH: one match
chr12:6534817-6536591 1775bp F : TGTAGTTGAGGTCAATGAAGGG R: ACATCGCTCAGACACCATG

B2M: two matches
chr15_KI270849v1_alt:38935-39704 770bp F: ACCTCCATGATGCTGCTTAC R: GGACTGGTCTTTCTATCTCTTGT

chr15:44715589-44716358 770bp F : ACCTCCATGATGCTGCTTAC R: GGACTGGTCTTTCTATCTCTTGT

GUSB: one match
chr7:65964383+65967782 3400bp F : GTTTTTGATCCAGACCCAGATG R: GCCCATTATTCAGAGCGAGTA

HPRT1: one match
chrX:134493572-134498669 5098bp F : GTATTCATTATAGTCAAGGGCATATCC R: AGATGGTCAAGGTCGCAAG

GNB2L1: 15 matches when the target used was Gencode Genes
ENST00000511566.5__RACK1:493-639 147bp F: TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000513060.5__RACK1:1094-1240 147bp F : TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000502548.5__RACK1:529-984 456bp F: TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000506312.3__RACK1:403-549 147bp F: TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000502844.5__RACK1:1121-1267 147bp F: TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000507756.5__RACK1:205-351 147bp F : TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA

ENST00000512805.5__RACK1:822-968 147bp F : TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000504325.5__RACK1:506-652 147bp F : TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000503081.1__RACK1:447-587 141bp F : TCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000510199.5__RACK1:757-903 147bp F : TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000514183.5__RACK1:355-501 147bp F : TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000504128.5__RACK1:132-278 147bp F : TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000511473.5__RACK1:541-687 147bp F : TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000376817.8__RACK1:384-530 147bp F : TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA
ENST00000507000.5__RACK1:392-538 147bp F : TGGTCTTCAGCTTGCAGTTAG R:
GCAAATACACTGTCCAGGATGA

TBP: one match
chr6:170554413-170556934 2522bp F : CAGCAACTTCCTCAATTCCTTG R:
GCTGTTTAACTTCGCTTCCG

RPL13A: one match
chr13:54441231+54441320 90bp F : TACTTCACTGTTTAGCCACGAT R:
CGAAGATGGCGGAGGTG

-Location of each primer by exon or intron:

RNA18S: 1-1
ACTB: exon 1-2
GAPDH: exon 2-3
B2M: exon 2-4
GUSB: exon 10-11
HPRT1: exon 6-8
GNB2L1: exon 3-5
TBP: exon 1-2
RPL13A: exon 1a-4

-What splice variants are targeted?
RNA18S: Detect the 5 sub-units SN1, SN2, SN3, SN4, SN5 of 18S, and sub-units of 45S: SN1, SN2, SN3 and SN5 and 28S sub-unit SN5.
ACTB: Detect all variants

GAPDH: Detect all variants
B2M: Detect all variants
GUSB: Detect all variants
HPRT1: Detect all variants
GNB2L1: Detect all variants
TBP: Only the transcript NM_003194
RPL13A: Only the transcript NM_001270491

qPCR oligonucleotides

-Primers and probes sequences:
They were all labeled with reporter and double-quencher dyes 5'6-carboxyfluorescein/ZEN/3' Iowa black FQ (5'6-FAM/ZEN/3'IBFQ) and were obtained from IDT (Integrated DNA Technologies, Skokie, IL, USA).

Gene name (abbreviation)	**Accession number**		**Sequence**
18s ribosomal RNA (RNA18S)	NR_003286	Forward Reverse probe	CGC CGC TAG AGG TGA AAT TCT CAT TCT TGG CAA ATG CTT TCG ACC GGC GCA AGA CGG ACC AGA
β-Actin (ACTB)	NM_001101	Forward Reverse probe	CCT TGC ACA TGC CGG AG ACA GAG CCT CGC CTT TG TCA TCC ATG GTG AGC TGG CGG
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_002046	Forward Reverse probe	TGT AGT TGA GGT CAA TGA AGG G ACA TCG CTC AGA CAC CAT G AAG GTC GGA GTC AAC GGA TTT GGT C
β2-Microglobulin (B2M)	NM_004048	Forward Reverse probe	ACC TCC ATG ATG CTG CTT AC GGA CTG GTC TTT CTA TCT CTT GT CCT GCC GTG TGA ACC ATG TGA CT
β-Glucuronidase (GUSB)	NM_000181	Forward Reverse probe	GTT TTT GAT CCA GAC CCA GAT G GCC CAT TAT TCA GAG CGA GTA TGC AGG GTT TCAC CAG GAT CCA C
Hypoxanthine ribosyltransferase 1 (HPRT1)	NM_000194	Forward Reverse probe	GTA TTC ATT ATA GTC AAG GGC ATA TCC AGA TGG TCA AGG TCG CAA G TGG TGA AAA GGA CCC CAC GAA GT
RACK1 (receptor for activated C kinase 1) or Guanine nucleotide-binding protein, b-peptide 2-like 1 (GNB2L1)	NM_006098	Forward Reverse probe	TGG TCT TCA GCT TGC AGT TAG GCA AAT ACA CTG TCC AGG ATG A TGG GTG TCT TGT GTC CGC TTC TC
TATA-box binding protein (TBP)	NM_003194	Forward Reverse probe	CAG CAA CTT CCT CAA TTC CTT G GCT GTT TAA CTT CGC TTC CG TGA TCT TTG CAG TGA CCC AGC ATC A
Ribosomal protein L13A (RPL13A)	NM_001270491	Forward Reverse probe	TAC TTC ACT GTT TAG CCA CGA T CGA AGA TGG CGG AGG TG AGG TCC TGG TGC TTG ATG GTC G

-Location and identity of any modifications: /

qPCR protocol

-Complete reaction conditions: Every qPCR were realized in duplicate and included non-template controls as well as no reverse transcription control for each gene. The reactions were performed in 96-well plates allowing sample maximization approach.

-Reaction volume and amount of cDNA/DNA: For each sample, 2 µl of cDNA was used in a total reaction volume of 12.5 µl (6.25 µl of master mix, 3.626 µl of RNAse free water, 0,625 µl of mix 20X primers + probe).

-Primer, (probe), $Mg2^{+}$, and dNTP concentrations: 500 nM forward primer, 500 nM reverse primer and 250 nM probe for all the tested genes except for RNA18S for which 300 nM were used for the primers and 175 nM for the probe. The master mix 2X of the Quantitect Probe PCR Kit contains dNTP mix including dUTP, 8mM MgCl2, ROX passive reference dye, Quantitect Probe PCR Buffer and HotStar Taq DNA polymerase.

-Polymerase identity and concentration: HotStar Taq DNA polymerase 2X

-Buffer/kit identity and manufacturer (+ plate and seal). The Taqman PCR step was achieved using the Quantitect Probe PCR Kit of Qiagen. The white plates used were Framestar 480/96 for Roche Lightcycler 480, with qPCR Adhesive seal (product code 4ti-0952) and were purchased at Bioké (Leiden, The Netherlands).

-Additives (SYBR Green I, DMSO, and so forth): /

-Complete thermocycling parameters: Amplification was performed on the LightCycler 480 Real-Time PCR (Roche, Pleasanton, CA, USA) during 45 cycles as follows: initial activation step: 95 °C for 15 min; denaturation stage: 94 °C for 15 s; annealing and elongation stage: 60 °C for 1 min.

-Manufacturer of qPCR instrument: LightCycler 480 Real-Time PCR (Roche, Pleasanton, CA, USA)

qPCR validation

-Specificity (gel, sequence, melt, or digest): see gel below as well as the fragment sizes in the QIAxcel (Qiagen, Hilden, Germany) report (electrophoresis profiles).

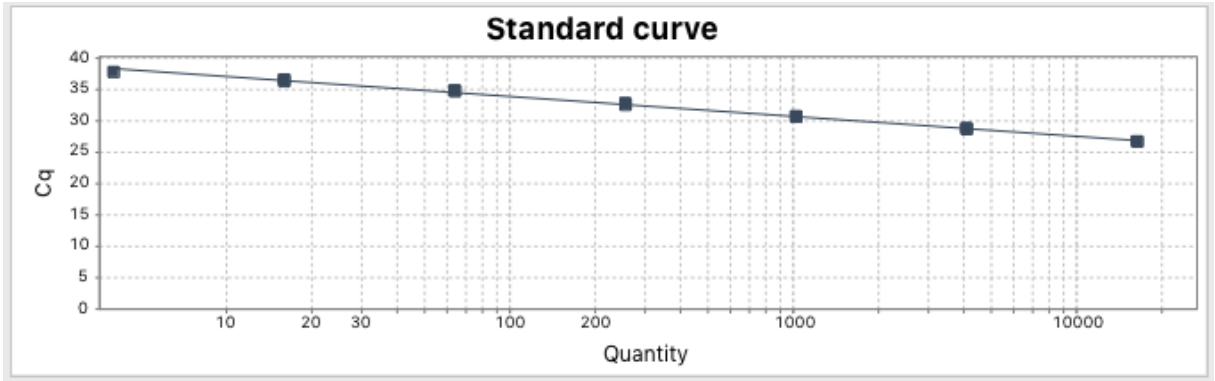
-For SYBR Green I, Cq of the NTC: /

-Calibration curves with slope and y intercept: see graphs below (screenshots from qbase^{+} software by Biogazelle).

-PCR efficiency calculated from slope: see graphs below (screenshots from qbase^{+} software by Biogazelle).

-r2 of calibration curve: see graphs below (screenshots from qbase^{+} software by Biogazelle).

GNB2L1

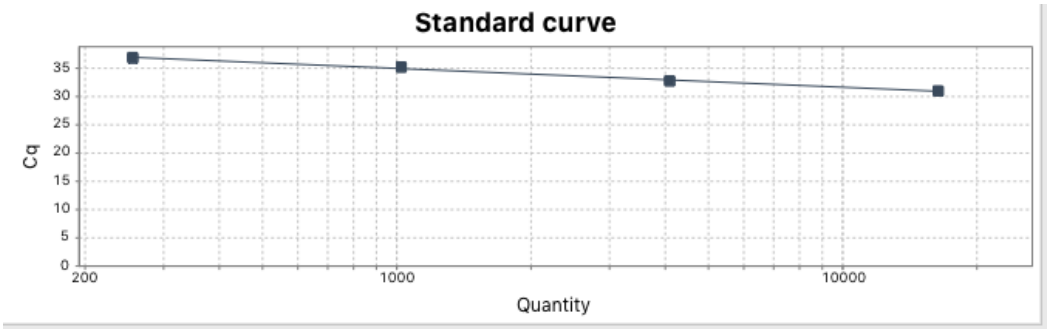


Computed Amplification Efficiency

E :	2,064 ± 0,027	r^2 :	0,996	slope :	-3,177 ± 0,058	Y-Intercept :	40,248 ± 0,162

Included	Run	Well	Sample	Quantity	Cq
☑	miqe2	B1	Sample 1	16.384,000	26,720
☑	miqe2	A1	Sample 1	16.384,000	26,700
☑	miqe2	A2	Sample 2	4.096,000	28,690
☑	miqe2	B2	Sample 2	4.096,000	28,840
☑	miqe2	A3	Sample 3	1.024,000	30,680
☑	miqe2	B3	Sample 3	1.024,000	30,710
☑	miqe2	A4	Sample 4	256,000	32,820
☑	miqe2	B4	Sample 4	256,000	32,550

HPRT1

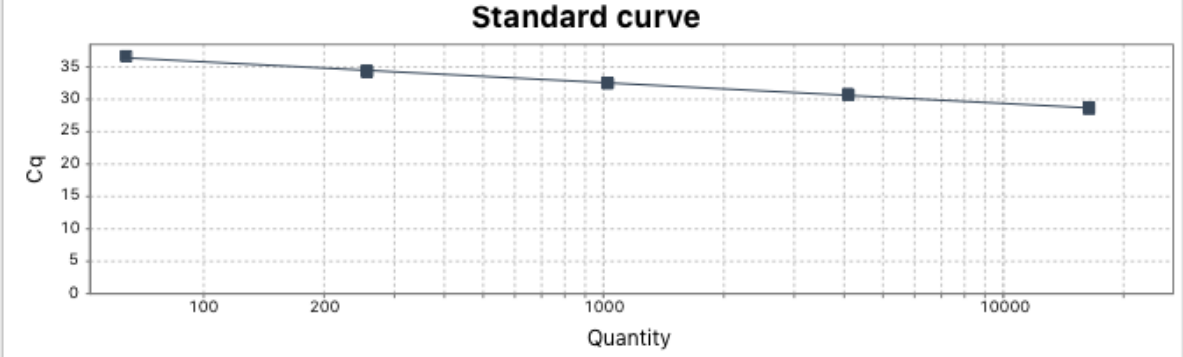


Computed Amplification Efficiency

E :	1,997 ± 0,046	r^2 :	0,993	slope :	-3,329 ± 0,11	Y-Intercept :	44,956 ± 0,373

ncluded	Run	Well	Sample	Quantity	Cq
☑	miqe2	D1	Sample 25	16.384,000	30,960
☑	miqe2	C1	Sample 25	16.384,000	30,970
☑	miqe2	D2	Sample 26	4.096,000	32,700
☑	miqe2	C2	Sample 26	4.096,000	32,780
☑	miqe2	D3	Sample 27	1.024,000	35,150
☑	miqe2	C3	Sample 27	1.024,000	35,230
☑	miqe2	D4	Sample 28	256,000	36,950
☑	miqe2	C4	Sample 28	256,000	36,710

GUSB

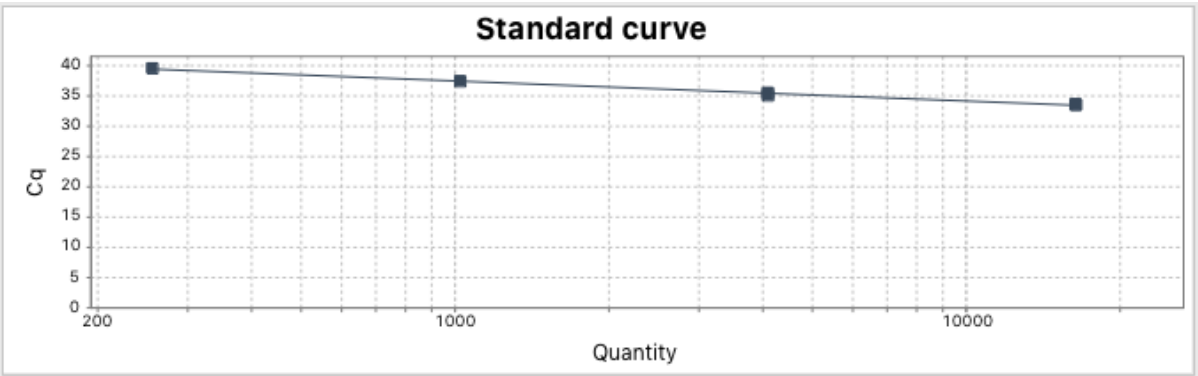


Computed Amplification Efficiency

E :	2,049 ± 0,032	r^2 :	0,997	slope :	-3,211 ± 0,07	Y-Intercept :	42,203 ± 0,228

Included	Run	Well	Sample	Quantity	Cq
☑	miqe2	E1	Sample 49	16.384,000	28,530
☑	miqe2	F1	Sample 49	16.384,000	28,770
☑	miqe2	E2	Sample 50	4.096,000	30,750
☑	miqe2	F2	Sample 50	4.096,000	30,680
☑	miqe2	F3	Sample 51	1.024,000	32,470
☑	miqe2	E3	Sample 51	1.024,000	32,540
☑	miqe2	E4	Sample 52	256,000	34,400
☑	miqe2	F4	Sample 52	256,000	34,200

TBP

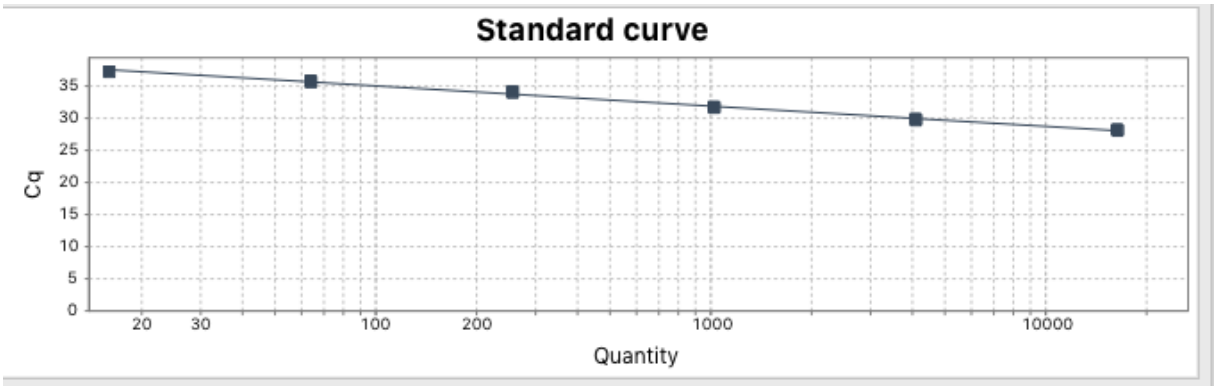


Computed Amplification Efficiency

E :	2,004 ± 0,058	r^2 :	0,993	slope :	-3,313 ± 0,138	Y-Intercept :	47,415 ± 0,493

Included	Run	Well	Sample	Quantity	Cq
☑	miqe2	H1	Sample 73	16.384,000	33,600
☑	miqe2	G1	Sample 73	16.384,000	33,530
☑	miqe2	H2	Sample 74	4.096,000	35,500
☑	miqe2	G2	Sample 74	4.096,000	35,070
☑	miqe2	H3	Sample 75	1.024,000	37,420
☐	miqe2	G3	Sample 75	1.024,000	36,330
☑	miqe2	H4	Sample 76	256,000	39,560
☐	miqe2	G6	Sample 78	16,000	40,000

GAPDH

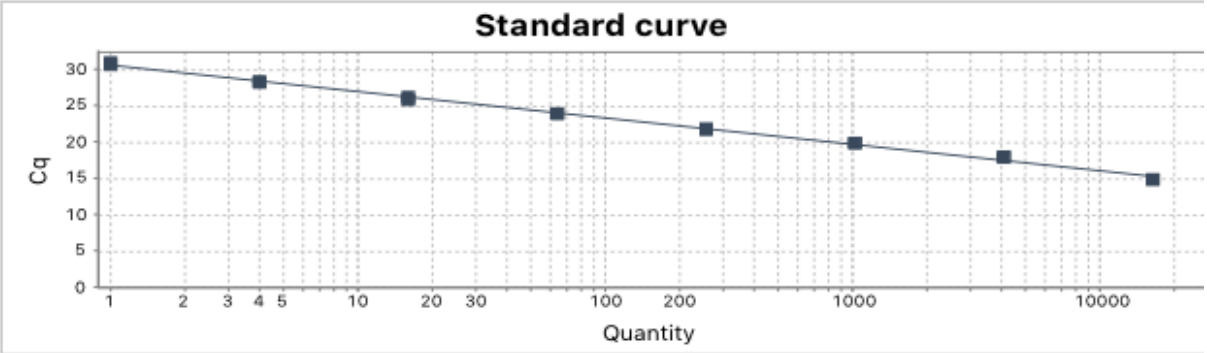


Computed Amplification Efficiency

E :	2,077 ± 0,035	r^2 :	0,995	slope :	-3,15 ± 0,073	Y-Intercept :	41,33 ± 0,219

ncluded	Run	Well	Sample	Quantity	Cq
☑	miqe4	A1	Sample 1	16.384,000	28,070
☑	miqe4	B1	Sample 1	16.384,000	28,220
☑	miqe4	B2	Sample 2	4.096,000	29,690
☑	miqe4	A2	Sample 2	4.096,000	29,860
☑	miqe4	B3	Sample 3	1.024,000	31,640
☑	miqe4	A3	Sample 3	1.024,000	31,750
☑	miqe4	A4	Sample 4	256,000	34,000
☑	miqe4	B4	Sample 4	256,000	34,120

RNA18S

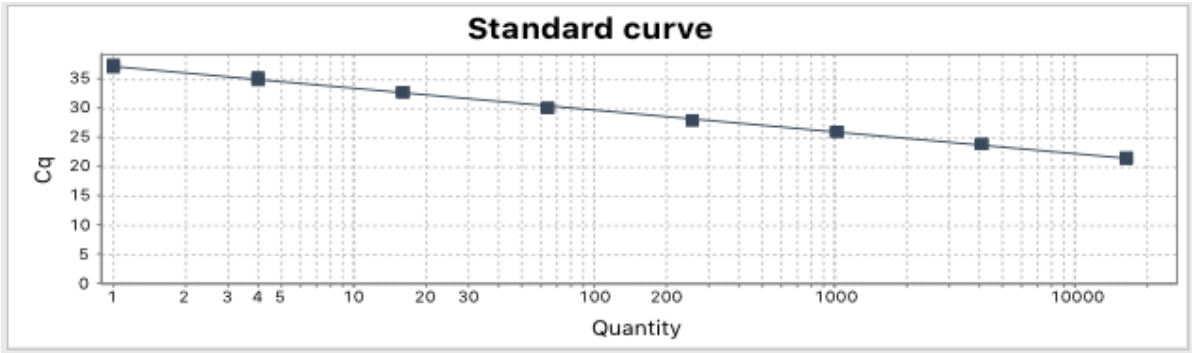


Computed Amplification Efficiency

E :	1,884 ± 0,019	r^2 :	0,997	slope :	-3,635 ± 0,057	Y-Intercept :	30,553 ± 0,143

Included	Run	Well	Sample	Quantity	Cq
☑	miqe5	B1	Sample 1	16.384,000	14,800
☑	miqe5	A1	Sample 1	16.384,000	14,820
☑	miqe5	B2	Sample 2	4.096,000	17,840
☑	miqe5	A2	Sample 2	4.096,000	17,980
☑	miqe5	B3	Sample 3	1.024,000	19,810
☑	miqe5	A3	Sample 3	1.024,000	19,860
☑	miqe5	B4	Sample 4	256,000	21,660

B2M

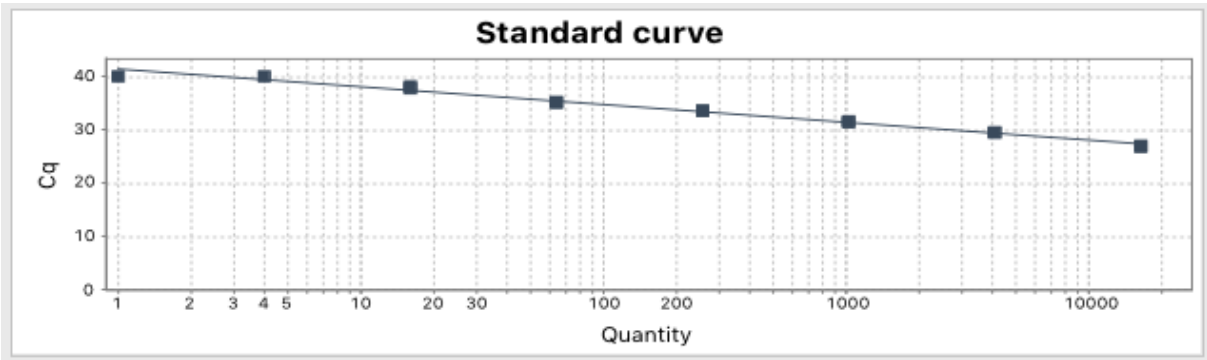


Computed Amplification Efficiency

E :	1,853 ± 0,013	r^2 :	0,998	slope :	-3,733 ± 0,044	Y-Intercept :	37,102 ± 0,111

Included	Run	Well	Sample	Quantity	Cq
☑	miqe6	B1	Sample 1	16.384,000	21,260
☑	miqe6	A1	Sample 1	16.384,000	21,540
☑	miqe6	A2	Sample 2	4.096,000	23,810
☑	miqe6	B2	Sample 2	4.096,000	23,820
☑	miqe6	B3	Sample 3	1.024,000	25,930
☑	miqe6	A3	Sample 3	1.024,000	25,930
☑	miqe6	A4	Sample 4	256,000	27,910

ACTB

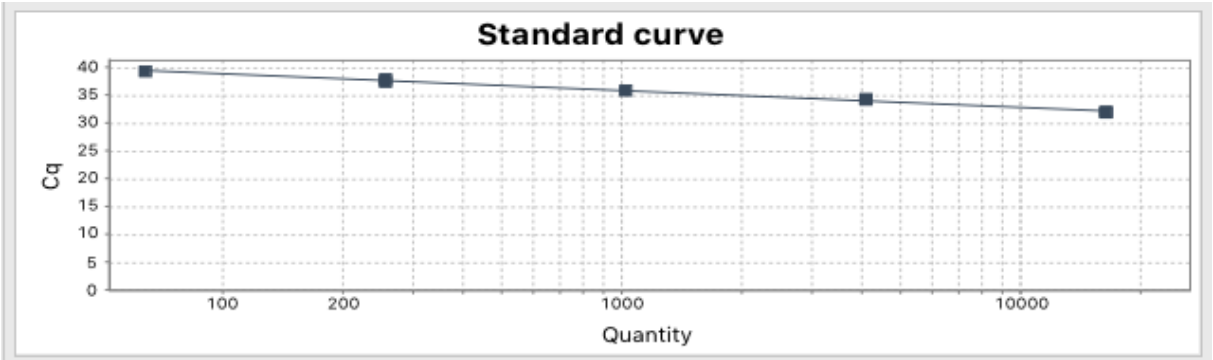


Computed Amplification Efficiency

E :	1,995 ± 0,048	r^2 :	0,985	slope :	-3,334 ± 0,117	Y-Intercept :	41,383 ± 0,315

Included	Run	Well	Sample	Quantity	Cq
☑	miqe6	D1	Sample 25	16.384,000	27,020
☑	miqe6	C1	Sample 25	16.384,000	26,770
☑	miqe6	D2	Sample 26	4.096,000	29,440
☑	miqe6	C2	Sample 26	4.096,000	29,500
☑	miqe6	C3	Sample 27	1.024,000	31,600
☑	miqe6	D3	Sample 27	1.024,000	31,380
☑	miqe6	D4	Sample 28	256,000	33,570

RPL13A



Computed Amplification Efficiency

E :	2,142 ± 0,057	r^2 :	0,993	slope :	-3,022 ± 0,105	Y-Intercept :	44,866 ± 0,344

Included	Run	Well	Sample	Quantity	Cq
☑	miqe6	F1	Sample 49	16.384,000	31,990
☑	miqe6	E1	Sample 49	16.384,000	31,850
☑	miqe6	E2	Sample 50	4.096,000	34,160
☑	miqe6	F2	Sample 50	4.096,000	34,260
☑	miqe6	E3	Sample 51	1.024,000	35,800
☐	miqe6	F3	Sample 51	1.024,000	34,220
☑	miqe6	E4	Sample 52	256,000	37,370

-Linear dynamic range: For each assay, a 4 times serial dilution of stock cDNA (pool of 4 cDNA) was used (dilution 1:1-1:4-1:16-1:64-1:256-1:1024-1:4096-1:16384). The linear dynamic ranges were as followed: Cq expressed as mean ± SD (/ indicates SD value was impossible to obtain).

pool dilution	RNA18S	ACTB	GAPDH	B2M	GUSB
1:1	14.81±0.01	26.89±0.18	28.14±0.11	21.40±0.20	28.65±0.17
1:4	17.91±0.10	29.47±0.04	29.77±0.12	23.81±0.01	30.71±0.05
1:16	19.83±0.04	31.49±0.16	31.69±0.08	25.93±0.00	32.50±0.05
1:64	21.75±0.13	33.55±0.02	34.06±0.08	27.88±0.04	34.3±0.14
1:256	23.88±0.04	35.13±0.15	35.71±0.13	29.99±0.05	36.64±/
1:1024	25.96±0.30	37.94±0.30	37.23±/	32.69±0.05	
1:4096	28.23±0.12			34.99±0.36	
1:16384	30.76±0.25			37.18±0.35	
Control RT-	35.06±0.05				
Control NTC					
r^2	0.997		0.995	0.998	0.997

pool dilution	HPRT1	GNB2L1	TBP	RPL13A
1X	30.96±0.01	26.71±0.01	33.56±0.05	31.92±0.10
4X	32.74±0.05	28.76±0.11	35.28±0.3	34.21±0.07
16X	35.19±0.06	30.69±0.02	37.42±/	35.8±/
64X	36.83±0.17	32.68±0.19	39.56±/	37.6±0.32
256X		34.79±0.06		39.24±/
1024X		36.46±0.22		
4096X		37.79±/		
16384X				
Control RT-				
Control NTC				
r^2	0.993	0.996	0.993	

-Cq variation at LOD: see tables above

-Evidence for LOD: see tables above

-If multiplex, efficiency and LOD of each assay: /

Data analysis

-qPCR analysis program (source, version): LightCycler 480 SW 1.5.1 62

-Method of Cq determination: The Absolute quantification of Cq was obtained by 2^{nd} derivative maximum of each curve for all samples.

-Outlier identification and disposition : We used qbase$^+$ qPCR analysis software (Biogazelle, Zwijnaarde, Belgium) and exclude the samples for which technical replicates differed for more than 1 Cq.

-Results for NTCs: see tables above

-Justification of number and choice of reference genes: /

-Description of normalization method: Using qbase$^+$ qPCR analysis software (Biogazelle, Zwijnaarde, Belgium), the relative quantitation was determined by the $E^{-\Delta\Delta Cq}$ method in accordance with the target specific amplification efficiency values.

-Number and stage (reverse transcription or qPCR) of technical replicates: duplicate, PCR step.

-Repeatability (intraassay variation): mean of difference between duplicates ± SD

RNA18S: 0.10 ± 0.07
ACTB: 0.15 ± 0.13

GAPDH: 0.29 ± 0.23
B2M: 0.16 ± 0.17
GUSB: 0.22 ± 0.29
HPRT1: 0.27 ± 0.23
GNB2L1: 0.18 ± 0.17
TBP: 0.31 ± 0.21
RPL13A: 0.23 ± 0.21

-Statistical methods for results significance: In the application example, the relative expression between groups of subjects was analyzed with Kruskal-Wallis test followed by Dunn's multiple comparisons test for multiple comparison and with Mann-Whitney test when 2 groups were compared. Correlations were tested with Spearman's rank correlation analysis. Differences were considered statistically significant when a two-sided p-value was < 0.05. See main body text for details about the 3 algorithms.

-Software (source, version) : See main body text for details about the 3 algorithms. Other statistical analyses were carried out with Graphpad Prism 7.0 (Graphpad Software San Diego, CA, USA).

Validation experiment

Primer sequences used for the validation set : see table below. They were purchased at Thermo Fisher Scientific, Wilmington, USA.

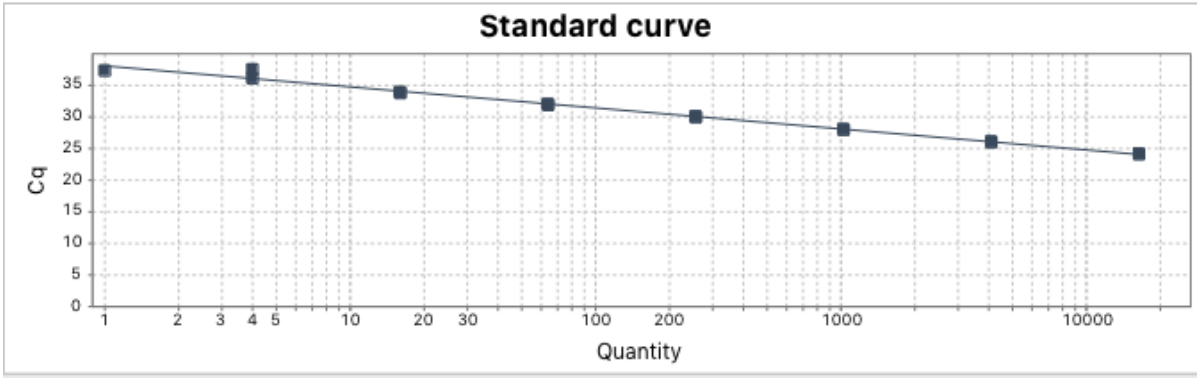
qPCR validation

-Specificity: see gel below as well as the fragment sizes in the QIAxcel (Qiagen, Hilden, Germany) report (electrophoresis profiles).

Gene name (abbreviation)	**Assay ID**
18s ribosomal RNA	Hs99999901_s1
β-Actin (ACTB)	Hs01060665_g1
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Hs02758991_g1
β2-Microglobulin (B2M)	Hs00984230_m1
Hypoxanthine ribosyltransferase 1 (HPRT1)	Hs02800695_m1
Guanine nucleotide-binding protein, b-peptide 2-like 1 (GNB2L1)	Hs00272002_m1
Ribosomal protein L13A (RPL13A)	Hs03043885_g1
Ribosomal protein L32 (RPL32)	Hs00851655_g1

Efficiency:

ACTB

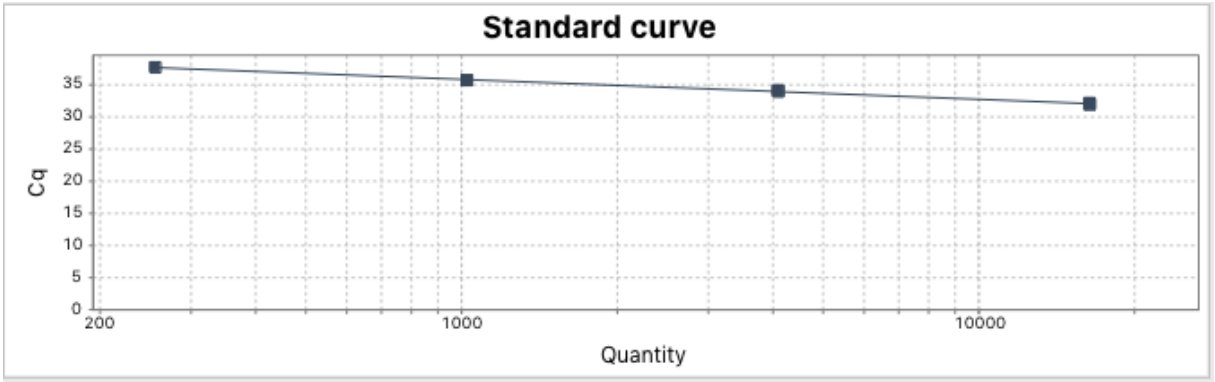


Computed Amplification Efficiency

E :	1,999 ± 0,039	r^2 :	0,99	slope :	-3,325 ± 0,093	Y-Intercept :	38,077 ± 0,242

ncluded	Run	Well	Sample	Quantity	Cq
☑	miqe3	E1	Sample 49	16.384,000	24,140
☑	miqe3	F1	Sample 49	16.384,000	24,160
☑	miqe3	E2	Sample 50	4.096,000	26,060
☑	miqe3	F2	Sample 50	4.096,000	26,050
☑	miqe3	E3	Sample 51	1.024,000	28,030
☑	miqe3	F3	Sample 51	1.024,000	28,000
☑	miqe3	E4	Sample 52	256,000	30,080
☑	miqe3	F4	Sample 52	256,000	29,960

HPRT1

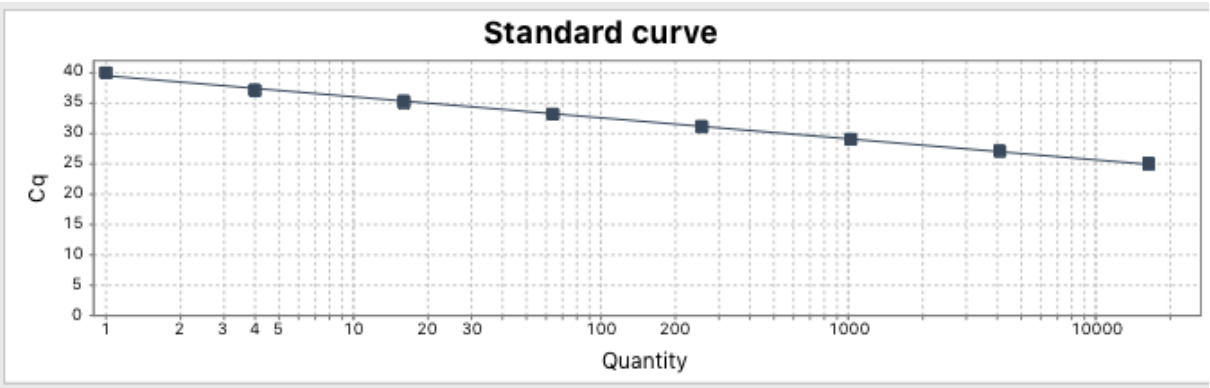


Computed Amplification Efficiency

E :	2,099 ± 0,039	r^2 :	0,997	slope :	-3,106 ± 0,078	Y-Intercept :	45,155 ± 0,272

ncluded	Run	Well	Sample	Quantity	Cq
☑	miqe3	D2	Sample 26	4.096,000	34,120
☑	miqe3	C2	Sample 26	4.096,000	33,940
☑	miqe3	D3	Sample 27	1.024,000	35,740
☑	miqe3	C3	Sample 27	1.024,000	35,720
☑	miqe3	D4	Sample 28	256,000	37,710
☐	miqe3	C4	Sample 28	256,000	38,450
☐	miqe3	C6	Sample 30	16,000	40,000
☐	miqe3	D8	Sample 32	1,000	32,770

RPL13A

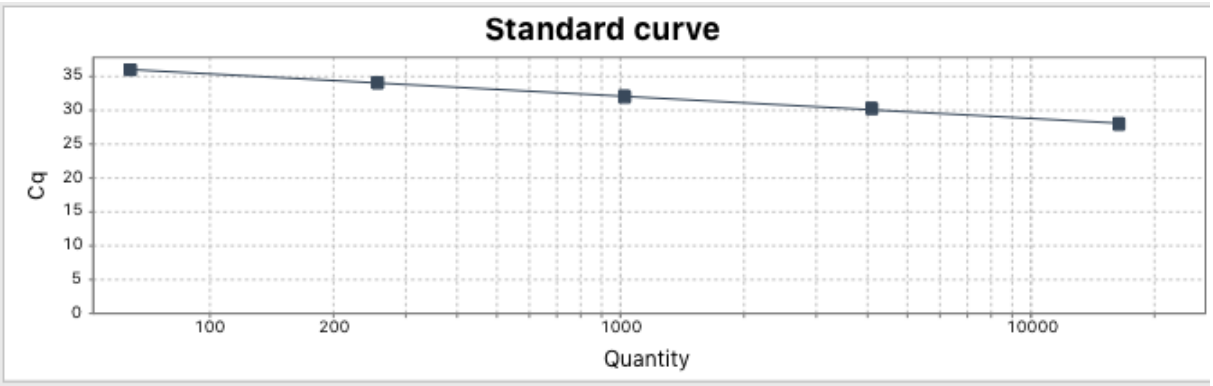


Computed Amplification Efficiency

E :	1,943 ± 0,017	r^2 :	0,998	slope :	-3,467 ± 0,046	Y-Intercept :	39,525 ± 0,115

Included	Run	Well	Sample	Quantity	Cq
☑	miqe3	H1	Sample 73	16.384,000	24,920
☑	miqe3	G1	Sample 73	16.384,000	25,110
☑	miqe3	H2	Sample 74	4.096,000	27,080
☑	miqe3	G2	Sample 74	4.096,000	27,110
☑	miqe3	H3	Sample 75	1.024,000	29,040
☑	miqe3	G3	Sample 75	1.024,000	29,060
☑	miqe3	H4	Sample 76	256,000	31,060
☑	miqe3	G4	Sample 76	256,000	31,180

GNB2L1

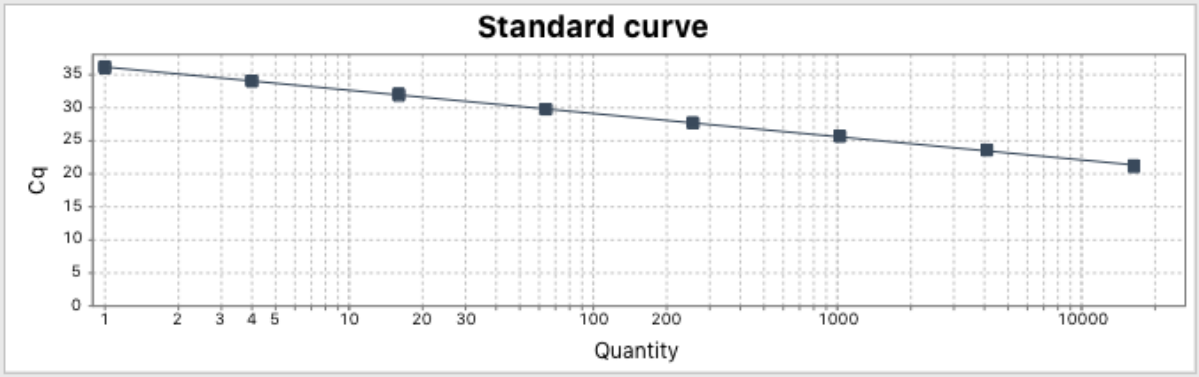


Computed Amplification Efficiency

E :	2,012 ± 0,022	r^2 :	0,998	slope :	-3,293 ± 0,05	Y-Intercept :	41,969 ± 0,158

Included	Run	Well	Sample	Quantity	Cq
☑	miqe3	A1	Sample 1	16.384,000	27,880
☑	miqe3	B1	Sample 1	16.384,000	28,070
☑	miqe3	B2	Sample 2	4.096,000	30,220
☑	miqe3	A2	Sample 2	4.096,000	30,300
☑	miqe3	A3	Sample 3	1.024,000	32,110
☑	miqe3	B3	Sample 3	1.024,000	31,900
☑	miqe3	B4	Sample 4	256,000	34,050
☑	miqe3	A4	Sample 4	256,000	34,060

B2M

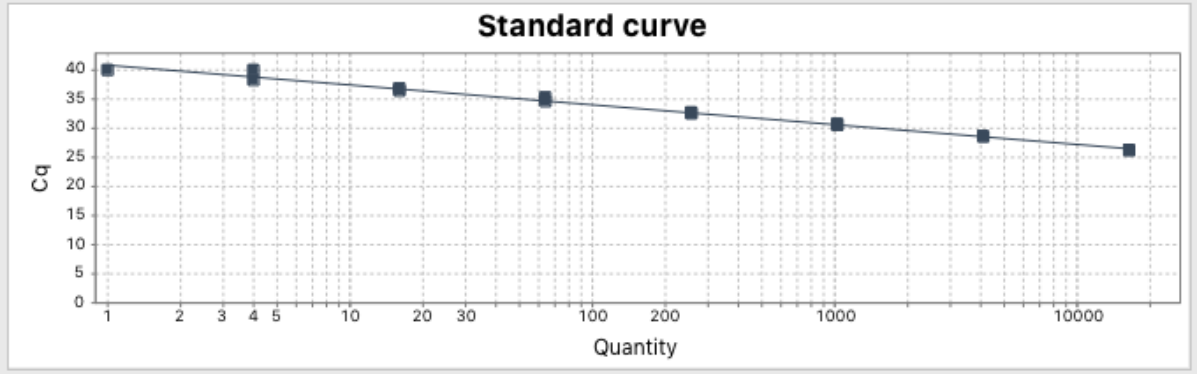


Computed Amplification Efficiency

E :	1,925 ± 0,009	r^2 :	0,999	slope :	-3,517 ± 0,025	Y-Intercept :	36,168 ± 0,062

Included	Run	Well	Sample	Quantity	Cq
☑	miqe4	D1	Sample 25	16.384,000	21,050
☑	miqe4	C1	Sample 25	16.384,000	21,250
☑	miqe4	D2	Sample 26	4.096,000	23,570
☑	miqe4	C2	Sample 26	4.096,000	23,640
☑	miqe4	D3	Sample 27	1.024,000	25,720
☑	miqe4	C3	Sample 27	1.024,000	25,670
☑	miqe4	C4	Sample 28	256,000	27,710
☑	miqe4	D4	Sample 28	256,000	27,690

GAPDH

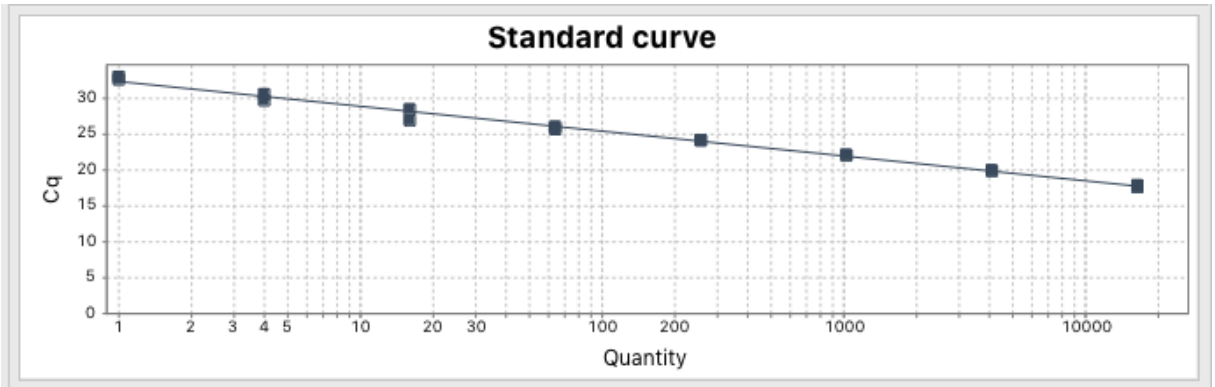


Computed Amplification Efficiency

E :	1,966 ± 0,038	r^2 :	0,99	slope :	-3,407 ± 0,096	Y-Intercept :	40,844 ± 0,251

Included	Run	Well	Sample	Quantity	Cq
☑	miqe4	F1	Sample 49	16.384,000	26,220
☑	miqe4	E1	Sample 49	16.384,000	26,250
☑	miqe4	E2	Sample 50	4.096,000	28,580
☑	miqe4	F2	Sample 50	4.096,000	28,640
☑	miqe4	E3	Sample 51	1.024,000	30,770
☑	miqe4	F3	Sample 51	1.024,000	30,560
☑	miqe4	F4	Sample 52	256,000	32,730
☑	miqe4	E4	Sample 52	256,000	32,530

RNA 18S

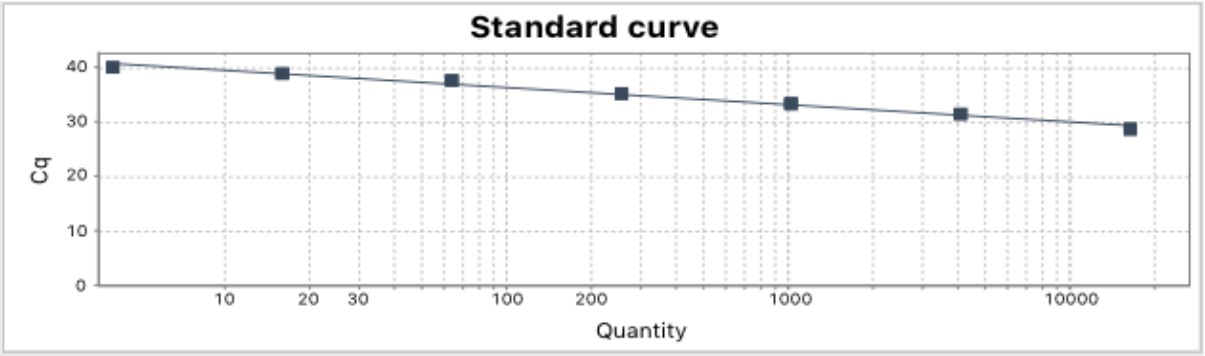


Computed Amplification Efficiency

E :	1,945 ± 0,031	r^2 :	0,992	slope :	-3,461 ± 0,083	Y-Intercept :	32,358 ± 0,208

Included	Run	Well	Sample	Quantity	Cq
☑	miqe4	H1	Sample 73	16.384,000	17,910
☑	miqe4	G1	Sample 73	16.384,000	17,640
☑	miqe4	H2	Sample 74	4.096,000	19,970
☑	miqe4	G2	Sample 74	4.096,000	19,880
☑	miqe4	H3	Sample 75	1.024,000	22,090
☑	miqe4	G3	Sample 75	1.024,000	22,100
☑	miqe4	H4	Sample 76	256,000	24,120
☑	miqe4	G4	Sample 76	256,000	24,200

RPL32

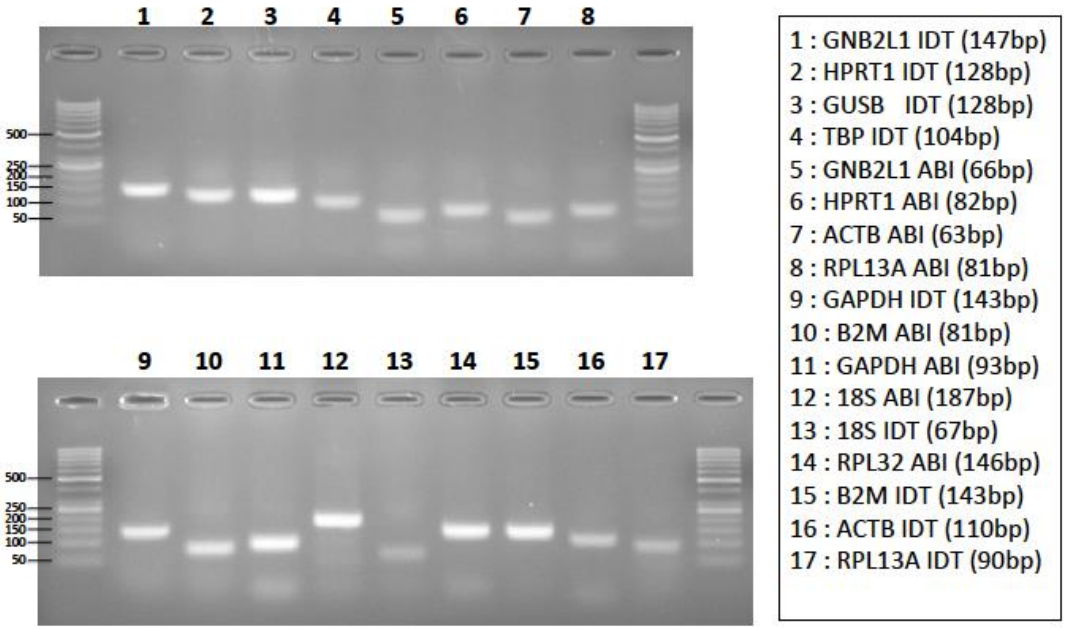


Computed Amplification Efficiency

E :	2,075 ± 0,054	r^2 :	0,985	slope :	-3,153 ± 0,113	Y-Intercept :	42,595 ± 0,305

Included	Run	Well	Sample	Quantity	Cq
☑	miqe5	D4	Sample 28	256,000	35,100
☑	miqe5	D5	Sample 29	64,000	37,520
☑	miqe5	C5	Sample 29	64,000	37,640
☑	miqe5	C6	Sample 30	16,000	38,790
☑	miqe5	D6	Sample 30	16,000	38,910
☑	miqe5	C7	Sample 31	4,000	40,000
☑	miqe5	D7	Sample 31	4,000	40,000

Specificity of the IDT and Thermo Fisher Scientific (ABI) primers. PCR products were loaded on a 3% agarose gel in TAE buffer. Ladder is O'GeneRuler DNA Ladder, 50-1000 bp (Thermo Fisher Scientific).



Specificity: see fragment sizes in the QIAxcel (Qiagen, Hilden, Germany) report (electrophoresis profiles) below. 15 bp and 3000 bp markers are the alignment markers used for the QIAxcel run.

Report Overview	
Report Date:	9/10/2019 11:36:31 AM
Experiment Name:	C190321036_2019-09-10_10-25-24
Cartridge ID:	C190321036
Instrument ID:	30610

Sample Header			
Position:	A1	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	GNB2L1-IDT	Run Date:	9/10/2019 10:25:24 AM

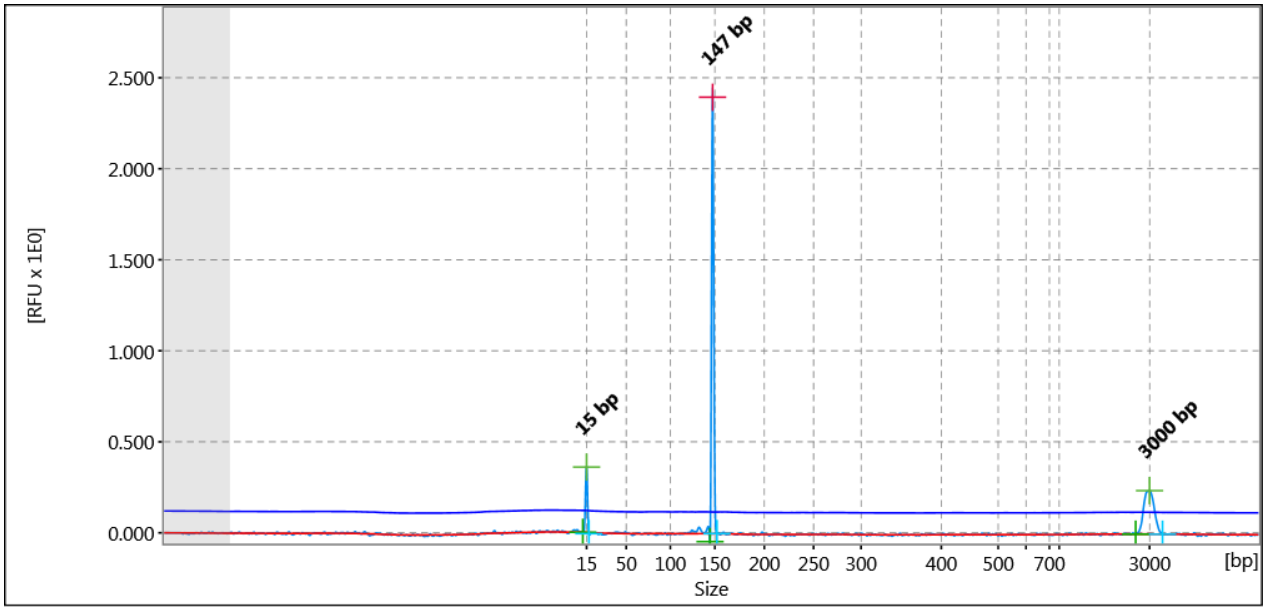


Figure: 1

Peak Calling Result Table: n/a

Sample Header			
Position:	A2	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	HPRT1-IDT	Run Date:	9/10/2019 10:25:24 AM

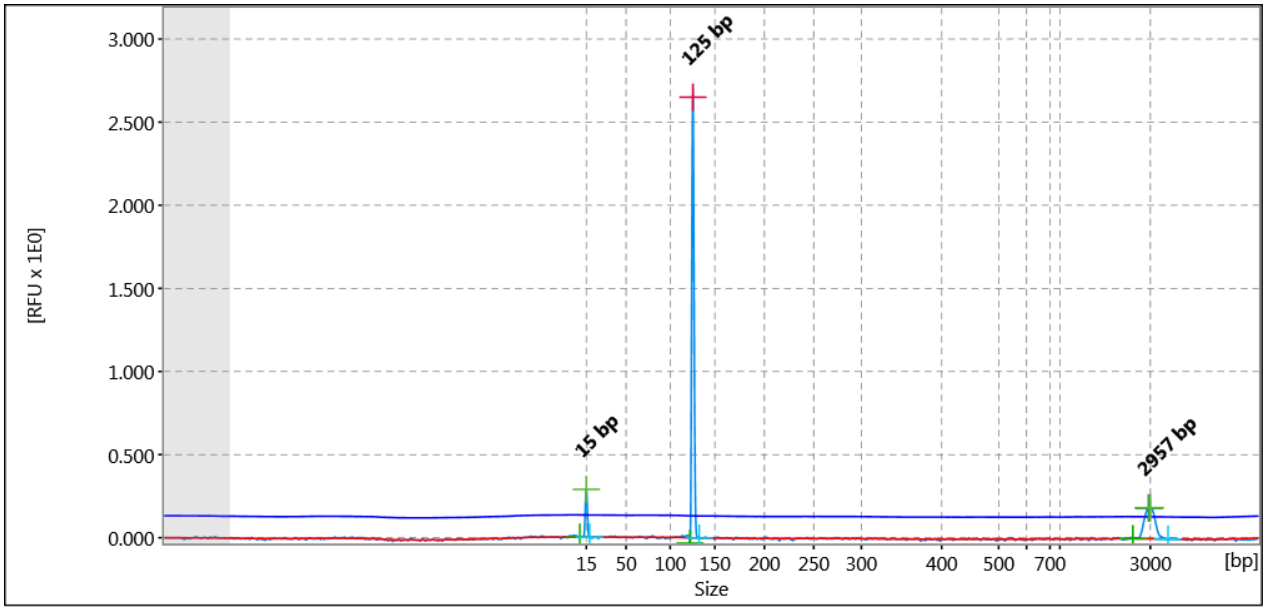


Figure: 2

Peak Calling Result Table: n/a

Sample Header			
Position:	A3	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	GUSB-IDT	Run Date:	9/10/2019 10:25:24 AM

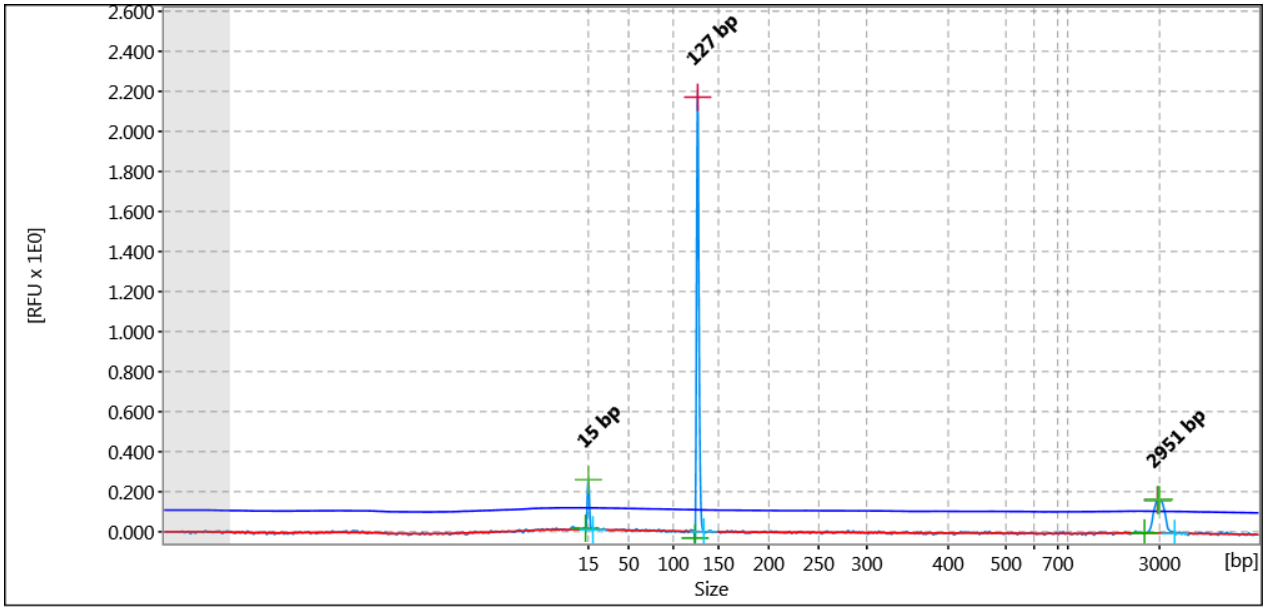


Figure: 3

Peak Calling Result Table: n/a

Sample Header			
Position:	A4	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	TBP-IDT	Run Date:	9/10/2019 10:25:24 AM

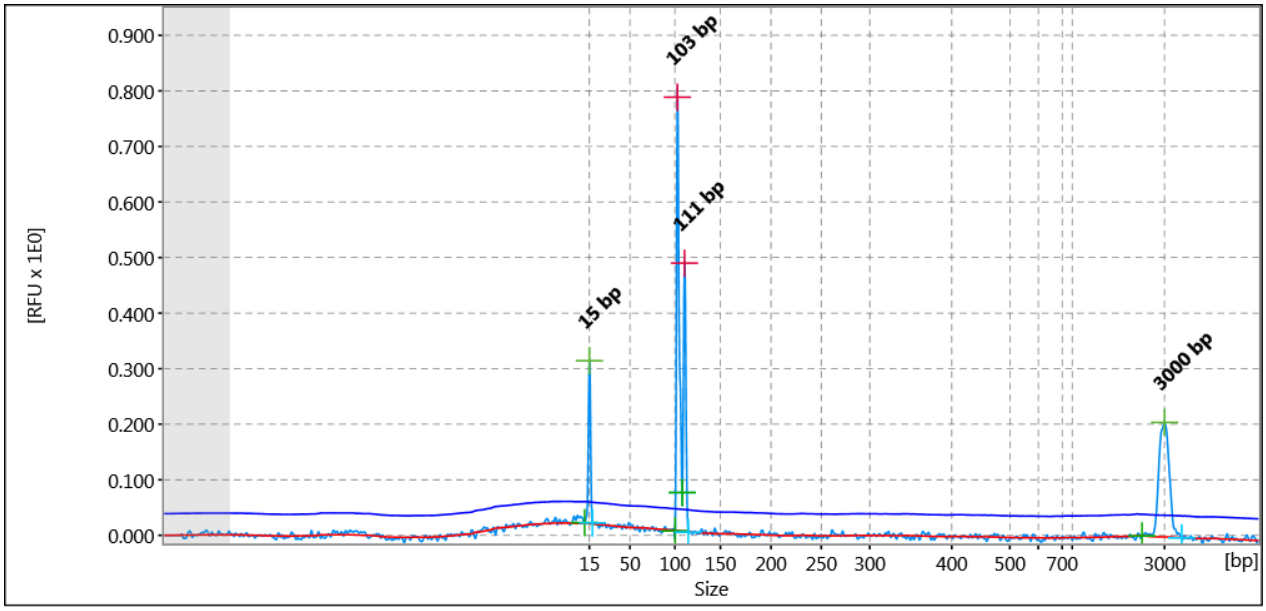


Figure: 4

Peak Calling Result Table: n/a

Sample Header			
Position:	A5	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	GNB2L1-ABI	Run Date:	9/10/2019 10:25:24 AM

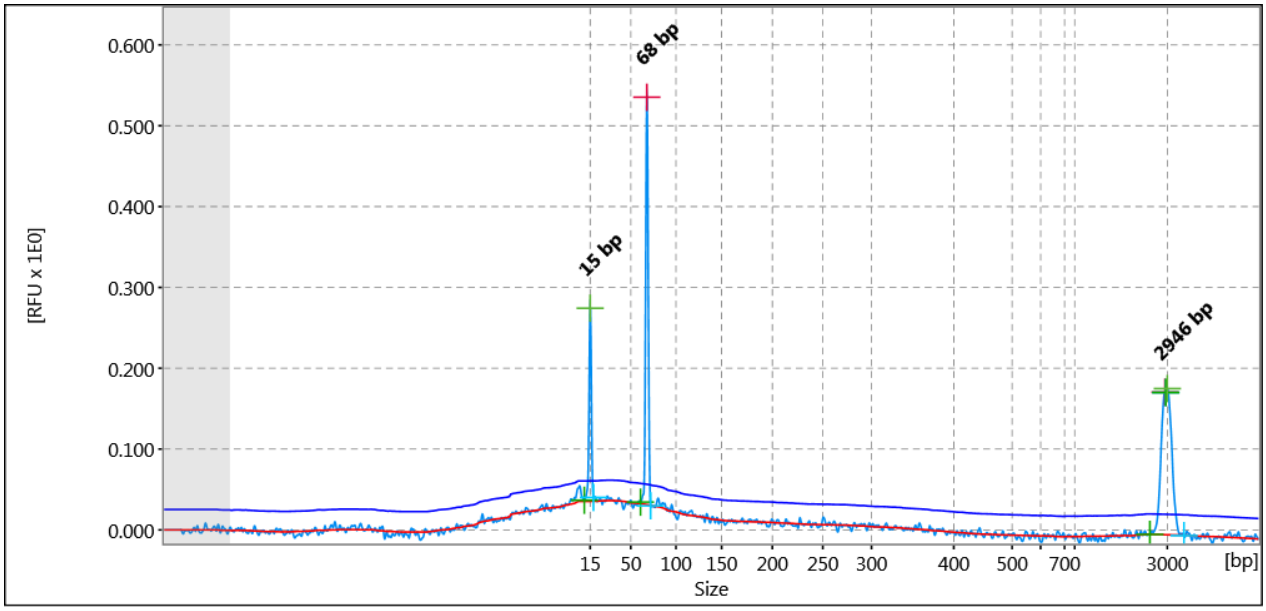


Figure: 5

Peak Calling Result Table: n/a

Sample Header			
Position:	A6	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	HPRT1-ABI	Run Date:	9/10/2019 10:25:24 AM

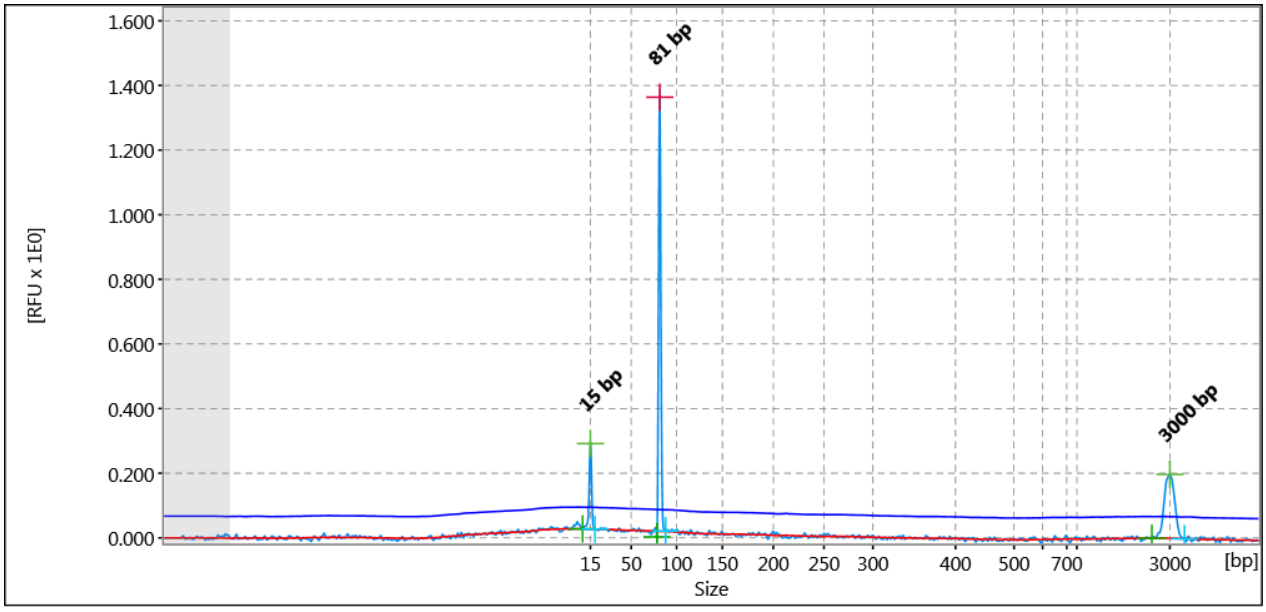


Figure: 6

Peak Calling Result Table: n/a

Sample Header			
Position:	A7	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	ACTB-ABI	Run Date:	9/10/2019 10:25:24 AM

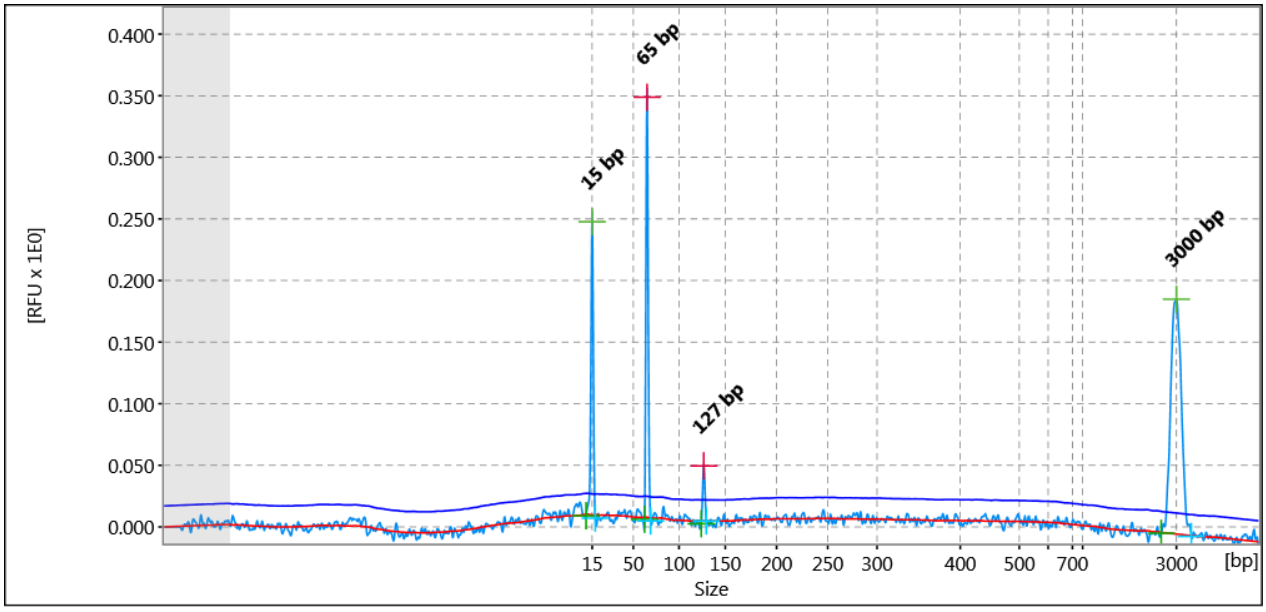


Figure: 7

Peak Calling Result Table: n/a

Sample Header			
Position:	A8	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	RPL13A-ABI	Run Date:	9/10/2019 10:25:24 AM

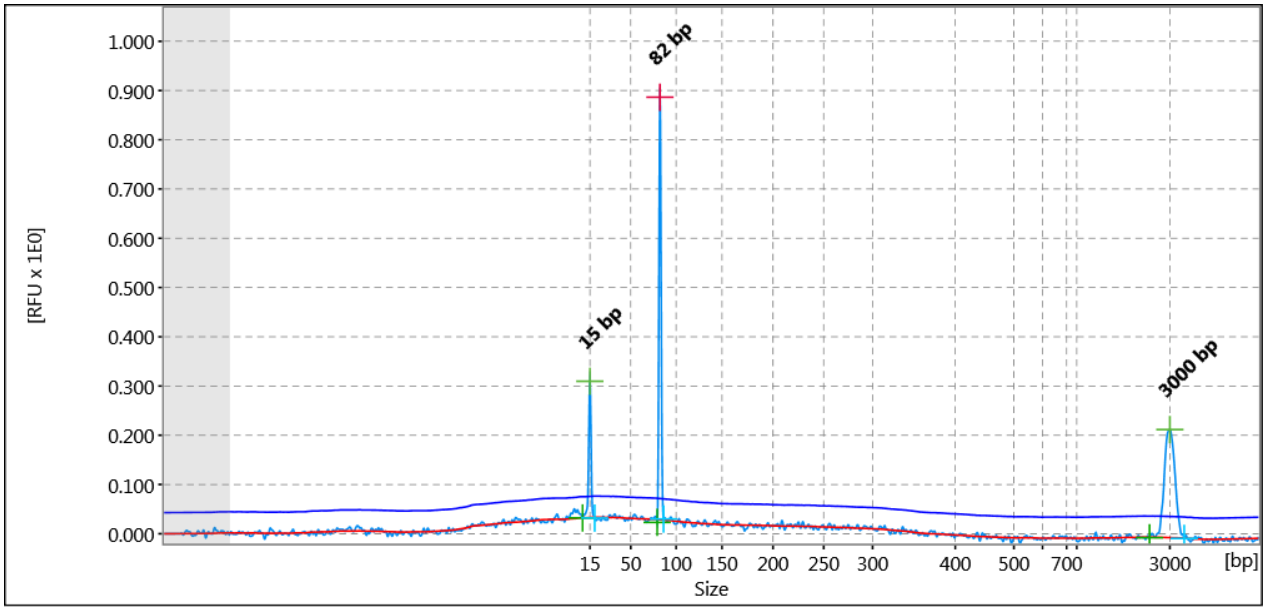


Figure: 8

Peak Calling Result Table: n/a

Sample Header			
Position:	A9	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	GAPDH-IDT	Run Date:	9/10/2019 10:25:24 AM

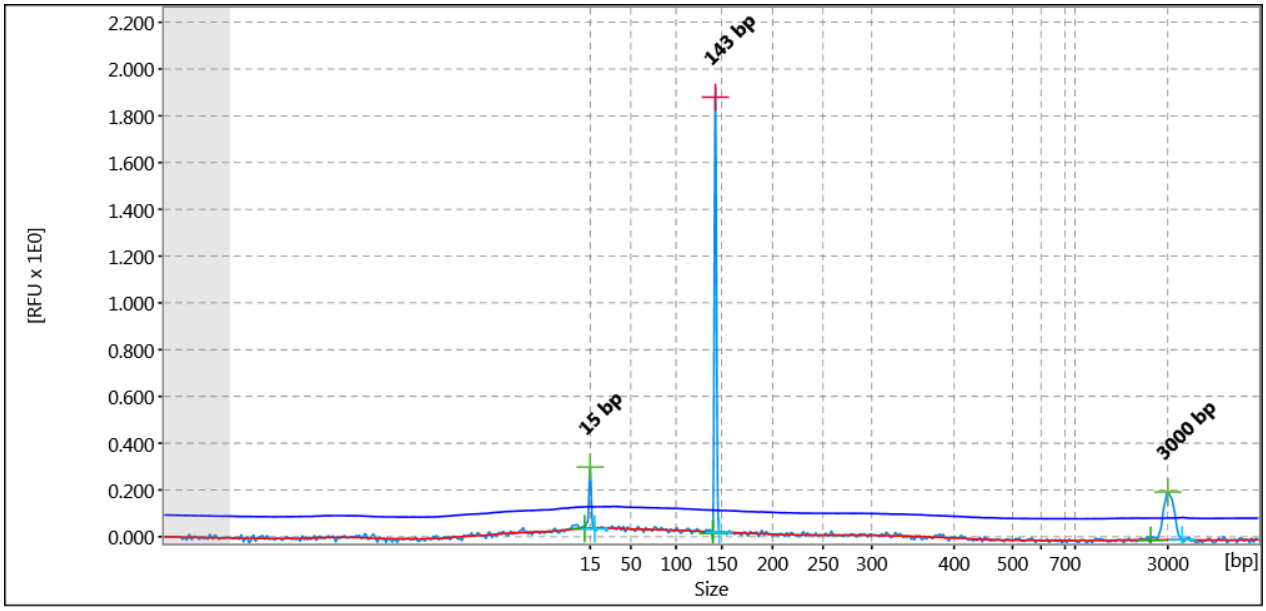


Figure: 9

Peak Calling Result Table: n/a

Sample Header			
Position:	A10	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	B2M-ABI	Run Date:	9/10/2019 10:25:24 AM

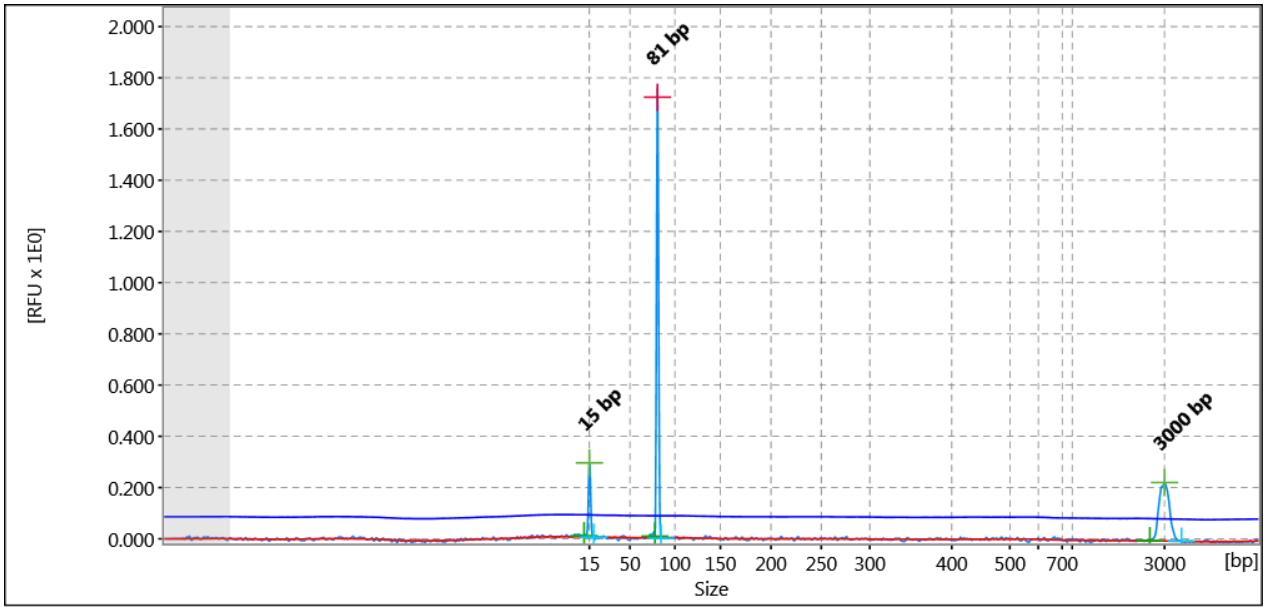


Figure: 10

Peak Calling Result Table: n/a

Sample Header			
Position:	A11	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	GAPDH-ABI	Run Date:	9/10/2019 10:25:24 AM

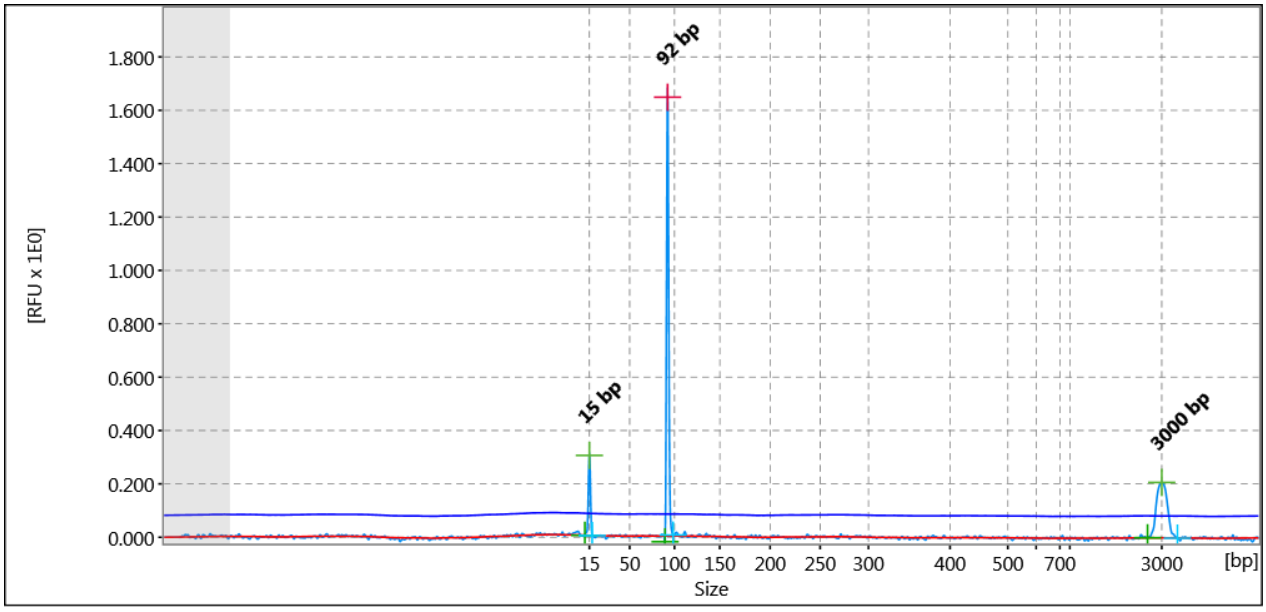


Figure: 11

Peak Calling Result Table: n/a

Sample Header			
Position:	A12	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	18S-ABI	Run Date:	9/10/2019 10:25:24 AM

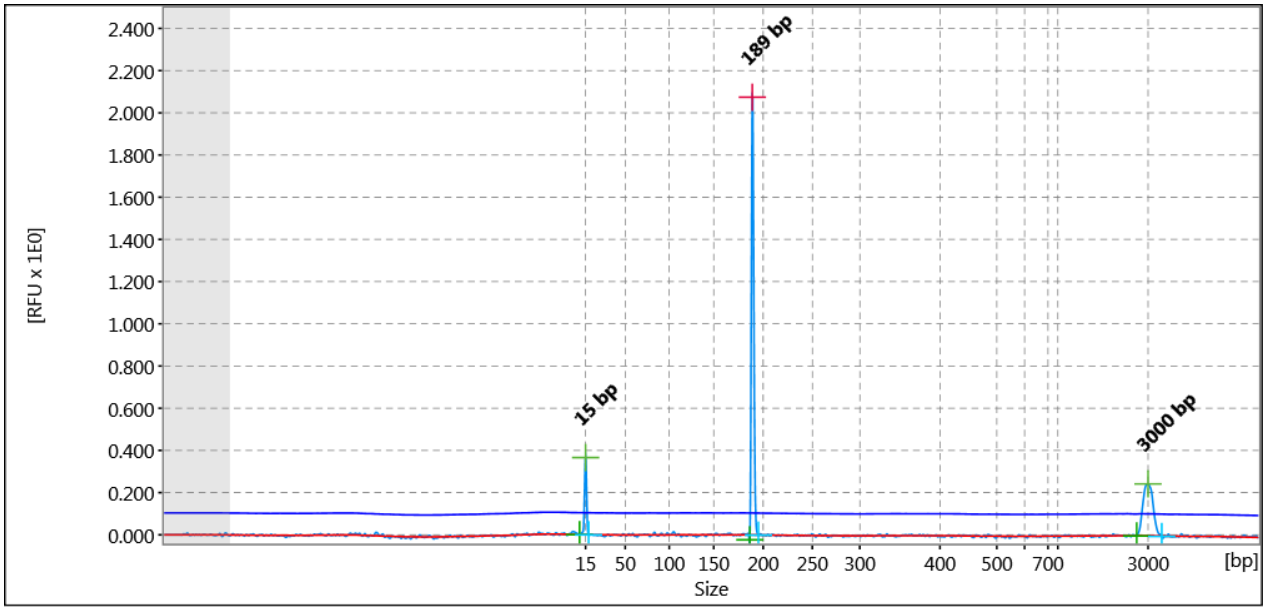


Figure: 12

Peak Calling Result Table: n/a

Sample Header			
Position:	B1	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	18S-IDT	Run Date:	9/10/2019 10:25:24 AM

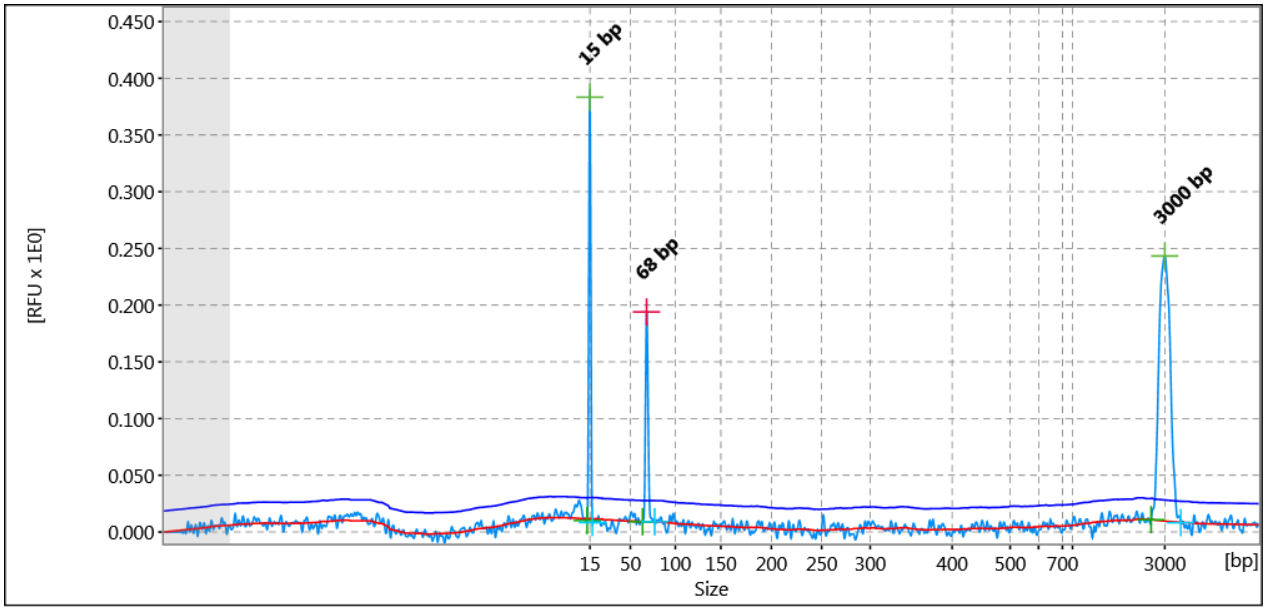


Figure: 13

Peak Calling Result Table: n/a

Sample Header			
Position:	B2	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	RPL32-ABI	Run Date:	9/10/2019 10:25:24 AM

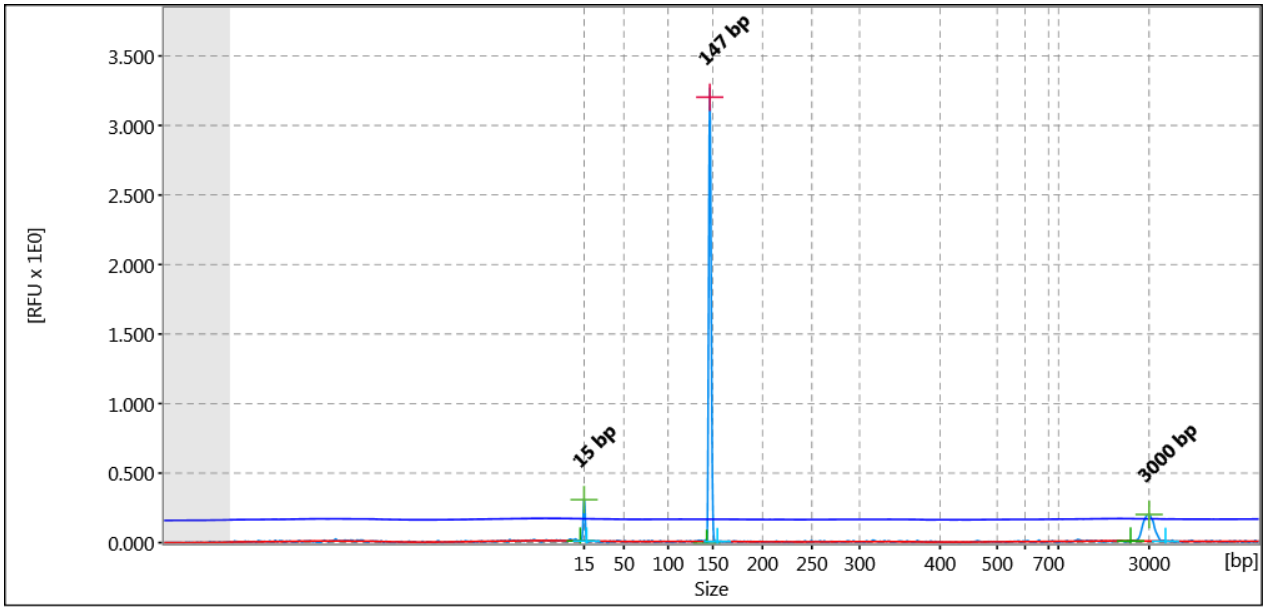


Figure: 14

Peak Calling Result Table: n/a

Sample Header			
Position:	B3	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	B2M-IDT	Run Date:	9/10/2019 10:25:24 AM

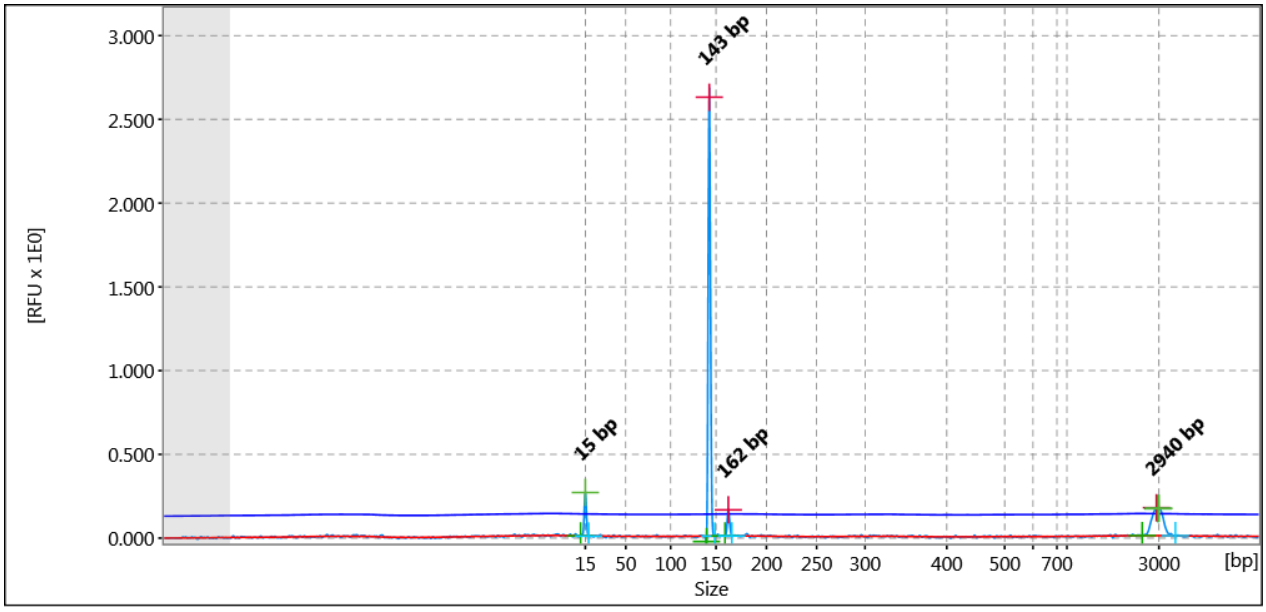


Figure: 15

Peak Calling Result Table: n/a

Sample Header			
Position:	B4	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	ACTB-IDT	Run Date:	9/10/2019 10:25:24 AM

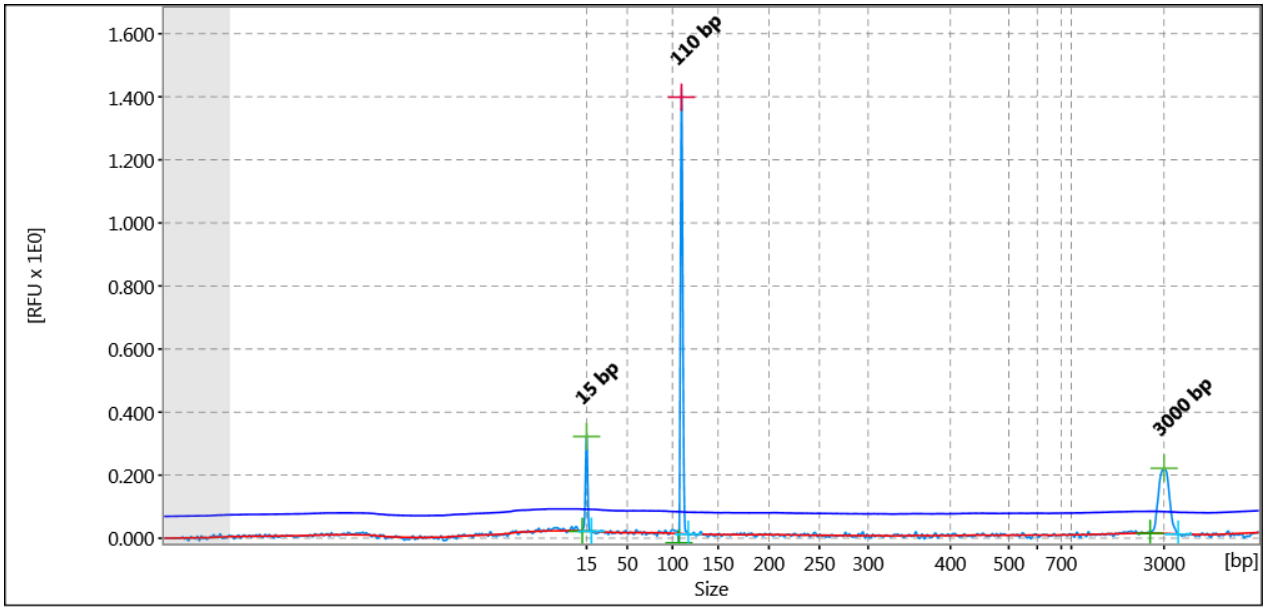


Figure: 16

Peak Calling Result Table: n/a

Sample Header			
Position:	B5	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:	RPL13A-IDT	Run Date:	9/10/2019 10:25:24 AM

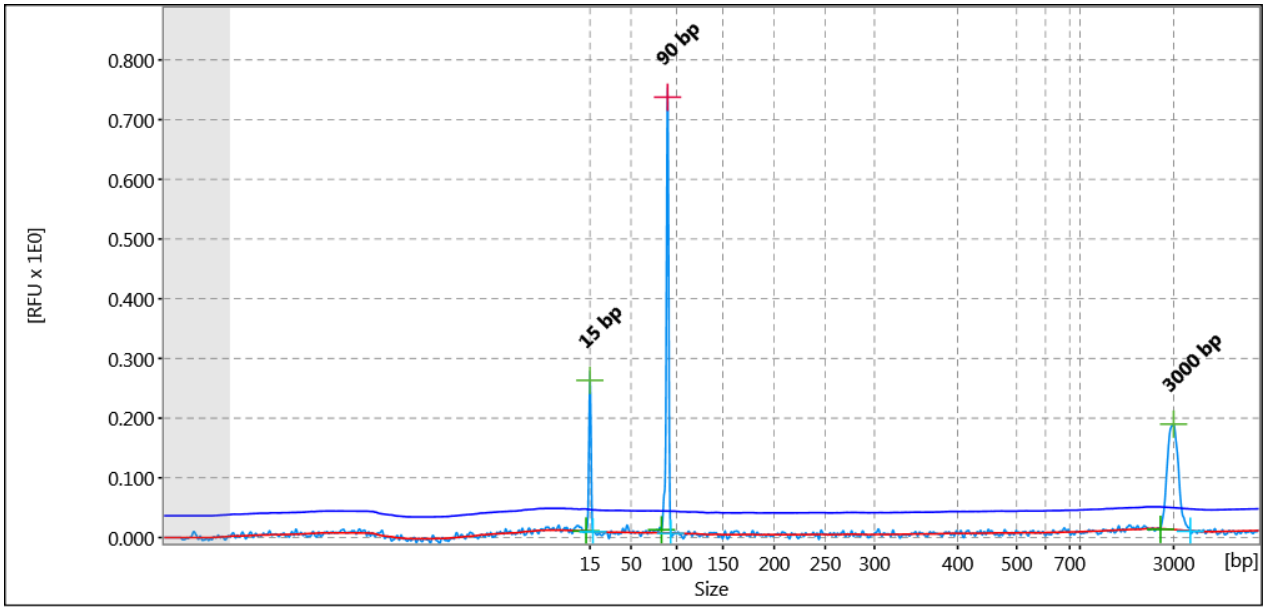


Figure: 17

Peak Calling Result Table: n/a

Sample Header			
Position:	B6	Plate ID:	C190321036_2019-09-10_10-25-24, R: 1, E: 1
Sample Info:		Run Date:	9/10/2019 10:25:24 AM

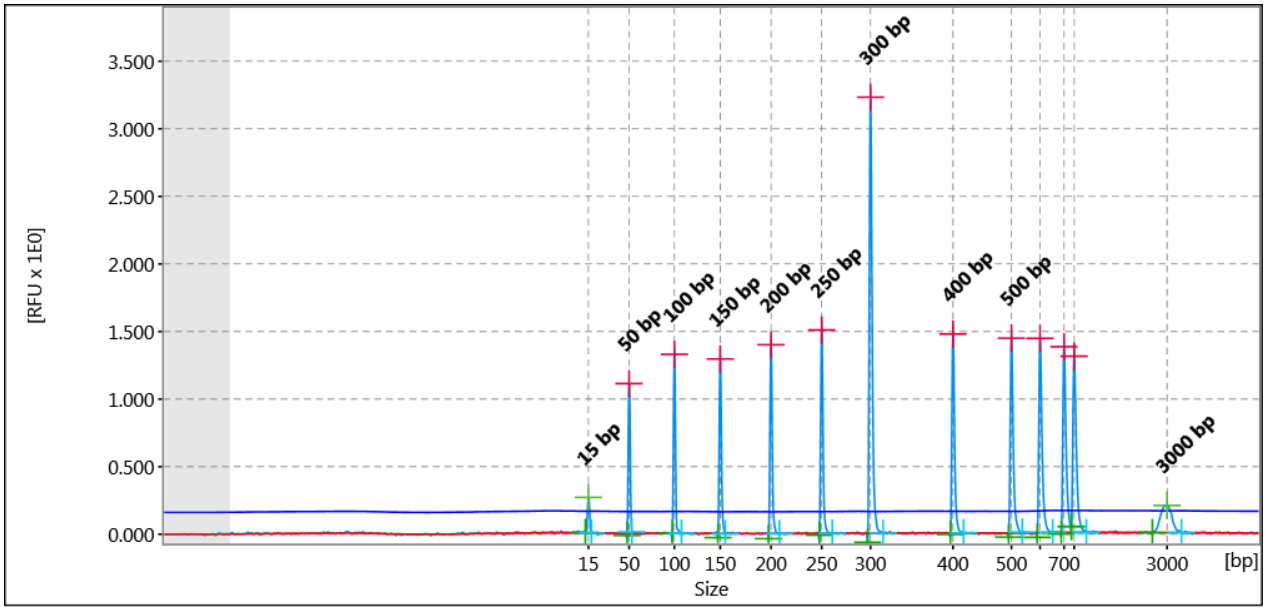


Figure: 18

Peak Calling Result Table: n/a

Online supplementary material. **Table 1: Demographic and functional characteristics of the validation set.**

	Control	COPD	Asthma	P value
Subjects (n)	9	5	5	
Age (years)	44 ± 14	61 ± 14	60 ± 9	<0.05
Sex (m/f)	3/6	3/2	3/2	0.51
Tobacco status (ns/es/cs)	5/1/3	0/3/2	1/2/2	0.19
Pack years	5 ± 7	28 ± 20	22 ± 26	0.06
Treatment				
ICS (yes/no)	0/9	5/0	0/5	<0.0001
LABA (yes/no)	0/9	5/0	0/5	<0.0001
LAMA (yes/no)	0/9	2/3	0/5	<0.05
SABA (yes/no)	0/9	0/5	3/2	<0.01
LTRA (yes/no)	0/9	1/4	0/5	0.23
Severity status		0 GOLD 1 4 GOLD 2 1 GOLD 3	4 mild-moderate 1 severe-refractory	
FEV1(% predicted)	110 ± 9	48 ± 11***$$$	96 ± 10	<0.0001
FEV1 post BD(% predicted)	110 ± 10	56 ± 12***$$	101 ± 11	<0.0001
FVC (% predicted)	113 ± 11	70 ± 21***$	97 ± 7	<0.001
FEV1/FVC (%)	82 ± 5	58 ± 10***$$	79 ± 6	<0.0001
Sputum				
Squamous cells (%)	7.0 (3.0-17.0)	0.0 (0.0-4.5)	7.0 (2.0-14.0)	0.06
Total non-squamous (10^6/g)	1.8 (1.0-2.5)	15.7 (5.7-22.7) **	2.3 (1.3-3.6)	<0.01
Viability (%)	70.0 (60.5-83.0)	76.0 (70.0-87.5)	79 (59.5-80.5)	0.74
Macrophages (%)	52.0 (43.3-71.8)	12.3 (4.7-15.9) *$	56.8 (47.6-59.6)	<0.01
Neutrophils (%)	41.0 (14.8-45.1)	82.8 (77.4-94.3) *$	36.6 (32.5-41.8)	<0.01
Lymphocytes (%)	2.6 (1.5-4.7)	0.4 (0.1-2.0)	2.4 (0.6-4.3)	0.14
Eosinophils (%)	0.2 (0.0-0.4)	0.8 (0.3-4.3)	0.4 (0.2-8.1)	0.07
Epithelial cells (%)	3.0 (2.5-11.7)	0.4 (0.1-2.5) *	2.2 (1.5-5.2)	<0.05

COPD: Chronic Obstructive lung disease, ns: non-smoker, es: ex-smoker, cs: current smoker, ICS: inhaled corticosteroids, LABA: long acting beta agonist, LAMA: long acting muscarinic antagonist. SABA: short acting beta agonist, LTRA: leukotriene receptor antagonist. FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity, post BD: post bronchodilation. Results are expressed as mean ± SD or median (IQR). * <0.05, ** <0.01, *** < 0.001 vs healthy subjects. $ <0.05, $$ <0.01, $$$ < 0.001 vs asthmatic patients.

Online supplementary material: Table 2: Demographic and functional characteristics of the patients

	Control	COPD	Asthma	P value
Subjects (n)	6	6	6	
Age (years)	30 ± 7	59 ± 16**	51 ± 16*	<0.01
Sex (m/f)	5/1	5/1	2/4	0.10
Tobaccostatus (ns/es/cs)	4/1/1	2/3/1	6/0/0	0.16
Pack years	3 ± 8	24 ± 25$^{\$}$	0 ± 0	<0.05
Treatment				
ICS (yes/no)	0/6	6/0	5/1	<0.001
LABA (yes/no)	0/6	6/0	2/4	<0.01
LAMA (yes/no)	0/6	2/4	0/6	0.10
SABA (yes/no)	0/6	1/5	0/6	0.35
LTRA (yes/no)	0/6	3/3	2/4	0.14
Severity status		0 GOLD 1 4 GOLD 2 2 GOLD 3	3 mild-moderate 3 severe-refractory	
FEV1(% predicted)	109 ± 14	46 ± 17***$^{\$\$}$	87 ± 12	<0.0001
FEV1 post BD(% predicted)	113 ± 16	53 ± 17***$^{\$\$}$	93 ± 14	<0.001
FVC (% predicted)	111 ± 12	67 ± 19**$^{\$\$}$	99 ± 14	<0.001
FEV1/FVC (%)	83 ± 6	53 ± 7***$^{\$\$}$	74 ± 10	<0.0001
Sputum				
Squamous cells (%)	24.5 (10.2-31.2)	1.5 (0.0-7.7) *	15.5 (2.2-22.0)	<0.05
Total non-squamous (10^6/g)	0.8 (0.3-2.7)	20.7 (13.6-101.9) **$^{\$}$	1.1 (0.7-4.7)	<0.001
Viability (%)	72.5 (60.2-77.2)	87.5 (76.7-95.7)	79.5 (68.0-92.7)	0.10
Macrophages (%)	72.7 (65.0-81.8)	1.2 (0.4-8.9) ***	30.8 (21.3-40.3)	<0.0001
Neutrophils (%)	12.6 (4.6-23.9)	97.5 (86.7-99.0) ***	45.9 (30.0-53.4)	<0.0001
Lymphocytes (%)	2.1 (1.3-3.5)	0.0 (0.0-0.4) *$^{\$}$	2.1 (1.1-3.8)	<0.01
Eosinophils (%)	0.0 (0.0-0.0)	0.4 (0.3-0.8)	9.1 (5.3-22.8) ***	<0.0001
Epithelial cells (%)	9.8 (3.7-14.9)	0.5 (0.0-1.9) *	5.4 (2.4-15.9)	<0.01

COPD: Chronic Obstructive lung disease, ns: non-smoker, es: ex-smoker, cs: current smoker, ICS: inhaled corticosteroids, LABA: long acting beta agonist, LAMA: long acting muscarinic antagonist. SABA: short acting beta agonist, LTRA: leukotriene receptor antagonist.

FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity, post BD: post bronchodilation. Results are expressed as mean ± SD or median (IQR). * <0.05, ** <0.01, *** < 0.001 vs healthy subjects. $ <0.05, $$ <0.01, $$$ < 0.001 vs asthmatic patients.

<u>Online supplementary material.</u> Table 3: Validation set: Housekeeping gene expression stability obtained with the 3 algorithms.

Gene	geNorm (M)	Rank	NormFinder	Rank	BestKeeper (r^+)	Rank	Final rank
GAPDH	0.578	1	0.21	1	0.97	1	1
GNB2L1	0.579	2	0.49	3	0.87	5	3
HPRT1	0.584	3	0.42	2	0.94	3	2
B2M	0.666	4	0.49	4	0.96	2	4
ACTB	0.761	5	0.65	6	0.8	8	6
RPL13A	0.831	6	0.58	5	0.9	4	5
18S	1.126	7	0.82	7	0.82	7	7
RPL32	1.428	8	1.1	8	0.83	6	8

<u>Online supplementary material.</u> Table 4: HPRT1 and GNB2L1 as reference genes in examples of other systems in humans.

System/organ/disease	Best reference genes	sources
Alveolar macrophages of COPD	GNB2L1, HPRT1, RPL32	[1]
Blood neutrophils	GNB2L1, HPRT1, RPL32, ACTB, B2M	[2]
Osteoarthritis synovium	HPRT1	[4]
Sepsis	HPRT1	[5]
Granulosa cells/ polycystic ovarian syndrome	HPRT1, RPLP0, HMBS	[6]
Bone marrow-derived mesenchymal stromal cells and dermal fibroblasts	HPRT1	[7]
Uterine sarcoma and carcinosarcoma tumors	HPRT1	[8]
Glioblastoma	HPRT1, TBP	[9]
HBV-related hepatocellular carcinoma	HPRT1, TBP	[10]
Tumor tissues	HPRT1	[11]
Bone mesenchymal stem cells from patients with avascular necrosis of the femoral head	HPRT1	[12]
Meniscus injury	HPRT1, TBP, GAPDH	[13]
Shoulder instability	HPRT1, B2M	[14]
Colon cancer	HPRT1, PPIA	[15]
Nasopharyngeal carcinoma	HPRT1, YARS	[16]
Non-small cell lung cancer	HPRT1	[17]

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