Multiplex Loss of Heterozygosity Analysis by Using Single or Very Few Cells

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Loss of heterozygosity (LOH) is an indication of tumor suppressor gene inactivation. However, loss of heterozygosity analysis has been limited to either a small scale or to very few genetic markers. To significantly increase the scale of study and to include a large number of markers in the analysis, experimental conditions were established for using single cells or single cell equivalent with 10 markers typed simultaneously. Under these conditions, the allele amplification failure rate was 3.7% when single tissue cultured human cells were used. When 30 cells from a 5- μ m paraffin-archived breast tumor tissue section were used, the failure rates were 0% for four of the five heterozygous loci and 10% for the fifth. Small amplification failure rates (6.1% and 6.7% on average) were observed when 5 or 10 cells from paraffin-archived breast tissue were used. These results indicate that with polymerase chain reaction (PCR) primers of high quality, it is possible to obtain reliable results by using single cells from fresh tissue or very few cells from paraffin-archived specimens. The results also show the importance of including replicates, using primers of high quality, and optimizing PCR conditions when a limited amount of material is used for the assay. The feasibility of LOH analysis with very few paraffin-embedded breast cancer tissues was demonstrated. (*J Mol Diagn* 2002, 4:172-177)

Loss of heterozygosity (LOH) in cancer cells was initially described in 1971¹ and is considered an indicator of tumor suppressor gene (TSG) inactivation in both cancer and pre-malignant cells. Because LOH affects chromosomal regions rather than single genes, it can be detected easily by examining polymorphic markers in these regions. Therefore, the correlation between TSG inactivation and the corresponding phenotypic effect may be studied without knowledge of TSGs, their precise chromosomal locations, and their protein products.

Cancer development is a multi-step process during which a large number of TSGs is inactivated. The cancer

research community, therefore, needs genetic approaches that allows LOH detection on a large scale. The results will allow one to exhaustively identify the TSGs affected by LOH and to include all known TSGs in the study. In this way, the mechanisms underlying cancer development can be understood in a comprehensive way. However, with the traditional one-marker-one-assay approach, a large number of assays would be required and, therefore, a large amount of material. With non-optimized detection methods, even more material is needed. Such a study may be possible for large invasive tumors. Recently, however, the development of diagnostic instruments and methods have made it possible to detect tumors at very early stage, and therefore, the size of tumors that are available for study is small. In many cases, in addition to the invasive components, microscopic precursors (*in situ* carcinomas) and transitional lesions (the so-called atypical hyperplasias) are recognized, which also are of great interest for LOH study. With microdissection, although these lesions can be isolated they yield very small samples. Obviously, the traditional one-marker-per-assay method is not feasible for the analysis of these lesions. Yet, systematic analysis of advanced (invasive) and coexisting proliferative lesions could help to understand the genetic mechanisms underlying cancer development in a comprehensive way. This is because some of the proliferative lesions may be the precursors of the more advanced malignancy and may represent the footprints of cancer development.

The other limitation on using a large number of markers is the amount of cost and effort. For example, if 1000 specimens are analyzed with 1500 markers (2×10^6 bases per marker evenly distributed in the human genome), 1,500,000 assays are needed. This translates into \$450,000 for the TaqDNA polymerase alone. If a laboratory has the ability of determining 200 markers per day, at least 32 years would be needed to complete this project.

Two approaches may be considered for addressing the above issues: (1) increasing the sensitivity of detection. With increased sensitivity, less material is required for each assay and therefore, more markers can be in-

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cluded for a given amount of material. However, when the number of cells available is limited, the number of included markers is also limited even when the sensitivity is markedly increased. For example, with 4000 cells available, it is impossible to perform LOH analysis with 6000 markers even if the sensitivity is high enough so that one cell can be used for each assay. Furthermore, increasing sensitivity alone cannot reduce the cost and effort; (2) including more than one marker in each assay. Because the marker sequences are at different locations in the genome, they can be amplified independently. By carefully designing primers for polymerase chain reaction (PCR), it is possible to include more than one marker in each assay. In this way, more markers can be included without a requirement for increasing the amount of starting material. By enhancing genotyping sensitivity and multiplex analysis, cost and effort can be reduced significantly. The present study was designed to approach the solution in both directions. The experimental conditions were established for the highest sensitivity with which single cells could be used for the analysis and for typing 10 markers simultaneously.

Materials and Methods

Sample Preparation

Single cells were isolated from a tissue cultured human cell line (MHO 4454, Coriell Institute for Medical Research). Samples with 5, 10, and 30 nuclei were isolated by microdissection from a 5- μ m paraffin-archived breast cancer tissue section after hematoxylin and eosin (H & E) staining. Samples for LOH analysis were prepared from a 10- μ m section. All samples were lysed in 3 μ l of lysis solution (200 mmol/L KOH and 50 mmol/L DTT (Note, all chemicals were purchased from Life Technologies or otherwise indicated.)) and neutralized with 3 μ l of neutralization buffer [200 mmol/L HCl, 900 mmol/L Tris-HCl (pH 8.3), and 300 mmol/L KCl].²

Genetic Markers and Multiplex PCR

Ten genetic markers consisting of single nucleotide polymorphisms (SNPs) were used for genetic analysis with single tissue culture cells or very few paraffin-embedded breast cancer tissues. For LOH analysis with paraffin-embedded breast cancer tissue, the marker C3 was replaced by GALNS since the latter has been shown to be associated with LOH in some breast tumors. All markers were restriction fragment length polymorphisms (RFLP) for *MspI* (New England Biolabs, MA). The polymorphic sequences of these marker loci were amplified by a two-round PCR protocol (Figure 1) which was a modification of the three-round PCR protocol by Lin et al.³

In the first round of PCR, 1X PCR buffer [100 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.1 mg/ml gelatin], the four dNTPs (100 μ mol/L each) and 1 unit of HotStart (Qiagen, CA) *TaqDNA* polymerase in a final volume of 50 μ l were used. Two locus-specific primers, O and R, for the 10 loci at a concentration of 20

Figure 1. Schematic presentation of the multiplex PCR protocol. Three loci are shown.

nmol/L were also included (Table 1). Other than the locus-specific sequences, the R primers also contained a 20-base universal "tail" at their 5'-ends. Amplification with the R primers attached the universal tail to all PCR products. PCR was performed on a MJPTC100 (MJ Research Inc., MA). Each PCR cycle consisted of 1 minute at 94°C, for denaturation; 2 minutes at 55°C followed by 5 minutes ramping from 55°C to 70°C for annealing and extension. Thirty-five cycles were performed. Amplification with the R primers attached the tail 1 sequence to all target fragments.

In the second round, 1 μ l of 100 $\times$ diluted first-round PCR product was re-amplified. All O primers were replaced with corresponding N (nested) primers (20 nmol/L) which were internal to the O primers to enhance the amplification yield and specificity. All R primers were replaced with a single universal primer that was identical to the universal tail. The length of the amplified fragments ranged from 99 to 202 bp, differing by 6 bp from their neighboring fragments so that the PCR products and the allelic products after restriction enzyme digestion could be easily separated by 8% polyacrylamide gels. The PCR profile used in the first round was used in the second round. Twenty-five cycles were performed.

Genotype Determination

The final PCR products were digested with the *MspI* restriction enzyme, and subjected to electrophoresis with 8% polyacrylamide gel electrophoresis.

Results

For genetic analysis, 10 SNP markers that were RFLPs for the restriction enzyme, *MspI*, were chosen. Single tissue cultured cells, and samples containing 5, 10, and 30 nuclei isolated from a 5- μ m paraffin archived breast cancer tissue section were prepared and used for the analysis. After the two-round multiplex PCR amplification as described in the Materials and Methods section, samples

Table 1. Primers Used for Genetic and LOH Analyses

Primer	Sequence (from 5' to 3')
CBS-dO*	ACAAAGTCGATCAGCAGTTG
Cbs-R	GCTGCTTCACCTTCGTGACGGCTGGCGGGACGAGTTTGCCT
Cbs-C	GGTCGTGGACACTCTACAGGGGGGTGATGACAGAATGTTCC
Gnas1-O	AGGCCACACAAGTCGGGGTG
Gnas1-R	GCTGCTTCACCTTCGTGACGGGCCCTCCAGCCAGTGCT
Gnas1-C	GGTCGTGGACACTCTACAGGCAGGCACGTTTCATCACACTCCG
G6pd-O	TCAAGAAGTACGAGAGCAGG
G6pd-R	GCTGCTTCACCTTCGTGACGGACATCCGAAACAGAGTGAG
G6pd-C	GGTCGTGGACACTCTACAGGAGGAGAGGAGAGCATCC
Apoc3-O	CCTTTTAAGCAACCTACAGG
APoc3-R	GCTGCTTCACCTTCGTGACGGGCACCGTTAAGGACAAGTTC
Apoc3-C	GGTCGTGGACACTCTACAGGGATTGCAGGACCCCAAGGAGCC
Alad-O	CCGGAGGACCGTTGCCTGGG
Alad-R	GCTGCTTCACCTTCGTGACGGTCCTTTGATTCTTCACCTTG
Alad-C	GGTCGTGGACACTCTACAGGTACCCACACCCACACAGGTC
Ldlr-O	CTGCCCTCAGCACCCAGCTTG
Ldlr-R	GCTGCTTCACCTTCGTGACGGGTCCAGTAGATGTTGCTGTGG
Ldlr-C	GGTCGTGGACACTCTACAGGCCTATGACACCGTCATCAGCCG
D15S664O	TCTCCCTGCTGGCTCCTGAG
D15S664R	GCTGCTTCACCTTCGTGACGGCTGAAACATTCTGCCTGATG
D15S664C	GGTCGTGGACACTCTACAGGCTTATCTGGCCAAACTGATCC
P53-O	CTCATGGGGTTATAGGGAGG
P53-R	GCTGCTTCACCTTCGTGACGGAAGTGGGGTCTCTGGGAGGA
P53-C	GGTCGTGGACACTCTACAGGAAGCCCCCTACTGCTCACC
D1S8-O	CTGATTCTGACACAGGTAG
D1S8-R	GCTGCTTCACCTTCGTGACGGTAGCCAATCGGAATTAGCTT
D1S8-C	GGTCGTGGACACTCTACAGGCTGATGCGTCGTTCCCGTAC
C3-O	TGAGTGCAAGATGACGGTGG
C3-R2*	GCTGCTTCACCTTCGTGACGGTGAGTCGGAAGGTAAGTCTGG
C3-C	GGTCGTGGACACTCTACAGGCCCCACCAGGTCTTCTGCTCCG
Galns-dO*	CGGGCTGTGAAAAGTTAGGG
Galns-Rd*	GCTGCTTCACCTTCGTGACGGCATCCTTCTCCAGGCACCT
Galnd-NS*	CGTCCCGCCGCCCCGCCCCACTCAGGCCAGGCCTAGAAT

*, These primers were substitutes of the originally designed primers because of their better amplification properties.

were digested with *MspI* followed by gel electrophoresis for genotype determination.

Genotype Analysis with Single Human Tissue Cultured Cells

The cell line from which the single cells were prepared was heterozygous for 3 of the 10 loci. Of the nine single cell samples analyzed, only one sample had a missing allele at locus 2 (Lane 3 in Figure 2). This represents a 1.85% amplification failure rate (note, each heterozygous locus contains two allelic sequences). Since such a small fraction would not be considered as actual LOH in practice, this result indicates that with the experimental conditions used in this study, LOH can be analyzed rather reliably in a multiplex way by using single cells. As shown by the results below, locus 2 had a generally higher amplification failure rate, indicating that the PCR primers used for this locus may need to be further improved and better multiplex amplification could be obtained.

Genotype Analysis with Cells from a Paraffin-Archived Tissue Section

Higher allele amplification failure rate is expected for single cells prepared from archived tissue sections because DNA in these cells may be highly degraded during

storage. In addition, since the thickness of the tissue section used for the analysis was only 5- μ m which is used for most pathological analyses, all nuclei must have been truncated under this thickness. For these reasons, 5, 10, and 30, instead of single cells, were used for the analysis. Part of the results are shown in lanes 4 to 12 of

Figure 2. Part of results from LOH analysis with single or very few cells. M: *MspI* digest of pBR322 DNA as molecular markers. As shown, the "+" allele of locus 2 is missing in **lanes 3 and 9** (lanes are counted from left to right), and the "-" allele is missing in **lane 12**. The "-" allele of locus 10 is missing in **lane 6**.

Table 2. Locus and Allele Amplification Failure Rates for Samples with Different Numbers of Cancer Cells from Paraffin-Embedded Tissue

Cells/sample Samples analyzed	Number (%) of failed samples (locus)				Number (%) of failed samples (allele)			
	5 24	10 28	30 20	All 72	5 48	10 56	30 40	All 144
Homozygous								
L1	2 (8.3)	2 (7.1)	1 (5.0)	5 (6.9)	—	—	—	—
L2	4 (16.7)	3 (10.7)	0 (0.0)	7 (9.7)	—	—	—	—
L3	2 (8.3)	1 (3.6)	0 (0.0)	3 (4.2)	—	—	—	—
L4	0 (0.0)	1 (3.6)	0 (0.0)	1 (1.4)	—	—	—	—
L5	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	—	—	—	—
Heterozygous								
L6	1 (4.2)	2 (7.1)	0 (0.0)	3 (4.2)	4 (8.3)	9 (16.1)	4 (10.0)	17 (11.8)
L7	0 (0.0)	1 (3.6)	0 (0.0)	1 (1.4)	4 (8.3)	6 (10.7)	0 (0.0)	10 (6.9)
L8	0 (0.0)	1 (3.6)	0 (0.0)	1 (1.4)	6 (12.5)	1 (1.8)	0 (0.0)	7 (4.9)
L9	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.1)	0 (0.0)	0 (0.0)	1 (0.7)
L10	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.1)	1 (1.8)	0 (0.0)	2 (1.4)
Sum	9 (3.8)	11 (3.9)	1 (0.5)	21 (2.9)	16 (3.3)	17 (3.0)	4 (1.0)	37 (2.6)

Figure 2. The allele and locus amplification failure rates are summarized in Table 2. The specimen was heterozygous for five markers (Table 2). For these five loci (10 allelic sequences), the allele amplification failure rates ranged from 0.0% to 18.1% for 5 and 10 cells. The average rates (3.3 and 3.0) for samples with 5 and 10 cells were not significantly different. However, when the cell number increased to 30, only one locus (locus 6 whose primers may have had a low quality) had a 10% failure rate, and the other four had no missing allele among the 20 samples included in the study. This result indicates that if PCR primers of high quality are used, it is possible to obtain very reliable results by using 30 paraffin-archived cells.

As a comparison, locus amplification failure rates are also included in Table 2. Locus amplification failure rates were generally lower than the failure rates for alleles simply because each locus contains two copies (two alleles) of the amplified sequences. Similar to the results for alleles, the locus failure rates for the samples with 5 or 10 cells were not significantly different. When 30 cells were used, amplification at one (locus 1) of the 10 loci failed in only one sample, which could be attributed to sample preparation. Since 20 samples were analyzed, this accounted for an overall 0.5% failure rate.

LOH Analysis with Cells from a Paraffin-Archived Tissue Section

To test the feasibility of using very small number of nuclei from paraffin-embedded tissue for LOH analysis, genotypes of normal and invasive carcinoma components of 10 tumors from 10- μ m tissue sections were determined for the 10 markers of which one marker, C3, was replaced by another marker, GALNS, on chromosome 16q24 that has been shown to be associated with LOH in a number of tumors.⁴⁻¹⁸ One of the 10 tumors was shown to have LOH in its invasive component. Twenty-five samples with 30 cells from the invasive component from this tumor were prepared. Eighteen of these samples were shown to be associated with LOH, six were shown to have significantly reduction of one allele and one was not detected

for the entire locus. Part of these results are shown in Figure 3. These results are consistent with those obtained with large number of cells (100 to 150) which indicates that the method that we described in the present study is highly reliable.

Discussion

An alternative method for using a limited amount of material for genetic analysis is called the whole genome amplification (WGA) that was initially described by Zhang et al¹⁹ and was further developed into more efficient forms very recently.^{20,21} When starting with low copy numbers, WGA may increase the copy number of the entire or major portion of the human genome for 3000 to 100,000-fold²¹ so that a limited amount of genomic DNA can be amplified to a sufficient amount for genetic analysis with a relatively large number of markers. The major difference between WGA and multiplex PCR is that WGA raises the copy number of the entire or the major portion of the genome but its product cannot be used for direct analysis. For genetic analysis, aliquots of the WGA product need to be further amplified so the target sequences

Figure 3. Part of the results from LOH analysis with 30 breast cancer cells. Eight samples with either complete LOH or allelic reduction for the GLANS (—) allele are shown on the left and results from genotyping with normal components from the same tissue specimen are shown on the right. A non-specific band that does not affect detection is indicated by the arrow.

may be analyzed. Multiplex PCR amplifies specific sequences that are a very small portion of the genome. Since the PCR product can be used directly for analysis, WGA is not needed when the capacity of the multiplex PCR meets the need of the study. In many cases, the two approaches can be used in a complementary way. Techniques reducing primer-primer interaction during PCR may increase the capacity of multiplex PCR. By using 5' universal "tails" as described in our previous publication³ and in the present paper, the capacity of multiplex PCR could be increased significantly while the yield and specificity remain sufficient for direct analysis.

As shown in Table 1 and discussed below, the experimental conditions used in this study allowed us to achieve the highest sensitivity. With these conditions single cells could be used very reliably for multiple LOH analysis with 10 markers. This was accomplished by using the four improvements below.

Using Nested Primers with a Two-Round Amplification Protocol

We have shown successful use of a two-round PCR protocol and nested primers for analyzing genetic markers in single haploid cells with single markers.²² Compared with the single-round PCR protocol in which two primers are used, our two-round PCR protocol involves using three primers. Because the presence of genomic DNA and formation of "primer-dimers," single-round PCR is often associated with a significant amount of non-specific amplification. This becomes more serious when multiple sets of primers are used in the same tube and very few target sequences are used as starting material. With our two-round PCR protocol, an aliquot of the first-round PCR products is used as the starting material for the second round of amplification. This transfer and dilution process leaves most genomic DNA in the original tube. Because one of the two primers used in the first round is replaced in the second round, primer-dimers and non-specific sequences containing the replaced primer sequence cannot be further amplified. Because of a significant increase in the copy number of the target sequences during the first round of amplification, very few PCR cycles (usually 25 cycles) are needed in the second round to amplify that target sequence to the detectable amount. Therefore, even if amplification of some non-specific sequences is initiated, these sequences cannot be amplified to a detectable amount.

Transferring a Reduced Aliquot from the First-Round PCR Product

Although taking 1 to 2 μ l aliquots from the first-round PCR product for the second round of amplification may generate detectable amounts of material from the target sequences, we showed that using 0.01- μ l aliquot may significantly improve the yield and specificity. This is presumably because, in this way, the amounts of genomic sequences, primer-dimers generated in the first

round and primer used in the first round could be significantly reduced. Although the amount of target sequences are also reduced in this way, the improved conditions may significantly improve the exponential amplification.

Using Universal Tail and Primer

One of the major factors limiting the level of multiplex amplification is primer-primer interaction. In many cases, primer-primer interaction becomes significant when more than three sets of primers are included in the analysis. By using universal primers, we were able to reduce the complexity of the primer sequences in the second round of amplification resulting in better yield.

Using "Hot-Start" TaqDNA Polymerase

The "hot-start" TaqDNA polymerase is not activated till the chemical group is dissociated from the enzyme at a high temperature. Therefore, with this enzyme, non-specific primer annealing and extension that may occur at the low temperature is avoided.

For the above reasons, the PCR products from multiplex amplification with 10 markers can be clearly resolved by gel electrophoresis. The assay involves PCR amplification, restriction enzyme digestion, and gel electrophoresis that can be performed easily by most laboratories. When the SNPs are not RFLPs, >98% of the polymorphic sites can be easily converted into RFLPs during the second round of PCR.²³ Multiplexing with 10 markers can significantly reduce cost and effort compared with the single locus-based assay. The high degree of sensitivity of this method allow us to perform LOH analysis with single or very few cells. This makes it possible to analyze microscopic lesions with a large number of markers, yielding sufficient data to trace the mechanisms of cancer development in a comprehensive way.

To determine whether the two rounds of PCR are sensitive enough for using single cells, it would be difficult to use single cell samples from archived tissue because DNA in these tissue may be highly degraded and because of truncation artifact in 5- μ m tissue sections. For this reason, tissue culture cells were used for the assay. We showed that the locus and allele amplification failure rates for the samples with single tissue cultured cells and for those with 30 cells from archived breast tumor tissue were very similar. This result suggests that the amount of template DNA in these two group of samples could be equivalent.

Truncated nuclei from tissue sections may also cause inaccuracy in genotype determination because with a small number of such nuclei, the copy number for the two allelic sequences of a given marker may not be equal. As a result, allele imbalance may be observed. This may be mistaken as the result of sample impurity and counted as LOH. Using thicker tissue sections may increase the proportion of the intact nuclei and increase the reliability of the experimental data. Therefore, tissue sections used

for the study should be as thick as the devices for preparation and microdissection allow.

Other than DNA quality, primer quality also has a significant influence on genotyping results. As shown in Table 1, loci 1 and 2 had higher locus amplification failure rates than others while loci 8, 9, and 10 did not have amplification failure even when five cells were used. Among the five heterozygous loci, allele amplification failure rate for locus 6 was higher than others. Even when 30 cells were used, this locus had a amplification failure rate of 10%.

Clearly, it is important to use a DNA template of adequate quantity and PCR primers of high quality. When the amount of the DNA is at borderline and/or the primer quality is not optimal, inclusion of replicate samples in the analysis would be an important means to ensure the reliability of the genotyping results. For example, if a primer set is associated with a 10% failure rate, the probability of all three replicates failing would be reduced to 0.1%.

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