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LIFE BEYOND LIFE – An Easy Way to Derive Lung Fibroblasts from Cadavers*

ABSTRACT: Several protocols have illustrated the possibility of deriving cells, such as fibroblasts, from different organs. These techniques generally concern organs sampled from living persons, but have already been described for cadavers, especially concerning the skin and tendons. We present, for the first time, an easy way to derive pulmonary fibroblasts from a lung tissue sampled from a cadaver and directly culture plated. The fibroblast output was checked daily. We obtained lung fibroblasts from 3 (60%) cadavers and 2 (100%) living persons. The fibroblast output took about 3 days for cells from living persons and took up to 39 days for those from cadavers. We did not clearly identify any parameters that could explain these differences. Nevertheless, these derived cells had the same features as the source cells, especially in terms of morphology and proliferation, and could potentially be used in different research domains such as forensic or regeneration medicine.

KEYWORDS: forensic science, explant, fibroblast, lung, senescence, forensic autopsy, postmortem delay

Postmortem specimens have been used for fundamental research for many years. Surprisingly, some living cells can be found after death. Indeed, body death is not synonymous with cell death, which occurs gradually and not simultaneously for all parts of the body, or even within the same part. Several authors have described how to harvest and work with living cells from the postmortem tissues at variable times after death (1,2). These studies have been conducted using adult or infant corpses to derive different types of cells, i.e. skin fibroblasts (2), tendon fibroblasts (1,3,4), and bladder cells (5). These specimens can be sampled in different ways from an organ (donor) or an autopsy. We hypothesized that living fibroblasts could be harvested from lungs sampled during an autopsy and then cultivated and expanded. We assessed the feasibility, the maximal time after death and if the cause of death could impact the success of the cell culture outcome. We developed conditions to successfully sample lungs from forensic autopsies to cultivate lung fibroblasts. We collected samples from forensic autopsies which are commonly performed in our hospital without requiring next of kin authorization.

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Materials and Methods

Population

Table 1 summarizes the characteristics of the study population. We obtained samples from four male subjects and one female subjects aged between 18 and 66 years, autopsied at the Forensic Institute of Montpellier (France). Three of these subjects had died of an asphyxial syndrome, secondary to hanging for two of them, but secondary to an undetermined event for the third one—probably the result of massive toxic absorption, but unfortunately no toxicological analysis had been performed. Another subject had died of cerebral damage caused by a direct gunshot wound, while the last one probably succumbed to cardiovascular deficiency.

The postmortem delay was defined as the number of hours between the estimated time of death and the discovery of the corpse. Different assessment parameters were used, such as the corpse and environmental temperature, rigor and livor mortis that ranged from 0 to 48 h.

The period between the discovery of the corpse and the onset of cell culture ranged from 20 h to 4 days. Meanwhile, the corpses were stored at 4°C before autopsy.

Lung slices from two living persons, who had given prior written informed consent, was removed during peripheral nodule surgery and used as controls (78-year-old female and 66-year old male). These slices were collected by the surgeon within the removed lobe, as far as possible from the nodule.

Cellular Studies

Explant Method—As previously described (6,7), we performed fibroblast derivation from about 0.5 cm^3 of lung. Briefly, after lung removal during autopsy, a piece of distal left upper

TABLE 1—*Patient characteristics and fibroblast release time.*

	Sex	Age (Years)	Cause of Death	Manner of Death	PMD (h)	DCD (h)	Fibroblast Obtained Y/N	Release Time (Days)
1	M	47	AS (Hanging)	Suicide	<12	60	Y	17
2	M	66	CV deficiency?	Natural	36–48	90	N	NC
3	F	33	AS (Hanging)	Suicide	<12	40	N	NC
4	M	35	AS (Toxic?)	Accidental?	<12	96	Y	39
5	M	18	Cerebral damage (gunshot)	Suicide	O	20	Y	3
6	M	66	NC		NC	NC	Y	4
7	F	78	NC		NC	NC	Y	3

Cases 1–5 concerned cadavers, while cases 6 and 7 concerned living persons. Manner of death is commonly used to classify the death as suicide, natural, accidental or homicide. PMD, Postmortem delay corresponds to the time period between the death and the discovery of the corpse; DCD, Discovery culture delay is the period of time between the discovery of the corpse and culture seeding. The delay between the culture onset and fibroblast migration out of the explant is called the "release time" in the table. AS, Asphyxial syndrome; CV, Cardiovascular; NC, Not concerned; Y, Yes; N, No.

lobe was taken and cut into six slices. These slices were incubated in a mixture of physiological serum and 10% streptomycin-penicillin for 1 h under agitation. The slices were then placed in 6-well plates under sterile conditions. After about 30 min to 1 h, 300 μL of RPMI medium supplemented with 10% fetal calf serum and a mix of previously described antibiotics were added. Lung tissues were incubated at 37°C and the medium was changed twice weekly. After 3 days of incubation, the fibroblast output from the lung explants was checked every other day. Outgrowth cells were subcultured in a T-25 tissue culture flask (corresponding to passage 0) with the same mixed medium, until they reached 80–100% confluence. Then the fibroblasts were passaged after 5-to-10 min incubation in a 0.1% trypsin/1% EDTA solution. The same medium was used for cell propagation.

Foreskin Fibroblast Culture—A generous gift of a foreskin fibroblast aliquot was cultured in the same medium and under the same conditions as our explant-derived fibroblasts.

Immunofluorescence Assay—At each fibroblast passage, a cell aliquot was spread on a cover slip. After cell attachment (about 24 h), they were fixed in a 10% formalin solution for 15 min at room temperature, washed twice in phosphate buffered saline (PBS) and also kept in PBS at 4°C until the immunofluorescence assay.

Cells on a cover slip were permeabilized in PBS-0.1% Triton solution for 10 min, washed twice for 5 min in a PBS-0.01% Triton solution. Then they were blocked by adding a solution of 1% bovine serum albumin (BSA) diluted in PBS for 1 h at room temperature. Primary antibody was added in the same solution at the appropriate concentration and left overnight at 4°C. After antibody removal, cells were washed 3 times for 5 min in a PBS-0.01% Triton solution. Secondary antibodies were used at 1/2000 dilution in a PBS-1% BSA solution and applied on cells for 1 h at room temperature. Three washes with PBS were then performed. Cells were subsequently incubated with 1 μg/mL of a DAPI solution for 5 min, washed twice in PBS and mounted with Vectashield© (Abcam, Cambridge, U.K.).

Goat anti-Vimentin antibody was used at 1/50 (sc-7557, Santa Cruz Biotechnology, Dallas, TX), rabbit anti-Ki67 antibody at 1/200 (ab 15580, Abcam), both respectively employed with an Alexa Fluor©-conjugated donkey anti-goat and anti-rabbit secondary antibodies (A-21447 and A-31572, Invitrogen, Carlsbad, CA).

Genetic Studies

DNA Extraction—We performed the DNA analysis to ensure that fibroblasts obtained from the lung explants were not contaminated. DNA was extracted from three different sources: (i) from a source lung tissue, (ii) from fibroblasts isolated from the corresponding explant, and (iii) from the oral mucosal cells obtained from a control volunteer. Lung tissues were incubated in lysis buffer for 30 min at room temperature and the obtained solutions were filtered. Fibroblasts of one well were gathered, centrifuged at 3000 g for 5 min at 4°C and the pellets were homogenized in lysis buffer. Mouth mucosal cells were collected with a swab.

DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Multiplex Polymerase Chain Reaction—A multiplex polymerase chain reaction (PCR) using the AmpFLSTR© Identifiler© PCR Amplification Kit (Life Technologies, Carlsbad, CA) was performed with 1 ng of the extracted DNA to amplify and label 16 different loci (15 microsatellite marker and one gender-determining marker) with four different fluorescent dyes. After migration on a 16-capillary *3130xl* Genetic Analyzer (Applied Biosystems, Foster City, CA), the results were analyzed using GeneMapper 4.0 (Life Technologies).

Results

Fibroblast Isolation from Explants

Day 0 corresponded to the cell seeding step in all assays. Fibroblasts from lung slices acquired from living persons during surgery were obtained within 3–4 days (Table 1).

Autopsy-harvested fibroblasts were obtained from three patients (60%). The fibroblast output required nearly 17 days (Table 1), except for patient no. 5. Figure 1 shows fibroblasts derived from a lung explants sampled at autopsy (case no. 1) and at surgery (case no. 7). No morphological differences were noted.

Immunofluorescence Assay

We performed different protein labeling procedures to ensure that the autopsied fibroblasts displayed features similar to those from living persons.

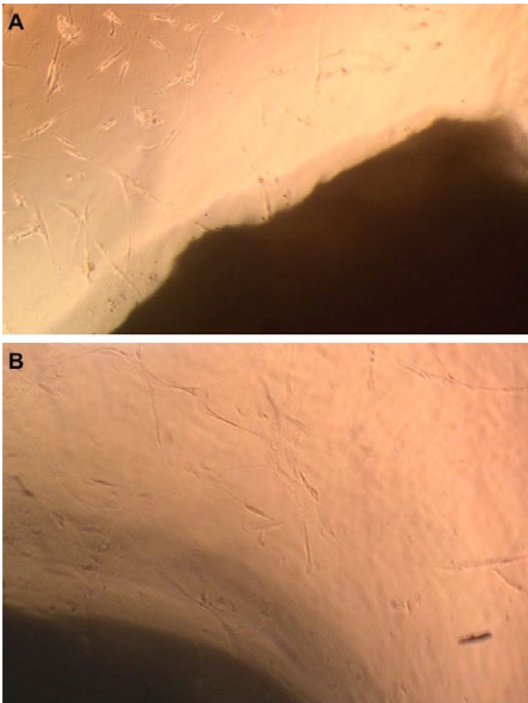


FIG. 1—*Fibroblast isolation. Fibroblasts migrating from an explant extracted from patient no. 1 (A) and from patient no. 6 (B) at D17 and D3, respectively, after explant plating. [Color figure can be viewed at wileyonlinelibrary.com]*

Figure 2 shows the fibroblasts from patients no. 1 and no. 7 labeled with anti-Ki67 and anti-Vimentin antibodies. The same staining was performed with foreskin fibroblasts as control. No qualitative or quantitative differences were observed.

DNA Analysis

DNA extracted from lung explants and derived fibroblasts perfectly matched and differed from our control, as shown in Fig. 3.

Discussion

In this study, the explant technique was used to isolate fibroblasts from the postmortem tissues. This technique has been widely implemented to derive fibroblasts (1,2), usually from the skin or tendon tissues. Many studies have described the lung explant technique to isolate the fibroblasts, but always using biopsies from living persons (7).

In this study, for the first time, we describe an easy way to derive lung fibroblasts from the postmortem tissues. We performed immunochemical analyses to characterize the fibroblasts extracted from cadavers and compare them with those from living persons.

We performed Vimentin labeling, which is widely used for fibroblast identification (8), and then verified the proliferation capacity using Ki67 labeling (9). The fibroblasts thus obtained seemed to display the same features as those obtained from living persons.

In our study, we observed the fibroblasts migrating out of explants at different times. Regarding biopsies sampled in living people, fibroblasts migrated from explants in 3 or 4 days, as previously observed (6,7). It seemed that neither the donor age nor the time between sampling and culturing modified this migration time. Conversely, the fibroblast derivation from cadaver samples sometimes required up to 39 days. Several parameters, such as the postmortem delay (PMD), could explain this difference.

Patient no. 5 died in the hospital, without any delay between the death and the corpse storage at 4°C, and fibroblasts were obtained after 3 days, as also observed with explants from living people. On the contrary, the fibroblasts obtained from patients no. 1 and no. 4, who died 12 h prior to the discovery of the corpse, were obtained 17 and 39 days after culture plating, respectively. We hypothesize that an excessively long PMD could limit or even block fibroblast release, as observed in case no. 2.

The time between the discovery of the corpse and the culture may also affect the culture yield. Indeed, after the declaration of death, if an autopsy was required, corpses were stored at 4°C until autopsy. This delay probably influenced fibroblast release since the sample from patient no. 4 showed fibroblast output within 39 days after 96 h storage at 4°C, whereas samples from patient no. 1 required 17 days with less storage time at 4°C. We sought to determine why we could not derive fibroblasts from samples processed earlier after the corpse discovery.

The literature states age-at-death as another important factor. In our study, age-at-death did not seem to play a role in fibroblast output. The cause of death could be another factor, but it was out of line with our observations.

Unfortunately, we studied a very small number of cases and we consider that it would be essential to diversify recruitment in terms of age, the PMD and cause of death to verify their potential impacts on fibroblast release.

Indeed, accurate determination of parameters likely to influence the output latency of fibroblasts may have potential forensic applications. Such parameters could significantly help determine the PMD or age-at-death, i.e. crucial indicators in forensic medicine.

Fibroblasts are widely used to study senescence (10). As previously shown by Hayflick, fibroblasts can potentially divide about 50 times before becoming senescent (11,12)—this is called replicative senescence. Some authors support the idea that the proliferation rate could depend on the biological age of the donor (13,14), but this observation remains controversial (15). In forensic cases, the corpse gender and age frequently need to be determined. Identification is a major concern in forensic medicine, and forensic anthropology was created for this purpose. Age is the most difficult but also the most informative indicator in determining the biological profile. Many techniques have been described to determine the age-at-death (16,17) using teeth (18), bones, such as pubic symphisis (19), or both (20), as well as ribs (21). Age-at-death estimation techniques tend to be less reliable for people beyond the age of 65.

Using the proliferation rate of lung biopsy derived fibroblasts to estimate the age-at-death could be an interesting, non-invasive alternative, especially for the elderly.

It is known that after a number of replication cycles, which could depend on the donor's age, fibroblasts enter senescence (11,12). This switch is due to sequential cellular events including telomere shortening (22). Interestingly, some authors have shown that the telomere length could depend on the donor's age and even gender (22,23), while some authors even consider that this parameter is an individual characteristic (24).

Telomere shortening triggers the activation of different genes such as *p53, p21* (25), or *p16ink4a* (26). Senescent fibroblasts

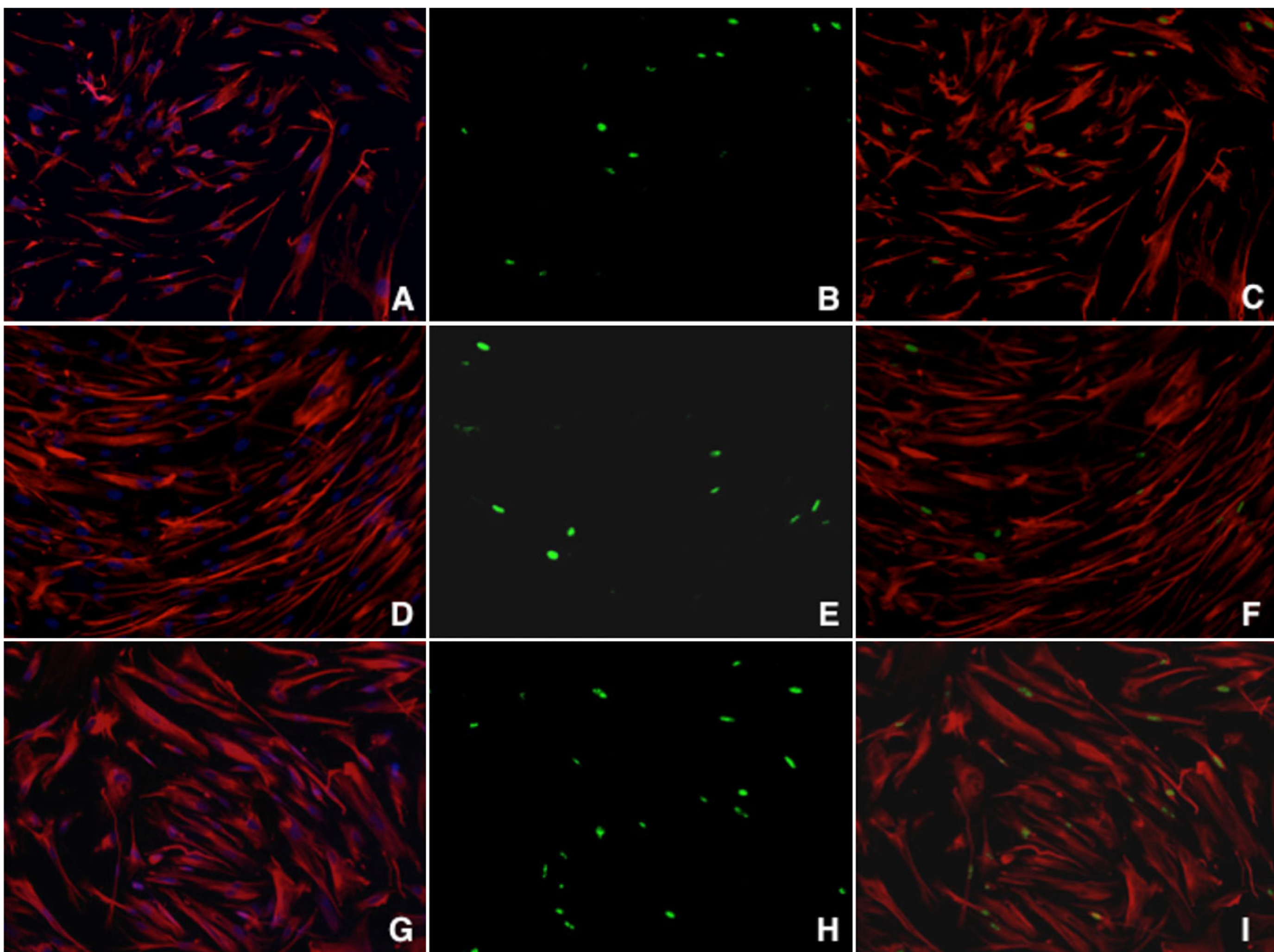


FIG. 2—*Fibroblast characterization. Fibroblasts isolated from an explant from the first patient no. 1 (A–C), patient no. 7 (D–F) and from foreskin fibroblasts (G–I). Fibroblasts stained with anti-Vimentin antibody (red) and DAPI (blue), (A, D, G) and stained with anti-Ki67 antibody (green) (B, E, H). (C, F, I) show merged images after double staining with anti-Vimentin (red) and anti-Ki67 (green) antibodies. [Color figure can be viewed at wileyonlinelibrary.com]*

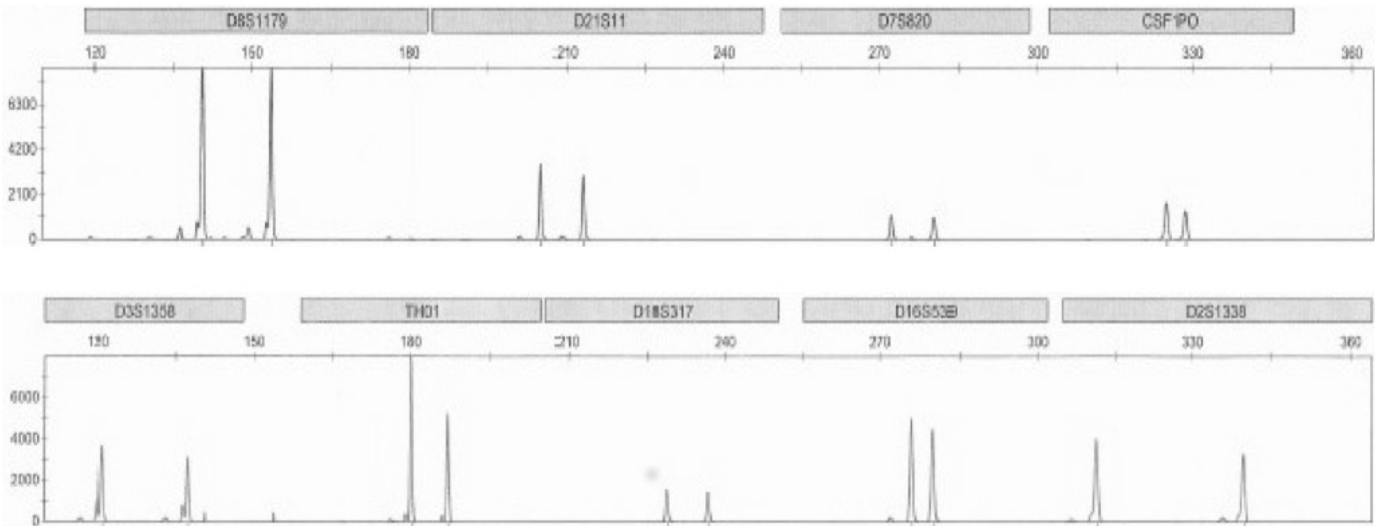
can thus be characterized by monitoring the expression of these genes (25–27).

When combined with proliferation rate and telomere length estimation, senescent phenotype characterization could provide an effective tool to determine age-at-death in a broad age range group. Unfortunately, senescent switching is not entirely related to the replication rate. Indeed, different "ways-to-senescence" have been described and it should be kept in mind that, in addition to age, different factors could play a role in senescence switching. For example, the smoking status has been shown to potentially favor senescence (28). Therefore, senescence characterization should not be used alone and must be combined with other age-at-death determination tools.

Since they present the same features as fibroblasts isolated from living persons, fibroblasts obtained with postmortem biopsies could be used in translational research.

For example, induced pluripotent stem cell (iPS) technology was described few years ago (29). This technique is used to reprogram any somatic cell into a pluripotent stem cell which has the same proprieties as an embryonic stem cell and therefore can differentiate into any cell type from the three germ layers (29). This was first described in studies on mice by Shinya Yamanaka's team (30) in Kyoto, and then in humans by the same team (31). Although other cell types such as human renal epithelial cells (32) have also been used, fibroblasts are the most commonly used cells (29). Fibroblasts are usually extracted from the skin biopsy explants from living persons (6,31). Necropsy derived fibroblasts have features similar to those obtained from living persons. As Basma et al. (33) showed that iPS could be generated from lung fibroblasts, it should be possible to use cadaver samples to generate iPS—prolonging life after death thus becomes a reality for any kind of cell, i.e. organs in the short term and potentially living entities in the longer term.

To conclude, we present a simple method to derive fibroblasts from lung tissues. We report, for the first time, the development of this technique for use with cadaver samples. The fibroblasts obtained thus displayed similar features to those obtained from living persons. As it is easier to obtain samples from forensic cases than from living persons, we intend to perform more in-depth larger scale studies to confirm the potential interest of



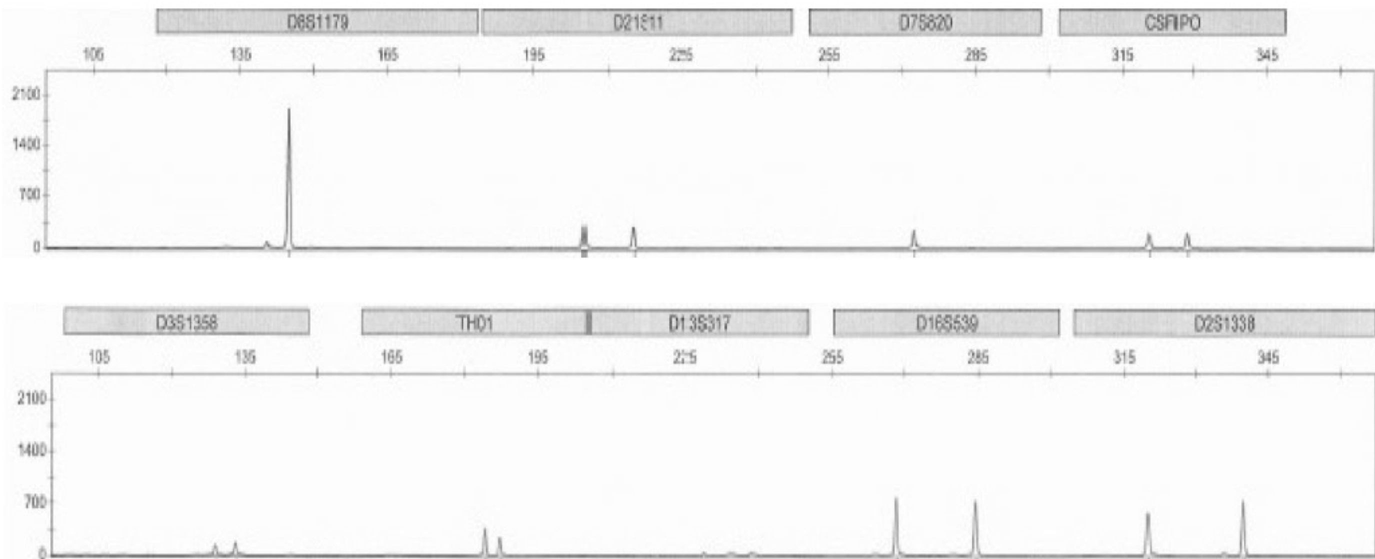


FIG. 3—*DNA profiles. DNA was extracted from three different sources and analyzed by Multiplex PCR. In (A) DNA was extracted oral mucosa cells of fibroblast manipulator, in (B) from fibroblasts isolated from an explant from patient no. 1. DNA was also extracted from a small piece of lung explant tissue (patient no. 1) and showed same profile as (B) (data not shown). The lung tissue and (B) displayed the same profile and differed from (A), confirming that (B) and the lung tissue were from the same person.*

fibroblasts in determining the age-at-death or even PMD in forensic science.

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