



# SUITABLE REFERENCE GENES FOR REAL-TIME PCR IN GENE EXPRESSION STUDIES WITH COLORECTAL, LUNG AND OVARY CLINICAL SAMPLES

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## INTRODUCTION

Classical housekeeping genes, such as 18S, GAPDH, HPRT1, GUSB, PGK1 are routinely used as endogenous references genes<sup>1-7</sup> to determine differentially expressed genes in cancer studies. However, recent reports show that they could present serious differences between tissues, cell lines or clinical samples, which may lead to unreliable results and consequently misinterpretations<sup>8</sup>. This study was focused on the selection of suitable reference genes for real time PCR in the clinical samples studied.

For this purpose It was performed a screening analysis of 154 clinical samples (48 of colorectal, 54 of lung and 52 samples of ovary) using a panel of 11 well-known endogenous genes. The endogenous gene accuracy was measured with *MinVarMedian* algorithm (very similar to *NormFinder*<sup>9</sup>), included in the SpotFire®+ StatMiner® Software. *MinVarMedian* algorithm is recommended for minimum variability and maximum stability normalization studies.

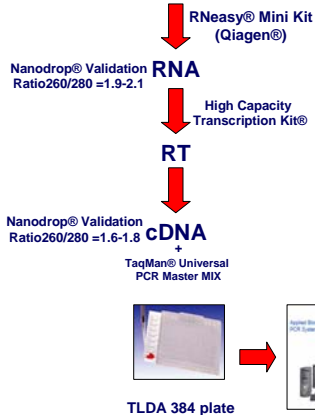
Dynamic comparison between the 11 candidates, let us to identify three suitable couples of endogenous genes on the clinical studied: B2m (beta-2- microglobulin)-POLR2A (DNA directed RNA polymerase II polypeptide A) genes in colorectal samples, ACTB (beta-actin)-IPO8 (importin 8) for lung and POLR2A-PP1A<sup>10</sup> (cyclophilin A) for ovary, with no statistical significant differences between normal and tumoral samples.

We have showed that the commonly used genes 18S (18S ribosomal RNA gene), GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and HPRT1 (hypoxanthine phosphoribosyl transferase 1) are not suitable to normalize the clinical samples studied, however, we have identified three plausible couples of endogenous genes with a high level of confidence. In a simultaneous analysis of three tissues, we have showed POLR2A and IPO8 were determined as the most stable genes in the clinical samples studied.

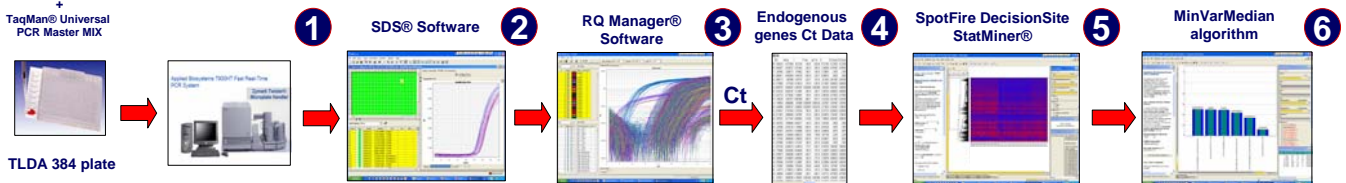
## METHODS

### Sample Analysis

Normal and Tumoral Tissues  
(colorectal, lung, and ovary)



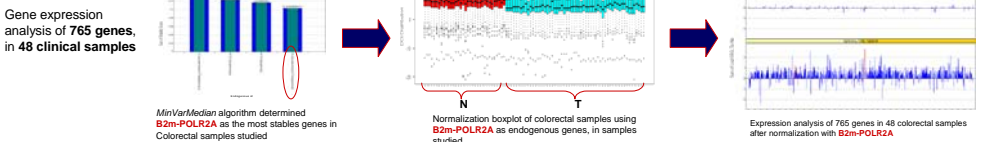
- 1 After cDNA validation by Nanodrop®, samples were loaded into a TLDA 384 plates format which included a special panel of 11 well-known endogenous genes (18S, ACTB, B2M, GAPDH, GUSB, HPRT1, IPO8, PGK1, POLR2A, PPIA and RPLP0). Applied Biosystems® 7900HT fast Real-Time System was used for PCR amplification.
- 2 Amplification signal ("reporter" signal of TaqMan® probe) was checked on each sample by SDS® Software.
- 3 Amplification signal was transformed into Ct values by the RQ Manager® Software.
- 4 Endogenous Ct data were analyzed with SpotFire® DecisionSite with StatMiner®. It was performed a previous data filtering based on Ct quality, outliers, detectability of genes and samples.
- 5 Gene expression stability were analyzed using *MinVarMedian algorithm* included in StatMiner®, based as *NormFinder* on correlation coefficients among genes. In *MinVarMedian algorithm* the influence of groups (Normal or Tumoral) is considered too.
- 6 Algorithms determined the best endogenous gene out of a panel of 11 previously included. Endogenous genes selected were used to normalize each tissue samples.



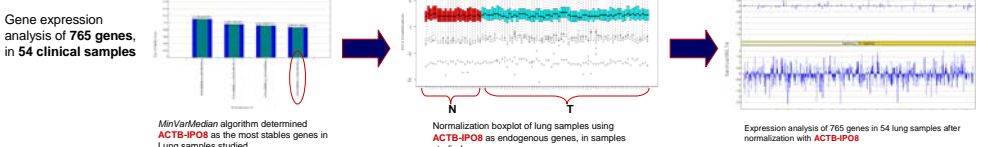
## RESULTS

- 6 After determination of the suitable endogenous genes, we performed a gene expression analysis in 154 clinical samples included, the results obtained showed statistical significant differences between normal and tumoral samples with a high level of confidence on biomarkers studied.

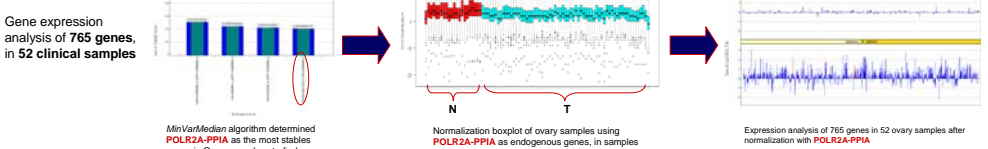
### Colorectal



### Lung



### Ovary



## CONCLUSIONS

•The protocol presented has demonstrated to be able to obtain an accurated endogenous genes analysis.

• Commonly used endogenous genes like 18S, GAPDH, HPRT1 GUSB, PGK1 are not suitable to normalize the clinical samples studied.

• Three couples of endogenous genes selected: B2m-POLR2A in colorectal, ACTB-IPO8 in lung and POLR2A-PP1A in ovary, have demonstrated to be very useful in gene expression profiling, allow us to determine several diagnostic biomarker in each tissue.

• POLR2A and IPO8 genes have demonstrated a very high stability score, it has been selected by the algorithm as the most stable endogenous genes in a simultaneous analysis of COLORECTAL, LUNG and OVARY clinical samples studied.

• In our opinion, suitable endogenous gene should be determined of a previous panel for each tissue and for a given group of samples, as something dynamic not fixed, because a small gene expression variations of endogenous genes between tissues, cell lines, etc could lead different results and consequently misinterpretations.

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