# CHAPTER 11

# Cell Line Characterization and Authentication

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Research and development involving the use of cell lines require precise knowledge of the purity and species of origin of the cell lines used. This can only be assured by periodic monitoring of cultured cell lines for possible contamination by other cells and for characteristics that authenticate the cell line identity. In the absence of such monitoring, inter- and intraspecies cell line contaminations are likely to occur in the laboratories of unsuspecting investigators and can result in the generation of mistaken conclusions with an attendant loss of investigators' time, effort, and resources. This chapter provides a history and an overview of the methods that have been developed for cell line authentication, the type of information each of these different methods provides, and how synthesis of that information can be used to characterize a cell line and confirm its identity. An effective cell line monitoring strategy is described that involves testing for a

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combination of genetic markers, including cell membrane species antigens, isoenzymes, chromosomes, and DNA fingerprints, and use of databases for each marker system to compare the results obtained with a test cell culture with results from an extensive panel of previously tested cell lines.

## I. Introduction

The requirement for cell line authentication has a history almost as long as cell culturing itself, presumably beginning at the time when more than one cell line could be cultured continuously. The need today is as great as it was in the early 1960s when cell banks were first established to provide characterized cell lines. Of the 2376 cell lines submitted to the authors for characterization since 1990, 166 (7%) were found to be cross-contaminated by cells of the same or different species. Because most facilities carrying out cell culture employ multiple cell lines, and because the complexity of experimental design and the broad use of cell lines, in scientific investigation and in the pharmaceutical and biotechnology industries continues to increase, the possibility of inadvertant admixture of cell lines during the course of day-to-day cell culture is always present.

As illustrated by the following examples, research and development involving the use of cell lines require precise knowledge of the purity and species of origin of the cell lines used.

1. When human cell lines are assayed in animals for tumorigenic potential, it is obviously of critical importance to know whether any tumors that arise derive from the inoculated cell line or from the host animal species.
2. When drugs are screened for antitumor effects using human cell lines of specified tumor origin, it is essential that the cell lines maintain the identity and characteristics of the tumor of origin and its earliest cell culture passages. Moreover, when different tumor cell lines of a specific tumor type are used to assess the effects of drugs on that particular tumor type, it is important to confirm that the tumor cell lines are, in fact, distinct representatives of that tumor type.
3. In the production of animal or human viruses for vaccines, baseline cell identification data are required on the cell lines used, and continued monitoring is necessary as the cell line passages mount to ensure that the cell lines do not change on passage.
4. When agents are used to transform a cell line, the characterization of both the untransformed parent cell line and its transformed or transfected derivatives is required in order to show that the transformed cells are indeed derived from the parent cell line. Quite a few cell "transformations" have proven to be due to contamination of the original cell line by cells from another species. Such contamination results in cell cultures that mimic cell

transformation because of the resulting alterations in cell morphology, cell growth rate, and other properties.

5. When cell hybridization and gene transfer experiments are carried out, their success can often be confirmed by isoenzyme and cytogenetic analyses of the resulting cell lines.
6. Finally, the expanding therapeutic use of such biological products as monoclonal antibodies requires quality control monitoring of the cell lines from which these are derived.

This chapter provides a history and an overview of the methods that have been developed for cell line authentication, the type of information each of these different methods provides, and how synthesis of that information can be used to characterize a cell line and confirm its identity.

## II. Chronology of Cell Line Authentication Efforts

The great increase in interest and use of cell culture systems that began with virologists in the early 1950s, and then spread to all fields of biology, led to a reexamination of the status of the many cell lines extant in the early 1960s. Use of species surface antigens (Simpson and Stulberg, 1963; Greene *et al.*, 1964) showed that interspecies contamination was fairly widespread. In 1967 the classic work of Gartler introduced to cell culturists the power of isoenzymic analysis as a means for identifying human cell lines, and showed that intraspecies contamination of human cell cultures had become a serious problem and that many of the human tumor cell lines in use at that time were actually derived from a single cell line, i.e., HeLa (Gartler, 1967, 1968). The development of chromosomal banding procedures by Caspersson *et al.* (1970), and the demonstration by Miller *et al.* (1971) that HeLa cells had marker chromosomes that were persistent and readily identifiable firmly established the power of chromosome analysis as a means of cell identification and detection of intraspecies contamination.

Numerous refinements and additions to these approaches to cell identification and cell purity have been obtained since the mid-1970s. To briefly note a few examples, Montes de Oca *et al.* (1969) used glucose-6-phosphate dehydrogenase and lactate dehydrogenase in tandem to distinguish between cell species by the distinctive isoenzyme mobilities expressed by each species. O'Brien *et al.* (1977, 1980) and Wright *et al.* (1981) expanded the number of human polymorphic isoenzymes in use as first proposed by Gartler. The result has been the development of an isoenzyme phenotype profile for each cell line that in many instances not only confers a unique genetic signature, but also serves to identify the cell line with a high degree of probability. Nelson-Rees *et al.* (1976, 1980) reported extensively on HeLa cell contaminations and identified marker chromosomes (Nelson-Rees *et al.*, 1975) uniquely possessed by a large series of tumor cell

lines. This latter approach has also been followed by several other investigators (Giovanella *et al.,* 1976; Satya-Prakash *et al.,* 1981). Noguchi *et al.* (1979), Siciliano *et al.* (1979), and Rutzky *et al.* (1980) characterized cells by the combined use of isoenzyme genetic signature and marker chromosome identification. Marker chromosomes have been shown to be nonrandom in several kinds of tumors (Wheng-Peng *et al.,* 1982) and have been used to identify cell cultures derived from these tumors. However, during long-term culture, evolution of new marker chromosomes may occur from ongoing chromosome rearrangements, which can confound attempts to resolve the relationships between cell lines. DNA fingerprinting, a methodology used for cell line identification (Gilbert *et al.,* 1990; Yan *et al.,* 1996), has been used to help resolve this type of problem. Fluorescence *in situ* hybridization (FISH) is another technique that has proven extremely useful in providing additional power to the analysis and interpretation of cytogenetic findings. In addition to labeled probes for total human, mouse, or hamster DNA, which are particularly useful in the analysis of interspecies hybrid cells, chromosomal DNA probes for specific whole chromosomes, centromeric regions of all chromosomes (useful for chromosome enumeration), or locus-specific regions have been used for a variety of purposes, including (i) detection and estimation of the size of translocations that cannot be readily determined by banding patterns alone (Pinkel *et al.,* 1988) and (ii) tracking of a specific chromosomal function in experiments using interspecies cell hybrids (Durnham *et al.,* 1985).

Because no one single method provides sufficient information to characterize and authenticate a cell line, the most effective approach is to test for a combination of genetic markers, including cell membrane species antigens, isoenzymes, chromosomes, and DNA fingerprints. Using this combined approach, the results obtained in one test provide a validation and amplification of the information obtained from each of the other tests.

## III. Markers Used for Cell Line Identification

### A. Species-Specific Immunofluorescence

The species of origin of cells present in a given cell culture can be determined conveniently and reliably using immunofluorescence to detect cell surface expression of species-specific antigens (Simpson and Stulberg, 1963).

Cells to be tested for species-specific immunofluorescence are centrifuged and washed, and separate aliquots are resuspended in appropriately diluted fluorescein isothiocyanate-labeled, heterologous species-specific antisera. The cells are incubated for an hour on a shaker and are then washed and mounted on slides in a wet suspension preparation of living cells. The slides are examined with a fluorescence microscope, and the reaction noted. A positive reaction is denoted by bright green peripheral fluorescence of the cell membrane. A minimum of two antisera are employed when testing the cell suspension. One of the antisera is that of the presumed species of origin of the cell culture. The other

antiserum is of a species that is a common cell contaminant (e.g., mouse, rat, human). The reactions with the two antisera are evaluated with respect to the intensity of reaction and the percentage of cells that react. In a pure culture, all of the cells react, and literally thousands of cells are scanned. The second antiserum is used as a negative control and as a test for contaminating cells of the species against which the antiserum was raised. In the latter case, it is possible to detect 1 contaminating cell in 1000 cells. If the reaction is negative for both antisera, or if the test antiserum stains only a portion of the cells, the results imply contamination of the culture with cells of a species different than that detected by either test or control antiserum. In that case, the cells are tested with a broad panel of additional species-specific antisera.

The authors have prepared a panel of heterologous antisera against cell lines or red blood cells of 20 different species commonly used in the laboratory. The degree of specificity has been extended by appropriate absorption and/or dilution of each antiserum. For example, Cercopithecus monkey cells and Rhesus monkey cells can be detected by anti-Cercopithecus antiserum. Absorption of the antiserum by Rhesus monkey cells leaves the Cercopithecus reactivity intact. Using absorption and/or dilution, these antisera can be used quite specifically in determining species that are closely related. The less closely related species do not cross-react.

## B. Isoenzyme Phenotyping

Isoenzymes are enzymes that exhibit interspecies and intraspecies polymorphisms that can be detected by differences in electrophoretic mobility. By using enzyme-specific colorimetric staining reactions of electrophoretically separated cell extracts, it is possible to readily determine the electrophoretic mobility of many different isoenzymes in a given cell extract.

As described in more detail elsewhere (Halton *et al.*, 1983), cells to be tested for isoenzyme phenotypes are centrifuged, washed, and resuspended volume for volume in a 10 m*M* Trisma base buffer, pH 7.4, containing 10 m*M* NaCl and 10 m*M* 2-mercaptoethanol. The cells are freeze–thawed six times in dry ice/methanol, and the resulting enzyme extract is centrifuged at 5000 rpm at 5°C to remove cell debris. The supernate is either tested immediately or stored frozen at −70°C until later testing. Electrophoretic mobilities of glucose-6-phosphate dehydrogenase, lactate dehydrogenase, purine nucleoside phosphorylase, and malate dehydrogenase are determined immediately on samples of the cell extract to assist in the determination of cell species. One microliter of sample is applied to a slot on electrophoresis universal agarose film for each of the four enzymes. The other slots are filled similarly with samples from other unknown cell lines under investigation and with appropriate control samples prepared from known cells. The films are placed in an electrophoresis chamber and run for 25 min. The films are then removed and stained. Results of the run can be analyzed immediately.

The results of a typical isoenzyme phenotype analysis are shown in Fig. 1. When analyzed together with the results of the immunofluorescence tests, these initial isoenzyme results provide useful information concerning species of origin. Because these four isoenzymes can distinguish between several primate species commonly used in the laboratory (i.e., human, Rhesus monkey, and Cercopithecus monkey) and can distinguish between rodent cell lines commonly encountered (i.e., Syrian and Chinese hamster, rat, and mouse), the initial electrophoretic results can reinforce the immunofluorescence species identification tests and may indicate the presence of a second species of cell when it is at least 10% of the total population. If the immunofluoresence reaction is negative or partially

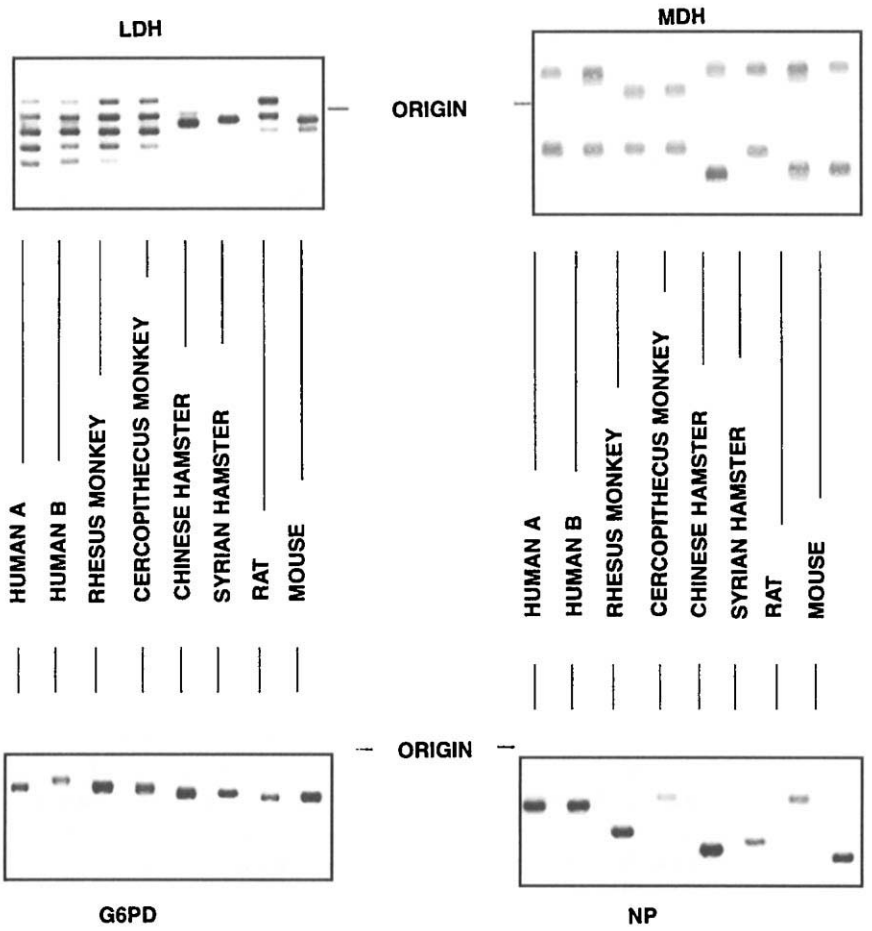


**Fig. 1** Comparison of various species for the electrophoretic mobilities of four different isoenzymes: lactate dehydrogenase (LDH), malate dehydrogenase (MDH), G6PD glucose-6-phosphate dehydrogenase (G6PD), and purine nucleoside phosphorylase (NP).

negative, the results of the initial electrophoretic run can be used to suggest antisera to be used in the next immunofluoresence test panel. They can also indicate the presence of an interspecies hybrid cell population.

Those cell lines that turn out to be human are subjected to additional isoenzyme analysis with isoenzymes that are polymorphic in humans. These include glucose-6-phosphate dehydrogenase, phosphoglucomutase-1 and -3, esterase-D, mitochondrial malic enzyme, adenylate kinase, and glyoxolase-1. This array of isoenzymes, even when the most frequent phenotypes are found in a cell line, have a frequency product in the order of 0.05, i.e., only 5% of cell lines would be expected to have this same isoenzyme genetic signature. When less frequently occurring phenotypes are encountered in a cell line, the frequency product is much lower and gives a measure of the uniqueness of that particular cell line.

## C. Chromosome Analysis

To prepare cultured cells for chromosome analysis, they are incubated with colcemid for 2 hr, suspended, centrifuged, resuspended in hypotonic solution, and fixed in a methanol–acetic acid solution. The fixed cells are then dropped onto cold slides to create spreads of metaphase chromosomes. The slides are stained using one of a variety of techniques, each of which provide different types of information. These techniques include Giemsa staining, trypsin–Giemsa banding, quinacrine mustard staining, C-banding, Hoechst 33258 banding, G-11 banding, and FISH.

Giemsa-stained metaphases are used for chromosome counts and ploidy assessment as described previously in detail (Peterson *et al.*, 1979). Because the chromosomes of normal cells exhibit a species-specific morphology and ploidy (number of chromosomes per metaphase), analysis of chromosome morphology in multiple examined metaphases from a given cell culture can serve as an additional check on the species of origin of the cultured cells. At least 100 metaphases should be examined per culture to check that all have chromosomes of the same cell species and to assess the presence of such nonspecific chromosome abnormalities as endoreduplication, chromosomal pulverization, chromosome or chromatid breaks, gaps, exchanges, or acentric fragments. Precise chromosomal counts are then made on at least 30 metaphases.

Having firmly established the species of origin, the ploidy of the cells in culture is then related to the ploidy of normal somatic cells of that species. Many cell lines are found to be aneuploid, with subpopulations of cells containing different numbers of chromosomes per cell. However, the ploidy of any given established cell line is usually a stable characteristic, thereby providing another useful cell line individualization marker.

Trypsin–Giemsa banding (Seabright, 1971) permits assignment of all chromosomes in the karyotypes. Between 5 and 15 karyotypes are prepared from banded chromosomes (a process greatly facilitated by use of an image analyzer) in order to provide an accurate assessment of normal, marker, and unassignable

chromosomes. One first determines the frequency with which each normal chromosome is found per karyotype, i.e., whether it is absent, monosomic, bisomic, trisomic, etc. Unassignable chromosomes are those that are neither normal nor consistently present from karyotype to karyotype. Their presence usually indicates that the chromosomal complement has not stabilized and that the culture may still be changing. In contrast, marker chromosomes are abnormal chromosomes that are consistently present from karyotype to karyotype (see Fig. 2). The composition of marker chromosomes is designated according to standard nomenclature (Anonymous, 1978), which denotes the portions of normal chromosomes contained in the markers and whether they represent translocations, deletions, transpositions, or other abnormalities. These stable chromosome abnormalities likely represent gross morphological features reflecting genomic alterations that have been selected for *in vivo* and/or *in vitro* because of the growth advantage they have provided these cells over cohort cells lacking such changes.

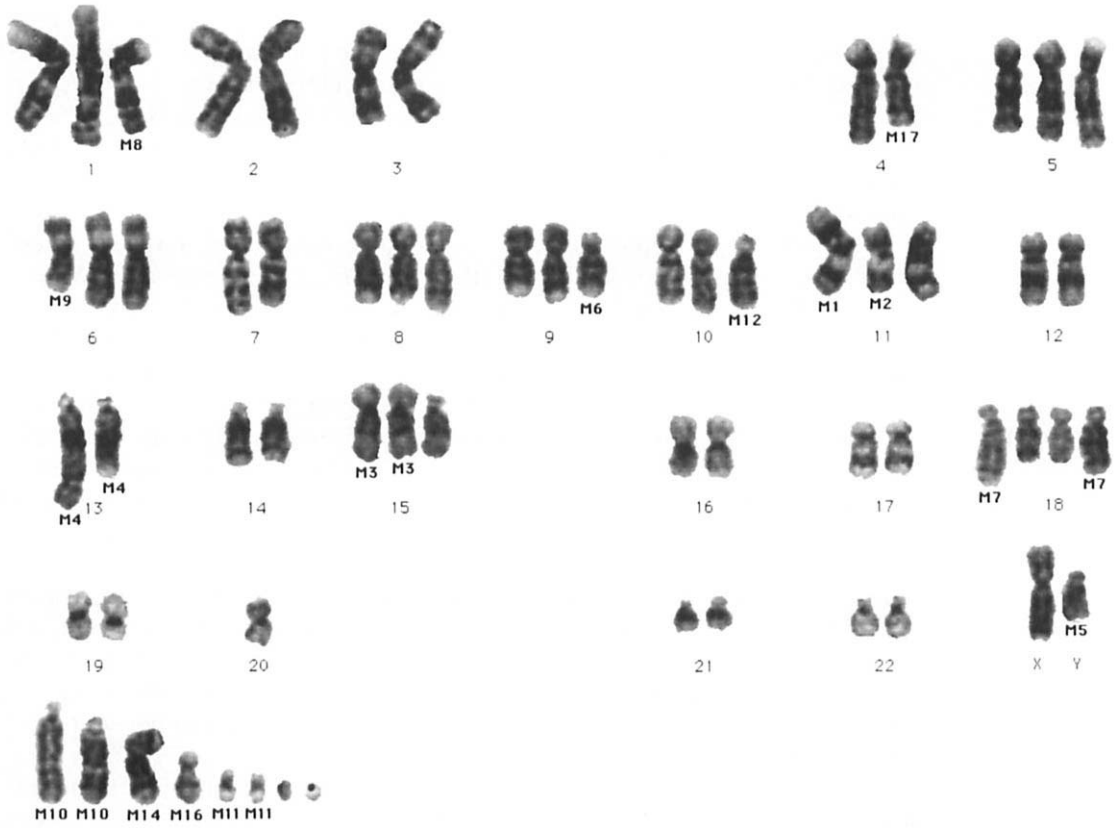


**Fig. 2** Human trypsin–Giemsa-banded karyotype showing normal chromosomes and marker chromosomes (designated as "M").

Other chromosome staining methods have more specific applications. Quinacrine mustard staining (Peterson *et al.,* 1973) easily shows whether the human Y chromosome is present. By differential fluorescent banding of several normal chromosomes, it also quickly differentiates between some primate species that have the same diploid number and similar morphology of chromosomes, e.g., gorilla and chimpanzee (Miller *et al.,* 1974). C-banding (Sumner, 1972) locates the centromere of mouse chromosomes, whereas Hoechst 33258 (Misawa *et al.,* 1977) and G-ll banding techniques (Friend *et al.,* 1976) are particularly useful in the study of interspecies hybrids. As shown in Fig. 3 (see color plate), interspecies hybrids are also detected readily by FISH using fluorescent probes for total human DNA (Fuscoe *et al.,* 1989).

FISH is a particularly powerful, albeit highly specialized, method that is used as an adjunct to standard cytogenetic analysis for the determination of ploidy and detection of aneuploidy for specific chromosomes in tumor cells, identification of marker chromosomes that elude specific identification by standard banding techniques, detection of deletions and amplifications of specific genes in tumors, and detection of species-specific chromosomes or chromosome fragments in metaphases of interspecies hybrid cells. FISH is particularly useful in the analysis of translocations when the size of the segment translocated cannot be determined readily by banding patterns alone (see Fig. 4, color plate) or when interpretation of the banding pattern can be confirmed by detection of a specific probe fluorescence. As an increasing number of probes become available, these probes will permit localization of specific gene regions on intact and translocated chromosomes more precisely, information of particular use in studies aimed at tracking the function of specific chromosomes or chromosome regions.

To carry out FISH analyses, chromosomes are prepared in a manner identical to that for standard staining procedures. Multicolor fluorescence-labeled probes are then used to stain the chromosomes. The chromosomes are counterstained with 4,6-diamino-2-phenylindole (DAPI), which gives a banding pattern similar to Giemsa banding. Slides are examined using a fluorescence microscope equipped with appropriate filter combinations. At least 25 metaphases are analyzed for each probe. After examining the chromosomes with a fluorescence microscope and taking photographs of individual metaphases, these same fluorescence-stained metaphases are counterstained with Wright's stain. The resulting G-banding on parallel stained metaphases is used to precisely locate the positive FISH signal on the chromosomes.

## D. DNA Fingerprinting

DNA fingerprinting is based on the existence of dispersed hypervariable regions of tandem-repetitive nucleotide sequences in the genome (Jeffreys *et al.,* 1985a). Polymorphism within a given species is due to the existence within that species of multiple different alleles, each of which encodes a different number of tandem repeats of the core nucleotide sequence characteristics of that region.

The most polymorphic tandem repeats are those with long core sequences (e.g., 15 bp) called "variable number of tandem repeats" (VNTR) or "minisatellites" (Jeffreys *et al.,* 1985b; Nakamura et al., 1987). Those with core sequences of a few base pairs (e.g., 1 to 5 bp) are called "short tandem repeats" (STR) or "microsatellites (Weber and May, 1989).

Several different approaches to DNA fingerprinting of cell lines have been described. One approach involves restriction fragment length polymorphism (RFLP) analysis by the Southern blot technique using multilocus VNTR probes that bind to several different hypervariable regions distributed on many different chromosomes. This procedure results in blots with a complex pattern of bands permitting ready distinction between different cell lines (Gilbert *et al.,* 1990). However, when mixtures of different cell lines occur, the band patterns are not analyzed easily. Moreover, the difficulty in resolving the size of each of the many bands makes this approach somewhat tedious, and the results are not readily tabulated. Hence, the creation of a cross-reference database is cumbersome. An alternative approach that overcomes these limitations is the use of RFLP analysis with single locus polymorphism (SLP) probes that detect highly polymorphic VNTR unique to single gene loci. Each single gene locus VNTR exhibits Mendelian inheritance, a high degree of heterozygosity, and a low mutation rate. When digested with restriction enzymes and analyzed by the Southern blot technique with SLP probes, these VNTR loci have the potential for generating a large number of unique size fragments characteristic of the many different alleles encoding that locus (Nakamura *et al.,* 1987). Given the high degree of heterozygosity at each of these loci, each cell line usually yields two bands that can be accurately and reproducibly sized by comparison with molecular size standards. Application of four or five different SLP probes to a single enzyme digest yields a virtually unique cell line fingerprint. However, although accurate and precise, this approach is complicated, labor intensive, and time-consuming. Moreover, interassay standardization for purposes of developing reference database information is problematic.

A more rapid method of DNA fingerprinting human cell lines involves polymerase chain reaction (PCR) amplification of single locus polymorphisms of multiple VNTR or STR origins (Yan *et al.,* 1996), a procedure commonly referred to as AmpFLP (amplified fragment length polymorphism) analysis. The authors' approach is to use crude DNA extracted from a cell line lysate as a template for PCR reactions with primers specific for each of six highly polymorphic STRs known to have a low incidence of mutation in normal humans. A high degree of precision is achieved by conducting electrophoretic separation of amplified DNA products on high-resolution agarose or polyacrylamide gels that give sharp, well-defined bands permitting distinction between bands differing by only three nucleotide base pairs in size and by using FLP-specific "allelic ladders" to identify individual alleles (see Fig. 5). Based on current knowledge of the allele frequency of different FLP loci, determination of the composite AmpFLP fingerprint of a cell line at six different FLP loci

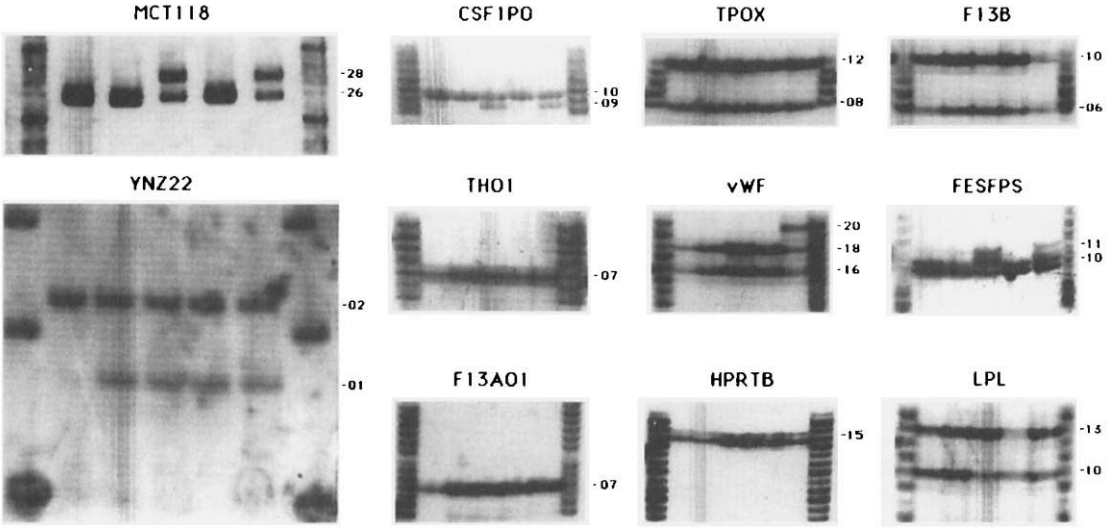


**Fig. 5** DNA fingerprinting of five "HeLa" cell lines at 11 FLP loci. The first and last lanes are the allelic ladders of the corresponding locus, except for locus YNZ22, which is the 100-bp DNA ladder (the 100-, 200-, and 300-bp bands are shown). The sample lanes from left to right are cell lines 117, 118, 119, 120, and 121. All five of the test cell lines are identical to each other and to standard HeLa cell line for alleles at seven FLP loci: vWF, THO1, F13AO1, TPOX, HPRTB, F13B, and LPL. This indicates with virtual certainty that the five test cell lines are all HeLa cell derivatives. At each of the other four FLP loci (MCT 118, CSF1PO, FESFPS, and YNZ22), cell lines 119 and 121, like the standard HeLa cell line, exhibit heterozygosity for the same two alleles. In contrast, cell lines 117, 118, and 120 exhibit only one of these same two alleles. These results show that, compared to the parental HeLa cell line, cell lines 117, 118, and 120 have lost heterozygosity at loci MCT118, CSF1PO, and FESFPS on chromosomes 1, 5, and 15, respectively. Additionally, cell line 117 appears to have lost heterozygosity at locus YNZ22 on chromosome 17, and cell line 121 appears to have gained an extra allele at locus vWF.

should result in a minimum discrimination power of 0.999. In the authors' experience, the DNA fingerprints of most cell lines are stable over prolonged periods of routine passage and thereby serve as highly reliable cell line identification markers. An exception to this rule occurs when cell lines are exposed to mutagenic agents, including radiation, chemical carcinogens, and certain viruses. Each of these treatments can result in genomic instability in cell lines reflected most commonly in the DNA fingerprint by loss of heterozygosity or by loss or gain of one or more tandem repeats at a given STR locus. When such changes in a DNA fingerprint occur, they are frequently accompanied by loss of heterozygosity in isoenzyme phenotypes and by changes in chromosome number and morphology (Hukku *et al.*, 1983). In such instances, using at least six or more STR loci in DNA fingerprinting provides an effective approach to elucidating the relatedness of the treated cell line to the putative parental line. In some instances, testing at additional STR loci may be required.

## IV. Conclusions

The optimal approach to cell line characterization and authentication is to apply multiple immunological and genetic marker systems to each cell culture to be tested and to analyze the results in the context of previously established marker databases. Each different marker system serves to confirm and extend the information provided by the other marker systems. Immunofluorescence results are checked against the results of the isoenzyme analysis, and these combined results in turn are checked against chromosome findings and results of DNA fingerprinting. The availability of computer databases for each marker system, such as those developed since the mid-1970s in the authors' laboratory, permits comparison of the results obtained with a test cell culture with results from previously tested cell lines. Given the precision and reliability of PCR-based AMP-FLP DNA fingerprinting, current efforts to develop a database containing DNA fingerprints of most of the commonly used extant cell lines should be of particular future value for the routine monitoring of cell line authentication. In the absence of such monitoring, it is virtually certain that inter- and intraspecies cell line contamination will occur periodically in the laboratories of unsuspecting investigators using cultured cell lines in their research, resulting in the unfortunate generation of false conclusions with an attendant loss of investigators' time, effort, and resources.

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