Urinary mRNA for the Diagnosis of Renal Allograft Rejection: The Issue of Normalization

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ABSTRACT

Urinary messenger RNA (mRNA) quantification is a promising method for noninvasive diagnosis of renal allograft rejection (AR), but the quantification of mRNAs in urine remains challenging due to degradation. RNA normalization may be warranted to overcome these issues but the strategies of gene normalization have been poorly evaluated. Here, we address this issue in a case-control study of 108 urine samples collected at time of allograft biopsy in kidney recipients with (n=52) or without (n=56) AR by comparing the diagnostic value of IP-10 and CD3ε mRNAs, two biomarkers of AR, after normalization by either the total amount of RNA, or by normalization by one of the three widely used reference RNAs – 18S, GAPDH and HPRT – or by normalization using uroplakin 1A (UPK) mRNA as a possible urine-specific reference mRNA. Our results show that normalization based on total quantity of RNA is not substantially improved by additional normalization and may even be worsened with some classical reference genes that are overexpressed during rejection. However, considering that normalization by a reference gene is necessary to ensure PCR quality and reproducibility, and to suppress the effect of RNA degradation, we suggest that GAPDH and UPK1A should be preferred to 18S or HPRT RNA.

INTRODUCTION

Quantification of urinary cell mRNAs is an emerging, noninvasive method to diagnose acute rejection (AR) of the renal allograft. A major achievement was the publication by Suthanthiran et al. of the prospective multicenter CTOT4 study validating a diagnostic formula using the quantification of interferon-inducible protein 10 (IP-10), CD3ε and 18S RNAs in the urine cell pellets of kidney recipients.(1)

Quantification of RNA species from urine samples remains challenging due to low amounts of degraded mRNAs.(2) Normalization of RNA quantification, which is a universal rule in quantitative polymerase chain reaction (PCR) analysis, seems even more critical for urine samples, as it avoids biases linked to variations in total RNA content, in storage conditions, RNA integrity, reverse transcription (RT) or PCR amplification efficiency.

Several normalization strategies have been used for the quantification of urinary mRNA levels. **1) Normalization by a reference gene**. If the chosen reference gene is expressed steadily and ubiquitously, the results amount to a quantification of the number of copies of mRNA per cell. It is a robust, easy, and widely used technique, but it is dependent on the quality of the reference gene: if its expression is not stable among the different diagnoses being explored, the results will correlate with the reference gene in addition to the gene of interest. **2) Normalization by the total amount of RNA**. This technique, developed by the Suthanthiran team,(1) consists in the standardization of the amount of total RNA for the RT step. Unfortunately, this strategy is dependent on the quantification of the total amount of RNA in the sample, which has limited accuracy in samples with very low RNA amount, or containing degraded RNAs. After RT and absolute quantification using a standard curve method, the results are expressed as the number of copy per microgram total RNA. **3) Normalization by a cell-type specific gene**. Here, the reference gene is specific to a cell type

shed steadily in urine, independent of the underlying disease. For example, the epithelial-specific aquaporin-4 mRNA has been suggested as a reference gene for the detection of glomerular disease.(3)

While a multicenter evaluation of a standardized protocol for gene expression profiling of urine samples was recently published and reported acceptable interlaboratory coefficients of variation, the need for improvement of the current method was acknowledged.(4) Here, we address the question of the method for mRNA normalization by comparing the diagnostic value of IP-10 and CD3ε mRNAs, two biomarkers of AR, after normalization by either the total amount of RNA, or by normalization by 18S, GAPDH or HPRT RNAs, three of the most widely used reference RNAs, or by normalization using uroplakin 1A (UPK) mRNA as a possible urine-specific reference mRNA.(5)

MATERIAL AND METHODS

We retrospectively included kidney transplant recipients from the Department of Kidney Transplantation at the Tenon and Necker hospitals (Paris, France). We examined 108 urine samples from those patients who had undergone either a diagnostic (for-cause, n=78) renal allograft biopsy or a scheduled (protocol, n=30) biopsy. Biopsies were scored according to the Banff 97 diagnostic categories for renal allograft biopsies using the Banff '09 update.(6, 7) This study included two groups of patients: one "acute rejection" group (n=52) with T cell mediated rejection (n=11), borderline changes (n=3), antibody mediated rejection (n=28) or mixed rejection (n=9) and one "no rejection" group without any feature of acute rejection (n=56). Patients' characteristics and histopathological features of the biopsy including Banff's score are detailed in **Supplemental Tables 1 and 2**, respectively. The study followed the French legislations for biomedical research, and the clinical database was duly declared to the *Commission Nationale Informatique et Libertés* (CNIL). All patients were informed of the aims and contents of the trial, and gave written consent, according to French law. Urine specimens for the mRNA profiling study were collected within 24 hours of the biopsy procedure.

Quantification of mRNAs

Absolute quantification of RNAs was performed using a standard curve method.(1) The standard curve is established using PCR generated 73bp mouse Bak amplicon as the standard. A detailed description of the absolute quantification method is provided in the Supplemental Material and Methods. Urine samples were centrifuged at 2000g for 10 minutes within 4 hours of collection. RNA was extracted from the pellet using the RNeasy mini kit (Qiagen, Courtaboeuf, France) and reverse-transcribed to cDNA using TaqMan®

Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA). Sequences of primers and fluorogenic probes for the measurements of levels of IP-10, CD3ε, and 18S RNAs have been previously reported.(1, 8) We designed oligonucleotides primers and fluorogenic probes for the measurement of GAPDH mRNA. The sequences and location of the primers and the probe are detailed in **Supplemental Table 3**. We used commercially available assays (Applied Biosystems, Carlsbad, CA) for the measurement of HPRT (ref Hs02800695_m1) and UPK1A (Hs01086736_m1) mRNAs. Except for 18S rRNA, PCR analysis was performed by a two-step process, a preamplification step followed by measurement of levels of RNAs using an ABI Prism 7500 Fast detection system.

In vitro modeling

We used a urothelial cell line from human bladder carcinoma (EJ138), immortalized human proximal tubular epithelial cells (HK-2 cell line), and human peripheral blood mononuclear cells (PBMCs) with or without activation by phytohemagglutinin A (PHA) for 48 hours. We counted the cells after letting them grow for two days. Various amounts of EJ138 and HK-2 cells and activated or resting PBMCs were mixed to mimic three clinical conditions: condition 1 (unstimulated PBMCs + urothelial cells), condition 2 (stimulated PBMCs and urothelial cells) and condition 3 (stimulated PBMCs + urothelial cells + epithelial cells). After centrifugation, RNA was extracted from the pellet using the RNeasy mini kit (Qiagen, Courtaboeuf, France). IP-10, GAPDH, UPK mRNAs and 18S rRNA were measured by quantitative PCR. Values of IP-10 mRNA per μg of total RNA were compared to IP-10 mRNA values after normalization by 18S, GAPDH or UPK RNAs.

Statistical analysis

Receiver operating characteristic (ROC) curves were performed using the pROC package of the R software.(9) Statistical analyses were performed using the JMP 11.0.0 and R software. We used Student's t-test for quantitative variables and exact Fisher's test for categorical data.

RESULTS

Confirmation of the diagnostic value of urinary RNAs to predict renal allograft rejection

We first attempted to validate the results of the CTOT4 study in our study population. Compared to the no-AR group, the CTOT4 formula «F= −6.1487 +0.8534 $\log_{10}$(CD3ε) + 0.6376 $\log_{10}$(IP-10) +0.1554 $\log_{10}$(18S)» increased in the AR group (P=0.0003), with a discrimination (area under the curve [AUC]=0.72; 95% confidence interval [95% CI], 0.61 to 0.82; P=0.0002) similar to previously published results (**Figure 1A** and **1B**).(1)

We then accessed the performance of each of the three RNAs involved in this formula. Compared to the no-AR group, absolute (i.e. not normalized to 18S rRNA) copy number of IP-10 (P=0.00002) and CD3ε (P=0.004) was indicative of AR (**Figure 1C-1F**). Interestingly, and as already reported by the CTOT4 study, (1) the copy numbers of 18S rRNA were also higher in patients with AR than in those who had biopsy specimens showing no AR (P=0.02) and may be considered as a diagnostic marker for AR (AUC=0.63, P=0.008) rather than a prototypical reference gene (**Figure 1G-H**).(10)

Together, these results, showing that absolute copy number per microgram total RNA of IP-10 and CD3ε RNAs provide good discrimination and that 18S rRNA copy number significantly differs between groups, question the usefulness of 18S as a reference gene.

Virtual modeling of the detection of cells of renal origin in urine

Our aim was to investigate innovative reference genes that would be used in urinary cell mRNA profiling. We hypothesized that urinary cells are typically of urothelial and renal origin. In case of renal disease, the urothelial cells should not be impacted, but variations would be expected in renal cell numbers, type, and pattern of marker expression. In order to assess all these variations, a perfect mRNA-based noninvasive tool should be able to

estimate the different cell types and the markers of cellular activation in a particular cell type. The normalization by a ubiquitous reference gene, such as 18S rRNA, GAPDH mRNA or HPRT mRNA, is adequate to assess changes of expression of markers by cells, but inadequate to assess the number of cells, as normalization by a ubiquitous gene is equivalent to normalization by the number of cells.

As urothelial cells would not be impacted by renal diseases, we reasoned that normalization by an urothelial-specific transcript should allow detection of variations in the number of cells of renal origin, whether or not there are variations in cell types and cellular activation. To test this hypothesis, we identified uroplakin 1A (UPK) as a urothelial-specific mRNA. **Figure 2A** shows different theoretical conditions characterized by variations in the total number of cells, the expression level of a biomarker candidate in expressing cells and the number of cells expressing the candidate biomarker. The effect of normalization by a ubiquitous or a urothelial gene is modeled in **Figure 2B** and **Supplemental Table 4** and suggests that the presence of other cells of renal origin interferes with the detection of the immune-related biomarker when normalization by 18S, GAPDH or HPRT RNAs, but not UPK mRNA is used.

Comparison of several normalization methods

We then evaluated whether an alternative strategy for mRNA normalization could improve the performance of the immune-related mRNAs to diagnose AR. We therefore evaluated the discrimination capacities of IP-10 and CD3ε mRNAs assessed by their absolute copy number, their normalized value using 18S, GAPDH or HPRT, three of the most widely used reference RNAs, or using the urothelial-specific mRNA UPK. Of note, the UPK copy numbers were similar in male and female urine samples (P=0.70) (**Figure S1**). We first

evaluated the relationships between the different RNA levels and found that they strongly correlated to each other (Spearman's correlation coefficient=0.59-0.94) except for UPK mRNA, whose correlation with the immune-related genes remained minimal (0.36-0.43)(**Figure 3**). Strikingly, IP-10 and CD3ε mRNAs were found to be highly correlated with the three usual reference genes, GAPDH, HPRT and 18S RNAs (**Figure 3**). In line with this observation, urinary GAPDH, HPRT and 18S RNA levels were marginally associated with the diagnosis of AR, while UPK mRNA level was independent of AR (**Figure S2**).

We then compared the discrimination capacities of IP-10 and CD3ε mRNA levels using the five different approaches for gene normalization (**Figure 4A** and **Supplemental Table 5)**. The different processes for IP-10 mRNA normalization yielded similar AUC values (P>0.05 for all comparisons) with the exception of the AUC of IP-10 mRNA normalized by HPRT, which was significantly lower than the AUC obtained after normalization by GAPDH mRNA (P=0.03)(**Figure 4A** and **Supplemental Table 5**). Comparing the five strategies used, IP-10 mRNA absolute copy number yielded the best AUC (0.76, 95% confidence interval [95% CI], 0.66 to 0.86, P=0.000008) for the diagnosis of AR. Focusing on the four tested reference genes, GAPDH normalization yielded numerically better results than HPRT, 18S and UPK normalization. Similar patterns were obtained with regard to the diagnostic value of CD3ε mRNA; absolute copy number and GAPDH normalization gave the best AUC values, while normalization by HPRT yielded significantly lower AUC (**Figure 4B** and **Supplemental Table 5**).

DISCUSSION

The PCR-based method developed by the Cornell group is now very close to implementation in clinical practice and standardization of the protocol is crucial to allow its general use in non-expert laboratories.(4) While urine samples are primarily characterized by extensive degradation of the RNA and issues about the reliability of gene expression profiling, the question of gene normalization has been poorly evaluated with inconsistencies in the methods used by independent teams. Though, there is increasing evidence that classical reference RNAs are not perfect, and should be re-evaluated through a systematic approach.(11)

Using an *in vitro* model reproducing the changes in urinary cell composition and proportion, our results suggest that biomarker levels normalized with conventional reference genes can fail to detect variations of markers expressed by cells that can themselves vary in number and type, a result that prompted us to investigate a urothelial-cell specific reference gene.

The strong correlation between the expression of the immune-related mRNAs and the reference mRNAs suggests that the cells associated with AR (expressing CD3ε and IP-10 mRNAs) are an important contingent of the total number of urinary cells, a result that may explain why the diagnostic values of IP-10 and CD3ε mRNAs are not improved at all by the normalization strategies that use a reference gene. This observation is in total agreement with Suthanthiran et al who reported that, if the inclusion of 18S rRNA in the CTOT4 formula did not weaken the predictive value, a signature based on CD3ε and IP-10 mRNA has a near-perfect correlation with the signature that used an additional normalization by 18S rRNA levels and a similar AUC (0.8447 vs. 0.8454).(12) Together with our results, these data support the view that a strategy based on normalization of the total amount of RNA

followed by an absolute quantification with the use of a standard curve as developed by the Cornell team is not improved, and may even be worsened, by additional normalization to a reference gene, at least in an experimented lab.

Normalization by a reference gene is necessary to suppress the effect of RNA degradation. RNA is very sensitive to degradation, especially in urinary samples.(2) Even when stored at -80°C (13) and when using RNA stabilizing solutions,(14) there is a systematic decrease of RNA amounts. Normalization with a reference gene accounts for this problem, as the decrease of the gene of interest (numerator) and the decrease of the reference gene (denominator) will counterbalance each other, ensuring a stable result. Thus, the use of reference genes remains important to ensure PCR quality and reproducibility, and to correct various systematic biases, which is essential for wide generalization of a test to different independent centers. However, reference genes should be chosen cautiously, depending on the situation.(15) If an additional level of normalization is necessary to export this technically demanding strategy, our comparative results suggest that the reference gene GAPDH mRNA may be preferred over that of HPRT and 18S genes as its expression level in urine is only marginally associated with an AR diagnosis. However, a prerequisite of a reference gene is its constant expression level in different conditions, and this characteristic is fulfilled by none of the three conventional reference genes. In this regard, UPK mRNA, whose expression level is not altered by AR, may be an attractive option that requires further evaluation. Finally, unlike 18S rRNA whose expression is huge in urine samples, GAPDH and UPK expression levels remain in a very similar range compared to the immune-related biomarkers and are included in the preamplification step that is necessary for the other RNAs to be detected by qPCR, thus providing an additional opportunity to adjust for preamplification variations.

There are several limitations to our study: firstly, it is exploratory in essence, which means that we did multiple comparisons to evaluate different normalization techniques, a method that can be associated with false discovery of significant results. However, our results mainly show the absence of difference between different methods (which is not induced by multiple testing), suggesting that the simplest and safest method should thus be used. Secondly, we focused our study on the normalization of IP-10 and CD3ε by four different reference genes for the detection of renal allograft rejection. IP-10 and CD3ε are among the most robust urinary markers to date, but with the use of genome-wide techniques it is anticipated that new candidate and reference genes will emerge. Given that there is no perfect normalization method, the best combination of genes of interest and a reference gene should always be reassessed in order to ensure that they fulfill particular diagnostic needs. Thirdly, our results are limited to evaluation of markers in urinary cell pellets as the use of circulating RNAs (a new and growing field) might require different normalization and strategies.

To conclude, we suggest that a strategy based on normalization of the total amount of RNA coupled to an absolute quantification with the use of a standard curve as developed by the Cornell team, is not substantially improved by additional normalization to a reference gene in an experimented lab. However, considering that normalization by a reference gene is necessary to ensure PCR quality and reproducibility, and to suppress the effect of RNA degradation, we suggest that GAPDH and UPK1A should be preferred to 18S or HPRT RNA.

ACKNOWLEDGEMENTS

DISCLOSURE

PG, AH, IB, YCXD and ER are the authors of a patent on UPK normalization.(5)

FIGURE LEGENDS

Figure 1: Diagnostic value of the CTOT4 formula and of the three RNAs that are included in this formula, separately. (A, C, E, G) Levels of the CTOT4 formula «F= −6.1487 +0.8534 $\log_{10}(CD3\varepsilon)$ + 0.6376 $\log_{10}(IP\text{-}10)$ +0.1554 $\log_{10}(18S)$» (A), IP-10 mRNA (C), CD3ε mRNA (E) and 18S rRNA (G) collected at time of renal allograft biopsy. (B, D, F, H) Corresponding receiver operating characteristic (ROC) curve analyses of the biomarker. The reported results were obtained from 108 paired urine and biopsy specimens (52 acute rejections and 56 samples with no rejection).

AUC, area under the curve; CI, confidence interval

Figure 2: The issue of RNA normalization in urine. The relative quantification of an RNA marker in urine will vary with changes in expression of the marker in the urinary cells, or with changes in the number of cells expressing the marker in urine. This relative quantification may also not vary (or vary in the opposite way) if changes in the number of other cells expressing the reference RNA counterbalance the changes in expression of the marker of interest. **(A)** The figure depicts four conditions characterized by various numbers and types of cells. For each condition, an estimation of the IP-10/18S and IP-10/UPK ratio is provided. For comparison, the ratios are reported to the one obtained in the healthy control (brackets). **(B)** Quantitative PCR analysis of IP-10 mRNA normalized or not by 18S, GAPDH or UPK RNAs in cell preparations containing or not PBMCs, renal epithelial cells and urothelial cells. The different conditions include a cell pellet containing urothelial cells, unstimulated PBMCs (basal state) or PHA-stimulated PBMCs (mimicking their activation state during rejection). A third condition included urothelial cells, PHA-stimulated PBMCs and proximal tubule epithelial cells (mimicking rejection with acute tubular necrosis). Gene expression was assessed using the absolute quantification method (see Material and Methods) and quantified as mRNA copies/µg of total RNA. The figure shows the relative expression level where the first condition is used as the reference. The quantification of non-normalized IP-10 mRNA, as well as IP-10/18S, IP-10/GAPDH and IP-10/HPRT ratios, but not IP-10/UPK ratio, were underestimated when proximal tubule renal epithelial cells were added suggesting that the presence of other cells of renal origin interferes with the detection of the immune biomarker when normalization by 18S, GAPDH or HPRT RNAs is used, but not when normalization by UPK mRNA is used (basal values normalized to 1).

Figure 3: Correlation matrix. The following scatterplot matrix represents all values of urine pellet RNAs plotted against each other. Patients in the acute rejection group are colored in red, and patients in the no-rejection group are plotted in blue. The correlation line is fitted to the plot with its 95% confidence interval. The Spearman's correlation coefficient (r_s) is shown in the upper left-hand side with a corresponding color code. This scatterplot shows that all tested RNAs, except UPK, strongly correlate to each other.

Figure 4: Consequences of normalization on the diagnostic value of the urinary biomarkers. Receiver operating characteristic (ROC) curves for urinary IP-10 (A) and CD3ε mRNA levels (B), normalized by the RNA amount (absolute copy number), or after additional normalization by UPK, HPRT, GAPDH and 18S RNAs.

AUC, area under the curve; UPK, uroplakin 1A

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Supplemental Material and Methods

Table S1: Patient characteristics

Table S2: Banff scores (mean ± SD) and percent of patients with histology scores >0 on biopsy

Table S3: Oligonucleotide primers and probes used for the quantification of RNAs

Table S4: Experimental conditions, total quantity of obtained RNA and copy numbers of IP10, 18S, GAPDH, HPRT and UPK RNAs in three in vitro experiments mimicking clinical situations.

Table S5: Discrimination of acute rejection by urinary biomarkers.

Figure S1: Effect of gender in UPK mRNA copy numbers in urinary cells. Absolute copy numbers of UPK mRNA in 108 urine specimens collected at time of allograft biopsy in 67 males and 41 females.

Figure S2: Diagnostic value of the four RNAs evaluated as reference gene. Absolute copy numbers of 18S rRNA (A), GAPDH (B), HPRT (C) and UPK (G) mRNAs in 108 urine specimens collected at time of allograft biopsy (52 acute rejections and 56 samples with no rejection). (B, D, F, H) Corresponding receiver operating characteristic (ROC) curve analyses of the RNAs as markers of acute rejection.

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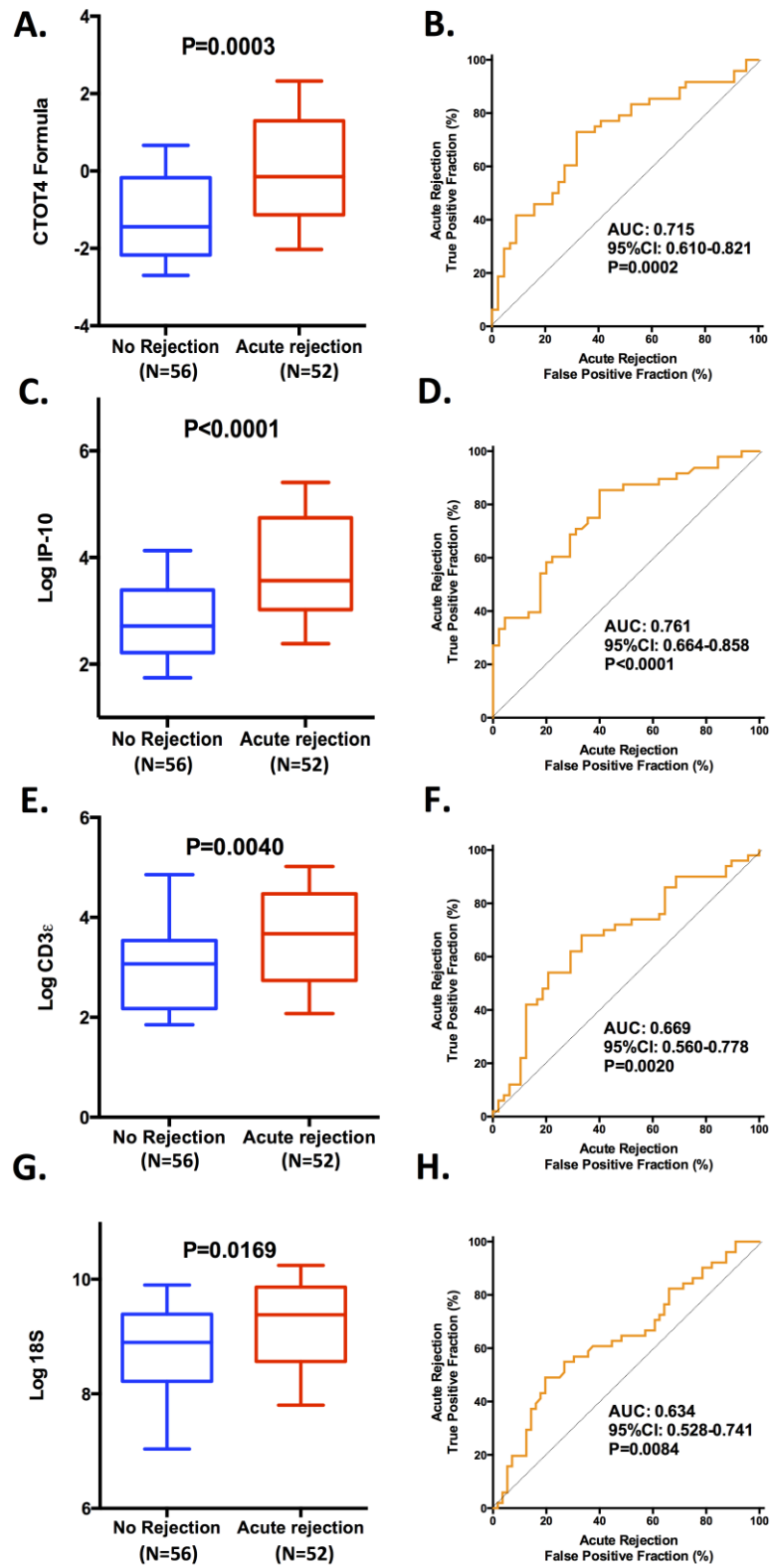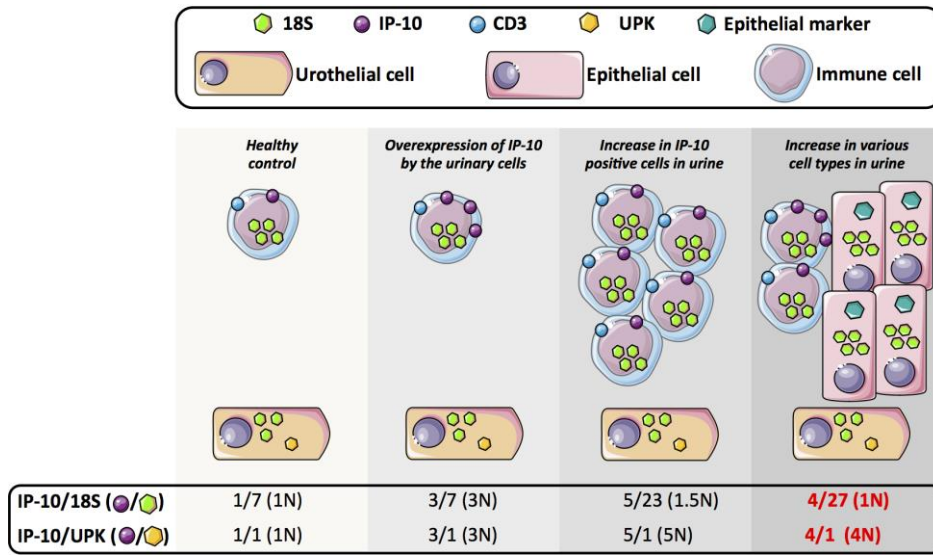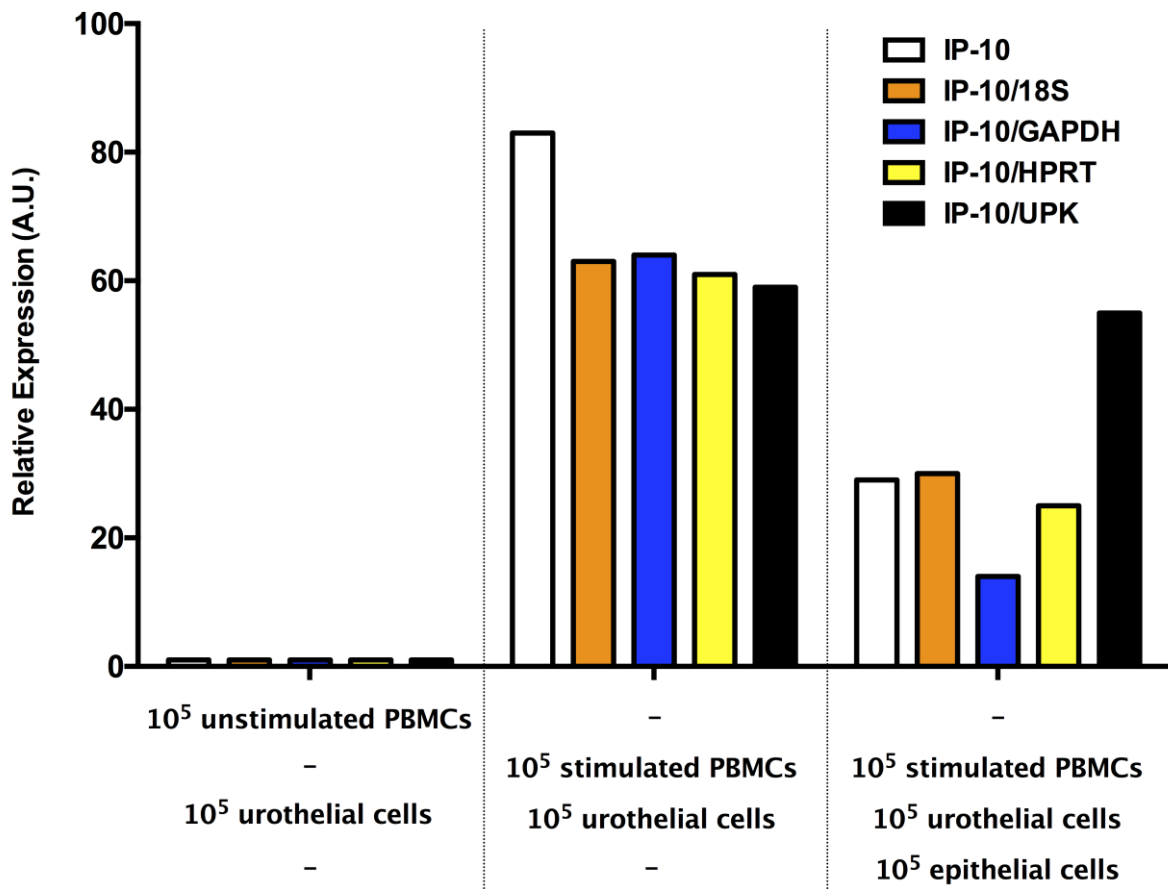
Figure 1

Figure 2

A.

B.

Figure 3

Figure 4

A.

UPK-normalized (AUC: 0.735; P<0.0001)
HPRT-normalized (AUC: 0.700; P=0.0004)
GAPDH-normalized (AUC: 0.745; P<0.0001)
18S-normalized (AUC: 0.733; P<0.0001)
Absolute copy number (AUC: 0.761; P<0.0001)

B.

UPK-normalized (AUC: 0.618; P=0.0228)
HPRT-normalized (AUC: 0.561; P=0.1549)
GAPDH-normalized (AUC: 0.690; P=0.0006)
18S-normalized (AUC: 0.639; P=0.0091)
Absolute copy number (AUC: 0.669; P=0.0020)