# Quantitative RT-PCR gene expression analysis of laser microdissected tissue samples

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**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) is a valuable tool for measuring gene expression in biological samples. However, unique challenges are encountered when studies are performed on cells microdissected from tissues derived from animal models or the clinic, including specimen-related issues, variability of RNA template quality and quantity, and normalization. qRT-PCR using small amounts of mRNA derived from dissected cell populations requires adaptation of standard methods to allow meaningful comparisons across sample sets. The protocol described here presents the rationale, technical steps, normalization strategy and data analysis necessary to generate reliable gene expression measurements of transcripts from dissected samples. The entire protocol from tissue microdissection through qRT-PCR analysis requires ~16 h.**



## INTRODUCTION

There is significant interest in the biomedical community in quantitative mRNA expression analysis of microdissected cells from animal models or clinical specimens. These measurements can be carried out as either a primary investigative tool or as a method to independently validate results from expression microarray experiments[1–15]. This report provides a comprehensive protocol for analyzing microdissected samples[5,16]. To date, this is the only available protocol detailing the use of quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for the comparative analysis of diseased and normal frozen microdissected tissues using an appropriate normalization strategy. The protocol is generally accessible to researchers with the potential application to all cell types from snap-frozen specimens, but as with any scientific methodology the user may need to modify conditions to meet their individual experimental requirements.

In our experience, qRT-PCR can be applied successfully to microdissected samples, although there are several challenges and caveats that need to be considered. Clinical specimens in particular require special diligence and care, as upstream processing steps can significantly influence downstream molecular results[17–21]. Owing to the potential importance of these caveats in influencing expression results, a brief overview of each issue is first provided followed by the detailed protocol for measuring mRNA.

### Tissue considerations

Investigators need to be aware of upstream influences on data derived from tissue specimens, especially those from the clinic. Potentially confounding patient-related issues include demographics, disease status and past and present therapies. In addition, one needs to be cognizant of changes that can occur in the clinic during surgery, tissue acquisition and in the pathology laboratory, including time to freezing, freezing method, tissue type, storage and the effects of endogenous proteases and RNases. More information on these topics and a general review can be found at the National Cancer Institute Office of Biorepositories and Biospecimen Research and is addressed by other researchers[22–34].

### Tissue handling, processing and embedding

It is important to consider the route the specimen takes from the time it leaves the patient[30] or the animal model to the purification of analytes, including how the tissue is handled until it is processed. Tissue handling steps are dependent on tissue sample size[35]. Biopsies are typically small and are snap-frozen or placed in fixative immediately after removal from the body. Alternatively, surgical specimens (e.g., entire prostate) are large and need gross processing into smaller pieces before snap-freezing or fixing. Snap-frozen tissue is embedded in optimum cutting temperature (OCT) compound and stored at −80 °C, and fixed tissue is almost always formalin-fixed and embedded in paraffin to form a tissue block and stored at room temperature (20–25 °C). Fixation and embedding provides for optimal histopathological review under light microscopy, although the biomolecules are compromised due to the cross-linking effects of formalin as well as due to the embedding process. In contrast, snap-frozen samples provide the highest quality DNA, mRNA and protein for analysis, although the histological detail of the sections is inferior to that of fixed and embedded samples. The majority of microdissection-based mRNA expression studies are performed on frozen samples. If the tissue is adequately preserved, it is feasible to recover sufficient RNA quantities (100–200 ng) to make accurate measurements without pre-amplification of the transcriptome[16]. If it is not possible to immediately freeze the tissue during collection, then RNase inhibitors such as RNALater can be used. Although this approach is useful for preserving RNA in bulk tissue, it may not be suitable for microdissection due to the effect on tissue histology. If microdissection is required, it is recommended that the effect of RNALater on tissue histology be investigated before using it on samples.

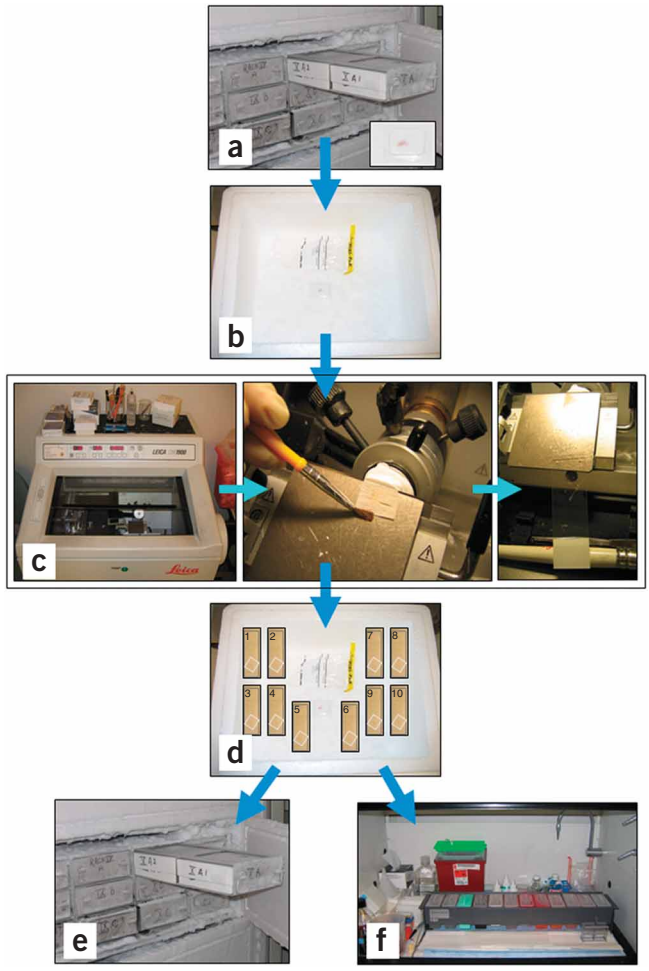


**Figure 1 |** Tissue sectioning and staining flowchart. (**a**) Tissue block is removed from the −80 °C biorepository and (**b**) placed immediately on dry ice. (**c**) The OCT-embedded tissue block is placed in the cryostat and adhered to a chuck using OCT. It is important to level the block to reduce facing off tissue loss. Prepare 8-µm-thick sections and place onto the labeled glass slide. Repeat nine times for a total of ten tissue sections. (**d**) Place the tissue sections immediately on dry ice. (**e**) Replace the tissue block back into the −80 °C biorepository. If tissue sections are not to be used immediately, store at −80 °C until use. (**f**) If tissue sections are to be used immediately, stain tissue sections 1, 5 and 10 by the traditional H&E staining method (**Box 1**). Follow with histopathological analysis to assess the cells of interest to be microdissected (**Box 2**). Keep the other sections on dry ice or store at −80 °C until ready to carry out microdissection (**Box 4**). Immediately before microdissection, use the same tissue staining station to stain sections for microdissection (**Box 3**).

### Tissue sectioning and staining

Once the tissue sample has been located in the database and retrieved from the freezer or archive, it is ready for sectioning onto glass slides (see Step 3 and **Fig. 1a–e**). The thickness that tissue sections are cut at varies depending on the experimental design, with 3–5 µm being typical[26,36–38] for ethanol-fixed paraffin-embedded and formalin-fixed paraffin-embedded (FFPE) tissues. However, we have found that cutting OCT-embedded frozen tissues at 8 µm gives excellent results for downstream RNA analysis, without increasing the tissue opacity or chance of dissecting contaminating cells. Frozen tissue sections are generally stored for no more than 1 month[36], and microdissection is carried out within a few weeks of cutting. However, improved RNA recovery has been observed from frozen tissue sections stored for $\leq$2 weeks. When cryostat sectioning, investigators should be aware of contamination issues that may be due to tissue carryover from other tissue blocks. Thus, clean uncharged slides, disposable cryostat blades, clean brushes and RNA clean protocols are recommended. To reduce tissue waste, it is recommended that the tissue be aligned as closely as possible with the cryostat blade before cutting tissue sections. In addition, investigators often prepare 10 recuts (or more) at a time, with sections 1, 5 and 10 hematoxylin and eosin (H&E) stained and coverslipped for review of the histopathology (see Step 4, **Box 1** and **Fig. 1f**).

Before microdissection, it is necessary to stain the tissue section to allow visualization of the cells of interest. H&E is a standard approach for visualization and is used widely. However, more recently methyl green was shown to have the least fluorescent interference for qRT-PCR[39]. Therefore, to investigate this more thoroughly, we carried out analysis of three different staining methods (H&E, hematoxylin and methyl green) on replicate histological sections before microdissection to determine whether any of the stains caused a detectable fluorescent interference of subsequent qRT-PCRs. No statistically significant $C_T$ value difference for the three stains was found using an F-test[16]. Therefore, H&E continues to be an excellent choice for staining tissue sections before laser capture microdissection (LCM).

### Pathology evaluation of tissue for microdissection

Tissue sections need to be reviewed and annotated before microdissection to histologically identify the desired cells for microdissection. Therefore, an evaluation of the tissue samples by a pathologist or a scientist trained in histological cell identification of frozen tissues is needed[36] before, during and after microdissection (see Step 5, **Box 2** and **Fig. 2**). Also of importance is the orientation of the specimen in the tissue block. This is particularly important so that the cells of interest are adequately represented on the slide. Pathology slide review includes the evaluation of the tissue integrity, histopathology, determination of the adequacy of the sample for microdissection based on the amount of the target cell population and annotation of the target cells on the slide. The pathologist also can give advice on the staining procedure that will help to better identify the cells of interest under the microscope during dissection.

### Tissue microdissection

Laser-based techniques have now made tissue microdissection a routine step in obtaining precise quantitative gene expression measurements from dissected tissue samples. LCM was invented at the National Institutes of Health (NIH) and developed through a cooperative research and development agreement with Arcturus Engineering to facilitate fast, simple and reliable tissue microdissection[37,40]. Several laser-based microdissection platforms are now available for procurement of pure populations of cells. A thorough review of the various microdissection platforms and protocols for LCM has been detailed previously in *Nature Protocols*[36].

### RNA recovery and assessment

Tissue samples may contain quantitative PCR (qPCR) inhibitory agents[41–46]. In our experience, OCT is a major factor in qPCR

## BOX 1 | FROZEN TISSUE SECTION STAINING FOR HISTOPATHOLOGY CONSIDERATION AND ARCHIVE

Frozen tissue section numbers 1, 5 and 10 should be H&E stained and coverslipped for histopathology consideration (**Box 2**) and archival reference. The fixation step (Step 1, 70% ethanol) should be at least 1 min for tissue morphology preservation. It is recommended to use the same fixative as the one used for tissue sections intended for microdissection (i.e., 70% ethanol). This assures the generation of similar tissue morphology for histological review. In general, 70% ethanol fixation is an acceptable fixative for tissue morphology preservation. However, other fixatives, such as formalin, can be used to improve tissue morphology preservation for histopathological assessment. Depending on the tissue, H&E should be modified a number of times to obtain the desired staining tone.

Prepare individual staining dishes with the following solutions and treat the slides for the described durations. All solution preparation and staining are conducted at room temperature. After each incubation, slides should be briefly drained and then moved to the next solution:

1. 70% Ethanol, 1–2 min
2. Deionized $H_2O$, 30 s
3. Mayer's Hematoxylin, 1 min
4. Deionized $H_2O$, 30 s
5. Scott's Bluing, 30 s
6. 70% Ethanol, 30 s
7. Eosin Y, 30 s
8. 95% Ethanol, 1 min
9. 95% Ethanol, 1 min
10. 100% Ethanol, 1 min
11. 100% Ethanol, 1 min
12. Xylene, 1 min
13. Xylene, 1 min
14. Xylene, 1 min
15. Coverslip using xylene-based mounting media
16. Allow slide to dry for 10–15 min. Be sure that the mounting media is dry before storing the slide sideways in a slide box.
17. Stained tissue section is now ready for histopathology review.

inhibition; therefore, it is important to extract and isolate RNA from the microdissected sample before qRT-PCR analysis. Many methods for RNA extraction and isolation are available on the market. Choice of the appropriate method depends on the type of starting tissue sample, irrespective of whether it is derived from cells in culture, bulk tissue, tissue scrapes or small cell quantities (e.g., microdissection). In addition, it is recommended that an RNA-only workspace is available for RNA extraction.

RNA purification methods for downstream qRT-PCR analysis have been discussed in detail earlier[47]. For a microdissection-based approach, samples are placed in a lysis buffer and stored at −80 °C. To be rigorous in ensuring the total RNA isolated is free of large DNA fragments, the sample is subjected to DNase during the extraction process because DNA is often a contaminant when using glass filter RNA extraction methods[2,16]. To reduce bias, total RNA from all microdissected samples in a given study

## BOX 2 | HISTOPATHOLOGICAL CONSIDERATIONS FOR TISSUE MICRODISSECTION

The goals of histopathology consideration before proceeding with tissue microdissection are (1) to evaluate the total amount of tissue and the amount of the target cells in the tissue section present in the block, (2) to study the histopathology of the tissue specimen and identify the target cells and (3) to plan the microdissection for each specimen, taking into account the heterogeneity of tissue samples.

Recommendations

1. Always make a regular H&E slide for histopathological analysis (**Box 1**) before proceeding with tissue microdissection. Traditional H&E staining (using longer times in each solution than H&E for LCM) and coverslipping of sections 1, 5 and 10 for histopathological analysis are recommended before beginning the study. These sections will serve as a permanent record of the tissue specimen status and show histological changes that occur in the deeper sections.
2. Always label the slides with the tissue block identification label, the number of the recut (e.g., 1, 2, 3) and the date the section was cut from the tissue block.
3. If you are unsure of the tissue histology represented in the traditional H&Es, consult a pathologist to review the slides to accomplish the general goals listed above.
4. Keep the traditional H&Es used for histopathological assessment with you when performing the tissue microdissection. These slides will help in identifying the cells of interest in the tissue section being used for microdissection. Both slides can be compared side by side.
5. Only dissect the cells that can be clearly identified. If there is any doubt, do not dissect it without consulting pathologist.

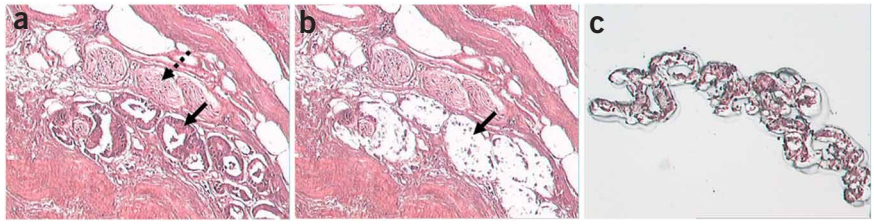


**Figure 2** | Frozen prostate epithelium laser capture microdissection (LCM) procurement sequence. (**a**) Roadmap before microdissection (solid arrow indicates the epithelium; dashed arrow indicates the nerve). (**b**) Post-microdissection (solid arrow indicates the lifted epithelial area). (**c**) Microdissected epithelial cells on the LCM cap (hematoxylin and eosin (H&E) stained. Original magnification ×100).

should be extracted using the exact same method and aliquoted before analysis and stored at −80 °C.

RNA quantitation methods are numerous and each technique has its own strengths and weaknesses[47,48]. However, it is imperative that the quantitation method chosen is appropriate for the range of RNA obtained during dissection, which is generally on the order of 5–20 pg per cell. As microdissection yields low quantities of RNA (**Table 1**), NanoDrop total RNA quantitation technique (NanoDrop Technologies) is an excellent choice owing to the small quantity of sample that is required for the assay.

The RNA quality in frozen tissue samples can vary due to the upstream effects of tissue handling, but are generally higher than ethanol-fixed paraffin-embedded- and FFPE-processed specimens. There are several schools of thought regarding RNA quality related to qRT-PCR that are reviewed elsewhere[48]. For assessing total RNA from frozen microdissected tissues, the Bioanalyzer system (Agilent Technologies) and RNA integrity number (RIN) provide an adequate assessment of total RNA quality (**Table 1**). This is best utilized in concert with NanoDrop quantitation to select the appropriate Bioanalyzer kit (either Pico- or Nano-Chip) as the recovered total RNA quantity varies per microdissection.

### qRT-PCR assay

As limited quantities of total RNA from clinical or animal model tissue samples are typically used as the starting template, it is recommended to carry out two-step qRT-PCR, with one reverse transcription (RT) tube per sample producing sufficient cDNA to carry out 12 qPCRs, allowing for triplicate analysis of three genes of interest and one endogenous control reference gene. This protocol is optimized for bias reduction; therefore, RNA pre-amplification is not used. However, if an investigator chooses to use an RNA pre-amplification step (see below), the protocol should be adjusted accordingly.

qPCR assays use one of two fluorescent detection chemistries. The first, SYBR Green, uses one pair of specific primers, and the fluorescent dye incorporates into the DNA during amplification[49]. The second uses one pair of specific primers and a gene-specific fluorescent probe that increases the specificity of the assay. The

**TABLE 1** | Representative total RNA quantity and quality from microdissected frozen tissue samples[a].

| Tissue | Neoplastic status | No. of LCM shots | Total RNA quantity (ng μl$^{-1}$) | Total RNA quality (RIN)[b] |
|---|---|---|---|---|
| *Epithelium* | | | Range = 8.4–20.42 | Range = 4.2–7.8 |
| Breast | Normal | 2,000 | 12.29 | 7.6 |
| | Tumor | 3,000 | 13.51 | 7.8 |
| Colon | Normal | 3,000 | 13.2 | 4.2 |
| | Tumor | 3,000 | 12.57 | 6.5 |
| Esophagus | Normal | 4,000 | 16.59 | 5.1 |
| | Tumor | 3,700 | 20.42 | 4.5 |
| Prostate | Normal | 3,000 | 8.4 | 6.2 |
| | Tumor | 3,000 | 10.8 | 5.6 |
| Urethra | Normal | 2,000 | 13.87 | 6.1 |
| *Stroma* | | | Range = 4.81–8.86 | Range = 4.8–6.9 |
| Breast | Normal | 3,000 | 6.53 | 6.8 |
| | Tumor | 2,400 | 8.86 | 6.5 |
| Prostate | Normal | 20,000 | 6.65 | 6.9 |
| | Tumor | 20,000 | 6.37 | 5.7 |
| Urethra | Normal | 3,000 | 4.81 | 4.8 |

LCM, laser capture microdissection; NCI, National Cancer Institute; RIN, RNA integrity number. [a]These data were generated at the Pathogenetics Unit, NCI, and are representative of frozen microdissected total RNA from these tissues. [b]Algorithm assigning value to RNA quality, as calculated by Bioanalyzer 2100 (Agilent Inc.).

most common probe detection chemistry is TaqMan (Applied Biosystems), in which the signal is generated by cleavage of the fluorescent quencher during the reaction cycle. This is the detection chemistry described in the protocol below; however, fluorescent probe expense may be an issue for some users.

For downstream qRT-PCR application, RNA quality of RIN $>5$ is considered good and RIN $>8$ is considered excellent[50]. In general, highly fragmented RNA from FFPE samples is not optimal for qRT-PCR amplification; however, our experience has shown that informative qRT-PCR results can be obtained from frozen samples that show some RNA degradation (RIN $2< x <5$). The use of random hexamers as primers for RT diminishes the effects of degradation by generating more complete cDNA coverage of genes than with oligo dT priming. A more complete review of issues related to RT and qPCR has been discussed elsewhere[47,48,51–58]. In addition, our experience suggests that single plexing of the qPCR (i.e., separate reactions from the same cDNA sample for the gene(s) of interest and the endogenous control reference gene) reduces the potential for competitive inhibition by the two primer and probe sets and subsequently improves detection capabilities by 2–3 $C_T$ values, which is important when analyzing transcripts that may be expressed at a lower level. Spiking the master mix with additional Taq polymerase (1 µl per qPCR) increases the detection capabilities by another 2–3 $C_T$ values. In addition, we have found that using larger qPCR volumes (50 µl), $C_T$ values were further improved. Smaller reaction volumes (e.g., 20 µl) without additional Taq polymerase spiking may be used successfully to obtain quantitative gene expression measurements; however, investigators must weigh the cost of larger reaction volumes and additional Taq polymerase to the potential decrease in detection capabilities for the specific experiment and transcripts to be detected.

The number of gene transcripts that can be analyzed from a typical microdissection from frozen tissue samples using qRT-PCR is limited. Moreover, the ability to analyze qPCR efficiency and standard curves is not possible owing to the small sample quantity generated by microdissected tissues[48]. Therefore, the use of a comparable positive control total RNA sample (i.e., if using microdissected frozen human esophageal tissue, the positive control would be purchased total human esophagus RNA) in the qRT-PCR is necessary.

As an example, a typical prostate epithelium microdissection of 3,000 laser shots ($\sim$10,000 cells) allows for the procurement of ±160 ng of total RNA, which is sufficient to analyze the transcripts of nine genes of interest and one endogenous control reference gene. For the prostate stroma, microdissection of 3,000 laser shots typically yields ±24 ng of total RNA, which is only an adequate template to analyze one gene of interest and one endogenous control reference gene. To procure $\sim$10,000 cells from the stroma, the microdissection laser shots are generally increased four times, depending on the cellular density.

### Normalization strategy

A normalization control is critical when attempting to quantitatively compare gene expression levels between two biological samples (e.g., diseased cells versus normal cells). Housekeeping genes such as *ACTB* and *GAPDH* have historically been used for normalization because they were presumed to be stably expressed in all cell types. In addition, cell analyte content (i.e., amounts of RNA, DNA or protein), numbers of cells and averages of multiple genes from expression arrays are sometimes used as normalization approaches. The majority of the qRT-PCR studies in the literature use a common set of housekeeping genes as endogenous controls for normalization. These mRNAs are generally effective, but their expression levels have been shown to vary among different cell types, samples and environments[59–70]. Therefore, universally stable endogenous control genes have not been found[71]. For example, *ACTB* has consistently stable levels of gene expression in cultured prostate cancer DU-145 and PC-3 cells[67]. However, our experience and the experience of others[72] have shown that the same *in vitro* expression levels are not always observed in *ex vivo* tissue samples. Therefore, a housekeeping gene such as *ACTB* may not be the most stable endogenous control gene for use in qRT-PCR studies using tissue samples. In addition to issues related to tissue type, disease states (e.g., cancer or other proliferative processes) and procurement methods may introduce variability in housekeeping gene expression[60,73–77]. Therefore, for each tissue type and experimental system, a reliable and accurate normalization strategy must be determined and validated.

To address this in prostate, our group analyzed three gene expression normalization strategies (LCM cell count, total RNA measurement and endogenous housekeeping genes) using microdissected frozen samples[16]. Briefly, the data showed that microdissection cell counting was not a reliable normalization method. Within replicate dissections, a sizeable variation of up to 1.84 $C_T$ values was seen, which could induce a bias of up to 3.6-fold between samples. As gene expression changes of approximately three- to four-fold (1.5–2 $C_T$ values) are considered as potentially biologically important[78], the use of microdissected cell count is not recommended as a normalization strategy.

The second normalization method analyzed was the use of total RNA quantity as an internal control for qRT-PCR from microdissected tissues. Total RNA was recovered and quantified by the NanoDrop method from microdissected cells from triplicate serial sections. The data showed that RNA quantitation alone produces a large variance, which prohibits using this approach for normalization.

Even though the data showed that neither microdissection cell count nor total RNA measurement are precise enough to serve as a normalization strategy for microdissected tissue, they are practical first and second steps in calibrating 'ballpark' RNA input levels that can be successfullyexamined by qRT-PCR.

The third normalization method analyzed was the use of endogenous housekeeping genes as an internal reference control for qRT-PCR from microdissected tissues. Endogenous housekeeping genes were found to offer an excellent representation of the cellular transcriptome, and the use of only one or two housekeeping genes (i.e., *PGK1* or *18s* and *HPRT* for paired samples) was precise enough to serve as an internal reference control for quantitative gene expression analysis. The need to normalize with only one or two housekeeping genes is important, as limited amounts of RNA are typically procured from microdissected tissue samples.

Several methods for relative quantitation exist, including the most commonly used $2^{-\Delta\Delta C_T}$ method[79,80]. This approach is recommended for dissected cells, as it allows for the maximal number of genes that can be analyzed from one sample.

Assuming comparable amplification efficiencies between the gene(s) of interest and the calibrator, this method eliminates the need for generating standard curves, as the gene of interest data are normalized to the calibrator (endogenous housekeeping reference control gene)[81].

## Experimental design

**General considerations.** Before beginning the protocol, the following general guidelines should be considered: (1) Use a clean dead-air hood that is dedicated for setting up RT and PCR reactions in preparing all qRT-PCs to avoid contamination. All pipettes, pipette tips, tubes, pens, min-centrifuge and vortex should be contained inside the hood. (2) Place all reagents on ice. (3) Protect all TaqMan probes and master mixes from light (i.e., keep in dark). (4) Mix (vortex or swirl, depending) and spin down all source vials before opening. (5) Always run a no template control (NTC), a no RT and a positive control ((+) C) for each RT and primer/probe set for qPCR. (6) In loading reaction tubes, load in the following order: (1) NTC, (2) no RT, (3) unknowns and (4) (+) C. This will minimize the potential for contamination carryover. (7) Aliquot source vials to minimize freeze–thaw cycles. (8) To prevent pipetting errors, prepare master mixes of reagents wherever possible to minimize the number of pipette steps made. (9) Use powder-free gloves to prevent optical reader interference. (10) Use a common positive control commercial total RNA for all qRT-PCRs for the same tissue type. This will allow a common reference from plate to plate, allowing data to be analyzed across batches.

**Tissues.** To obtain total RNA from microdissected tissues, the following considerations must be taken into account: (1) Sample has been snap-frozen properly before storage. (2) Tissue block is maintained at optimal temperature during sectioning. (3) Cryostat and all sectioning and staining consumables are free of contaminating tissues. (4) Once the tissue section is cut from the block, mount the section on an uncharged glass slide at room temperature. An uncharged slide will allow the tissue to more easily be procured/lifted off the glass slide during the microdissection process. The glass slide with the tissue section on it should then be placed immediately on dry ice. Do not let the tissue section defrost. (5) Before choosing the tissue block for use in the study, do a scrape of the tissue section[35] and do RNA extraction and isolation followed by quantitation and qualitation analysis to assess RNA integrity[16]. (6) Frozen tissue sections should not be stored for more than 2 weeks before use. (7) Once the tissue section is out of the freezer, not more than 30 min should elapse before placing the microdissected tissue into the lysis buffer.

**RNA isolation and measurement.** RNase-free techniques need to be followed stringently to prevent experiment failure due to contamination. This can be accomplished by (1) wearing powder-free disposable gloves, (2) avoiding touching RNase-contaminated surfaces, (3) changing gloves often, (4) cleaning bench tops and hood surfaces with an RNase decontaminant solution before use, (5) using a dedicated RNA bench and equipment and (6) using reagents and consumables that are free of contaminants (e.g., RNases, other RNA or DNA, PCR products, thawed tissue fragments or chemical contaminants).

Extract and isolate RNA using a total RNA extraction kit that is appropriate for the amount of microdissected tissue procured. In our experience, the PicoPure RNA Isolation Kit (Arcturus/Molecular Devices), a glass filter-based method, provides the largest and most reproducible total RNA yields from microdissected tissue samples.

After RNA purification, DNase treatment for 15 min is recommended[2,16]. For most genes, a DNase step is not necessary. It is best to design and use cDNA-specific primers and probes to reduce the need for a DNase treatment, but for some pseudogenes a DNase step is necessary. Therefore, it is generally recommended to add a DNase treatment to the RNA extraction if the kit chosen does not include one. In this protocol, DNase treatment is recommended to prevent interference by the PicoPure RNA Isolation Kit (Arcturus/Molecular Devices) components that may cause artifacts during downstream Agilent 2100 Bioanalyzer assessment of quality and genomic DNA (gDNA) contamination. However, if investigators choose not to use a DNase step, downstream quality assessment using the Agilent 2100 Bioanalyzer may be confounded.

Immediately after total RNA extraction and before storage at −80 °C, one must be sure to aliquot samples into volumes appropriate for quantitation (1 μl), qualitation (2 μl) and multiple RT reactions (4 μl each). It is recommended to use an elution buffer volume of 20 μl to recover sufficient total RNA to make the previous aliquots. Place total RNA aliquots at −80 °C until ready for use in RT reaction.

This RNA extraction and isolation protocol eliminates the presence of qRT-PCR inhibitors in the total RNA sample; however, qRT-PCR inhibition due to template overload is still possible.

After total RNA purification, quality and quantity measurement is recommended[16]. As very small total RNA quantities are obtained from microdissected tissues (**Table 1**), it is difficult to adjust or equalize total RNA quantities across samples before qRT-PCR. However, it is important to know the starting quantities and qualities of total RNA in order to 'ballpark' starting template input,[16] and it is recommended to quantify total RNA using the NanoDrop ND-1000 spectrophotometer (260/280 nm; NanoDrop Technologies). This method only requires 1 μl of sample and can be used with concentrations as low as 2 ng $\mu l^{-1}$. As total RNA concentrations per microliter from microdissected tissue samples (**Table 1**) generally fall below the quantity detection limits of the Bioanalyzer (>25 ng $\mu l^{-1}$; Agilent Technologies), this method of total RNA quantitation is not sensitive enough for routine use.

Before measuring total RNA quality, several considerations for measuring quality of total RNA from microdissected tissues need to be taken into account. When generating total RNA from microdissected tissues, the quantity of total RNA recovered per sample varies depending on many variables, including, but not limited to, the subject, tissue type, tissue processing and storage. RNA qualitation of total RNA from microdissected tissue samples yields quantities too small to routinely use conventional gel electrophoresis to determine RNA integrity. Therefore, it is recommended to use the Agilent 2100 Bioanalyzer (Agilent Technologies) because it only requires 1 μl of sample for the analysis of 18S and 28S rRNA and provides an RIN. Total RNA concentrations per microliter of elution buffer often fall in the range of one of the detection capabilities of the Agilent Bioanalyzer Pico- and Nano-Assays. The Agilent RNA 6000 Pico Kit has a qualitative range of 50–5,000 pg $\mu l^{-1}$ and is not suitable for quantitation. The Agilent RNA 6000 Nano Kit has a qualitative range of 5–500 ng $\mu l^{-1}$ and a quantitative range of 25–500 ng $\mu l^{-1}$. As such, it is imperative to

carry out NanoDrop quantitation before the Bioanalyzer qualitation analysis to determine which kit (i.e., Pico or Nano) is appropriate. In addition, one method of quantitation and one method of qualitation should be used throughout the study for all tissue samples. Therefore, as RNA quantities vary and are predominantly $<25$ ng $\mu l^{-1}$ (the minimum for Bioanalyzer quantitation analysis is $\geq 25$ ng $\mu l^{-1}$), NanoDrop is the preferred technique for RNA quantitation. In summary, it is recommended that investigators should carry out total RNA quantitation of samples using the NanoDrop quantitation technique for RNA and also using appropriate Agilent RNA 6000 kit (i.e., use Agilent 6000 Pico Kit for samples with total RNA quantities $<5$ ng $\mu l^{-1}$ or use Agilent 6000 Nano Kit for samples with total RNA quantities $>5$ ng $\mu l^{-1}$).

**Controls.** An LCM negative control is prepared for each tissue section by making 3,000 laser shots in a tissue-free portion of the tissue section. This sample is processed throughout the entire experiment in parallel with microdissected tissue samples to control for contaminating RNA.

The choice of positive control total RNA origin (i.e., organism, organ and tissue type) is dependent on the tissue samples being analyzed, but is typically from the same organism and the tissue type, e.g., positive control of total human prostate RNA for experimental microdissected frozen human prostate tumor and normal tissue samples. However, if the desired tissue type is not commercially available, a good alternative is to use organism-specific universal RNA.

Once initial positive control concentrations are determined, the investigator should choose the three positive controls that are closest in concentration to the total RNA amount from the microdissected sample.

An endogenous control reference gene(s) that shows stable expression in the samples being analyzed is used in the third step of the normalization strategy[16]. It is recommended that if a single endogenous control reference gene can be used for the study, that just one is used, see Erickson *et al.*[16] for a discussion of this topic. Primer and probe design has been discussed extensively in a previous issue of *Nature Protocols*[48]. TaqMan chemistry-based primer/probe sets (e.g., AB Assays-on-Demand, AB Assays-by-Design or other TaqMan chemistry primer/probe sets) are recommended for use with RNA from microdissected samples because of improved specificity over primer-only-based detection chemistries (e.g., SYBR). For microarray gene expression profile validation, it is recommended to design all primer/probe sets to amplify the same region of the cDNA sequence as the gene-specific microarray probe set used to derive the data.

Using a single endogenous control housekeeping gene, it is possible to analyze up to nine genes of interest from a single microdissection (**Fig. 3**). For both the genes of interest and the endogenous control reference gene(s), non-primer-limited primer/probe sets can be used in single-plex reactions. While carrying out multi-plex reactions, it is recommended to use primer-limited primer/probe sets for amplification of the endogenous control gene, as this limits amplification of the gene once the signal threshold is reached. In multi-plex reactions, choose different reporter dyes for the genes of interest (e.g., FAM) and the endogenous control reference gene (e.g., VIC/HEX). For single-plex reactions, it is not necessary to choose different reporter dyes or use primer-limited primer/probe sets. However, if using the endogenous control reference gene(s) for other types qRT-PCR experiments, it may be more cost-effective to use VIC-labeled primer-limited primer/probe sets.

**qRT-PCR.** Total RNA yield from microdissected tissue determines the number of genes that can be analyzed by qRT-PCR. Each qPCR requires 4 ng of total RNA starting template that was used for RT. For example, if the microdissection of epithelium yielded $\sim$160 ng of total RNA, this would allow for the analysis of nine genes of interest and one endogenous control housekeeping gene. However, if the microdissection of the stroma yielded $\sim$24 ng of total RNA, only one gene of interest and one housekeeping gene can be analyzed. Stroma should be analyzed for cellular density and the number of laser shots adjusted to increase the total RNA yield.

Total RNA samples from microdissected tissues are limited and expensive. Therefore, it is imperative that primers and probe sets are validated to ensure that they specifically amplify their intended target sequence linearly and reproducibly before using the microdissected RNA samples. This can be accomplished using a commercially available tissue-specific total RNA to carry out serial dilutions. The serially diluted RNA is then applied to the qRT-PCR protocol using the specific primers and probe sets. The $C_T$ values, PCR efficiencies and standard curves are evaluated. Following primer and probe set specificity and sensitivity, cDNA generated from the RT portion of the protocol can be used for qPCR assays, which should be carried out in triplicate as technical replicates to assess precision of assay.

qRT-PCR of microdissected tissues is carried out in two steps (RT and qPCR) to maximize the number of genes that can be analyzed from one sample. During RT, random hexamers should be used as reverse primers to counter the effects of mRNA degradation. Carry out RT and qPCR reactions of matched disease and normal tissue sample total RNA at the same time. Three technical replicates of qPCR are used to assess precision of the assay, and endogenous control gene primer/probe sets are analyzed for each sample and control. This qPCR protocol described here uses 50 PCR cycles instead of the usual 40 cycles, although with microdissected samples we find that more than 90% of genes do not have $C_T$ values $>40$. A total of 50 cycles are used for qPCR to ensure that the NTCs for a gene of interest is at least 10 $C_T$ values later than the sample gene of interest expression in cases where low levels of gene expression (e.g., 37 $C_T$ values) are encountered. This ensures that if the NTCs indicate 'no $C_T$' at 50 cycles, the qPCR reagents are free of contamination. In addition, the observation of 10 $C_T$ difference comparing no-RT with RT-positive samples means there is negligible contamination (0.1%) of gDNA[82,83]. However, as PCR efficiency changes with the length of the experiment, it is recommended to test the in-run PCR efficiency of gene-specific primer/probe sets at the $C_T$ range of the gene of interest using a standard cDNA dilution series. In our experience, adding additional Taq polymerase is valuable in lowering $C_T$ values and improving technical replicate standard deviations[16]. However, investigators should be aware that changes such as Taq concentrations can change PCR efficiencies.

**Analysis.** To ensure standardization of analysis techniques, one qPCR method and software analysis method should be used

throughout an entire study. Although beyond the scope of this protocol, a detailed review of qRT-PCR data analysis for amplification plots, primer/probe efficiencies, standard curve generation and general qRT-PCR analysis has been described in a previous issue of *Nature Protocols*[48].

The normalization strategy dictates the analysis method that can be used. On the basis of the optimal normalization strategy for qRT-PCR from microdissected tissues[16] when comparing diseased and normal tissues, data are analyzed by relative quantitation analysis using the $2^{-\Delta\Delta C_T}$ method[84]. This is not the only analysis method; however, investigators should assess if their study requires absolute quantitation using a standard curve. It is always recommended to assay the PCR efficiencies of each gene primer/probe set to be used in relative or absolute quantitation studies. In summary, all samples are analyzed relative to an endogenous control housekeeping gene from the same sample total RNA to generate the normalized $\Delta C_T$ value. Subsequently, normalized disease tissue qPCR data are analyzed relative to normalized matched normal tissue qPCR data to generate the $\Delta\Delta C_T$ value.

## Conclusion

The protocol described in this report allows for quantitative analysis of mRNA derived from microdissected animal model tissues or from clinical specimens. However, it is important that investigators factor tissue-related variables, especially those inherent to clinical samples, into their experimental design and data analysis. Precise expression measurements from phenotypically or molecularly defined cell populations in tissue samples likely will have significant value for both laboratory researchers and clinical investigators.

## MATERIALS

### REAGENTS

- Dry ice ! CAUTION Dry ice can burn the skin on contact, and vapors can cause asphyxiation. Take appropriate precautions when using dry ice.
- OCT compound (Tissue-Tek, Sakura Finetek, cat. no. 4583) for cryopreservation
- Frozen tissue specimens sectioned at 8 µm ▲ CRITICAL Do not store tissue sections for more than 1 month (≤2 weeks is preferred) before microdissection. ! CAUTION Follow established protocols for safely working with bloodborne pathogens for all human and animal biological samples. For animal samples, follow all Institutional Animal Care and Use Committee protocol regulations. For human samples, follow all established Institutional Review Board clinical protocol regulations, including patient consent and sample anonymization.
- Mayer's Hematoxylin solution (Sigma, cat. no. MHS128) ! CAUTION Contact with the solution is hazardous.
- Scott's Blueing Solution (Fisher, cat. no. CS410-4D) or alkaline water ▲ CRITICAL The reddish color of Mayer's Hematoxylin will turn blue with mild alkaline treatment, such as that of Scott's Blueing Solution. Omitting the alkaline treatment step will make histopathological analysis of tissue and cell morphology difficult.
- Eosin Y solution (Sigma, cat. no. HT110116) ! CAUTION Contact with the solution is hazardous; flammable.
- Absolute (100%) ethanol (molecular grade, Sigma, cat. no. E7023) ! CAUTION Contact with the solution is hazardous; flammable. ▲ CRITICAL In humid climates (>40%), monitor the water content of the ethanol, and change the solution as often as necessary. It is generally recommended to change all solutions in staining process every week or more frequently if staining >20 slides.
- 95% and 70% ethanol. Prepare with Milli-Q-filtered water (Millipore) ▲ CRITICAL If preparing solutions in batches, do not store for more than 1 week at 4 °C.
- Xylene (Sigma Aldrich, cat. no. 247642) ! CAUTION Vapor and contact are hazardous; vapor is harmful or fatal; use appropriate safety measures for working with and disposing of hazardous materials. ▲ CRITICAL Xylene substitutes (e.g., Citra-Solv) may diminish microdissection efficiency of some tissue types.
- RNA extraction buffers (PicoPure RNA Isolation Kit, Arcturus/Molecular Devices, cat. no. KIT0204)
- RNase-free DNase Set (50) (Qiagen, includes RDD buffer and DNase 1; cat. no. 79254)
- RNA quality measurement (see REAGENT SETUP): RNA 6000 Pico Series II Assay (Agilent Technologies, cat. no. 5067-1513) and/or RNA 6000 Nano Series II Assay (Agilent Technologies, cat. no. 5067-1511)
- RNase/DNase-free water (Ambion Applied Biosystems, cat. no. AM9937)
- Tissue-specific total RNA (Ambion Applied Biosystems, cat. nos. vary depending on the species and organ) to use as positive control template
- RT-PCR Grade Water (Ambion Applied Biosystems, cat. no. AM9935)
- RT assay: RT reagents (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, cat. no. 4368813; TaqMan Reverse Transcription Reagents, Applied Biosystems, cat. no. N8080234), Random hexamers (Applied Biosystems, cat. no. N8080127). Additional information for other RT reagents has been covered in a previous issue of *Nature Protocols*[48].
- Primer/probe sets for genes of interest and endogenous controls: Assays-on-Demand TaqMan MGB Probe (Applied Biosystems, inventoried or made to order, cat. no. 4331182 or 4351372), Assays-by-Design: Primers (Applied Biosystems, cat. no. 4304970) and TaqMan MGB Probe (Applied Biosystems, cat. no. 4316034). Primer and probe design considerations have been covered in a previous issue of *Nature Protocols*[48].
- TE buffer pH 7.0–8.0 (e.g., 1× TE Buffer pH 8.0, Quality Biological, cat. no. 351-011-131)
- qPCR assay: 2× TaqMan Universal Master Mix (Applied Biosystems, cat. no. 4304437), AmpliTaq Gold DNA Polymerase, LD (Applied Biosystems, cat. no. 4338857). Other companies such as Stratagene offer qPCR reagents. Additional information for non-TaqMan chemistry-based qPCR assay reagents has been covered in a previous issue of *Nature Protocols*[48].

### EQUIPMENT

- Protective personal wear, including lab coats, powder-free latex and/or nitrile gloves and safety glasses
- Biohazard or medical pathological waste container
- −80 °C freezer
- −20 °C freezer
- 4 °C refrigerator
- Cryomolds (Tissue-Tek, Sakura Finetek, cat. no. 4557)
- Cryostat (Leica CM 1900 UV, Leica Microsystems)
- Uncharged slides and coverslips of the size appropriate for the tissue specimen to be sectioned. There are many distributors for these consumables (Fisher Scientific, cat. nos. NC9744786 and 22-037-169).
- LCM system (PixCell II, Arcturus/Molecular Devices). Considerations for the choice of LCM systems have been discussed in a previous issue of *Nature Protocols*[36].
- Adhesive pads (Post-it Note, 3M)
- CapSure Macro LCM Caps (Arcturus/Molecular Devices, cat. no. LCM0211) ▲ CRITICAL As tissue is in direct contact with the cap, it is important to use the adhesive pad to remove any tissue debris. If extraneous tissue is not carefully removed, it will be available for RNA extraction and may contaminate the sample.
- Microcentrifuge tubes: MicroAmp 500 µl Thin-walled PCR Reaction Tubes (Applied Biosystems, cat. no. 9N801-0611) or Safe-Lock 500 µl Eppendorf Tubes (Brinkmann Instruments, cat. no. 2236361-1) ▲ CRITICAL To prevent lysis buffer leakage during pre-extraction heat incubation, the use of either of these tubes is recommended.
- Oven (Hybaid Mini MK-II hybridization oven, Hybaid, cat. no. Mini Oven MK II)
- Agilent 2100 Bioanalyzer (Agilent Technologies, cat. no. G2940CA)
- NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies)

- Dedicated PCR Hood (AirClean 600 PCR Workstation, AirClean Systems, cat. no. AC632DB Dead Air Box)
- Microfuge tubes: 1.7 ml DNase/RNase-free microfuge tubes (CoStar, cat. no. 3560), 0.65 ml DNase/RNase free microfuge tubes (CoStar, cat. no. 32090) and 0.65 ml DNase/RNase free PCR thin-walled tubes (e.g., PGC, cat. no. 502-075). Considerations for the choice of plasticware have been discussed in a previous issue of *Nature Protocols*[48].
- Barrier filter pipette tips (CLP Direct Barrier Tips, cat. nos. BT10XL, BT20, BT100, BT200 and BT1000).
- PCR thermocycler (MJ Research PTC-200 thermocycler, MJ Research)
- qPCR thermocycler (Stratagene Mx3000P Real-Time PCR machine, Stratagene; Applied Biosystems 7500 Real-Time PCR System, Applied Biosystems). Considerations for the choice of a qPCR thermocycler have been discussed in a previous issue of *Nature Protocols*[48].

**REAGENT SETUP**

**RNA qualitation reagents** For RNA 6000 Pico Series II Assay (Agilent Technologies, cat. no. 5067-1513) and/or RNA 6000 Nano Series II Assay (Agilent Technologies, cat. no. 5067-1511), remove the reagent from the freezer for 30 min to bring to room temperature before use.

**Primer/probe sets** For primer/probe sets delivered as individual components, prepare a 100-µl 20× working solution in TE buffer using TaqMan Gene Expression qPCR assays according to calculations detailed in the table below:

| Component | Stock concentration (µM) | Volume (µl) | Final working concentration (µM) |
|---|---|---|---|
| Forward primer | 100 | 18 | 18 |
| Reverse primer | 100 | 18 | 18 |
| MGB Probe | 100 | 5 | 5 |
| 1× TE Buffer | | 59 | |

Store all primer/probe stocks and working solutions at −20 °C until ready for use. For primer/probe sets that are delivered in the pre-prepared working solution, aliquot into 60 µl volumes and store at −20 °C until ready for use.

## PROCEDURE

### Frozen tissue sectioning and staining for histopathological consideration ● TIMING 1.5–3 h

**1|** Select the frozen tissue sample to be used in the study from the database of available tissue specimens (**Fig. 1a**).
▲ CRITICAL STEP Assess that the tissue specimen has been promptly snap-frozen and embedded in OCT. Rapid processing of the tissue specimen limits RNase activity and subsequent RNA degradation. If the frozen tissue specimen has not been previously embedded in OCT, do so at this time. The tissue block is now ready to have tissue sections cut onto glass slides.

**2|** Remove the selected tissue block(s) to be sectioned from the freezer and place on dry ice (**Fig. 1b**).

**3|** Prepare 10 tissue sections by sectioning the tissue block onto the standard histology glass slide using a cryostat and then place on dry ice (**Fig. 1c** and **d**).
▲ CRITICAL STEP It is recommended to cut OCT-embedded tissue blocks at 8 µm. This thickness allows for excellent histology visualization and is technically easy to cut. Decreased tissue thicknesses are technically difficult to cut an intact tissue section, whereas increased tissue thicknesses increase tissue opacity and the possibility of microdissecting contaminating cells. Frozen tissues most often can be sectioned onto non-charged slides; however, small tissue samples tend to fall off the slides while undergoing staining or microdissection; therefore, it is recommended that small tissue be sectioned onto charged slides.
! CAUTION Tissue blocks preserve biomolecules better than tissue sections. Do not store tissue sections for more than 2 weeks after cutting from the tissue block before microdissection (**Fig. 1e**). Longer storage increases biomolecule deterioration and makes the tissue unsuitable for the downstream RNA analysis.

**4|** H&E stain and coverslip tissue sections 1, 5 and 10 (**Fig. 1f** and **Box 1**).

**5|** Obtain histopathology consult to review tissues and select the cells of interest for microdissection procurement (**Box 2**).

### Tissue section staining and microdissection ● TIMING 30 min

This reflects the maximum time for staining and microdissection of a given slide. The number of slides that can be microdissected in this time is dependent on the ease of recognition of cells to be dissected and the number of cells present on the tissue section.

**6|** Stain the tissue section for microdissection (**Box 3**).

**7|** Microdissect the cells of interest (**Box 4**, **Fig. 2**).
▲ CRITICAL STEP The first step in normalization is to ensure that the same number of shots (assuming similar cellular density) are microdissected for both the cells of interest (e.g., diseased epithelium) and the calibrator cells (e.g., normal epithelium)[16]. It is recommended to aim for about 10,000 cells per 3,000 shots for each of the tissue samples to be procured by microdissection if there is no previous experience with RNA quantity and quality of this particular tissue type/cell type to be microdissected. If there is no previous experience, perform a test microdissection followed by RNA quantity and quality assessment to determine the number of cells per number of laser shots that will be required for the study tissues.
? TROUBLESHOOTING

**8|** Place the LCM cap on dry ice and proceed directly to pre-RNA extraction; prepare LCM negative control or dissection of another area of the tissue with another cap.
▲ CRITICAL STEP If more than one cap needs to be used to procure the necessary number of cells for a single cell type sample (e.g., 20,000 shots for prostate stroma), pre-RNA extraction should begin for the first cap (cap A) before moving to microdissection

## BOX 3 | FROZEN TISSUE SECTION STAINING FOR MICRODISSECTION

To improve cell adhesion to the LCM cap, create a 'rougher' tissue surface before staining by melting the frozen tissue section. This is achieved by placing the underside of the slide on the back of the hand for ~30 s after the slide containing the tissue section is removed from the freezer. Times in parentheses should be used in laboratories located in humid environments. In addition, the longer times in absolute ethanol and xylene are recommended, even in semi-arid environments, to achieve strong dehydration to facilitate lifting cells from the glass slide.
**! CAUTION** In our experience, the times in ethanol and xylene baths do not affect the quality of RNA. However, always use RNase-free water for the staining baths and always use fresh solutions and clean jars.

Prepare individual staining dishes with the following solutions and treat slides for the described durations. All solution preparation and staining are conducted at room temperature. After each incubation, slides should be briefly drained and then moved to the next solution:
1. 70% Ethanol, 15 s (30 s)
2. Deionized $H_2O$, 15 s
3. Mayer's hematoxylin, 30 s
4. Deionized $H_2O$, 15 s
5. Scott's Bluing, 15 s
6. 70% Ethanol, 15 s
7. Eosin Y, 2–5 s
8. 95% Ethanol, 15 s (30–60 s)
9. 95% Ethanol, 15 s (30–60 s)
10. 100% Ethanol, 15 s (30 s–2 min)
11. 100% Ethanol, 15 s (30 s–2 min)
12. Xylene, 60 s
13. Xylene, 60 s
14. Xylene, 60 s
15. Completely remove xylene from tissue by air-drying for approximately ~2 min (5 min).
**! CAUTION** The use of an air gun is not recommended as it may remove tissue.
16. Stained tissue section is now ready for microdissection.
**! CAUTION** No more than 30 min should elapse from staining until tissue is microdissected and placed into the lysis buffer.

with the second cap (cap B). Begin microdissection using cap B while cap A is heat-incubated (see Step 12). An LCM negative control is prepared for each tissue section by making 3,000 laser shots in a tissue-free portion of the tissue section. This sample is processed throughout the rest of the protocol in parallel with microdissected tissue samples to control for contaminating RNA.
**! CAUTION** If time is running out (i.e., time elapsed from the beginning of staining to the end of microdissection for a single slide is quickly approaching the 30 min time limit), it is best to place cap A on dry ice while proceeding to microdissection with

## BOX 4 | FROZEN TISSUE MICRODISSECTION METHOD

Appropriate frozen tissue processing, sectioning and staining for microdissection (**Box 1**) should be observed. Histopathology consultation should be used to ensure that the cells of interest are the only cells procured (**Box 2**). If in doubt, do not procure those cells.

Microdissection
1. Keep the glass slide with the stained tissue section on the laser microscope stage.
2. Keep a LCM cap (cap) onto the tissue. Use microscopic visualization (lowest magnification) to guide the cap placement onto the tissue.
3. Take the road map image. This is done initially while visualizing the tissue section under the microscope.
4. Before beginning microdissection, focus the laser (infrared or UV) and set up the appropriate laser parameters (power, duration of the pulse). Test the laser in a free space within the cap area, with no tissue, to evaluate the laser parameters.
5. Fire the laser over the cells to be dissected until the desired number of laser shots has been taken.
6. Take a pre-dissection image. This is done before lifting the tissue.
7. Lift the cap up from the tissue to remove the laser-targeted cells of interest.
8. Take a post-dissection image. This is done after lifting the cells of interest from the remaining tissue section.
9. Take a cap image. This is done to visualize the dissected cells.
10. Check the efficiency of tissue lifting onto the cap under a light microscope. Record the percentage of lifting by noting the overall average percentage of the laser spots occupied by dissected cells. In addition, the presence of any contaminating cells should be noted.
11. If contaminating cells are present on the cap, clean the cap with a sterile adhesive paper (e.g., Post-it Note, 3M). This is accomplished by gently placing the cap on top of the sticky border of the paper a couple of times.
12. Re-examine the cap by light microscopy. If contaminating cells are still present, repeat Steps 10 and 11 until no contaminating cells are present.
13. Place the cap onto a microcentrifuge tube containing lysis buffer. Lysis buffer is provided in the RNA extraction kit.

cap B. In this instance, pre-RNA extraction batch processing can be carried out once all caps containing microdissected cells for a single tissue section have been collected.

**Pre-RNA extraction ● TIMING 1 h 40 min**

**9|** Add 50 µl extraction buffer into labeled microfuge tube.

**10|** Place the cap containing microdissected cells onto microcentrifuge tube containing lysis buffer. Lysis buffer and all reagents and consumables for RNA extraction and isolation are found in the PicoPure RNA Isolation Kit (Arcturus/Molecular Devices).
▲ **CRITICAL STEP** If multiple caps (e.g., cap A, cap B and cap C) containing microdissected cells were generated for one tissue cell type sample, the caps can be processed sequentially in the single volume of extraction buffer[36]. For cap A, follow Steps 10 through 14. Discard cap A and transfer the lysate to a clean labeled microfuge tube. Repeat Steps 10 through 15 for additional caps.

**11|** Invert a tube and flick it to cause the buffer to cover the cap.

**12|** Heat at 42 °C for 30 min.

**13|** Mix by pulse vortex.

**14|** Centrifuge for 2 min at 800*g* at room temperature.

**15|** Discard the cap and close the microfuge tube containing cell extract.
▲ **CRITICAL STEP** If multiple pre-RNA extraction cell extracts for one tissue cell type sample were batch processed from multiple caps (e.g., cap A, cap B and cap C), instead of sequential processing as described in Step 10, pool all cell extracts into a clean labeled microfuge tube and record total volume of pooled cell extracts. This volume will be in multiples of 50 µl depending on the number of single cap-derived cell extracts that were pooled (e.g., 3 caps = 150 µl total cell extract volume).

**16|** Keep in a −80 °C freezer for 1 h or until ready to extract RNA. It is recommended to incorporate this step for all microdissected tissue samples for RNA extraction and subsequent transcript analysis. This ensures equal treatment of all tissue samples to be analyzed and reduces bias that may be incorporated by not all microdissected tissues being subjected to −80 °C freezing.
■ **PAUSE POINT** Samples may be stored at −80 °C at this point until ready to extract RNA.

**RNA extraction ● TIMING 45 min**

**17|** Remove microfuge tubes containing cell extract from the pre-RNA extraction step from the −80 °C freezer.

**18|** Place in wet ice to transfer to an RNA clean bench for RNA extraction.

**19|** Pre-condition the RNA purification columns: add 250 µl of conditioning buffer to the RNA purification columns, incubate at room temperature for 5 min, and then centrifuge the columns at 16,000*g* for 1 min at room temperature.

**20|** Add 50 µl of 70% ethanol to the microfuge tube containing the cell extract from pre-RNA extraction. This results in a 1:1 ratio of 70% ethanol:cell extract.
▲ **CRITICAL STEP** If multiple pre-RNA extraction cell extracts for one tissue cell type sample were batch processed from multiple caps (e.g., cap A, cap B and cap C), as described in Step 17, use the recorded pooled cell extract volume to determine the necessary volume of 70% ethanol (e.g., 150 µl pooled cell extract requires 150 µl of 70% ethanol).

**21|** Add the cell extract and ethanol mixture to the RNA purification columns.
▲ **CRITICAL STEP** There is no limit to the amount of RNA the column can handle. However, the column can only handle 200 µl of ethanol:cell extract at one time. Therefore, for volumes >200 µl, repeat Steps 21 and 22 using sequential aliquots of the ethanol:cell extract that are ≤200 µl until all the ethanol:cell extract has been loaded to the column and spun down.

**22|** Centrifuge at 100*g* for 2 min at room temperature. Flow-through collection tube is large enough to collect all flow-through between Steps 22 and 33, and between Steps 35 and 38. Therefore, it is not necessary to discard flow-through between these steps. If processing an ethanol:cell extract volume >200 µl, discard flow-through after the final time repeating Step 23.

**23|** Centrifuge at 16,000*g* for 30 s at room temperature.

**24|** Add 100 µl of wash buffer 1 (W1) to the columns.

**25|** Centrifuge at 8,000*g* for 1 min at room temperature.

**26|** Add 40 µl of DNAse solution (35 µl RDD buffer and 5 µl DNAse) to the columns.

**27|** Incubate at room temperature for 15 min.

**28|** Add 40 µl of W1 to the columns.

**29|** Centrifuge at 8,000*g* for 15 s at room temperature.

**30|** Add 100 µl wash buffer 2 (W2) to the columns.

**31|** Centrifuge at 8,000*g* for 1 min at room temperature.

**32|** Add another 100 µl of W2 to the columns.

**33|** Centrifuge at 16,000*g* for 2 min at room temperature.

**34|** Transfer each of the columns to a labeled microfuge tube.

**35|** Add 10 µl of elution buffer to the columns.
▲ **CRITICAL STEP** To improve RNA elution, it is recommended to elute the RNA in two 10-µl volumes instead of using one 20-µl volume. Depending on experimental tissues being used, an investigator may choose to elute in one 20-µl volume and still recover adequate quantities of total RNA.

**36|** Centrifuge at 1,000*g* for 1 min at room temperature.

**37|** Centrifuge at 16,000*g* for 1 min at room temperature to elute the RNA.

**38|** Repeat Steps 35 through 37.

**39|** Remove the column from the labeled microfuge tube. The elution buffer containing the total RNA sample will be collected at the bottom of the microfuge tube.

**40|** Save the column and flow-through until after RNA quantitation analysis.

**41|** Aliquot each total RNA sample as follows for quantitation, qualitation and qRT-PCR analysis (**Fig. 3**). Pipette 3 µl of total RNA sample into a microfuge tube for quantitation and qualitation analysis. Aliquot the remaining total RNA into 4 µl volumes by pipetting into labeled microfuge tubes for qRT-PCR analysis.

**42|** As total RNA samples are aliquoted, place on either dry or wet ice depending on downstream analysis. If time permits, it is recommended to immediately proceed to RNA quantitation and qualitation at this point using the 3-µl aliquot intended for quantitation and qualitation analysis; therefore, place these samples on wet ice. If total RNA measurement cannot be conducted promptly, place samples on dry ice and move to the −80 °C freezer until ready to use.
▲ **CRITICAL STEP** Place total RNA aliquots intended for qRT-PCR analysis in the −80 °C freezer for 1 h or until ready to run qRT-PCR. It is recommended to incorporate this step for all total RNA samples for qRT-PCR analysis. This ensures equal treatment of all tissue samples to be analyzed and reduces bias that may be incorporated by not subjecting all RNA samples to −80 °C freezing.
■ **PAUSE POINT** Samples may be stored at −80 °C at this point until ready for qRT-PCR analysis.

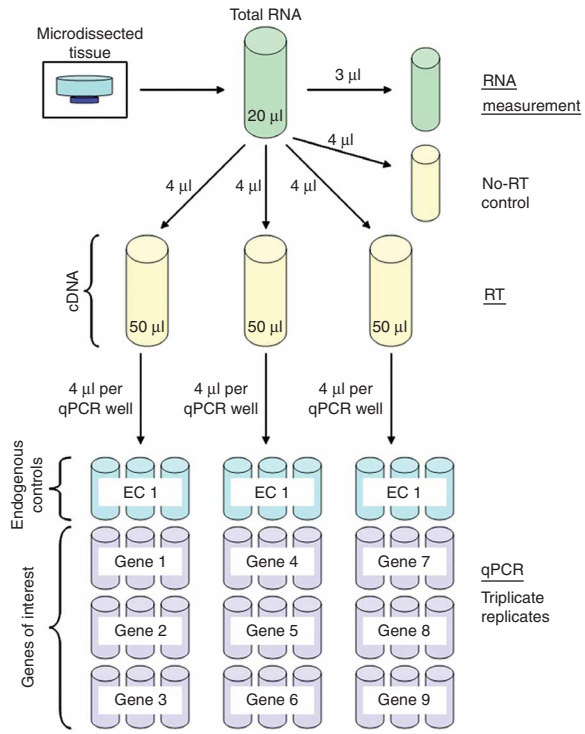


**Figure 3** | qRT-PCR from microdissected tissue process flowchart. Beginning with a typical dissection of ~10,000 cells (3,000 laser shots), total RNA is extracted and isolated. This process yields ~160 ng of total RNA in a volume of 20 µl. The total RNA is aliquoted into four 4-µl volumes and one 3-µl volume. The 3-µl volume is used for RNA measurement. The remaining aliquots are used for qRT-PCR with one aliquot used as the no-RT control and the remaining three aliquots are used in RT to generate three 50-µl volumes of cDNA. Each of these 50-µl volumes is used for qPCR triplicate replicate analysis of three genes of interest and one endogenous control housekeeping gene. The genes of interest may be different between the three subsamples, but the same endogenous control housekeeping gene must be analyzed from each of the RT tubes.

**Preparation of positive control total RNA dilutions ● TIMING 20 min**

**43|** Prepare dilution series of commercially available total RNA sample covering the expected ranges of total RNA concentrations recovered from microdissected tissue samples, as follows. These will be used as positive controls throughout total RNA measurement, primer/probe amplification efficiency assay and qRT-PCR analysis of samples from microdissected tissues. All dilutions, other than 1:1, are prepared in RNase/DNase-free microcentrifuge tubes using RNase/DNase-free molecular-grade water ($H_2O$).

| Dilution | Total RNA concentration (ng $\mu l^{-1}$) | Recipe |
|---|---|---|
| 1:1 | 1,000 | 100 µl of 1,000 ng $\mu l^{-1}$ of total RNA |
| 1:5 | 200 | 20 µl of 1:1 in 80 µl of $H_2O$ |
| 1:10 | 100 | 10 µl of 1:1 in 90 µl of $H_2O$ |
| 1:20 | 50 | 5 µl of 1:1 in 95 µl of $H_2O$ |
| 1:50 | 20 | 10 µl of 1:5 in 90 µl of $H_2O$ |
| 1:100 | 10 | 10 µl of 1:10 in 90 µl of $H_2O$ |
| 1:200 | 5 | 10 µl of 1:20 in 90 µl of $H_2O$ |
| 1:500 | 2 | 10 µl of 1:50 in 90 µl of $H_2O$ |

**44|** Pulse vortex and pulse centrifuge all dilutions at room temperature and place on wet ice.

**45|** Aliquot 3 µl of diluted samples to use as a positive control total RNA measurement and place on wet ice. As samples are aliquoted, place on either dry or wet ice depending on time constraints. If time permits, it is recommended to immediately proceed to RNA quantitation and qualitation at this point using the 3-µl aliquot intended for quantitation and qualitation analysis; therefore, place these positive control dilution samples on wet ice. If total RNA measurement cannot be carried out promptly, place positive control dilution samples on dry ice and move to the −80 °C freezer until ready to use.

**46|** Prepare 20-µl aliquots of diluted samples for future use in total RNA measurements and qRT-PCR analysis. Place positive control dilution samples on dry ice and move to the −80 °C freezer until ready to use.

■ **PAUSE POINT** Samples may be stored at −80 °C at this point until ready for downstream analysis.

**47|** Aliquot 4 µl of commercially available RNase/DNase-free molecular grade water to use as a negative control and for blanking the system.

▲ **CRITICAL STEP** Use water that does not have diethyl pyrocarbonate (DEPC) or any other chemical compound in it. The quantitation method is spectrophotometry-based; therefore, any additional compound that may be in the water used as a blank or negative control will confound the data and will not give accurate representation of the total RNA concentration found within the sample aliquots.

**Total RNA quantity analysis ● TIMING 2 min per sample**

**48|** Place 3-µl aliquots of all controls (see Step 45) and total RNA samples from the microdissected tissue samples (from Step 42) on wet ice. Mix the controls and samples by vortexing and spin down by centrifugation. Place the tubes back on wet ice.

**49|** Use 1 µl of aliquot from Step 48 to follow NanoDrop manufacturer's protocol for RNA quantitation.

▲ **CRITICAL STEP** To prevent contamination carryover and ensure accurate data analysis, analyze samples and controls in the following order: (1) negative control water, (2) all experimental total RNA samples from microdissected tissues, (3) positive control RNA samples and (4) negative control water. The use of the negative control water at the end of quantitation analysis to determine whether residual total RNA may be present in the NanoDrop equipment. If negative control water analysis indicates the presence of total RNA, clean the analysis equipment and re-measure total RNA quantities of all samples and controls.

**? TROUBLESHOOTING**

**50|** Place remaining 2 µl of RNA sample back on wet ice after quantitation analysis.

**Total RNA integrity quality analysis ● TIMING 50 min per chip plus an initial 30 min to bring kit reagents to room temperature**

**51|** Decide which total RNA qualitation assay to use (i.e., BioAnalyzer RNA 6000 Pico Assay or RNA 6000 Nano Assay) for each individual sample, depending on sample total RNA concentrations (ng $\mu l^{-1}$) determined in Step 49.

**52|** Bring appropriate kit reagents to room temperature in the dark for 30 min.

**53|** Use the remaining 2 µl aliquot (from Step 50) that is on wet ice for quality analysis following the manufacturer's protocol, with the following modification: After preparing the gel–dye mix, incubate all samples and controls and ladder at 70 °C for 2 min. Then quench on ice for 2 min, vortex and spin down. Load the sample within 10 min. Continue with manufacturer's protocol until the completion of quantitation analysis.

▲ **CRITICAL STEP** Owing to potential evaporation during the sample denaturation, it is recommended to use the remaining 2 µl of total RNA aliquot for heat incubation. This ensures that a full 1 µl is available to meet sample requirement volume for qualitation

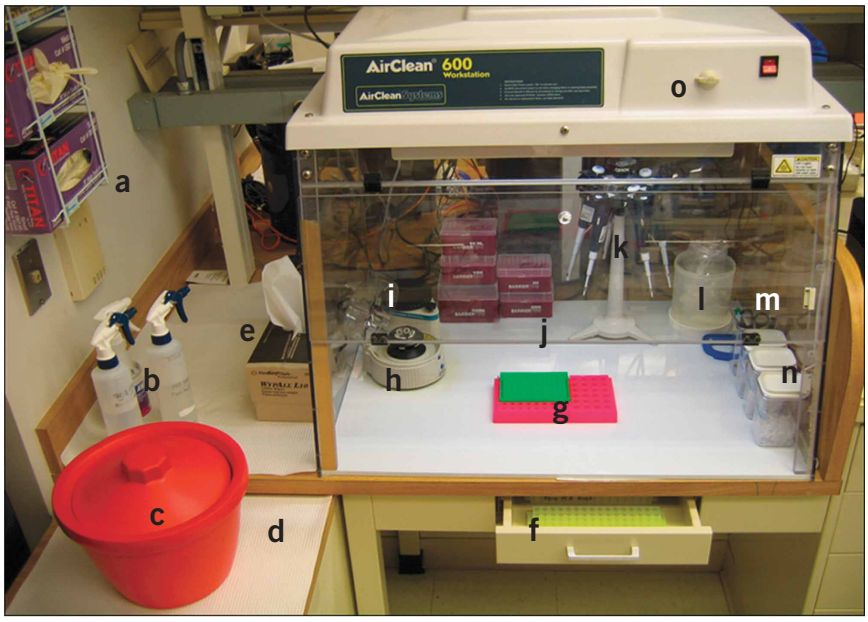


**Figure 4 |** Dedicated RT-PCR hood setup. The clean dead-air hood with UV sterilization capability used for setting up RT and PCR reactions is imperative in preventing contamination. All necessary equipment should be dedicated and remain beside or in the hood at all times, depending on the specific use. Dedicated equipment and consumables presented in this figure outside the hood remains outside the hood. This includes (**a**) gloves, (**b**) 10% bleach, 70% ethanol and RNase Away solutions, (**c**) ice bucket, (**d**) blotter paper, (**e**) paper towels and (**f**) tube racks in the drawer that provides a dark environment for thawing out light-sensitive reagents. The dedicated equipment and consumables presented inside the hood remain inside the hood at all times except when adding sterile pipette tip boxes, tubes and clean waste bags or removing empty pipette tip boxes or full waste bags. This includes (**g**) tube and plate racks, (**h**) mini-centrifuge, (**i**) vortexer, (**j**) pipette tips, (**k**), pipettes, (**l**) waste receptacle with bag inside, (**m**) pens, scissors and tube cap sealing tool and (**n**) tube containers with sterile tubes. The UV bulb is located in the top of the dead-air hood and the timer is located outside the hood (**o**). cDNA tubes (after RT) should be quenched on ice in the dedicated ice bucket. The tubes containing reagents or cDNA may be brought into the hood for use and removed after use. After each use and between RT and PCR, the hood should be cleaned and UV-sterilized to prevent contamination.

analysis. Accurate pipetting is critical, as only 1 µl of the sample is loaded into the chip wells. During chip and sample loading process, ensure that no air bubbles are formed as this may confound qualitation analysis.

**54|** Review data for each sample and controls. Assess ladder, peaks, electropherograms and RINs. RNA quality of RIN $>5$ is considered good quality total RNA and RIN $>8$ is considered excellent total RNA quality[50].

■ **PAUSE POINT** All sample aliquots (see Steps 42 and 45) are stored at −80 °C and do not need to be removed until ready for downstream analysis.

**? TROUBLESHOOTING**

**Primer/probe-specific qPCR assay efficiency determination ● TIMING 3.5 h**

**55|** Test assay efficiency, sensitivity and reproducibility of all primer/probe sets using the positive control total RNA dilution series prepared in Step 43. The qRT-PCR assay will be carried out in a two-step reaction; therefore, specific primer/probe qPCR test assays will be conducted using the same parameters as the experimental assay parameters for template, RT and qPCR reagent concentrations. See Steps 57–70 for experimental protocol for RT and qPCR. For one RT tube of each positive control dilution series sample, 12 qPCR assays will be able to be carried out. Using three qPCR technical replicates per cDNA sample and primer/probe set, four primer/probe sets can be analyzed. Usual convention is to test three genes of interest and one endogenous control primer/probe set per RT tube-generated cDNA sample.

▲ **CRITICAL STEP** All qRT-PCR setup should be carried out in a dedicated PCR hood providing a clean controlled environment (**Fig. 4**; **Box 5**).

**56|** Analyze each qPCR assay individually by evaluating efficiencies and standard curves. The detection limits will be determined by the lowest dilution sample concentration that generates repeatable $C_T$ values within the three technical replicates. A review of qPCR efficiencies and standard curves has been discussed in a previous issue of *Nature Protocols*[48].

**qRT-PCR assay; RT ● TIMING 1.5–2 h**

**57|** Prepare cDNA by RT of all individual total RNA samples and controls (NTC, no-RT and (+) C) using TaqMan RT Reagents (Applied Biosystems, cat. no. N808-0234), with random hexamers as the RT primers. Random hexamers have been shown to minimize the potential effects of starting template degradation and to improve the qPCR analysis of short amplicons ($<200$ bp).

▲ **CRITICAL STEP** All qRT-PCR setup should be carried out in a dedicated PCR hood providing a clean controlled environment (**Fig. 4**; **Box 5**).

▲ **CRITICAL STEP** Before planning and running the RT, plan the qPCR plate setup and determine the number of qPCRs (**Fig. 5**) that will be run from each of the samples and controls to determine the number of RT reactions that will be needed.

## BOX 5 | CLEANING PROCEDURES FOR MAINTENANCE OF THE RT-PCR PREPARATION HOOD

To maintain a clean environment for qRT-PCR setup, it is imperative to use a dedicated dead-air hood and dedicated equipments such as pipettes, pipette tips, tubes, racks, vortexer and mini-centrifuge. PCR hood and/or reagent/template contamination may adversely affect the results of the qRT-PCR experiment. This contamination potential can be negated by optimal hood and equipment cleaning as described below.

- Regular cleaning should take place before and after each experiment is completed.
- Weekly cleaning and maintenance should also take place.
- Additional barrier tips, caps, tubes, extra trash bags bottles of 10% bleach, 70% ethyl alcohol, RNase Away, and paper towels and gloves should be located within reach of the hood.
- Any time one goes in and out of the PCR hood, gloves must be changed.
- While working in the PCR hood, it is necessary to wear the designated clean PCR hood lab coat with elastic cuffs.
- For UV light use, make sure all pipette tips are unstacked and the centrifuge lid is open. This allows UV to reach all areas of potential contamination during the UV sterilization process.
- Fresh 10% bleach solution should be prepared for each weekly cleaning.
- As supplies dwindle in the PCR hood, they should be replaced as soon as possible.

Regular cleaning

1. Use in succession: 10% bleach followed by 70% ethanol on the inside of the hood to
   a. wipe all sides and back of the hood;
   b. wipe bottom of the hood;
   c. wipe outside of all containers;
   d. wipe around both centrifuge and vortex;
   e. wipe outsides of pipette tip boxes;
   f. wipe down pipettes;
   g. wipe sides of the hood;
   h. wipe all parts of the pull-up access panel on both front and back; and
   i. wipe and dry PCR-only ice bucket.
2. Turn UV light switch all the way to start.
3. Replace plastic-backed paper mats ('diapers') on the outside counter of the PCR hood as needed.

Weekly maintenance

In addition to regular single use cleaning, perform the following each week:

1. Follow all bleach and ethanol steps using RNase Away.
2. Replace plastic-backed paper mats on outside counter of the PCR hood. Tape down mats to counter area to prevent it from sliding.
3. Mark mats with 'FOR PCR ONLY' to remind others to respect the integrity of the PCR clean area.
4. Compile list of consumables that are running low/need to be ordered (tips, tubes, caps, plates and plastic bags). Keep two boxes minimum of the consumables at all times. When the second to last box of consumables is opened, an order should be placed.
5. Record date weekly maintenance was completed by writing on the tape and placing on top of the hood.

UV light

The UV integrity is good for 1,000 h per bulb. One hour is clocked/used every time the light is turned on, even if the light is on for only 5 min.

1. Change UV light every 6 months to a year, depending on use. If heavy use of UV, record each time UV is turned on and change when 1,000 uses has occurred.
2. Update the tape on the hood with the date UV light has been changed.

**58|** In a 1.7- or 2-ml microfuge tube, prepare a master mix of the following RT reaction components multiplied by the number of reactions plus one (for pipetting loss) as outlined in the table below. Vortex and spin down all RT master mix components and templates before use.

▲ **CRITICAL STEP** Prepare no-RT master mix for each of the samples. Do not add RT to the RT master mix, instead add 1.25 µl additional $H_2O$ to the master mix. Prepare one additional RT reaction with (+) C to be analyzed with the no-RT controls.

| Reagent | Volume to add per reaction (µl) |
|---|---|
| 10× Buffer | 5.00 |
| $MgCl_2$ | 11.00 |
| 10 mM dNTP | 10.00 |
| RNase Inhibitor | 1.00 |
| Random Hexamers | 2.50 |
| Multiscribe RT Enzyme | 1.25 |
| $H_2O$ | 15.25 |

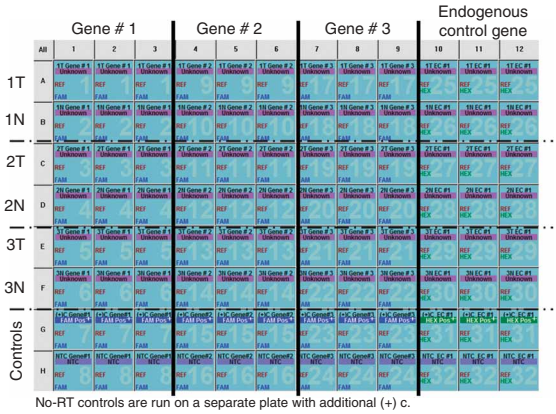


**Figure 5 |** Example qPCR 96-well setup. Using this layout, three genes of interest and one endogenous control housekeeping gene can be analyzed by qPCR from six samples and appropriate control cDNAs (one RT tube per template). This setup may be used to analyze additional genes of interest from the microdissected sample using the same endogenous control housekeeping gene. Example cDNA samples (e.g., 1N, 1T, 2N, 2T, 3N and 3T) are from three cases of matched tumor (T) and normal (N) tissues. To prevent the introduction of bias, it is important to process both diseased and normal tissue samples at the same time. Plate 2 would include genes of interest 4–6. Plate 3 would include genes of interest 6–9. All no-RT controls are run on a separate plate using (+) C and NTCs.

**59|** Vortex and spin down the master mix.

**60|** Add 46 µl of RT master mix to labeled PCR tubes.

**61|** Add 4 µl $H_2O$ to the NTC RT reaction tube. Add 4 µl of template total RNA per RT reaction tube and no-RT control reaction tube. Add 4 µl (+) C total RNA to the positive control RT reaction tube. The final reaction volume will be 50 µl. Vortex and spin down, avoiding bubbles.

**62|** Run all RT reactions in a PCR thermocycler using the following protocol as suggested by the manufacturer (Applied Biosystems):

| Cycles | Temperature (°C) | Time (min) |
|---|---|---|
| 1 | 25 | 10 |
| | 48 | 30 |
| | 95 | 5 |

**63|** Immediately place the samples on ice and spin down after 2 min.

**64|** Use RT product cDNA immediately in qPCRs or store at −20 °C.
▲ **CRITICAL STEP** If analyzing nine genes of interest from one microdissected sample, prepare three RT reactions from each of the microdissected RNA samples (**Fig. 3**). The first of three cDNA samples will be used in the first qPCR run. Place the other two cDNA samples on wet ice until use. All three qPCR runs should be carried out consecutively on the same day as RT reaction. After running all three qPCR plates, analyze all no-RT controls and the additional (+) C RT cDNA in an additional qPCR plate.
■ **PAUSE POINT** If this cannot be carried out, store the remaining cDNA samples at −80 °C until use.

**qRT-PCR assay; qPCR ● TIMING 3 h per 96-well plate (1 h for setup, 2 h for qPCR)**

**65|** Conduct single-plex qPCR analysis of all individual cDNA samples and controls (NTC, no-RT and (+) C) generated from the above RT using 2× TaqMan Universal Master Mix and AmpliTaq Gold, and specific primer/probe sets (Applied Biosystems). For each of the primer/probe sets, prepare a master mix in a 1.7- or 2-ml microfuge tube of the following qPCR components multiplied by the number of reactions plus one (for pipetting loss) as outlined in the table below. Vortex and spin down all cDNA templates and qPCR master mix components, except for the 2× TaqMan Universal PCR Master Mix, before use.
! **CAUTION** The 2× TaqMan Universal Master Mix is viscous and will bubble upon vortexing. Mix gently instead of vortexing.

| Reagent | Volume to add per reaction (µl) |
|---|---|
| 2× TaqMan Universal PCR Master Mix | 25.00 |
| AmpliTaq Gold | 1.00 |
| $H_2O$ | 17.50 |
| FAM or VIC primer/probe set | 2.50 |

**66|** Mix gently and spin down the master mix.
! **CAUTION** Do not vortex the qPCR master mix, as microbubbles will form, interfering with the qPCR optical detection analysis.

**67|** Add 46 µl of the qPCR master mix to the designated wells in a 96-well plate (**Fig. 5**).

**68|** Add 4 µl of $H_2O$ to the NTC qPCR wells. Add 4 µl of template cDNA per RT reaction tube and no-RT control reaction tube. Add 4 µl (+) C of cDNA to the (+) C wells. Pipette up and down, avoiding bubbles, when adding the template to the master mix to mix thoroughly.
! **CAUTION** Do not depress the pipette all the way down, as this may cause bubbles to occur in the well.

## BOX 6 | BIOLOGICAL FOLD-CHANGE EXPRESSION CLASSIFICATIONS

Biological fold change (relative quantitation) of a diseased tissue sample compared with a normal tissue sample can be calculated using the relative quantitation $2^{-\Delta\Delta C_T}$ method[84]. Expression classifications (adapted from Xu *et al.*[78]) can be used to define general thresholds of overexpression, underexpression or no change for a gene of interest.

- Overexpression in disease tissue (D) compared with normal tissue (N) (D > N), defined as
  1+ (1.5- to 5-fold),
  2+ (5.1- to 10-fold),
  3+ (10.1- to 20-fold) and
  4+ (> 20-fold).
- Underexpression in disease tissue (D < N), defined as
  1– (1.5- to 5-fold),
  2– (5.1- to 10-fold),
  3– (10.1- to 20-fold) and
  4– (> 20-fold).
- No change (D = N), refers to the difference of gene expression between D and N as < 1.5-fold.

**69|** Place optical caps or film over the wells of the plate. Mix gently and spin down.

**70|** Run all qPCRs in a qPCR thermocycler using the following protocol:

| Cycles | Temperature (°C) | Time |
|---|---|---|
| 1 | 50 | 2 min |
| 50 | 95 | 15 s |
| | 60 | 1 min |

▲ **CRITICAL STEP** A total of 50 cycles are used for qPCR to ensure that the NTCs for a gene of interest is at least 10 $C_T$ values later than the sample gene of interest expression in cases where low level of gene expression (e.g., 37 $C_T$ values) are encountered. This ensures that if the NTCs indicate 'no $C_T$' at 50 cycles, the qPCR reagents are free of contamination. In addition, the observation of the 10 $C_T$ difference comparing no-RT with RT-positive samples, negligible contamination (0.1%) of gDNA contamination is confirmed[82,83].

■ **PAUSE POINT** Upon qPCR data collection, data are saved at this point, and can be analyzed later.

**? TROUBLESHOOTING**

**Data analysis** ● **TIMING 2.5 h**

**71|** Data from qRT-PCR of microdissected tissue samples are normalized using the endogenous control housekeeping gene expression. Therefore, relative quantitation is the analysis method that must be used. The recommended method for relative quantitation is the $2^{-\Delta\Delta C_T}$ method[84] summarized in the following table. A brief discussion of biological fold-change expression classifications can be found in **Box 6**.

**? TROUBLESHOOTING**

| Steps | Formula | Definitions |
|---|---|---|
| 1. Gene expression normalization within a sample | $\Delta C_T = X - \mathrm{EC}$ | For a given sample (diseased or normal), $X$ is the gene of interest $C_T$ value and EC is the endogenous control $C_T$ value |
| 2. Gene expression comparison | $\Delta\Delta C_T = \Delta C_T x - \Delta C_T \mathrm{cb}$ | For a given case (comparing diseased (D) and normal (N) tissues from the same organism), $x$ is the sample of interest and cb is the calibrator. The calibrator can be D or N sample if different questions are asked |
| 3. Biological fold change | $2^{-\Delta\Delta C_T}$ | Translates the $\Delta\Delta C_T$ value to assign biologic significance in terms of biological fold change. If $\Delta\Delta C_T$ is a positive number after calculating $2^{-\Delta\Delta C_T}$ transform by $1/\times$ to obtain the underexpression value |

● TIMING

Steps 1–5, 1.5–3 h
Steps 6–8, 45–55 min
Steps 9–16, 1 h 40 min
Steps 17–42, 45 min
Steps 43–47, 20 min
Steps 48–50, 2 min per sample
Steps 51–54, 50 min per chip plus an initial 30 min to bring the kit reagents to room temperature
Steps 55 and 56, 3.5 h
Steps 57–64, 1.5–2 h
Steps 65–70, 3 h per 96-well plate
Step 71, 2.5 h

? TROUBLESHOOTING

Troubleshooting qRT-PCR data are a complex process when working with tissue samples. Standard total RNA controls, such as commercially available, high-quality total RNA from tissue, should be used for qRT-PCR, including primer/probe optimization and efficiency assessment, as well as for standard curve generation. It is recommended that the total RNA chosen for this purpose should be from the same organ as the one being analyzed in the qRT-PCR study. However, when working with animal model or clinical tissue samples, RT and qPCR only represent the last two steps of a series of protocols that have been applied sequentially to generate the quantitative gene expression data. Incorporating appropriate controls during each step, these steps leading to qRT-PCR include tissue collection, tissue processing, tissue sectioning and staining, microdissection and RNA extraction and isolation. Assuming proper techniques have been used for collecting the samples (in particular, reasonable reduction of the time from tissue removal to freezing), as well as for adequate snap-freezing techniques, the issue of little or no RNA recovery can still be faced.

In general, sets of tissues from different animals or patients are analyzed in each experiment. Therefore, the situation varies if the negative results are observed in all or just in one or a few samples. In the former case, general explanations are found, such as an improper RNA extraction technique. Investigators can even use high-quality RNA to 're-extract' and make sure its integrity is intact throughout the extraction. It is ideal to use a RNA quality- and quantity-'proved' tissue control side by side with the experimental samples while performing the extraction. This 'proved' tissue serves as a 'tissue extraction positive control.' DNase treatment is one common source for RNA degradation. Another general problem could be improper storing such as freezer malfunctions, or storing the tissues too long in the freezer. It has been observed that after storage for a year, RNA can be compromised even at −80 °C.

If only a few individual samples show poor RNA recovery or higher than expected $C_T$ values are seen, general experimental protocol issues can be ruled out. In this case, the most typical explanation is RNA integrity in the individual sample. This can often be attributed to RNA degradation before freezing or poor tissue processing. In addition, certain organ types may also contain higher level of RNases. Brain tissue inherently contains less RNases than other tissues, with pancreas, spleen and intestinal mucosa having the most[34]. Another step in the protocol to examine is the dissection process. Issues of cell procurement (lifting on the cap) during the laser process as well as cellular density of the dissection area of interest need to be taken into account. In the case of variable cell density, it has been observed that 5–6 times more laser shots are needed to obtain equivalent total RNA yields in the stroma compared with those in the epithelium.

Finally, troubleshooting issues can also arise at the phase of the analysis of the data. This includes, but is not limited to, the use of inappropriate normalization strategies, high variability (i.e., large standard deviations) between qPCR replicates that is sometimes observed in low-expressed transcripts and the appropriate data analysis strategy (e.g., relative quantitation for data obtained from qRT-PCR of microdissected tissues). Furthermore, a key point in the analysis is to define a threshold of what fold change ($2^{-\Delta\Delta C_T}$) is meaningful between samples (e.g., paired diseased and normal tissue samples). The natural trend is to consider a larger fold change as biologically more relevant. However, smaller differences in expression can still be important in disease progression. For instance, the loss of function of just one allele (haploinsufficiency) may be critical in several neoplastic and non-neoplastic processes[85–87].

General qRT-PCR and LCM troubleshooting issues have been addressed earlier in a previous issues of *Nature Protocols*[36,48]. Additional troubleshooting advice specifically related to qRT-PCR of microdissected tissue samples can be found in **Table 2**.

**TABLE 2** | Troubleshooting table.

| Problem | Possible reason | Solution |
|---|---|---|
| Step 7: LCM | | |
| No cells captured in the LCM cap | Tissue section mounted on a charged (plus) slide | Mount the tissue section on uncharged slides for LCM |
| | Tissue section on the slide are not sufficiently dry | Change solutions including ethanol and xylenes. Now increase the times of dehydration in ethanol 95% and 100% and xylene at 2 min for each bath |
| Contamination of the LCM cap with nondissected extra tissue | Tissue section could be too dry | Check the dissected cells in the cap under the microscope. If there is extra tissue, remove it with a sterile adhesive tape or with a similar notepaper. Gently place the cap with the film on the sticky border of the sterile adhesive tape or similar notepaper a couple of times. Eliminate the used sterile adhesive tape or similar notepaper. Now, examine the cap again under the microscope. The extra tissue will be removed from the cap (attached to the sticky paper) while the dissected cells will remain embedded in the film and will not be removed by the sticky paper |
| Steps 49 and 54: RNA quantity and quality | | |
| No quantity or quality data reported by NanoDrop or Bioanalyzer analysis, respectively | Tissue section stored in a freezer too long | Use the tissue section within 2 weeks of sectioning and freezing |
| | RNase-rich tissue (e.g., pancreas, duodenum, colon) | Stain and dissect as quickly as possible. Add RNase inhibitors to staining solutions |
| | Cell not captured by LCM in the cap | Always examine lifting of captured cells on the cap under the microscope after LCM before proceeding to RNA extraction |
| | Nanogram quantity of sample appropriate for the Bioanalyzer chip chosen (Pico vs Nano) | Use the appropriate chip for the quantity of total RNA determined by NanoDrop |
| | Wash RNA through the column during RNA extraction | Pool all flow-through and re-extract all the flow through and recover the RNA |
| | Total RNA not aliquoted appropriately leading to multiple free-thaw degradation | Aliquot total RNA before initial storage at −80 °C |
| | Tissue block assessment by scraping tissue section and performing RNA extraction, and quantity and quality assessments before microdissection was not done | Perform tissue block assessment before microdissection |
| Low RIN number | Tissue or RNA degradation | Continue with qRT-PCR analysis. Use random hexamers in RT step to minimize effects of degradation. Determine acceptable RIN range for the tissue type being used in the study. For prostate, esophagus and urethra, we have found that even low RINs (RIN $>2$) produce specific repeatable qRT-PCR results |
| Steps 70 and 71: qRT-PCR | | |
| No $C_T$ values reported | Poor quality and quantity or total RNA template used for RT | See above RNA Quantity and Quality section |
| | RT failed to generate cDNA | Check positive control. If positive control for a particular primer/probe set did generate $C_T$ values and the endogenous control gene analyzed for the sample generated $C_T$ values, then it can be concluded that cDNA was generated and the sample may not be expressing the transcript of interest within qRT-PCR detectable quantities. If not, repeat RT |
| No $C_T$ values reported in one paired sample and not the other | Only ran qPCR for 40 cycles | Use RT-generated cDNA to run qPCR for 50 cycles |
| | Identical number of LCM shots per number of cells not collected for RNA extraction | Use comparable number of cells per laser shots for RNA extraction and downstream qRT-PCR. If comparable numbers of cells and total RNA were used for downstream qRT-PCR, transcript in one sample (e.g., a diseased sample or a normal sample) may not be expressed within detectable levels. At this point, it is imperative to ascertain that the endogenous control qRT-PCR for that sample did generate a $C_T$ value. If not, see No $C_T$ values reported section above |
| Step 71: Analysis | | |
| Underexpression or downregulation of gene expression unable to be determined | $\Delta\Delta C_T$ value is positive | Multiply positive $\Delta\Delta C_T$ value by $1/\times$. Then perform $2^{-\Delta\Delta C_T}$ fold-change calculation using this number. This fold-change value will represent the underexpression compared to the calibrator |

LCM, laser capture microdissection; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RIN, RNA integrity number; RT, reverse transcription.

## ANTICIPATED RESULTS

**Figure 2** depicts the specific capture of the cells of interest from a heterogeneous cellular environment by LCM using the PixCell II (Arcturus/Molecular Devices).

**Table 1** describes the quantity and quality of RNA yields from typical microdissections from various tissues. Attention to cellular density per laser shot is important to estimate in advance the total RNA recovery (e.g., prostate stroma compared with breast stroma number of laser shots to obtain similar quantities of total RNA).

**Figure 6** presents an example of the amplification plot results from the qRT-PCR analysis of epithelial microdissected prostate tumor and matched normal tissue samples.

A review of an example $C_T$ value ranges that can be anticipated for endogenous control housekeeping genes and the corresponding RNA quantity/quality measurements can be found in Erickson *et al.*'s[16] *Laboratory Investigation* paper. Other examples of $C_T$ value ranges have been published earlier[4,16] and can be reviewed for further information.

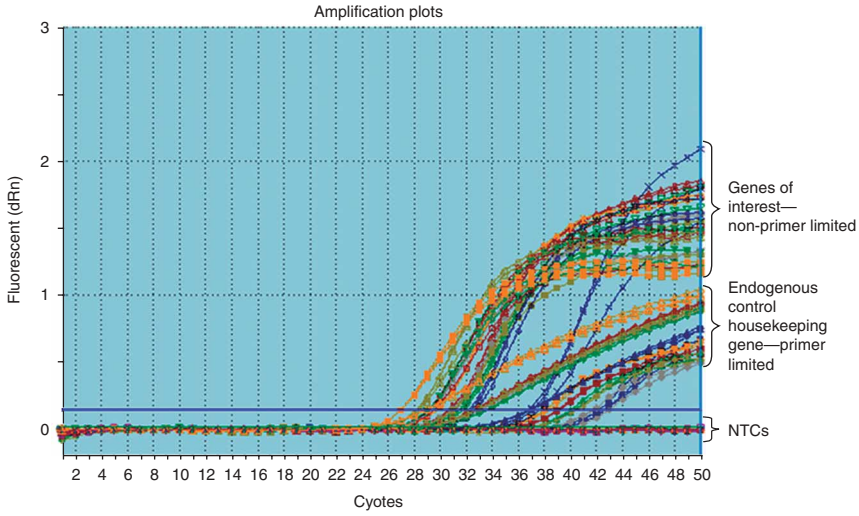


**Figure 6** | Typical TaqMan qPCR amplification plots for 96-well setup. $\Delta R_n$ versus cycles linear view. Primer/probe concentrations are optimized for highest $\Delta R_n$ and lowest $C_T$ values for microdissected tissue sample templates. All samples endogenous control housekeeping gene analysis used VIC/HEX-labeled primer-limited primer/probe sets. Genes of interest were FAM-labeled non-primer limited primer/probe sets. NTCs were all negative as evidenced by no amplification crossing the threshold resulting in no $C_T$ values. Triplicate technical replicates are good with similar amplification efficiencies and standard of pipetting.

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