For protein assays, we mixed 20 μmol/L dye (0.03 or 0.12 mmol/L), sodium oxalate (0 or 0.06 mmol/L dye and 1.0 mmol/L sodium oxalate without SDS) in all combinations. Sodium oxalate concentration (with omission of SDS) eliminates detectable interference (Table 1). We confirmed the sodium oxalate decreases the interference (7) whereas decreased dye combined with increased sodium oxalate (minus SDS) eliminates detectable interference.

In conclusion, the extent of aminoglycoside interference in the PRM assay is dictated by the composition of the dye reagent. SDS increases the interference (6), whereas sodium oxalate decreases the interference (7), and these effects are compounded by changes in the concentration of the dye. Thus, increased dye combined with the omission of sodium oxalate (plus SDS) gives very high interference, whereas decreased dye combined with increased sodium oxalate (minus SDS) eliminates detectable interference.

### Table 1. Effect of varying the composition of the PRM reagent on the absorbance produced by the aminoglycosides in the absence of protein. *

<table>
<thead>
<tr>
<th>Dye, mmol/L</th>
<th>0.12</th>
<th>0.12</th>
<th>0.06</th>
<th>0.03</th>
<th>0.03</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS, mg/L</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sodium oxalate, mmol/L</td>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Mean absorbance at 600 nm in the presence of:

| Neomycin, 1 g/L | 0.792 | 0.549 | 0.210 | 0.029 | <0.005 |
| Gentamicin, 1 g/L | 0.733 | 0.460 | 0.138 | 0.024 | <0.005 |
| Tobramycin, 10 g/L | 0.803 | 0.590 | 0.270 | 0.036 | <0.005 |
| Paromomycin, 10 g/L | 0.798 | 0.436 | 0.121 | 0.019 | <0.005 |
| Kanamycin, 10 g/L | 0.801 | 0.183 | 0.060 | 0.017 | <0.005 |
| Geneticin, 10 g/L | 0.855 | 0.138 | 0.037 | 0.014 | <0.005 |
| Streptomycin, 10 g/L | 0.143 | 0.042 | 0.026 | 0.011 | <0.005 |
| Amikacin, 10 g/L | 0.541 | 0.055 | 0.021 | 0.010 | <0.005 |
| Dihydrostreptomycin, 10 g/L | 0.255 | 0.044 | 0.020 | <0.005 | <0.005 |

* The results were generated by a mixture of 20 μL of aminoglycoside and 1 mL of dye reagent (n = 4; CV <10%). The Watanabe reagent contains 0.06 mmol/L dye and 1.0 mmol/L sodium oxalate without SDS (2).

### References


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becomes systematically depleted of its lithogenic substances (1). This effect can be large enough to be clinically relevant (2). However, only the altered urine can be assessed by urine collection, which leads to a urinalysis that does not sufficiently reflect the patient’s true metabolic status. The excessively low concentrations and excretion values of the lithogenic urinary constituents may lead to “optimistic” misinterpretation of the patient’s actual health status and overestimation of treatment success.

Apart from geometric and physiologic simplifications, the previously used model assumes that the differential volume function of the kidneys amounts to 50%. Consequently, both the stone-free and the stone-forming kidney contribute equally to the total urine volume. However, even in healthy individuals, the differential function varies by ±6%.

Parenchymal defects, such as tumors, renal arteriopathy, and posttraumatic failures, or morphologic abnormalities, such as pyeloureteral junction stenosis and vesico-ureteral-renal reflux, can lead to a dramatic decrease in renal function, causing a reduction in the glomerular filtration rate. Patients suffering from these diseases often present with calcium nephrolithiasis and proteinuria, occasionally with progression to nephrocalcinosis, urinary concentrating defects, and renal insufficiency caused by tubular atrophy, intestinal fibrosis, and glomerulosclerosis.

The process of kidney stone formation follows fundamental physical principles such as mass conservation. In a recently introduced model (1), we evaluated the influence of in vivo-growing uroliths on urinary composition. The material from which the stones are formed originates completely from the urine streaming through the kidneys; therefore, the urinary concentrations of the lithogenic components, such as calcium and oxalate, in the excreted urine must be depleted by the mass deposited in the stone(s). As long as no method exists to measure those concentrations in vivo, mathematical models describing the depletion process are invaluable for quantitatively estimating the effect. These models can be used to “correct” measured concentration values of lithogenic urinary constituents.

In the example calculations below, we refer to the mineral phase calcium oxalate monohydrate (COM), which is currently the most common stone type formed in individuals in developed countries (3).

When the differential function of a kidney is 50% and only one kidney is affected by urolithiasis, the stone-growth-related depletion effect can be estimated according to Eq. 1 (1):

\[ c_i = \frac{1}{2} \left( \frac{V}{\tau} \right) + \frac{1}{\nu} \left( 1 + \frac{V}{\nu \phi c} \right) c \]  

(1)

with \( c_i \) as the proximal (with respect to the stone) concentration of, e.g., oxalate, and \( c \) as the distal concentration (i.e., the measured concentration); \( c_i \) is the concentration that maintains stone formation (\( c_i \approx c \)). The term \( V/\tau \) is the mean growth rate of the stone (mm³/day) within the observation period between \( t = 0 \) and \( t = \tau \), and \( V \) denotes the total stone volume that has been formed within the period \( \tau \). The variable \( \phi \) is the mean urinary flow rate, and \( \nu \) represents the volume of a stone composed of 1 mol of substance.

The former model (1) assumes that urine excretion is equally distributed between the stone-bearing and stone-free kidneys. Because this is not necessarily the case in totally stone-free individuals, our model can be refined by considering the distribution of excretion between the kidneys. As the depletion effect takes place only in the stone-forming kidney, an asymmetric differential function influences the correction of measured values.

For quantitative determination of the differential-function-dependent depletion effect, we have to enhance our formalism of measured concentration \( c \) and initial concentration \( c_i \) by introducing the exposure fraction (\( \gamma \)) for urine to stone(s), with \( 0 \leq \gamma \leq 1 \). \( \gamma \) denotes the volume fraction of a, e.g., 24-h urine that is flowing past the stone(s); therefore, \( 1 - \gamma \) is the volume fraction formed by the stone-free kidney, or the urine fraction excreted without contact to stone(s).

Assuming identical flow rates of \( \phi \) through both kidneys, Eq. 14 of Laube et al. (1) becomes:

\[ c = \frac{V}{\tau} \left( 1 + \frac{1}{\nu \gamma c_i} \right) \left( \gamma c_i + (1 - \gamma) c_i \right) \]

(2)

By transforming and resolving this equation in a way analogous to that described in detail in Laube et al. (1), we find for the initial concentration \( c_i \):

\[ c_i = \left\{ \frac{1}{2} \left[ 1 + \frac{V}{\nu \gamma c} \right] \right\} \left( \frac{V}{\tau} \right) + \frac{1}{\nu} \left( 1 + \frac{V}{\nu \phi c} \right) \]

\[ + \left[ \frac{1}{2} \left( 1 + \frac{V}{\nu \phi c} \right) \right]^{\gamma} \left( \frac{V}{\tau} \right) \left( 1 + \frac{V}{\nu \gamma c} \right) \]

\[ + \left[ \frac{1}{2} \left( 1 + \frac{V}{\nu \phi c} \right) \right]^{\gamma} \left( \frac{V}{\tau} \right) \left( 1 + \frac{V}{\nu \gamma c} \right) \]

\[ c \]  

(3)

The above equation (Eq. 3) is an important improvement of our former model because it offers a more general approach. For \( \gamma = 1 \), all urine is streaming through the stone-bearing kidney, and Eq. 2 reproduces case 2 of our former model (1). Case 2' from our former model can be directly deduced for \( \gamma = 0.5 \) (1). In that case, only one-half of the urine is produced by the stone-forming kidney. For the theoretical situation of \( \gamma = 0 \), all urine originates from the stone-free kidney; therefore, the measured concentration \( c \) equals \( c_i \).

However, if the value of the stone formation rate (\( V/\tau \)) is positive, a lower limit for \( \gamma \) exists, which is established
by the positiveness of the numerator in Eq. 2; this assures the existence of the numerical solution of Eq. 3.

The interpretation of \( \gamma \) can be further generalized. The model permits its interpretation not only as being the volume fraction formed by the stone-bearing kidney, but also allows it to be regarded as the urine fraction that actually passes the growing stone. Thus, the case when not all of the urine produced by the stone-bearing kidney comes in contact with the stone is also covered by the improved depletion model.

With this generalized interpretation, not only can the differential function be mapped to our model, but also all limitations through partial exposure of stone(s) to lithogenic substances. Thus, the introduction of limitations through partial exposure of stone(s) to lithogenic components distal to in vivo-growing uroliths, \( S \) of minor relevance. However, for a more asymmetric strategy. Thus, the depletion effect should be considered when interpreting data from the clinical metabolic work-up of stone-bearing patients.

To calculate values of \( \phi \), in dependence of \( \phi \) and \( V/\tau \), the following reasonable values for the variables \( \tau \), \( V \), \( \phi \), \( \nu \), and \( c \) are chosen: growth rate \( (V/\tau) = 1 \), 2, 5, 10, or 20 mm\(^3\)/day. These growth rates result after a period of 1 year in uroliths indicated by radii of 4.43, 5.59, 7.58, 9.55, and 12.03 mm, respectively. The molar volume of COM \( (\nu) = M/\delta = 65.82 \text{ cm}^3/\text{mol} \), where \( M = 146.1 \text{ g/mol} \) and \( \delta = 2.22 \text{ g/cm}^3 \) as the molar weight and the density of COM, respectively. The mean urine flow rate \( \phi \) during period \( \tau \) amounts to 1500 cm\(^3\)/day, a typical value observed in stone-forming persons. The measured urinary oxalate concentration \( (c) \) is set to be 0.37 mmol/L. The latter value is calculated from \( \phi \) and the established limit value of the oxalate excretion of 0.56 mmol/day, which is applied to distinguish normooxaluric urines from "mild" hyperoxaluric ones (5, 6).

To gain an overview of the effect of exposure fraction \( (\gamma) \) on the extent of the urinary depletion effect caused by in vivo-growing uroliths, \( \gamma \) is varied nearly within the entire theoretical value range, i.e., from 0.01 to 1. However, only in rare cases will a patient's differential function fall below 0.25 or exceed 0.75.

The top panel in Fig. 1 illustrates the importance of the presented refinement to the depletion model with a fixed \( \gamma = 0.5 \) (1). This graph shows the dependence of the ratio of the results of the refined and the former model \( (\gamma = 0.5) \) on \( V/\tau \). As expected, all functions show for \( \gamma = 0.5 \) a ratio indicated by the value 1. The slopes of the functions increase with the value of \( V/\tau \). Within the range of normal fluctuation for the differential function, i.e., \( 0.44 \leq \gamma \leq 0.56 \), the refined model discloses relative deviations of up to 4\%. From a clinical point of view, these deviations are of minor relevance. However, for a more asymmetric differential function, the deviations get much larger; at \( \gamma < 0.3 \) or \( \gamma > 0.8 \), the deviations in the results obtained with the refined model compared with those obtained with the basic model increase, at stone formation rates >15 mm\(^3\)/day, an order of magnitude, which is of increasing relevance.

The bottom panel in Fig. 1 displays the dependency of the degree of underestimation of a measured urinary oxalate concentration of 0.37 mmol/L on the values of \( \gamma \) and \( V/\tau \). It is obvious that the degree of underestimation increases strongly, in particular for high rates of stone formation, with both, \( \gamma \) and \( V/\tau \).

Our results document the importance of the step-by-step generalization of theoretical concepts. Consideration of a variable differential kidney function demonstrates how stone-forming processes depend on often-neglected medical variables.

The potential difference between urinary concentrations of lithogenic components distal to in vivo-growing stone material and the measured concentrations can be on the order of tens of percentages. Lack of awareness of this fact may lead to misinterpretation of a patient's health status and, consequently, to an unfavorable treatment strategy. Thus, the depletion effect should be considered when interpreting data from the clinical metabolic work-up of stone-bearing patients.

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**Fig. 1.** Ratio between corrected \( c_2 \) values in the refined vs the former model \( (\gamma = 0.5) \) (top), and percentage of underestimation of concentration \( c_2 \), from a measured urinary oxalate concentration \( c_1 \) of 0.37 mmol/L in dependence of the exposure fraction \( \gamma \) (bottom).
Comparison of Reverse Transcriptases in Gene Expression Analysis, Anders Ståhlberg,1,2* Mikael Kubista,1,2 and Michael Pfaffl3 (1Department of Chemistry and Biosciences, Chalmers University of Technology, Gothenburg, Sweden; 2TATAA Biocenter, Gothenburg, Sweden; 3Physiology Weihenstephan, Center of Life and Food Sciences, Technical University of Munich, Munich, Germany; * address correspondence to this author at: Department of Chemistry and Biosciences, Chalmers University of Technology, Medicinaregatan 7B, 405 30 Gothenburg, Sweden; fax 46317733910, e-mail anders.stalberg@tataa.com)

In most measurements of gene expression, mRNA is first reverse-transcribed into cDNA. The reverse transcription reaction is not very well understood, and it is expected to be the uncertain step in gene expression analysis. It can introduce errors produced by effects of mRNA secondary and tertiary structures, variation in priming efficiency, and properties of the reverse transcriptase (1–5). The aim of this work was to study the yield, reproducibility, and sensitivity of some commercially available reverse transcriptases on low to intermediate expressed genes by use of quantitative real-time PCR (QPCR).

Total RNA extraction, reverse transcription, and QPCR were performed as described in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue9/ (6, 7). All reverse transcription reactions were run in replicates of four, using starting material from the same RNA pool prepared from bovine spleen, liver, or jejunum, which eliminated sample-to-sample variation (8). Only results for RNA from spleen are shown. Liver and jejunum gave similar results, which are provided in the online Data Supplement. To determine absolute reverse transcription yields, we added an artificial RNA MultiStandard (Robo-screen) to samples (9, 10). Eight reverse transcriptases were studied: Moloney murine leukemia virus RNase H− (MMLVH; Promega); MMLV (Promega); avian myeloblastosis virus (AMV; Promega); Improm-II (Promega); Omniscript (Qiagen); cloned AMV (cAMV; Invitrogen); ThermoScript RNase H− (Invitrogen); and SuperScript III RNase H− (Invitrogen). Reverse transcription with AMV, MMLV, and Omniscript was performed at 37 °C, whereas with cAMV, Improm-II, and MMLVH it was performed at 45 °C, and with ThermoScript and SuperScript, it was performed at 50 °C.

The cDNA synthesis yields of the intermediate to highly expressed β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes and the low expressed genes 5-hydroxytryptamine 1a receptor (HTR1a), HTR1b, HTR2a, and HTR2b were measured by QPCR using SYBR Green I detection chemistry (7). The mean threshold cycle (Ct) and corresponding SD for all combinations of genes and reverse transcriptases are shown in Fig. 1. Because of the exponential behavior of PCR, a difference of 1 cycle in Ct between runs that differed only in the reverse transcriptase used corresponded to twofold difference in reverse transcription yield (assuming 100% PCR efficiency). For HTR1a, HTR1b, and HTR2b, the reverse transcription yields obtained with the eight reverse transcriptases were similar, whereas for GAPDH and, in particular, for HTR2a and β-actin, substantial variations were observed (Fig. 1). For example, for HTR2a, the Ct was 32.3 cycles when SuperScript III was used, whereas it was 38.8 cycles when AMV was used. This corresponds to a 238.8/32.3 = 91-fold difference in reverse transcription yield. For HTR2b, the difference in yield with the two enzymes was only 238.8 – 32.3 = 1.14, which is 14%.

Primer hybridization relies on access to the appropriate target site in the mRNA and may vary substantially because of mRNA folding (11, 12). Reverse transcription yields could vary among the reverse transcriptases in a highly gene-dependent way as a consequence of mRNA secondary and tertiary structures. Large variation is expected for mRNAs with tight structures in which access to primer target sites is restricted. Our data suggest that this may be the case for β-actin, GAPDH, and HTR2 with our choice of primers. The reverse transcriptase that performed best for these genes was SuperScript III, which was used at 50 °C. A higher annealing temperature is often claimed to improve reverse transcription yields by reducing the degree of mRNA secondary structure, but ThermoScript, which also was used at 50 °C, did not perform particularly well. Furthermore, we found no advantage when we used reverse transcriptases without RNase activity (MMLVH, SuperScript III, and ThermoScript), which also is claimed by some vendors to improve transcription efficiency. For the six genes studied, SuperScript III gave the overall highest yield, followed by...