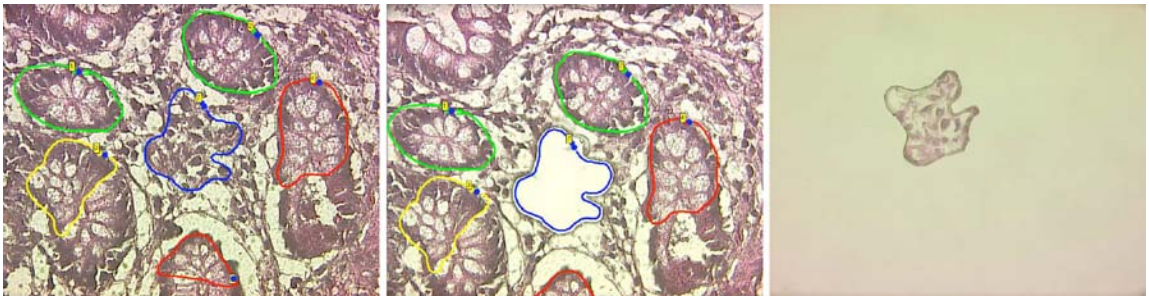
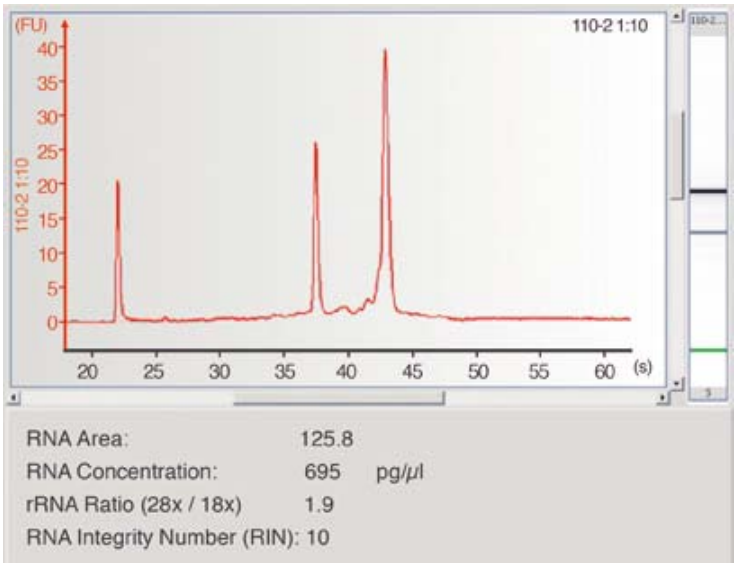
P.A.L.M.
Microlaser Technologies





Laser Microdissection and Pressure Catapulting (LMPC)

PROTOCOLS

RNA Handling

Contents

1 Introduction

1.1 Some remarks on RNA

RNA is a biological macromolecule with many different functions. Messenger RNA (mRNA), transcribed from DNA, serves as a template for synthesis of proteins. This protein synthesis is carried out by ribosomes, which consist of ribosomal RNA (rRNA) and proteins. Amino acids for protein synthesis are delivered to the ribosome on transfer RNA (tRNA) molecules. RNAs are also part of riboproteins and ribozymes.
Analysis of RNA can provide a good reflection of an organism's gene expression profile. Gene expression profiling of material isolated by microdissection has become a very important method for analyzing cellular behavior in a micro scale and is used in research and clinical applications.
Therefore the isolation of high quality RNA is crucial for all subsequent steps and the success of the overall experiment.

1.2 The DOs and DON'Ts on handling RNA

RNA degradation is a common reason for failing experiments. RNA is prone to digestion by a wide variety of endogenous and exogenous RNases. These RNases are present on almost all objects that come into contact with human skin and are difficult to inactivate. Even minute amounts are sufficient to destroy RNA. Some precautions can make the difference between an intact and degraded RNA prep (www.ambion.com) and therefore between successful and unsuccessful experiments.

DOs

- Designate a special area for working with RNA
- Clean benches with special cleaning solutions e.g. RNaseZap (AMBION, #9780)
- Wear gloves and change them frequently
- Use sterile, disposable plasticware
- Glassware should be treated with **D**i**E**thyl**P**yro**C**arbonate (DEPC) and oven baked at 180°C for at least 4 hours before use
- Use filtered pipette tips
- Solutions (water or other solutions) should be prepared with 0.1% DEPC
- Use RNase-free reagents, tubes and tips
- For best results use either fresh samples or samples that have been snap frozen on dry ice or in liquid nitrogen; all required reagents should be kept on ice
- Store prepared RNA, aliquoted in ethanol or RNA elution buffer at -80°C
- To avoid condensation of moisture during thawing, the slides should be frozen at -80°C and rethawed in a tightly sealed container (e.g. 50 ml Falcon tube)
- In general use protocols (e.g. staining) with short incubation times on ice

DON'Ts

- Don't breath on samples; some researchers wear masks
- Don't touch anything with bare hands
- Don't autoclave pipette tips, as water vapor may contain RNases
- Don't allow frozen tissue to thaw
- Don't resuspend RNA in DEPC water; residual DEPC can inhibit downstream reactions

2 First steps of sample preparation

2.1 Preparation of slides

2.1.1 Samples on PALM MembraneSlides

PEN-membrane covered 1 mm	#1440-1000
PEN-membrane covered 0.17 mm	#1440-1500
PEN-membrane covered 1 mm, nuclease free	#1440-1600

MembraneSlides are special slides covered with a membrane on one side. This membrane is easily cut together with the sample and acts as a stabilizing backbone during catapulting. Therefore even large areas are catapulted by a single laser impulse without affecting the morphological integrity. Use of MembraneSlides is especially important for isolating single cells, chromosomes as well as living cells or small organisms.

P.A.L.M. offers slides (1 mm, 0.17 mm) covered with **p**oly**e**thylene **n**aphthalate (PEN)-membrane. This PEN-membrane is highly absorptive in the UV-A range, which facilitates laser cutting. The membrane can be used for all kind of applications.
When working with low magnifying objectives like 5x, 10x or 20x, regular 1 mm thick glass slides and 0.17 mm glass slides can be used. To keep this flexibility for higher magnifications (40x or 63x) P.A.L.M. recommends using long distance objectives.
With those you have the possibility to adapt the working distance to the different glass slides by moving the correction ring on the objective.

Due to the short working distance of the 100x magnifying objectives only 0.17 mm thin cover glass slides can be used.
Note: The thin (0.17 mm) MembraneSlides are not resistant against heat. Use other treatments to remove RNases.

PALM MembraneSlides NF (nuclease free) are certified to be free of DNase, RNase and human DNA.
In addition to PEN-MembraneSlides, P.A.L.M. offers for special processes, i.e. for isolation of chromosomes, PET- or POL-membrane covered slides. If you need information about these slides, please contact salesteam@palm-microlaser.com.

2.1.1.1 Treatment to remove RNases

PALM MembraneSlides are shipped without any pretreatment.

- To ensure RNase-free MembraneSlides, heat MembraneSlides at 180°C for 4 hours to completely inactivate RNases.
- An alternative for decontamination of slides is the treatment with RNase ZAP (AMBION, #9780). First dip the slides for a few seconds into pure RNase ZAP, followed by two separate washings in **D**i**E**thyl**P**yro**C**arbonate (DEPC)- treated distilled water and drying at 37°C for 30 minutes up to 2 hours.
- PALM MembraneSlides NF (nuclease free) are certified to be free of DNase, RNase and human DNA. Using these slides treatments to remove nucleases are not necessary.

2.1.1.2 UV treatment

To overcome the hydrophobic nature of the membrane it is advisable to irradiate with UV light at 254 nm for 30 minutes (e.g. in a cell culture hood). The membrane gets more hydrophilic, therefore the sections (paraffin- and cryosections) adhere better. Positive side effects are sterilization and destruction of potentially contaminating nucleic acids.

2.1.1.3 Poly-L-Lysine treatment

Additional coating of the slide with Poly-L-Lysine (0.1% w/v) only will be necessary for poorly adhering materials (e.g. brain sections) and should be performed after UV treatment. Distribute a drop of the solution on top of the membrane. Let air-dry at room temperature for 2-3 minutes. Avoid any leakage underneath the membrane, as this might result in impairment of Laser Pressure Catapulting.

2.1.2 Samples on glass slides

With PALM MicroBeam almost every kind of biological material can be microdissected and catapulted directly from glass slides. Even archival pathological sections can be used after removing the cover slip and the mounting medium.

To facilitate easy catapulting additional adhesive substances or “Superfrost + charged slides” should only be applied when absolutely necessary for the attachment of poorly adhering special material (e.g. some brain sections or blood vessel rings).
For catapulting higher laser energy is needed.

2.1.2.1 Treatment to remove RNases

Treatments of glass slides to remove RNases are identic with the procedure for PALM MembraneSlides (2.1.1.1).

3 Mounting samples onto slides

3.1 Frozen sections

- **Sectioning**

Sections are mounted onto MembraneSlides the same way as routinely done using glass slides. To allow subsequent cutting and catapulting a coverslip and standard mounting medium must not be applied. Freezing media like OCT or similar may be used but should be kept to a minimum and have to be removed before laser cutting.
For optimal RNA retrieval take a pre-cooled MembraneSlide, warm the back of the slide shortly with your finger (gloves!), transfer section as routinely done by touching with the warmed area and let the slide remain in the cryostat for 2-3 minutes.

- **Fixation**

After mounting the sections there are many possibilities to fix the material.
P.A.L.M. recommends the dehydration in ice-cold 70% ethanol for 2-3 minutes.

- **Removing the tissue freezing medium**

If OCT or another tissue freezing medium is used, it is important to remove it before Laser Microdissection, because these media will interfere with laser efficiency. Removing of the medium is easily done by dipping the slide 5-6 times in ice-cold RNase-free water. If the sections will be stained, the supporting substance is normally removed "automatically" in aqueous staining solutions.

3.2 Paraffin embedded (FFPE) sections

Sections are mounted onto MembraneSlides the same way as routinely done using glass slides. Floating the section on warm water as well as hot plate techniques can be applied. After mounting, let dry the slides overnight in a drying oven at 56°C. To allow cutting and catapulting a coverslip and standard mounting medium must not be applied.

- **Deparaffination**

Paraffin will reduce the efficiency of the laser, sometimes completely inhibiting cutting and catapulting. If you are working with unstained sections it is therefore very important to remove the paraffin before laser cutting and pressure catapulting. If applying standard staining procedures deparaffination is routinely included in any protocols.

- **Minimal procedure:**

1.	Xylene	2 times for 3-10 minutes
2.	Ethanol 100%	1 minute
3.	Ethanol 96%	1 minute
4.	Ethanol 70%	1 minute

Note: The thin (0.17 mm) MembraneSlides are not as resistant against organic solvents and should be handled according to the Minimal procedure (see above).

3.3 Cytospins

Cytospins can be prepared on glass slides or on MembraneSlides. After centrifugation with a cytocentrifuge let the cells air-dry. Then fix for 5 minutes in 100% methanol. Allow the cytospins to dry at room temperature before staining.

3.4 Blood and tissue smear

Distribute a drop of (peripheral) blood or material of a smear over the slide. Be careful to avoid injuries in the membrane, which would lead to leakage during fixation or washing steps and therefore will impair the laser pressure catapulting process.
Let smears air-dry shortly and fix them for 2 up to 5 minutes in 70% ethanol.

4 Staining procedures

For isolation of high quality RNA, use only freshly prepared staining solutions and take notice of our tips on handling RNA (pp 15).

4.1 Frozen sections

Most standard histological stainings (like HE, Methyl Green, Cresyl Violet, Nuclear Fast Red) can be used when you are interested in RNA.
Note: Using frozen sections endogenous RNases may be active after the short fixation step. Therefore it is recommended to keep all incubation steps as short as possible. Please use RNase-free water and solutions for any steps. All required reagents should be kept on ice.

Commonly used protocols are the Cresyl Violet and Hematoxylin/Eosin staining.

4.1.1 Cresyl Violet

This short staining procedure colors the nuclei violet and the cytoplasm weak violet.

- **Procedure:**

1. After fixation dip slide for 60 seconds in 1% cresyl violet acetate solution (*)
2. Remove excess stain on absorbent surface
3. Dip into 70% Ethanol
4. Dip into 100% Ethanol
5. Air-dry shortly (1-2 minutes)

(*) Dissolve solid cresyl violet acetate (e.g. ALDRICH #86,098-0) at a concentration of 1% (w/v) in 100% EtOH at room temperature with agitation/stirring for several hours to overnight. Some unsolubilized powder is normal. Filter the staining solution before use.

4.1.2 Hematoxylin/Eosin (HE)

HE-staining is used routinely in most histological laboratories and does not interfere with RNA preparation. The nuclei are stained blue, the cytoplasm pink/red.

- **Procedure:**

1. After fixation quickly dip slide 5-6 times in RNase free distilled water
2. Stain 1 minute in Mayer's Hematoxylin solution (e.g. SIGMA, #MHS-32)
3. Rinse 1 minute in DEPC-treated tap water or blueing solution
4. Stain 10 seconds in Eosin Y (e.g. SIGMA, #HT110-2-32)
5. Perform a quick increasing ethanol series (70%, 96%, 100%)
6. Air-dry shortly

4.2 Paraffin embedded (FFPE) sections

After deparaffination continue with the staining procedure of your choice. Standard staining procedures can be used for frozen and FFPE sections (see above).

5 Storage

Stained slides can be used immediately or stored at -80°C before LMPC.
To avoid excess condensation of moisture during thawing, the slides should be frozen and rethawed in a tightly sealed container (e.g. 50 ml Falcon-tube).

6 Laser Microdissection and Pressure Catapulting (LMPC) Procedures

Please, additionally have a look at PALM MicroBeam user manual.

6.1 Tips for improvement of morphological information

For LMPC (Laser Microdissection and Pressure Catapulting) embedding and glass covering of the specimen is inapplicable. Thus, the rough open surface of the section/material often results in impaired view of morphology.

6.1.1 PALM Diffusor CM

#1210-0320

PALM Diffusor CM can be inserted into PALM CapMover as any holder and swivels over the sample. The opaque glass diffuses the incident microscope light, which smoothens the harshness of contrast and, depending on material and staining, even minute details as nuclei and cell boundaries show up. Even slight differences in color become visible. For more details and handling, please see PALM Diffusor CM instruction manual.

6.1.2 PALM AdhesiveCaps

500 µl opaque	#1440-0250
200 µl opaque	#1440-0240

The white/opaque filling of PALM AdhesiveCaps clearly improves visualization of morphological information of the samples at the object plane due to enhanced color balance and contrast, which makes the view comparable to those of coverslipped tissue sections.
Two different microfuge tube sizes with these filled caps are available from P.A.L.M.
For more details and handling, please see PALM AdhesiveCaps product information.

6.1.3 PALM LiquidCover Glass N

#1440-0600

The polymeric and low viscose PALM LiquidCover Glass N completely embeds the tissue and smoothens the rough tissue surface, resulting in enhanced morphology.
The coverslip-like surface, that is formed, not only improves the optical characteristics of the specimen, but also protects it against environmental influences (e.g. moisture and associated RNase activity). For more details and handling, please see PALM LiquidCover Glass N instruction manual.

6.2 Collection devices

6.2.1 PALM AdhesiveCaps

500 µl opaque	#1440-0250
500 µl clear	#1440-0255
200 µl opaque	#1440-0240
200 µl clear	#1440-0245

The intention of PALM AdhesiveCaps is to allow LMPC (Laser Microdissection and Pressure Catapulting) without applying any capturing liquid into the caps prior to LMPC. This minimizes RNase activity.
Beside the quick relocation of the catapulted samples in the cap due to instant immobilization there is no danger of evaporation and crystal forming during extended specimen harvesting.
Furthermore the filling of the caps allows a low energy catapulting due to the short distance between slide and collection device.
For more details and handling, please see also PALM AdhesiveCaps product information.

Note: P.A.L.M. recommends AdhesiveCaps as collection device for all RNA experiments.

6.2.2 Other microfuge tubes

In case you don't like to work with PALM tubes use commercially available RNase free plasticware (e.g. ABgene #AB-0350; 0.5 ml tubes).

If there are only no RNase free tubes available use the following procedure to remove RNases.

- **Treatment of microfuge tubes to remove RNases**

Add 0.1 ml DEPC to 100 ml of double distilled water to get a 0.1% DEPC solution (DEPC: e.g. ROTH #K028.1)

1. Stir for 5-6 h at room temperature to dissolve the DEPC
2. Soak the reaction tubes into the DEPC solution, take care that the tubes are completely covered with liquid (not blistered!) and incubate overnight at room temperature
3. Autoclave the tubes together with the solution for 20 minutes at 121°C to inactivate the DEPC
4. Discard the liquid carefully and thoroughly. Dry the tubes at 50°C-80°C.
5. Use the tubes as usual

Note: DEPC is toxic and should be used under a hood!!!

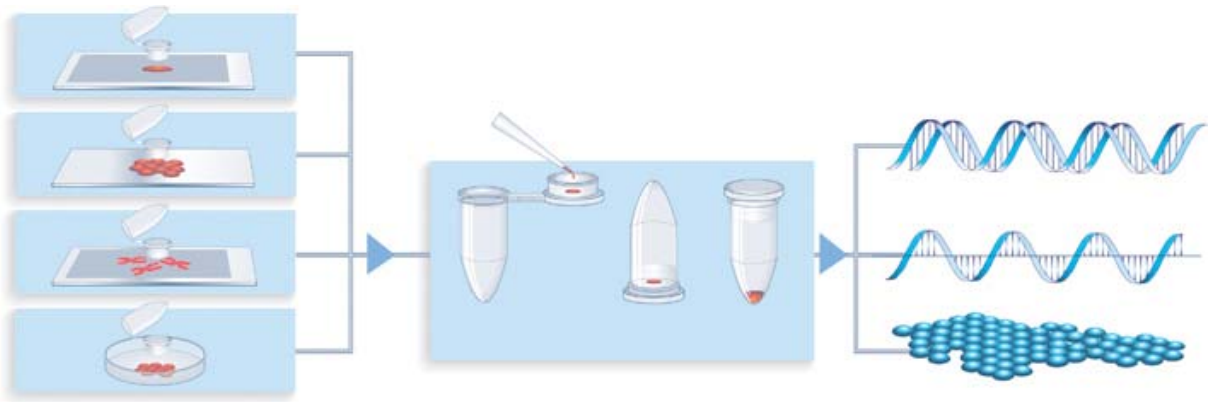
6.3 Collection procedures

Please have a look at PALM MicroBeam user manual.

6.3.1 "Dry" collection (PALM AdhesiveCaps)

Note: P.A.L.M. recommends PALM AdhesiveCaps as collection device for all RNA experiments. Capturing without liquid minimizes RNase activity.

After LMPC add lysis buffer of your own choice (e.g. QIAGEN: 350 µl RLT buffer) and centrifuge the lysis mix after an "upside down" incubation (30 minutes).



Then the routine RNA extraction procedure can be attached.

6.3.2 "Liquid" collection (other microfuge tubes)

Pipette 20 µl lysis buffer into the cap. The catapulted cells or cell areas will stick onto the wet inner surface of the cap and will not fall down after the catapulting procedure.
Be aware that aqueous solutions will dry out after a while.
When using glass mounted samples it may be advisory to put more liquid (up to 40 µl) into the cap since the smaller "fragments" produced by multiple LPC points cannot be catapulted so straight to the center of the cap as areas on membrane can be catapulted.

6.4 Capture check – looking into the cap to see the catapulted samples

To control the efficiency of catapulting it is possible to have a look into the collection device (e.g. microfuge cap) with the 5x, 10x, 40x and 63x objectives. By using the software function "go to checkpoint" the slide is moved out of the light path and the cap can be lowered further towards the objectives for looking inside.

7 Downstream Applications

7.1 Preparation of RNA

7.1.1 RNA from frozen sections

For capturing microdissected samples from frozen sections we recommend PALM AdhesiveCaps (500 µl opaque: #1440-0250; 500 µl clear: #1440-0255; 200 µl opaque: #1440-0240; 200 µl clear: #1440-0245).
The RNeasy® Micro Kit (QIAGEN, #74004) in our hands results in a good yield of RNA. But in contrast to most other laser microdissection companies you can go on straight forward with RNA extraction by using your extraction procedure of choice.

After LMPC adjust the sample volume to 350 µl with lysis buffer (QIAGEN, RLT buffer) and incubate upside down for 30 minutes.
Vortex the digestion mix thoroughly. Tissue fragments left undisrupted represent RNA lost. Then the lysate is spun down in a bench top centrifuge (5 minutes, 13400 rcf; e.g. Eppendorf 5415D: 12000 rpm) and samples can then be stored for later use at -80°C.

7.1.2 RNA from FFPE sections

For capturing of microdissected samples we recommend PALM AdhesiveCaps (500 µl opaque: #1440-0250; 500 µl clear: #1440-0255; 200 µl opaque: #1440-0240; 200 µl clear: #1440-0245).
Note: For FFPE samples an additional Proteinase K digestion step is necessary. Do not forget to inactivate the Proteinase K after the digestion (90°C for 10 minutes).

After LMPC add 20 µl of lysis buffer containing Proteinase K (150 mM NaCl, 100 mM Tris pH 7.5, 0.5% Igepal, 0.5 µg/µl Proteinase K) directly onto the microdissected sample, which is still located in the cap.
Proteinase K digestion is performed in an "upside down" position (pp 10) at 55°C overnight in an incubator to avoid evaporation of the lysate. The time necessary for complete digestion depends on the kind and on the number of catapulted cells.
If not proceeding immediately, store the samples at -20°C or -80°C.

All components of the following extraction procedure except glycogen are from the "Purescript RNA isolation kit", (GENTRA, USA, #212010). Mainly follow the standard protocol of the Purescript Kit. This procedure is very simple and allows high-end concentrations of RNA due to an effective precipitation step. Also some loss of the minute RNA amounts on columns is avoided.

Protocol in brief:

1. Add 300 µl *cell-lysis-solution* to the 20 µl of the digested lysate from above, vortex thoroughly and transfer to a 1.5 ml microfuge tube
2. Incubate at room temperature for 30 minutes
3. Add 100 µl of *protein-precipitation-solution,* mix carefully by inverting the tube at least ten times
4. Incubate on ice for 30 minutes
5. Centrifuge at maximum speed for 15 minutes in a cooled centrifuge (e.g. Eppendorf 5417C, 14000 rpm, 4°C)
6. Carefully transfer the supernatant into a fresh tube (*Do not disturb the white DNA-/protein-pellet!*). Discard the pellet together with the used tube
7. Add 0.5 µl of a glycogen solution (20 mg/ml) as carrier substance to the transferred supernatant
8. Add 300 µl isopropanol and mix carefully but thoroughly by inverting the tube fifty times
9. Centrifuge for 25 minutes as above (4°C, max. speed). Mark the orientation of the tubes in the rotor!
10. Remove supernatant carefully and discard it (*The pellet is mostly not visible! Look for the mark on the tube and remove the liquid with a thin pipette tip on the opposite side of the expected pellet position!*)

11. The pellet is “washed” briefly by adding 200 µl of pre-cooled ethanol (70%, -20°C)

12. Centrifuge for 15 minutes as above (4°C, max. speed). (*Keep the orientation of the tubes in the rotor as before!*)

13. Remove all supernatant carefully and discard it (*The pellet should now be visible as a small point of about 1 mm; remove the liquid with a thin pipette tip on the opposite side of the pellet position!)*

14. The pellet is now air dried for 20-30 minutes at room temperature *(Do not use vacuum drying!)* and then resolved in 10 µl of *RNA-Hydration-Buffer* for at least 30 minutes at 4°C or in ice. The RNA-solution can now be used for cDNA-synthesis or stored at -20°/-80°C

We normally use half of the solution in a RT-reaction of 20 µl (e.g. Transcriptor First Strand cDNA Synthesis Kit, ROCHE, #04 379 012 001) using random-oligomers (instead of oligoT) as primers for the cDNA synthesis.

7.2 Quality control of RNA

The most common method used for assessing the integrity of total RNA is to run the RNA sample on an agarose gel. In general, at least 200 ng of RNA must be loaded onto the gel. To analyze RNA samples with concentrations down to 50 pg/µl, the Agilent 2100 Bioanalyzer is an alternative to traditional gel-based analysis and provides information about RNA quality (degradation, purity) and quantity (www.chem.agilent.com).
A prognosis of the expected amount of RNA in a tissue is difficult to give since many factors like species, cell/tissue-type, fixation, staining, fragmentation, extraction procedure and others will influence the outcome.

8 General remarks on RNA (distribution, content, RNase activity)

A typical mammalian cell contains 10-30 pg total RNA (mRNA, rRNA, tRNA). The majority of RNA molecules are tRNAs and rRNAs. mRNA represents only 1-5% of the total cellular RNA.
Approximately 360 000 mRNA molecules are present in a single cell, corresponding to approximately 12 000 different transcripts with a typical length of 2 kb. Some mRNAs comprise as much as 3% of the mRNA pool whereas others account for less then 0.01% (QIAGEN, Bench guide).

RNA distribution in a typical mammalian cell

Total RNA per cell	~ 10-30 pg
rRNA (28S, 18S, 5S)	80-85%
tRNAs, snRNAs, low MW species	15-20%
mRNAs	1-5%
Total RNA in nucleus	~ 14%
DNA:RNA in nucleus	~ 2:1
mRNA molecules per cell	~ 2 x 10^5 to 1 x 10^6
Typical mRNA size	1900 nt

RNA content in various cells and tissues

		Total RNA (µg)	mRNA (µg)
Cell cultures (10^7 cells)	NIH/3T3	120	3
	HeLa	150	3
	COS7	350	5
Mouse tissue (100 mg)	Brain	120	5
	Heart	120	6
	Intestine	150	2
	Kidney	350	9
	Liver	400	14
	Lung	130	6
	Spleen	350	7

Also the RNase activity varies dramatically across different tissues (Krosting J, Latham G, AMBION, Inc.). A comparison of total RNase activities for 8 different mouse tissues showed that total RNase activity spans a 181,000-fold range from pancreas to brain, which points out the importance of RNase control.

Quantitative hierarchy of RNase activity in mouse tissues (AMBION, Inc.)

Mouse tissues	Fold increase relative to brain
Pancreas	181,000
Spleen	10,600
Lung	5,300
Liver	64
Thymus	16
Kidney	8
Heart	2
Brain	1

9 PALM LabTips for working with RNA

For best RNA quality we use frozen sections on PALM MembraneSlides. Frozen sections should not be stored for more than a few days at -80°C. Freezing should be performed after staining and drying.
A prognosis of the expected amount of RNA is difficult to give since many factors will influence the outcome (see above). From mouse liver frozen sections we usually are able to retrieve 5-20 pg RNA per cell (calculated from extractions of 1000 cells and analysis with an Agilent Bioanalyzer; Agilent Application Note 5988-EN on our website or at www.chem.agilent.com).
Note: Quantitative results from an analysis with the RNA Pico kit are dependent on the salt content of the sample.
Archival tissues are often formalin-fixed and paraffin-embedded. RNA extraction from these tissues is not effective because of the cross linking properties of aldehydes. Other methodologies for preservation of high molecular-weight RNA in FFPE tissue are described by Vincek et al. 2005 (see website: www.palm-microlaser.com/publications) and Olert et al. 2001 (*Pathol Res Pract*, 197: 823-826).

Summarized recommendations:

- Keep attention to DOs and DON´Ts on handling RNA (pp 3)
- Take PALM AdhesiveCaps as collection device for all RNA experiments (pp 9)
- Choose a short staining procedure for tissues with high content of endogenous RNases (e.g. Cresyl Violet) (pp 7)
- QIAGEN RNA extraction kit results in good RNA yield (quality and quantity) from frozen sections in our lab (pp 11)
- GENTRA RNA extraction kit results in good RNA yield (quality and quantity) from FFPE tissue in our lab (pp 12)

10 Other protocols:

10.1 DNA

DNA protocols on request.

10.2 Chromosomes

Protocols on request.

10.3 Live cells

Live cell protocols on request.
Please note: There is also a review brochure available dealing with laser micro-manipulation of live cells (P.A.L.M. Scientific Edition No. 11, ISBN No. 3-9808893-0-0).

For questions, comments or protocol requests please contact:

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