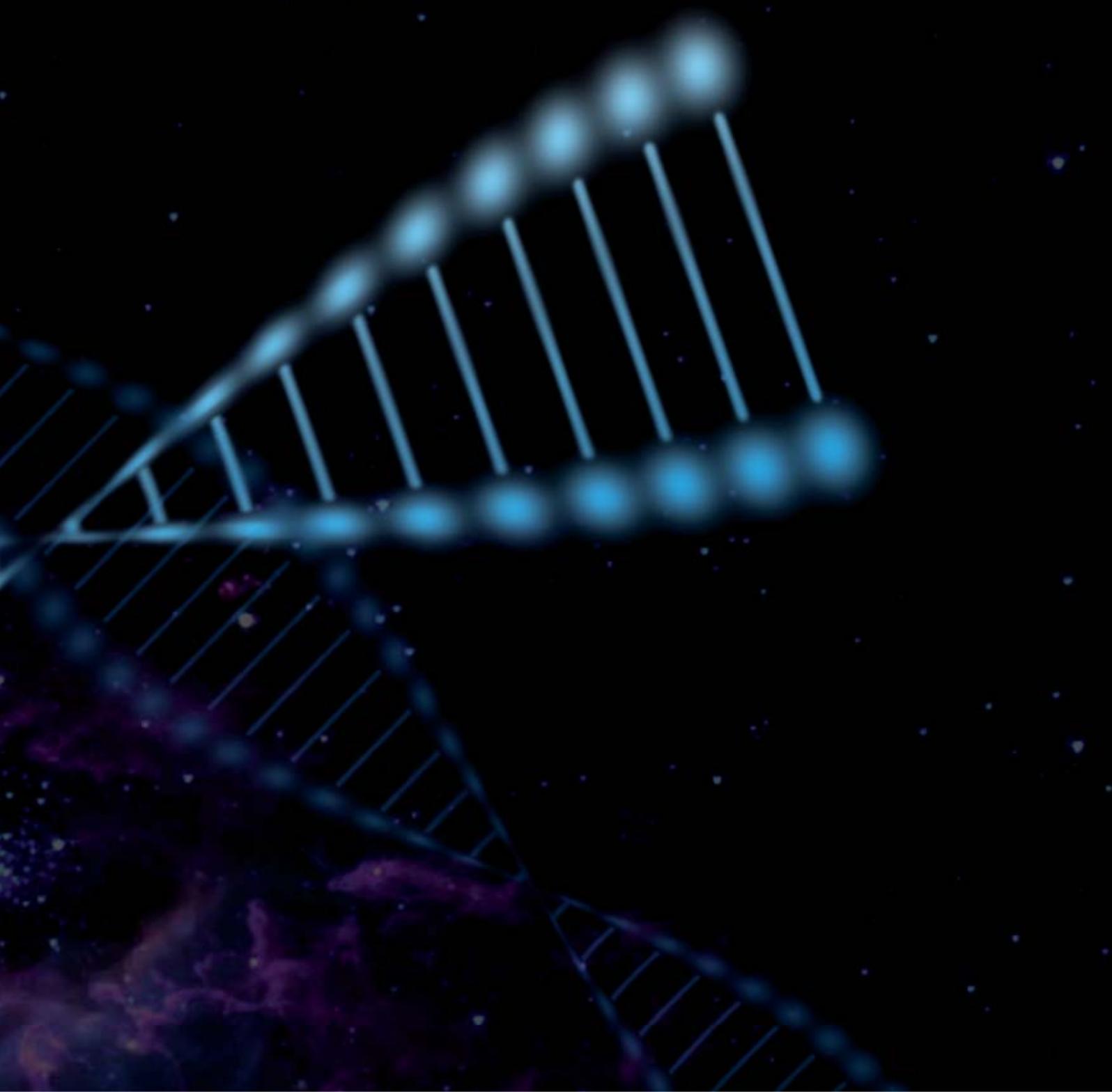


Carl Zeiss MicroImaging

PALM Protocols - DNA handling





We make it visible.

PALM Protocols

DNA handling

Non contact Laser Capture Microdissection

Content

5	Introduction - Some remarks on DNA
6	Preparation of slides – Samples on MembraneSlide
8	– Samples on glass slides
8	– Archived Samples: removing the coverslip
9	Treatment of slides – Heat treatment
9	– UV treatment
9	– Poly-L-Lysine treatment
10	Mounting samples onto slides
10	Frozen sections
10	Formalin Fixed Paraffin Embedded (FFPE) sections
11	Cytospins
11	Blood and tissue smear
12	Staining procedures
12	Storage
12	Formalin Fixed Paraffin Embedded (FFPE) sections
12	Frozen sections – Cresyl Violet
13	– Hematoxylin/Eosin (HE)
13	– Methyl Green
13	– Methylene Blue
13	– Toluidine Blue
13	– Nuclear Fast Red
14	Non-contact Laser Capture Microdissection (LCM) Procedure
14	Tips to improve morphological information
14	Diffusor CM
15	AdhesiveCap opaque
15	Liquid Cover Glass
16	Collection devices
16	AdhesiveCap
16	Other microfuge tubes
16	AmpliGrid AG480F
17	Collection procedures
17	„Dry“ collection (AdhesiveCap)
18	„Wet“ collection (other microfuge tubes)
19	„Wet“ collection onto Slide48 (AmpliGrid AG480F)
19	Capture check – looking into the cap to see the lifted samples
20	Downstream Applications
20	DNA isolation from FFPE sections
20	DNA isolation from frozen sections
21	PCR setup – Standard PCR (20 µl) in a capillary cycler
22	– High volume PCR (50 µl) in a 96-well block cycler
22	– Low volume PCR (1 µl) in an Eppendorf
22	Mastercycler with In situ Adapter

Introduction

Some remarks on DNA

Human Genome Project has shown new insights to poorly understood biological phenomena. As a result of this expansion of genomics into human health applications, the field of genomic medicine was born. Genetics is playing an increasingly important role in the diagnosis, monitoring, and treatment of diseases.

Further areas that stand to benefit from these results include biomedical and biological research, toxicology, drug design, forensics, animal and plant genetics, and many others.

In all special fields the methods for molecular testing must be able to determine and analyze DNA sequences accurately and rapidly, and whenever possible easy to use, highly automated, and minimized.

Beside these requirements on the molecular genetic method, the step before
- *generation of a defined sample as source for the analysis* -
is of prime importance.

Non-contact Laser Capture Microdissection (LCM) from Carl Zeiss is state of the art for sample preparation.

Preparation of slides

– Samples on MembraneSlide



*Regular glass slide (1 mm thick) => 1,
thin slide (0.17 mm thick) => dot,
DuplexDish and FrameSlide => between dot and 0.*

MembraneSlide is a glass slide covered with a membrane on one side.

This membrane is easily cut together with the sample and acts as a stabilizing support during lifting. Therefore even large areas are lifted by a single laser pulse without affecting the morphological integrity of the tissue. Use of MembraneSlide is especially recommended for isolating single cells or chromosomes as well as live cells or small organisms. Carl Zeiss MicroImaging (CZMI) offers slides (1 mm, 0.17 mm) covered with Polyethylene Naphthalate (PEN)-membrane or Polyethylene Teraphthalate (PET)-membrane.

PEN-membrane is highly absorptive in the UV-A range, which facilitates laser cutting. The membrane can be used for all kind of applications.

MembraneSlide NF (nuclease free) is certified to be free of DNase, RNase and human DNA.

In addition to PEN-MembraneSlide, CZMI also offers PET-membrane covered slides. These slides are helpful for special processes, i.e. fluorescence applications. Even weak fluorescence signals can be detected with PET-slides, due to the low signal to noise ratio.

Alternatively the PET-membrane can be attached to a metal frame (FrameSlide PET). The frame structure of FrameSlide PET is resistant to microwave treatment or pressure cooking. The special bonding is inert and adapted to heat treatment (up to 95°C) so that the membrane does not ruffle during the heating process. If you need further information about these slides, please contact:

E-Mail: palm-labs@zeiss.de

When working with low magnifying objectives like 5x or 10x, both regular 1 mm thick glass slides and 0.17 mm glass slides can be used. To keep this flexibility for higher magnifications (20x, 40x or 63x) CZMI recommends using long distance objectives.

With those the working distance can be adapted to the different glass slides by moving the correction collar on the objective. (see picture above)

Due to the short working distance only 0.17 mm thin cover glass slides can be used with the 100x magnifying objectives.



- MembraneSlide 1.0 PEN* - Order No. 415101-4401-000 (white)
- MembraneSlide 1.0 PEN NF* - Order No. 415101-4401-600 (white)
- MembraneSlide 0.17 PEN* - Order No. 415101-4401-500 (uncolored)
- MembraneSlide 50x1.0 PEN* - Order No. 415101-4401-005 (doublewidth)
- MembraneSlide 1.0 PET* - Order No. 415101-4401-050 (blue)
- FrameSlide PET* - Order No. 415101-4401-055 (metal)

Preparation of slides – Samples on glass slides

With PALM MicroBeam almost every kind of biological material can be microdissected and lifted directly from regular glass slides, also see Application Note: „AutoLPC from glass slide result in good quality RNA“. Even archival sections can be used after removing the coverslip and the mounting medium.

To facilitate lifting 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). In those cases higher laser energy is needed for lifting.

Archived Samples: removing the coverslip

Depending on the applied mounting medium (whether it was xylene based or water soluble) the whole slide should be completely submerged in the respective solvent.

1. standing up in a glass jar filled with either pure xylene or warm water (30-50°C)
2. time needed for the coverslip to swim off may range from hours to days
3. air-dry the slide

Note: It is very important NOT to use any force to push off the coverslip because this might damage the section! Wait till it falls off by itself! The necessary time depends on the age of the sample and the dryness of the mounting medium. Fresh slides (only days old) can be decovered much faster.

From the dry glass slide sample material can be lifted directly by “AutoLPC” function of PALM RoboSoftware.

Treatment of slides

Slides are shipped *without* any pretreatment.

To remove nucleases and DNA, MembraneSlides and glass slides can be treated in the same way.

MembraneSlide NF (nuclease free) is certified to be free of DNase, RNase and human DNA. Treatments to remove nucleases and contaminating DNA are therefore not necessary using these slides.

Heat treatment

To ensure nuclease-free MembraneSlides (1mm) or glass slides, heat slides at 180°C in a drying cabinet for 4 hours to completely inactivate nucleases.

Note: The thin (0.17 mm) MembraneSlides are not resistant against heat. Use other treatments to remove nucleases.

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- as well as cryosections) adhere better.

Positive side effects are sterilization and destruction of potentially contaminating nucleic acids.

Poly-L-Lysine treatment

Additional coating of the slide with Poly-L-Lysine (0.1% w/v, e.g. SIGMA, #P8920) 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 slide.

Let air-dry at room temperature for 2-3 minutes.

Avoid any leakage of the membrane, as this might result in impairment of Laser Capture Microdissection.

Mounting samples onto slides

Frozen sections

Sectioning

Sections are mounted onto MembraneSlides the same way as routinely done using glass slides. To allow subsequent cutting and lifting by the laser 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.

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 the medium is easily done by dipping the slide 5-6 times in water. If the sections are stained in aqueous solutions, the supporting substance is normally removed "automatically" by the water containing steps.

Formalin Fixed Paraffin embedded (FFPE) sections

Sectioning

Floating the section on warm water (40°C) as well as hot plate techniques can be applied.

After mounting the section let the slides dry overnight in a drying oven at 56°C to improve the adhesion of the sections to the membrane. To allow laser cutting and lifting a coverslip and standard mounting medium must not be applied.

Archival sections with mounting medium and coverslip have to be processed as described to remove the coverslip (see page 8).

Deparaffination

Paraffin will reduce laser efficiency, sometimes completely inhibiting cutting and lifting. If you are working with unstained sections it is therefore very important to remove the paraffin before laser cutting and lifting. 1 mm MembraneSlides can be handled like normal glass slides.

Deparaffination Procedure

1. Xylene	5 minutes, 2 times (2 minutes minimum)
2. Ethanol 100%	1 minute
3. Ethanol 96%	1 minute
4. Ethanol 70%	1 minute

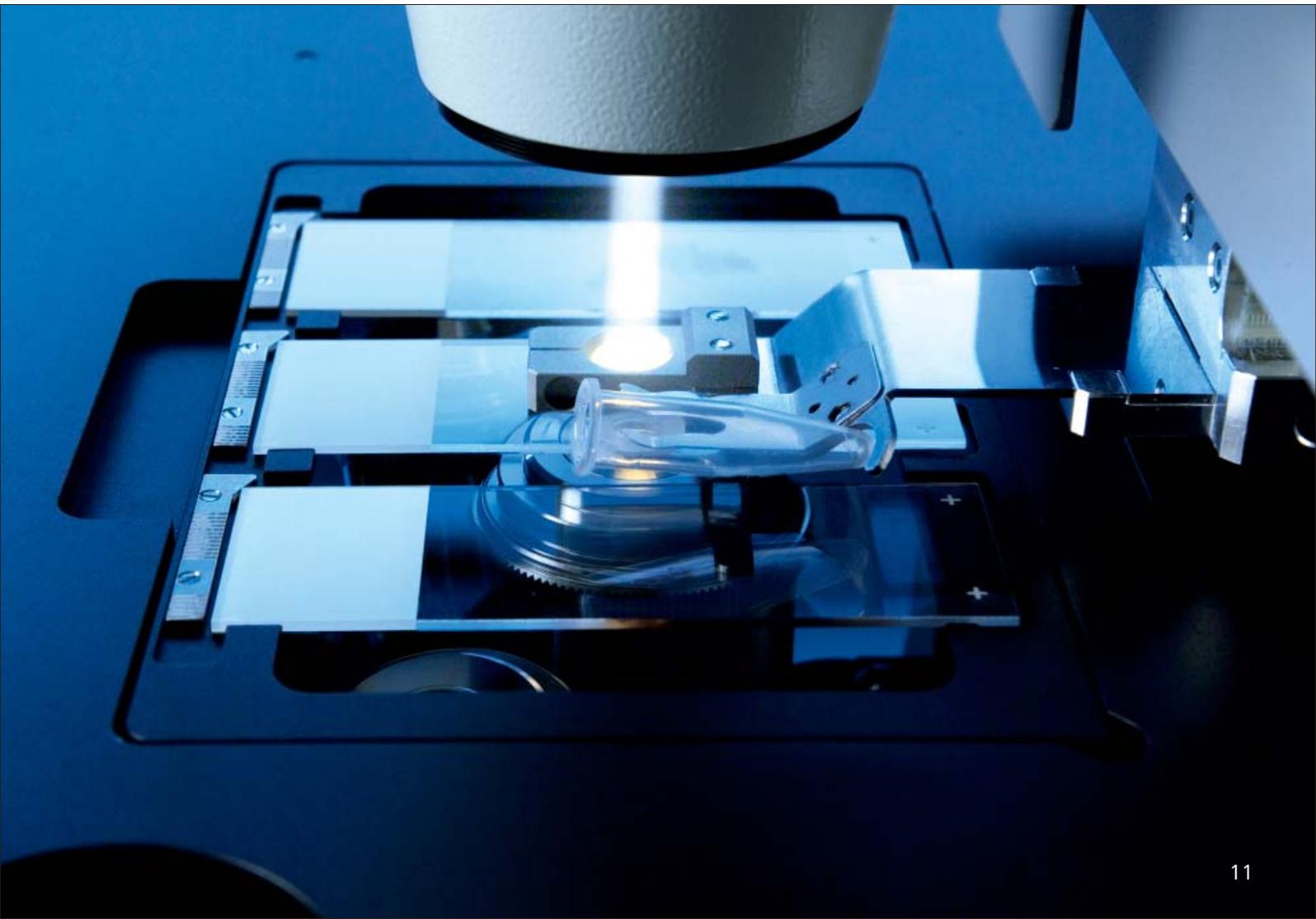
Note: The thin (0.17 mm) MembraneSlides are not as resistant against organic solvents and should only be handled according to a minimal procedure. Especially longer treatment in xylene will resolve the glue holding the membrane to the slide.

Cytospins

Cytospins can be prepared on glass slides or on MembraneSlides.
After centrifugation in a cytocentrifuge let the cells air-dry at room temperature.
Then fix for 2 minutes in 70% ethanol and air-dry again before staining.

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 would impair the Laser Capture Microdissection process.
Let smears air-dry shortly and fix them for 2 up to 5 minutes in 70% ethanol.



Staining procedures

For isolation of high quality DNA use freshly prepared, autoclaved solutions.

Formalin Fixed Paraffin Embedded (FFPE) sections

After deparaffination (see page 10) continue with the staining procedure of your choice. Most staining procedures for frozen sections can be applied for FFPE sections (for recommendations see 'Frozen sections').

Frozen sections

Most standard histological stainings (e.g. HE, Methyl Green, Cresyl Violet, Nuclear Fast Red) are compatible with subsequent DNA isolation.

At PALM Laboratories we usually perform the Cresyl Violet or Hematoxylin/Eosin (HE) staining.

Storage

Stained slides can be used immediately or stored. If the slides have to be stored in a freezer before LCM, the slides should be frozen in a tightly sealed container (e.g. two slides back to back in a 50 ml Falcon-tube) to avoid excess condensation of moisture during thawing.

For rethawing the container should not be opened before it is completely warmed up again to ambient temperature.

Cresyl Violet

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

Staining Procedure

1. after fixation (2 min, 70% Ethanol) dip slide for 30 sec into 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 min)

(*) Dissolve solid cresyl violet acetate (e.g. ALDRICH #86,098-0) at a concentration of 1% (w/v) in 50% EtOH at room temperature with agitation/ stirring for several hours to overnight. Filter the staining solution before use to remove unsolubilized powder. Sometimes Lot to Lot variations in the purchased cresyl violet powder can lead to weaker staining results if the dye content is below 75%.

Note: In most cases this cresyl violet staining procedure will be sufficient for cell identification. If an enhancement of the staining is desired, a reinforcement by two additional steps in 50 % ethanol (first, before staining in cresyl violet; second, after the staining in cresyl violet) is possible.

Ambion offers the LCM Staining Kit (#1935) which also contains a cresyl violet dye. When using this kit we strongly recommend to omit the final xylene step of the Ambion instruction manual because xylene makes the tissue very brittle and reduces the adhesion of the section to the PEN-membrane.

Hematoxylin/Eosin (HE)

HE-staining is used routinely in most histological laboratories. The nuclei are stained blue, the cytoplasm pink/red.

Staining Procedure

1. after fixation (2 min, 70% Ethanol) dip slide 5-6 times in distilled water
2. stain 1-2 minutes in Mayer's Hematoxylin solution (e.g. SIGMA, #MHS-32)
3. rinse 1-2 min in distilled 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 (1-2 min)

Toluidine Blue

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

Staining Procedure

1. after fixation (2 min, 70% Ethanol) dip slide 5-6 times in distilled water
2. stain 30 seconds in Toluidine Blue solution (0.1 % in water; SIGMA, #T-0394)
3. rinse in distilled water
4. perform a quick increasing ethanol series (70%, 96%, 100%)
5. air-dry shortly (1-2 min)

Methyl Green

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

Staining Procedure

1. after fixation (2 min, 70% Ethanol) dip slide 5-6 times in distilled water
2. stain 5 minutes in Methyl Green solution (DAKO, #S1962)
3. rinse in distilled water
4. air-dry shortly (1-2 min)

Methylene Blue

The nuclei are stained dark blue.

Staining Procedure

1. after fixation (2 min, 70% Ethanol) dip slide 5-6 times in distilled water
2. stain 5-10 min in Methylene Blue solution (0.05 % in water; SIGMA, #31911-2)
3. rinse in distilled water
4. air-dry shortly (1-2 min)

Nuclear Fast Red

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

Staining Procedure

1. after fixation (2 min, 70% Ethanol) dip slide 5-6 times in distilled water
2. stain 5 to 10 minutes in Nuclear Fast Red solution (DAKO, #S1963)
3. rinse in distilled water
4. air-dry shortly (1-2 min)

Non-contact Laser Capture Microdissection (LCM) Procedures

Please, additionally have a look into the PALM MicroBeam user manual.

Tips to improve morphological information

For LCM 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. Diffusor, AdhesiveCap as well as Liquid Cover Glass are comparable to the coverslip for enhanced visualization.

Diffusor CM

Holders for PALM RoboMover and PALM CapMover II are equipped with diffusors. 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 Diffusor CM product information.



PALM CombiSystem



Diffusor CM - Order No. 415101-2100-320

AdhesiveCap opaque

The white/opaque filling of AdhesiveCap clearly improves visualization of morphological information of the samples due to enhanced color balance and contrast, which makes the view comparable to those of coverslipped tissue sections. Two different microfuge tube sizes (200 μ l, 500 μ l) with these filled caps are available from CZMI. For more details and handling, please see AdhesiveCap product information.



*AdhesiveCap opaque - Order No. 415101-4400-250 (500 μ l)
AdhesiveCap opaque - Order No. 415101-4400-240 (200 μ l)*

Liquid Cover Glass

The polymeric and low viscose Liquid Cover Glass completely embeds the tissue and smoothens the rough tissue surface, resulting in enhanced morphology. For more details and handling, please see Liquid Cover Glass product information.



Liquid Cover Glass - Order No. 415190-9020-000

Collection devices

AdhesiveCap

The intention of AdhesiveCap is to allow LCM (Laser Capture Microdissection) without applying any capturing liquid into the caps prior to LCM.

This minimizes nuclease activity.

Beside the quick relocation of the lifted samples inside the cap due to instant immobilization there is no risk of evaporation and crystal formation of the buffer during extended specimen harvesting. For more details and handling, please see also AdhesiveCap product information.

Note: CZMI recommends AdhesiveCap as a collection device.

Other microfuge tubes

Other commercially available plasticware can be used, too.

(e.g. ABgene #AB-0350; 0.5 ml tubes)

AmpliGrid AG480F

Using the SlideCollector 48 in conjunction with AmpliGrid technology from Advantix enables working in a higher throughput LCM (48 samples simultaneously).

The AmpliGrid technology allows DNA analysis in an extremely low volume (1 μ l) directly on chip.

Please, see the brochure:



AdhesiveCap opaque - Order No. 415101-4400-250 (500 μ l)

AdhesiveCap opaque - Order No. 415101-4400-240 (200 μ l)

AdhesiveCap clear - Order No. 415101-4400-255 (500 μ l)

AdhesiveCap clear - Order No. 415101-4400-245 (200 μ l)

Microdissection from Carl Zeiss

On-Chip Single Cell Analysis for PALM MicroBeam

Contemporary techniques in molecular biology have begun to solve a vast number of previously unanswered questions. However, the inherent reliability of an analytical method is always limited by the quality of the available starting material.

PURITY
PALM MicroBeam from Carl Zeiss enables recovery of ultra-pure starting material from tissue preparations as well as from cell cultures in vitro (first step). Using the PALM RoboPicker high-throughput capture device in unique conjunction with the AmpliGrid slide it is possible to control up to 48 discrete reaction sites.

LOW VOLUME
With the AmpliGrid technology from Advantix, DNA amplification and cycle sequencing are possible in an extremely low volume reaction format. The reaction is carried out on-chip by using single cells as the template source (second step).

The combination of both technologies opens new doors to the study of genomics.

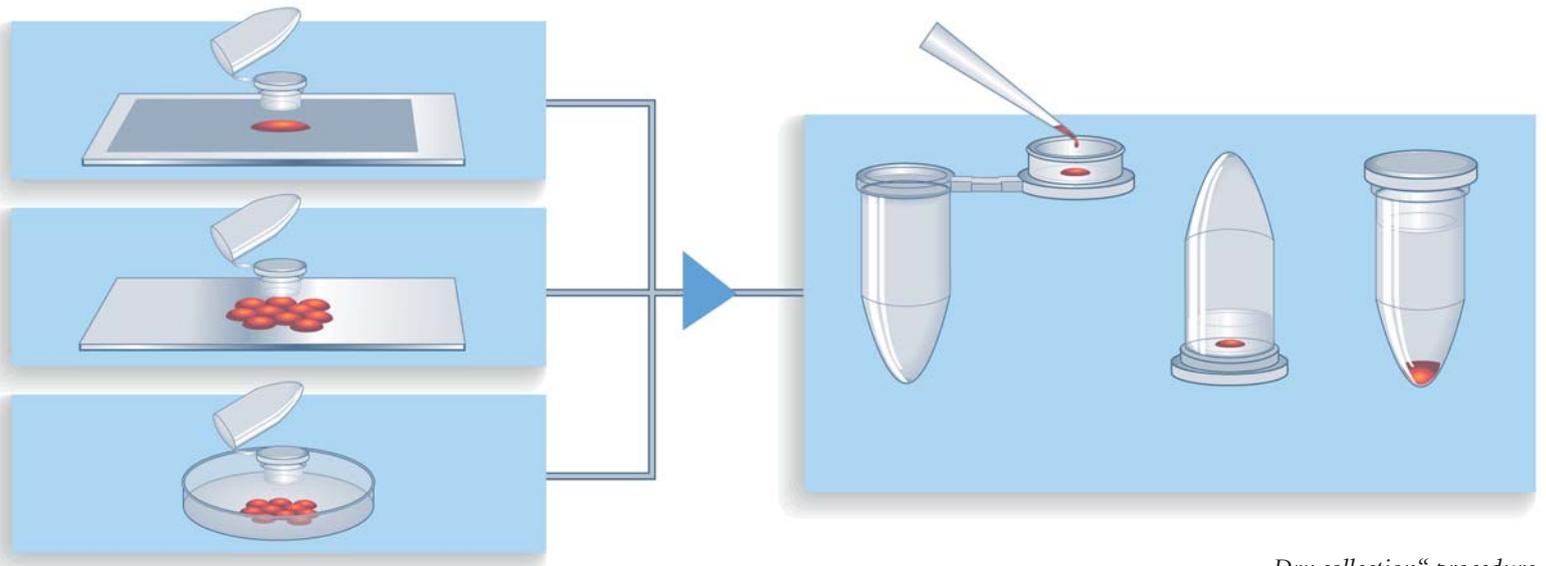
No elaborate template preparation.
Accurate to a single cell.
Pooling of cells not necessary.
Pool the results of up to 48 individual cell reactions.

Single cells were captured onto AmpliGrid Ac480F reaction reaction slide (Advantix).
For the 552 opening the PowerBlock (Promega) in one and the same time reaction for 48 samples.
AmpliGrid 480 Primer Kit 5000 (Applied Biosystems).

For Single Cell Genotyping,
Sequencing and Expression Analysis:
Reliable, Safe, Reproducible

ZEISS
We make it visible.

On-chip Single Cell Analysis for PALM MicroBeam.



„Dry collection“ procedure

Collection procedures

Please have a look into the PALM MicroBeam user manual.

„Dry“ collection (AdhesiveCap)

Note: CZMI recommends AdhesiveCap as collection device for most experiments.

1. put the AdhesiveCap into the collector
check the right position of the correction collar (see page 6)
2. perform non-contact LCM of selected cells
3. after LCM add 15 µl lysis buffer to the sample inside the cap
(QIAamp® DNA Micro Kit #56304)
4. add 10 µl Proteinase K (20 mg/ml) and mix by pulse-vortexing for 15 sec
5. place the tube in an “upside down” position in an incubator at 56°C for 2 - 18 h with occasional agitation
6. centrifuge the tube at full speed for 5 min (Hettich Centrifuge, Universal 32R)

If not going on immediately, store the samples at -20°C.

Note: The time necessary for complete Proteinase K digestion depends on the kind and the amount of collected material. After the Proteinase K digest the QIAamp® DNA Micro Kit #56304 (page 21, step 4) can be attached.

Collection procedures

„Wet“ collection (other microfuge tubes)

When using “unfilled” routine microfuge tubes it is necessary to fill a liquid into the cap to improve the adhesion of the captured cells.

The detergent Igepal CA-630 in the capturing buffer allows to smear out a small amount of liquid in the whole cap area.

Note: All kinds of aqueous solutions will run dry with extended working time.

Prerequisites - Capturing Buffer

0.05 M EDTA pH 8.0	20 µl
1 M Tris pH 8.0	200 µl
Igepal CA-630 (SIGMA #I-3021)	50 µl
(Proteinase K 20 mg/ml)	(100 µl)
ddH ₂ O	fill up to 10 ml
Proteinase K Solution	20 mg/ml (Qiagen #19131)

Final Concentration: 20 mM Tris, 0.1 mM EDTA, 0.5% Igepal, 1% Proteinase K

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

Note: The time necessary for complete Proteinase K digestion depends on the kind and the amount of collected material. After the Proteinase K digest and the inactivation step the routine downstream application of choice can be continued with.

If not going on immediately, store the samples at -20 °C.

Collection Procedure

1. take an autoclaved microfuge tube
2. pipette 3-15 µl of Capturing Buffer without Proteinase K or DNase-free water in the middle of the cap
3. put the cap/tube into the collector
check the right position of the correction collar (see page 6)
4. perform non-contact LCM of selected cells
5. centrifuge the tube at full speed for 5 min (Hettich Centrifuge, Universal 32R)
6. add 10-15 µl Capturing Buffer containing Proteinase K and mix by pulse-vortexing for 15 sec
7. incubate the tube at 56°C for 2-18h with occasional agitation
8. centrifuge the tube at full speed for 5 min (Hettich Centrifuge, Universal 32R)
9. final heating step at 90°C for 10 min to inactivate Proteinase K
10. centrifuge the tube at full speed for 5 min (Hettich Centrifuge, Universal 32R)

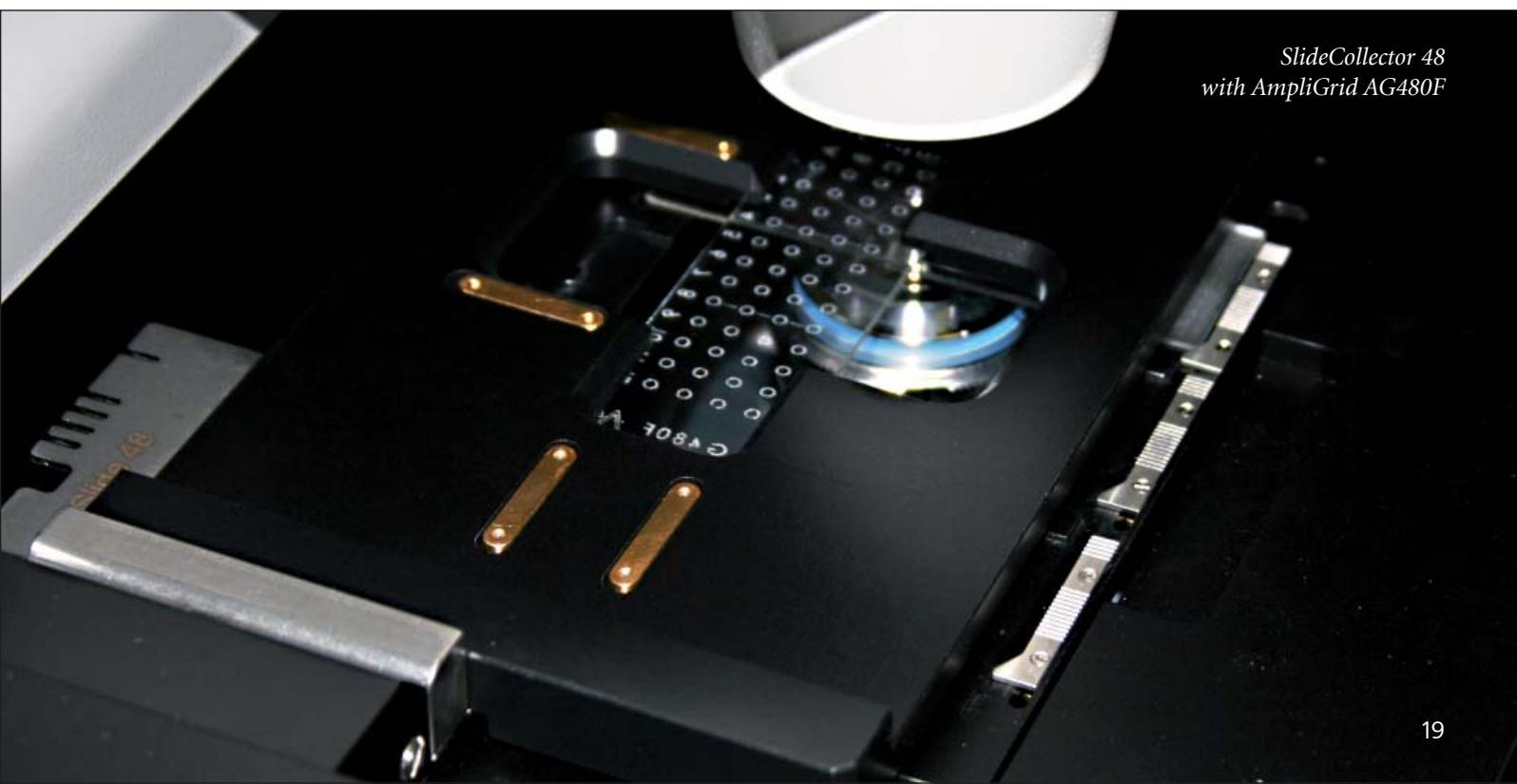
„Wet“ collection onto Slide48 (AmpliGrid AG480F)

A preloading of 48 ReactionSites of the AmpliGrid with 1 μ l liquid (e.g. 1% Glycerol in water) enables elongation of the working time and increases adhesion of the captured samples. The LCM process onto 48 reaction sites can be operated automatically and is controlled by PALM RoboSoftware. Using Slide48 technology, DNA amplification doesn't require any template transfer and preparation. Analysis (PCR, cycle sequencing) can be performed on the same reaction site of the AmpliGrid AG480F (see page 23): Low volume PCR (1 μ l) in a Eppendorf MasterCycler.

Capture check – looking into the cap to see the lifted samples

To control and document the efficiency of lifting it is possible to have a look into the collection device (e.g. microfuge cap) with the 5x, 10x, 20x, 40x and 63x objectives.

By using the software function “go to checkpoint” the slide is moved out of the light path and the objective lifted for looking inside.



*SlideCollector 48
with AmpliGrid AG480F*

Downstream Applications

DNA isolation from FFPE sections

Deparaffination and staining are done according to standard procedures for slides (please see page 10,12).

Note: Proteinase K digestion step is essential for formalin fixed samples.

The time necessary for optimal Proteinase K digestion depends on many factors like tissue type, fixation procedure or element size of lifted material. An overnight digestion (12-18 hours) is a good starting point for optimization but shorter digestion times may be tested as well. To our experience at least 3 hours digestion should be applied with any extraction procedure and material.

For subsequent DNA extraction from FFPE sections PALM Laboratories prefer the QIAamp DNA Micro Kit (#56304), please see DNA isolation from frozen sections.

DNA isolation from frozen sections

To capture microdissected samples from frozen sections we recommend the use of AdhesiveCap.

For DNA isolation any procedure of choice can be used.

In our hands the QIAamp® DNA Micro Kit (#56304) combined with AdhesiveCap results in good yield and quality of DNA. This QIAamp® DNA Micro Kit is designed for use of small amounts of tissue. The subsequently described protocol is suitable even for single cells.

Note: Incubating the QIAamp MinElute Column loaded with water for 5 min at room temperature before centrifugation generally increases DNA yield.

Diluted solutions of nucleic acids (e.g. dilution series used as standards) should be stored in aliquots and thawed once only. We recommend storage of aliquots in siliconized tubes if possible.

This avoids adsorption of nucleic acids to the tube walls, which would reduce the concentration of nucleic acids in solution.

Applying the components of the QIAamp® Micro Kit for isolation of genomic DNA from Laser Microdissected tissues:

1. Add 15 µl ATL to the microdissected sample in the AdhesiveCap.
2. Add 10 µl Proteinase K and mix by pulse-vortexing for 15 sec.
3. Place the 0.2 ml tube in an “upside down” position at 56°C in an incubator for 2-18h with occasional agitation.
Note: The time necessary for complete Proteinase K digestion depends on the kind and the amount of collected material.
4. Add 25 µl Buffer ATL and 50 µl Buffer AL, close the lid and mix by pulse-vortexing for 15 sec. To ensure efficient lysis, it is essential that the sample and Buffer AL are thoroughly mixed to yield a homogeneous solution.
5. Add 50 µl ethanol (96-100%), close the lid, and mix thoroughly by pulse-vortexing for 15 sec. Incubate for 5 min at room temperature (15-25°C).
If room temperature exceeds 25°C, cool the ethanol on ice before adding to the tube.
6. Briefly centrifuge the 0.2 ml tube to remove drops from the lid.
7. Carefully transfer the entire lysate to the QIAamp MinElute column without wetting the rim, close the lid, and centrifuge at 6000 x g (e.g. Eppendorf 5415D: 8000 rpm) for 1 min. Place the QIAamp MinElute Column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
If the lysate has not completely passed through the column after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute Column is empty.
8. Carefully open the QIAamp MinElute Column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute Column in a clean 2 ml collection tube, and discard the collection tube containing flow-through.
9. Repeat step 8.
Note: Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol - coming into contact with the QIAamp MinElute Column. Take care when removing the QIAamp MinElute Column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute Column.
10. Centrifuge at full speed (20,000 rpm x g; 14,000 rpm) for 3 min to dry the membrane completely. This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.
11. Place the QIAamp MinElute Column in a clean 1.5 ml microcentrifuge tube (not provided) and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute Column and apply 20 µl distilled water to the center of the membrane. Ensure that distilled water is equilibrated to room temperature (15-25°C). Dispense distilled water onto the center of the membrane to ensure complete elution of bound DNA.
Note: QIAamp MinElute Columns provide flexibility in the choice of elution volume. Choose a volume according to the requirements of the downstream application. Remember that the volume of eluate will be up to 5 µl less than volume of elution solution applied to the column.
12. Close the lid and incubate at room temperature (15-25°) for 1 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min.

Downstream Applications

PCR setup

Depending on the *concentration* of the isolated DNA the suitable setup for the amplification has to be selected:

The *standard volume PCR (20 µl) in a capillary cycler* is useful for highly concentrated DNA eluates, because the maximal input of target DNA in the reaction setup is limited. Only 30-50% of the eluate can be analysed.

For low concentrated DNA eluates, e.g. from a single microdissected cell, the *high volume PCR (50 µl) in a 96-well block cycler* is recommendable, as 100% of the eluate can be used for the reaction setup.

The *low volume PCR (1 µl) in an Eppendorf Mastercycler* allows a direct analysis without separate DNA isolation and transfer step. The method offers the advantage of the combination of LCM and low volume PCR on the same slide.

Standard PCR (20 µl) in a capillary cycler

QuantiFast SYBR Green PCR (QIAGEN #204052) in our hands results in exact amplification products.

PCR Procedure

1. Thaw 2x QuantiFast SYBR Green PCR Master Mix, template DNA, primers, and water. Mix the individual solutions.
2. Prepare a reaction mix according to setup. Due to the hot start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

Note: We recommend starting with the Mg²⁺ concentration as provided in 2x QuantiFast SYBR Green PCR Master Mix.

Reaction Setup

2x QuantiFast SYBR Green PCR Master Mix	10 µl
Primer A (10 µM)	0.5 µl
Primer B (10 µM)	0.5 µl
Template DNA	≤ 100 ng/reaction
distilled water (PCR clean)	variable
Total reaction volume	20 µl

3. Mix the reaction mix thoroughly and dispense appropriate volumes into PCR capillaries.
4. Add template DNA (≤100 ng/reaction) to the individual capillaries containing the reaction mix.
5. Program the cycler according to conditions

Capillary Cycler conditions (exemplary)

Step	Time	Temp.	Ramp rate
PCR initial activation	5 min	95°C	fast mode
Two-step cycling			
denaturation	10 sec	95°C	fast mode
combined			
annealing/extension	30 sec	60°C	fast mode
number of cycles	32		

6. Place the PCR capillaries in the cycler and start the cycling program.
7. Optional: Check the specificity of the PCR product(s) by agarose gel electrophoresis

High volume PCR (50 µl) in a 96-well block cycler

The input of the whole eluate (20 µl) to the PCR reaction mix requires an increased total reaction volume of 50 µl.

PCR Procedure

1. Thaw PCR buffer, dNTPs, template DNA, primers, and water. Mix the individual solutions. Keep samples on ice during reaction setup or while programming the cycler.
2. Prepare a reaction mix according to setup:

Reaction Setup

10x Buffer	5 µl
dNTP-Mix (2 mM each)	5 µl
Primer A (10 µM)	1 µl
Primer B (10 µM)	1 µl
template DNA	variable
Qiagen HotStarTaq Polymerase	0.5 µl
distilled water (PCR clean)	variable
Total reaction volume	50 µl

3. Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes.
4. Add template DNA (≤ 100 ng/reaction) to the PCR tubes containing the reaction mix.
5. Program the cycler according to conditions.

Block Cycler conditions (exemplary)

Step	Time	Temp.
denaturation	30 sec	95°C
annealing	30 sec	50°C
extension	30 sec	72°C
number of cycles	35	

6. Place the PCR tubes in the cycler and start the cycling program.
7. Optional: Check the specificity of the PCR product(s) by agarose gel electrophoresis

Depending on the experiment a second subsequent nested PCR in a capillary cycler or in a 96-well block cycler based on the first PCR product, and internal primers can be attached.

*1 *Advalytix protocols:*
www.advalytix.com/images/download

Low volume PCR (1 µl) in an Eppendorf Mastercycler

DNA amplification and cycle sequencing, for example of a single cell, are possible in an extremely low volume reaction format (1 µl) with the Slide48/AmpliGrid technology. After lifting the cell onto the chip analysis can be performed directly on-chip without any template preparation.

PCR Procedure

1. Thaw PCR buffer, dNTPs, template DNA, primers, and water. Mix the individual solutions. Keep samples on ice during reaction setup or while programming the cycler.
2. Prepare a reaction mix according to setup:

Reaction Setup (see Advalytix protocols *1)

AmpliTaq Gold	0.1 µl
10x GeneAmp Buffer I with 15mM MgCl ₂	0.1 µl
Primer (5 pmol/µl each)	0.1 µl
dNTP-Mix (2.5 µM each)	0.1 µl
distilled water (PCR clean)	0.6 µl
Total reaction volume	1.0 µl

3. Mix the reaction mix and dispense 1 µl to each microdissected cell on the reaction site of the AmpliGrid slide.
4. Cover the PCR droplet with 5 µl of sealing solution.
5. Place the loaded AmpliGrid on the Eppendorf Mastercycler.
6. Program the cycler according to conditions.

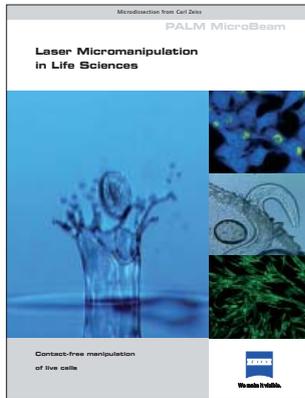
Eppendorf Mastercycler conditions (example)

Step	Time	Temp.
PCR initial step	10 min	95°C
denaturation	40 sec	94°C
annealing	30 sec	56°C
extension	30 sec	72°C
number of cycles	32	10 min 72°C

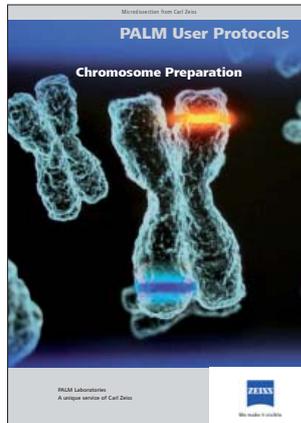
Depending on the experiment a sequencing reaction on an Eppendorf Mastercycler with capillary electrophoresis analysis or a check of the specificity of the PCR product(s) by gel electrophoresis can be attached.

Other brochures and protocols

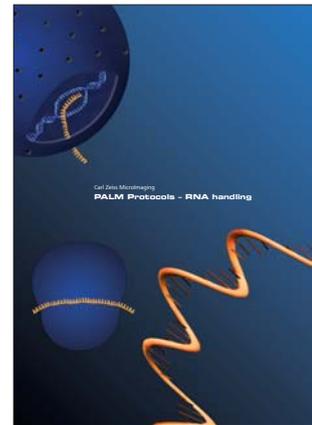
Live cells



Chromosomes



RNA



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PALM Protocol - DNA handling

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