Protocols

Specimen preparation
Specimen isolation and collection
Downstream applications

Specimen preparation.. 4

- Preparation of slides.. 4
  - Samples on glass slides
  - Samples on membrane slides
    - UV treatment
    - Poly-L-lysine treatment
    - Treatment to remove RNases

- Mounting sections onto slides.. 5
  - Paraffin embedded sections
  - Frozen sections
  - Cytospins
  - Blood and tissue smear

- Staining of the sections... 6
  - DEPC treatment of water and solutions
  - Histological staining methods
    - Hematoxyline/Eosin (H&E, HE)
    - Methylene Blue
    - Methyl Green
    - Nuclear Fast Red
  - Fluorescence
    - DAPI staining

Specimen isolation and collection.................................... 8

- Laser cutting (microdissection) of the samples
  - *Tricks for improvement of the morphology sight of sections*
    - *Ethanol*
    - *Glycerol in PBS*
- Laser Pressure Catapulting of the samples
- Looking into the cap to see the catapulted samples
- Getting the collected cells from the cap into the tip of the tube

Downstream Applications.. 10

DNA... 10

- Preparation of DNA from catapulted samples
- Protocol for DNA amplification (example)
  - PCR protocol for sex determination (Amelogenin); nested PCR

RNA... 12

- Some special tips for working with RNA
- Preparation of frozen sections
- Preparation of RNA from catapulted samples
  - Proteinase K digestion
- RNA extraction: Alternative I.. 14
  - Buffers, Enzymes and Solutions
  - RNA extraction
  - Ethanol precipitation
- RNA extraction: Alternative II... 15
  - Buffers, Enzymes and Solutions
  - RNA extraction

- Reverse Transcription: Alternative I... 16
  Omniscript / Sensiscript

- Reverse Transcription: Alternative II.. 17
  TaqMan Gold RT-PCR

  Protocol for cDNA amplification (example)............................. 17

- **Chromosome preparation** on request..................................... 18

- **Isolation of Living cells** on request... 18

Protocols

Specimen preparation
Specimen isolation and collection
Downstream applications

Specimen preparation

➢ Preparation of slides

When working with low magnifying objectives, including 40x and 63x long distance objectives, regular 1 mm thick glass slides can be used. Due to the short working distance of the 100x magnifying objectives 0.17 mm thin cover glass slides have to be used.

Samples on glass slides

With the 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 adhesion of special material (e.g., brain sections).

Samples on membrane slides

Membrane slides are special slides covered with a membrane. The membrane is easily cut together with the sample and acts like a stabilizing backbone during catapulting. Therefore even large areas are catapulted by a single laser pulse without affecting the morphological integrity. The membrane facilitates catapulting of even large areas with one single laser pulse. This feature is especially important for isolated single cells, chromosomes and also living cells or small organisms.

PALM offers slides with two different membranes (**PEN** and **POL**). The **PEN (polyethylene naphthalate) membrane** is 1.35 µm thick and is highly absorptive in the UV-A range, which facilitates laser cutting. The PEN-membrane can be used for all applications.

The **POL (polyester) membrane** is 0.9 µm thick and less sensitive to the UV laser, e.g., a higher laser energy is required for cutting. Thus, it is possible to perform laser ablation of unwanted specimen with moderate laser energy without immediate cutting the membrane. After "cleaning" of the surrounding a higher laser energy is required to circumcise and catapult the selected specimen.

UV treatment

To overcome the hydrophobic nature of the membrane it is advisable to irradiate with UV light at 254 nm for 30 minutes. The membrane gets more hydrophilic, therefore the sections (paraffine and cyrosections) will obtain a better adherence. Positive side effects are sterilization and destruction of potentially contaminating nucleic acids.

Poly-L-lysine treatment

Additional coating with poly-L-lysin (0.1% w/v) will be only necessary for special materials (e.g. brain sections) and should be performed by distributing a drop of the solution on the membrane. Let it air dry at room temperature for 30 minutes. Avoid any leakage underneath the membrane, as this might result in impairment of Laser Pressure Catapulting.

Treatment to remove RNases

To ensure RNase-free membrane-slides, we dip them for a few seconds into RNase-ZAP (Ambion, #9780, 250 ml), followed by two separate washings in DEPC-treated water (see page 6) and drying at 37°C for 30 minutes up to 2 hours. Subsequently the standard UV-treatment (see above, page 5) is performed as usual.
NOTE: Find special tips for work with RNA at page 12 (Applications: RNA).

➢ Mounting sections onto slides

Sections are mounted onto membrane slides the same way as routinely done with glass slides. For cutting and catapulting a coverslip or standard mounting medium must not be applied.

Paraffin embedded sections

Mount the sections onto the slide as routinely done. Afterwards let air-dry at room temperature or dry at up to 56°C overnight in a drying oven.

Deparaffinization

Paraffin will reduce the efficiency of laser cutting; sometimes it will make it impossible to cut and catapult. If working with unstained sections it is therefore very important not to forget removing the paraffin before laser cutting and laser pressure catapulting. If applying staining procedures deparaffinization is routinely included in any protocols.

Procedure:

Xylene	2 times for 2 minutes
Ethanol absolut	1 minute
Ethanol 96%	1 minute
Ethanol 70%	1 minute
rinse with water	

Prolonged Xylene treatment could resolve the membrane fixation; Xylene treatment should therefore be kept as short as possible.

Frozen sections

Fixation

After mounting the sections there are many possibilities to fix the material. If RNA preparations are planned with these frozen sections we at PALM recommend ethanol fixation. This is performed by dipping the mounted sections for 5 minutes into 70% ethanol.

Removing the freeze supporting substance

If OCT or another tissue freezing medium is used it is important to get rid of the media on the slide before Laser Microdissection, because these media will interfere with laser efficiency. Removing of the medium is very easily done by gently washing the slide for about 2 to 3 minutes in water. Let air-dry at room temperature or dry at 37°C in the drying oven. If the sections are stained, the supporting substance is removed „automatically" in the aqueous staining solutions.

Frozen sections should always be allowed to dry for 15 up to 30 minutes at room temperature before use.

Cytospins

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

Blood and tissue smear

Distribute a drop of (peripheral) blood or material of a swab smear over the slide.
Let smears shortly air-dry and fix them for 2 up to 5 minutes in 70% ethanol.

➢ Staining of the sections

Using frozen tissue one has to be aware of endogeneous RNases that may still be active after short fixation steps. Therefore it is recommended to keep all incubation steps of histochemistry as short as possible. Please use RNase-free water and solutions.

DEPC treatment of water and solutions

RNases in aqueous liquids can be destroyed with 0.1% DEPC (diethylpyrocarbonate).
Caution! DEPC is highly toxic and should be handled under a fume hood. Wear gloves!
1 L of bidestilled H_2O (or solution) + 1 ml DEPC (e.g., Roth, #K028.1) are stirred for 6-8 hours at room temperature; then let incubate overnight in a fume hood. The next day residual DEPC is removed by autoclaving. Store treated solution at room temperature.

NOTE: All chemical substances containing amino groups (e.g. TRIS, MOPS, EDTA, HEPES, etc.) cannot be treated directly with DEPC. Prepare these solutions in DEPC-treated H_2O.

Histological staining methods

Hematoxylin/Eosin (H&E, HE)

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

Procedure:

10 minutes Mayer's hematoxylin solution
10 minutes rinsing in running tap water
5 minutes Eosin Y
increasing Ethanol series
let air-dry

HE-staining of Cryosections for <u>RNA preparation:</u>

3 minutes Mayer's hematoxylin solution
3 minutes rinsing in RNase-free tap water
30 seconds Eosin Y
increasing Ethanol series
let air-dry

Methylene Blue

The nuclei are stained dark blue.

Procedure:
5-10 minutes Methylene Blue solution (0.05% in water; SIGMA, #31911-2)
rinsing in Aqua dest
let air-dry at room temperature

Methyl Green

The nuclei are stained dark green, the cytoplasm light green.

Procedure:
5 minutes Methyl Green solution (DAKO, #S1962)
rinsing in Aqua dest
let air-dry at room temperature

Nuclear Fast Red

The nuclei are stained dark red, the cytoplasm lighter red.

Procedure:
5 to 10 minutes Nuclear fast red solution (DAKO, #S1963)
rinse in A. dest
let air-dry at room temperature

Fluorescence

DAPI staining

DAPI 1 mg/ml, Sigma (#D9542)

Buffer: 100 mM NaCl
10 mM EDTA
10 mM Tris, pH 7

1. DAPI (0.25 µg/ml) in H_2O (two absorption maxima: 349 nm and 263 nm) or
2. DAPI (0.25 µg/ml) in buffer (extinction maximum 364 nm, emmision maximum 454 nm)

Deparaffinization as usual (see page 2)
Dip into 200 mM KCl
Incubate in darkness with DAPI for one hour
Let dry in darkness

Specimen isolation and collection

➢ Laser cutting (microdissection) of the samples

Tricks for improvement of the morphology sight of sections

Ethanol

Go forward to search an interesting area on the section and pipet about 5 µl of ethanol onto this area. You may use 70% or 100% ethanol. When using absolute ethanol a little destaining of the section may happen, but the drying is much quicker.

Observe the area at the screen. The depiction of the cells is improved immediately after having contact with the ethanol. Now mark the cells or cell area with the software tool. Ethanol is evaporating rapidly; now catapult the marked cells or cell areas.

Glycerol in PBS

The same effect is produced by 1% glycerol in PBS (phospate buffered saline). The time period until drying of the section is longer than with ethanol. Handling of pipetting, drying, marking and cutting is the same as shown with ethanol above.

➢ Laser Pressure Catapulting of the samples

Catapulting into the cap

Pipette 2 to 3 µl bidistilled water, buffer or light weight mineral oil (PCR oil) into the inner ring of the cap. The catapulted cells or cell areas will stick onto the inner surface of the cap and will not fall down after the catapulting procedure.

If it is planned to prepare RNA it may be advantageous to pipette a RNA protective solution into the cap. Such kind of solution (for example RNAlater from Ambion) stabilizes the RNA and inhibits its degradation. It may also be advantageous to catapult directly into RNA lysis buffer, as this buffer contains RNA stabilizing chemicals.

When using membrane mounted samples the dissected membrane acts as a backbone for the selected area/cell and can therefore be catapulted with a single laser shot from a remaining „bridge" at the border. The morphological integrity is completely preserved with this procedure.

When using glass mounted samples it may be advisory to put more liquid into the cap since the smaller „flakes" produced by multiple LPC points cannot be catapulted so straight to the centre of the cap as areas on membrane.

➢ Looking into the cap to see the catapulted samples

To control the efficiency of catapulting it is possible to look into the special PALM 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. Normally most catapulted areas/cells can be found within the small inner ring of the PALM caps.

➢ Getting the collected cells from the cap into the tip of the tube

Appropriate lysis buffer is added to the PCR tube and after closure placed upside down. (For future RNA isolation/analysis the tube is placed on ice.) After microdissection, the fluid is shortly spun down in a bench centrifuge (1 minute, 13000 rpm) and samples can then be stored for later use. When RNA isolation/analysis is intended samples are stored at –80°C in a freezer.

Downstream Applications

DNA

➢ Preparation of DNA from catapulted samples

For DNA-prep from paraffin sections we usually use a Proteinase K containing catapult buffer. This step is not necessary for frozen sections.

Catapult Buffer	0.5 M EDTA pH 8.0		20 µl
	1 M Tris pH 8.0		200 µl
	Tween 20		50 µl
	(Proteinase K 20 mg/ml)		(1000 µl)
	ddH_2O	9.73 ml	(8.73 ml)

Proteinase K solution 20 mg/ml (Qiagen GmbH, Hilden, Germany)
Catalog number #19131

Always prepare a fresh mixture of Catapult Buffer and Proteinase K.

(a) take an autoclaved PALM cap
(b) pipet 2-3 µl of Catapult Buffer, PCR oil or DNase-free water in the middle of the cap
(c) put the cap into the cap holder
(d) perform laser microdissection and laser pressure catapulting of selected cells or cell areas
(e) remove the cap from the cap holder and put it onto a 0.5 ml microfuge tube
(f) centrifuge the tube at full speed for 1-2 minutes; discard the cap
(g) add 10 µl Catapult Buffer containing Proteinase K onto the cells (which are now in the tip of the tube)
(h) incubate at 55°C for about 12 hours (may be shortened). Heat the samples immediately after digestion for 10 minutes at 99°C to inactivate Proteinase K. At best perform digestion in a thermal cycler with a heating lid.

If not going on immediately store the samples in the fridge at 4°C.

➢ Protocol for DNA amplification (example)

Buffers, Enzymes and Solutions

dNTP solution (100 mM each; MBI Fermentas, St. Leon-Roth, Germany)
Catalog number #R0181 (working solution: 2 mM each)

Thermo-Start DNA polymerase for all PCRs. Buffer and $MgCl_2$ are derived hereof.

Thermo-Start DNA Polymerase (250 units, 5 units/µl; ABgene, Hamburg, Germany).
Catalog number #AB-0908

PCR protocol for sex determination (Amelogenin); nested PCR

For single cells start the first PCR using the **entire** amount of extract after digestion.

Primer sequences, outer primers: Sexing F atc aac ttc agc tat gag g
Sexing R tag aac caa gct ggt cag

1st PCR

The product size of the DNA fragment is 330 bp for the X chromosome and 340 bp for the Y chromosome.
The 5´primer sequence is located in the exon/intron boundary of exon 1 and the 3´primer is located in intron 1.

2 mM dNTP-Mix	2.0 µl
10x Buffer	2.0 µl
25 mM $MgCl_2$	1.6 µl
50 µM 5´outer primer	0.5 µl
50 µM 3´outer primer	0.5 µl
5 units/µl Thermo Start Taq	0.1 µl
DNA (entire extract)	10 µl
H_2O double destilled	3.3 µl
total volume	20.0 µl

Cycle number	Denaturation	Annealing	Extension
1 cycle	15 min at 94°C		
35 cycles	30 sec at 94°C	30 sec at 54°C	40 sec at 72°C
1 cycle			10 min at 72°C

cool down to 4°C

2nd PCR (nested)

The product size of the nested DNA fragment is 106 bp for the X chromosome and 112 bp for the Y chromosome.

Primer sequences, inner primers: Sexing F ccc tgg gct ctg taa aga ata gtg
Sexing R atc aga gct taa act ggg aag ctg

2 mM dNTP-Mix	2.0 µl
10 x Buffer	2.0 µl
25 mM $MgCl_2$	1.6 µl
50 µM 5´inner primer	0.5 µl
50 µM 3´inner primer	0.5 µl
5 units/µl Thermo Start Taq	0.1 µl
1st PCR reaction product (variable)	
H_2O double destilled	(ad 20 µl)
total volume	20.0 µl

Cycle number	Denaturation	Annealing	Extension
1 cycle	15 min at 94°C		
30 cycles	30 sec at 94°C	30 sec at 62°C	40 sec at 72°C
1 cycle			10 min at 72°C

cool down to 4°C

RNA

➢ **Some special tips** for working with RNA

Working with RNA is more demanding than working with DNA, because of the chemical instability of the RNA and the ubiquitous presence of RNAses.

- It makes sense to designate a special area for RNA work only.
- Clean benches with 100% ethanol.
- Always wear gloves. After putting on gloves, do not touch surfaces or equipment to avoid reintroduction of RNAses to decontaminated material.
- Use sterile, disposable plasticware.
- Use filtered pipetter tips.
- Glassware should be baked at 180°C for 5 hours. (RNAses can maintain activity even after prolonged boiling or autoclaving!)
- Purchase reagents that are RNAse-free.
- All solutions should be made with DEPC-(diethylpyrocarbonate) treated H_2O.
- Treat all used material with DEPC.
- For best results use either fresh samples or samples that have been quickly frozen in liquid nitrogen or at -80°C. (This procedure minimizes degradation of RNA by limiting the activity of endogeneous RNAses.) All required reagents should be kept on ice.
- Store RNA, aliquoted in ethanol or elution buffer, at -80°C. Most RNA is relatively stable at this temperature. Store prepared slides also at -80°C.
- RNA is not stable at elevated temperatures, therefore avoid high temperatures (> 65°C) since these affect the integrity of RNA.

DEPC treatment of water and solutions see at page 6.

➢ Preparation of frozen sections

Prepare your sections onto the slide as you do routinely. Fix in 70% ethanol for 15 seconds, dip in DEPC-water, perform your preferred staining (e.g., hematoxylin), dip into 50%, 70% and 100% ethanol, respectively. Subsequently let air dry for 5 minutes. The slides can now be used at once (even if they are somewhat wet) or deep frozen at –80°C.

Procedure

For cryosections we perform catapulting of the cells into a buffer without Proteinase K. If using paraffin sections for catapulting, please use a buffer containing Proteinase K like mentioned in the DNA protocol.

Best use freshly cut specimens.

➢ Preparation of RNA from catapulted samples

To ensure RNase-free membrane-slides, we at PALM dip them for a few seconds into RNase-ZAP (Ambion, #9780, 250ml), followed by two separate washings in DEPC-treated water and drying at 37°C for 30 minutes up to 2 hours. Afterwards the standard UV-treatment is performed as usual (see page 5) shortly before use.

To reduce the chance of contamination with exogenous RNases, we use only special reagents and solutions for RNA isolation, reverse transcription and RT-PCR. All used solutions and tubes are prepared with DEPC treated water. We also recommend the use of filter tips.

Best results are obtained using freshly prepared cryosections.

Catapult Buffer:	0.5 M EDTA pH 8.0	20 µl
	1 M Tris pH 8.0	200 µl
	Tween 20	50 µl
	(Proteinase K 20 mg/ml)	(1000 µl)
	ddH_2O DEPC treated, autoclaved	9.73 ml (8.73 ml)

Proteinase K solution 20 mg/ml (Qiagen GmbH, Hilden, Germany)
Catalog number #19131 (only used for paraffin sections)

Always prepare a fresh mixture of Catapult Buffer with Proteinase K.

1. take an autoclaved PALM cap
2. pipet 2-3 µl of Catapult Buffer into the middle of the cap
3. put the cap into the cap holder
4. perform laser microdissection and laser pressure catapulting of selected cells or cell areas
5. remove the cap from the cap holder and put it onto a 0.5 ml microfuge tube containing lysis buffer
6. centrifuge the sample at full speed for 2 minutes immediately after catapulting

If using **cryosections** you can go on straight forward with RNA extraction by using e.g. the RNeasy Mini Kit (Qiagen, #74104).

If using **paraffin sections** it is recommended to perform a Proteinase K digestion step before starting the RNA extraction.

Proteinase K digestion

a) After centrifugation pipet 11 µl of Catapult Buffer containing Proteinase K onto the catapulted cell(s) (which are now in the tip of the tube).
b) Vortex gently. Digest for 2 - 18 hours at 55°C(*) followed by a heating step at 99°C for 10 min to inactivate Proteinase K.
c) At best use a thermal cycler with a heating lid for digestion. If not going on immediately, store the samples in the fridge at 4°C.

(*)The time necessary for complete digestion depends on the kind and on the number of catapulted cells.

Procedure
We perform catapulting of the cells directly into DEPC water containing RNAse Inhibitor or into Catapult Buffer without Proteinase K. (NOTE: If using paraffin sections for catapulting, please use a buffer containing Proteinase K.)

Best use freshly cut specimens.

➢ RNA extraction: Alternative I

Preparation of total RNA from microdissected cell samples using the *RNeasy Mini Kit*

Buffers, Enzymes and Solutions

- *RNeasy Mini Kit* (50 rct., Qiagen, Hilden, Germany)
  Catalog number #74104
- *RNase-free DNase Set* (50 rct., Qiagen, Hilden, Germany)
  Catalog number #79254
- *RNAseOUT Recomb. RNase Inhibitor* (Invitrogen, Groningen, NL)
  Catalog number #10777-019
- *Random Primers* (1.5 mM, Invitrogen, Groningen, NL)
  Catalog number #48190011 (working solution: 25 µM)
- *Glycogen* MB Grade 20 mg/ml (1ml, Roche, Mannheim, Germany)
  Catalog number #901 393

All solutions have to be treated with DEPC!

RNA extraction

- After centrifugation of the cells prepare a fresh solution of 1 ml Buffer RLT (included in the RNeasy Kit) with 10 µl ß-Mercaptoethanol. Pipet 350 µl of the buffer onto the cells.
- incubate the mixture for 30 minutes at 42°C
- go ahead with the RNeasy protocol step 5
- to elute the RNA from the RNeasy column use 30 µl of RNase free water to minimize the volume

Instead of the default amount (30-50 µl) it is also possible to use 100 µl water for highest yield. Then concentrate the RNA with an ethanol precipitation step (see the following steps).

Ethanol precipitation

- pipette 5 µg of glycogen to the eluated 100 µl RNA, mix gently
- add 10 µl of sodium acetate (3 M, pH 5.0) to the mixture, mix gently
- pipet 250 µl ethanol abs. to the mixture, invert the tube for several times and centrifuge for 30 minutes at full speed
- after centrifugation you can see a white pellet at the bottom of the tube, discard the supernatant carefully by a pipetting step. Add 250 µl of 100% ethanol to the pellet
- centrifuge for 15 minutes. Remove the supernatant carefully. CAVE: The pellet may be very slacky!
- Add 100 µl of ethanol abs. to the pellet, centrifuge for 15 min, discard the supernatant
- let the pellet air-dry for 10 minutes under a sterile hood
- dissolve the pellet in 10 to 15 µl RNAse free water
- incubate for 30 minutes on ice to make sure that the pellet has resolved completely

The RNA is now ready to use for Reverse Transcription.
If you do not plan to perform the Reverse Transcription step now, freeze the RNA at –20°C or –80°C.

➢ RNA extraction: Alternative II

Preparation of total RNA from microdissected cell samples using the *Absolutely RNA Nanoprep Kit*

Buffers, Enzymes and Solutions

- *Absolutely RNA Nanoprep Kit* (50 rct., Stratagene Europe, Amsterdam, NL)
  Catalog number #400753

- *RNAseOUT Recomb. RNase Inhibitor* 40 U/µl (5000 units, Invitrogen, Groningen, NL)
  Catalog number #10777-019

- *Proteinase K solution* 20 mg/ml (2 ml, Qiagen GmbH, Hilden, Germany)
  Catalog number #19131

- Catapult Buffer:

0.5 M EDTA pH 8.0	20 µl
1 M Tris pH 8.0	200 µl
Tween 20	50 µl
(Proteinase K 20 mg/ml)	(1000 µl)
ddH$_2$O DEPC treated, autoclaved	9.73 ml (8.73 ml)

  All solutions have to be treated with DEPC!
  If using Proteinase K in the Catapult Buffer, please always prepare a fresh mixture of Catapult Buffer and Proteinase K.

- DEPC water with RNAse Inhibitor
  100 µl water with 1 µl RNAse Inhibitor (40 units/µl)

RNA extraction

- After centrifugation of the cells prepare a fresh solution of 100 µl Lysis Buffer (included in the Nanoprep Kit) with 0.7 µl ß-Mercaptoethanol for each sample. Pipet 100 µl of this mixture onto the cells (or the PK digest)
- incubate the mixture for 30 minutes at 42°C
- go ahead with the Nanoprep protocol step 3
- to elute the RNA from the Nanoprep column use at least 10 µl of the elution buffer; consider that you need 2-3 µl RNA for a 'no RT control'

The RNA is now ready to use for Reverse Transcription.
If you do not plan to perform the Reverse Transcription step now, freeze the RNA at –20°C or –80°C.

Reverse transcription: Alternative I

Reverse Transcription with the *Sensiscript RT Kit or Omniscript RT Kit*

- *Sensiscript RT Kit* (50 rct., Qiagen, Hilden, Germany)
  Catalog number #205211

- *Omniscript RT Kit* (50 rct., Qiagen, Hilden, Germany)
  Catalog number #205111

- *Thermo-Start DNA Polymerase* (250 units; ABgene, Hamburg, Germany)
  Catalog number #AB-0908

- *dNTP solution* (100 mM each; MBI Fermentas, St. Leon-Roth, Germany)
  Catalog number #R0181 (working solution: 2 mM each)

- *Random Primers* (1.5 mM, Invitrogen, Groningen, NL)
  Catalog number # 48190011 (working solution: 25 µM)

Sensiscript Reverse Transcriptase is designed for use with less than 50 ng RNA. This amount corresponds to the entire amount of RNA present, including any rRNA, mRNA, viral RNA and carrier RNA.

For more than 50 ng RNA the *Omniscript Reverse Transcriptase Kit* from Qiagen is used. It is designed for an amount of 50 ng to 2 µg of total RNA.
Before starting dilute RNase inhibitor to a concentration of 10 units/µl in ice-cold 1x buffer RT (dilute an aliquot of the 10x buffer RT in RNase-free water). 0.5 units/µl final concentration should be used in the assay.

Leave 3 µl of the diluted RNA for a ‚no RT control' of the RT-PCR.

- prepare a fresh master mix on ice according to the manufacturer´s protocol. Add the RNA template to the tubes containing the master mix. Incubate for 1 hour at 37°C. Heat the mixture for 5 minutes to 93°C to inactivate Reverse Transcriptase. Cool down rapidly.

The cDNA is now ready to use for RT-PCR.

➢ Reverse transcription: Alternative II

Reverse Transcription with the *TaqMan Gold RT-PCR Kit*

- *TaqMan Gold RT-PCR Kit* (5000 units, Applied Biosystems, Weiterstadt, Germany)
  Catalog number #N808-0234
- *Thermo-Start DNA Polymerase* (250 units; ABgene, Hamburg, Germany)
  Catalog number #AB-0908
- *dNTP solution* (100 mM each; MBI Fermentas, St. Leon-Roth, Germany)
  Catalog number #R0181 (working solution: 2 mM each)
- *Random Primers* (1.5 mM, Invitrogen, Groningen, NL)
  Catalog number #48190011 (working solution: 25 µM)

Leave 3 µl of the diluted RNA for a 'no RT control' of the RT-PCR

- prepare a fresh master mix on ice according to the manufacturer´s protocol. Add the RNA template to the tubes containing the master mix.
  Incubate for 10 min at 25°C, then for 30 min at 48°C. Heat the mixture for 5 minutes to 95°C to inactivate Reverse Transcriptase. Cool down to 4°C rapidly.

The cDNA is now ready to use for RT-PCR.

➢ Protocol for cDNA amplification (example)

RT-PCR

As a control we at the PALM laboratory prefer amplifying the porphobilinogen deaminase gene (PBGD) for this is a houskeeping gene without showing any pseudogenes.

We usually make a nested PCR to get a better yield of PCR product.

Use 3 µl of the RT reaction if starting with 50 cells. For more or less than 50 cells you have to test what amount of cDNA template is best for using in RT-PCR. (We also used 1–10 µl of the cDNA template.)

It is recommended to additionally perform some controls for every PCR:
‚no RT control' (omitting RT reaction) with or without DNase digestion, genomic DNA control to check for pseudogenes, only PCR mix without any nucleic acid-template (water control).

To reduce the chance of contamination with exogenous nucleic acids, we only use special reagents and solutions for RNA isolation, reverse transcription and RT-PCR. All used solutions and tubes were prepared with DEPC treated water. We also recommend the use of safeseal tips.

Best results are obtained using freshly prepared cryosections.

Chromosome preparation

Protocols on request

Isolation of living cells

Protocols on request
Please note: There is also a review brochure availabe dealing with live cell laser micromanipulation.

For questions, remarks or protocol requests please contact PALM's Service & ApplicationLaboratory

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Service Line: +49-(0)8158-9971300