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Review

Quantitative mRNA expression analysis in kidney glomeruli using microdissection techniques

Ward De Spiegelaere[1], Pieter Cornillie[1], Mario Van Poucke[2], Luc Peelman[2], Christian Burvenich[3] and Wim Van den Broeck[1]

[1]Department of Morphology, [2]Department of Nutrition, Genetics and Ethology and [3]Department of Comparative Physiology and Biometrics, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

Summary. The introduction of new tools for molecular analysis, such as RT-qPCR and microarrays, has provided researchers with powerful applications to study renal disease and development. However, the high cellular heterogeneity of the renal tissue complicates the molecular analysis of specific cells and cell groups such as glomerular or tubular cells. In the past, glomerular sieving and manual dissection were used for the isolation of glomeruli. However, these techniques cannot be used for the isolation of specific glomeruli or for the co-isolation of additional tissue fractions. In recent decades, new microdissection techniques such as laser-assisted microdissection have been developed. These applications allow the isolation of small cell groups from heterogeneous tissue for molecular analysis, including microarray and RT-qPCR. Although very promising, some drawbacks are associated with these techniques. The isolated sample material is generally small and requires sensitive assays. In addition, the long sample processing time may result in a considerable loss of RNA integrity. Careful optimization and rigorous quality analysis should overcome these drawbacks. In the present paper, the recent literature on the application of microdissection techniques in kidney research is reviewed, together with a discussion of the critical issues that are essential for the application of quantitative mRNA expression analysis with RT-qPCR on microdissected samples.

Key words: Glomerular sieving, Laser microdissection, Real time PCR, Gene expression analysis, Glomerular isolation, Kidney

Offprint requests to: Ward De Spiegelaere, Ghent University, Faculty of Veterinary Medicine, Department of Morphology, Salisburylaan 133, B-9820 Merelbeke, Belgium. e-mail: Ward.DeSpiegelaere@UGent.be

Introduction

Methods for mRNA expression analysis such as microarray, reverse transcription polymerase chain reaction (RT-PCR) and reverse transcription quantitative real time PCR (RT-qPCR) can produce valuable information on the expression of mRNA in specific tissues (Patterson and Potter, 2004; Neusser et al., 2008). Until recently, mRNA expression in the developing and diseased kidney was mainly studied in whole tissue lysates of renal biopsies, without the possibility of a precise localization of the mRNA expression pattern at the cellular level (Henger et al., 2004). Moreover, the heterogeneous nature of this tissue renders the subsequent analysis extremely difficult (Neusser et al., 2008). In recent decades, various methods have been developed to isolate specific cell groups from heterogeneous tissues. In this review, an overview of these techniques is given along with some practical considerations for the downstream applications with RT-qPCR. The quality assessments described are in line with the recently published MIQE guidelines (Minimum Information for Publications of Quantitative Real-Time PCR Experiments) that provide authors and reviewers the minimum information that must be reported to ensure correct interpretation and repeatability of qPCR experiments (Bustin et al., 2009).

Glomerular sieving

The specific isolation of kidney glomeruli by glomerular sieving is a well established technique that has already been used for several decades (Norgaard, 1976). The sieving technique involves disruption of the kidney cortex by pressing the tissue through a metal sieve. Subsequently, glomeruli are isolated by centrifugation (Krakower and Greenspon, 1951) or by

pouring the suspension through sieves with different mesh sizes (Gang, 1970). Recently, an innovative isolation method has been developed in which the kidney is perfused with magnetic dynabeads of 4.5 µm diameter prior to disruption (Takemoto et al., 2002). After perfusion, the kidney is excised and minced into small pieces, digested with collagenase and filtered through a sieve with a pore size of 100 µm. Glomeruli can be obtained from the filtrate with a magnetic particle concentrator which attracts the dynabeads present in the glomerular capillaries. Glomeruli obtained by this technique are morphologically intact, and except for the early vesicle stage, all stages of glomerulogenesis, i.e. from the S-stage until mature glomeruli, can be isolated.

With the sieving technique, a considerable amount of RNA can be isolated for further analysis (Cui et al., 2005). The RNA in lysates of sieved glomeruli is well preserved and ideal for subsequent mRNA expression analysis because of the fast processing (Takemoto et al., 2002). The glomerular sieving method has already been successfully used for mRNA expression profiling, proteomic analysis and isolation of glomerular cells for primary cell culture (Katsuya et al., 2006; Tryggvason et al., 2007; Sakai et al., 2009; Sun et al., 2009). The sieving method is also an interesting method for the downstream isolation of single cells, such as podocytes, allowing the molecular analysis of one type of glomerular cells only (Schröppel et al., 1998; Kretzler et al., 2002). However, the sieving technique cannot be used for the isolation of specific subpopulations of kidney glomeruli such as diseased glomeruli or glomeruli from only one developmental stage, and neither does it allow the co-isolation of additional cell types from the kidney (Table 1). In addition, contamination of nonglomerular renal tissue can bias the results obtained with the sieving method (Steinmetz et al., 2007). Yaoita et al. (1991) report that 5% to 10% of the isolated material obtained by the sieving method originates from tubular cells or from the Bowman's capsule. These percentages can even rise to 39% (Norgaard, 1987; Yaoita et al., 1991).

Laser-assisted microdissection (LAM)

A range of microdissection techniques that enable the dissection of single cells and small tissue fractions from heterogeneous tissues have been developed by using laser technology (Fig. 1). Laser capture microdissection (LCM) was the first laser-assisted microdissection (LAM) technique developed (Emmert-Buck et al., 1996). The LCM system, ArcturusXT™ (Molecular Devices) uses an infrared laser in combination with a thermoplastic polymer to isolate specific cell fractions (Fig. 1A). The thermoplastic polymer is attached to a small cap that is moved on top of the tissue section. A laser beam melts this polymer on top of the region of interest, and the cells adhering to the polymer are isolated by lifting the cap from the tissue section (Fig. 2). A range of alternative LAM microscopes are now available, most of which use an ultraviolet laser. For laser cutting microdissection with the LMD7000 (Leica), the tissue section is placed on a UV-absorbable membrane instead of on a glass slide (Fig. 1B). The UV laser is used for cutting the contours of the region of interest, after which it drops down into an underlying receptacle. For laser pressure catapulting (LPM) with the PALM Microbeam (Zeiss) the tissue sections are adhered to glass slides, a UV laser cuts the contours of the region of interest and then this region is catapulted into a receptacle by using an additional laser (Fig. 1C). Detailed reviews of these techniques can be found elsewhere (De Preter et al., 2002; Murray, 2007; Sluka et al., 2008; Kenngott et al., 2010).

The strength of the LAM techniques is that a morphological evaluation is combined with a specific isolation at the cellular level. This enables the isolation of only one type of glomeruli or only a specific portion of the glomerulus (Stemmer et al., 2006; Ratliff et al., 2007). However, it does not allow the isolation of only one specific cell type from the glomerulus. LAM also allows the parallel isolation of additional cell fractions from the kidney, such as renal tubular cells or cells from the macula densa (Kurita et al., 2009; Zhang et al., 2009). Moreover, cells can also be isolated based on their molecular characteristics by performing immunohistochemical staining prior to the microdissection procedure (Kohda et al., 2000; Kinnecom and Pachter, 2005).

The LAM technique has already been extensively used in kidney research for conventional RT-PCR, but

Table 1. Schematic representation of the advantages and disadvantages of glomerular sieving and laser-assisted microdissection

	Glomerular sieving	Laser assisted microdissection
sample preprocessing	samples are obtained from fresh biopsies	samples must be preprocessed for sectioning and staining
specificity	only glomeruli can be isolated and contamination of additional renal components is possible	different tissues can be specifically isolated and contamination can be prevented
RNA yield	high amounts of RNA	only nanograms of RNA
RNA integrity	RNA integrity is generally good	sufficient RNA integrity can only be obtained after thorough optimization of the procedure

the application of RT-qPCR is more complicated (Blakey and Laszik, 2004). This due to some drawbacks that come with the LAM technique (Table 1), namely the small amount of obtained sample material and the long sample processing times which can seriously affect RNA integrity (Inoue et al., 2003).

Total RNA quantity

Due to the small amount of RNA isolated from microdissected tissue, most techniques for total RNA quantification fall short. Recently, new photometric methods have been developed for quantitative analysis of picograms of RNA from as little as 1 µl of sample material. For example, the NanoDrop™ total RNA quantification (NanoDrop Technologies) and the Nanophotometer™ (Implen) are well suited for quantifying RNA from microdissected samples.

Single glomeruli provide sufficient material for RT-PCR on a small set of genes (Tanji et al., 2001), but more glomeruli should be isolated when a larger set of genes needs to be tested. The isolation of 30 glomeruli from 7 µm thick cryo-sections should result in an amount of 10 ng total RNA (Woroniecki et al., 2006). Alternatively, the amount of isolated RNA can be increased by an RNA amplification step prior to reverse transcription, so that even single cell lysates can be used for subsequent RT-qPCR on different target sequences (Raj and van Oudenaarden, 2009). Although the RNA-amplification step can introduce extra bias that might interfere with the subsequent analysis, this technique has been successfully used on as little as 1 ng of highly degraded RNA with gene specific primers without significant changes in the relative quantities (Theophile et al., 2008).

RNA integrity

Good RNA integrity is important for mRNA expression analysis with RT-qPCR or microarray because a compromised RNA integrity can bias quantified data or make expressed genes undetectable (Perez-Novo et al., 2005). RNA is a very unstable molecule that is easily degraded by RNase enzymes. These enzymes are almost omnipresent and extremely stable (Fleige and Pfaffl, 2006). LAM samples are exposed to the deteriorating actions of RNases during the time-consuming protocols at room temperature and the tissue staining steps in aqueous solutions. This leads to an inferior RNA integrity of LAM samples in comparison to that of whole tissue lysates (Kerman et al., 2006). Therefore, a thorough optimization of the tissue processing and a careful evaluation of the RNA integrity are of utmost importance to get reliable results.

Initially, RNA integrity analysis was mainly performed by running isolated RNA on an agarose gel on which the ribosomal 18S and 28S bands was analyzed. This technique requires a considerable amount of RNA, which is not available in microdissected samples. Recently, some instruments have been

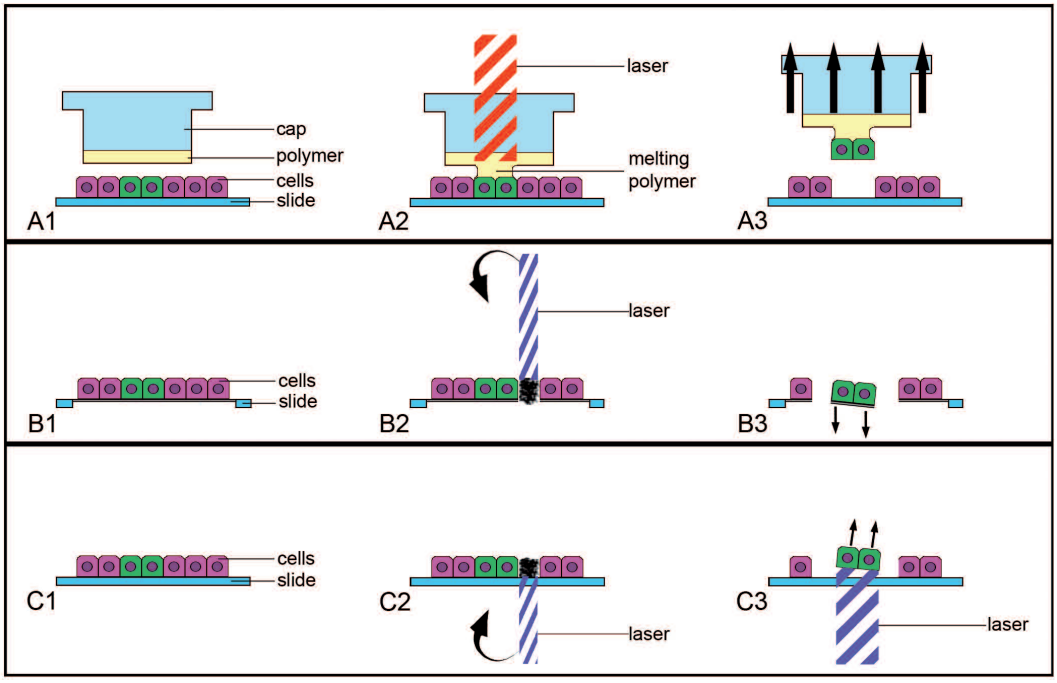

Fig. 1. Overview of three different mechanisms for laser-assisted microdissection: laser capture microdissection **(A)**, laser cutting microdissection **(B)** and laser pressure catapulting **(C)**. Laser capture microdissection starts with a tissue section on a glass slide. A cap with a thermoplastic polymer is placed on top of the section **(A1)**, the laser melts the thermoplastic polymer on the region of interest **(A2)** and this region is lifted up together with the cap **(A3)**. Laser cutting microdissection starts with a tissue section on a special membrane (**B1**), the laser ablates the contours of the region of interest together with the underlying membrane **(B2)**, after which this region drops into an underlying receptacle **(B3)**. Laser pressure catapulting starts with a tissue section on a glass slide **(C1)**. A laser beam ablates the contours of the region of interest **(C2)**, and a second laser catapults this region into a receptacle which is placed above the slide **(C3)**.

developed that use microfluidics for assessing RNA integrity, e.g. the Agilent 2100 Bioanalyzer (Agilent), the Experion™ Automated Electrophoresis System (Bio-Rad) and the LabChip® System (Caliper). These devices can analyze very small amounts of RNA and only require 1 µl of sample material, allowing the assessment of RNA integrity from small samples, as obtained with LAM. Because of the controversy on the use of the 18S/28S ratio as a measure for RNA integrity, Agilent, Bio-Rad and Caliper have developed comparable tools for a standardized evaluation of RNA integrity, i.e. the RNA Integrity Number (RIN, Bioanalyzer, Agilent), the RNA Quality Indicator (RQI, Experion, Bio-Rad) and the RNA Quality Score (RQS, LabChip, Caliper). With these tools the RNA integrity is not only determined from the 18S/28S ratio, but from the whole electrophoretic trace of the RNA (Fleige and Pfaffl, 2006). The software of these systems uses an algorithm to classify RNA integrity by attributing a number from 1 to 10 to the samples, 1 being totally degraded and 10 being intact RNA. The RIN, RQI and RQS estimates are reproducible and allow the comparison between different RNA samples and between different experiments. This method is becoming the gold standard for quality assessment of RNA (Bustin et al., 2009). An alternative assay for RNA-integrity is the 3'-5' ratio assay (Nolan et al., 2006a). In this assay, mRNA is reverse transcribed with Oligo (dT) primers. Three primer pairs are designed for a gene transcript that is highly expressed in the sample. One of the primer pairs is positioned at the 3' end, another at the 5' end, and a third is positioned in the centre of the mRNA strand. The ratio of these amplicons after PCR reflects the success of the reverse transcription reaction to transcribe the entire fragment. A ratio of 1 indicates intact RNA, while a higher ratio indicates RNA fragmentation (Nolan et al., 2006a).

Unfortunately, significant RNA degradation is inevitable when paraffin embedded material is used or when microdissection is preceded by time demanding staining protocols such as immunofluorescent staining (Wang et al., 2006). The RNA integrity obtained from frozen sections should have a RIN value of around 8, but RNA obtained from methacarn fixed tissue and formalin fixed tissue will have a much lower integrity, ranging between 4 and 2 respectively (Cox et al., 2008). Even though degraded RNA is inevitable in certain circumstances, valuable data can still be extracted from highly degraded RNA isolates. As such, the quality loss may impair the detection of small differences in mRNA expression due to a higher variation in the data, but large differences can still be distinguished (Rogerson et al., 2008). Furthermore, it is crucial that all samples in a given experiment have a similar RNA integrity. Effective relations can be distorted when comparing samples with a different RNA integrity, because not all mRNA strands are equally susceptible to degradation (Strand et al., 2007).

In order to minimize RNA degradation during the processing steps, all products and disposables should be RNase free and solutions should be made in DEPC-treated water or water that is certified to be free of nucleases. Glassware can be made RNase free by heating at 180°C for 2 hours or more. All surfaces and all equipment that cannot be dry heat sterilized should be cleaned with a solution of 100 mM NaOH in EDTA or with commercially available RNase inactivating solutions such as RNase AWAY® (Molecular BioProducts), RNase-OFF™ (Takara) or RNaseZap® (Ambion). Processing time in watery solutions must be made as short as possible because RNases are active in aqueous medium, whereas their activity is decreased in a dry state or in solutions containing more than 70% alcohol (Port et al., 2007; Clement-Ziza et al., 2008).

When working with degraded mRNA samples, it is important to use random primers or gene specific primers for the reverse transcription step, instead of oligo (dT) primers. The latter can only anneal to the poly-A tail of mRNA, making it impossible to transcribe broken fragments that are positioned upstream of a fractured region in fragmented RNA strands (Farragher et al., 2008). An adequate primer design is also important when working with degraded RNA. Primers that span small amplicons (70 to 250 base pairs) result in more stable Cq values in degenerated samples, because

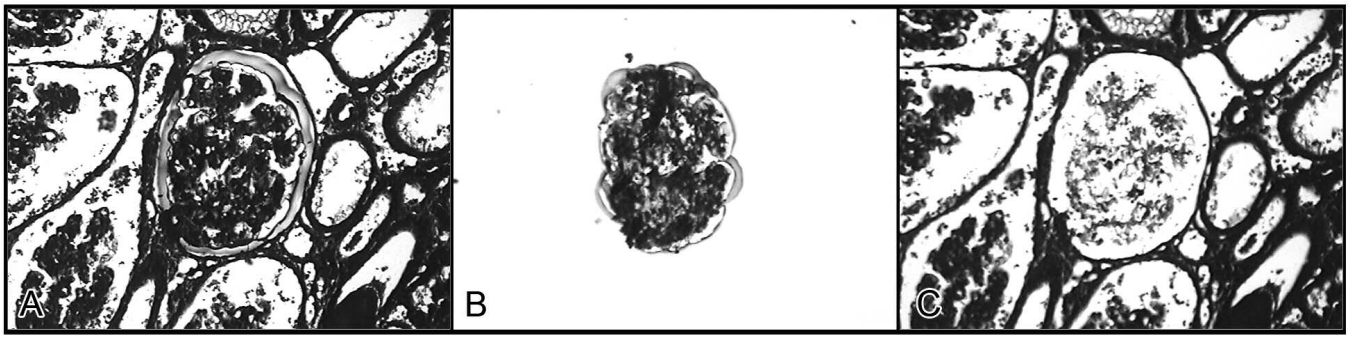

Fig. 2. Laser capture microdissection of glomeruli from an embryonic kidney. The laser melts the thermoplastic polymer on top of the region of interest, i.e. the glomerulus **(A)**. After lifting the cap, the glomerulus adheres to the cap **(B)** while the surrounding tissue remains on the slide **(C)**.

the chances of a break between primers that span a small amplicon are relatively small (Antonov et al., 2005; Fleige and Pfaffl, 2006).

Tissue fixation and processing

The optimal way to preserve good RNA integrity is by snap freezing the tissue and storing the samples at -80°C. This method is most frequently used with LAM techniques and has proven to preserve a sufficient RNA integrity for downstream molecular analysis (Erickson et al., 2009). Some investigators have tested the influence of a commercially available RNA stabilization buffer (RNAlater, Ambion) as an additional fixative to further enhance the RNA integrity of LAM-samples, but the results of these investigations are not equivocal (Stemmer et al., 2006; Botling et al., 2009). The major drawback of frozen tissue is the compromised morphological quality of the sections, which hampers the identification of specific cells on the basis of their morphological characteristics. Chemical fixation followed by paraffin embedding forms an alternative to snap freezing, because it provides a superior morphological quality. However, as already mentioned, chemical fixation and paraffin embedding can be detrimental for RNA integrity, due to the properties of the fixative and the long processing times required for dehydration and paraffin embedding. Although formaldehyde is regarded as the ideal chemical fixative for morphological analysis, RNA integrity of formaldehyde fixed tissue is generally very low (Hewitt et al., 2008). A range of alternative cross-linking and precipitating fixatives have been analyzed for their influence on RNA-integrity (Buesa, 2008; Goldsworthy et al., 1999). In these investigations, precipitating fixatives, such as alcohol and acetone based fixatives, provide a better RNA integrity than formaldehyde fixatives. A modified methacarn solution (8 parts methanol and 1 part acetic acid) has been identified as the fixative that gives a similar morphological quality to formaldehyde fixatives, while preserving an adequate RNA integrity for subsequent molecular analysis (Cox et al., 2006, 2008; Lee et al., 2006; Sluka et al., 2008). However, formaldehyde fixation is often the only option when working with archival tissues or with diagnostic samples (Cohen et al., 2002). Because of the great value of archival tissues, a large effort has been made to get reliable mRNA expression results from formaldehyde fixed tissues, and numerous investigations report that RNA can be isolated and quantified reliably from formaldehyde fixed samples (Farragher et al., 2008; Specht et al., 2001).

The paraffin embedding procedure can also seriously affect RNA integrity. During paraffin embedding, the tissue is dehydrated in an increasing alcohol series, then immersed in an organic solvent such as xylene, and subsequently immersed in hot (60°C) paraffin for an extended period of time. Both the long processing time for paraffin embedding as well as the high temperature at which these processes take place can be detrimental for RNA. In alcohol based fixatives the tissues are already partly dehydrated, which results in shorter processing times. Alternative embedding materials that work at lower temperatures than conventional paraffin have also been examined such as low temperature paraffin (melting point 52-54°C; Lykidis et al., 2007), polyester wax (melting point 38°C; Sluka et al., 2008), or cold temperature plastic resin embedding (polymerization at 4°C; Finkelstein et al., 1999). The use of alternative embedding materials is a promising strategy for obtaining better RNA integrity in the samples. However, a comprehensive investigation of the influence of different embedding materials has not been performed to date. Therefore, the influence of the embedding procedure on RNA integrity should be carefully monitored before each experiment.

Sectioning and staining

A proper staining procedure is crucial for the morphological identification of tissue components eligible for microdissection. However, staining procedures usually involve the use of aqueous solutions at room temperature, and this can seriously affect RNA integrity. At present, the standard stain for histology as well as for LAM is hematoxylin, alone or in combination with eosin, but methyl green or nuclear fast red staining have been reported to better preserve RNA integrity (Burgemeister et al., 2003). RNA degradation can effectively be decreased by including RNase inhibitors to the staining solution (Kube et al., 2007). Alternatively, alcohol-soluble stains have been reported to better maintain RNA integrity. A staining procedure with alcohol-soluble stains is less time-consuming because less rehydration and dehydration steps are required, and the presence of more than 70% alcohol inhibits RNase activation (Port et al., 2007). Although much research has already been performed on the influence of the staining procedure on RNA integrity, the results of these investigations are not equivocal. In one investigation, the RNA integrity was better preserved with alcohol-soluble stains, including eosin, methyl green and cresyl violet in comparison to the HistoGene LCM Frozen Section Staining Kit (Molecular Devices; Clement-Ziza et al., 2008). However, in another investigation cresyl violet in a 100% alcohol solution was shown to affect RNA integrity more than the conventional hematoxylin & eosin staining in aqueous solution (Stemmer et al., 2006). A strict evaluation of the staining protocol is therefore very important when designing the LAM procedure.

PCR inhibitors

Samples may contain agents that inhibit the PCR reaction, and these agents can either come from the tissue or they can be introduced by the processing steps prior to the PCR (Radstrom et al., 2004). PCR inhibitors

can strongly bias the subsequent PCR efficiency and also the downstream analysis of the RT-qPCR data (Suslov and Steindler, 2005; Bustin et al., 2009). Samples with a low amount of cDNA will be more prone to the action of PCR inhibitors than samples with a higher amount of cDNA, e.g. from whole tissue lysate (Flekna et al., 2007). Several methods have been described to check for the presence of inhibitors in the PCR reaction. The presence of inhibitors can be assessed by calculating the PCR efficiency (Tichopad et al., 2003) or by adding positive control nucleic acids in the sample after sample purification. Recently, the SPUD-assay has been described, in which a specific sequence of the *Solanum tuberosum* phyB gene is introduced in the PCR mix as a positive control, allowing the detection of inhibitors in all tissue samples with the exception of tissue from *Solanum tuberosum* (Nolan et al., 2006b).

Various techniques that eliminate PCR inhibitors exist for RT-PCR, such as increasing the concentration of target cDNA or the addition of reaction facilitators, such as bovine serum albumin (BSA) (Comey et al., 1994; Radstrom et al., 2004). Although these techniques can be effective, they require intensive optimization and are not always suitable for small sample amounts. Choosing between different polymerases and pre-PCR processing kits can significantly reduce the interference of inhibitors (Erickson et al., 2009). In case the aforementioned techniques prove to be ineffective, inhibitors can be removed from small samples by an extra purification step of the cDNA before the actual PCR, without significant loss of cDNA (De Spiegelaere et al., 2008). Alternatively, Guescini et al. (2008) have developed the Cy-0 method that allows relative quantification of qPCR data without requiring the prior assumption that the PCR efficiency is equal among all the samples (Guescini et al., 2008).

Normalization

Information about the amount of isolated material is crucial for analysing quantitative mRNA expression data. Estimates such as the total number of isolated cells or the measured amount of total RNA have been proven to be inaccurate for minimizing non-biological variation in the results, because these measurements are often not accurate enough and they cannot correct for the extra variation introduced during the subsequent processing and PCR (Huggett et al., 2005; Erickson et al., 2007). However, RNA quantity as well as the isolated surface or the amount of isolated cells can be used as broad estimates, because it is advisable to use comparable quantities of RNA in each sample (Bustin et al., 2009). Currently, the use of reference genes for sample normalization is supported by most authors and is suggested in the recently published MIQE guidelines (Bustin et al., 2009). In practice, most reference genes have been reported to show some variation, depending on the tissue origin or the experimental conditions (Thellin et al., 1999; Radonic et al., 2004). This implies that the most stable reference genes should be identified for every experiment by quantitative analysis of a set of candidate reference genes. Consequently, the samples are best normalized by using a set of 3 or more reference genes that have shown the least amount of variation from an initial set of around ten reference genes (Vandesompele et al., 2002; Erkens et al., 2006). Different algorithms and software programs that calculate the most stable reference genes are freely available on the Internet and are reviewed elsewhere (Vandesompele et al., 2009). The normalization procedure based on the use of more than one reference gene consumes a considerable amount of already scarce sample material obtained by LAM, but it is indispensable to obtain reliable data. Although as few as two reference genes can serve as a good standard (Erickson et al., 2007), based on our own experience we would suggest using an initial set of at least six to eight candidate reference genes which have been selected from the most stably expressed genes by prior investigations on a similar tissue type or by screening the expression database, Genevestigator (Hruz et al., 2008). From these candidates, a set of three reference genes can then be picked for normalization.

Conclusion

The new microdissection techniques for the isolation of renal subsamples are promising tools for investigating the molecular basis of renal development and disease processes, and glomerular sieving is a robust technique, by which a large number of glomeruli can be isolated. A drawback of this technique is contamination of nonglomerular tissue and the inability to co-isolate different tissue types or specific subpopulations of glomeruli. With LAM, contamination can be avoided, and different subsamples of specific cell groups can be isolated individually. However, a thorough optimization of the LAM procedure is indispensible to overcome difficulties due to the small sample size and the loss of RNA integrity of LAM-isolates. Recent innovations in tissue processing, as well as in the novel development of qualitative and quantitative analysis of RNA have improved the ease of use and the reliability of RT-qPCR on microdissected samples.

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