

Laser Microdissection and RNA Analysis

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Summary

Microdissection techniques have become an important tool to link histomorphology and pathophysiological events using modern methods of molecular biology. They allow isolation of cell clusters or even single cells precisely under optical control from complex tissue structures for further analysis of DNA, RNA, and proteins. In particular, the fragile RNA molecules can be preserved during microdissection so that gene expression and regulation measurement become feasible in a cell type-specific manner within complex tissues. This report focuses on and outlines the procedures for RNA investigation, from tissue fixation, sectioning, and staining to downstream applications (RT-PCR, mRNA quantification, and mRNA preamplification). Standards for the preparation of RNA from frozen and formalin-fixed tissues are presented. Specific protocols are given for both the isolation of RNA from small numbers of cells (50 cells) as well as for larger cell numbers. While most of the procedures are identical for the microdissection systems, special features of each technique are mentioned.

Key Words: Laser microdissection; gene expression, formalin fixation; immunohistochemistry; immunofluorescence; mRNA quantification; real-time PCR; RNA preamplification; LCM.

1. Introduction

The analysis of commonly used tissue homogenates leads inevitably to an average measurement of the biochemical components (such as, nucleic acids and proteins) from various cell types. There is a high risk that changes in mRNA expression of an individual cell type may be masked by the surrounding cells. To overcome tissue heterogeneity, cells and cell types must be isolated selectively for further analysis. Microdissection and micromanipulation techniques were consequently developed (1–3) and, particularly, laser-based

microdissection systems proved to allow simple, rapid and precise retrieval of target cells (4).

When planning a microdissection study from tissue harvesting to the analytical steps the isolation of nucleic acids comprise a number of steps that may cause fragmentation and degradation of RNA. Poor quality of RNA again results in a lower recovery of an already limited amount of input material.

The type of tissue preparation, storage, and fixation have a major impact on analytical success and influence the workup of microdissection studies. First, the time span between onset of ischemia and fixation should be kept as short as possible, as endogenous RNases and ribozymes may be activated or are active for their targets. Next, the fixation of fresh tissues requires several hours, depending on the type of fixative, rate of penetration into the tissue, and size of the tissue sample. This time also influences the molecular quality of the tissue (i.e., due to extensive crosslinking and strand scissions by formaldehyde). On the other hand, persistent formalin fixation for 48 h and more may remarkably change measurement of relative mRNA expression (5). Finally, the embedding procedure may cause an adverse effect on nucleic acids, i.e., high temperature exposure during submersion in melted paraffin.

Snap-freezing of unfixed tissue in liquid nitrogen-cooled isopentane circumvents these problems and results in the best available product for further molecular applications. This advantage, however, is contrasted by a poorer quality in morphology with possible split artifacts and higher costs for tissue storage at -80°C .

Promising trials have involved new strategies for optimal tissue processing, i.e., fixation of small tissue specimens in 70% ethanol followed by embedding in paraffin or low-melting polyester (6). Alternatively, methacarn fixation (60% methanol, 30% chloroform, 10% glacial acetic acid) and paraffin embedding were also shown to yield reasonable quantity and quality of mRNA and protein, significantly superior to that obtained from cross-linking fixatives (7). Low temperature embedding in plastic resin was suggested to obtain a higher degree of integrity of nucleic acids (8), but low-melting polyester also has the disadvantage of drying out and cracking after storage. Finally, there are no data on the integrity of cellular morphology and nucleic acids after long-term storage of tissues processed this way.

In consequence, there is no optimal preparation strategy available. If microdissection is intended, especially with full-length cDNA synthesis, tissue specimens should be frozen airproof at -80°C or in liquid nitrogen. If fixation is desired (because of easier storage and handling), a non-crosslinking fixative should be combined with low-temperature embedding. Only when immunolabeling has to be combined with microdissection should a mild neu-

tral buffered-formalin fixation be considered, accepting a higher degradation rate of nucleic acids. In this case, 4.5% neutral buffered formalin (e.g., Roti-Histofix, Roth, Karlsruhe, Germany) is appropriate. Fixation time of small specimens should not exceed 12 h to limit crosslinking and shearing of nucleic acids.

While the conditions of fresh sample preparation can be controlled, archival tissues are, most often, formalin-fixed and paraffin-embedded (FFPE). Due to easy storage and very good long-term preservation of morphology, such tissue specimens have accumulated over a long time period and represent a valuable resource of tissue. To make these tissues available for molecular approaches, procedures have to be developed to use this type of material. Several reports have adapted existing protocols to the fixation-inherent problems. Due to the crosslinking of all tissue components, proteinase K digestion was shown to be indispensable for releasing nucleic acids from the cells.

2. Materials

2.1. Sectioning

1. Cryotome for frozen tissues, e.g., CM 3000 (Leica, Bensheim, Germany) with feather microtome blade (PFM, Cologne, Germany).
2. Microtome for FFPE tissues, e.g., Jung SM 2000C (Leica).
3. Tissue Tek[®] O.C.T.[™] Compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands).
4. Parafilm[™]M[™] laboratory film (American National Can[™], Chicago, IL).
5. Glass slides, e.g., Superfrost[®] Plus (Menzel-Gläser, Braunschweig, Germany).
6. Poly-L-lysine and/or 3-aminopropyltriethoxysilane (APES).

2.2. Routine Histological Staining

If not mentioned otherwise, reagents are purchased from Sigma Aldrich, in “molecular biology” quality.

1. Hematoxylin, e.g., Mayer’s acidic hematoxylin (Division Chroma, Münster, Germany).
2. Xylene.
3. Ethanol, 70%, 90%, 100%.
4. RNase-free H₂O (DEPC-treated).

2.3. Immunofluorescence/Immunohistochemistry

2.3.1. Immunofluorescence

1. Primary antibody.
2. Secondary antibody, coupled with fluorophore (e.g., FITC).
3. Tris-buffered saline (TBS): 50 mM Tris-HCl, 150 mM NaCl, pH 7.5.

2.3.2. Immunohistochemistry

1. Primary antibody.
2. Secondary “link” antibody, e.g., mouse immunoglobulins 1:40 (Dako Diagnostica, Hamburg, Germany).
3. Enzyme-carrying antibody, e.g., APAAP Mouse Monoclonal 1:50 (Dako).
4. Antibody Diluent Chem Mate™ (Dako).
5. Staining complex, e.g., new fuchsin (Division Chroma), naphthol, levamisole in TBS, pH 8.8 (300 mM Tris-HCl, 750 mM NaCl).
6. TBS : 50 mM Tris-HCl, 150 mM NaCl, pH 7.5
7. Reaction buffer for 50 cells: 52 mM Tris-HCl, pH 8.3, 78 mM KCl, 3.1 mM MgCl₂.

2.4. RNA Extraction

1. RNA lysis buffer I: 4 M GTC, 25 mM sodium citrate, 0.5% sarcosyl, 0.72% 2-mercaptoethanol, 100 mM Tris-HCl, pH 7.5 (add 2-mercaptoethanol immediately before use).
2. RNA lysis buffer II: 200 μL 1 M GTC, 0.5% sarcosyl, 0.72% 2-mercaptoethanol, 20 mM Tris-HCl, pH 7.5 (add 2-mercaptoethanol immediately before use).
3. RNA lysis buffer III: 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0), 2% sodium dodecyl sulfate (pH 7.3).
4. Water saturated phenol, pH 4.3 (light-sensitive, toxic, at 4°C).
5. Chloroform.
6. Isoamylalcohol.
7. Isopropanol.
8. Ethanol 75%.
9. Rnase-free H₂O (DEPC treated).
10. Proteinase K (aliquots at -20°C).
11. Glycogen 20 mg/mL (e.g., Roche Diagnostics, Mannheim, Germany).
12. RNase-free DNase I (e.g., Ambion, Austin, TX) (at -20°C).

2.5. Reverse Transcription

Purchased from Applied Biosystems, Foster City, CA; stored at -20°C.

1. GeneAmp® 10X PCR Buffer II
2. 25 mM MgCl₂.
3. 50 μM Random hexamers .
4. 20 U/μL RNase-inhibitor.
5. 50 U/μL MMLV-reverse transcriptase.
6. dNTPs (Eurobio, Raunheim, Germany).

2.6. Qualitative/Quantitative PCR

Purchased from Applied Biosystems; stored at -20°C.

1. GeneAmp® 10X PCR Gold Buffer II.
2. 25 mM MgCl₂.

3. dNTPs.
4. Oligonucleotide primers, probe.
5. AmpliTaq[®] Gold 5 U/mL.
6. Polymerase chain reaction (PCR) cycler, e.g., GeneAmp[™] 2400 PCR cycler (Applied Biosystems).

2.7. RNA Preamplification

1. Smart[™] PCR cDNA Synthesis Kit (BD Clontech, Palo Alto, CA; stored at -20°C).
2. Advantage[®] 2-PCR Kit (BD Clontech; stored at -20°C).
3. QIAquick[®] PCR Purification Kit (Qiagen, Hilden, Germany).

3. Methods

The methods described here outline the sectioning, routine staining, immunospecific labeling, and RNA preparation from (1) frozen tissue specimens and (2) FFPE tissue specimens. Depending on the introduced cell amount, specific protocols are provided in both cases for few-cell approaches as well as for higher cell scales. For microdissection the major techniques are (1) laser capture microdissection (LCM), (2) laser microdissection and micromanipulation (LMM), and (3) laser pressure catapulting (LPC). Details are given in **Subheading 3.1.3**.

While special features of each system are mentioned, most procedures can be used with any system. Afterwards, reverse transcription (RT) and PCR are described for the different quantities of cells. Furthermore, a reliable protocol for RNA preamplification is given that increases the initial amount of cDNA from microdissected cells. This allows sufficient material to be obtained for cDNA array and virtual Northern blot hybridization.

3.1. Frozen Tissue

3.1.1. Sample Preparation, Sectioning, and Routine Staining

1. Fresh tissue samples are frozen in liquid nitrogen-cooled isopentane and stored at -80°C . To keep them airproof, the specimens are wrapped in parafilm[®] and placed in suitable cryo-tubes. Tissue fragments 125 mm^3 are prepared in the cryotome at -20°C to avoid thawing of the total specimen (*see Note 1*).
2. For slide preparation new disposable knives are used, while all other contact areas are cleaned with 70% ethanol followed by 0.1 M NaOH. Cryosections are cut at maximal thickness (5–10 μm), allowing a sufficient precise microscopic recognition, and mounted on glass slides. While nonadhesive glass slides are sufficient for short staining protocols, immunolabeling procedures require slides precoated with poly-L-lysine and/or 3-aminopropyltriethoxysilane (APES) to ensure attachment (*9,10*). In the case of LPC, sections are mounted onto a 1.35–1.5-mm polyethylene membrane treated with 1% poly-L-lysine or 8% APES solution in acetone. Prior to this procedure, the membrane must be fixed wrinkle-free to the glass slides (*11*).

Table 1
Routine Staining Procedure for Frozen Tissue Sections

	Frozen tissue
100% EtOH	optional 30 s
95% EtOH	—
70% EtOH	—
0.1% Mayer's hematoxylin	30–60 s
Rinse in DEPC water	2 × 5 s
Eosin (optional)	10–30 s
Rinse in DEPC water	2 × 5 s
70% EtOH	15 s
95% EtOH	15 s
100% EtOH	15 s

3. To prevent RNA degradation and contamination, standard precautions should be followed (*see Note 2*).
4. Sectioning should be performed immediately before staining. The routine staining procedure is presented in **Table 1**. After staining, the sections are stored in 100% ethanol to keep the tissue soft for LMM and micromanipulation. In contrast, using membrane and LPC, the ethanol has to evaporate. For LCM in particular, the sections are finally dehydrated in xylene for 2 min twice before air-drying (**12**). The time between staining and microdissection should be kept as short as possible. We have not seen any disadvantageous effect of routine hematoxylin staining on RNA recovery. Additionally, methyl green, nuclear fast red, and eosin stains are also recommended (**13,14**).

3.1.2. Immunolabeling

1. Precise characterization of defined cell types within complex tissues often requires immunolabeling. Several articles have reported on factors influencing the quality of nucleic acids following this process. Total staining time, number of incubation steps, and staining complex/enzymatic reaction were seen to be crucial for RNA recovery from unfixed and alcohol-fixed tissues (**15**). RNA is degraded rapidly during exposure to the aqueous phase so that labeling must be accomplished within a few minutes (**12,16,17**). Moreover, enzymatic conversion of the staining complex takes time and may affect negatively quality and quantity of RNA (**14,15**). In consequence, immunofluorescence staining is preferable to immunohistochemical staining. The total incubation time should not exceed 10–15 min in unfixed sections (*see Note 3*). This may be achieved by reduction of the antibody incubation steps. Therefore, moderate increases in the antibody working concentrations are often necessary. After acetone or 100% ethanol treatment for 1–5 min, sections are rinsed in Tris-buffered saline (TBS;

pH 7.5) briefly. Depending on the applied antibodies, incubations last about 1–5 min. Optionally, RNase inhibitor (RNasin, 400 U/mL, Promega, Mannheim, Germany) can be added to the antibody solutions.

2. Using micromanipulation, the labeled sections must be kept under ethanol continuously until isolation, as drying leads to marked unspecific background signals.
3. If immunohistochemical staining is essential, the procedure is described as for FFPE tissue in **Subheading 3.2.2**. However, the total staining time should not exceed 20 min for unfixed or ethanol-fixed tissue sections, as RNA is continuously degraded during this time.

3.1.3. Microdissection

1. The laser beam either dissects the tissue directly or focally melts a thermoplastic membrane to form a composite with the tissue. In the latter case, the cells become adherent to the film and both can be removed at once. This type of microdissection uses a pulsed low-energy infrared laser and is called laser capture microdissection (LCM; Arcturus Engineering, Mountain View, CA).
2. In the first case, a pulsed ultraviolet laser is coupled to an inverted microscope. After microdissection, the tissue islet or cells can be procured with a sterile needle mounted on a motorized micromanipulator (laser microbeam microdissection and micromanipulation, LMM; PALM Microlaser Technology, Bernried, Germany; **19**).
3. Alternatively, cells can be catapulted by the laser beam into the cap of a reaction tube positioned above. This is called laser pressure catapulting (LPC) (PALM; **20**).
4. A third system also equipped with an ultraviolet laser for microdissection has become available recently. Using an upright microscope, the dissected area falls into a cap that is positioned below (Leica Microsystems, Wetzlar, Germany; **21**).
5. Applying LMM, the stained sections are transferred from 100% ethanol to the microscope table without drying. One drop of 100% ethanol onto the uncovered section improves the histomorphology remarkably. Moreover, the tissue and cells remain flexible and adhere tightly to the needle, which can be lifted easily after rapid evaporation of the alcohol.
6. When LPC is performed from sections mounted on the membrane, any liquid must be removed between membrane and glass slide; otherwise the adhesion forces may prevent the catapulting.
7. For LCM the sections need to dry sufficiently to allow the local fusion with the film. On the other hand, strong adhesion of the section to the glass by prolonged drying may prevent removal of the cells.

3.1.4. RNA Preparation

3.1.4.1. LOW CELL NUMBER (50 C ELLS)

1. The requirements for microdissection may differ markedly. For some approaches, single cells must be isolated, while other studies require a hundred or even thou-

Table 2
Recommended Treatment for the Investigation of RNA From
Microdissected Cells Harvested From Frozen Tissue Sections

	50 cells	>50 cells
• Resuspension in	reaction buffer, 10 μ L	4 M GTC-buffer, 200 μ L
• Proteinase K digestion; 0.5 μ g/ μ L final conc.	optional; 30 min, 58°C	—
• Denaturation	7 min, 95°C	—
• Extraction	—	phenol/chloroform
• DNase digestion	optional; 2 U, 30 min, 37°C	optional; 2–10 U, 30 min, 37°C
• Denaturation	7 min, 95°C	7 min, 95°C

sands of cells. Usually, nucleic acids have to be extracted for further application. However, isolation of a few cells allows proceeding without an extraction step. The limit is 50 to 100 cells (22,23). Up to this number, cells can be directly transferred to a reaction buffer (see Materials), suitable for necessary digestions, reverse transcription, and PCR.

- Alternatively, a PCR buffer can be used. In case of LPC, the buffer can be prefilled into the cap of a reaction tube to collect the catapulted cells. In case of the Leica system, the cells drop into the buffer-filled cap. RNase inhibitor can be added (4% v/v).
- To disrupt the cell membrane of intact cells (e.g., from cytopins), addition of a nonionic detergent, e.g., Igepal CA-630 or Tween-20, can be advantageous. Its concentration should not exceed 1% v/v; otherwise further enzymatic reactions may be inhibited. We usually snap-freeze the samples, followed by thawing three times, and centrifuge the samples at 10,000g for 1 min to destroy the cell structure. Afterwards, the samples are kept in liquid nitrogen.
- When tissues with abundant connective tissue are investigated or proteins are expected to be bound to RNA, proteinase K ($\leq 0.5 \mu\text{g}/\mu\text{L}$; 53–60°C) digestion may improve the results. The incubation time should not exceed 30–60 min. For denaturation of the enzyme, 7 min at 95°C are necessary which can be used simultaneously for denaturation before cDNA synthesis. **Table 2** summarizes the preparation steps.

3.1.4.2. HIGHER CELL NUMBER (>50 CELLS)

- While fewer than 50 cells can be processed without extraction, this step is necessary for larger numbers of cells. After microdissection of frozen tissue, the cells are resuspended in 200 μ L of lysis buffer containing 4 M guanidine thiocyanate

(GTC), 25 mM sodium citrate, 0.5% sarcosyl, 0.72% 2-mercaptoethanol, and 20 mM Tris-HCl, pH 7.5. After incubation for 10 min at room temperature, 20 μ L 2 M sodium acetate, 220 μ L phenol (pH 4.3), and 60 μ L chloroform/isoamyl-alcohol (24:1) are added.

2. The samples are vortexed and centrifuged for 15 min at 4°C.
3. The aqueous layer is collected, 1 μ L glycogen (10 mg/mL) added and then the samples are precipitated with 200 μ L isopropanol.
4. Samples are frozen for 1 h at -20°C and centrifuged for 15 min at 12,000g.
5. The pellets are washed with 75% ethanol, air-dried and finally resuspended in 10 μ L H₂O (**Table 2**).
6. Use of silica-columns for RNA extraction is an interesting alternative, but up to now, the elution requires a considerably higher total volume (at least 30–40 μ L). This again has to be reduced by speed vacuum centrifugation. Recently, the first columns with lower binding capacity but also lower elution volume were released (i.e., Qiagen).

3.1.5. DNase Digestion

1. Employing RNase-free DNase I digestion (2–10 U, \leq 30 min, 37°C) depends on the subsequent analysis and should be limited to those cases when intron-spanning primers are useless, such as presence of pseudogenes or investigation of an intron-free gene.
2. The use of RNA for cDNA libraries or array hybridization requires this kind of digestion as well.
3. A subsequent precipitation to eliminate DNase is optional and depends on the volume of the sample and the planned analysis. Its presence may impair further enzymatic reactions.

3.2. FFPE Tissue

3.2.1. Tissue Preparation, Sectioning, and Routine Staining

1. FFPE tissue specimens are cooled at 4°C for 1–2 hours for better sectioning. The slides are prepared on a microtome (e.g., Jung SM 2000R, Leica).
2. Contact areas are cleaned with 70% ethanol followed by 0.1 M NaOH.
3. The sections (5–10 μ m) are mounted on adhesive-coated glass slides (e.g., Superfrost®Plus). They are made at maximal thickness, but still allow precise microscopic cell recognition.
4. For drying, the sections are stored at room temperature for 24 h.
5. In the case of LPC, sections are mounted onto a 1.35–1.5- μ m polyethylene membrane treated with 1% poly-L-lysine or 8% APES solution in acetone.
6. The routine staining procedure is presented in **Table 3**. Analogous to frozen tissue, the sections are stored in 100% ethanol after staining to keep the tissue soft for LMM and micromanipulation.
7. When using membrane and LPC or LCM, the ethanol has to evaporate, e.g., by dehydration in xylene for 2 min twice before air drying.

Table 3
Routine Staining Procedure
for FFPE Tissue Sections

	FFPE tissue
Dewax with xylene	2 × 5 min
100% EtOH	30 s
95% EtOH	30 s
70% EtOH	30 s
0.1% Mayer's hematoxylin	30–60 s
Rinse in DEPC water	2 × 5 s
Eosin (optional)	10–30 s
Rinse in DEPC water	2 × 5 s
70% EtOH	30 s
95% EtOH	30 s
100% EtOH	30 s

8. Apart from hematoxylin staining, methyl green, nuclear fast red, and eosin stains are also recommended.

3.2.2. Immunolabeling

1. For microdissection of archival FFPE tissue, even prolonged immunohistochemical staining could be combined successfully with RNA analysis of a few or single cells.
2. Limit the antibody incubation times to 5 min in combination with the alkaline-phosphatase monoclonal antialkaline phosphatase (APAAP) technique, slightly modified from Cordell et al. (24): After 10 min of deparaffinization in xylene, the slices are immersed in acetone and acetone/Tris-buffered saline (TBS; 1:1) for 10 min each.
3. For antibody dilution, TBS or alternatively the DAKO Chem Mate™ antibody diluent is used.
4. The samples are incubated at room temperature with the primary antibody for 5 min.
5. After short washing in TBS, second “link” antibody and third antibody mouse-APAAP complex (Dako, 1:50) are applied analogously.
6. Alkaline phosphatase substrate reaction is performed at pH 8.8 with new fuchsin (100 µg/mL) and levamisole (400 µg/mL) for 20 to 25 min at room temperature.
7. Afterwards, sections are counterstained with hematoxylin for 45 s, rinsed in water, immersed in 70%, 95%, and 100% ethanol, and stored in 100% ethanol for LMM or dried for LPC/LCM.
8. Thus, for LMM the staining complex has to be resistant to alcohol. Moreover, the complex should not interfere with further reactions; neither DAB nor new fuchsin showed any deleterious effects (14,25).

Table 4
Recommended Treatment for Investigation of RNA From
Microdissected Cells Harvested From FFPE Tissue Sections

	50 cells	>50 cells
Resuspension in	reaction buffer, 10 μ L	1 M GTC buffer, or Tris/EDTA buffer, 200 μ L
Proteinase K digest; 0.5 μ g/ μ L final conc.	indispensable; 6–10 h, 58°C	indispensable; 12–16 h, 58°C
Denaturation	7 min, 95°C	—
Extraction	—	phenol/chloroform
DNase digestion	optional; 2 U, \leq 30 min, 37°C	optional; 2–10 U, \leq 30 min, 37°C
Denaturation	7 min, 95°C	7 min, 95°C

9. High-temperature antigen retrieval treatments should be omitted, as they can adversely affect recovery of nucleic acids.

3.2.3. RNA Preparation

3.2.3.1. LOW CELL NUMBER (50 C ELLS)

1. In analogy to frozen tissue we differentiate a low cell scale (\leq 50 cells) from higher cell numbers. Up to this amount the isolated cells can be transferred into the 10- μ L reaction buffer (**Table 4**).
2. Regardless of the cell number, microdissected cells from FFPE tissue must be digested by proteinase K to release the nucleic acids from the cross-linking network.
3. Therefore, 1 μ L with 5 μ g/ μ L proteinase K (final conc: 0.5 μ g/ μ L) is added and incubated for 6 to 10 h at 58°C.
4. Afterwards, the enzyme is denaturated for 7 min at 95°C; this can be used as an initial denaturation step for cDNA synthesis.
5. For some applications a DNase digestion may be advantageous (*see Subheading 3.1.5.*).

3.2.3.2. HIGHER CELL NUMBER (>50 CELLS)

1. Greater amounts of microdissected cells are resuspended in 200 μ L of a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0), and 2% sodium dodecyl sulfate (pH 7.3), and are then digested with proteinase K (0.5 mg/ mL end concentration) at 58°C for 12–16 h (*see Note 4*).
2. RNA is then extracted by the aforementioned phenol/chloroform procedure (**26**).

3. Alternatively, the microdissected cells are suspended in 200 μL 1 M GTC, 0.5% sarcosyl, 0.72% 2-mercaptoethanol, and 20 mM Tris-HCl, pH 7.5.
4. Adding 0.5 $\mu\text{g}/\mu\text{L}$ proteinase K to the buffer, the samples are digested for 12–16 h (58°C) and phenol/chloroform extraction follows (27).
5. The first technique turned out to be most sensitive and is feasible for lower cell amounts and low copy genes.
6. On the other hand, higher DNA contamination has to be taken into account. The GTC preparation results in lower DNA contamination and is recommended especially when introns are absent and thus intron spanning primers cannot be applied or pseudogenes are present (*see Note 5*).

3.3. cDNA Synthesis

1. cDNA synthesis is performed shortly after digestion/extraction or sole denaturation. Especially when processing a small number of cells, microdissection, digestion, and reverse transcription (RT) should be performed directly and cells not stored until RT has been completed.
2. Microdissected cells collected in the reaction buffer, as well as RNA dissolved in H_2O , are heated to 70°C for 10 min and then cooled on ice for 5 min.
3. With a preceding digestion step, denaturation of the enzyme simultaneously serves to denature the RNA; thus the 70°C step can be omitted. cDNA synthesis from 10 μL H_2O -diluted RNA is performed with 4 μL MgCl_2 (25 mM), 2 μL 10X buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 1 μL dNTP (10 mM each), 1 μL random hexamers (50 μM), 0.5 μL RNase inhibitor (10 U), and 1 μL MMLV reverse transcriptase in a total volume of 19.5 μL (*see Note 6*).
4. Due to the presence of MgCl_2 in the cell buffer, only 2 μL MgCl_2 are added, resulting in a volume of 17.5 μL for few-cell approaches without RNA extraction. Samples are incubated at 20°C for 10 min followed by 42°C for 60 min. The reaction is stopped by heating to 95°C for 5 min and then cooled to 4°C (*see Note 7*).
5. The cDNA from extracted RNA can be applied to several PCRs. In the case of few-cell analyses, samples are split into two identical volumes for further PCR reactions (i.e., for target gene and standard gene analysis). Three or more PCR reactions from one sample reduce the respective recoveries (22).

3.4. PCR for Qualitative mRNA Analysis

1. To distinguish DNA and RNA, intron-spanning primers should be constructed.
2. Selecting an intron with 1000 bp, amplification of DNA can be prevented by suitable PCR conditions. In consequence, short amplification products for cDNA (150 bp) spanning a large intron guarantee optimal PCR efficiency (*see Note 8*). The amplicon length is particularly crucial for the investigation of archival FFPE tissue and is ideally limited up to 100 bp.
3. Due to degradation and fragmentation of RNA, increasing length of the PCR product just to 150–300 bp results in a considerably lower recovery or even total loss (26,28).

4. In a final volume of 50 μL , a standard PCR master mix for one sample consists of 5 μL PCR 10X buffer, 4 μL MgCl_2 , forward and reverse primer in a final concentration of 300 nM, 1 μL dNTP with 10 mM each 0.5 μL AmpliTaq Gold and cDNA. Up to 10 μL of the cDNA may be transferred to the PCR reaction.
5. Because of an inhibitory effect of the RT product on the polymerase activity, not more than 20% of the PCR volume should consist of the cDNA.
6. Typical PCR conditions are: 95°C for 6 min followed by 45 to 50 cycles with 95°C for 20 s and annealing temperature for 30–60 s (see **Note 9**).
7. If the mRNA detection fails after first amplification (i.e., low-copy genes from either a few cells or a single cell), the PCR product can be reamplified in a second “nested” PCR (**20**).

3.5. PCR for Quantitative mRNA Analysis

1. Real-time PCR quantification of mRNA molecules has become a reliable analysis, even in combination with few microdissected cells (**29**). This has been shown for frozen tissue (**30–32**) as well as FFPE tissue (**26,33,34**).
2. To determine the regulation of gene expression quantitative assays are inevitably needed. Moreover, interaction of mRNA and binding proteins can be assessed by this technique (**35**).
3. Valid measurement over a large range of initial starting quantities is obtained with known sensitivity of PCR. While absolute quantification by comparing the target to constant dilution series requires a remarkable effort, relative quantification (ΔC_T) is often sufficient to measure gene regulation. Based on **Eq. 1**, the target gene sequence is normalized to an internal reference gene (i.e., almost unregulated “housekeeping gene” mRNA) or to an external standard.

$$\frac{T_0}{R_0} = K \cdot (1 + E)^{(CT,R - CT,T)} \quad (1)$$

T_0 : initial number of target gene copies; R_0 : initial number of reference gene/standard copies; E : efficiency of amplification; CT,T : threshold cycle of target gene; CT,R : threshold cycle of reference gene/standard; K : Constant.

4. In preliminary experiments it must be shown that the amplification efficiency of the target and the reference primer/probe sets—both may vary between 0 and 1—are approximately equal. This has to be determined by calculating the slope of the dilution series. K is assumed to be equal within a definite fluorogenic-labeled primer/probe system and thus does not affect the comparison of the ratios.
5. Apart from the aspects mentioned above (short PCR products), special requirements mainly concern the construction of the primer/probe system. The primers are selected to span a long intron to prevent amplification of DNA by suitable PCR conditions. Additionally, in case of a single probe, it is placed centrally onto the exon-exon transition. In case of two probes, one is positioned centrally onto the exon-exon transition. This assures a complete detachment of the probe from the nucleic acid in case of partial DNA binding.

6. Alternatively, SYBR-Green may be applied for real-time quantification since it is less expensive and, moreover, works independently from any sequence.
7. However, some aspects have to be considered: (a) the amplification of a single PCR product has to be ensured; (b) as primer-dimers may affect the amplification efficiency their generation must be minimized and a sufficient efficiency rate has to be determined by a dilution series; (c) the analysis must be performed and calculated at a temperature level where primer-dimers are already melted.

3.6. RNA Preamplification

1. RNA preamplification techniques were introduced to increase the total mRNA amount without affecting the representative expression profile. This allows sufficient RNA to be obtained for several single gene analyses, virtual Northern blotting, or cDNA microarray hybridization even from microdissected cells.
2. For array hybridization, protocols were suggested using either T7-based linear amplification (36–38) or PCR-based amplification (39–41). Both of these techniques preserve the expression profile sufficiently.
3. Due to reproducibility, reliability, and efficiency we considered the SMART™ PCR technique to be superior and modified the original protocol by introducing the entire cDNA to PCR amplification (42). The principle of this kind of unspecific amplification is described in ref. 43.
4. To generate cDNA profiles representative for the input mRNA, it is crucial to stop the PCR cycling before amplification of any gene reaches its plateau phase. Depending on the initial amount of RNA, timely termination of the PCR has to be evaluated in preliminary experiments; otherwise the expression profile would be inevitably changed.
5. After microdissection the RNA is extracted as described in **Subheading 3.1.4.2.**, resuspended in 10 μL H_2O and DNase digested (1 U, 30 min, 37°C).
6. The extraction is repeated and RNA is finally diluted in 4 μL 1 H_2O .
7. Total RNA is reverse-transcribed using the SMART™ PCR cDNA Synthesis Kit (BD Clontech) with slight modifications: 4 μL total RNA, 1 μL CDS Primer (diluted to a concentration of 5 μM) and 1 μL SMART II oligonucleotide (diluted to a concentration of 5 μM) are mixed and incubated at 70°C for 8 min.
8. After short spinning, 2 min on ice and 2 min at 42°C, a master mix containing 2 μL 5X buffer, 1 μL DTT (20 mM), 1 mL 1 dNTP (10 mM) and 0.5 μL RNase H⁻MMLV reverse transcriptase (PowerScript™, BD Clontech) is added and incubated at 42°C for 1 h.
9. Afterwards, cDNA is mixed with 38.5 μL TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA) and purified by the QIAquick™ PCR Purification Kit (Qiagen).
10. Therefore, 250 μL buffer PB are added to the cDNA to load a column. According to the manufacturer's protocol, the columns are washed once.
11. For elution, 45 μL elution buffer (EB; 10 mM Tris-HCl, pH 8.5) is applied to the center of the column, incubated for 2 min, and centrifuged.
12. To improve the recovery, this step is repeated using the first eluate again.

13. From the eluted cDNA (~44 μL), 2 μL can be separated for further determination of the amplification factor.
14. For PCR-based amplification, the remaining 42 μL cDNA are mixed with the reagents of the Advantage™ 2 PCR Kit (BD Clontech): 5 μL 10X buffer, 1 μL PCR Primer (10 μM), 1 μL dNTP (10 mM) and 1 mL Advantage 2 Polymerase Mix. PCR conditions are 95°C for 1 min, followed by the evaluated amount of cycles with 95°C for 15 s, 65°C for 30 s, and 68°C for 3 min.
15. The resulting PCR product is purified using the QIAquick columns as described above. 44 μL elution buffer are applied twice for elution and 2 μL may be separated for determination of the amplification factor.
16. If necessary the final volume is reduced by SpeedVac centrifugation.
17. To determine the factor of preamplification 2 μL nonamplified cDNA, as well as 2 μL amplified PCR product, are introduced to real-time PCR reactions.
18. Applying a primer/probe set for a representative gene the ratio of CT values is a measure for the preamplification efficiency (*see Subheading 3.5.*).

4. Notes

1. Even under these conditions we could observe a slight deterioration of amplifiability during months of storage especially when analyzing RNA from single or a few cells. Thus, ideally, tissue not older than 3–6 mo is applied in this case.
2. To avoid the potential danger of contamination and false-positive results, tissue preparation, microdissection, extractions, and especially RT/PCR preparations should be carried out in different rooms and separated from the amplification and postamplification settings. To prevent transmission of contaminant every workbench should be equipped with its own set of tools. Disposable gloves should be worn throughout the procedure and removed when leaving the work site. Positive air displacement pipets with sterile filter tips help to prevent liquid and aerosol contamination. Plasticware must be kept sterile, glassware and buffers should be autoclaved and RNase-free solutions are to be used (i.e., DEPC-treated H₂O).
3. Applying a mild formalin fixation (short perfusion or ~2 h immersion) allowed us to increase the labeling time and, moreover, to obtain a sufficient RNA recovery. However, the samples have to be treated afterwards similar to FFPE tissue, including proteinase K digestion.
4. RNA extraction from FFPE tissue with oligo-dT-based techniques (supermagnetic beads, columns) resulted in a somewhat lower recovery. This might be due to the high RNA fragmentation, so that only poly-A tails with the linked 3' ends are caught. All other mRNA fragments are lost.
5. However, regardless of the extraction technique, the use of RNA derived from routine FFPE tissue remains problematic for hybridization to cDNA arrays and especially oligonucleotide arrays. Moreover, when employing this RNA for construction of cDNA libraries and for RNA preamplification techniques, the results have to be considered with reservation due to the high amount of degradation and fragmentation.

6. Random hexamers are preferable, especially when working with FFPE-derived RNA. Due to degradation, all mRNA fragments are reverse-transcribed. In case of oligo-dT priming, only the poly A tail-carrying mRNA fragments are transcribed.
7. Depending on the requirements, RT-PCR can be performed at once using enzyme mixtures (reverse transcriptase and DNA polymerase). For analysis of a low cell number, the application of recombinant *Thermus thermophilus* enzyme for one-step RT-PCR is not recommended, as both reverse transcriptase and polymerase activities are often lower than those of the respective single enzymes.
8. To distinguish DNA and RNA when intron-spanning primers cannot be applied, samples have to be split after proteinase digestion/extraction to one +RT (reverse transcriptase added) and one –RT (no enzyme added). The RT master mix should differ only in the enzyme that is replaced in –RT (mock) samples by H₂O; all other reagents and incubation steps remain identical. Subsequent quantitative PCR allows determination of the RNA portion in relation to DNA by calculating the ratio of the threshold cycles.
9. Several internal controls should be incorporated. While many preparation steps may lead to mistakes and negative results, highest sensitivity opens the risk of contamination and false positive results. Suitable negative controls should comprise especially the microdissection procedure and PCR reactions (buffers); positive controls are necessary to assess cDNA synthesis and PCR amplification. The use of dUTP and uracil-*N*-glycosylase digestion for PCR reaction is helpful and should be applied routinely but cannot replace controls.

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