Species Identification and Confirmation of Human and Animal Cell Lines: A PCR-Based Method

BioTechniques 32:432-440 (February 2002)

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ABSTRACT

Misidentification and cross-contamination of cell lines are major problems of cell cultures that can make scientific results and their reproducibility unreliable. This paper describes a PCR-based method for easily identifying or confirming the species of origin of cell lines by using a panel of oligonucleotides specific for the nine animal species most common in cell culture laboratories. A panel of 35 human and animal cell lines, whose species of origin were previously confirmed by isoenzyme assay, was studied with nine species-specific primer pairs that specifically anneal to DNA sequences coding for human, cat, dog, mouse, rat, horse, rabbit, African Green monkey cytochrome c oxidase subunit I (cox I), and one primer pair specific for the cytochrome b gene of Chinese hamster. The amplified fragments were analyzed by electrophoresis in ethidium bromide-stained 2% agarose gels. The method is simple, rapid, highly sensitive, and useful for routinely monitoring the species identity of cell cultures.

INTRODUCTION

Continuous cell lines are widely used tools in research laboratories around the world. These cell lines are often exchanged between laboratories without any guarantee of their real origin, their names are often truncated and misspelled, and records are not always kept of the history of the ampoules. Thus, cell cultures can be grown, maintained, and used for years without confirming even the species of origin.

However, the scientific community does not recognize misidentification of cell lines as a major concern. Little effort is spent in laboratories to exclude contamination of cell lines with cells from another individual or species, and subsequent overgrowth has occurred (20,24). In fact, the researchers give more attention to the contamination by microorganisms (6,7,12,18,20,23). Unlike bacterial and fungal contamination, cell cross-contamination is not readily detectable, and the morphology and behavior of the cells in culture can remain unchanged. This is true also for mycoplasma contamination, which is not easily detected, but the awareness of the consequences of this is growing in the scientific community, and a number of papers have been published in the last few years on falsification of data because of mycoplasma contamination (4,26,30). On the contrary, the literature on cell cross-contamination is limited in quantity, and the scientific community is not sufficiently responsive about problems related to this type of contamination.

The risk of cell-culture overgrowth by unrelated cells was first recognized in

1957 (5,20). Since then, cross-contamination has been detected by karyology, transplantation, and hemagglutination experiments (2,34), immunofluorescence (31), and DNA fingerprinting (22, 32). In 1966, the isoenzyme polymorphism was implemented as a genetic marker (11), and G6PD polymorphism clarified the problem of HeLa cell contamination (8,9). Biochemical analysis of isoenzyme polymorphism is currently considered to be the standard method in quality control of cell line identification and interspecies contamination and is routinely used by the main Biological Resource Centers around the world (i.e., ATCC, ECACC, DSMZ, and Riken) (13,14,25,33), in conjunction with DNA fingerprinting for intraspecies cross-contamination detection (5,19,21,32).

Very few laboratories perform isoenzyme assay or fingerprinting on a regular basis because of the cost and complexity of the assays. In addition and most importantly, journal editorial boards, study sections, and others supporting fine research frequently do not insist that certification of cell line species and origin be included. In recent years, PCR has become a widespread tool commonly used in most research laboratories (29). The assays described could easily be added to strengthen quality-control programs in even the most modest cell culture facility.

MATERIALS AND METHODS

Cell Cultures

A panel of 35 Interlab Cell Line Col-

lection (ICLC)-certified continuous cell lines (27,28) was used in this work. Table 1 shows the list of cell lines with the available origin data. Among the cell lines, normal, virus-transformed, and tumoral cell lines are well represented. The age of the donor ranges from embryo stage to adult, and, within the species, different strains/ethnic origins have been taken into account. The cell lines were grown in 75-cm² plastic culture flasks in their respective culture media (Invitrogen, Milano, Italy and Biochrom KG, Berlin, Germany) in the absence of antibiotics and supplemented with 10%–20% heat-inactivated (30 min at 56°C) FCS (Mascia Brunelli S.p.A., Milano, Italy) and when relevant with other supplements, according to the protocols given in the ICLC Catalogue of Human and Animal Cell Lines, available at www.iclc.it. Flasks were incubated in a humidified 5% CO₂ and 95% air atmosphere. All the cell cultures were mycoplasma free, as assessed by at least two methods: bisbenzimidazole (Hoechst 33258) DNA fluorescence staining (1,3) and PCR analysis (16).

Isoenzyme Analysis

The species of origin of all the cell lines, as certified by the depositor, was verified and confirmed by isoenzyme analysis with the reagents and equipment provided by the AuthentiKit™ System (Innovative Chemistry, Marshfield, MA, USA) (17). The electrophoretic mobility of at least two different isoenzymes from a panel of seven (AST, G6PD, LD, MD, MPI, NP, and Pep B) was determined for each cell line (Table 1). Briefly, a pellet containing 5–10 × 10⁶ cells was resuspended in an equal volume of Cell Extraction Buffer (Tris Buffer, EDTA, and detergent in deionized water, pH 8.0), allowed to stand for approximately 15 min, and then centrifuged for 5 min at 2000× *g* at 4°C. The resulting supernatant containing extracted proteins was retained, and 1–3 µL were loaded onto thin agarose gels and subjected to electrophoresis for 25 min at 160 V. The gel was covered with the enzyme reagent specific for one of the enzymes being analyzed and then incubated at 37°C for 5–20 min. The result was the formation of an insoluble band to mark the location of the en-

zymes in the gel. The enzyme migration distances were measured and compared with values of standardized migration distances for the different species.

PCR Analysis

Sample preparation. One milliliter of cell culture suspension with 5 × 10⁵ cells was centrifuged at 12 800× *g* for 6 min. The resulting pellet was washed with PBS (Invitrogen) and centrifuged at 12 800× *g* for 6 min. The pellet was then resuspended in 100 µL extraction buffer consisting of 10 µL 10× PCR Buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 0.01% gelatin) (GeneCraft, Münster, Germany), 2.5 mM MgCl₂ (GeneCraft), 4.5 µL 10% Nonidet™ P-40 in sterile distilled water (Roche Molecular Biochemicals, Mannheim, Germany), 4.5 µL 10% Tween® 20 in sterile distilled water (Sigma-Aldrich S.r.l., Milano, Italy), 0.6 µL proteinase K (Invitrogen) from a 10 mg/mL solution in sterile distilled water, and 70.4 µL sterile distilled water. The sample was incubated for 1 h at 60°C, followed by 10 min at 95°C. Samples were stored frozen at -20°C.

Negative control. For each experiment, a “negative cell pool” containing 5 × 10⁴ cells from each of eight cell lines representing eight different species, with the exclusion of the species under investigation, was used as negative control of PCR. As an example, the “no dog” pool will contain cells of human, mouse, rat, monkey, cat, rabbit, horse, and hamster origin. The following cell lines are used: Caski, Mab 62B1PC, B104, V-79, Vero, Cf2Th, PG-4 (S+L-), SIRC, and E.Derm (Table 1). In some experiments, additional control lanes with 5 × 10⁵ cells from single selected species were added.

Positive control. In each experiment, a “positive cell pool” was used as positive control of PCR, containing 5 × 10⁴ cells of each of the following nine cell lines from the nine different species for which the primer pairs had been prepared: Caski, AKR/14C, JTC-27, V-79, Vero, Cf2Th, PG-4 (S+L-), SIRC, and E.Derm.

Primers. Oligonucleotide primer pairs, GenBank® information, and amplification product sizes are listed in Table 2. Oligonucleotides were supplied

by TIB Molbiol S.r.l. (Genova, Italy). Cytochrome c oxidase subunit I (cox I) sequence for *Homo sapiens*, *Rattus norvegicus*, *Canis familiaris*, *Oryctolagus cuniculus*, *Mus musculus*, *Felis catus*, *Equus caballus*, *Cercopithecus aethiops* and cytochrome b sequence for *Cricetulus griseus* were employed to select highly specific amplification primers. We used software for multiple sequence alignment analysis to evaluate the polymorphic positions (10).

Amplification. For amplification, the reaction mixture (100 µL) contained 79 µL PCR mixture [5 µL each primer (forward and reverse) (10 pmol/µL), 10 µL 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 0.01% gelatin, 15 mM MgCl₂], 200 µM dNTP (Hybaid, Teddington, UK), 10 µL DMSO (Sigma-Aldrich S.r.l.), 39 µL sterile distilled water], 20 µL sample, and 2.5 U *Taq* DNA polymerase (GeneCraft). Reaction mixtures were covered with 50 µL light mineral oil (Sigma-Aldrich S.r.l.) and heated to 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min. In the last cycle, the extension step was prolonged to 5 min. Amplification was carried out in an OMN-E™ programmable thermal cycler (Hybaid). At least one negative control was incorporated into each experiment. The amplification product (10 µL) was run on 2% agarose gel (Bio-Rad Laboratories S.r.l., Milano, Italy), stained with ethidium bromide (Invitrogen), visualized under UV light, and photographed.

Sensitivity of the PCR Detection

To determine the sensitivity of the PCR amplification, DNA extracts from cultures of 1 × 10⁵ cell lines were diluted 1/5, 1/12.5, 1/25, 1/125, and 1/625 with sterile distilled water, down to an amount of DNA corresponding to 160 cells, and tested with the respective species-specific primer pairs. The following cell lines were used: MPP89, L929, GS-9L, V-79, Vero, Cf2Th, PG-4 (S+L-), SIRC, and E.Derm.

To verify the ability of the assay to detect interspecies cross-contamination, cells from a rat cell culture (RBL-1) were mixed with different propor-

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Table 1. List of the Cell Lines Tested

Cell Line Name	Species	Identified by Isoenzyme Analysis ^a	Strain/Ethnic Group	Sex	Age	Cell Type
CaSki	human	AST, MPI	Caucasian	female	40 years	cervix carcinoma, epidermoid
HCT-15	human	AST, MPI	Black	female	31 years	colon adenocarcinoma
HeLa S3	human	AST, G6PD, MD, NP, Pep B				cervix carcinoma, epitheloid
IM-9	human	AST, MPI				B-lymphoblast
MCF7-432	human	G6PD, NP	Caucasian	female	69 years	breast adenocarcinoma
ME-180	human	AST, G6PD, Pep B	Caucasian	female	66 years	cervix carcinoma, epidermoid
MPP89	human	AST, MPI	Caucasian	male	67 years	mesothelioma
SW1353	human	AST, MPI	Caucasian	female	72 years	bone chondrosarcoma
THP-1	human	G6PD, NP		male	1 year	leukemia, acute monocytic
U-937	human	AST, NP, Pep B	Caucasian	male	37 years	pleural effusion, lymphoma, histiocytic
3T3	mouse	G6PD, MD, NP	Swiss albino		embryo	fibroblasts
AKR/14C	mouse	MD, NP	AKR/J			thymocytes
B16-F10	mouse	NP, Pep B	C57BL/6			melanoma
Hepa 1-clc7	mouse	MD, Pep B	C57L/J			hepatoma
L929	mouse	AST, G6PD, MD, NP, Pep B	C3H/An	male	100 days	connective tissue
Mab 62B1PC	mouse	MD, MPI	BALB/c			hybridoma
Mab 8A-PC	mouse	MD, MPI	BALB/c			hybridoma
P19	mouse	MD, Pep B	C3H.He		embryo	teratocarcinoma
B104	rat	MD, NP	BDIX		adult	brain neuroblastoma
C6	rat	AST, MD, NP				glial tumor
FAO	rat	MD, NP				hepatoma
GS-9L	rat	AST, MD				glioma
JTC-27	rat	MD, NP				liver hepatoma
RBL-1	rat	MD, NP	Wistar			leukemia, basophilic
CHO	Chinese hamster	AST, MD		female		ovary
CHO-SSR1	Chinese hamster	MD, NP		female	adult	ovary, transfected
V-79	Chinese hamster	MD, NP		male		lung fibroblasts
COS-7	African Green monkey	MD, NP				kidney, SV40 transformed
Vero	African Green monkey	AST, MD, NP, Pep B			adult	kidney
Cf2Th	dog	G6PD, MD				thymus
DH82	dog	AST, G6PD, Pep B	Golden retriever	male	10 years	monocyte-macrophage
PG-4 (S+L-)	cat	G6PD, MD			fetal	brain, M-MSV transformed
S+L-CAT2	cat	AST, G6PD, LD				kidney, MSV transformed
SIRC	rabbit	AST, G6PD, Pep B				cornea
E. Derm	horse	N P		female	4 years	dermis

^aAST, aspartase aminotransferase; G6PD, glucose-6-phosphate dehydrogenase; LD, lactate dehydrogenase; MD, malate dehydrogenase; MPI, mannose phosphate isomerase; NP, nucleoside phosphorylase; Pep B, peptidase B

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Table 2. Species-Specific Primers

Species	Gene GenBank Accession No.	Position	Direction	Sequence	Size of Amplified Product (bp)
Human	cox I J01415	5967–5988	for: 5'	TTCGGCGCATGAGCTGGAGTCC	228
		6173–6194	rev: 5'	TATGCGGGGAAACGCCATATCG	
Mouse	cox I J01420	5895–5916	for: 5'	ATTACAGCCGTA CTGCTCCTAT	150
		6023–6044	rev: 5'	CCCAAAGAATCAGAACAGATGC	
Rat	cox I NC001665	6022–6043	for: 5'	CGGCCACCCAGAAGTGTACATC	196
		6196–6217	rev: 5'	GGCTCGGGTGTCTACATCTAGG	
Chinese hamster	Cytochrome b AB033693	196–216	for: 5'	GTGACCCATATCTGCCGAGAT	293
		468–488	rev: 5'	CATTCTACTAGGGTGGTGCCC	
African Green monkey	cox I AF312703	320–339	for: 5'	CCTCTTTCCTGCTGCTAATG	222
		522–541	rev: 5'	TTTGATACTGGGATATGGCG	
Dog	cox I U96639	5466–5487	for: 5'	GAAGTAGGTCAGCCCGGTACTT	153
		5597–5618	rev: 5'	CGGAGCACCAATTATTAACGGC	
Cat	cox I NC001700	7413–7434	for: 5'	TTCTCAGGATATACCCCTTGACA	180
		7571–7592	rev: 5'	GAAAGAGCCCATTGAGGAAATC	
Rabbit	cox I NC001913	5587–5608	for: 5'	CGGGAAGTGGCTTGTCCCCCTG	151
		5716–5737	rev: 5'	AACAGTTTCAGCCAGTCCCCGCC	
Horse	cox I NC001640	5456–5475	for: 5'	CCCTAAGCCTCCTAATCCGT	235
		5671–5690	rev: 5'	AGGAATGATGGGGGAAGTAA	

tions (0%, 1%, 2%, 5%, 10%, 25%, 50%, and 100%) of cells from an African Green monkey cell line (VERO): DNA extracts of 1×10^5 cells from each mixture were then tested with the monkey-specific primer pairs.

RESULTS

PCR Amplification with Species-Specific Primer Pairs

Human cell lines. Ten human cell lines were used for testing the specificity of primers designed to recognize a region of human cox I gene (Tables 1 and 2). Specific amplification was detected in all human cell lines and in the positive control, while no amplification was detected in the negative controls. Figure 1B shows a representative result.

Mouse cell lines. DNA from eight murine cell lines was analyzed by PCR using primers specific for the mouse cox I gene region. The murine origin was confirmed for all eight cell lines tested (Figure 1C shows a representative re-

sult), while no amplification was detected in negative controls. The primer specificity was confirmed by PCR amplification of DNA from the positive cell pool and the “no mouse negative cell pool” of cell lines (Figure 1A).

Rat cell lines. Six cell lines were tested with specific DNA PCR amplification of a fragment from the rat cox I gene region. The rat origin was confirmed for all the six cell lines (Figure 1D shows results relative to four cell lines). Amplification was detected also in the positive cell pool (Figure 1A), while no amplification was detected in the negative controls (Figure 1, A and D).

Chinese hamster cell lines. Three hamster cell lines were used for testing the specificity of primers specific for the Chinese hamster cytochrome b gene. Specific amplification was detected in all the hamster cell lines and in the cell pool used as positive control. No amplification was detected in the negative controls (Figure 1E).

African Green monkey cell lines. Two monkey cell lines were used for testing the specificity of primers de-

signed to recognize a fragment of the African Green monkey cox I gene. Specific amplification was detected in both the cell lines and in the cell pool used as positive control. No amplification was detected in the negative controls (Figure 1F).

Dog cell lines. DNA from two cell lines was analyzed by PCR with a pair of primers specific for the dog cox I region. Specific amplification was detected in both cell lines and in the positive control. No amplification was seen in the cell pool used as negative control (Figure 1, A and G).

Cat cell lines. DNA from two cell lines was analyzed by PCR with a pair of primers that amplify a fragment from the cat cox I region. Specific amplification was detected in both cell lines and in the positive control, while no amplification was detected in the negative control (Figure 1, A and G).

Rabbit cell lines. DNA from SIRC cell line was analyzed by PCR with a pair of primers specific for the rabbit cox I region. Specific amplification was detected in the cell line and in the cell

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pool used as positive control. No amplification was detected in the cell pool used as negative control (Figure 1H).

Horse cell lines. DNA from the E.Derm cell line, previously tested with four isoenzymes (LD, MD, NP, and Pep B) but confirmed only with NP, was analyzed by PCR with a pair of primers that amplify a fragment from the horse cox I region. Specific amplification was detected in the cell line and in the positive control, while no amplification was detected in the negative controls (Figure 1, A and I).

Sensitivity of the PCR Detection

The detection limits for species identification were determined by PCR amplification, as described in Materials and Methods. The lower limit of detection was determined to be about 4×10^3 cells for Chinese hamster, 160 cells for African Green monkey, and 800 cells for the other species tested (Figure 2).

The ability of the assay to detect interspecies cross-contamination was verified by an experiment where rat cells were mixed with different proportions of monkey cells and tested by PCR with the monkey-specific primer pairs. The specific band is evident in all lanes where the monkey cell line is present, down to the lower concentration tested (1%), and no band is detected in the negative control (100% rat) (data not shown).

DISCUSSION

A PCR-based method for defining or confirming the species of origin for human and animal cell lines and for detecting interspecies cross-contamination and misidentification is presented in this paper. Specific primer pairs have been designed for either cox I gene or cytochrome b gene of nine different species (human, cat, dog, mouse, rat, horse, rabbit, African Green monkey, and Chinese hamster) among the most widely used in research laboratories (Table 2).

A panel of 35 cell lines was tested (Table 1) whose species of origin had been previously confirmed by isoenzyme biochemical analysis. In all the experiments, the cell lines, when tested

with the relevant primer pair, showed a band of amplification corresponding to the expected size of the fragment amplified, while no amplification was seen when primer pairs specific for different species were used.

The sensitivity of the assay was determined for all the species tested by dilution assays (Figure 2): a distinct band is detectable at a dilution ranging between 4×10^3 (hamster) and 1.6×10^2 cells (monkey). In a cross-contamination assay, a band of amplification was

detected in the presence of down to 1% of contaminating cells (data not shown). On the basis of these results, positive and negative cell pools have been prepared as positive and negative controls.

When the method described in this paper is compared to the isoenzyme biochemical analysis, some considerations can be done. The isoenzyme analysis is considered the most reliable test for identification of species of cell lines and is widely used by the cell line repositories around the world. The

Figure 1. PCR amplification with species-specific primer pairs. Amplified fragments (see Table 2 for size and respective gene region) were detected by ethidium bromide staining after agarose gel electrophoresis. Lane 1, molecular weight marker (phi X 174/*Hae*III DNA, 1 µg; Invitrogen). (A) PCR amplification of DNA extracted from negative and positive cell pools. Lanes 2, 4, 6, 8, and 10, negative cell pools (dog, cat, horse, mouse, and rat, respectively); lanes 3, 5, 7, 9, 11, positive cell pools (dog, cat, horse, mouse, and rat). (B) Human-specific PCR. Lanes 2–5 and lanes 9–12, HeLa S3, MCF7-432, ME-180, THP-1, U-937, CaSki, SW1353, and IM-9; lanes 6 and 13, negative cell pool; lane 7, positive cell pool; lane 8, African Green monkey COS-7. (C) Mouse-specific PCR. Lanes 2–5, AKR/14C, Mab 8A-PC, L929, and 3T3; lane 6, rat C6; lane 7, human HCT-15; lane 8, negative cell pool. (D) Rat-specific PCR. Lanes 2–5, B104, JTC-27, FAO, and C6; lane 6, mouse 3T3; lane 7, human U-937; lane 8, negative cell pool. (E) Chinese hamster-specific PCR. Lanes 2–4, V-79, CHO, and CHO-SSR1; lane 5, mouse Mab 8A-PC; lane 6, human U-937; lane 7, negative cell pool; lane 8, positive cell pool. (F) African Green monkey-specific PCR. Lanes 2 and 3, Vero and COS-7; lane 4, negative cell pool; lane 5, positive cell pool; lane 6, human HeLa S3; lane 7, mouse L929. (G) Dog- and cat-specific PCR. Lane 2, DH82; lane 3, Cf2Th; lane 4, dog negative cell pool; lane 6, S+L-CAT2; lane 7, PG-4 (S+L-); lane 8, cat negative cell pool. (H) Rabbit-specific PCR. Lane 2, negative cell pool; lane 3, positive cell pool; lane 4, SIRC; lane 5, distilled water. (I) Horse-specific PCR. Lane 2, E.Derm; lane 3, mouse Mab 8A-PC; lane 4, rat JTC-27; lane 5, human U-937; lane 6, negative cell pool.

analysis of a cell line with 3–5 isoenzyme substrates can identify or confirm the species of origin of the cell line and can detect and identify interspecies cross-contamination, provided the percentage of the contaminating cells is over 25% (15) or 11% (25). On the other hand, it requires specific equipment and expensive reagents, and very few research laboratories regularly perform the test on their cell lines for a routine checking. Furthermore, the isoenzyme assay does not always allow one to confirm the species of a cell line. For example, for the equine E.derm cell line, only one enzyme (NP) gave a result compatible with the expected origin (Table 2).

The PCR has become a routine technique in a large majority of the biomedical research laboratories, and the ap-

plication of this technique to the authentication of the cell lines implies neither dedicated equipment nor expensive reagents.

The present assay allows one to confirm the species of origin of a cell line by a single PCR amplification, and a cross contamination by a defined species can be detected by a single assay with a sensitivity ranging between 1% and 4% of contaminating cells. Furthermore, the equine origin of the cell line E.derm could be easily confirmed by this assay.

Analysis of the catalogs of the main culture collections worldwide shows that the cell lines of human, mouse, and rat origin cover 77%–92% of the material available. It can be postulated that these are also the species mostly used

Figure 2. Sensitivity of the PCR amplification. Amplified fragments (see Table 2 for size and respective gene region) were detected by ethidium bromide staining after agarose gel electrophoresis. Lane 1, phi X 174/*Hae*III DNA size standard; lanes 2–7, serial dilutions of cell DNA in distilled water; lane 2, DNA extracted from 1×10^5 cells (non-diluted); lane 3, extract diluted 1/5; lane 4, 1/12.5; lane 5, 1/25; lane 6, 1/125; lane 7, 1/625; lane 8, distilled water.

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in research laboratories. When interspecies cross-contamination is suspected, the cell line can be checked with the relevant primer pairs, depending on the species of cell lines mostly used in the laboratory. Thus, a simple assay with 3–4 primer pairs, designed to serve as a routine check of the origin of the cell lines, could help in rapidly answering the question whether the cell culture has been overgrown by a cell line of different species or a mislabeling has happened. The use of this test in conjunction with assays able to detect intraspecies contamination (DNA profiling systems, such as multiplex STR profiling) (21) will give an acceptable confidence on the quality of the cell lines used in most laboratories.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. Nadia Ricchetti for her secretarial help. The work has been funded by the Italian Ministry of University (MURST) in the frame of a project on new assays for cell line authentication.

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Received 31 July 2001; accepted 9 October 2001.

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