

Microdissection for Detecting Genetic Aberrations in Early and Advanced Human Urinary Bladder Cancer

Arndt Hartmann, Robert Stoehr, Peter J. Wild, Wolfgang Dietmaier, and Ruth Kneuchel

Summary

Laser microdissection is an essential method for the investigation of the multistep carcinogenic process in the urinary bladder. Reliable detection of tumor-specific alterations which can be compromised by the presence of normal cells, requires microdissection of pure tumor cell populations (>80%) to detect loss of heterozygosity (LOH) by either fluorescence *in situ* hybridization (FISH) or sequence analysis. Multiple molecular methods need to be performed in the course of studying often-small lesions. This chapter describes in detail the use of laser microdissection, whole-genome amplification by improved primer extension preamplification (I-PEP)-polymerase chain reaction, and subsequent LOH, FISH, and sequencing analysis in the investigation of urothelial tumors and their precursor lesions. The combination of the described methods allows a wide spectrum of molecular investigations of tumor cells and helps to understand the fundamental alterations involved in urothelial carcinogenesis.

Key Words: Bladder cancer; whole-genome amplification; oncogene; tumor suppressor gene.

1. Introduction

Bladder cancer has two hallmarks that make it an interesting model for cancer research. Transitional cell carcinomas are mostly multifocal, recurring tumor entities, and are endoscopically accessible. Multifocal tumors, which are mostly derived from the urothelium (>95%), are found at the same stage or different stages of tumor progression (1). Recent methods of fluorescence-guided tumor diagnosis allow better detection, especially of precancerous and flat, e.g., nonpapillary, urothelial lesions (2,3). Because the hypothesis for molecular carcinogenesis of human bladder cancer was mainly derived from

From: *Methods in Molecular Biology*, vol. 293: *Laser Capture Microdissection: Methods and Protocols*
Edited by: G. I. Murray and S. Curran © Humana Press Inc., Totowa, NJ

manifest or even advanced tumor stages, the investigation of genetic aberrations in early tumor stages was considered to be interesting (4).

The assessment of genetic changes in advanced invasive tumors is important and is described in several chapters of this book. Besides the marked inflammatory reaction in early stages of bladder cancer (pT1), the high stromal component in muscle-invasive tumors (pT2) has to be eliminated as well. In addition, precancerous lesions of the bladder typically show an abrupt transition from normal to dysplastic urothelium; even a pagetoid growth pattern may occur, i.e. intraepithelial low quantity areas of highly abnormal cells (**Fig. 1A** = CIS abrupt; **B** = CIS pagetoid). Therefore, laser microdissection provides new options for gaining pure cell populations in tumors with low amounts of tumor cells.

2. Materials

2.1. Laser Microdissection

1. PALM Robot-Microbeam (PALM, Wolfratshausen, Germany).
2. Stereo microscope DRC (Zeiss, Germany).
3. Microlance3 sterile needles (Braun, Germany).
4. Polyethylen membrane (1.35 μm ; PALM).
5. Glass object slides (Menzel, Germany).
6. Poly-L-lysine (0.1%).
7. TESA[®] house and garden universal cover strip (Beiersdorf, Germany).
8. Xylene.
9. Ethanol (70%, 96%, 100%).
10. H₂O (Millipore, Bedford, MA).
11. Methylene blue solution (1%).

2.2. DNA Isolation and Whole-Genome Amplification (WGA)

1. SpeedVac SC110 (Savant Instruments Inc., NY).
2. QIAamp DNA Mini Kit (Qiagen, Germany).
3. Proteinase K (Merck, Darmstadt, Germany).
4. Tween-20 (Merck).
5. 1X Expand HiFi buffer No. 3 (Roche, Mannheim, Germany).
6. Expand[™] High Fidelity PCR System (Roche).
7. 15-mer random primer (NNNNNNNNNNNNNNNN, synthesized by Proligo, France).
8. Thermocycler PTC100 (MJ Research, MA).

2.3. Fluorescence In Situ Hybridization

1. Carnoy's solution (3 parts methanol, 1 part acetic acid).
2. Maxi Prep DNA Isolation Kit (e.g. NucleoBond[®] PC 500 Kit, MN GmbH, Dueren, Germany).

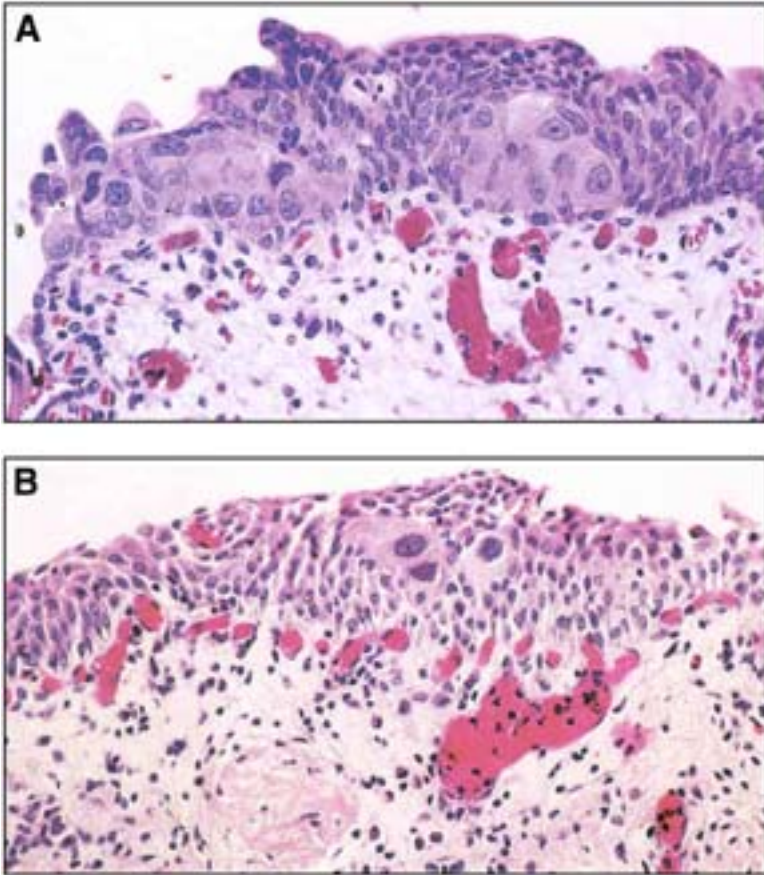


Fig. 1. Carcinoma *in situ* of the bladder. **(A)** Carcinoma *in situ* with distinct areas of atypical cells within normal urothelium. **(B)** Extreme variant with single atypical cells in normal urothelium, called pagetoid CIS. Photomicrographs (hematoxylin and eosin staining) are taken from the WHO classification on urothelial tumors (5).

3. Standard nick translation kit (e.g., Roche).
4. Xylene.
5. Carlsberg solution: 0.1% proteinase K solution (20 mg/mL), 1.4% NaCl (5 M), in 10 mL Tris-HCl base (0.1 M), pH 7.2.
6. Ethanol (70%, 85%, 96%, 100%).
7. PBS-buffer: 8 g NaCl, 1.15 g Na₂HPO₄, 0.2 g KCl, 0.24 g KH₂PO₄, make up to 1 L with H₂O.
8. 20X standard saline citrate (SSC): 175.32 g NaCl, 88.24 g sodium citrate (dihydrate), make up to 1 L with H₂O, pH 5.3.

9. 2X SSC, pH 7.0–7.1.
10. 2X SSC; 0.3% NP40, pH 7.0–7.5.
11. C100T: citric acid with 0.5% Tween-20.
12. Proteinase K (0.8 µg/mL).
13. Methylene blue solution (1%).
14. RNase solution: 1 mM Na-EDTA, 0.3% NP40 (10%), 1% RNase (10 mg/mL) in 10 mL of 10 mM Tris-HCl, pH 7.5.
15. Zytospin centrifuge.

2.4. Loss of Heterozygosity Analysis

1. Oligonucleotide primers specific for the appropriate microsatellite markers.
2. PCR reagents including Taq polymerase, dNTPs, MgCl₂.
3. 10X TBE: 0.9 M Tris base, 0.9 M boric acid, 4% 0.5 M EDTA, pH 8.0.
4. Gel mix for 6.7% polyacrylamide gel: 7.5 M urea, 18% acrylamide (37%), 10.8% 10X TBE.
5. *N,N,N',N'*-Tetramethylethylenediamine (TEMED).
6. Ammonium persulfate (APS, 10%).
7. Polyacrylamide gel electrophoresis (PAGE) equipment.
8. Loading buffer: 10 mL formamide, 0.01 g xylene cyanol FF, 0.01 g bromophenol blue, 200 µL EDTA (0.5 M, pH 8.0).
9. Silver staining reagents: 10% ethanol, 1% nitric acid, 0.012 M silver nitrate, 0.28 M sodium carbonate including 0.1% formaldehyde, 10% acetic acid.
10. Whatman filter paper.
11. Gel dryer.

2.5. Gene Sequencing

1. Oligonucleotide primers specific for the individual gene of interest.
2. PCR reagents including Taq polymerase, dNTPs, MgCl₂.
3. Agarose gel equipment.
4. PEG Mix: 26% PEG8000, 0.6 M sodium acetate, pH 5.5, 6.6 mM magnesium chloride.
5. 100% ethanol, 70% ethanol, 3 M sodium acetate, pH 4.6.
6. PRISM Ready Dye Terminator Cycle Sequencing Kit (Applied Biosystems GmbH, Weiterstadt, Germany).
7. Applied Biosystems 373 DNA sequencer.

3. Methods

The methods described below outline (1) laser microdissection of relevant tumor areas, (2) DNA isolation and whole-genome amplification, (3) fluorescence *in situ* hybridization (FISH), (4) LOH analysis, and (5) gene sequencing. Emphasis is put on laser microdissection of fresh-frozen material; FISH analysis has been described in frozen and wax-embedded material, respectively.

3.1. Laser Microdissection

With many precancerous lesions and early-stage tumors presenting as intraluminal formations, conventional manual microdissection under a stereomicroscope using sterile needles is the method of choice for the majority of cases. However, laser microdissection is appropriate if accurate segregation of low cell amounts is required, for example: (1) small areas of dysplasia or carcinoma *in situ*, (2) denuding carcinoma *in situ*, (3) intraepithelial migration of tumor cells, (4) invasive tumors with surrounding fibrosis and inflammation, and (5) small high-grade foci (G3) in well-differentiated carcinomas, e.g., at the invasive interface.

3.1.1. Fresh-Frozen Material

1. Genomic DNA should be prepared from 5- μ m frozen sections.
2. Pure tumor cell populations (>80% of tumor cells) are obtained using a PALM Robot-MicroBeam laser microdissection device.
3. For laser microdissection, the specimens are mounted on a 2 \times 3 cm polyethylene membrane (1.35 μ m), which is taped onto a supporting object slide (TESA house and garden universal cover strip) (*see Notes 1 and 2*).
4. Adhesion of tissues to the polyethylene membrane can be preserved by preparation of the membrane with 80 μ L of poly-L-lysine before tissue sections are applied (*see Notes 3–5*).
5. After staining with 1% methylene blue, the selected tumor region is cleared of nontumorous cells (e.g., inflammatory cells) and microdissected precisely following its irregular shape.
6. With one single laser shot the entire microdissected membrane tissue area is then ejected and catapulted toward the collector, i.e., the lid of the reaction tube (*see Note 6*).
7. Catapulted specimens in the lid are morphologically well preserved, and can be documented with a digital camera.

3.1.2. Wax-Embedded Tissue

1. Before microdissection, 5- μ m histologic sections are dewaxed (1 h at 65°C) followed by incubation in xylene at room temperature for 2 \times 15 min.
2. The sections are then rehydrated in ethanol (100%, 96%, 70%; at least 2 \times 5 min each).
3. After incubation for 5 min in H₂O, specimens are stained with 1% methylene blue and used for microdissection.

3.2. DNA Isolation and Whole-Genome Amplification

The investigation of small premalignant lesions containing few aberrant cells often requires a preamplification of the DNA using whole-genome amplifica-

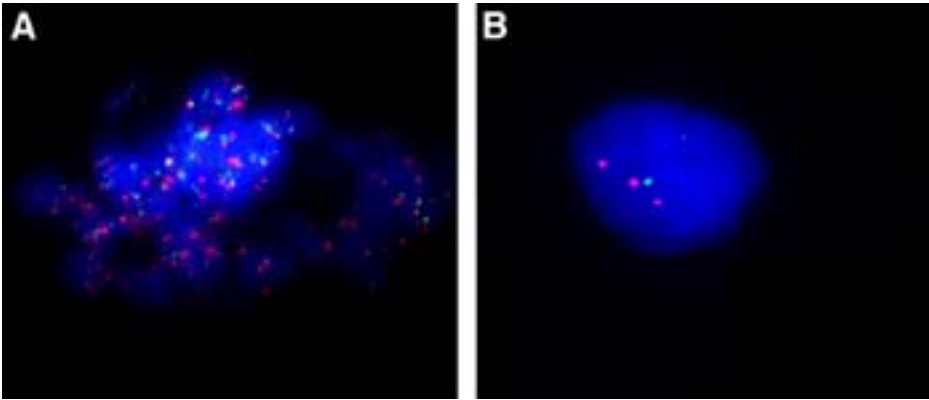


Fig. 2. FISH analysis of bladder tumor specimens. (A) Microdissected cells of a pT1G3 bladder tumor. Red signals: centromeric probe for chromosome 8; green signals: locus specific probe (BAC) for chromosome 8p11–12; blue staining: DAPI counterstaining of the nucleus. Tumor cells show a high-level amplification of chromosome 8. (B) Bladder tumor with aneuploidy of chromosome 8 and deletion of chromosomal region 8p11–12. Three red signals, but only two green signals, can be seen.

tion (WGA). The combination of microdissection and WGA allows multiple analysis of small cancer precursor lesions, providing additional information about the first chromosomal events in carcinogenesis. Details of the method used, i.e., improved primer extension preamplification polymerase chain reaction (I-PEP-PCR) including data on minimal cell numbers required are described in detail in Chapter 8 of this book.

3.3. FISH

The detection of chromosomal losses is of great importance in the molecular analysis of human malignancies. Regions frequently affected by deletions might contain important tumor suppressor genes (TSGs), which may play an essential role in carcinogenesis. Comparative genomic hybridization (CGH) has low resolution and gives only an overview of the chromosomal changes occurring in tumor cells. Multicolor FISH (*see Fig. 2*) allows the investigation of specific deletions using BAC clones and (for chromosome enumeration) commercially available centomere probes. The reliable detection of deletions requires microdissection from thick (>20 μm) sections to avoid artefacts because of sectioning of nuclei and loss of chromosomal material. The method described here is for formalin-fixed and wax-embedded tissue, but also works in tissue microdissected from frozen sections or in touch preparations of fresh tumor tissue. For these cases pretreatment can be omitted from the protocol,

and digestion is performed by using C100T incubation for approx 90 min. BAC clones can be selected from several commercially available BAC libraries and labeled by nick translation. Several pretreatment kits are available from suppliers of FISH probes.

3.3.1. Probe Preparation

1. Centromeric probes already labeled with fluorescent dyes (e.g., FITC, Texas Red) are commercially available from several suppliers (e.g., Vysis, Abbott Laboratories, Berkshire, UK) and are ready to use following the manufacturer's instructions.
2. BAC clones can be used for the investigation of specific chromosomal regions.
3. After isolation from bacterial culture, digoxigenin-11-dUTP labeling of the BAC using standard nick translation is necessary.
4. The BAC can then be visualized using fluorescent immunostaining.

3.3.2. Pretreatment of Tissue Sections

1. Incubate the slides for 25 min at 72°C.
2. Afterward, remove the wax from the tissue.
3. Incubate the slides 2 × 10 min in fresh xylene at room temperature (RT) and, for dehydration, a few minutes in 100% ethanol.
4. After drying at RT, slides are put in freshly prepared 2X SSC for 2 min at 73°C.
5. After washing for 5 min in PBS buffer at RT, incubate slide in proteinase K solution for 15 min at 39°C.
6. Wash the slides in for 5 min in PBS buffer at RT followed by a dehydration of the tissue in graded ethanol (70%, 85%, 100%, each for 1 min at RT).
7. Slides are dried at RT.

3.3.3. Denaturing of Probe and Tissue DNA

1. All steps described here should be performed in the dark.
2. Denature the probe(s) for 5 min at 73°C in a water bath.
3. Apply the probe to the tissue section and cover the tissue with a cover slip.
4. Seal the cover slip with a vulcanized rubber and heat the slide for 9 min at 96°C.
5. Incubate the slide overnight at 37°C in a humid chamber.

3.3.4. Washing the Slides

1. Incubate the slides for 30 s in 2X SSC/0.3% NP40 at RT to detach cover slip.
2. Carefully remove cover slip.
3. Wash slides in 2X SSC/0.3% NP40 for 2 min at 73°C followed by an incubation for 1 min in deionized water at RT.
4. Carefully dry the slides and add 10 µL DAPI in Vectashield mounting medium (Vector Laboratories, Burlingame, CA).
5. Store the slide in the dark at 2–8°C (*see Note 7*).

3.3.5. Microdissected Formalin-Fixed, Wax-Embedded Tissue

1. After removal of wax, incubate slides in 80% ethanol, methylene blue, and water, each for approx 15 s at RT.
2. Carefully microdissect the cells of interest and immediately transfer the tissue into 50- μ L Carlsberg solution (on ice).
3. Incubate the cells for 30 min at 37°C to digest the cytoplasm of the cells.
4. Add 50 μ L RNase to the cells and incubate another 15 min at RT.
5. Apply the cell suspension to a standard cytospin centrifuge.
6. Air-dry the cells for 5 min.
7. Incubate the cells for 10 min in Carnoy's solution at RT for fixation.
8. Air-dry the cells and store the slides at -20°C/-80°C until use.

3.4. LOH Analysis

LOH analysis using microsatellite markers (highly polymorphic repetitive DNA sequences) located within specific chromosomal segments is a different method that allows a detailed deletion analysis of a chromosomal region of interest. The investigation of a large number of microsatellite markers of a specific chromosomal region allows the narrowing of a minimally deleted region facilitating the detection of a putative TSG.

The detection of chromosomal losses in malignant tumors gives new insights into the correlation between chromosomal alterations and tumor progression and prognosis. The investigation of premalignant tissue offers the chance to detect the deletions occurring early in the carcinogenic process. The following description provides a short protocol for the rapid establishment of a microsatellite analysis.

3.4.1. Selection of Microsatellite Markers

1. The first prerequisite for the LOH analysis is the selection of a panel of microsatellite markers localized within the chromosomal region of interest (*see Note 8*).
2. Most available microsatellite markers with amplification primers and information about size of amplicon and literature data can be found at the Genome Database (www.gdb.org).

3.4.2. Thermogradient PCR

1. It is advisable to determine optimal PCR conditions before starting LOH analysis.
2. The establishment of PCR amplification conditions can be done using a gradient-thermocycler (*see Notes 9–11*).
3. A standard PCR protocol (25 μ L reaction volume containing 0.2 mmol/L dNTP, 0.3 μ mol/L of each primer (forward and reverse primer), 1.5 mmol/L MgCl₂, 0.5 U DNA Taq polymerase, 50–100 ng template DNA) should be used.
4. The following cycle conditions are satisfactory in most instances: 3 min preheating at 95°C, 35 cycles of 1 min at 95°C for denaturation—1 min at specific tem-

perature range (gradient) for primer annealing—1 min at 72°C for primer elongation, 10 min at 72°C for final elongation, cooling to 10°C for termination of PCR reaction.

5. PCR conditions (annealing temperature, MgCl₂ concentration) resulting in a specific PCR product should be used for further analysis.

3.4.3. Microsatellite Amplification

1. PCR amplification of the microsatellite markers chosen for the LOH analysis should be performed according to optimized PCR conditions (*see Subheading 3.4.2.*).
2. If a gradient-thermocycler is not available, PCR protocol and cycle conditions shown above should be used.
3. Optimal annealing temperature should be assessed by several test PCR reactions with varying temperatures and, if necessary, varying MgCl₂ concentrations (0.25 M–2.5 M).

3.4.4. Polyacrylamide Gel Electrophoresis

1. For separation of PCR products standard polyacrylamide gel electrophoresis (PAGE) equipment (e.g., Sequi-Gen Sequencing Cell, Bio-Rad, Hercules, CA) can be used.
2. For a standard 32.5 × 38 cm denaturing gel (6.7% PAA, 1 mm thickness) the following protocol is recommended: add to 80 mL Gel-Mix 1 mL APS and 100 μL TEMED to start polymerization of the gel; complete polymerization process takes 60 min.
3. Before sample loading mix equal amounts of PCR reaction and loading buffer and denature the sample for 5 min at 95°C.
4. After denaturation place the sample on ice immediately.
5. Electrophoresis should be started after preheating to 40–50°C for about 5 min (2500 V).
6. PAGE should be performed at a temperature of 55°C for best results.

3.4.5. Silver Staining

1. After PAGE, silver staining of the gel is performed to detect PCR products (**6**).
2. The following procedure is recommended:
 - a. 5 min 10% ethanol.
 - b. 3 min 1% nitric acid.
 - c. Rinse with water three times.
 - d. 0.012 M silver nitrate for at least 20 min (up to 1 h) in complete darkness.
 - e. Rinse with water several times.
 - f. Incubate gel with 0.28 M sodium carbonate with 0.1% formaldehyde several times until PCR products become visible (*see Fig. 1*).
 - g. Stop reaction with 10% acetic acid for 2 min.
 - h. Rinse with water several times.
3. After staining, transfer gel to Whatman filter paper and dry gel.

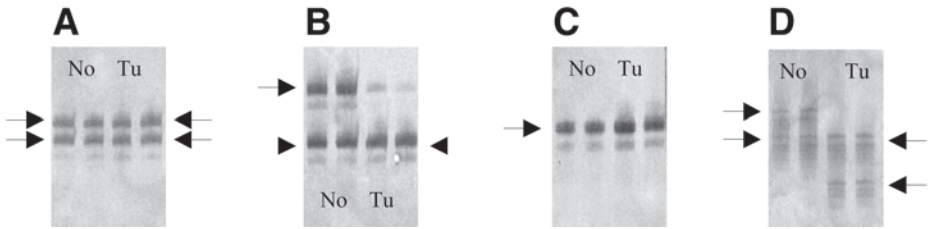


Fig. 3. Microsatellite analysis. No, normal urothelium; Tu, papillary bladder tumor. (A–C) microsatellite marker D8S591 on chromosome 8p; (D) microsatellite marker D8S255 on chromosome 8p. (A) Informative patient sample. Two alleles can be distinguished (see arrows), no deletion in the tumor. (B) Informative patient sample, loss of one allele in the tumor → chromosomal deletion (LOH). (C) Patient sample is not informative, the two alleles cannot be distinguished, a normal to tumor comparison is not possible. (D) Informative patient, band-shift in the tumor → microsatellite instability (MSI).

3.4.6. Evaluation of LOH Analysis

1. Direct comparison between normal tissue and premalignant or tumor tissue reveals chromosomal alterations (see Fig. 3).
2. Informative cases are scored as allelic loss (LOH) when intensity of the signal for the tumor allele is decreased to at least 50% relative to the matched normal allele (Fig. 1B).
3. To prevent errors because of preferential amplification of one allele during the PCR reaction, all LOH analyses should be run in duplicate. Following independent PCR reactions for the DNA aliquots detected LOH should be verified in a second, independently performed PCR reaction.

3.5. Gene Sequencing

Sequencing analyses have revealed a large number of mutations in tumor-related genes. Activating mutations in oncogenes (e.g., FGFR3) or knockout of tumor suppressor genes (e.g., *p53*) by single-point mutations, deletions, insertions, or chromosomal rearrangement are well studied in most human malignancies. Both aberrant constitutive activation of oncogenes and loss of function of TSGs caused by gene mutations are important genetic alterations in carcinogenesis. Sequence analysis is therefore an essential part of tumor characterization. The method described below is a short protocol for automated genomic sequencing (using DNA from snap-frozen or formalin-fixed, wax-embedded tissue). The described protocol is also adapted for DNA after WGA (see Notes 12–14).

3.5.1. PCR Amplification and PEG Precipitation

1. For amplification of the target region for sequence analysis, PCR conditions described above are recommended (*see Subheading 3.4.2.*).
2. Before the PCR product can be used for sequencing, PEG (polyethyleneglycol) precipitation is necessary for purification.
 - a. Add equal amount of PEG Mix to PCR reaction; mix well.
 - b. Incubate at room temperature for 20 min.
 - c. Centrifuge for 30 min at 15,000g.
 - d. Remove supernatant carefully (do not disturb pellet).
 - e. Add 100 μ L 100% ethanol without mixing.
 - f. Centrifuge for 10 min at 15,000g.
 - g. Remove supernatant.
 - h. Dry pellet (SpeedVac, 5 min).
 - i. Resuspend DNA pellet in 20 μ L water and mix well.
3. After PEG precipitation a DNA aliquot (3 μ L) should be used for agarose gel electrophoresis to control precipitation and to assess DNA concentration.

3.5.2. Sequencing Reaction

1. For sequence analysis, a PRISM Ready Dye Terminator Cycle Sequencing Kit (Applied Biosystems GmbH, Weiterstadt, Germany) is advisable using the manufacturer's protocol.
2. Before sequencing reactions are analyzed (Applied Biosystems 373 or 377 sequencer), sequencing products must be purified using an ethanol precipitation.
 - a. Add 55 μ L 100% ethanol and 2 μ L sodium acetate to sequencing reaction (20 μ L volume); mix well.
 - b. Incubate at room temperature for 20 min in complete darkness.
 - c. Centrifuge for 30 min at 15,000g.
 - d. Remove supernatant.
 - e. Dry pellet for 5 min at room temperature (cup upside down).
 - f. Add 500 μ L 70% ethanol without mixing.
 - g. Centrifuge for 10 min at 15,000g.
 - h. Remove supernatant.

3.6. Comparison of Methods Used

The most important prerequisite to achieving reliable results from the application of the methods described above is the analysis of a pure cell population without contamination. The use of laser microdissection ensures a purity of >90% of the microdissected cells and is essential for the investigation of small preneoplastic lesions, flat malignancies (e.g., CIS), or invasive tumors. An advantage of the methods described is the applicability to both snap-frozen and formalin-fixed, wax-embedded tissue, allowing the investigation of archi-

val material. In addition, the methods complement one another. FISH analysis is ideal for detecting chromosomal deletions, losses of whole chromosomes, and amplifications of specific chromosomal regions. Gene-specific probes enable the investigation of tumor tissue for high-level amplification of known oncogenes or deletions of tumor suppressor genes. The use of several gene-specific and centromer-specific probes labeled with different colors (multicolor FISH) allows the analysis of interrelated members of a whole signalling pathway. However, FISH needs a standardized protocol and a very experienced observer. Weak hybridization, tissue damage, destroyed or overlapping nuclei, or splitting of signals could complicate scoring of the signals.

LOH analysis is one of the most frequently used methods for detection of chromosomal alterations in cancer research. Direct comparative analysis of polymorphic microsatellite markers from normal and tumor tissue shows chromosomal deletions and microsatellite instability (MSI), genomic changes found in most malignancies. Using LOH analysis, amplification of specific genes can not be determined. This restricts the field of application to detection of allelic losses or MSI. Analysis of LOH is an essential tool for definition of a minimal deleted chromosomal region in a tumor and therefore is excellent preparatory work for FISH analysis. Both methods can also be used for verification of each other. To achieve reliable results from microsatellite analysis, minimal contamination of the tumor cells with stromal or inflammatory cells, for example, is acceptable. Using laser-assisted microdissection, it should be very easy to achieve a clean cell population for analysis.

Another crucial point is the absolutely necessary verification of the results from LOH analysis. Poor DNA quality and a low DNA quantity could cause a preferential PCR amplification of only one allele, mimicking an allelic loss. This leads to a misinterpretation of the result and the detection of a “false-positive” LOH. In our experience at least 10–20 cells from frozen sections and at least 100 cells from wax-embedded tissue should be used for PCR analysis to avoid preferential amplification. Our own experience showed a frequency of this PCR failure in about 5–10% of all LOH analysis. This is a particular problem when trying to define a minimally deleted region on a chromosome. To avoid this source of error each identified deletion or MSI should be verified by a second independently performed PCR reaction.

Gene sequencing is an ideal complement to the methods described above. Mutation analysis of a (putative) TSG (e.g., *p53*) that is located within a chromosomal region showing allelic deletion in LOH or FISH analysis could reveal specific mutations within the coding region or splice sites of this gene, causing a complete knockout. Sequence analysis of known oncogenes which showed no significant alterations by FISH analysis (e.g., no high-level amplification) might discover activating mutations.

Genomic sequencing can be used for both snap-frozen and formalin-fixed, wax-embedded tissue. It is a more sensitive method than LOH analysis. Again it is very important to verify all found mutations with a second, independent generated PCR amplicon. To confirm the results, sequencing of both DNA strands is highly advisable. Often a PCR failure in one strand mimics a mutation that is not visible in the antisense strand. Also the quality and quantity of the DNA are important factors. Using formalin-fixed tissue, often only short PCR amplicons can be generated (max. approx <500 bp) caused by degradation of the DNA during the fixation. The problem of low DNA quantity can be solved using WGA before sequencing analysis. It has been shown (7) that there is only a minimal risk of incorrect amplification by PCR during WGA. However, it is advisable to split the DNA in half before WGA to provide the possibility of repeating the whole process (WGA and gene sequencing) for verification.

Taken together, all three described methods are ideal for the analysis of both snap-frozen and formalin-fixed, wax-embedded tissue. The combined application allows the rapid detection of the most important alterations in tumors and provides the basis for more intensive investigations.

4. Notes

1. The polyethylene membrane should be cut in pieces of 2×3 cm with a surgical blade between two sheets of paper on a metal surface. After putting the object slide into 100% ethanol, the polyethylene membrane adheres to the object slide easily by capillary effects. The membrane should be dry and as flat as possible before fixation with a cover strip (0.5×2 cm) at each ending.
2. For wax-embedded tissue, fixation of the polyethylene membrane with the cover strip (TESA house and garden universal) is indispensable before removal of the wax with xylene.
3. Poly-L-lysine must always be handled under ventilated conditions.
4. The slides prepared with a polyethylene membrane and poly-L-lysine should be completely dry before tissue sections are applied.
5. To avoid unintentional fixation of the polyethylene membrane, poly-L-lysine should not contaminate the space between the membrane and the object slide.
6. At least 200 cells are prepared from each specimen to prevent preferential monoallelic amplification.
7. The scoring of FISH signals can be hampered by a strong and diffuse background staining. In this case agitation of the slides during the washing steps decreases the background.
8. The microsatellite markers used for LOH analysis should show a high rate of heterozygosity. This minimizes the problem of too many noninformative samples during deletion analysis.
9. For the initial optimization of PCR conditions use DNA from cultured cells or blood to avoid wasting tumor DNA.

10. If only weak PCR signals are visible after PAGE, PCR cycle number can be increased up to 50 rounds to achieve a larger amount of PCR amplicons.
11. PCR amplification from formalin-fixed, wax-embedded tissue is sometimes unsuccessful. Initial PCR preheating for 5–10 min at 95°C often improves results.
12. Evaluation of the sequencing results should be done very carefully. Often only the graphs indicate the presence of a mutation that is not visible in the shown sequence (\geq high degree of contamination with cells from normal tissue).
13. A nested PCR can be used to obtain reliable sequencing results if only a small amount of DNA is available for analysis.
14. The results from the analysis described above are ideal preparatory work for the application of additional techniques. Mutations detected by genomic sequencing can be used for allele-specific PCR analysis (8). Immunohistochemical staining of tissue specimens allows direct analysis of an oncogene or tumor suppressor gene on protein level in close correlation to mutational status.

References

1. Epstein, J. I., Amin, M. B., Reuter, V. R., and Mostofi, F. K. (1998) The World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder. Bladder Consensus Conference Committee. *Am. J. Surg. Pathol.* **22**, 1435–1448.
2. Filbeck, T., Pichlmeier, U., Knuechel, R., Wieland, W. F., and Roessler, W. (2002) Clinically relevant improvement of recurrence-free survival with 5-aminolevulinic acid induced fluorescence diagnosis in patients with superficial bladder tumors. *J. Urol.* **168**, 67–71.
3. Zaak, D., Kriegmair, M., Stepp, H., et al. (2001) Endoscopic detection of transitional cell carcinoma with 5-aminolevulinic acid: results of 1012 fluorescence endoscopies. *Urology* **57**, 690–694.
4. Veltman, J. A., van Weert, I., Aubele, M., et al. (2001) Specific steps in aneuploidization correlate with loss of heterozygosity of 9p21, 17p13 and 18q21 in the progression of pre-malignant laryngeal lesions. *Int. J. Cancer* **91**, 193–199.
5. Mostofi F. K, Davis C. J., and Sesterhenn, I. A. (1999) Histological typing of urinary bladder tumors. WHO, International Histological Classification of Tumors. Springer, New York.
6. Schlegel, J., Stumm, G., Scherthan, H., et al. (1995) Comparative genomic in situ hybridization of colon carcinomas with replication error. *Cancer Res.* **55**, 6002–6005.
7. Dietmaier, W., Hartmann, A., Wallinger, S., et al. (1999) Multiple mutation analyses in single tumor cells with improved whole genome amplification. *Am. J. Pathol.* **154**, 83–95.
8. Stoehr, R., Knuechel, R., Boecker, J., et al. (2002) Histologic-genetic mapping by allele-specific PCR reveals intraurothelial spread of p53 mutant tumor clones. *Lab Invest.* **82**, 1553–1561.