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Journal
Methods in Molecular Genetics, 8(C)

ISSN
1067-2389

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Publication Date
1996

DOI
10.1016/s1067-2389(96)80055-0

Peer reviewed

[22] Gene Amplification as Marker for Studying Genomic Instability

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Introduction

It has long been recognized that genomic instability is widespread in tumor cells. Evidence for instability is manifested in the continually changing karyotype that is evident in the chromosome analysis of tumor biopsy materials. Many types of changes are evident on both gross and molecular levels. Three categories of abnormalities are detected in tumor cells. Aneuploidy, which involves the gain or loss of a whole chromosome, probably results from defects in the machinery that is responsible for the proper segregation of chromosomes. A second category, gene rearrangements includes such changes as deletions, amplifications, inversions, translocations, and chromosome breaks. All these aberrations share the common defect of improper chromosome breakage and religation. Finally, on a molecular level we know that point mutations occur. The generation of these base changes involves the action of repair enzymes such as exonucleases and ligases.

Several years ago it was postulated that one of the first steps in the generation of transformed cells could be the acquisition of increased genomic instability (1). The karyotypic changes that result could then fuel the phenotypic changes that culminate in transformation. More recently, data that support this hypothesis have become available. To study genomic instability (or conversely, the maintenance of genomic integrity) it would be helpful to focus the efforts on one type of abnormality. As the molecular basis of this change is determined, its involvement in other types of abnormalities could be examined. Our laboratory has adopted this approach in our study of genomic integrity of human cells.

Some years back we posited that the in-depth genetic and molecular analysis of one type of genome rearrangement might give insights into the cellular processes that maintain genomic integrity or promote genomic lability. To this purpose we initiated studies that examined the regulation of gene amplification. We have used amplification ability as a marker of genomic instability and as a prototype of how the acquisition of genetic instability may be involved in neoplastic initiation and progression.

Gene amplification is an excellent marker for genomic instability for a number of reasons. For several decades, the manifestations of gene amplification, homogeneously staining regions and double minute chromosomes, have been observed in neoplastic tissue, although at the time the molecular basis of these chromosomal abnormalities was not known. In the 1970s, studies by Biedler and Spengler (2) associated these chromosomal abnormalities with drug resistance; Alt and co-workers (3) found

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that the basis of the resistance in this instance was an increase in gene copy number (gene amplification). These observations prompted investigations into the identity of the sequences that are carried on homogeneously staining regions and double minute chromosomes in human tumor biopsy materials and led to the discovery that oncogenes are often amplified, especially in certain tumor types. Additionally, gene amplification is an example of a rearrangement that is easy to measure on a molecular level. Unlike random chromosomal breaks of translocations, amplification of a targeted sequence can be measured using colony formation and easily verified by determination of gene copy number. Finally, some studies have suggested a relationship between the frequency and extent of oncogene amplification and the progression of malignancy.

A powerful method for studying the frequency of gene amplification is to assay the generation of drug-resistant colonies. In this type of clonogenic assay only the cells that have acquired an increase in gene copy number will survive the selection protocol and produce colonies. Analysis of the colonies then indicates the status of the genome.

One could choose to study genetic instability using *N*-(phosphonoacetyl)-L-aspartate (PALA) resistance and the ensuing amplification of the *CAD* gene because, unlike methotrexate resistance which may occur through multiple mechanisms, the major mechanism of resistance to PALA is through amplification of the *CAD* gene (4). The *CAD* gene encodes the multifunctional CAD enzyme, and PALA inhibits the aspartate transcarbamylase activity of this enzyme. Thus, in asking whether tumorigenic cells amplify DNA sequences more often than nontumorigenic cells, one can use the incidence of resistance to PALA as an indirect indicator of the ability of a cell to amplify DNA. This chapter describes the measurement of *CAD* gene amplification frequency in monolayered mammalian cells.

Measurement of incidence of amplification is a more rapid determination than measurement of rate. The incidence measurement entails characterization of the cell lines for plating efficiency, population doubling time, initial sensitivity to the drug, and quantification of the initial gene copy number. Once the cell line is characterized it is challenged at a stringency of selection equivalent to $9 \times LD_{50}$ (50% lethal dose), and the number of resistant colonies is tabulated. Gene copy number, karyotype, and cytometric analysis of DNA content are determined to verify that gene amplification (not polyploidy) is the mechanism of resistance. One can use this method with most cell lines to estimate their propensity to amplify.

Measuring Incidence of Gene Amplification

The advantages of using this method are several. First, this assay is rapid. It consists of determination of the plating efficiency and LD_{50} followed by subsequent determination of the incidence of drug resistance (percent survival) at $9 \times LD_{50}$. Although

the time required for these measurements is dependent on the doubling times of the cells, determination for the lines generally required approximately 2 months. Second, this assay is direct. Because amplification is the major reported mechanism for resistance to PALA, complications in interpreting the underlying basis of resistance appear to be avoided by using this drug, thus allowing direct assessment of amplification ability from the incidence of drug resistance. Third, this assay is quantitative; it allows the comparison of cell lines with differing growth properties or sensitivities to the drug. Finally, this assay determines the incidence of gene amplification, in contrast to other studies that measure rate (5). Both values are determined by (a) the rate at which the cells are generated, (b) their intrinsic stability, and (c) their growth rates relative to the rest of the cell population. This assay, which measures incidence, accounts for these variables and provides a sensitive and direct measurement of gene amplification. Rate measurements, however, have an additional component of time and the preceding three variables should be taken into account at each step in the selection (rate determination).

Measurement of the incidence of gene amplification has an added advantage in that it can measure *CAD* gene amplification in cell populations that have a limited capacity for proliferating. This is particularly important in analyzing mortal cell populations. A single cell must double 6 times, at most, to be visible as a drug-resistant colony (>50 cells). This is in contrast to the second method of analyzing amplification potential: the use of the Luria–Delbrück fluctuation analysis. In the Luria–Delbrück fluctuation assay one uses the conditions previously determined for the incidence measurements and applies them to the estimation of the amplification rate. Plating efficiency and LD_{50} determination are particularly relevant here. The fluctuation analysis requires propagation of the population before placing the cells in selection. To accurately measure an amplification rate on the order of 10^{-6}, a population of 100 cells must be expanded to 10^6–10^7 cells. A total of 18–25 population doublings is required. At this point, the cells are placed in selection and an additional six cell doublings are required to produce a visible colony. Both methods have given comparable results with the cell lines examined (6).

In designing the incidence assay to measure amplification ability in these lines, one wants to develop as rapid an assay as possible. To this end, one would choose to use a single-step selection protocol and determine the minimal concentration of selective drug that allowed the correlation of drug resistance with amplification ability. Cells placed in a low stringency of selection ($3 \times LD_{50}$) produced colonies within 1 week. Rechallenging these colonies with the same drug concentration during clonal propagation revealed that a large proportion of the cells were killed, with the remainder (those used for molecular analyses) growing very slowly. This result indicates that these cells were not completely resistant to PALA but were merely capable of tolerating the low drug concentrations. In contrast, cells placed in a higher stringency of selection ($9 \times LD_{50}$) take 2–4 weeks to emerge as colonies. Cells from virtually all of these colonies were resistant to killing by the drug and grew in the

drug at a rate similar to that of unselected cells during clonal propagation. These observations are consistent with our results showing that subclones resistant to concentrations of PALA are equivalent to $3 \times LD_{50}$ were not amplified, while those grown at $9 \times LD_{50}$ were amplified in each case (7). It is important to note that at drug concentrations greater than $9 \times LD_{50}$, greater amplification can result, and the relationship between the incidence of drug resistance and tumorigenicity can remain. Placing cells at drug doses that are very high, however, often eliminates the appearance of colonies. It is probable that a single amplification event can provide only a limited degree of resistance.

Two variables are especially important in the determination of incidence of amplification ability: the plating efficiency and LD_{50} of each line. The plating efficiency, the baseline number of colonies obtained without selection (defined as 100% survival), is the value to which the number of colonies obtained after drug selection is compared. Overestimating or underestimating this number would lead to an incorrect determination of the incidence of drug resistance. Likewise, accurate determination of the LD_{50}, the pivotal value for the final stringency of selection, is required.

Measuring Rate of Gene Amplification

The Luria–Delbrück fluctuation analysis is a statistical method that allows one to distinguish between variant cells arising by rare spontaneous mutations and variant cells arising through adaptation to an environmental selection (8). Figure 1 shows a schematic diagram of a fluctuation experiment and the hypothetical results one could obtain when analyzing a spontaneous mutation. A large parent population of cells is procured and the experimental samples are divided into two categories. The left arrow indicates the first category, which contains replicate samples in which an appropriate aliquot of cells from the parent population is plated directly into selection medium and analyzed for the number of resistant colonies that emerge. The colonies on these plates will represent rare resistant mutants. The number of colonies on each plate should follow a Poisson distribution and the mean number of colonies per plate will reflect the prevalence of resistant mutants in the parental population. Replicate platings from the same parent population should show variation due to random sampling only; the variance from these replicate samples should equal the mean. This category of samples is used to demonstrate that the method of sampling and plating has not introduced any fluctuations into the results besides random sampling error.

The right arrow in Fig. 1 indicates replicate samples of the second category. A small aliquot of cells (small enough to assure no preexisting mutants are present) is plated and allowed to propagate under nonselective conditions for a given amount of time. When the individual populations have reached the same cell density plated in category 1, they are transferred to fresh plates for an even distribution of cells and

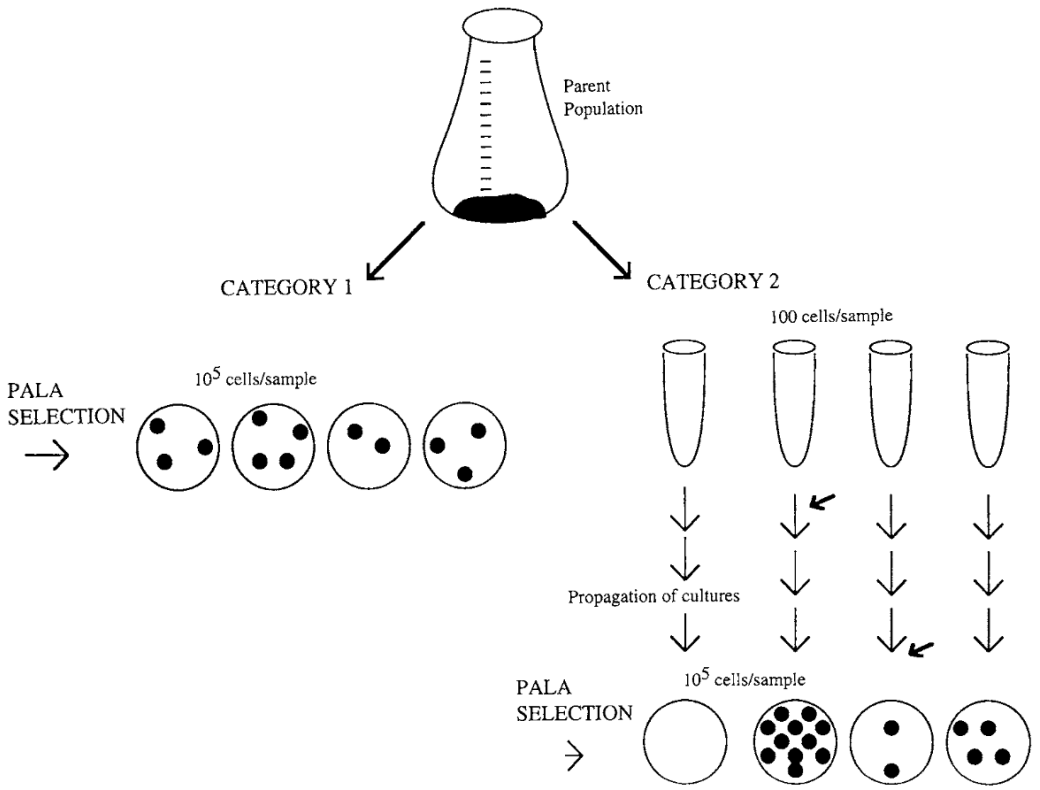

FIG. 1 Luria–Delbrück fluctuation analysis.

placed under selection (in this case PALA). If the drug-resistant cells are the result of exposure to the PALA (i.e., adaptive), each cell should have a probability for survival in selective media and the appearance of resistant mutants should be similar in all plates. The variation from plate to plate will be consistent with the Poisson model. If the events are spontaneous, as each parallel culture expands it will have a given probability for generating resistant mutants with each cell division. In some cultures the event (amplification) will occur early (as indicated by the small horizontal arrow in the second sample) and many of the progeny of the resistant cell will be present to form drug-resistant colonies. In others, the event will occur during one of the last cell divisions (as indicated by the small horizontal arrow in the third sample) and few drug-resistant progeny will result. The appearance of mutants will be random and the contribution to the surviving colonies when the cells are placed in selection will vary greatly depending on when in the propagation of the population the mutation occurred. Statistical analysis of this variation allows one to calculate the rate of appearance of spontaneous mutants.

Variables

In analyzing cells for their incidence of *CAD* gene amplification, particular attention should be paid to the following considerations.

Analysis of Multiple Subclones

Clonal heterogeneity exists in most populations for any marker that is being analyzed. The analysis of *CAD* gene amplification is no exception. Previous work has shown that populations that have been passaged for an extended period of time exhibit heterogeneity in their ability to amplify their dihydrofolate reductase gene (9). Newly subcloned populations, on the other hand, are fairly homogeneous. The determination of amplification frequency should be conducted with several isolates of each subclone.

Characterization of Subclones

As noted earlier, several characteristics of a cell line must be determined before a successful selection may be carried out. The growth rate of the cellular populations as well as the plating efficiencies of the subclones must be determined as described in Methods.

Determination of LD_{50}

Of paramount importance in designing a selection study is the initial subclonal sensitivity to the drug. A common parameter that is used for characterization is the mean lethal dose at which 50% of the population is eliminated by the drug (LD_{50}). To accomplish this, predetermined numbers of cells should be placed in various concentrations of PALA and incubated. The concentration of PALA that achieves a 50% kill of the population will be designated as the LD_{50}.

Initial CAD Gene Copy Number

To study *CAD* gene amplification, we must know the initial *CAD* gene copy number in the unchallenged population. DNA should be isolated from each cell line as described in Methods. *CAD* gene copy number and arrangement in the genome should be analyzed by Southern hybridization and slot blots. Selective hybridizations with

additional markers should allow one to determine if the chromosome carrying the *CAD* gene is present in single or multiple copies, thereby allowing discrimination between amplification and aneuploidy. Karyotypic analysis of the cell lines should be performed. Alternatively, one can use the sensitivity of fluorescent *in situ* hybridization to visualize the endogenous gene number. Both procedures are described under Methods.

Density Effects

For many cell lines, the density effects of plating increasing numbers of cells are negligible. If cells generate 1 to 2 colonies when 100 cells are plated, they generate between 10 and 20 colonies when 1000 cells are plated and between 100 and 200 colonies when 10^4 cells are plated. However, there are two considerations in this regard. The first is that if the cells are too close together (i.e., too close to confluence) a proper selection pressure cannot be achieved and a density effect is evident. Cells should always be plated at a density that is well below subconfluence. If the frequency of amplification is so low that millions of cells need to be analyzed, fewer cells in a greater number of plates will give a better determination than a greater number of cells in fewer plates. The second consideration has to do with cell volume. Cells vary in size and the area they occupy on the plating surface. In order for a proper selection to occur, the cells should be well spaced. This means that cells that occupy a greater area on the plate will need to be plated at a lower density to obtain accurate colony measurements.

Characterization of PALA-Resistant Mutants for CAD Gene Amplification

The PALA-resistant subclones that emerge from the selections should be characterized for their *CAD* gene copy number as described in Methods. The most direct route for determining if *CAD* gene amplification is the basis of PALA resistance in a cell population is to quantitate its *CAD* gene copy number through hybridization with labeled probes. Normalization to the copy number of an endogenous gene is an important part of this analysis because variations in DNA concentration in individual samples could lead to false positives for amplification of the gene in question. This conceptual control becomes even more critical in tumorigenic cells because of the reported increase in frequency of polyploid cells and cells that endoreduplicate; two (or more) complete genomes would be present. Two additional classes of chromosomal abnormalities can also lead to an increase in gene copy number and must be ruled out before amplification as a mechanism of resistance is claimed: (a) formation of a polyploid cell line could be resistant to PALA without selective amplification of its *CAD* gene; (b) formation of a cell that is aneuploid for the chromosome carrying the *CAD* gene.

We can determine the formation of polyploid cells through karyotypic analysis of the PALA-resistant cell lines. Cells are collected during mitosis from parental cell lines and from the PALA-resistant sublines. Chromosome counts are performed on fixed preparations. Mean chromosome counts for each population will reveal whether polyploid cells have given rise to the resistant cell line. One can anticipate that the majority of cells will not be polyploid. One can detect the presence of a trisomic cell through hybridization with a panel of gene probes. Hybridization with selected gene markers will allow determination of the chromosome dosage in each cell line.

Frequency of Gene Amplification

The frequency (or incidence) of gene amplification can be determined by graphing the relative plating efficiency (RPE) versus the concentration of PALA expressed in relative LD_{50}. The RPE is the number of PALA-resistant colonies normalized to the number of colonies that have emerged in the nonselected population. As indicated previously, the determination of gene amplification frequency is made with the number of colonies observed at $9 \times LD_{50}$ to ensure that one is dealing with true drug-resistant colonies. If the protocol of the experiment has not provided for cells to be plated directly at $9 \times LD_{50}$, this number can be extrapolated from data that bracket this dose. Figure 2 depicts representative data from a typical selection experiment.

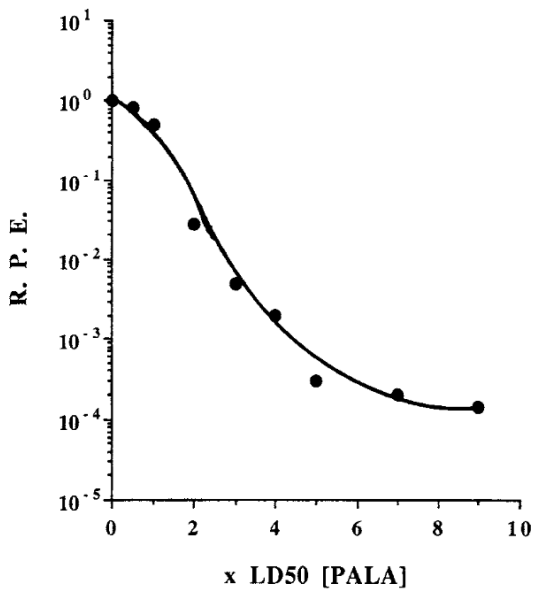

FIG. 2 Relative plating efficiency (RPE) versus concentration of PALA to determine frequency of gene amplification.

Methods

All cell lines should be grown in α-modified Eagle's medium (α-MEM) without deoxynucleotides, supplemented with 10% (v/v) dialyzed fetal calf serum, penicillin, and streptomycin and kept at 5% CO_2. It is critical that the serum be adequately dialyzed so as to remove low molecular weight ribo- and deoxyribonucleotides. If these molecules are present, the metabolic selection does not proceed because nucleotides are provided exogenously, short-circuiting the selection. Exponentially growing cells should be used for all experiments. Stock cell lines should be used for a maximum period of 3 months, at which time frozen aliquots of cells should be thawed for use in subsequent experiments to minimize changes resulting from extended propagation. As an additional control, dialyzed serum from a single lot should be used in all experiments.

Plating Efficiencies and Cell Cycle Times

To determine the plating efficiencies of unselected lines, 100 cells of each will be seeded into 100-mm-diameter dishes containing complete medium in a uniform monolayer. After 6–10 hr this medium will be replaced with fresh medium to remove any unattached cells. During this period of time, cells have an adequate amount of time to attach but not enough time to divide (forming two cells). If cells are too close to each other, adequate selection cannot proceed and calculations are confounded. Colonies should appear within 5–7 days; they will be fixed with 3:1 (v/v) methanol–acetic acid, stained with 2% (w/v) Giemsa (Gurr's), and only those colonies with more than 50 cells will be scored. The population doubling time for each line can be determined by plating cells into 100-mm-diameter dishes at a density of 5×10^4 cells/dish. Each day, plates should be trypsinized and the total number of cells determined. The population doubling time is the time required for cells to double during exponential growth in complete medium.

Drug Selections

For all selection experiments, cells should be seeded into complete medium at the appropriate density, allowed to attach for 6–10 hr, and then exposed to PALA by replacing this medium with medium supplemented with the drug; PALA can be obtained from the Drug Evaluation Branch of the National Cancer Institute (Bethesda, MD). After 3 days the medium should be changed to remove dead cells and their toxic products. Except for additional replacements with fresh selection medium each week, cells should grow undisturbed until colonies are visible: colonies selected in low PALA concentrations ($3 \times LD_{50}$) should become visible within 6–9 days; colo-

nies resistant to higher concentrations should require 2–4 weeks of growth. Colonies should then be fixed, stained, and counted as described above. The incidence of PALA resistance (percent survival) is the proportion of attached cells that give rise to resistant colonies, and thus is relative to the plating efficiencies of the cells in medium without drug (100% survival). To minimize variation between experiments, these plating efficiencies will be determined independently and in triplicate for each drug selection experiment. The LD_{50} values represent the concentration of PALA that allows 50% survival and are estimated by interpolation from values of percent survival in increasing concentrations of PALA; these values should be determined in triplicate at both the beginning and end of experiments.

Subcloning PALA-Resistant Lines

Cells from individual PALA-resistant colonies should be scraped from the bottom of 100-mm-diameter culture dishes using a sterile micropipette tip and collected in a 25-μl drop of PALA medium. These cells can be transferred to a 24-well cloning dish (Cat. No. 3047; Falcon, Oxnard, CA) and expanded in the same concentration of PALA-containing medium (3 or 9 $\times$ LD_{50}) until a total of 4×10^7 cells has been obtained. A small portion of these cells (10%) should be frozen in 90% (v/v) dialyzed serum–10% (v/v) dimethyl sulfoxide; genomic DNA can be isolated from the remainder (see below).

Molecular Analyses

Genomic DNA can be isolated as described in Brown *et al.* (9). Briefly, cells are lysed by the addition of 0.01 *M* Tris-HCl (pH 8), 0.01 *M* EDTA, 0.01 *M* sodium chloride, 0.02% sodium dodecyl sulfate (SDS), treated with proteinase K, extracted with phenol–chloroform (1 : 1, v/v), and treated with RNase A. DNA is quantitated spectrophotometrically and the indicated amounts electrophoresed on 0.7% (w/v) agarose gels after digestion with *Eco*RI. DNA is transferred to nitrocellulose (10) and hybridized to probes at 65° C under standard conditions: 10–20 ng of probe DNA per milliliter, 0.45 *M* sodium chloride, 0.045 *M* sodium citrate, 0.5% (w/v) SDS, 2× Denhardt's solution, and salmon sperm DNA (50 μg/ml). Radioactive probes can be prepared by either nick translation or randomly primed DNA synthesis (multiprime system; Amersham, Arlington Heights, IL) using [^{32}P]dCTP.

DNA Cytofluorometric Analysis

Cells can be prepared for analysis by the procedure of Gray and Coffino (11) with the following modifications: fixed cells are treated with RNase A (5 mg/ml) for

30 min at 37° C, and then stained with propidium iodide (0.5 mg/ml) for 30 min at room temperature. Cell suspensions ($2-5 \times 10^6$ cells/ml) are kept on ice and analyzed at a flow rate of approximately 500 cells/sec using a Becton-Dickinson (Mountain View, CA) FACS IV with an argon laser (488 nm). The average cellular DNA contents are relative to a karyotypically determined diploid ($2n$) cell standard.

Chromosome Analysis

Chromosome spreads can be prepared and stained by the method of Nelson-Rees *et al.* (12). Mid-log-phase populations of cells are exposed to Colcemid (5–10 μg/ml) for 1 hr or more and removed from the plate. Cells are collected by centrifugation and resuspended in hypotonic KCl plus sodium citrate and incubated at room temperature for 30 min. Cells are fixed in 3:1 (v/v) methanol–acetic acid and dropped onto a wet glass slide. For banding, slides are dried for 2 days and briefly incubated with 0.01% (w/v) highly purified trypsin on ice. The trypsin is neutralized with 10% (v/v) calf serum and the slides are stained with 2% (w/v) Giemsa. Approximately 20–50 spreads per cell line should be examined. Currently the fastest and most definitive method for verifying amplification of a specific locus is FISH (fluorescent *in situ* hybridization). It is routinely used on both rodent and human preparations. We use the *in situ* hybridization protocols of Trask and Hamlin (13) to localize the amplified *CAD* genes in these cells as follows.

Fluorescent in Situ Hybridization

Pretreatment

Initially, slides must be prepared and baked overnight at 60° C to anchor chromatin to the slides. See slide making protocol for details. Throughout the FISH protocol the slides will be immersed in liquid. Handle the slides gently to prevent sample loss. Do not shake vigorously. Slides aged 2 days to one or 2 months give the best results.

RNase A Treatment

RNase (100 μg/ml) in 2× SSC (1× SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate) for 1 hr at 37° C removes transcripts and prevents sequestering of probe and high background. The treatment is followed by a dehydration series in ice-cold 70, 85, and 100% fresh ethanol for 5 min each (3 min each is sufficient if pressed for time). Air dry the slides on the benchtop for at least 2 hr and use immediately or store in a slide box until needed. Slides are good for approximately 1 or 2 months.

Denaturation of chromatin is in 70% (v/v) formamide (FA) (nucleic acid grade;

BRL, Gaithersburg, MD)–2× SSC for 2 min at 72° C. The H_2O bath should be set so that the temperature is 1° C above 72° C for each slide because each slide added decreases the temperature of the solution by 1° C. Prewarm the denaturing solution in a 15-ml conical tube for at least 15 min before use. Add 5 ml of denaturing solution to a hybridization chamber and immediately drop the slide in and begin timing. Denaturation time is critical. Less than 2 min may not denature the chromatin, and the probe will not bind. More than 2 min may overdenature the DNA, breaking the backbone. When overdenatured, the site complementary to the probe may be degraded, the chromosomes look hairy, and propidium iodide (PI) does not intercalate as well, so PI intensity is significantly decreased. After precisely 2 min, quickly remove the slides and dip into ice-cold 70% (v/v) ethanol, followed by 85 and 100% as before. Slides are then air dried for at least 10 min before the next step. (Stock denaturing solution can be stored at 4° C.)

Proteinase K (PK) treatment degrades both cytoplasmic and nuclear proteins, decreasing background and facilitating more efficient probe binding. For hamster cells PK (60 ng/ml) in 20 n*M* Tris–2 m*M* $CaCl_2$ (pH 7.4) for 4 min at 37° C worked best. For human cells, PK (600 ng/ml) for 3–4 min worked best. The time and concentration of PK treatment may vary depending on the cell line. Proteinase K incubation is followed by a quick room temperature rinse in the Tris-$CaCl_2$ solution to remove PK. Dehydration in the ethanol series follows. The 70 and 85% (v/v) solutions from the denaturing step can be reused, but the 100% ethanol must be fresh. Air dry the slides on the benchtop for 5 min.

Hybridization Mixture

I.		II.	
Formamide	(50%, v/v)	Formamide	(55%, v/v)
SSC	(2×)	SSC	(1×)
Dextran sulfate	(10%, w/v)	Dextran sulfate	(10%, w/v)
Salmon sperm DNA	(5 μg/μl)	Salmon sperm DNA	(5 μg/μl)
Competitor DNA	(5 μg/μl)	Competitor DNA	(5 μg/μl)
Biotinylated probe	(2 μg/μl)	Biotinylated probe	(2 μg/μl)

Mixture II is recommended for chromosome-specific repetitive probes. It is more stringent (T_m of DNA is lower) than mixture I. Each added percent formamide decreases the T_m of DNA by 0.7° C. Salts and SSC, increase the T_m of DNA. Dextran sulfate is for volume exclusion to increase effective concentration of the mixture components. Salmon sperm DNA is for nonspecific blocking. Competitor DNA should be from the same species as the probe to block repetitive sequences in the probe. For most effective blocking, the blocking DNAs should be fragmented to 200–600 bp. Probe concentration can be increased if necessary, but 2 μg/μl is the minimum concentration for adequate signal. *Note:* Total DNA in the reaction should not exceed 10 μg.

Hybridization Procedure

Repetitive Sequence or Small Unique Sequence Probes

1. Denature the probe mix (70° C for 5 min, simultaneously with 5-min air drying of slides).
2. Ice quench (<2 min).
3. Apply 100 μl of probe to each $\frac{1}{2}$ slide and coverslip.
4. Seal the edges with rubber cement and place in a moist chamber at 37° C.

Unique Sequence Probes with Repetitive Sequences That Must Be Suppressed

1. Denature.
2. Incubate at 37° C for 5 min to 1 hr.
3. Follow steps 3 and 4 from above.

Posthybridization Washes/Fluorescein Isothiocyanate Staining

The purpose of the posthybridization washes is to remove excess probe and nonspecifically bound probe. The fluorescein isothiocyanate (FITC) staining is a sandwiching technique. A layer of avidin-DN (Vector Laboratories, Burlingame, CA) is added to the biotinylated probe followed by biotinylated anti-avidin antibody (Vector Laboratories) and another layer of avidin-DN (see Table I).

The washes need to be at the temperature specified before slides are immersed. The 50% (v/v) formamide–2× SSC can be reused two or three times. Store at 4° C between uses. The blocking solution must be made fresh each time. Five milliliters of solution in the hybridization chamber covers the slides well. The avidin-DN and anti-avidin solutions can be prepared beforehand (5 ml), but add the avidin or anti-avidin just before needed. The FITC conjugated to the avidin is very light sensitive. Cover the incubation chambers with foil. The solutions can be used for four to six slides. Store in foil-covered conical tube at 4° C between uses.

TABLE I Posthybridization Washes

Step	Washes	Temperature (°C)	Time (min)	Reagents
1	3	42	5 each	Formamide (50%, v/v), 2× SSC, pH 7
2	3	42	5 each	SSC (2×)
3	1	Room temperature	Quick rinse	SSC (4×)
4	Blocking	37	30	SSC (4×), 3% (w/v) BSA
5	Avidin	37	30	Avidin-DN (5 μg/ml) 4× SSC, 1% (w/v) BSA, 0.1% (v/v) Tween 20
6	3	37	5 each	SSC (4×), 0.1% (v/v) Tween 20
7	3	37	5 each	BN
8	Anti-avidin antibody	37	30	Biotinylated anti-avidin antibody (5 μg/ml) in PBS
9	Repeat step 7			
10	Repeat step 5			
10	Repeat step 7			

After the first two steps, the procedure can be stopped at any following step. Store the slides in a Coplin jar of BN (a blocking solution composed of 8.41 g sodium bicarbonate, 5 ml Nonidet P-40 added to 995 ml of distilled water) at 4° C until needed. If stopped after the avidin-DN step, cover the jar with foil. If stopped after the initial blocking step, another blocking step is necessary before resuming the detection and washes.

Mounting

The mounting medium is a glycerol-based antifade solution with 0.4 μg of PI/ml. Propidium iodide is a DNA intercalator for counterstaining the DNA. Apply 50 μl of antifade/PI to each slide and coverslip. Let the slides incubate in the dark for at least 15 min before viewing, or store in a dark box at 4° C until time permits viewing. Do not expose the slides to direct light; the signal will fade.

Viewing

For scanning, the 10× objective and green light, filter cube G, to see PI-stained chromatin. Once a good spread is located, center the spread between the cross-hatches, add oil, and switch to the 100× objective. Focus the spread and try to identify by shape the chromosome where the signal may be. Switch to the blue light, filter cube B-2, to view the FITC and PI simultaneously. Refocus if necessary and photograph. Spend only the minimum amount of time necessary under the blue light because the signal bleaches very quickly, and the PI bleaches somewhat. Also, surrounding spreads are being affected as one views a particular spread. A spread is much dimmer after it has been viewed.

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