29 General Protocol for Loss of Heterozygosity Detection

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Contents

29.1 Introduction and Purpose 171

29.2 DNA Extraction from FFPE for LOH Analysis 172
29.2.1 Precautions 172

29.3 LOH Analysis: Basic Method 172
29.3.1 PCR Amplification 172
29.3.2 Silver Stain Detection and Interpretation of the Results 173
29.3.3 Considerations and Pitfalls 174

29.4 LOH Analysis: Capillary Electrophoresis Method 174
29.4.1 PCR Reaction 175
29.4.2 Detection by Capillary Electrophoresis and Interpretation of Results 176

References 177

29.1 Introduction and Purpose

Cancer cells often show losses, gains, or rearrangements of chromosomes, and most often the loss of genetic material involves loci harbouring tumour suppressor genes (TSGs) [1]. Many techniques have been developed over time to assess gains and losses of genetic material. Among these, Fluorescence In Situ Hybridization (FISH), Comparative Genomic Hybridization (CGH), and Multiplex Ligation-dependent Probe Amplification (MLPA) can now be consistently used also in formalin-fixed and paraffin-embedded (FFPE) material.

Because of their wide distribution and polymorphic length in a given population, microsatellite sequences (MS) are suitable as markers of deletion at specific chromosomal loci. In fact, the loss of one marker allele but the retention of the other one, the so-called loss of heterozygosity (LOH), is rather frequent because in both sporadic and inherited cancers, the other hit is usually a point mutation [2]. LOH has been extensively adopted for identification of TSGs by deletion mapping and is currently used in the clinical setting as an indicator for disease stratification, prognosis, and response to therapy [3–7]. In designing an LOH assay, prior knowledge of the Smallest Region of Overlapping (SRO) deletions is required and at least two MS markers within this region should be chosen. MS should be polymorphic enough and with elevated heterozygosity (index ≥0.7)[1] in order

[1]Heterozygosity refers to the fraction of individuals in a population that is heterozygous for a particular locus. The expected heterozygosity (*He*) for a given locus is calculated as

$$He = 1 - \sum_{i=1}^{m} (fi)^2 \tag{29.1}$$

where m is the number of alleles at the target locus, and fi is the allele frequency of the ith allele at the target locus.

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to minimize the number of uninformative cases where the constitutional DNA is homozygous for the marker. Microsatellite markers can be chosen consistently with the requirements above by referring to suitable web resources.[2] In order to get more reliable results, tri- and tetranucleotide repeats are preferred to dinucleotide repeats as the latter are peculiarly prone to replication slippage during PCR amplification. Since the major problem with FFPE tissues is the extent of degradation of the extracted nucleic acids, it is important to design primers that produce a PCR product shorter than 200–250 bp [8]. Moreover, it is preferable to avoid the choice of markers in which the larger and the smaller allele are too different in size. This strategy is used to avoid the preferential amplification of the shorter allele, which could be interpreted as a deletion of the larger one [9, 10].

This protocol offers a method for LOH assessment in DNA obtained from FFPE samples by providing a standard workflow for PCR amplification and detection of PCR products. Since DNA from FFPE could be quite variable in quality, a control PCR for the assessment of amplifiability and integrity of sample DNA is also suggested. Herein two technical approaches to LOH analysis are proposed:

- A basic method involving singleplex amplification of two microsatellite markers mapping at the 9p21 locus (CDKN2A) and subsequent poliacrylamide gel electrophoresis (PAGE) run and silver stain detection of PCR products;
- A singleplex PCR amplification of the same two markers coupled with capillary electrophoresis analysis of PCR products.

29.2 DNA Extraction from FFPE for LOH Analysis

Total DNA can be extracted following the protocol described in microsatellite instability analysis.

[2]Genomic position of the marker can be retrieved from the NCBI GenBank database at http://www.ncbi.nlm.nih.gov/nucleotide/ (Accessed 31 March 2010). Heterozygosity and allele frequencies can be retrieved from the CEPH database at http://www.cephb.fr/en/cephdb/browser.php (Accessed 31 March 2010).

29.2.1 Precautions

For LOH analysis, DNA from both the tumoral and normal tissue of the same patient is required. If both normal and tumour tissues are present in the same paraffin block, microdissection is necessary. Since LOH can be missed because of contamination from normal tissue, a minimal tumour fraction of at least 70% is suggested [11]. To this purpose, manual microdissection by exclusion [12] or laser capture microdissection should be the methods of choice (see Chaps. 4 and 6).

29.3 LOH Analysis: Basic Method

Microsatellite markers amplifications are performed as singleplex PCR. PCR products are then separated on an acrylamide gel, visualized by means of silver staining, and quantified by the use of a gel imaging system. Herein a method for the detection of LOH at the 9p21 locus (CDKN2A) [13] is described. Genetic alterations involving the 9p21 region are common in human cancers, and LOH assessment of this locus may be relevant for prognosis in lung adenocarcinoma and head and neck cancer patients [14].

29.3.1 PCR Amplification

29.3.1.1 Reagents

- *10X PCR buffer with $MgCl_2$*: We report the standard composition of PCR buffer: 15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris pH 8.3 at 25°C
- *dNTPs stock solution pH 8, 100 mM each* (e.g., Amersham): Dilute the dNTP stock solution to prepare 10 mM solution of each dNTP in sterile water
- *Primers*: Lyophilized primers should be dissolved in a small volume of distilled water or 10 mM Tris pH 8 to make a concentrated stock solution. Prepare small aliquots of working solutions containing 30 pmol/µl to avoid repeated thawing and freezing. Store all primer solutions at –20°C (see Table 29.1)
- *AmpliTaq Gold* (e.g., Applied Biosystems, N8080247), 5 U/µl

Table 29.1 LOH primers[a]

Marker name	Genomic position	Sequence 5′–3′	T°m	Range(bp)	Het.[b]
IFNA	9p22	F: TGCGCGTTAAGTTAATTGGTT	61.7	138–150	0.72
		R: GTAAGGTGGAAACCCCCACT	62.3		
D9S171	9p21	F: AGCTAAGTGAACCTCATCTCTGTCT	61.2	159–177	0.80
		R: ACCCTAGCACTGATGGTATAGTCT	59.5		

[a]Purchased from Sigma, lyophilized. Resuspended at 300 pmol/µl in 10 mM Tris pH 8.

[b]Heterozygosity, see footnote 1

F forward primer, *R* reverse primer

- *Negative control* for LOH PCR amplification: see footnote 4 in Chap. 28
- *Positive control* for LOH PCR amplifications: DNA from normal human lymphocytes, 50 ng/µl[3]

29.3.1.2 Equipment

- Adjustable pipettes; range: 2–20 µl, 20–200 µl, 100–1,000 µl
- *Nuclease-free aerosol-resistant pipette tips*
- *0.2 and 1.5 ml nuclease-free microtubes*
- *Sterile laminar flow hood*
- *Tabletop refrigerated centrifuge* suitable for centrifugation of 0.2 ml tubes
- *Thermal cycler*
- *Electrophoresis unit for agarose gel*
- *UV transillumination unit*

29.3.1.3 Method

Operate in a sterile laminar flow hood. Prepare a different master mix for each microsatellite marker (singleplex). PCR is performed in a final volume of 50 µl, containing:

- 5 µl 10X PCR Buffer.................... 1X final
- 1 µl dNTP 10 mM each................ 0.2 mM final
- 0.5 µl forward primer, 30 pmol/µl.................................... 0.3 pmol/µl final
- 0.5 µl reverse primer, 30 pmol/µl .... 0.3 pmol/µl final
- 0.25 µl AmpliTaq Gold, 5 U/µl.... 0.025 U/µl final
- 1 µl of diluted sample DNA
  or
- 1 µl of undiluted negative control or
- 1 µl of positive control for amplification.
- H_2O to volume
  - Overlay reaction mixture with 20 µl of mineral oil.
  - Thermal cycling: 94°C 10'+5 × (94°C 60", 55°C 60", 72°C 60")+40 × (94°C 30", 55°C 30" 72°C 30")+72°C 5′.
  - Gel visualization: mix 10 µl of PCR product with 2 µl of 6× loading buffer[4]; load on a 2% agarose gel prepared with TBE 1×, containing 0.5µg/ml ethidium bromide.[5] Include a 100 bp marker ladder (e.g., Amersham 27400701). Run at 80 V constant until bromophenol blue reaches 1/2 of the gel. Inspect under a UV source. A single band should be visible in the sample and in the positive control lanes.

29.3.2 Silver Stain Detection and Interpretation of the Results

29.3.2.1 Silver Stain Detection

The same reagents and equipment described in microsatellite instability analysis can be used (see Chap. 28). The white light transilluminator should be coupled with a gel imaging system (e.g., Bio-Rad Versadoc). Band staining is quantified by means of an optical

[3]The use of a high-quality DNA, e.g., a DNA extracted from a cell line, is recommended.

[4]6× loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in H_2O.

[5]Ethidium bromide is a potentially carcinogenic compound. Always wear gloves. Used EtBr solutions must be collected in containers for chemical waste and discharged according to the local hazardous chemical disposal procedures.

density (O.D.) reader. Image acquisition and analysis should be performed according to the manufacturer's instructions.

29.3.2.2 Interpretation

We score an allele as lost if its band signal is reduced by at least 50% with respect to the other allele (Fig. 29.1) [15]. Microsatellite alleles might show very complex band patterns after PAGE separation and silver staining. Polymerase slippage during elongation generates, in addition to the main allele, products referred to as shadow-bands [16, 17]. Sometimes this can make the identification of the true allele cumbersome.

29.3.3 Considerations and Pitfalls

The complete loss of an allele is rarely found in LOH studies, because of contaminating normal tissue and/or genetic heterogeneity within the tumour. Furthermore, preferential amplification of shorter alleles may occur. For these reasons, LOH should be considered on a quantitative basis as a comparison between allelic ratios in neoplastic tissue and in normal control (allelic imbalance, A.I.) [15].

$$\text{A.I.} = \frac{\text{O.D. tumor allele 1/O.D. tumor allele 2}}{\text{O.D. normal allele 1/O.D. normal allele 2}} \quad (29.2)$$

When this ratio gives a value higher than one, A.I. is set in an inverted form. A.I. ranges from 0 to 1, indicating a condition from total loss to retained heterozygosity, respectively [11].

Quantification of nucleic acids by means of silver stain should be cautiously evaluated, particularly regarding stain saturation which could result in overestimation of weaker bands. For this reason, [α-^{32}P] dCTP labeling of nucleic acids or primer coupling with fluorochromes in a genescan setting should be preferred for the quantitative analysis of PCR products. Moreover, thanks to the greater sensitivity of the two latter methods with respect to silver staining, PCR amplification can be performed using a minor number of cycles. This allows an easier interpretation of band pattern, as intensity of the shadow or stutter bands decreases by reducing the numbers of PCR cycles [16].

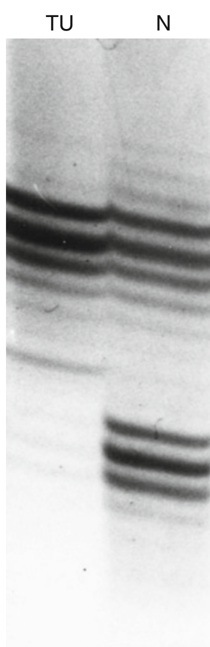

Fig. 29.1 Visualization of a representative case with LOH in a silver-stained polyacrylamide gel. An allele is scored as lost if its band signal is reduced by at least 50% with respect to the other allele (*N* normal tissue, *TU* tumour sample)

29.3.3.1 Troubleshooting

One or both microsatellite markers fail to amplify: repeat amplification using a different quantity of DNA. Perform the amplification of a control gene (e.g., β-globin, see the protocol for Microsatellite Instability) to check the amplifiability of the sample DNA and to establish the optimal quantity for amplification. Repeat LOH analysis running the sample in duplicate/triplicate. If only one of two/three replicas amplifies, stochastic amplification is suspected and results should be cautiously evaluated. If no replica amplifies, a homozygous deletion of the entire locus can be suspected.

29.4 LOH Analysis: Capillary Electrophoresis Method

Generally, LOH analysis should be performed by methods allowing quantification of both size and extent of amplification products. Fluorescence-based PCR analyzed by capillary electrophoresis optimally fulfills these criteria. The same reagents and equipment described in microsatellite instability analysis with capillary electrophoresis can be used.

Table 29.2 LOH primers

Marker name	Genomic position	Sequence	T°m	Range(bp)	Het.[a]
IFNA	9p22	F: HEX-TGCGCGTTAAGTTAATTGGTT	61.7	138–150	0.72
		R: GTAAGGTGGAAACCCCCACT	62.3		
D9S171	9p21	F: FAM-AGCTAAGTGAACCTCATCTCTGTCT	61.2	159–177	0.80
		R: ACCCTAGCACTGATGGTATAGTCT	59.5		

[a]Heterozygosity, see footnote 1

F forward primer, *R* reverse primer

29.4.1 PCR Reaction

29.4.1.1 Reagents

Note: Reagents from specific companies are reported here, but reagents of equal quality purchased from other companies may be used

- *10X PCR HotMaster™ Buffer with 25 mM $MgCl_2$* (5 Prime GmbH)
- *Commercial 25 mM stock solution of each dNTP* (Applied Biosystems): dilute the dNTP stock solution to prepare 2.5 mM solution of each dNTP in sterile water.
- *Primers*: Lyophilized primers should be dissolved in a small volume of distilled water or 10 mM Tris pH 8 to make a concentrated stock solution. Prepare small aliquots of working solutions containing 5 pmol/µl to avoid repeated thawing and freezing. Store all primer solutions at –20°C (see Table 29.2).
- *HotMaster™ DNA Taq polymerase* (5 Prime GmbH), 5 U/µl
- *Negative control* for LOH PCR amplification: see footnote 4 in Chap. 28.
- *Positive control* for LOH PCR amplifications: DNA from normal human lymphocytes, 50 ng/µl.[6]

29.4.1.2 Equipment

- *Adjustable pipettes*: range: 2–20 µl, 20–200 µl, 100–1,000 µl
- *Nuclease-free aerosol-resistant pipette tips*
- *0.2 and 1.5 ml nuclease-free microtubes*
- *Sterile laminar flow hood*
- *Tabletop refrigerated centrifuge* suitable for centrifugation of 0.2 ml tubes
- *Thermal cycler*
- *Electrophoresis unit for agarose gel*
- *UV transillumination unit*

29.4.1.3 Method

Operate in a sterile laminar flow hood. Prepare a singleplex master mix for each microsatellite marker.

- PCR is performed in a final volume of 20 µl, containing:
  - 4 µl 10X PCR HotMaster™ Buffer.................................... 2X final
  - 2.4 µl dNTP 2.5 mM each, ... 3 mM final
  - 0.4 µl Forward primer, 5 pmol/µl, ... 0.1 pmol/µl final
  - 0.4 µl Reverse primer, 5 pmol/µl .... 0.1 pmol/µl final
  - 0.6 µl HotMasterTM DNA Taq polymerase 5 U/µl .... 0.15 U/µl final
  - 3 µl of 10 ng/µl DNA sample
  - or
  - 1 µl of undiluted negative control-or
  - 1 µl of positive control for amplification
  - H_2O to volume
- Thermal cycling: 94°C 2' + 35 × (94°C 20", 55°C 10", 65°C 30") + 65°C 7'.
- Gel visualization: Mix 10 µl of PCR product with 2 µl of 6× loading buffer[7]; load on a 2% agarose gel prepared with TBE 1×, containing 0.5µg/ml ethidium bromide. Include a 100 bp marker ladder (e.g., Amersham 27400701). Run at 80 V constant until bromophenol blue reaches 1/2 of the gel. Inspect under a UV source. One band should be visible in the sample and in the positive control lanes.

[6]The use of a high-quality DNA, e.g., a DNA extracted from a cell line or blood (see Chap. 10), is recommended.

[7]See Footnote 4.

29.4.2 Detection by Capillary Electrophoresis and Interpretation of Results

29.4.2.1 Detection by capillary electrophoresis

Reagents, equipment, default run conditions and sample preparation are the same as in the case of microsatellite instability analysis (see Chap. 28).

29.4.2.2 Interpretation

Raw data are analyzed by GeneScan™ software and peak heights are obtained (Fig. 29.2). Loss of the alleles can be precisely determined by calculating the ratio of the peak heights of normal and tumour alleles according to the following formula:

$$\text{LOH} = \frac{\text{Peak height of normal allele 2 / Peak height of normal allele 1}}{\text{Peak height of tumour allele 2 / Peak height of tumour allele 1}} \qquad (29.3)$$

When this ratio gives a value higher than one, LOH is defined as the reciprocal of the formula above.

LOH is strongly indicated by ratios less than 0.5.

Polymerase slippage during elongation generates products referred to as stutter-peaks. Additional fragments are one to four repeat units shorter than the allele, and when the size of the two alleles differs by one repeat unit, the stutter from the longer allele will contribute significantly to the main peak of the short allele. Such contribution from the stutter peak can be corrected. The height of a stutter peak compared to its main peak is calculated and then the main peak's height, as it would have been without contribution from the neighboring allele's stutter, can be estimated [18].

29.4.2.3 Troubleshooting

- The detected signal is too low to evaluate the results. Solution: increase the injection time or decrease the dilution of the PCR products.
- The detected signals go off-scale. Solution: decrease the injection time or increase the dilution of the PCR products.
- No peaks are visible on electropherogram after separation:
  - PCR failed to amplify microsatellite marker. Solution: optimize PCR conditions
  - Fragments reach the detector outside the detection time. Solution: adjust the detection time.

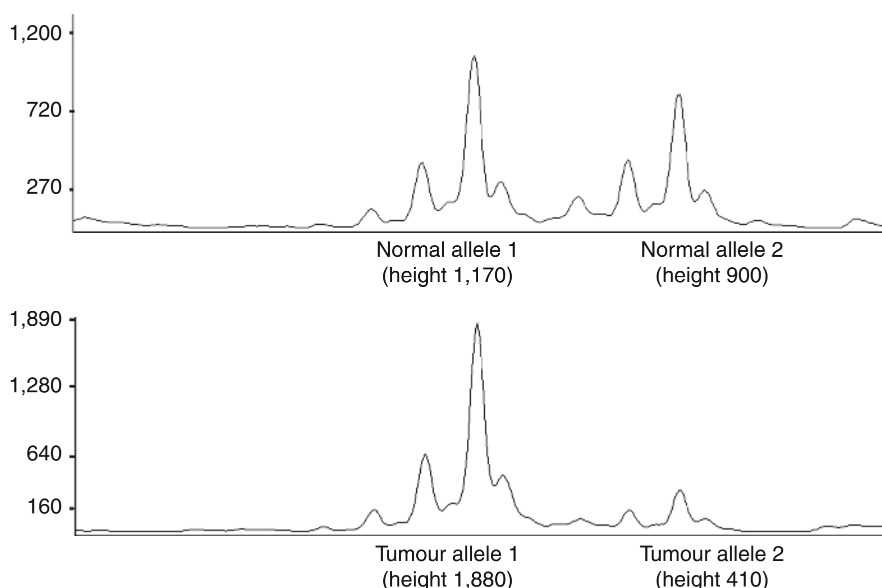

Fig. 29.2 Graphical representation of LOH by GeneScan software. LOH is calculated as the ratio between the allele ratios in tumour and normal DNA. The peak heights are measured in relative fluorescence units. In this example, $\text{LOH} = (900 / 1{,}170) / (410 / 1{,}880) = 3.52$, but since the ratio is greater than 1, the LOH value is set to be the inverse. LOH = 0.28 is showing a reduction of one allele's intensity, from normal to tumour DNA, by 72% relative to the other allele

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