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ORIGINAL ARTICLE

Establishment of a protocol for large-scale gene expression analyses of laser capture microdissected bladder tissue

M. Horstmann · B. Foerster · N. Brader · H. John · C. Maake

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Abstract
Purpose Lower urinary tract symptoms (LUTS) can be caused by structural and functional changes in different compartments of the bladder. To enable extensive investigations of individual regions even in small bladder biopsies, we established a combination protocol consisting of three molecular techniques: laser capture microdissection microscopy (LCM), RNA preamplification and quantitative polymerase chain reaction (qPCR).
Methods Urinary bladders of ten mice were resected and frozen immediately or after a delay of 15 min. Cryosections were obtained and smooth muscle was isolated using the LCM technique. Then, RNA was extracted, including protocols with and without DNase digestion as well as with and without the addition of carrier RNA. Extracted RNA was either used for reverse transcriptase (RT)-PCR plus qPCR or for a combination of RNA preamplification and qPCR.
Results Our data showed that with RNA preamplification, 10 µg cDNA can be regularly generated from 2.5 ng RNA. Depending on expression levels, this is sufficient for hundreds of pPCR reactions. The efficiency of preamplification, however, was gene-dependent. DNase digestion before preamplification lead to lower threshold cycles in qPCR. The use of partly degraded RNA for RNA preamplification did not change the results of the following qPCR.
Conclusions RNA preamplification strongly enlarges the spectrum of genes to be analyzed in distinct bladder compartments by qPCR. It is an easy and reliable method that can be realized with standard laboratory equipment. Our protocol may lead in near future to a better understanding of the pathomechanisms in LUTS.

Keywords LUTS · Gene expression profiling · BPH

M. Horstmann (✉) · B. Foerster · H. John
Department of Urology, Kantonsspital Winterthur, Brauerstr. 15, 8401 Winterthur, Switzerland
e-mail: marcushorstmann@gmx.ch; marcus.horstmann@ksw.ch

N. Brader · C. Maake
Institute of Anatomy, University of Zurich, Winterthurerstr. 190, 8057 Zurich, Switzerland

Introduction

Many studies dealing with gene expression take advantage of the ease, speed, and reliability of the reverse transcriptase (RT)-polymerase chain reaction (PCR) and its further development, the quantitative RT-PCR (qPCR) [1–3]. The amplification potential of PCR techniques allows for the detection and/or quantification down to a few specific transcripts. This feature makes them useful tools for investigations not only of low expressed genes, but also of specimens of limited size.

One of the challenges in relative quantitative expression studies (e.g., comparing "healthy" versus "diseased" samples), however, is to draw biologically meaningful conclusions if samples contain different populations of cells with each a specific pattern of expressed genes. Without morphological analysis, the random prevalence of a distinct tissue component within a sample may be easily misinterpreted in PCR as a change in expression levels. To overcome problems associated with heterogeneous tissues, the technique of laser capture microdissection microscopy (LCM) had been introduced [4, 5]. Briefly, in LCM, cells of interest are identified from microscopic sections, cut by a laser beam under microscopic control and eventually isolated from the surrounding tissue for separate analysis.

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We have previously shown that LCM provides new possibilities also for bladder research. As in other organs, the wall of the bladder is composed of various specific tissue compartments, including the urothelium, suburothelial connective tissue (containing interstitial cells, nerve fibers, and vessels), or the *muscularis* with the *m. detrusor*. Using LCM to selectively procure smooth muscle, we were able to demonstrate by qPCR changes in the expression levels of a distinct isoform of the smoothelin gene in the detrusor of patients with overactive bladders [6]. In a further LCM study, expression changes of connexin45 in the bladder of patients with bladder outlet obstruction could be reported [7].

However, since the sequencing of the human genome, detailed information about thousands of genes is available [8]. Furthermore, knowledge on interactions between gene products constantly expands—paving the road for a broader and more functional approach toward understanding pathologic pathways in disease development. It is therefore of great interest to use these data sources for extended studies in human bladder research—especially with respect to advances brought by LCM.

Unfortunately, biopsies from the operating theater pose a series of potential problems. Samples are mostly very small and/or available in restricted numbers, and RNA quality is compromised in many cases. LCM/qPCR-based studies on those tissues may provide highly specific gene information, but are not feasible if information about expression levels of extended pathways or large functional cascades are required.

For microarray studies on limited sample amounts, RNA preamplification methods had recently been introduced, including, for example T7 RNA polymerase-driven in vitro transcription, linear isothermal RNA amplification, or Switch Mechanism At the 5′ end of Reverse Transcript (SMART) [9–16]. This prompted us to evaluate in a mouse model as to whether RNA preamplification may also be useful for large-scale gene expression studies on LCM procured minute sample material of the urinary bladder.

Materials and methods

Animals

Adult female mice ($n = 10$) were euthanized, and bladders were dissected and snap-frozen ($n = 5$). In a subset of animals ($n = 5$), bladders were snap-frozen after a delay of 15 min at room temperature.

Laser capture microdissection microscopy and RNA extraction

Samples were prepared for LCM as described before [7]. Briefly, frozen samples were cryocut at 10 µm and placed on polyethylene membrane slides (MMI, Glattbrugg, Switzerland). After dehydrating sections through an ascending series of ethanol and finally xylene, areas of detrusor smooth muscle were isolated (2 mm^2/sample) by means of the UVcut laser capture microdissection microscope (MMI) without previous staining and collected on adhesive caps (MMI). Extraction of total RNA was performed with the RNeasy Micro Kit (Qiagen, Basel, Switzerland) according to the instructions of the manufacturer. In some experiments, addition of polyA carrier RNA or on-column DNase digestion (both components of the kit) was omitted. RNA was finally eluted in 17 µl RNase-free water in LoBind tubes (Eppendorf, Hamburg, Germany). RNA quantity and quality were assessed on-chip and by means of the RNA integrity number (RIN) algorithm, respectively (Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA, USA). RNA samples were stored at −80 °C.

RNA preamplification

For preamplification, the WT-Ovation Pico RNA Amplification System (NuGEN, San Carlos, CA) was applied. First- and second-strand synthesis was performed according to the provided manual, using an input of 2.5 ng total RNA. The isothermal linear amplification step was done with the SPIA system included in the kit (NuGEN), resulting in a volume of 160 µl amplified complementary DNA (cDNA). SPIA-amplified cDNA was subjected to a cleanup step with the QIAquick PCR purification kit (Qiagen), giving a volume of 30 µl. Concentrations of amplified cDNA were measured with the Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and size distribution of amplified products was assessed by the Eukaryotic Total RNA Nano Kit (Agilent) on the Bioanalyzer (Agilent).

Reverse transcriptase (RT) reaction

RT reactions were performed with 2.5 ng total RNA and either the QuantiTect RT kit (Qiagen) or the Superscript II RT system (Invitrogen, Basel, Switzerland), both yielding 20 µl. The QuantiTect RT kit comprises a DNA digestion solution (DNA wipeout, Qiagen) and a premade primer mix consisting of random hexamers and oligo(dT). The Superscript II RT reaction contains no DNAse digestion step and applies random hexamer primers (6.25 ng/µl, Sigma, Basel, Switzerland) only. Negative controls included omission of RT enzymes.

Polymerase chain reaction (PCR)

For standard PCR reactions, intron-spanning primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH); smoothelin (SMTN); ATPase, Ca++ transporting, cardiac

muscle, slow twitch 2 (ATP2A2) were designed with the Primer3 software (http://frodo.wi.mit.edu/primer3; Table 1) and synthesized (Microsynth, Balgach, Switzerland). 25 µl PCR reactions consisted of 1 µl cDNA, 1× PCR buffer (Applied Biosystems), 200 µM dNTP (Sigma), 0.4 µM each sense and antisense primer and 1.25 U TaqPolymerase (Applied Biosystems). The cycling program (T3000, Biometra, Goettingen, Germany) was as follows: 4 min at 94 °C, 35 cycles with 30 s at 94 °C, 30 s at 58 °C, 45 s at 72 °C, and finally 4 min at 72 °C. PCR products were visualized by gel electrophoresis (1× GelRed, Biotium, Hayward, CA, 1.2 % agarose, Sigma, in 1× Tris–borate-EDTA buffer) at 100 V. A DNA ladder served as marker (Bench Top Ladder 100 bp, Promega, Dübendorf, Switzerland).

Quantitative PCR (qPCR)

Primer/probes (Table 1) were either commercial TaqMan assays (Applied Biosystem; in the case of beta-2-microglobulin, B2M and phospholamban, PLN) or based on the human Universal ProbeLibrary System (Roche; ATP2A2 and SMTN). In the latter case, primers were designed with the Probe Library software (www.roche-applied-science.com) and synthesized by Microsynth. Both TaqMan and ProbeLibrary probes are labeled with FAM. Protocols for qPCR were performed in triplicates in each 10 µl: TaqMan assays with 1× Absolute QPCR low ROX mix (ABgene, Dietikon, Switzerland), 0.225 µM each sense and antisense primer (Applied Biosystems), 0.9 µM FAM-labeled TaqMan probe (Applied Biosystems) and 1 µl cDNA (amplified or non-amplified); ProbeLibrary assays with 1× Absolute QPCR low ROX mix (ABgene), 0.1 µM FAM-labeled Universal ProbeLibrary probe (Roche), 0.4 µM each sense and antisense primer and 1 µl cDNA (either amplified or non-amplified). qPCR was run in an Applied Biosystems 7500 Fast Real-time PCR System (Applied Biosystems) under the following conditions: 15 min at 95 °C and 40 cycles with 15 s at 95 °C and 1 min at 60 °C. Analyses were performed with the REST software (http://www.gene-quantification.de).

Results

In all samples, smooth muscle bundles were clearly identifiable in phase contrast mode of the microscope (Fig. 1). Histologic staining of sections before LCM was thus not necessary.

Even under optimal freezing conditions, we found that after LCM and RNA extraction, RNA was never undegraded (i.e., RIN 10).

Starting with approximately 2.5 ng total RNA, the preamplification protocol yielded about 10 µg cDNA.

Table 1 Primer design for PCR and for quantitative PCR

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	Data base number
mGAPDH	ACCCAGAAGACTGTGGATGG	GGTCCTCAGTGTAGCCCAAG	ENSMUSG00000057666
mSMTN	GCCAGGCAGAGAAGAAGAAA	ACTCCGAAGCTGGTAGAACG	ENSMUSG00000020439
mATP2A2	TGTACCCCAAACAAGCCAAG	CCAGGTGCATCTCCTCTCTC	ENSMUSG00000029467
Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	Probe (5′–3′)	Data base number
mB2M	ACTGATACATACGCCTG CAGAGTT	CTCGATCCCAGTAGACG GTCTT	AGCATGCCAGTATG GCGGAGCC-	ENSMUSG00000060802
mPLN	CAGACCTGCAACATGCC AACT	GCAGCGGTGCGTTGCT	AGCTTAAGCCGAG CACTCCGTCATG-	ENSMUSG00000038583
mATP2A2	TGGCAAAGAAAAATGCT ATCG-	GAAGTACAACCAAGGGT CTCCA	CTGCCTTC	ENSMUSG00000029467
mSMTN	GCGTGAGCTCCGACAAA G	CGTAGCCTCCGTTCTCGTT	GAGACCAG	ENSMUSG00000020439

m mouse, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *SMTN* smoothelin, *ATP2A2* ATPase, Ca++ transporting, cardiac muscle, slow twitch 2, *B2 M* beta-2-microglobulin, *PLN* phospholamban, *bp* base pairs. Date base numbers are from http://www.ensembl.org

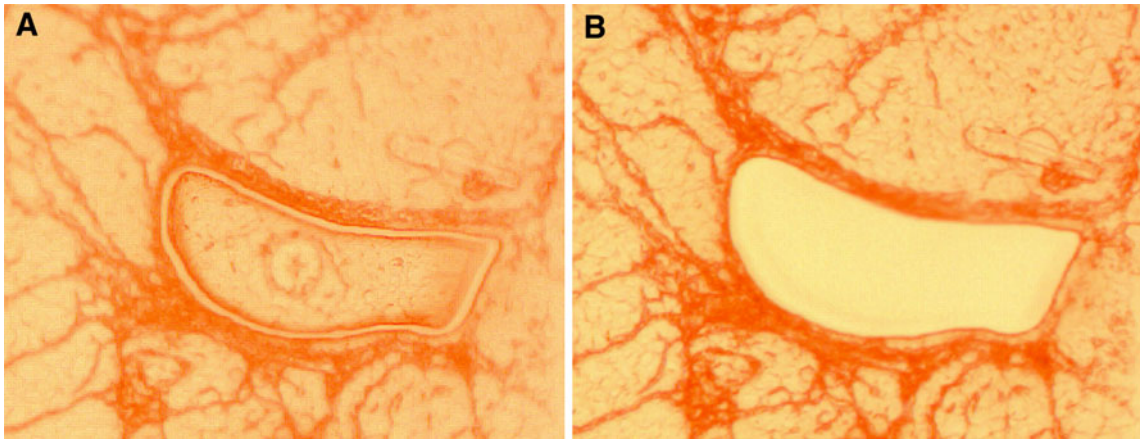

Fig. 1 Laser capture microdissection using the microscope's phase contrast mode. **a** 10 µm section of the frozen, unstained bladder with a detrusor smooth muscle bundle cut by the laser; **b** section after the removal of the cut smooth muscle bundle. Please note that septa of connective tissue remain in the section. Original magnification 100×

Concentrations of cDNA from independent biological replicas after standardized LCM, extraction, and preamplification were always in the same range (353.91 ng/µl ± 15.07).

When comparing qPCR data from preamplified and non-preamplified RNA (extracted from the same LCM sample), we found that the pattern of gene expression within this sample is conserved after the preamplification procedure, that is, genes with lowest threshold cycles (CTs) without preamplification have also the lowest CTs after preamplification. However, not all transcripts are preamplified with the same efficiency. As investigated with examples of three high and low expressed genes and after correction for cDNA input, relative expression levels of B2M were about 73,000-fold, of SMTN about 129,500-fold, and of ATP2A2 about 20,900-fold increased in preamplified versus non-preamplified cDNA from the same sample (Fig. 2a, b). The RNA preamplification method yielded highly reproducible results in qPCR, since independent experiments (LCM, extraction, preamplification) on the same sample resulted in almost identical CT values (Fig. 2c).

Measurements of RNA quantities of samples with and without carrier showed comparable yields. However, bioanalyzer measurements after preamplification indicated that the addition of carrier leads to a distortion of the size distribution curve that is used to estimate amplification efficiency. However, addition of carrier did not affect qPCR results after preamplification.

To investigate the effect of DNase treatment, sample duplicates with or without DNase digestion were extracted. With DNase treatment, RIN was significantly higher compared to non-DNase-treated samples (6.9 ± 0.58 vs. 4.6 ± 0.87; $p = 0.008$). To exclude co-amplification of genomic DNA during RNA preamplification, RT-PCT with three intron-spanning primer pairs was performed. Gel analysis showed that in non-DNase-treated samples, no DNA was present after preamplification (Fig. 2d). After qPCR and compared to preamplified DNAse-treated samples, preamplified non-DNase-treated samples showed higher CT values. As detected with three exemplary genes (B2M, SMTN, and ATP2A2), this phenomenon was gene-dependent and amounted to between 1 and 3 CTs.

The influence of RNA quality on preamplification was evaluated by comparing qPCR data from RNA with different RIN. As shown with B2M and PLN, CTs were identical (B2M) or almost identical (PLN) between input RNA with RIN 5.9 and RIN 1.8 (Fig. 2e, f). The dynamic range of qPCR was conserved in both setups.

Discussion

The pathoetiology of lower urinary tract symptoms (LUTS) is far from being clear—although expression changes of several distinct genes have been implicated in the disease processes [17–19]. While the availability of sequence information for the whole human genome raised hope to eventually obtain a global overview on underlying molecular alterations in LUTS, it remains technically challenging to subject samples from the operating theater to large-scale high-throughput techniques like, for example microarrays. This holds especially true if those samples are further processed for LCM, ending up with very specific but even smaller amounts of potentially higher degraded material. However, PCR-based methods tolerate partly degraded RNA to some extend. Unfortunately, in most cases, the number of genes that can be analyzed from LCM procedures by qPCR is restricted to 5−10 (depending on expression levels). Based on RNA preamplification, we here report a robust and reproducible method that enables

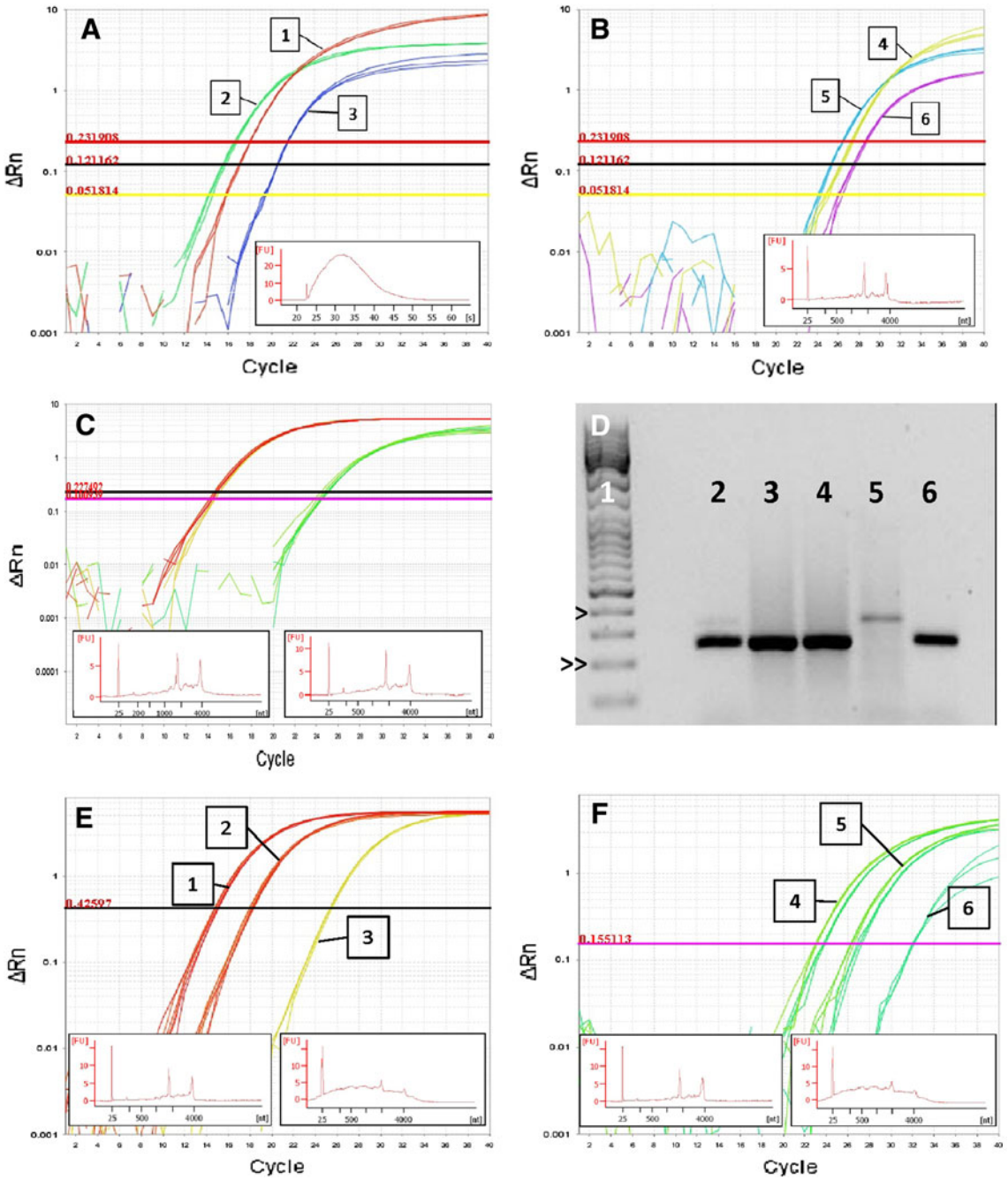

Fig. 2 Quantitative PCR: comparison of a preamplified batch (1:100, **a**) and a non-preamplified batch **b** of RNA from the same microdissected sample. Beta-2-microglobulin (*1* and *4*); smoothelin (*2* and *5*); ATPase, Ca++ transporting, cardiac muscle, slow twitch 2 (*3* and *6*). Thresholds: B2M (*black*), SMTN (*red*), ATP2A2 (*yellow*). Included are also RNA quality measurements by Bioanalyzer of **a** (spectrum) and **b** (RIN 6.8) **c** quantitative PCR with primer/probes for beta-2-microglobulin (*red* and *yellow*) and phospholamban (*light* and *dark green*) using the same sample, but two independent microdissections, extractions and RNA preamplifications (RNA quality measurements by Bioanalyzer: RIN 7.0, *left* and RIN 6.8, *right*). **d** RT-PCR using intron-spanning primers for ATP2A2 (expected size with intron: 319 bp, without intron: 241 bp) with the following cDNA: (*1*) DNA ladder; (*2*) no DNase digestion, no RNA preamplification, with RT enzyme; (*3*) with DNase digestion, with RNA preamplification, with RT enzyme; (*4*) no DNase digestion, with RNA preamplification, with RT enzyme; (*5*) no DNase digestion, with RNA preamplification, no RT enzyme; (*6*) with DNase digestion, no RNA preamplification, with RT enzyme. *Arrow*: 400 bp; *double arrow*: 200 bp. **e** and **f** quantitative PCR of partly degraded RNA samples: **e** B2M, *red*: RIN 5.9 (*left* RNA quality measurement), *orange*: RIN 1.8 (*right* RNA quality measurement); *1*: preamplified, cDNA 1:10; *2*: preamplified, cDNA 1:100; *3*: non-preamplified. **f** PLN, *light green*: RIN 5.9 (*left* RNA quality measurement), *dark green*: RIN 1.8 (*right* RNA quality measurement); *4*: preamplified, cDNA 1:10; *5*: preamplified, cDNA 1:100; *6*: non-preamplified

large-scale qPCR investigations of LCM tissues, suitable for application on partly degraded human (bladder) biopsies.

Several RNA amplification methods are available and are mostly used in conjunction with DNA micorarrays [14–16, 20]. We have chosen the same linear isothermal ribo-SPIA preamplification kit (WT-Ovation Pico System, NuGEN) with which we perform our DNA microarray studies and that has been shown by others to reliably work in qPCR for small amounts of RNA from cell lines [21–23].

With an input of about 2.5 ng RNA (an amount that can easily be obtained from laser microdissected smooth muscle of small bladder biopsies), our RNA preamplification resulted in about 10 μg cDNA. With our protocol, the qPCR detection of hundreds of genes from a given laser dissected samples is feasible, enabling extensive investigations on molecular pathways. Larger quantities of cDNA in qPCR reactions have the further advantage that CTs are lower, meaning that low expressed genes may be reliably detectable.

Of major concern is the preservation of differential expression after preamplification. While we found that the general patterns of high- and low-transcribed genes are retained after preamplification, it was apparent that the amplification potential is not identical for all RNA species. Such observations had been reported before for different preamplification methods and are thus not related to the laser capture microdissection procedure. For this reason, it is not advisable to compare the expression ratio of different genes within a preamplified sample by qPCR. However, our data indicate that a given transcript is preamplified with the same efficiency in the same tissue type of different individuals. Therefore, the important analysis method of relative qPCR (i.e., quantitative comparison of gene expression in two or more sets of samples) is practicable for preamplified samples.

The relevance of intact RNA for down-stream applications has been stressed in many publications [24]. However, especially with regard to human biopsies, where longer ischemic times cannot always be avoided, it would be of great advantage if the preamplification method is applicable to RNA with compromised quality. In contrast to earlier reports, we found that partly degraded RNA may be successfully preamplified and used for qPCR. Clearly, it is important to opt for a RT method that not only reverse transcribes from the polyA tail and to design primers that produce short amplicons. Our data show, however, that the use of partly degraded RNA requires some careful considerations. Since apparently preamplification may in some cases be slightly more efficient from undegraded RNA, in following PCR-based gene expression studies only samples with similar initial RIN can be compared. Inclusion of biopsies with different RNA integrity within an expression study may thus result in more or less false results. This observation stresses the necessity of standardized protocols for sample acquisition and handling.

The preamplification kit manual suggests a DNase treatment; however, others have shown that this is not necessary [22]. We here confirmed that genomic DNA is in fact not co-amplified with RNA. Despite the conclusion that DNase treatment is apparently not mandatory, we found that the addition of this step resulted in considerably lower CTs in subsequent qPCR and is therefore nonetheless recommended. Since we also observed higher RIN in DNase-treated versus non-DNase-treated samples, it is likely that the higher proportion of less degraded RNA has lead to a better preamplification efficiency and thus to a CT shift to the left.

Our data are in accordance with a previous study providing evidence that a purification step after preamplification is not necessary [22]. However, we decided to incorporate a quick cleanup in our protocol, mainly because this results in a higher concentrated and likely more stable preamplified cDNA solution. This is especially important if samples will be used for long-term studies and/or will be used for investigations on low expressed genes.

In our RNA extraction protocol established for laser micordissected bladder tissues, a polyA carrier is routinely added with the aim to improve RNA recovery and stability. It was therefore of interest as to whether this carrier is compatible with the preamplification procedure (the manual explicitly precludes the application of yeast tRNA carrier). Comparing protocols both with and without the addition of polyA carrier, we found no significant difference in the outcome of qPCR. Nevertheless, in samples with carrier, the spectrum as measured with the Bioanalyzer after preamplification presented with a distorted curve. With regard to quality controls after preamplification, we therefore suggest to omit polyA carriers.

Due to relatively high costs associated with LCM, RNA preamplification, and probe-based qPCR strategies, our protocol is mainly practicable for samples where a maximum of information output must be obtained from the available minimum of RNA input. We are also aware that the bladder wall may be (partly) dissected by stripping the urothelium. However, the method described in this paper is not intended to be used with samples where the urothelium can be easily stripped. Rather, the LCM technique has its advantages for cases, where only very small samples are available (such as human biopsies), which makes stripping of the urothelium a difficult task. For certain research questions, it may also be necessary to procure pure cell populations. In the muscularis of the bladder, for example the exclusion of interstitial cells may be required, which are located between detrusor bundles and are not removed by stripping of the urothelium. Furthermore, the LCM

technique described is not restricted to smooth muscle. It may also be used for other compartments of the bladder wall, such as, for example the suburothelial connective tissue that cannot be obtained by stripping.

Taken together, RNA preamplification is a reliable tool that enables researchers to massively enlarge the investigative spectrum of genes in human LCM samples using robust molecular techniques and expertise available in many laboratories. The combination of LCM, RNA preamplification, and qPCR may importantly contribute to more detailed and extended investigations of human bladder samples for specific research approaches. Eventually, this may lead to a better understanding of the pathogenesis of LUTS [25, 26].

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Conflict of interest None.

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