Leica Protocols

Preparation protocols for Leica laser microdissection

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1. Preparation of membrane-coated glass slides

Membrane-coated slides (1mm for 20-40x; 0.17mm for 100x magnification [oil]) can be prepared as follows:

Gloves must be worn at all times.

Conventional microscopic slides are cleaned meticulously with acetone/ethanol and left to dry in a dust-free environment.

Slides are dipped in distilled water and mounted with appropriately sized membranes
(approx. 21mm x 42mm). Take care of wrinkles.

Make sure to leave at least 2mm uncovered glass surface around the edges.

After complete evaporation of water from the uncovered slide areas the membranes are sealed around the edges with clear nail varnish and left to dry for at least 1 hour at room temperature (RT).

Optional: Siliconising of glass slides by dipping into dimethyldichlorosilane and drying for 10 min. at 40°C. Siliconising of glass slides may improve efficiency of cutting/falling for larger samples.

Optional: apply nail varnish only to two sides of the foil and put the slide in an oven at 40 °C overnight. Then fix the other two sides of the foil with the nail polish. This will improve the drying and make sure that there is no water between foil and slide.

Leica offers prepared PEN foil slides ready for step 2

Immunohistochemical detection requires use of LEICA membrane-coated slides.

2. Applying specimen to slide

Optional: For reduction of electrostatical charge of the foil and possible destruction of RNases the slide is incubated for 30 min. in a UV chamber (max. power). UV will improve the fixation of the foil.

Optional (frozen sections): For better attachment of tissue/cells to foil tissue adhesive can be applied as follows. Apply shortly before use one drop of diluted tissue adhesive (TA, Diagnostic Products Corporation, van Golsteinlaan26, 7339 GT Apeldoorn, the Netherland) to foil. Spread gently over foil with pipette tip and remove excess TA. Perform powerful shaking with the arm such that only a thin film of TA remains on the foil. Allow the TA to dry for 15 min. at 37°C.

Collect tissue section (+/- 5 µm) on the foil. Allow tissue section to dry for 30 min. or 1h. respectively at room temperature.

3. Staining of sections on membrane-coated slides

3.1 Paraffin-embedded tissue

Dewax in xylol for 45 seconds (max. 3 minutes) , as otherwise the adhesive fixing the foil to the specimen slide will dissolve. As an alternative, intermedium substitutes such as Paraclear can be used instead of xylol.

For rehydration, the sections are immersed for 30 seconds each in absolute ethanol (3x), 96% ethanol (1x), 70 % ethanol (1x) and finally in distilled water.

Nucleus staining with haemalum (Mayer technique) for 5 min.

Rinse in tap water

Differentiate: depending on the recipe of the haemalum used, differentiation must be made in 0.5-1% HCL alcohol under microscope control (Mayer´s haemalum, prepared with the following recipe: dissolve 1g haematoxylin in 1000ml distilled water, then add, one after the other: 200 mg sodium iodate, 50 g potash alum, 50 g chloral hydrate and 1 g citric acid, does not require differentiation)

Bleach nuclei blue in tap water for 5 to 10 min. (microscope control)

The staining of the cytoplasm can be done with eosine or erythrosine for 1 min. A 1:1 mixture of the two solutions is recommended.

Rinse the sections well in distilled water then air- dry. If necessary, it is also possible to differentiate in 70-96 % ethanol and dry after returning to distilled water.

3.2 Fresh frozen tissue

Follow protocol for paraffin sections after rehydration step.

Alternative protocol:
Stain tissue section for 1 min. with haematoxyline or 10 seconds with methyl green or toluidin blue and rinse in sterile water. There are indications that methyl green or toluidin blue give better PCR results than haematoxylin. Carefully remove excess water by placing a filter on the sample and gentle striking with a finger. Allow to dry further for 30 min. at 37°C.

4. Immunostaining

4.1 Paraffin-embedded tissue

4.1.1 Optional heat-induced epitope retrieval (HIER)

Transfer the rehydrated 3µm paraffin sections into 1000ml HIER citrate pH 6.0 buffer.

10x stock	7,65g Citric acid 48,2g Sodium citrate water ad 2000ml

Bring to the boil in a pressure cooker for 1-3 minutes, let equilibrate to room temperature and transfer into TBS pH 7.4 (50 mM Tris, 150 mM NaCl).

4.1.2 Alkaline Phosphatase Immunolocalization

Incubate sections in DAKO Protein Block (No. X0909) to reduce unspecific binding. Incubate the section in 100mµl TBS/0.5 % BSA with the first antibody for 1 h at room temperature or 37°C (alternatively overnight at 4°C).

Wash section once with TBS-Tween 0.01%, once with TBS.

Incubate the section in 100mµl TBS/0.5 % BSA with the second biotinylated antibody for 30min at room temperature or 37°C.

Wash section once with TBS-Tween 0.01%, once with TBS.

Incubate the section in 100µl Streptavidin-Alkaline Phosphatase conjugate (ABC-Kit Vector No. AK5000) for 30 min at 37°C.

Wash section once with TBS-Tween 0.01%, once with TBS.

Develop with Sigma Fast Red chromogen under microscopic control.

Wash section once with distilled water.

Counter stain with Mayer's Haemalum.

Bleach nuclei blue in tap water for 5 to 10 min. (microscope control)

Coverslip the section in Aquatex.

4.2 Fresh frozen tissue

Frozen tissue is cut at preferably 3-5µm thickness in a cryostat.

Sections are fixed in acetone 0.1% NP40 for 5 min.

Proceed to incubation in DAKO Protein Block (Nr.: X0909) to reduce unspecific binding as above under 4.1.

5. Preparation of microdissectate for DNA analysis

Microdissectates (frozen or paraffin-embedded) are collected individually or pooled in 20-100µl of lysis buffer (10 mM Tris-HCl pH 8.0, 1% Tween-20).

After capping the solution is spun for 15 s and 1-5µl of Proteinase K stock solution (e.g. 100mg/ml) is added.

The microdissectate solution is kept at 55°C for a minimum of 60min (preferably o/n).

Proteinase K is heat inactivated 99°C for 10 min.

Use 1-10µl aliquots in subsequent PCR analyses.

Alternatively spin column extraction (QIAGEN DNA blood/tissue kits) can be used.

6. Preparation of microdissectate for RNA analysis/ cDNA synthesis

It is recommended to wear gloves, to work, if not otherwise stated on ice and to use RNase-free tubes and DEPC-water.

Microdissectates (frozen or paraffin-embedded) are collected individually or pooled in 20-100µl of QIAGEN RNAeasy Mini Kit (Cat. No. 74 106) LGT/ß-ME buffer dispensed centrally in the lid of an 0.5ml Eppendorf cup.

After capping the solution is spun for 15 s and transferred into 1.5ml Eppendorf cup.

Follow QIAGEN RNAeasy protocol for further procedures.

Elute into 30µl of distilled water and keep at -80°C

6.1 First strand cDNA synthesis of RNA purified from microdissected tissue

Customary precautions for RNA handling should be observed.

Per reaction the Reverse Transcription Master Mix consists of

4 µl	dNTP-Mix; 10 mM each	(Applied Biosystems N8080260)
4 µl	MgCl2; 25mM	
2 µl	GeneAmp 10x Buffer II	(Applied Biosystems N8080010)
1 µl	Random hexamer primers	(Applied Biosystems N8080127)
1 µl	MuLV reverse transcriptase	(Applied Biosystems N8080018)
1 µl	RNAse Inhibitor (optional)	(Applied Biosystems N8080119)

Distilled water ad 13µl, mix at RT and use up to 8µl of the RNA solution (see 5.) in a 20µl total reaction volume.

Leave 10min at RT, then move to 42°C for 60min.

Use 2-10µl aliquots in subsequent PCR analyses.

7. PCR (Polymerase Chain Reaction)

(Mullis et al. 1987, Meth. Enzymol 155, 335-350 ; Sambrook et al. 1989, Molecular cloning: A laboratory manual, Cold Spring Harbour, New York; Cold Spring Harbour Laboratory Pres

The PCR is used to amplify defined DNA sequences using special oligonucleotid primer. The cyclic reaction is carried out in three steps: The denaturation of the DNA by heat, the sequence specific annealing of the primer and the polymerisation by the heat stable (Taq)-polymerase.

For example:

PCR reaction:

- 1x Taq-buffer complete with 1.5 mM MgCl2
- dNTP-mix (à 200µM)
- (Taq)-polymerase (1u)
- Primer 1 (2 µM)
- Primer 2 (2 µM)
- Template DNA
- A.dest to 50 µl

cycle	Denaturing	Annealing	polymerisation
1	95°C, 2 min.		
30	94°C, 30sec.	50°C, 45sec.	72°C, 1min
1			72°C, 5min

An aliquot of the amplified DNA is ready for agarose gel analysis

8. Protein-Analysis

The cells of the section allow also protein analysis, e.g.

- SDS-PAGE
- westernblotting
- 2-D gel elektrophoresis.

For example the cells can be directly cutted into SDS-sample buffer (10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0,05% (w/v/) Bromphenolblue, 2 mM Tris-base), heated and loaded to a normal polyacrylamid gel (Laemmli, U.K. 1970, Nature 227, 680-685).
For two-dimensional gel electrophoresis IEF-buffer (e.g. 9.5 M urea, 2% NP40, 5% 2- mercaptoethanol) (O´Farrell 1975, J Biol Chem, 250, 4007-4021; O´Farrel, Goodman 1977, Cell, 12, 1133-1142; Shoeman, Schweiger 1982, J Cell Sci, 58, 23-33) can be used.
Westernblot analysis can be carried out following standart procedure (e.g.Towbin, Staehelin, Gordon, 1979, Proc Natl Acad Sci USA, 76, 4350-4354)

9. In situ hybridisation

Standart protocols for in situ hybridisation. The foil is heat stable for more than 120 °C

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10. Cytospins

Spin cells with a centrifuge to the slide using standart protocol. It is also possible to spin cells to Menzel Superfrost slides without foil.
Human white blood cells:
Cytospin methanol fixed human white blood cells on microdissection slide. A drop of fixed cells is pipetted into 1 ml of 50% acetic acid (to remove cytoplasmic proteins) contained in a specially designed centrifuge bucket. After centrifugation, the slide is air dried and Giemsa stained according to the above mentioned procedure

11. Blood smear

One drop of freshly collected full blood is smeared on a foiled microdissection slide, air dried overnight and Giemsa stained according to the above protocol.

12. Chromosome preparation

Chromosomes from white blood cells from human peripheral blood
Cells were cultured for 72 hours, then treated with colcemid (to arrest cells in metaphase), then treated with a hypotonic solution (to let them swell), and finally fixed in methanol/acetic acid (3+1, v/v). Fixed cells were dropped on the foiled microdissection slides and were air dried overnight. Then they were stained with Giemsa staining solution (1:20 diluted in Gurr buffer, which is a standard buffer used for Giemsa staining made by dissolving commercially available tablets) for 3 minutes. After washing in water they were air dried.

13. Living cells

It is recommended to UV treat the PEN foil for sterilization. Then cells can be grown in culture medium at the foil. Single cells can be selected by laser cutting.