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ISOENZYME ANALYSIS AS A RAPID METHOD FOR THE EXAMINATION OF THE SPECIES IDENTITY OF CELL CULTURES

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SUMMARY

One of the major problems in cell culturing is the misidentification or cross-contamination of authentic continuous cell lines. We applied a rapid and efficient isoelectric focusing (IEF) technique for the routine analysis to detect interspecies contamination of cell cultures and for the identification of unknown animal cell lines. The method is based on the isoelectric separation of a specific set of intracellular enzymes which can be used to distinguish between cell lines of human, murine, or other mammalian origin. By means of preformed agarose gels, standardized conditions and equipment, this technique is especially applicable for routine work and allows the analysis of a large number of unknown samples with reproducible results. One hundred seventy-seven cell lines which have been sent to the Department of Human and Animal Cell Cultures at the DSM (Deutsche Sammlung von Mikroorganismen and Zellkulturen) were analyzed for species authentication; only three cell lines were found not to be of the presumed species. Our study strongly emphasizes standardized IEF as an efficient and rapid method for routinely monitoring the authenticity of cell lines.

Key words: isoenzymes; cell culture collection; cell identification; quality control; cell lines.

INTRODUCTION

Over the past years the use of permanent cell cultures has become increasingly important in biomedical research and biotechnology (5,19,30). The three major problems of contamination in cell culturing are contamination with microorganisms, cross-contamination with other eukaryotic cells, and misidentification. Microbial contamination, especially those caused by mycoplasmas, may occur quite frequently (up to 50%) (15,20,23,39), but can eventually be successfully eliminated applying different techniques (8 and references therein). Also, the detection of a "false" cell line was quite frequent in the past (18,24,36). The consequences of the use of a wrong cell line may indeed be deleterious: the cell line has to be discharged and the data obtained from the cell line may be of doubtful significance.

Several methods for monitoring cell culture authentication have been devised and reviewed (16,29,37). They include detection of immunologic markers (3,17,34), electrophoretic methods (4,11,22,27), karyologic examination (25,41), and very recently, DNA fingerprinting (2,10,12,35,38). Isoenzyme analysis uses the genetic polymorphism of enzymes. Polymorphic enzymes (allelic isozymes, allozymes) are genetically controlled variants of an enzyme resulting from point mutation(s) of the structural gene. These mutation(s) may yield electrophoretically resolvable phenotypes (11,42). The determination of the allozyme phenotype of a group of polymorphic enzymes gives rise to an enzyme pattern specific for a given cell line and can even be applied in the discrimination between cells from the same species (28,42). However, for speciation, an enzyme locus need not to be polymorphic. Isoenzymes (homologous enzymes) exhibiting different electrophoretic mobilities suffice to discriminate between cell lines from different species.

Here we describe the application of a recently developed, standardized isoelectric focusing (IEF) for species verification of cell lines submitted to the DSM-Department of Human and Animal Cell Cultures over the last 3 yr. The study documents a) the low number of incorrectly specified cell lines submitted to our cell repository, and b) the feasibility of this standardized IEF for routine work in all kinds of cell culture laboratories, even in laboratories working only with a small number of cell lines from different species.

MATERIALS AND METHODS

Cells and cell culture. All cell lines investigated are continuous mycoplasma-free cultures and are available as certified cell lines from the German Collection of Microorganisms and Cell Cultures (DSM, Braunschweig). Cells were cultivated without antibiotics according to the protocols given in the DSM-Catalogue of Human and Animal Cell Lines (1). Cells were cultivated at 37° C in a humidified atmosphere containing 5 to 10% CO_2 in appropriate culture media (GIBCO-BRL, Eggenstein, Germany) supplemented with heat-inactivated fetal bovine serum (varied between 5 and 20%) (Sigma, Deisenhofen, Germany). The cell cultures were monitored for mycoplasma infection (1,40) and examined by cytogenetics (1,33) and DNA fingerprinting analysis (1,12). The type of the hematopoietic cell lines was determined by immunophenotyping (1,32). Morphologic features of the cell lines were evaluated on cytocentrifuge slide preparations stained with May-Grünwald-Giemsa stain.

Isoelectric focusing. Sample extraction and IEF were carried out with the reagents and equipment provided by the AuthentiKit-System (Innova-

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tive Chemistry Inc., Marshfield, MA). Typically 10^7 cells were collected from an exponentially growing culture (viability usually 90% or higher), washed twice and sedimented for 5 min at 1000 $\times g$. The pelleted cells were resuspended in the same volume (10 to 30 μl) of Cell Extraction Buffer (a hypotonic buffer containing mild detergents) and stored at −20° C. Before the analysis the cells were lysed by repeated freezing and thawing followed by centrifugation for 10 min at 2000 $\times g$. The supernatant, containing soluble intracellular enzymes, was collected and diluted 1:1 with Enzyme Stabilizer. One to three microliter of the enzyme solution were applied on precast 1% agarose gels, and electrophoresis was carried out in Michaelis buffer (sodium barbital; Serva, Heidelberg, Germany) at 5° C for 20 min and 160 V. After electrophoresis, insoluble formazan was generated by the reaction of one of the following enzymes. Nucleoside phosphorylase (NP), EC 2.4.2.1; glucose-6-phosphate dehydrogenase (G6PDH), EC 1.1.1.49; malate dehydrogenase (MDH), EC 1.1.1.37; mannose phosophate isomerase, EC 5.3.1.8; peptidase B (PEP B), EC 3.4.11.4; aspartate aminotransferase (AST), EC 2.6.1.1; lactate dehydrogenase (LDH), EC 1.1.1.27, with appropriate substrates that mark the localization of the respective enzyme in the agarose gel.

Species evaluation of the IEF gels. All unknown samples were analyzed for the electrophoretic patterns of at least three different isoenzymes. The unknown samples were defined as of human, mouse, rat, or hamster origin when all enzymes migrated identically to a reference cell line from the same (presumed) species and migrated significantly different from the isoenzymes of the three other species mentioned above. Due to the close similarity of isoenzymes among primates, the respective non-human cell lines could be distinguished from human cells only by the two isoenzymes NP and AST.

Results and Discussion

With the dramatic increase in the number of cell lines being developed and distributed throughout the scientific community, the risk of inter- and intraspecies cross-contamination has risen proportionately. International cell culture repositories, like the DSM, fulfill the task of providing researchers with certified biological material of guaranteed quality and identity. In the past, starch gel electrophoresis was successfully carried out for species identification of a wide variety of different cell cultures, including rarely used cell lines such as those from buffalo, mink, dolphin, and minnow (22,28). By means of analysis of 16 polymorphic enzymes, Wright et al. (42) were able to distinguish even between 70 individual human tumor cell lines.

Recently, highly discriminative agarose gel systems have been developed (11). We applied one of those methods, namely the commercially available Authentikit-system. This kit contains all the equipment necessary, including material from certified human and mouse cell lines (obtained from the American Type Culture Collection, Rockville, MD), which serve as internal controls. By means of this kit we were able to rapidly screen more than 170 cell lines for interspecies cross-contamination. These cell lines were sent during the past years to our department for cell accessioning and characterization and were submitted from other cell repositories (16%), from the original investigator's laboratory (37%), or from third parties (47%). Cell extracts of each cell line were prepared and every single cell line was analyzed for the presence of at least three different isoenzymes. The majority of the investigated cell lines were either human or murine (Table 1). Therefore, we have chosen those isoenzymes that clearly exhibit different isoelectric mobilities for these species in our gel system and evaluated the unknown samples as outlined in Materials and Methods. IEF of MDH and AST easily distinguish human from mouse, rat, and hamster cells (a representative example is given in Fig. 1 *A,B*) as well as several other, rarely used species (data not shown). In most species the genes for MDH and AST are encoded on two autosomal loci, one for a cytoplasmic, the other for a mitochondrial polypeptide. Both enzymes are dimeric, but each dimer is composed of identical subunits, so one can detect a cytoplasmic and a mitochondrial variant. The mitochondrial form of both MDH and AST migrated toward the cathode (Fig. 1 *open arrow*); the cytoplasmic enzymes migrated to the anode. To differentiate between the rodents, MDH and AST are not useful (Fig. 1 *A,B*). However, we applied one or both of these isoenzymes, first to ensure that the given sample was not of human origin; then we determined the isoelectric points of NP and PEP B. The latter turned out to be the best isoenzyme for the distinction of rodent cell lines. As shown in Fig. 1 *C*, the isoelectric points of the rat, mouse, and hamster PEP B differ markedly. Interestingly, in several of the mouse cell lines analyzed we found two bands after staining the IEF gels for PEP B. The higher frequency of two PEP B-like bands observed and the subsequent analysis of other isoenzymes, combined with cytogenetic data, clearly ruled out the possibility of cross-contamination of these cell lines. Actually, the mouse cell lines or mouse hybridomas had been established from different mouse strains, such as Balb/c, various C57-strains, C3H, or others. It might be possible that in the respective cell lines either two independent genes encoding two PEP B-like enzymes or two actively transcribed alleles for PEP B exist.

TABLE 1

NUMBER OF CELL LINES AND SPECIES ANALYZED BY IEF

Species	No. Correct	No. False
Man	126	1
Mouse	32	1
Rat	10	1
Others[a]	9	—

[a] Includes monkey, hamster, and bovine cell lines.

When analyzing hamster cells it is important to know that most existing cell lines were established from Chinese hamster (*Cricetulus griseus*) or from Syrian hamster (*Mesocricetus auratus*), which are different species. From the panel of our enzymes, MDH and PEP B were the best ones to distinguish the two hamster cell lines, BHK-21 and CHO, whereas AST, LDH, and NP were ineffective (data not shown).

Figure 2 shows a representative example of an IEF stained for NP from several species. IEF of NP is useful to discriminate human from mouse, hamster, and non-primate monkey cell lines. The isoelectric points of human and rat NP, however, are too close for an unequivocal differentiation between these two species.

Halton et al. (11) distinguished eight species within a panel of 89 cell lines analyzed, using mainly LDH and G6PDH. However, we believe that the determination of the G6PDH isoenzyme profile is only of limited value for speciation of rodent and primate cells: hamster, mouse, and rat exhibit almost identical G6PDH isoenzyme mobilities; the same is true for primate cells. Inasmuch as human G6PDH exists in two 'ethnographic' variants, type A (African and Melanesian) and B (Caucasian), one can use this enzyme to distinguish a HeLa-like cell line from non-HeLa cells (6).

Vertebrate LDH is a tetrameric enzyme, composed of subunits A and B, giving rise to five possible isoenzymes, AAAA, AAAB, AABB, ABBB, and BBBB. Therefore, one could be tempted to rely

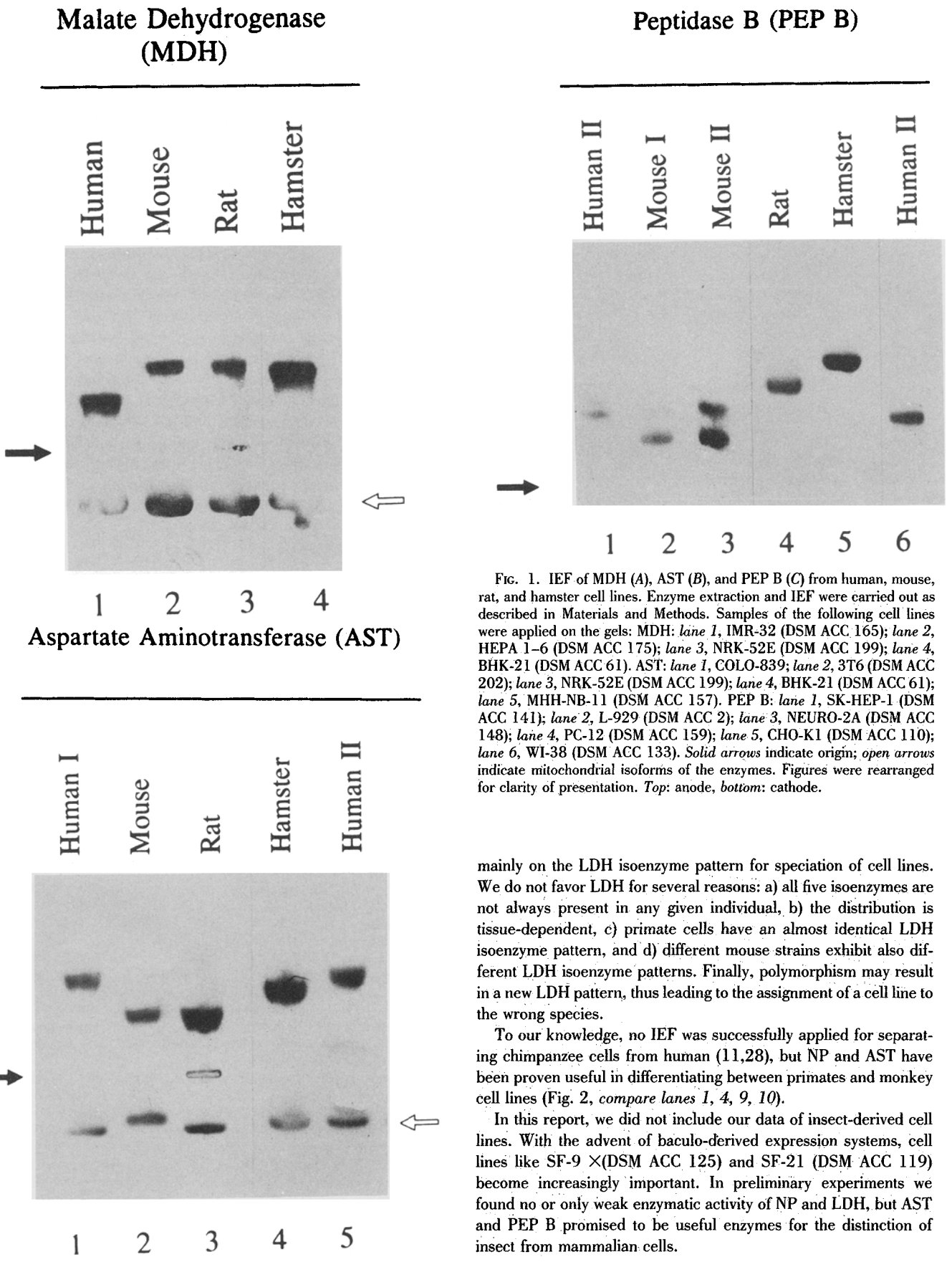

FIG. 1. IEF of MDH (*A*), AST (*B*), and PEP B (*C*) from human, mouse, rat, and hamster cell lines. Enzyme extraction and IEF were carried out as described in Materials and Methods. Samples of the following cell lines were applied on the gels: MDH: *lane 1*, IMR-32 (DSM ACC 165); *lane 2*, HEPA 1–6 (DSM ACC 175); *lane 3*, NRK-52E (DSM ACC 199); *lane 4*, BHK-21 (DSM ACC 61). AST: *lane 1*, COLO-839; *lane 2*, 3T6 (DSM ACC 202); *lane 3*, NRK-52E (DSM ACC 199); *lane 4*, BHK-21 (DSM ACC 61); *lane 5*, MHH-NB-11 (DSM ACC 157). PEP B: *lane 1*, SK-HEP-1 (DSM ACC 141); *lane 2*, L-929 (DSM ACC 2); *lane 3*, NEURO-2A (DSM ACC 148); *lane 4*, PC-12 (DSM ACC 159); *lane 5*, CHO-K1 (DSM ACC 110); *lane 6*, WI-38 (DSM ACC 133). *Solid arrows* indicate origin; *open arrows* indicate mitochondrial isoforms of the enzymes. Figures were rearranged for clarity of presentation. *Top*: anode, *bottom*: cathode.

mainly on the LDH isoenzyme pattern for speciation of cell lines. We do not favor LDH for several reasons: a) all five isoenzymes are not always present in any given individual, b) the distribution is tissue-dependent, c) primate cells have an almost identical LDH isoenzyme pattern, and d) different mouse strains exhibit also different LDH isoenzyme patterns. Finally, polymorphism may result in a new LDH pattern, thus leading to the assignment of a cell line to the wrong species.

To our knowledge, no IEF was successfully applied for separating chimpanzee cells from human (11,28), but NP and AST have been proven useful in differentiating between primates and monkey cell lines (Fig. 2, *compare lanes 1, 4, 9, 10*).

In this report, we did not include our data of insect-derived cell lines. With the advent of baculo-derived expression systems, cell lines like SF-9 ×(DSM ACC 125) and SF-21 (DSM ACC 119) become increasingly important. In preliminary experiments we found no or only weak enzymatic activity of NP and LDH, but AST and PEP B promised to be useful enzymes for the distinction of insect from mammalian cells.

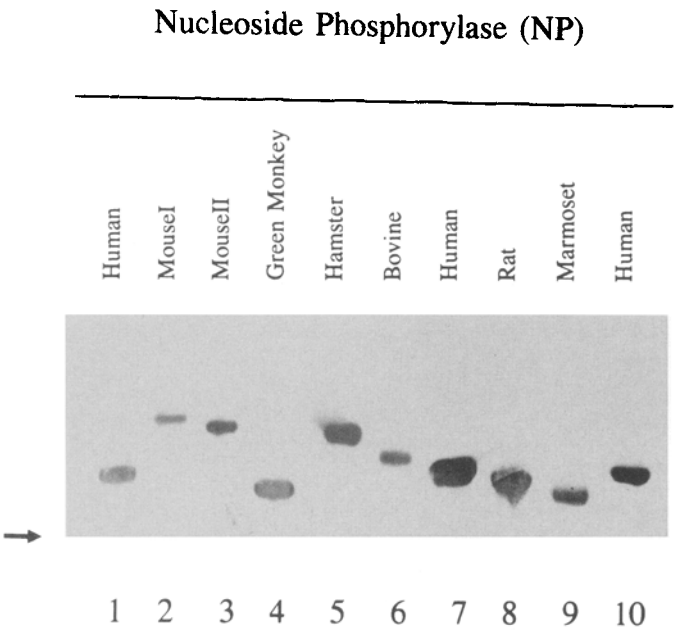

FIG. 2. IEF of nucleoside phosphorylase. Samples were from the following species: human (*lanes 1, 7, 10*), mouse (*lanes 2, 3*), green monkey (*lane 4*), Chinese hamster (*lane 5*), rat (*lane 8*), marmoset (*lane 9*), and an unknown sample specified by the depositor as bovine (*lane 6*). *Arrow* indicates origin; *top*: anode, *bottom*: cathode.

We found that only 3 out of 177 cell lines investigated were not of the species as indicated by the depositor. Although this is a very small number, the risk of contamination of cultures by unrelated cells is a potential problem. That this is generally not a rare event, and thus negligible, is documented by the reports of those laboratories to which cultures were submitted especially for cell line identification (26,28,37). Hukku et al. (18) reported that 35% of 275 cell lines examined were not as indicated by the donor laboratories. In an earlier study, 16% out of 466 cell lines obtained from 65 laboratories were found to be incorrectly defined (25). Although in most cases the contamination resulted in overgrowth of one culture, a few examples are also known where two cell lines grew continuously as mixed culture (11,26,37). We compared two different passages of one falsely specified cell line and discovered two species (rat and mouse) in the early passage and only one false species (rat) in the late passage.

The reason for the overall small number of falsely specified cell lines submitted to our collection might be because most of the cell lines we studied were obtained from the originator, the originator's laboratory, or from an international cell bank (58%). Also, 42% of the human cultures were of hematopoietic origin. These cells were usually thoroughly characterized by a series of specific cell surface markers, which often simplifies the discovery of a false cell line.

Several reports presented evidence that many permanent tumor cell lines established originally in different laboratories were in fact HeLa cells (18,24,31,36). However, a few cases of contamination with non-HeLa cells are also known (7,14). DNA fingerprinting carried out in our department suggests that one of the three incorrectly specified cell lines we discovered could also be a mix-up with HeLa-like cells.

Although IEF is excellent for the rapid detection of interspecies cross-contamination, only a combination of several methods is recommended to identify the correct species of a falsely specified cell line. Especially, computer-based data analysis (13) of DNA fingerprinting could open a straightforward method for individualization of a cell line; however, it should not be exclusively used in cell monitoring (21). Recently, we reported the successful combination of several techniques to identify an incorrectly defined cell line (9). As a cell repository, we routinely carry out IEF analyses together with DNA fingerprinting, cytogenetics, and immunologic analyses for the authentication of cell lines. However, because in many cell laboratories the equipment for DNA fingerprinting or cytogenetic examinations or both, techniques that are time consuming and require experienced personnel, might not always be available, we recommend IEF as a routine and efficient method for the rapid verification of cell lines.

In summary, we have demonstrated the feasibility of standardized agarose IEF for a fast and efficient identification of interspecies contamination in cell cultures. We emphasize that the increase in the number of cell lines in a laboratory or institute should also be accompanied by strict quality control assessments to monitor cell identification and authentication, and we recommend IEF for routine analysis in cell culture laboratories. Nevertheless, the use of good laboratory practices (i.e. working in safety cabinets with only one cell line) and the acquisition of only certified cell cultures will be helpful steps in this endeavor.

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