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Positional Approaches to Cancer Genetics

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1. Introduction

Loss of genetic material accumulates during tumor development as cancer cells select for the physical removal or functional inactivation of genes whose encoded proteins regulate normal cellular behavior. The hallmark indication for this type of gene, now termed a tumor suppressor gene, is a genetic event resulting in loss or deletion of chromosomal material. Position-oriented approaches have taken advantage of the intimate involvement of tumor suppressor gene inactivation through deletion to localize, identify, and demonstrate the involvement of these genes in carcinogenesis. Since deletion of genetic material exceeds genomic amplifications in most cancer types, loss of gene function appears to play a prominent role in tumor formation. However, despite the intimate involvement of tumor suppressor genes in the neoplastic process, relatively few of these genes have been identified, leaving the identity of the majority unknown. Thus, the use of position-based strategies for mapping and isolating tumor suppressor genes remains a prime tool in their identification.

>AU: 1. No callouts found for Notes 3, 4, 7, & 8.

2. Refs 1, 10-14, 16, 17, 19 not cited in text. Need all refs cited in numeric order.

In this chapter we discuss the positional approaches used in the localization and identification of tumor suppressor genes. This process can be broadly divided into the following 4 steps leading to the identification and validation of these genes: (a) genome-wide surveys to identify the chromosomal location of genes potentially involved in tumor development; (b) refinement and/or confirmation of the chromosomal region of loss or deletion; (c) cloning genes from consistent regions of loss; and (d) validating the candidate gene as a tumor suppressor through the identification of truncating or missense mutations as well as by growth and tumor suppressing assays.

Any strategy that detects genome-wide loss of genetic material could conceivably be used as a position-oriented approach to map the location of potential tumor suppressor genes. At the chromosomal level of resolution, traditional cytogenetic analysis, spectral karyotyping (SKY), and comparative genomic hybridization (CGH) have been used extensively for these purposes. All three of these chromosome-based approaches have a unique set of strengths and weaknesses and are therefore quite

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complementary in defining regions of loss in the genome. Comprehensive analyses based on these strategies and performed on collections of tumors have identified nonrandom areas of loss within particular cancers, and areas of loss common to multiple tumor types. In certain instances, the identification of these areas as "hot spots" for loss of genomic material has facilitated the identification of cancer suppressor genes at these sites.

Once a chromosomal region has been identified as a target of deletion, the region is narrowed by mapping using fluorescence *in situ* hybridization (FISH) or by searching for smaller regions of loss in tumors in comparison to normal tissue from the same patient. The latter process is termed loss of heterozygosity (LOH) and employs a variety of molecular strategies to detect regional loss in tumors by comparison to normal tissue from the same patient. These techniques initially used restriction fragment length polymorphism (RFLP), but now commonly employ polymerase chain reaction (PCR)-based strategies to identify each chromosomal allele present in the heterozygous condition from normal tissue and loss of one allele in tumor material, indicating deletion. The PCR-based protocol is discussed below. Like the cytogenetic approaches described above, nonrandom loss of a region or allele can infer disruption of a tumor suppressor gene and thereby indicate its chromosomal location. The identification of "hot-spot" areas of LOH has also facilitated the identification of several cancer suppressor genes.

Ideally, a small region of homozygous loss can be identified in the tumor material using LOH, and a physical map spanning the region can be constructed from which candidate genes can be cloned. Strategies such as exon trapping or the use of microarray analysis can aid in the identification of genes mapping to a particular regions lost from the tumor genome. The uses of arrayed genes or expressed sequence tags (ESTs) has the further advantage of identifying genes regulated by the tumor suppressor, thereby unraveling the biology of the gene. Another extension of this approach examines directly genes or ESTs that occur in regions of homozygous or hemizygous deletion for the presence of mutations. This electronic database approach is described under **Subheading 3.** As more genes and expressed sequences are identified and mapped to subchromosomal regions that are frequently deleted in cancers, determining their involvement in tumor progression will become an increasingly important strategy in the identification of this class of cancer-causing genes. In many instances only a small portion of sequence need be obtained, and then the intact gene can be pieced together and then screened for the presence of mutations. On-line databases such as those of The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) or The Institute for Genome Research (http://www.tigr.org/) are useful when using this approach.

>AU: Subheading correct?

Candidate cancer suppressor genes identified by either approach must be shown to be biologically relevant to the pathogenesis of the cancer being studied. Therefore, in addition to the identification of large-scale deletions in tumors, further confirmation is provided by the discovery of nonsense and missense mutations that either truncate the protein or affect its normal cellular function in primary tumors and cell lines. Approaches such as direct sequencing of single-stranded conformational polymorphism or coupled in-vitro transcription and translation (also called the protein truncation test) can aid in the identification of mutations.

2. Materials

2.1. Giemsa Trypsin Banding

1. Gurr buffer tablets (pH 6.8): BDH Chemicals, 50-tablet lots.
2. Gurr buffer stock solution: 1 Gurr tablet and 1000 mL of sterile distilled water.
3. Hanks' balanced salt solution, with phenol red (1×) (Ca/Mg-free): Gibco, 500-mL lots.
4. Leischman's stain (Gurr): BDH Chemicals, 25-g lots.
5. Leischman's stain stock solution: 0.8 g Leischman's stain and 500 mL methyl alcohol. Incubate at 37°C overnight and filter.
6. Leischman's stain working solution: 10 mL Leischman's stain stock solution and 30 mL Gurr buffer stock solution. Good for only 2 h.
7. Methyl alcohol anhydrous: Mallinckrodt AR, 4-L lots.
8. Sodium phosphate dibasic: Columbus Chemical Industries, ACS powder, 500-g lots.
9. Sodium phosphate dibasic (0.4 N): 5–6 g sodium phosphate dibasic and 100 sterile distilled water.
10. Trypsin-EDTA (10×): Sigma Chemical Co (cat. no. T-9395), 100-mL lots. Aliquoted into 5 mL batches and frozen at −20°C.
11. Trypsin EDTA working solution: 5 mL trypsin EDTA and 50 mL Hanks' with 2 drops of 0.4 *N* Na_2 HPO_4. Good for 2 working days.

>AU: 100 (unit)?

2.2. SKY Analysis

1. Applied Spectral Imaging Probe mix of fluorescently labeled spectral probes: SKY/M10 for mouse and SKY/H10 for human.
2. MilliQ Deionized water for all solutions.
3. 0.01 *M* HCL: Add 0.5 mL of 1 *M* HCL to 49.5 mL deionized (DI) H_2O in a Coplin jar and heat to 37°C.
4. Pepsin stock: Make 100 mg/mL in sterile H_2O. Store at −20°C. Add 6–15 µL to 0.01 *M* HCL solution.
5. Phosphate-buffered saline (PBS) 1× (Gibco/BRL): Stored at room temperature (RT). Make up 3 Coplin jars at RT.
6. 1× PBS/MgCl: Add 50 mL of 1.0 *M* $MgCl_2$ to 950 mL of 1× PBS. Make up 1 Coplin jar at RT.
7. 1% Formaldehyde: Add 2.7 mL of 37% formaldehyde to 100 mL of 1× PBS/$MgCl_2$. Make up 1 Coplin jar at RT
8. Ethanol series: Make up 2 Coplin jars each of 70%, 80%, and 100% ethanol, one at RT and one at 4°C.
9. Denaturation solution: Add 35 mL formamide, Fluka deionized catalogue no. 47671, Sigma; 10 mL deionized H_2O and 5 mL 20× SSC. pH to 7.0. Put aliquot of 100 µL/slide in a tube and warm to 72°C.
10. Water baths: One bath at 72°C and one at 37°C.
11. 50% Formamide/2× SSC: Add 15 mL 20× SSC, 60 mL DI H_2O, and 75 mL formamide, pH to 7.0. Make up 3 Coplin jars and warm to 45°C.
12. 1× SSC: Add 12.5 mL of 20× SSC to 237.5 mL of DI H_2O. Make up 2 Coplin jars warmed to 45°C.
13. 4× SSC/0.1% NP-40: Add 100 mL of 20× SSC, 400 mL DI H_2O, and 0.5 mL NP-40 and mix well. Make up 6 Coplin jars and warm to 45°C. Denature probe (vial #1) 10 µL/slide in a microfuge tube in a floating rack at 72°C for 7 min. Then transfer to 37°C for at least 1 h. Vectashield mounting medium with DAPI and antifade, Vector cat. no. H-1200.

>AU: "Make up" OK?

>AU: Spell out SSC first time

2.3. Pretreatment of Slides for CGH

Use MilliQ DI water for all solutions.

1. RNase A: To remove RNA from the target slides. Boehringer cat. no. 109169, 10-mg stock solution: 20 mg/mL sterile water, boil for 15 min, cool to RT, make aliquots, store at −20°C.
2. 5 g Pepsin (Sigma): Stock solution: 10% = 100 mg/mL, dissolve in sterile water, keep on ice, make 50-L aliquots, store at −20°C.
3. PBS/$MgCl_2$: 50 mL of 1*M* $MgCl_2$ + 950 mL of 1× PBS.
4. 1% Formaldehyde: Formamide from Fluka, cat no. 47671. Prepare in 1× PBS/$MgCl_2$. Add 2.7 mL of 37% formaldehyde to 97.3 mL of 1× PBS/$MgCl_2$.
5. 2× SSC: 50 mL 20× SSC, 450 mL H_2O, room temperature.

2.4. Postfixation for CGH

1. PBS at room temperature.
2. 1% Formaldehyde: Add 2.7 mL of 37% formaldehyde to 97.3 mL of 1× PBS/$MgCl_2$.

2.5. CGH

Use MilliQ DI water for all solutions.

1. CGH nick translation kit (nick translation enzyme, 10× nick translation buffer, dTTP, dCTP, dATP dGTP, nuclease-free water, unlabeled and Spectrum Green-labeled control DNA, Vysis cat. no. 32-801-3000).
2. Spectrum Red dUTP, Vysis cat. no. 30-803400.
3. Spectrum Green dUTP, Vysis cat. no. 30-803200.
4. Human COT-1 DNA 32-800028.
5. Salmon sperm DNA, 1 mg/mL.
6. 3 *M* sodium acetate.
7. 100% ethanol.
8. Nick translation kit with direct-labeled nucleotides, Vysis cat. no. 32-801-3000.
9. LSI/WCP hybridization buffer, Vysis cat. no. 30-804826 (dextran sulfate, 2× SSC, and formamide, pH 7.0).

2.6. Hybridization

2.6.1. Solutions for CGH

1. 70% Fluka formamide: 35 mL formamide, 10 mL of 20× SSC, 5 mL distilled H_2O, pH to 7.0, heat for 30 min to 74°C.
2. Ethanol, 70%, 80%, 100%.

2.7. CGH Detection

1. 0.4× SSC 0.3% NP 40: To 10 mL 20× SSC, add H_2O so that final volume is 500 mL, heat for 30 min at 74°C.
2. 2× SSC 0.1% NP-40: 50 mL 20× SSC: 450 mL H_2O, 0.5 mL NP-40, room temperature.

Washing of the slide should be done with extensive agitation of the slide by hand, because of the large amounts of DNA.

2.8. Less Stringent Wash Solutions for CGH

1. 50% Formamide, Fluka brand from Fisher Scientific, cat. no.

2. Formamide/SSC: 30 mL 20× SSC, 120 mL sterile water, 150 mL formamide. Adjust pH to 7.4 (200 mL FA/SSC requires 500 µL of 1*M* HCL). Heat for 30 min at 43°C.
3. 2X SSC 0.1% NP40: 50 mL 20X SSC, 450 mL distilled water, 0.5 mL NP 40. Heat to 37°C for 30 min.
4. Vectashield mounting medium with DAPI and antifade (Vector).

>AU: HCl?

>AU: cat. no.?

2.9. Labeling of BAC, P1, or Yac Clones

1. Nick translation kit (nick translation enzyme, 10× nick translation buffer, dTTP, dCTP, dATP dGTP, nuclease-free water, unlabeled and Spectrum Green-labeled control DNA, Vysis cat. no. 32-801-3000.
2. Texas Red dUTP, Molecular Probes cat. no. C-7608.
3. Spectrum Green dUTP, Vysis cat. no. 30-803200.
4. CGH hybridization reagents (CGH hybridization buffer, 20× SSC, NP40, and DAPI II counterstain Vysis cat. no. 32-801023.
5. Human COT-1 DNA, Vysis cat. no. 32-800028.
6. Nick translation kit with direct-labeled nucleotides, Vysis cat. no. 32-801-3000.
7. Vectashield mounting medium with DAPI and antifade, Vector cat. no.
8. LSI/WCP hybridization buffer, Vysis cat. no. 30-804826 (dextran sulfate, SSC, and formamide pH 7.0).

>AU/ED: Style(s)—BAC vs Bac? Yac vs yac vs YAC (p. 346)?

>AU: cat. no.?

2.10. Bac Hybidization

1. Vysis Nick Translation kit.
2. 70% Formamide/2× SSC.
3. 70%, 80%, 100% ethanol.
4. Hybridization buffer.

2.11. PCR-Based Copy

1. PCR buffer.
2. dNTP mix.
3. Flourescently labeled STS.
4. Automated DNA sequence analyzer.

2.12. Exon Trapping

1. Genomic DNA for this protocol can be obtained from cosmids, plasmids, phage, yacs, Bacs, or Pacs.
2. Restriction enzymes: BstXI and one or more of the following for cloning in vector pSPL3: BamHI, SstI, NotI, XhoI, XmaIII, PstI.
3. PSPL3 (Gibco)
4. 7.5 *M* ammonium acetate.
5. 100% ethanol.
6. TE buffer, pH 8.
7. Calf intestinal alkaline phosphatase (CIP).
8. T4 DNA ligase.
9. Competent *Escherichia coli.*
10. LB medium and plates with 100 µg/mL ampicillin.
11. COS-& cells.
12. DMEM supplemented with L-glutamine, pen/strep, and 10%10% FBS.
13. Cationic lipid (your choice).
14. Trizol (Gibco).

15. PBS lacking calcium and magnesium.
16. DEPC-treated water.
17. 20 μ*M* primer SA2:5′-ATCTCAGTGGTATTTGTGAGC-3′.
18. 5× first-strand buffer.
19. 0.1 M DTT.
20. 10 m*M* dNTP mix in DEPC water.
21. 200 U/μ*M* MLVH reverse transcriptase.
22. 2 U/μL Rnase H.
23. Taq DNA polymerase and 10× buffer.
24. 50 m*M* $MgCl_2$.
25. 20 μ*M* primer dUSA4:5′-CUACUACUACUACACCTGAGGAGTGAATTGGTCG-3.
26. 20 μM primer SUSD2:
27. 5′-CUACUACUACUAGTGAACTGCACTGCACTGTGAAAGCTGC-3′.
28. 1.5% Agarose gel.
29. 6-Well tissue culture plates.
30. Polystyrene microfuge tubes.
31. TA cloning kit.

2.13. Microarray

1. dNTP mix: 25 m*M* dA/G/TTP, 10 m*M* dCTP. For 100-μL volume, use 25 μL 100 m*M* dATP, 25 μL 100 m*M* dGTP, 25 μL 100 m*M* dTTP, 10 μL 100 m*M* dCTP, 15 μL sterile dH_2O.

2.14. Slide Wash Solutions

1. Wash 1: .5× SSC, 0.01% SDS.
2. Add 12.5 mL 20× SSC and 0.5 mL 10% SDS into a total vol. of 500 mL dH_2O (487 mL dH_2O). Measure volumes in grad. cylinder, *not* wash bottles.
3. Wash 2: 0.06× SSC, 0.01% SDS.
4. Add 1.5 mL 20× SSC and 0.5 mL 10% SDS into a total vol. of 500 mL dH_2O (498 mL dH_2O). Measure volumes in grad. cylinder, *not* wash bottles.
5. Wash 3: 0.06× SSC.
6. Add 1.5 mL 20× SSC into a total vol. of 500 mL dH_2O (498.5 mL dH_2O), and filter with a 0.22-μm filter. Measure volumes in grad. cylinder, not wash bottles.

2.15. Preparation of 6% Polyacrlyamide Gels (100-mL volume) for SSCP

1. 20 mL Acrylamide (49:1).
2. 6 mL 10× TBE.
3. 200 μL Ammonium persulfate (10%).
4. 20 μL TEMED.
5. qs to 100 mL ddH_2O.

2.16. Preparation of Stop Solution (50-mL volume) for SSCP

1. 10 mL 95% Formamide.
2. 10 mg Xylene cyanol.
3. 10 mg Bromophenol blue.
4. 200 μL 0.5 *M* EDTA.
5. 10 μL 10 *M* NaOH.
6. qs to 50 mL ddH_20.
7. Mix completely and aliquot into 1.5-mL Eppendorf tubes. Store at −20°C.

2.17. PTT Analysis

1. QIA quick PCR columns (Qiagen, Santa Clarita, CA).
2. TNT T7 Quick Coupled Transcription/Translation reaction (Promega, Madison, WI).
3. 0.3 m*M* magnesium acetate.
4. 10–15% discontinuous SDS-PAGE gel.
5. Amplify (Amersham Corp. Arlington, IL).
6. Phosphoimager and screens.

3. Methods

The analysis of chromosomes by the individual banding pattern allows a genome-wide scan of the karyotype. Staining methods allow the unambiguous identification of each individual chromosome. The study of cytogenetic changes in malignancies has been especially useful in the understanding of the pathogenesis of the disease. Genetic alterations in cancer commonly occur in chromosomal regions that regulate growth and development. Identifying these chromosomal regions is the first step in determining the critical genes for the initiation and progression of cancer. The analysis of these changes is the first step of isolating the specific genes that are involved in the progression of cancer. The identification of cytogenetic changes in tumors is limited by quality of the chromosome morphology. This technique is most powerful when it is done in combination with molecular cytogenetic methods.

3.1. Giemsa Trypsin Banding (see Note 1)

This procedure involves the proteolytic treatment of the chromosomes to produce a differential staining in the chromosomes arms. This treatment allows pairing of homologs and the detection of chromosomal rearrangements. The biochemical nature of the reactions involved has not yet been completely defined.

1. Immerse aged slide in working trypsin solution for 20–90 s at 20–25°C.
2. Rinse in tap water.
3. Immerse in working Leischman's stain for 1.5–2.5 min.
4. Rinse in tap water.
5. Dry the stained slide by blowing the compressed air at moderate force over the surface of the slide.
6. Examine the slide for quality of bands and staining. If slide is adequate for analysis and photography, place on slide warmer.
7. Adjust trypsin treatment times and/or stain times to find the best banding for the meatphase preparation.
8. Banded slides should be left on slide warmer for 5–10 min, then cover-slipped and allowed to dry before screening and analysis.

3.2. Spectral Karyotyping (SKY)

Nonrandom chromosome abnormalities associated with the development of tumors can target genes that are altered during carcinogenesis. Specific chromosome break points have also been associated with genes that confer susceptibility as well as resistance to cancer. Karyotypic analysis of tumor metaphase preparations is hindered by low mitotic index and complicated translocations as well as small rearrangements that are difficult to identify by traditional chromosome banding. Although comparative genomic

hybridization has made it possible to characterize the loss and gain of chromosomal material, this method cannot identify translocations. The accuracy of the identification of karyotypic alterations has been greatly increased by the development of spectral karyotyping. Spectral karyotyping is a novel imaging method that combines spectroscopy and imaging. This type of approach allows analysis of the full spectrum of light at all pixels of the image. The spectral-based approach can simultaneously identify 100% of the chromosomes in a metaphase spread. The loss, gain, or translocation of any chromosome in a metaphase spread can be characterized in one experiment *(2)*.

The spectral paints used to detect the chromosomes are prepared using flow-sorted chromosomes labeled with a combination of 5 fluorochromes by degenerate oligonucleotide-primed PCR *(2,3)*. All 46 chromosomes are labeled a unique color, which is analyzed and karyotyped using computer imaging and Applied Spectral Imaging software (ASI, Carlsbad, CA). The analysis of the slides is done using a microscope attached to a spectral cube (Applied Spectral Imaging) and a filter cube (SKY1, Chroma Technology, Brattleboro, VT). The attached spectral cube and filter allow for the simultaneous excitation of all dyes and the measurement of their emission spectra. The spectral measurements are analyzed using ASI software. A classification color is assigned based on the best match for each chromosome. A color image is then created in which every pixel is displayed in the color that corresponds to the chromosome-specific emission spectrum *(4)*. The spectral classification is the basis for chromosome identification and SKY.

1. Warm 50 mL of 0.01 *M* HCL with 6 µL pepsin stock (may need up to 15 µL depending on cytoplasm on slide) to 37°C in a Coplin jar.
2. Incubate slides in pepsin solution for 1–3 min for human and up to 1 min for mouse.
3. Wash slides in 1× PBS at room temperature for 5 min. Do a second wash in PBS for 5 min at room temperature.
4. Wash slides in 1× PBS/$MgCl_2$ at room temperature for 5 min.
5. Place slides in a Coplin jar with 1% formaldehyde for 10 min at room temperature.
6. Wash slides in 1× PBS for 5 min, agitating the slide during the wash.
7. Dehydrate slides in 70%, 80%, and 100% ethanol for 2 min each. Air-dry slide completely.

3.2.1. Denaturation

1. Warm denaturation solution (70% formamide/2× SSC, pH 7) to 72°C in a tube in a water bath. Warm the slide in a hybridization oven to 72°C.
2. Add 100 µL denaturation solution warmed to 72°C directly onto slide, add coverslip, and place in a hybridization oven at 72°C for 30–45 s. Shake off the cover slip and immediately put slide in 70% ethanol. It is important to do only one slide at a time.
3. Place slides in ice-cold 70%, 80%, and 100% ethanol for 2 min each, then air-dry completely.
4. Add 10 µL of denatured probe (vial #1) to the hybridization area and cover with a 22 × 22-cm glass cover slip, being sure the probe spreads evenly. Immediately seal edges with rubber cement. Let slide on slide warmer for 4 h to overnight for rubber cement to cure.
5. Dampen paper towels with water and place the folded towels at the bottom of a Petri plate to assemble the humidified chamber. Place Q-tips over the paper towels, then lay the slides directly over the Q-tips. Seal the Petri plate with aluminum foil. Transfer slides to a humidified chamber at 37°C for 72 h (from time the probe is added).

3.2.2. Wash/Detection After Hybridization

1. Remove slides from humidified chamber and carefully remove cover slip.

2. Transfer slides to a Coplin jar with 50% formamide/2× SSC, prewarmed to 45°C for 5 min. Repeat this twice for 3 full washes, 5 min each.
3. Wash slides in a Coplin jar of 1× SSC prewarmed to 45°C for 5 min. Agitate the slides by hand for each wash. Repeat this once for 2 full washes.
4. Wash slides in a Coplin jar with 4× SSC/0.1% NP-40 prewarmed to 45°C for 2 min.
5. Pipette 60–80 µL of blocking reagent (vial #2) to hybridization area and cover with a 24 × 30-mm cover slip, being sure it spreads evenly. Lay flat and incubate at 37°C for 30 min in a humidified chamber.
6. Carefully remove cover slip and add 60–80 µL of buffer 1 (vial #3) and cover slip. Incubate for 45 min in a humidified chamber at 37°C.
7. Carefully remove cover slip and wash slides in 4× SSC/0.1% NP-40 prewarmed to 45°C for 5 minutes. Repeat this with fresh wash for 3 full washes with agitation.
8. Add 60–80 µL of buffer 2 (vial #4) to hybridization area and cover slip. Incubate at 37°C in a humidified chamber for 45 min. *Repeat step 8 with new wash.*
9. Pipet about 20 µL of DAPI/antifade solution (vial #5) over the hybridization area, being sure to completely cover area evenly with no bubbles. Apply 24 × 60-mm cover slip to cover whole hybridization area. Store at −20°C until ready to view.

>AU: Step 8, OK?

3.3. Comparative Genomic Hybridization Introduction (see Note 2)

Neoplastic transformation is a complex process involving both positive and negative regulatory elements. Determination of loss of heterozygosity by allelotype studies alone underestimates the loss of genetic material. Using computer imaging systems, the degree of chromosomal loss and gain in frozen and archival tumor samples can be measured by comparative genomic hybridization (CGH). The degree of loss and gain of chromosomal material of archival samples can be estimated within 10 megabases using CGH software *(5)*. The CGH analysis can be followed by further FISH analysis of the common break-points with specific BAC, cosmid, and YAC probes *(6)*. The precise mapping of the alteration of a single gene can be accomplished using small probes, possibly using YAC, BAC, and cosmid clones *(7)*. The defined break-points identified in tumor cells can then be followed back to earlier changes both in dividing populations and in interphase cells using the specific genetic region probes within those regions.

>AU: Change(s) OK?

3.3.1. Pretreatment of Slides

1. Equilibrate slides in 2× SSC at RT.
2. RNase A treatment:
   a. Dilute the RNase A stock 1:200 (in 2× SSC).
   b. Apply 100 µL to 24 × 60-mm^2 cover slip, touch slide to cover slip, incubate at 37°C for 45 min.
   c. Remove cover slips and wash 3 times for 5 min in 2× SSC, RT, with shaking (Coplin jar).
3. Pepsin treatment:
   a. Prepare solution: Make 0.01 *M* HCl by adding about 1 mL of 1 *M* HCl to 99 mL of dH_2O, prewarmed at 37°C. Add 10–50 (µ) 1 pepsin first, then add 100 mL prewarmed 0.01 *M* HCl. Mix well and adjust pH to 2.0 with sodium hydroxide.
   b. Incubate slides at 37°C in Coplin jar for 4–10 min.
   c. Wash 2 times for 5 min each in 1× PBS, at room temperature, with shaking.
   d. Wash 1 time for 5 min at room temperature with 1× PBS/$MgCl_2$.

>AU: 100 µL, OK?

>AU: Change(s) OK?

3.3.2. Pretreatment Procedure

1. Make solution of 1% formaldehyde in 1× PBS/$MgCl_2$.

2. Incubate for 10 min at room temperature.
3. Wash 1 time for 5 min in 1× PBS, at room temperature, with shaking.
4. Dehydrate slides in 70%, 90%, and 100% ethanol for 3 min each.
5. Air-dry slides.

3.3.3. Probe Preparation

1. In a microfuge tube cooled on ice, combine the following:
   a. 1–3 μg DNA.
   b. 2.5 μL of 0.2 m*M* Spectrum Green dUTP or Spectrum Orange/Red dUTP.
   c. 5 μL of 0.1 m*M* dTTP.
   d. 10 μL of dNTP mix (0.3 m*M* dGTP, 0.3 m*M* dATP, 0.3 m*M* dCTP).
   e. 5 μL of 10× nick translation buffer
   f. 10 μL of nick translation enzyme cocktail
2. Vortex briefly.
3. Incubate 6–12 h (usually 8 h for YACs and BACs) at 15°C.
4. Stop reaction by heating to 70°C for 10 min—incubation and stop can be done in a thermal cycler.
5. Chill on ice or store at 4°C until ready to use probe.
6. Determine probe size by running 8–10 μL of the sample on a 2% agarose gel with ethidium bromide. The majority of the smear should be between 500 and 3000 bp for best hybridization; if the probe is larger, you can add more enzyme and incubate at 15°C for longer and stop reaction by 70°C for 10 min and rerun sample on a gel.
   It is important that the length of the test and the control DNA are the same.
7. From the smear, determine the amount of product that is required for a probe (usually 10–30 μL of product).
8. To a small centrifuge tube, add: 100 ng/labeled DNA product (usually 10–30 μL), 3 μL COT-1 DNA, for blocking, 0.5 μL salmon sperm, for blocking, 0.1 vol of 3 *M* sodium acetate, 2.5 vol of 100% ethanol.
9. Vortex briefly and incubate on Dry Ice for 15–30 min. Spin at >12,000 rpm for 30 min at 4°C. Carefully pour off supernatent. Dry pellet completely in a speed vacuum centrifuge for 5–8 min.
10. To the pellet add 3 μL nuclease-free water and 7 μL Vysis hybridization buffer (dextran sulfate, SSC, and formamide, pH 7.0).
11. Mix well and allow resuspending at least overnight at −20°C before hybridizing.

3.3.4. Assembly of Probe Mixture

1. Combine in a 1.5-mL tube:
   a. 10 μL (200 ng) Spectrum Green test DNA (nick translated).
   b. 1 μL (100 ng) Spectrum Red total genomic reference DNA.
   c. 10 μL (10 μg) Cot-1 DNA.
   d. 0.5 μL (10 mg/mL) Salmon sperm.
2. Add 2.1 μL (0.1 vol) 3 *M* sodium acetate. Then add 52.5 μL (2.5 vol) of 100 % ethanol and vortex briefly. Let stand on Dry Ice for 15–30 min.
3. Centrifuge at 12,000 rpm at 4°C for 30 min.
4. Pour off supernatent and dry pellet for 10–15 min under vacuum at ambient temperature.
5. Resuspend pellet in 3 μL nuclease-free H_2O and 7 μL hybridization buffer.
   Store at −20°C at least overnight.

3.3.5. Procedure

1. Select slides with well-spread, long chromsomes with very little cytoplasm. Choose slide and mark hybridization area with diamond pen. The area is 22 × 22.

>AU: Units?

2. Warm in a H_2O bath set to 74 ± 1°C with a Coplin jar of denaturation solution (70% formamide).
3. Immerse slides in denaturation solution for 3–5 min.
4. Dehydrate slides in ethanol for 2 min each, 70%, 80%, 100% ethanol.
5. Denature probe by placing it in a float in the 74°C water bath for at least 5 min.
6. Completely dry slides by wiping bottom and then put on slide warmer.
7. Apply 10 µL of probe to hybridization area while on slide warmer.
8. Apply cover slip and be sure probe spreads evenly.
9. Immediately seal with rubber cement, make sure it is completely sealed, and place immediately in the incubator. Place slides in a humidified box at 37°C for 2–4 d.

3.3.6. Washing Slide, Stringent Wash

1. Warm the water bath and a Coplin jar with a solution of 0.4× SSC/0.3% NP to 74 ± 1°C for at least 30 min.
2. Prepare a Coplin jar of room-temperature 2× SSC/0.1% NP-40.
3. Remove rubber cement and cover slip from slides carefully and immediately place in Coplin jar at 74°C of 0.4× SSC/0.3% NP-40 for 1–3 s and agitate slide.
4. Put slide in the room-temperature 2× SSC/0.1% NP-40 for 5–60 s with agitation to remove the probe. Metaphase preparations with a lot of cytoplasm will require the longer washing time.
5. Air-dry slide in darkness.
6. Apply 10 µL of DAPI II counterstain (Vectashield mounting medium with DAPI and antifade, Vector) to hybridization area and cover slip.
7. Detection of the losses and gains requires a fluorescent microscope, a sensitive camera, and computer software to quantitate the changes as detailed by Kallioniemi et al., 1992 ***(8)***.

3.3.7. Procedure for Less Stringent Wash

1. Make 3 Coplin jars of 50% formamide/2× SSC pH 7.0 and warm to 43°C in a H_2O bath. Make 3 Coplin jars of 2× SSC and warm to 37°C in a H_2O bath. Make 1 Coplin jar of distilled H_2O at room temperature.
2. Place slides in first 50% formamide jar at 43°C for 5 min. Repeat for 3 full washes.
3. Place slides in a 2× SSC at 37°C for 5 min. Repeat for 3 full washes in fresh 2× SSC.
4. Rinse slides in distilled deionized room temperature H_2O three times.
5. Apply 19 µL DAPI II counterstain (Vectashield).

3.4. Refinement of the Region of Consistent Abnormality. Mapping by Fluorescent In Situ Hybridization (FISH)

BACs or PACs containing an average of 100 kbp can be useful in refining regions lost during the tumorigenic process. They may be particularly useful in mapping regions of deletion or chromosome alteration by labeling DNA from these reagents with fluorescent tags and hybridizing to metaphase chromosomes obtained from cell lines or tumor material to map consistently altered regions of the genome. Narrowing the region of consistent abnormality can aid in the identification of candidate cancer suppressor genes by refining the search to a single BAC or PAC.

3.4.1. Procedure for Nick Translation of BAC, YAC, P1, or Cosmid Clones

1. In a microfuge tube cooled on ice, combine the following:
   a. 1–3 µg Extracted P1, BAC, cosmid, or YAC DNA. Prepare 0.2–1-µg/µL solution of extracted DNA in Tris-EDTA (10 m*M* Tris base, 1 m*M* EDTA, pH 8.5) buffer.

b. 2.5 µL of 0.2 m*M* Spectrum Green dUTP or Spectrum Orange/Red dUTP.
c. 5 µL of 0.1 m*M* dTTP.
d. 10 µL each of dNTP mix, 0.3 m*M* dGTP, 0.3 m*M* dATP, 0.3 m*M* dCTP.
e. 5 µL of 10× nick translation buffer.
f. 10 µL of nick translation enzyme cocktail.

2. Vortex briefly.
3. Incubate 6–12 h (usually 8 h for YACs and BACs at 15°C.
4. Stop reaction by heating to 70°C for 10 min.
5. Remove unincorporated nucleotides by gel filtration using a Sephadex G-50 spin column equilibrated in 10 m*M* Tris base, 1 m*M* EDTA, and 0.1% SDS (the SDS prevents the probe from sticking in the column).
6. Chill on ice or store at 4°C until ready to make probe. Incubations and stop reactions can be done in a thermal cycler.
7. Determine probe size by running 8–10 µL of the sample on a 2% agarose gel with ethidium bromide. The majority of the smear should be around 300 and 3000 bp for best hybridization. If the probe is larger than 300 bp, you can add enzyme and incubate at 15°C for longer and stop reaction by 70°C for 10 min and rerun sample on a gel. Probe fragments that are larger may produce bright fluorescent speckles across the hybridization area.

3.4.2. FISH Hybridization with BAC, P1, YAC, and Cosmid Region-Specific Probes

1. Mark slide hybridization area.
2. Denature slide in 70% formamide/2× SSC at 78°C for 7 min.
3. Make probe hybridization mixture from the nick translation reaction of about 10–20 µL nick translation product per slide.
4. Dehydrate slides in ethanol series (70%, 80%, and 100%) for 2 min each.
5. During dehydration denature probe mix at 78°C for 7 min (optional: pre-anneal the probe for 1–2 hours at 37°C prior to hybridization).
6. Immediately apply probe (10–20 µL) to hybridization area, apply a 22 × 22-cm glass cover slip, and seal with rubber cement.
7. Incubate at 37°C for 36–72 h.

3.4.2.1. WASH

1. Wash slides in 50% formamide/2× SSC at 45°C for 5 min each. Do 3 full washes in fresh solution in clean jars.
2. Wash slides in 0.1× SSC at 60°C for 3 full washes at 5 min each.
3. Wash slides in 4× SSC/0.1% NP-40 for a dip (1–3 s) at 45°C. Dip in RT tap water. Then air-dry.
4. Apply DAPI (Vectashield mounting medium with DAPI and antifade, Vector) to cover the hybridization area and cover slip.

3.5. Loss of Heterozygosity (LOH) to Map Homozygous Deletion

LOH is a molecular approach used to identify regions of consistent loss or to narrow a region of deletion in tumor material in comparison to normal tissue from the same individual. Mapping regions of loss using this approach has successfully localized nonfamilial cancer suppressor genes to particular chromosomes and further refined the region of deletion to chromosomal bands or even smaller genetic regions. Under ideal circumstances, a small region of homozygous loss can be identified in the tumor genome and a physical map spanning the region can be constructed from which candidate genes can be

cloned. Illustrative examples demonstrating the success of this approach are the cloning of INK4A/p16 in 9p21, RB1 in 13q14, and PTEN in 10q23. These successes demonstrate the usefulness of LOH in cancer suppressor gene identification for the future.

The techniques used to detect LOH have traditionally been molecular-based approaches such as restriction fragment length polymorphism (RFLP) or PCR-based amplification of polymorphic tandem repeats (STS, sequence-tagged-sites) to identify the alleles present in each respective chromosome. RFLP utilizes the normal variation in sequence that exists between chromosomal alleles to detect changes in the ability of restriction enzymes to cut DNA. Usually the variation is observed as fragments of different size produced after digesting DNA with a restriction enzyme. The PCR-based approach identifies polymorphic variation that occurs in the number of tandem repeats present in the maternally and paternally derived chromosomes. Isotopic labels have been widely used for both strategies; however, the latter technique now commonly employ fluorescently labeled PCR primers, with the product being visualized using fluorescence detectors. Fluorescently labeled STS primer sets or MapPairs are commercially available from Research Genetics, Inc. (http://www.resgen.com/).

STSs or MapPairs generally amplify under uniform PCR conditions; however, specific reaction conditions for each marker are described in GeneMap 99 (http://www.ncbi.nlm.nih.gov/genemap99/), from the Genome Data Base (http://www.gdb.org/) or can be obtained from a reference cited therein. Markers spaced at desired intervals along the length of the chromosome can be obtained through the GDB or GeneMap 99. Each marker should be accessed twice and examined by two independent evaluators prior to determining LOH.

Any standard procedure for DNA extraction from tumor samples and matched normal lymphocytes can be used to isolate DNA of sufficient quality for use in this procedure.

1. Prepare the following buffer and set up the PCR reaction as follows:
   a. Buffer conditions: 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 10 m*M* Tris-HCL, pH 9.3.
   b. Protocol: 10 ng template: 5 p*M* of each primer, 4 n*M* of each dNTP, 0.025 U/µL of Taq. Total volume 20 µL (*see* **Note 9**).
2. Heat reaction to 95°C for 5 min.
3. PCR thermalcycler program: 94°C for 40 s; 55/56°C for 30 s; 72°C for 40 s. Do 35 cycles followed by 2 min at 72°C.
4. From 1 to 5 µL of PCR reaction can be run on an ABI sequence analyzer. Fluorescent size markers should be added to the samples and used as an internal control to determine the approximate location and size of the PCR products.

3.6. Cloning Candidate TSG Genes from the Region of Consistent Abnormality

3.6.1. Exon Trapping

Exon trapping or exon amplification identifies expressed DNA sequences present in a segment of genomic DNA by selecting for functional splice sites found in that genomic DNA *(9)*. No prior knowledge is required regarding tissue-specific gene expression, and the protocol is amenable to complex genomes. An added advantage of this approach is that it can identify constitutive exons and alternative exons but cannot be used to identify genes that lack introns.

The procedure requires the use of an "exon trap" vector called pSPL3 (Gibco), which contains an artificial mini gene consisting of three parts. The first is a segment of the

simian virus 40 (SV40) containing an origin of replication and promoter. The second elements are two splicing-competent exons flanking an intron sequence that contains a multiple cloning site. The third part is an SV40 polyadenylation site.

The portion of genomic DNA to be screened for the presence of exons is inserted into a restriction site in the multiple cloning site followed by transfection into a mammalian cell line such as monkey COS-7 cells. Transcription of RNA occurs from the SV40 promoter and the transcript undergoes splicing driven by the machinery of the host cell. Any exon contained in the genomic fragment becomes attached between the upstream and downstream minigene exons. RT-PCR with primers that are specific for the minigene exons are used to confirm successful exon trapping. Trapped exons produce a PCR product that is larger than that of vector alone. The identity of the inserted product can be determined through sequencing using primers specific to the minigene sequence. Sequencing, followed by a database search, is the most direct way to obtain information from the trapped sequences. Since trapped exons are also putative cDNAs, they can be used as probes on cDNA libraries, Southern blots, Northern blots, or for FISH.

Exon trapping has proven to be an important part of positional cloning. Demonstrations of the successful use of exon trapping include the identification of at least 30 genes, including the Menkes disease gene, the NF-2 gene, and the HD gene ***(9,15,18)***.

Day 1–3: DNA Preparation and Subcloning

1. Digest approximately 1 µg of both genomic and pSPL3 DNA in separate microfuge tubes with the same restriciton enzyme or enzyme combinations. The pSPL3 multiple cloning site contains unique restriction sites for EcoRI, SstI, XhoI, NotI, ZmaIII, PstI, bamHI, and EcoRV. Single or double digests can be used (your choice).
2. Phenol extract DNA followed by ethanol precipitation (1/2 vol of 7.5 *M* ammonium acetate and 2 vol of 100% ethanol). Wash twice using 70% ethanol.
3. Dissolve the pellet in TE buffer, pH 8.0. The final concentration should be ~250 ng/mL.
4. If only a single enzyme is used, pSPL3 should be dephosphorylated with CIP (according to supplier's protocols). This step is not necessary if two different restriction enzymes are used.
5. Subclone genomic fragments into pSPL3 using T4 DNA ligase according to supplier's protocol.
6. Transform *E. coli* with ligated DNA.
7. Incubate 500 µL of cells with 5 mL of LB containing ampicillin overnight at 37°C.
8. Plate 10 and 100 µL of ligation reaction onto LB/ampihicillin plates to determine the degree of ligation. Place at 37°C overnight.
9. Isolate plasmid containing insert from the 5-mL culture by any standard miniprep procedure.

Day 4–5: Lipid-Mediated Transfection

10. On the afternoon before transfection (~15 h prior), plate COS-7 cells in DMEM supplemented with 10% FBS into 6-well plates to achieve 70–80% confluency (~4–6 × 10^5 cells).
11. Add an optimum amount of lipid (determined from supplier's protocol) reagent to 100 µL of serum-free medium in a sterile polystyrene tube, mix gently, and incubate at room temperature for 5 min.
12. For each transfection, add 1 to 100 µL of serum-free medium in separate polystyrene tubes. Combine lipid and DNA solutions with gentle mixing and incubate at room temperature for 15 min.

13. Aspirate medium from COS-7 cells and add 2 mL of 37°C serum-free DMEM.
14. Add an additional 1 mL of serum-free DMEM to the lipid/DNA mix.
15. Remove medium from the plates and add the entire lipid/DNA mix of one tube to the plate; incubate for 6 h at 37°C.
16. Following the 6-h incubation, add an additional 1 mL of DMEM medium supplemented with 10–20% FBS and incubate an additional 24 h at 37°C.
17. Rinse cells with PBS lacking calcium and magnesium then add 1 mL of Trizol (Gibco). Prepare RNA according to manufacture's protocol. Resuspend in 50 µL DEPC-treated water.

Day 6: RT-PCR

18. Add the following components in a polypropylene microfuge tube: 2 µg RNA, 1 µL 20 m*M* oligonucleotide SA2, and DEPC water to 8 µL.
19. Heat to 70°C for 10 min, ice for 10 min, and centrifuge briefly.
20. Add 3 µL 5× reaction buffer: 2 µL 0.1 *M* DTT, 1 µL mixed dNTP stock (10 m*M* of each dNTP).
21. Mix gently, centrifuge briefly, and equilibrate for 2 min to 37°C.
22. Add 1 µL MMLV H-RT and mix gently.
23. Incubate at 37°C for 1 h, then incubate at 55°C for 5 min.
24. Add 1 µL of RNase H, mix gently, and incubate at 55°C for 10 min.
25. Add 15 µL of DEPC water (final volume 30 µL).

Primary PCR

26. Combine the following reagents in a polypropylene tube:
    a. 5 µL of RT reaction mix.
    b. 5 µL of 10× Taq buffer.
    c. 1.5 µL 50 m*M* $MgCI_2$.
    d. 1 µL dNTP mix (10 m*M* of each in sterile water).
    e. 2.5 µL 20 m*M* primer SA2.
    f. 2.5 µL 20 m*M* primer SD6.
    g. Sterile water to 47.5 µL final volume.
27. Heat reaction to 95°C for 5 min, then add 2.5 µL of Taq (1 U/mL).
28. Run the following amplification cycles: 6 cycles of 1 min at 95°C, 1 min at 60°C, 5 min at 72°C; 1 cycle of 10 min at 72°C, followed by a soak at 55°C.
29. Add 25 U BstXI and incubate overnight at 55°C. This step eliminates vector-only sequence and sequences using the cryptic splice-donor site.
30. Add an additional 5 U of BstXI, incubate 2 h more at 55°C.

Secondary PCR

31. Combine the following reagents in a polypropylene PCR tube:
    a. 1 µL of a 1:10 dilution of the primary PCR product.
    b. 78 µL of sterile water.
    c. 10 µL of 10× Taq DNA polymerase.
32. Heat to 958C for 5 min, then add 3 lL of Taq DNA polymerase.
33. Perform the following amplification cycle: 30 cycles of 30 s at 558C, 2 min at 728C; 1 cycle of 10 min at 728C, followed by a soak at 48C.
34. Electrophorese 10 lL of PCR product with dye in a 1.5% agarose gel and identify reactions that contain PCR products.
35. PCR products containing exon can be sequenced using the AD2 and UAP primers and the PCR product can be cloned using the TA cloning kit (according to supplier's protocol, Promega).

3.7 Microarray Analysis

A major obstacle in postional cloning of TSG is identifying the specific mutated gene from within a large physical contig. The completion of the human genome has incited technologic advances that are geared toward efficiently exploiting these sequences and the associated physical resoureces to identify human disease genes. Here we describe the application of DNA microarray technology to a defined genomic region (physical map) to identify: (a) exons without *a priori* sequence data and (b) the TSG based on differential gene expression in a tumor. Rather than arrayed cDNA elements, this approach utilizes an arrayed genomic library within the genetic interval of interest. The feasbility of this approach has been demonstrated by Stephen et al. *(6)* who used such a strategy to identify the Niemann-Pick type C gene.

3.7.1. Total RNA Isolation with Trizol (see Note 10)

Prep. notes: Turn centrifuge on to 4°C.

1. For a confluent monolayer of cells, use 5 mL of Trizol for each T75 flask or 10 mL of Trizol for each T175 flask (generally yields 1 mg total RNA).
2. Lyse cells by repeated pipetting, making sure that the mixture has been sufficiently homogenized. *This is an important step.*
3. Incubate at room temperature for 5 min.
4. At this point the homogenized sample can be stored at −80°C (for up to 1 mo) for later use.
5. Add 0.2 mL of chloroform per 1 mL of Trizol (ex.: 5 mL of Trizol:1 mL of chloroform).
6. Shake tubes vigorously by hand for at least 15 s.
7. Incubate at room temperature for 3 min.
8. Spin samples at no more than 12,000 × g for 15 min at 4°C.
9. Transfer the clear aqueous phase to a fresh tube.
10. Add 0.5 mL of isopropanol per 1 mL of Trizol (ex.: 5 mL of Trizol:2.5 mL of isopropanol).
11. Invert tubes several times.
12. Incubate at room temperature for 10 min.
13. Spin at no more than 12,000 × g for 10 min at 4°C.
14. A gel pellet should now be visible on the bottom of the tube.
15. Discard the supernatant and wash the pellet with 1 mL of 75% ethanol in DEPC H_2O per 1 mL of Trizol (ex.: 5 mL of Trizol:5 mL of 75% EtOH).
16. Mix the sample by vortexing and centrifuge at no more than 7500 × *g* for 5 min at 4°C.
17. Remove the supernatant and briefly air-dry the pellet.
18. Resuspend the pellet in DEPC H_2O and keep on ice (store long-term at −80°C). Try to resuspend so that final conc. is > or = 2 μg/μL (in general use 100–300 μL).
19. Read samples at 260 nm to determine approximate RNA concentration and run 1 μg on a 1% agarose gel with ethidium bromide (look for the presence of 28s and 18s bands).

3.7.2. Poly A+ Isolation with Oligotex (Oligotex mRNA batch Protocol)

Prep. notes: Set centrifuge to 4°C, put Oligotex suspension in 37°C block, heat another block to 70°C (heat OEB or DEPC H_2O here), and place buffer OBB in 37°C incubator (to dissolve precipitant.)

Ex.: Preparation of 1 mg total RNA, starting material at 10 μg/μL.

20. Thaw total RNA on ice.
21. 100 μL of total RNA (for 1 mg total) + 400 μL of RNase-free water for a total volume of 500 μL.

22. Add 500 µL buffer OBB (make sure this solution has not precipitated upon storage; if it has, heat at 37°C to dissolve).
23. Add 55–85 µL of Oligotex suspension (for 1 mg of total RNA, 70 µL works well)—make sure to vortex suspension just before adding.
24. Mix the contents by inverting the tube.
25. Incubate the sample for 5 min at 70°C (linearizes mRNA).
26. Make sure the cap is still tight, and invert the tube to mix. Incubate at room temperature for 10 min (mRNA attaches to beads).
27. Spin for 2 min at 15,000 rpm at 4°C.
28. Remove the supernatant by pipetting (at this point it is okay to leave 50 µL of supernatant in the tube).
29. Resuspend the pellet in 1 mL of buffer OW2 by pipetting and vortexing.
30. Spin for 2 min at 15,000 rpm at 4°C.
31. Remove the supernatant by pipetting (once again, it is okay to leave 50 µL of supernatant in the tube).
32. Again resuspend the pellet in 1 mL of buffer OW2 by pipetting and vortexing.
33. Spin for 2 min at 15,000 rpm at 48°C.
34. This time, remove all supernatant (be careful not to disturb the pellet; use a p10 pipet to remove the residual supernatant).
35. Resuspend the pellet in 30 lL DEPC H20 (at 708C) by repeated pipetting.
36. Spin for 2 minutes at 15,000 rpm at 4°C.
37. Transfer the supernatant containing the eluted poly A+ RNA to a clean RNase-free tube and place on ice (we usually remove only 25 µL at this point, so as not to disturb the pellet).
38. Again wash the pellet in 70°C DEPC H_20 to elute RNA, but this time use 25 µL.
39. Spin for 2 min at 15,000 rpm at 4°C.
40. Transfer the remaining supernatant to the tube on ice containing the eluted poly A+ RNA.
41. Finally, spin this tube for 2 min at 15,000 rpm at 4°C to pellet any residual Oligotex particles and transfer this solution to another clean RNase-free tube and place on ice (for long-term storage place at −80°C).
42. Read the samples at 260 and 280 nm to determine concentration and purity. The 260 nm/280 nm ratio should between 1.7 and 2.0 (a ratio of 2.0 represents a pure preparation). If not, the procedure can be repeated on the sample by following the miniprep protocol (using 15 µL of Oligotex).

>AU: OK as written?

>AU: Give 15,000 rpm x *g*

>AU: rpm x *g*

>AU: rpm x *g*

>AU: rpm x *g*

>AU: rpm x *g*

3.7.3. Probe Preparation

This procedure can be performed in an Eppendorf thermocycler, heat blocks, hybridization oven.

Prep. notes: mRNA conc. must be at least 0.35 µg/µL; if it is not, lyophilize in a speed vacuum briefly to volume below 11.4 µL. Heat block to 70°C, turn on 42°C instrument.

43. Use 4 µg RNA.
44. Add DEPC H_2O to a volume of 11.4 µL.
45. Add 1 lg of oligo dT (2 lL stock at 0.5 mg/mL).
46. Heat 5 min at 70°C (turn block to 65°C), place on ice for 30 s, flash-spin.
47. Incubate 10 min at 25°C.
48. Add 14.6 µL of Cy3 or Cy5 master mix (per reaction tube).
49. Add 6 µL of 5× 1st strand buffer: µL 0.1 *M* DTT, 3 µL Cy3 or Cy5 dCTP, 0.6 µL dNTPs (25 m*M* dA/G/TTP and 10 m*M* dCTP), 2 µL RNasin (Gibco), 14.6 µL.
50. Once master mix has been added to mRNA, keep covered from light.
51. Add 2 µL of Superscript RT II.
52. Incubate for 2 h at 42°C.

53. Spin down tubes after incubation.
54. Pool the two tubes (Cy3 and Cy5 labeled).
55. Add 2.65 µL of 25 m*M* EDTA (to stop reaction).
56. Add 3.3 µL of 1 *M* NaOH (to degrade RNA strands).
57. Incubate for 10 min at 65°C.
58. Add 3.3 µL 1 *M* HCl (to neutralize reaction).
59. Add 5 µL 1 *M* Tris, pH 6.8.
60. Can store temporarily at −80°C at this point, if necessary.

3.7.4. Probe Purification

Perform all steps in minimal light.

3.7.4.1. Microcon 30

Label 2 collection tubes per probe.

61. Insert column into supplied tube.
62. Add 400 µL of dH_2O.
63. Apply the probe mixture (about 80 µL) onto the column and mix well by pipetting.
64. Spin at room temperature (25°C) for 8 min at 12,000 rpm (*see* **Note 11**).
65. Remove column from assembly, add an additional 20 µL of dH_2O, and place it upside down in a new tube (cut cap off of this tube).
66. Spin for 1 min at 15,000 rpm to collect probe (cap strap should now face outward).
67. Remove column and store tube on ice.

>AU: Give 12,000 rpm x *g*

3.7.4.2. QIAquick Nucleotide Removal Kit

Label column, elution tube, 0.5-mL final tube.

68. Add 5 vol of buffer PN to 1 vol of the reaction sample (ex.: If 50 µL of sample is recovered from the microcon, use 250 µL of buffer PN).
69. Transfer this solution to a QIAquick spin column in a 2-mL collection tube.
70. Spin at room temperature for 1 min at 6000 rpm.
71. Discard flow-through and place column back into the same tube.
72. Add 750 µL of buffer PE (be sure to add ethanol to buffer PE before use).
73. Spin at room temperature for 1 min at 6000 rpm.
74. Discard flow-through and place column back into the same tube.
75. Spin at room temperature for 1 min at 13,000 rpm to remove residual ethanol.
76. Place column in a clean 1.5-mL tube.
77. Add 30 µL of dH_2O to the center of the column.
78. Let stand for 2 min at room temperature.
79. Spin at room temperature for 1 min at 13,000 rpm.
80. Remove column and transfer probe to 0.5-mL tube for lyophilization.
81. Lyophilize sample in a speed vacuum until dry (about 1 min per microliter of sample); do not overdry the pellet.
82. Wrap tube containing the sample in foil and store in the dark at −20°C for temporary storage (<24 h), −80°C for long-term storage.

3.7.5. Slide Fixation

For non-amino-linked DNAs printed onto silane-coated slides.

83. Once dry, UV crosslink (0.3 J or 3000 µJ × 100) using Stratalinker.

84. Rinse in 0.1 % SDS for 1 min.
85. Rinse 2× in dH_2O for 1 min.
86. Air-dry.

3.7.6. Hybridization

Perform all steps in minimal light.

87. Denature slide (DNA side facing up) in a beaker of 95°C water for 3 min.
88. Place in a 50-mL tube containing ice cold ethanol for 15 seconds.
89. Spin-dry at 500 × *g* for 1 min (place slide in a 50-mL tube with a Kimwipe in the bottom).
90. Add 30 µL of 2× SSC to each of the hybridization chamber's lower grooves.
91. Set denatured slide into chamber DNA side up and seal for the time being.
92. Retrieve probe from −20°C and resuspend pellet in (for slides printed 10/00):
    a. 9.0 µL of dH_2O.
    b. 5.0 µL of 20 × SSC.
    c. 4.0 µL of 1% SDS.
    d. 2.0 µL of Cot-1 DNA (10 mg/mL).
93. Transfer mixture to a screw-cap tube (do not place probe mixture on ice once resuspended, as the SDS will precipitate).
94. Boil 3 min.
95. Immediately flash-spin sample and apply solution to the slide resting in the hybridization chamber, using the reference slide as a guide.
96. Gently place a cover slip over the array, try to avoid large air bubbles (small bubbles are not a concern because they will disappear).
97. Seal the hybridization chamber and carefully place it in a 64°C water bath overnight (14–18 h).

3.7.7. Washing

Perform all steps in minimal light.

98. Remove hybridization cassette containing slide from water bath, and place slide in the first wash. Carefully remove the cover slip from the slide by continuously dipping into the wash tube (or remove the cover slip from the tube if it has already come off).
99. All wash steps are performed at room temperature on a rocker in 50-mL conical tubes:
    First wash: 5 min, 0.5× SSC, 0.01% SDS.
    Second wash: 5 min, 0.06× SSC, 0.01% SDS.
    Third wash: 2× 2 min, 0.06× SSC filtered.
100. Spin-dry at 500 × *g* for 1 min (place slide in a 50-mL tube with a Kimwipe in the bottom).
101. Store the slide in a slide box until scanned (ScanArray/GenePix).

3.8. Database Searches for Homology to Human Genes or ESTs

When the region of loss or consistent abnormality has been narrowed to approximately 1 cM, genes or EST mapping can be identified by sequencing databases according to the following outline.

1. Either Blast sequence the region or search databases to identify gene ESTs from these regions.
2. Retrieve sequences of known STS from Genbank and Blast them against genomic sequences. Various Blast databases can be found at http:www.ncbi.nlm.nih/BLAST/blast_databases.html. For additional information see NCBI Blast tutorial and Blast course.
3. When genomic sequence is available, the following type of database search can be conducted:

a. Mask the retrieved genomic sequences. The presences of particular types of repetitive elements and vector sequences can distort the Blast results and gene prediction. In particular, L1 elements are often predicted as genes.
b. To avoid these problems, prescreen for repetitive elements within the sequences using a program such as RepeatMasker or Censor. These programs are designed to replace sequence segments that match any of the elements common to your organism (i.e., Alu) with the same number of asterisks or N's.
   RepeatMasker1: http://aurora.bwh.harvard.edu/cg9-bin/RepeatMasker.cgi
   RepeatMasker2: http://ftp.genome.washington.edu/cgi-bin/RepeatMasker
   VecScreen: http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html

4. 5× First-strand buffer (250 m*M* Tris-CI, pII 8.3, 375 m*M* KCI, 15 m*M* $MgCl_2$). This buffer is supplied with the SuperScript Reverse Transcriptase: 2 l 0.1 *M* DTT, 1 µL 10 m*M* dNTP mix in DEPC water, 1 µL Rnasin (Rnase inhibitor from Promega), DEPC water to 19 µL.
5. For prediction of potential genes or ORFs in the retrieved genomic sequences, use a gene prediction program such as FGENESH, which can be found in the Sanger center. However, proceed with caution when using these programs, since gene prediction programs are designed to predict and can give false predictions. Therefore, be sure to compare several predictions using programs based on different prediction algorithms.
6. Genes and ESTs can be screened for genomic sequences, by Blasting masked sequences against the dEST database. When genomic sequences are not available or the region of consistent alteration is too large, the following steps can be helpful in identifying candidate genes.
   a. Check candidates in GenMap 99 and other relevant mapping databases.
   b. Assembled sequences of candidates, based on mapped EST clusters, can be retrieved from other assembly databases such as:
      DOTS (http://.cbil.upenn.edu/DOTS*/dotsweb?page5blast)
      MIPS (http://www.mips.biochem.mpg.de/proj/human/human_blast.html)
      TBI (http://www.dfkx-heidelberg.de/tbi/Welcome.html)
      TIGR (http://www.ncbi.nlm.nih.gov/BLAST.theblast.html)
      STACK (http://ziggy.sanbi.ac.za/stack/stacksearch.html)
   c. The analysis and evaluation of candidiate genes and EST clusters can be based on the following criteria:
      The map position of the clone (GenMap 99)
      Expression in relevant tissues (UniGene)
      Protein similarities (ExPASy Proteomics tools)
   d. In order to find available genomic sequence and homologies of specific candidates to other genes, Blast sequence of chosen candidates against relevant databases.

There are several advantages to using available genomic sequence. First, when genomic sequence is available, new polymorphic STSs can be made to further refine the region of consistent alteration. Second, when genomic sequence is available, it is easier to determine the candidate genomic structure and organization (i.e., intron/exon borders), which can be helpful in designing primers for screening genomic DNA. Once a candidate gene is identified, the next key step is to identify disease-causing alterations associated with the tumor suppressor gene.

3.9. Testing Candidate Genes: Mutational Analysis of the Putative TSG

Direct detection of mutations is not simple, for many reasons that are related both to genes themselves and to current technologies. It is the scanning of large genes for base changes that is the rate-limiting step. Complete sequencing of large genes can also be

time-consuming, costly, and tedious, and mutations can be missed. The alternative approach is to use scanning methods that allow rapid analysis of exon and intron boundaries, then to use limited sequencing to confirm and identify the mutation in a fragment. This approach avoids the labor and cost of sequencing large numbers of negative samples. A variety of methods for detecting unknown mutations currently exist. This section covers single-strand conformation polymorphism (SSCP) analysis, the protein truncation tests, and direct DNA sequencing.

3.9.1. Search for Mutations by PCR-SSCP

3.9.1.1. Preparation of Samples for SSCP analysis

It is critical to use optimized PCR conditions that minimize unwanted extra products because these can result in artifact bands that interfere with interpretation of the SSCP results. DNA samples and PCR should be done according to standard methods; however, the following pointers could help in generating the best SSCP results.

1. Use only highly purified, salt-free template DNA in the PCR reaction.
2. Use primers that contain no partial mismatches in the target sequence.
3. Optimize reagent and primer concentrations for each amplification reaction.
4. Determine thermal cycler settings that eliminate nonspecfic priming.
5. Use the minimum number of PCR cycles to obtain a sufficient quantity of DNA, usually 30 cycles (or fewer) on 100 ng of genomic DNA.

The primers used for amplification can be radiolabled with ^{32}P prior to PCR, or ^{32}P-dCTP can be added to the PCR cocktail (5 µCi) before aliquotting to individual reaction tubes. Following PCR amplification, it is a good idea to check the success of the PCR products on a 3% agarose gel to ensure amplification

3.9.1.2. Gel Preparation and Pouring

1. Glass plates must be clean and free of soap residues or dried gel. To remove residues, apply ethanol to both plates and wipe dry.
2. To ensure that the gel will not stick to the glass plates, treat one of the plates with Gel Slick solution or a similar antistick product. (If the plates were previously silanized, the coating must be removed completely prior to applying a fresh coat of antistick solution).
3. Assemble the glass plates according to the manufacturer's instructions (use 0.4-mm spacers).
4. Either polyacrylamide or MDE matrix can be used to cast gels.
5. Pour into sequencing gel apparatus and allow to solidify. For 10% glycerol gels, add 5.0 mL of pure glycerol to the mixture and q.s with ddH_2O to 100 mL.
6. Place all samples on ice, remove 5.0 µL of hot sample, and mix with 45 µL of stop solution.
7. Heat-denature at 94°C for 2 min.
8. Transfer samples directly to ice. (Quick-spin samples to ensure that all the product is at the bottom of the tube).

3.9.1.3. Electrophoresis

1. Rinse the top of the gel thoroughly with running buffer.
2. Prerun gel for at least _, no longer than an hour. Gels to be run at 4°C should be placed at 4°C for 1 h prior to rerunning and rerun at 4°C.
3. Load 10 µL of sample on to the gel and electrophorese at 20 W for 5 h at room temperature.
4. The gels should be removed promptly and placed on transfer paper (Whatman 3MM filter paper), covered with saran wrap, and dried at 80°C for 30 min.

>AU: — units?

5. Perform autoradiography or phosphor imaging analysis using standard techniques.

3.9.2. Protein Truncation Test (Coupled In-Vitro Transcription and Translation) (see **Notes 5** and **6**)

Coupled in-vitro transcription and translation (also known as the protein truncation test; PTT) is a convenient single-tube reaction to determine if mutations or deletions produce proteins that differ in size when compared to the normal product. This procedure is highly effective for detecting mutations that lead to the termination of mRNA translation and subsequently protein truncation. The types of mutations that can be detected are: nonsense mutations in which there is a single nucleotide substitution that produces a stop codon (TGA, TAA, or TAG); as frameshift mutations in which one or more nucleotides are either inserted or deleted if the number of bases is not divisible by 3, the altered reading fame frequently results in a stop codon or mutations at a splice site. PTT has been especially successful for detecting mutations in genes in which the frequency of missense mutations is low.

3.9.2.1. PTT or Coupled In-Vitro Transcription and Translation (see **Notes 12** and **13**)

1. First-strand cDNA synthesis (start with 1–5 µg of total RNA or 50–500 ng of mRNA). >AU: Pls. review
2. Combine the following components into a nuclease-free microcentrifuge tube: 1 µL or 50–250 ng of random primers, 1 µL of oligo dT primers (stock solution 20 umo), 1–5 µg total RNA, sterile distilled water to 12 µL.
3. Heat mixture to 70°C for 10 min and chill on ice.
4. Collect the contents of the tube by brief centrifugation and add:
   a. 5× first-strand buffer (250 m*M* Tris-Cl, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$). This buffer is supplied with the SuperScript Reverse Transcriptase.
   b. 2 µL 0.1 *M* DTT.
   c. 1 µL 10 m*M* dNTP mix in DEPC water.
   d. 1 µL Rnasin (RNase inhibitor from Promega).
   e. DEPC water to 19 µL.
5. Mix contents of the tube gently and incubate at 42°C for 2 min.
6. Add 1 µL (200 U) of SUPERSCRIPT II and mix by gently swirling the pipet tip in the tube.
7. Incubate 50 min at 42°C. Inactivate the reaction by boiling for 10 min. Spin down contents.
8. Add the following to a PCR reaction tube for a final reaction volume of 100 µL: 10 µL 10× PCR buffer (200 m*M* Tris-HCl [pH 8.4], 500 m*M* KCl), 3 µL 50 m*M* $MgCl_2$, 2 µL 10 m*M* dNTP mix, 2 µL amplification primer 1 (10 µ*M*), 2 µL amplification primer 2 (10 µ*M*), 1 µL Taq DNA polymerase (2–5 U/µL), 2 µL cDNA (from first-strand reaction), 80 µL autoclaved, distilled water. Mix gently. Heat reaction to 94°C, 45 s at 55–65°C (depending on the primer sequence in your gene), and 1 min at 72°C. A final 10-min cycle at 72°C followed by a soak at 4°C is also common. >AU: Pls. review all units.
9. Purify the PCR product using the QIAquick columns (Qiagen, Santa Clarita, CA) according to the supplier's recommendations.
10. After amplification and purification, 100 ng of product is used in a TNT T7 Quick Coupled Transcription/Translation reaction (Promega, Madison, WI) with the addition of 0.3 m*M* magnesium acetate. For detection, a labeled amino acid is included. The label can be either a radionucleotide such as ^{35}S, which is visualized by autoradiography, or biotin for detection by chemiluminescence (see Promega TNT Quick Coupled Transcription/Translation protocol for details.
11. The resultant proteins are run out on a SDS-PAGE gel for sizing against normal control products and protein markers.

3.9.3 Search for Mutations by Direct DNA Sequencing

Direct sequencing of PCR products based on the Sanger dideoxy chain-termination method is a final step of any mutation scanning procedure, as all the methods described above are capable of detecting mutations with varying efficiencies, but none defines precisely the nature of the change. Major improvements have been achieved with recent automated capillary electrophoresis instruments and multicolor fluorescent detection.

1. Optimize PCR conditions for the region of interest in the putative TSG sequence. Amplify PCR products from samples of interest (i.e., cell lines, archival tumor material, blood samples, etc.) under the optimal conditions (total volume is 50 µL).
2. Verify PCR products by 2% agarose-EtBr gel electrophoresis.
3. Purify PCR products by using GFX PCR, DNA, and Gel Band Purification Kit (Amersham Pharmacia). Or, extract PCR product bands from low-melting-point agarose by using the same kit. Elute DNA in an appropriate volume of sterile nuclease-free water.
4. Check the concentration of the purified PCR products on 2% agarose-EtBr gel using Precision Molecular Mass Standard (Bio-Rad).
5. Adjust PCR products to an appropriate concentration (10 ng/100 bases) so that 5 µL can be used per reaction.
6. Sequence samples with the dideoxy termination method on an ABI 373A DNA sequencer.
7. Align sequences using CLUSTAL W (EMBL, http://www.ebi.ac.uk).
8. 1 min in (the radioactive room), inject the solution of probe to the bag that has the prehybridization solution and membrane, hybridize overnight.

4. Notes

>AU: Pls. add text callouts for Notes 3, 4, 7, 8

1. For GTL banding the following may be helpful:
   a. Times of trypsin treatment vary with each case, the age of the slides to be banded, and the technique used to make the slides.
   b. Chromosomes are undertreated when they stain homogeneously; when banding is present, but bands appear to blur into each other; when banding is present, but a darkly stained bar is noticeable between the chromatids.
   c. Chromosomes are overtreated when they appear swollen, with a "crust" around the chromatids; when they appear fuzzy or have a cobwebbed appearance.
   d. Due to variation in the stages of condensation of chromosomes within a specimen, some spreads may appear over- or undertreated, while others appear perfect for analysis. It is the technologist's judgment as to whether treatment or staining time is right for the particular length and morphology of chromosome he or she are working with.
2. If the amount of tumor DNA is not sufficient for CGH, the DNA can be amplified by degenerated oligonucleotide-primer (DOP)-PCR (Telenius et al., 1992).
3. The primers used for amplification can be radiolabled with ^{32}P prior to PCR, or ^{32}PdCTP (5 µCi) can be added to the PCR cocktail before aliqouting to individual reaction tubes. Following PCR amplification, it is a good idea to check the success of the PCR products on a 3% agarose gel to ensure amplification.

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4. We have found that a 30% stock solution of acrylamide with a 49:1 (acrylamide:bis) ratio works extremely well for SSCP analysis. Store the solution in a foil-covered bottle. Prior to preparing the stock solution, treating the acrlyamide with amberlite solution can improve the quality of the acryl amide. Add dissolved acryl amide to a beaker containing 100 g of dry Amberlite MB-1 resin and stir overnight in the cold room. Filter through Whatmen #1 filter paper.
5. The PTT protocol is useful for detecting truncating mutations, i.e., disease-causing and not missense mutations, which often represent non-disease-related sequence variation. Large

stretches of coding sequence (up to 5 kb) can be screened, but a 1.3–1.6-kb cDNA yields the best results. This protocol works equally well with large single exons derived from a DNA template or from multiple exons using an RNA template. The latter can significantly reduce the workload. The length of the truncated protein pinpoints the position of the mutation, thereby facilitating its confirmation by sequencing analysis. If RNA is used as a template, any abnormalities of message splicing can potentially be detected. Compared to other mutation detection techniques, PTT can detect mutated alleles present at 5–10% in a sample.

This technique is not applicable to genes in which there are low levels of truncating mutations. For example, *APC, BRCA1, BRCA2,* and *Dystrophin* all have approximately 90–95% truncating mutations, but *NFI* and *RTS* have only 50% and 10% truncating mutations, respectively. Unfortunately, archival material is usually DNA and not RNA, which further limits the usefulness of this technique. Transcripts carrying truncating mutations can also be highly labile, but this can be overcome by using DNA-based PTT. A final limitation of this technique is that it cannot detect mutations occurring outside the coding region that regulate expression and RNA stability.

6. Troubleshooting PTT:
   a. False negatives due to a failure to amplify the mutated allele or only detecting very small deletion/insertions or missense mutations might be caused by (1) mutations in the primer binding site; (2) very small in-frame deletions/insertions undetectable by the mobility shifts; (3) mutation may only occur germline or be present as somatic mosaicism; (4) large insertions, translocations, and inversions that enlarge the region under analysis beyond amplifiable length. The solution is to use different percentage gels and overlapping primer sets.
   b. False positives or alternate splicing give rise naturally to different size transcripts and can cause artifacts during RT or PCR procedures. These usually disappear in the huge amount of correctly amplified fragments, but problems can occur if the errors happen in the first 1–3 rounds of amplification, producing substantial amounts of artifactual products. A solution is to perform two independent RT and PCR reactions and check at each stage of the process to allow for identification of this potential problem.
7. In some tumors, a situation occurs in which there is physical loss of one allele of the gene with the second allele remaining wild type. This condition, which is termed haploinsufficiency, results in the suppressor protein being expressed at reduced levels that are insufficient to block tumor progression. Under these circumstances, mRNA expression analysis becomes important and protocols such as semiquantitative RT-PCR and Northern blot analysis are useful for quantifying gene expression levels. The ultimate validation of tumor suppressor gene function is reintroduction of the putative cancer suppressor gene into cancer-derived cell lines lacking endogenous expression and observing a reversion to a less tumorigenic, more "normal" phenotype. Protocols describing the functional testing of tumor suppressor genes, including transfection studies, are discussed in detail in other chapters of this volume.
8. Troubleshooting for SSCP analysis can be found at www.bioproducts.com/technical/sscp-analysiswithmdegelsolution.shtmL.
9. For each PCR reaction, to determine LOH occurring in tumor DNA, a control should be run using normal lymphocyte DNA from the same patient.
10. If using 13-mL round-bottom tubes, spin at 9000 rpm.
11. Note that the cap strap should be aligned toward the center of the rotor.
12. The cDNA can now be used as a template for PCR amplification to produce a product for use in the PTT assay. A specifically designed tailed sense primer should contain the following regions: a 5′-end containing a T7 RNA-polymerase promoter sequence that is needed to facilitate the in-vitro production of RNA, followed by a 5–7-bp spacer, and finally a eukaryotic translation initiation sequence (Kozak sequence), which includes an ATG start codon

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that will facilitate the initiation of protein synthesis. The 3′ portion of the primer will contain gene-specific sequence, allowing amplification reads inframe from the ATG. The gene specific 3′ primer should have a similar annealing temperature to that of the 5′ primer.

13. Large deletions, duplications, and splicing mutations may be detected by agarose gel electrophoresis at this stage.

References

1. Hack, M. A. and Lance, H. J. (eds.) (1980). *ACT Laboratory Manual,* pp. 38–62.
2. Schröck E., du Manior, S., Veldman, T., et al. (1996). Multicolor spectral karyotyping of human chormosomes. *Science* **273**, 494–497.
3. Telenius, H., Pelmear, A. H., Tunnacliffe, A., et al. (1992). Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. *Genes Chromosomes Cancer* **4**, 257–263.
4. Veldman, T., Vignon, C., Schröck, E., Rowley, J. D., and Ried, T. (1997). Hidden chromosome abnormalities in haematological malignancies detected by multicolor spectral karotyping. *Nat. Genet.* **4**, 406–410.
5. Vulpe et al. (1993) Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. *Nat. Genet.* **3**, 7–13.
6. Thompson, C. T. and Gray, J. W. (1993). Cytogenetic profiling using fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH). *J. Cell. Biochem.* **17**, 139–143.
7. Joos, S., Fink, T. M., Rätsch, A., and Lichter, P. (1994). Genomic mapping and chromosome analysis: the potential of fluorescence *in situ* hybridization. *J. Biotechnol.* **35**, 135–153.
8. Ried, T., Schröck, E., Ning, Y., and Wienberg, J. (1998) Chromosome painting: a useful art. *Hum. Mol. Genet.* **7**, 1619–1626.
9. Kallioniemi, A., Kallioniemi, O. P., Sudar, D., et al. (1992). Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* **258**, 818–821.
10. Buckler et al. (1991) Exon amplification: a strategy to isolate mammalian genes based on RNA splicing. *Proc. Natl. Acad. Sci. USA* **88**, 4005–4009.
11. Church and Buckler (1999) Gene amplification by exon amplification. *Meth. Enzymol.* **303**, 83–99.
12. Church et al. (1993) Identification of human chromosome 9 specific genes using exon amplification. *Hum. Mol. Genet.* **2**, 1915–1920.
13. Church et al. (1994) Isolation of genes from complex sources of mammalian genomic DNA using exon amplification. *Nat. Genet.* **6**, 98–105.
14. Datson et al. (1994) Specific isolation of 3′-terminal exons of human genes by exon trapping. *Nucleic Acids Res.* **22**, 4148–4153.
15. Datson et al. (1996) Scanning for genes in large genomic regions: cosmid-based exon trapping of multiple exons in a single product. *Nucleic Acids Res.* **24**, 1105–1111.
16. Den Dunnen et al. (1999) Cosmid-based exon trapping. *Meth. Enzymol.* **303**, 100–110. HD Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* **72** 971–983.
17. Stephen, D. A., Chen, Y., Jiang, Y., et al. (2000). Positional cloning utilizing genomic DNA microarrays: the Nieman-Pick Type C gene as a model system. *Mol. Genet. Metab.* **70**, 10–18.
18. Den Dunnen, J. T. and Can Ommen, G.-J. B. (1999) The protein truncation test: a review. *Hum. Mutat.* **14**, 95–102.
19. Trofatter et al. (1993) A novel moesin-, ezrin-, radaxin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell* **72**, 791–800.

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Tumor Suppressor Genes

Volume 1: Pathways and Isolation Strategies

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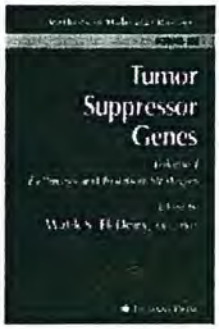

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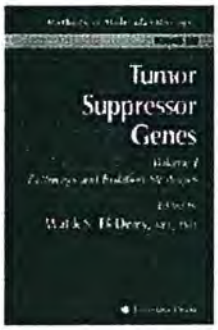

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