Cryosections: Laser Pressure Catapulting and downstream RNA examinations

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.

Buffers, Enzymes and Solutions

Enzymes and buffers are derived from the *Sensiscript RT-Kit*
Thermo-Start DNA Polymerase is used for all PCRs (Abgene, Hamburg, Germany).

- *RNeasy Mini Kit* (Qiagen)
  Catalog number # 74104 (50Rkt.)
- *RNase-free DNase Set* (Qiagen GmbH, Hilden, Germany)
  Catalog number # 79254 (50Rkt.)
- *Sensiscript RT Kit* (Qiagen GmbH, Hilden, Germany)
  Catalog number # 205211 (50Rkt.)
- *Omniscript RT Kit* (Qiagen GmbH, Hilden, Germany)
  Catalog number # 205111 (50Rkt.)
- *Thermo-Start DNA Polymerase* (250 units; ABgene, Hamburg, Germany)
  Catalog number # AB-0908
- *dNTP solution containing all four dNTPs (2mM each)*
- *RNAseOUT Recomb. RNase Inhibitor* (Invitrogen)
  Catalog number # 10777-019
- *Random Primers* (Invitrogen)
  Catalog number # 48190011
- *Proteinase K solution* 20mg/ml (Qiagen GmbH, Hilden, Germany)
  Catalog number # 19131
- *Catapult BUFFER:*

0,5M EDTA pH8,0	20µl
1M Tris pH8,0	200µl
Tween 20	50µl
(Proteinase K 20mg/ml	100µl)
ddH_2O DEPC treated, autoclaved	9,63ml

All solutions have to be treated with DEPC!

METHODS

Preparation of total RNA from microdissected cell samples using the *Sensiscript RT Kit*

Procedure
We perform catapulting of the cells into a buffer without Proteinase K. If using paraffine sections for catapulting please use a buffer containing Proteinase K like mentioned in the DNA protocol.

Best use freshly cut speciments, not older than 1 day.

Microdissection and Laser Pressure Catapulting

(a) take an autoclaved PALM cap
(b) pipette 2-3 µl of Catapult Buffer into the middle of the cap
(c) put the cap into the cap holder
(d) perform laser microdissection and laser pressure catapulting of wanted 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 sample immediately after catapulting at full speed for 2 minutes
(g) look into the cap again to make sure that the cells are no longer in the cap

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

Proteinase K digestion

a) After centrifugation pipette 11 µl of Catapult Buffer onto the catapulted cell(s) (which are now in the tip of the tube) to get a volume of 13µl
b) Vortex gently. Digest for 2 - 18 hours at 55°C followed by a heating step at 99°C for 10 min to inhibit Proteinase K activity
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.

If using *cryosections* you can go on straight forward with RNA extraction by using the **RNeasy Kit**

- After centrifugation of the cells make a fresh solution of 1ml buffer RLT (included in the RNeasy Kit) with 10µl ß-Mercaptoethanol. Pipette 350µl of the buffer onto the cells.
- incubate the mixture for 30 minutes at 42 °C
- go ahead with the RNeasy protocol step 3
  (it is recommended to perform a DNA digestion step)
- to elute the RNA from the RNeasy column pipette 100µl of RNAse free water instead of the default amount
- to minimize the volume of RNA precipitate the RNA immediately with an ethanol precipitation step (see the following protocol)

Ethanol precipitation

- pipette 5µg of glycogen to the eluated 100µl RNA, mix gently
- add 5µl of sodium acetate 0,5M pH5,0 to the mixture, mix gently
- pipette 250µl 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, discare the supernatant carefully by a pipetting step. Add 250µl of 70% ethanol to the pellet
- centrifuge for 15 minutes. Discare the supernatant carefully. CAVE: The pellet may be very slacky!
- Add 100µl of ethanol abs., centrifuge for 15 min, discare the supernatant carefully
- let the pellet airdry for 10 minutes under a sterile hood
- dissolve the pellet in 10 to 15µl 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

Reverse Transcription with the Sensiscript RT Kit

Sensiscript Reverse Transcriptase is designed for use with less than 50ng RNA. This amount corresponds to the entire amount of RNA present, including any rRNA, mRNA, viral RNA and carrier RNA.
For more than 50ng RNA we use the Omniscript Reverse Transcriptase Kit from Qiagen. It is designed for an amount of 50ng to 2µg 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). 0,5units/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 manufactured 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 inhibit Reverse Transcriptase activity. Cool down rapidly.

RT-PCR

The cDNA is now ready to use for RT-PCR.
We at PALM prefer amplifying the porphobilinogen deaminase gene for this is a houskeeping gene without any pseudogenes.

Every time we make a nested PCR to get a better yield of PCR product.

Use 3 µl of cDNA 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. Sometimes we used 1 – 10µl of the cDNA template.

1st PCR: PBGD For 1 and PBGD Rev 2
Fragment size cDNA 210 bp
DNA 561 bp

Cycle number	Denaturation	Annealing	Extension
1 cycle	15 min at 94°C		
35 cycles	30 sec at 94°C	30 sec at 60°C	40 sec at 72°C
1 cycle			10 min at 72°C

cool down to 4°C

2nd PCR: PBGD For 3 and PBGD Rev 4
Fragment size cDNA 138 bp
DNA 489 bp

Cycle number	Denaturation	Annealing	Extension
1 cycle	15 min at 94°C		
35 cycles	30 sec at 94°C	30 sec at 56°C	40 sec at 72°C
1 cycle			10 min at 72°C

It is recommended to additionally use some controls for every PCR:
„no RT control‟ with or without DNase digestion, DNA control for checking of pseudogens, only mix without nucleic acids.