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LAB PROTOCOL

A minimally invasive lab protocol for fibroblasts isolation from 1-mm skin punch biopsies in pediatric patients

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Abstract

Skin biopsies provide a valuable source of culturable cells for a broad range of clinical and biomedical research applications. However, conventional methods for establishing primary cell cultures typically require relatively large tissue specimens obtained through invasive procedures that involve intradermal anesthesia, suturing, and subsequent wound care. These factors are of particular concern when working with pediatric populations. To address these limitations, we developed a simplified protocol that reduces patient discomfort by minimizing the punch biopsy diameter to as little as 1 mm. Despite the markedly smaller sample size, this method consistently enables the successful establishment of primary fibroblast cultures, with no complications reported to date. This streamlined approach is compatible with basic cell culture facilities and provides a reliable source of fibroblasts for downstream applications. Overall, this protocol represents a meaningful advancement in both pediatric research and clinical practice.

1. Introduction

Patient-derived cell cultures constitute experimental models that faithfully represent human biology. They are indispensable for elucidating physiological processes, their disruption in disease and for establishing robust molecular diagnostics. However, obtaining suitable patient-derived primary cells remains a major practical challenge in biomedical research, as not all human cell sources are equally accessible or physiologically representative. While peripheral blood mononuclear cells (PBMCs) are easily procured, providing a common source of germline DNA for genetic testing

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or RNA, proteins and metabolites for omic studies, they are generally unsuitable for long-term culture, precluding functional assays. Moreover, their utility is limited when a non-hematopoietic genomic or enzymatic profile is essential. For instance, primary culturable cells are mandatory for the definitive diagnosis of certain inborn errors of metabolism, the accurate detection of low-level somatic mosaicism, and for the establishment of an uncompromised cell lineage in patients with hematological malignancies or in those who have undergone allogeneic or stem cell transplantation, where acquired somatic mutations can entirely confound germline analysis [1]. Furthermore, many biomedical applications require an uncompromised lineage of patient-derived cells with long-term in vitro expansion potential rather than readily available samples. For instance, the functional validation of genetic variants within the patient's native genetic background depends on the availability of such cells. Therefore, in these scenarios, human dermal fibroblasts (HDFs) derived from skin biopsies emerge as the preferred robust primary cell model, offering the necessary genetic stability and cultivability for high-fidelity studies.

Alternative cell models, although more convenient, present important limitations. While immortalized cell lines offer unlimited division and ease of maintenance, their utility in translational studies is severely restricted by accumulated genetic drift and alterations inherent to the immortalization process, which often misrepresent native physiological processes [2]. Similarly, induced pluripotent stem cell (iPSC)-based approaches, although capable of generating disease-relevant cell types [3], remain technically complex, time-consuming, and prone to incomplete differentiation and the retention of epigenetic memory [4,5]. Primary cells, conversely, are sourced directly from the patient, preserving both their native genetic background and crucial epigenetic signals, thereby providing a highly accurate representation of in vivo disease conditions, despite replicative senescence [6]. Consequently, obtaining culturable primary cells via skin biopsy is still a reference standard for high-fidelity biomedical research.

Within this framework, HDFs obtained from skin biopsies stand out as a robust and versatile primary model. They are mesenchymal cells derived from the embryonic mesoderm that reside within the dermal layer of the skin. In culture, HDFs exhibit a characteristic spindle-shaped morphology and express canonical stromal protein markers, including Vimentin (a general mesenchymal intermediate filament), Fibronectin, and TH-1 (CD90) [7]. Recent advances in single-cell transcriptomics have revealed the functional and transcriptional heterogeneity of fibroblasts [7,8], with distinct subtypes exhibiting different marker expression profiles and specialized roles in tissue homeostasis, wound healing, fibrosis, and aging [9–13]. Compared to peripheral blood, where transcriptional activity is dominated by a limited subset of highly expressed genes, fibroblasts exhibit a far more complex transcriptional landscape [14]. This transcriptomic diversity renders fibroblasts particularly valuable for biomarkers discovery and for the functional characterization of gene variants, as they express a broader and more physiologically relevant repertoire of transcripts than blood-derived cells. Nonetheless, as gene expression is tissue-specific, it is essential to verify that the gene of interest is actually expressed in the skin before performing a

biopsy; this can be readily assessed using public databases such as Genotype-Tissue Expression (GTEx) Project [15] or UniProt [16] to ensure the biological relevance of the fibroblast model for the specific study.

Despite the acknowledged value and extended use of primary HDFs, conventional skin biopsy protocols (typically using 3–4 mm punches) raise ethical and practical concerns in research settings. Standard procedures include intradermal anesthesia, post-procedural suturing and wound care. The inherent discomfort, coupled with the potential lack of outcome guarantee, results in an unfavorable risk-to-benefit ratio, particularly in pediatric cohorts [17]. Furthermore, skin biopsies are generally reserved for definitive histopathological evaluation, a requirement for which these larger specimens are necessary. However, the use of large specimens solely for cell culture often leads Institutional Review Boards (IRBs) to question the justification for such an invasive sampling method when no histopathology is intended. This constraint limits the widespread use of HDFs in research. To overcome this critical limitation, minimally invasive 1-mm punch biopsies have recently emerged, primarily for direct molecular or omic analysis [18,19]. Although unsuitable for histopathological evaluation, we demonstrate that these reduced-biopsies provide sufficient material for the successful establishment and expansion of primary HDFs cultures. Thereby, the use of these reduced-biopsies, as a standard for HDFs culture, may broaden the potential scope of diagnostic and research applications of primary HDFs cultures, making the acquisition of high-fidelity cellular collections ethically and practically feasible.

Once the tissue explant (TE) has been obtained, the next critical step involves its processing to cultivate fibroblasts. For this purpose, two well-established strategies are commonly employed: *enzymatic dissociation* and *direct explant culture*. The enzymatic approach typically entails gentle overnight digestion with dispase to separate the epidermis and dermis, followed by approximately one hour of collagenase treatment to degrade the extracellular matrix and release fibroblasts [20]. This method enables the rapid recovery of primary HDFs; however, it is technically demanding, as deviations in enzyme concentration or incubation time may reduce cell viability. Furthermore, the multiple handling steps involved increase the risks of contamination and sample loss. Alternatively, the direct explant culture technique relies on mechanically fragmenting the TE into small pieces and allowing them to adhere to the culture plate through partial desiccation. Fibroblasts then migrate and proliferate from the tissue fragments. This direct explant culture is considerably simpler and less susceptible to contamination. Its main limitations are the longer initial times and the potential failure of the tissue fragments to adhere properly. The latter may prevent the establishment of the culture [21]. However, the successful performance of this approach results in a TE releasing additional fibroblasts upon successive passages.

Building on these approaches, we developed and validated a protocol for the isolation of HDFs from 1-mm TE. This technique not only minimizes invasiveness and patient discomfort, but also aligns experimental procedures with stringent ethical standards, without compromising the quality or reproducibility of the resulting primary cell cultures. To promote its adoption in both clinical and research environments, we further streamlined the direct explant culture method, reducing procedural complexity and failure risks. In conclusion, these refinements have demonstrated 98% culture success rates, strengthening the applicability of primary HDFs models for translational and personalized medicine research. Furthermore, it has already been used in an experimental setting successfully [22].

2. Materials and methods

The protocol described in this peer-reviewed article has been published on protocols.io [23], and is included for printing as S1 File with this article. As stated above, it has also been used in research published in a peer-reviewed journal [22]. A description of equipment needed to perform the 1-mm skin biopsy, as well as cell culture and molecular biology techniques, can be found in the “Reagents and Materials” section.

This protocol was derived from a study reviewed and approved by the Comité de Ética de la Investigación de Cádiz (CEI Cádiz) (Protocol Code: FPS-CMER-2022; Registration No. 110.22) in accordance with the provisions of Spanish Law 14/2007, of July 3, on Biomedical Research and adheres to the General Data Protection Regulation (EU Regulation 2016/679, GDPR). Once the ethics committee approved the significant protocol modification on September 28, 2023, we

took the first skin biopsy from our first control volunteer on October 25, 2023. The last sample included in the study was taken on August 1, 2025. Written informed consent was obtained from all participants prior to their inclusion in the study. By adhering to these internationally recognized guidelines, this protocol seeks to uphold the highest ethical standards in biomedical research while safeguarding the rights, dignity, and well-being of all participants.

3. Expected results

The analyzed cohort is part of a study focused on the functional evaluation of genetic variants of uncertain significance (VUS) in pediatric patients harboring clinical phenotype of RASopathy and their family members. Accordingly, the current reduced-biopsy protocol was specifically designed to be minimally invasive and robustly suitable for this sensitive population. The study included 51 participants of both sexes and across a wide age range, comprising 65% healthy controls, 25% affected patients and 10% of VUS carriers (Fig 1). Despite the inherent clinical and genetic heterogeneity of this cohort, all HDFs cultures were successfully established and demonstrated high viability, validating the robustness and broad applicability of the developed protocol.

Skin biopsies were obtained from the upper right region of the back at subscapular level. This site was selected as it contains the thickest dermis [24] and is subjected to minimal mechanical stress, thereby minimizing post-procedural discomfort. Maintaining a consistent sampling site across individuals is essential to ensure reproducibility, given the presence of distinct fibroblast subtypes and transcriptional heterogeneity in different skin regions.

Across all applications of this method, no instances of infection, suture requirement, excessive bleeding, or specific wound care were observed or reported. Only one intervention required repetition due to sample contamination after overnight storage at 4ºC. Collectively, these findings demonstrate a 98% success rate and underscore the reliability of the protocol.

Two methodological changes have enhanced the risk-to-benefit ratio, thereby increasing its potential for routine clinical and research implementation. The first is the replacement of intradermal for topic anesthesia (e.g., EMLA). This modification is highly pertinent for pediatric and teenage donors, for whom needle phobia associated with routine medical

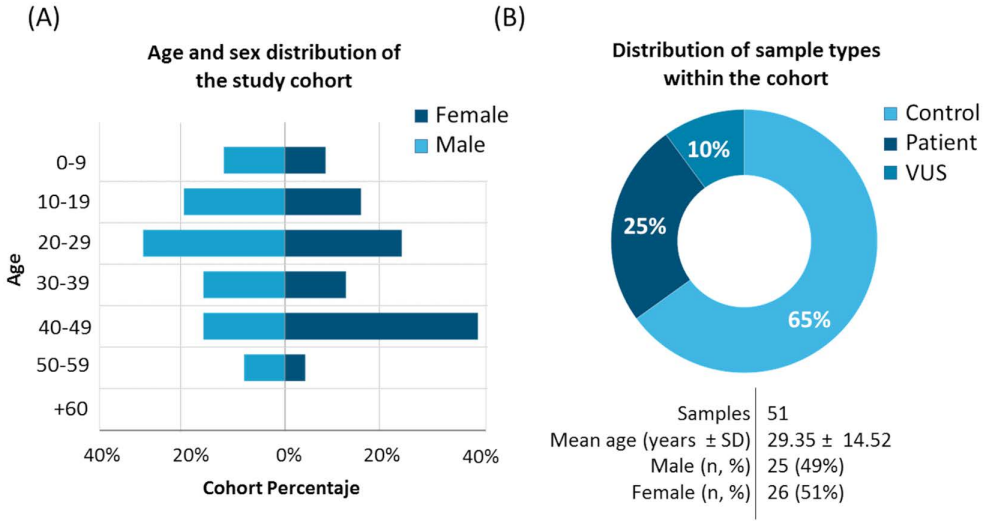

Fig 1. Demographic characteristics of the study cohort. The left graph represents the age distribution of participants by sex. The accompanying table summarizes the number of samples, sex distribution (% male and female), and mean ± SD of age. The right graph represents the health status distribution of participants.

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procedures, such as vaccination or blood collection, is a frequent concern. Eliminating painful intradermal injections minimizes procedural distress, enhancing patient compliance and facilitating recruitment.

The second refinement is the reduction of the biopsy punch size to 1 mm. This effectively addresses a major concern in conventional methods. The smaller punch, with a diameter comparable to a standard blood collection needle, reduces invasiveness and patient discomfort, eliminating the post-procedural sutures and wound management. The resulting reduced wound typically closes spontaneously within hours and is often imperceptible the following day. In contrast, biopsies obtained with 3–4 mm punches require stitches, more extensive wound care, are susceptible to infections, and frequently result in noticeable scarring (Fig 2, left).

Due to its minimal invasiveness, this procedure could be performed during routine follow-up visits by trained physicians at the point of care, reducing patient burden. Moreover, the associated protocol is straightforward, cost-effective, and feasible in any facility with basic cell culture equipment, promoting wide accessibility and scalability for biomedical studies.

Beyond improving the clinical feasibility of skin sampling, the introduction of the 1-mm punch also streamlined the subsequent cell culture process. In our opinion, the clean and uniform excision produced by the punch facilitates efficient adhesion of the TE to the culture surface, as straight and regular edges adhere more efficiently than irregular ones. Moreover, the soft and pliable nature of the dermal tissue, combined with the high surface area-to-volume ratio characteristic of the 1-mm TE, facilitates localized dehydration. This process generates strong capillary and surface tension forces that actively promote the stable adherence and slight deformation (flattening) of the cylindrical explant (Fig 2, right) against the bottom of the culture well, thereby maximizing the total tissue-to-surface contact area and promoting efficient cellular migration. Therefore, it is unnecessary to determine tissue orientation under a microscope during plating. This simplification further reduces handling time and plausibly, risk of contamination, making the protocol feasible in laboratories equipped with only basic cell culture facilities.

The 1-mm TE provided sufficient material for establishing HDFs cultures that reached confluence in a six-well plate within approximately one month, yielding sufficient cell mass for experimental applications or cryogenic preservation. Consistent with previous reports, the yield and proliferative capacity of the isolated HDFs in our cohort were influenced by inherent donor characteristics such as age and health status and individual genetic background [25]. While the expected variability was observed, such as a higher proliferative capacity in younger donors compared to older ones, these

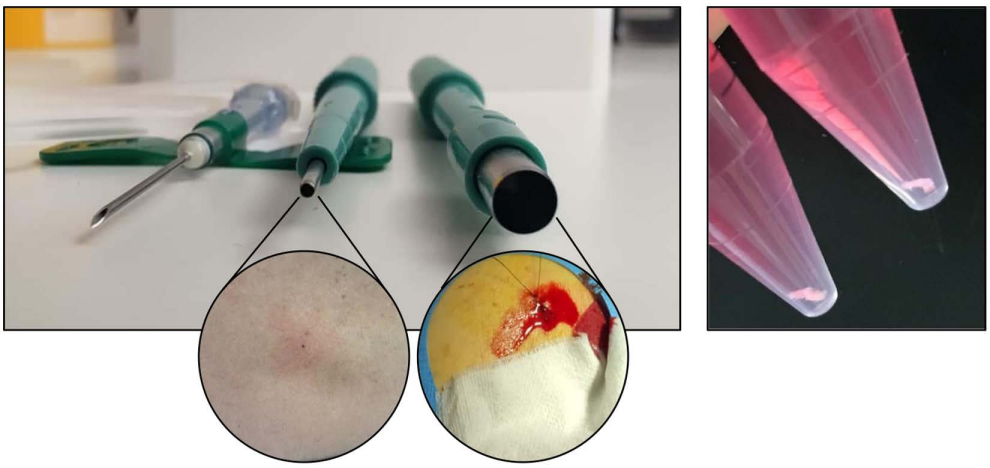

Fig 2. Comparison of biopsy punch size and handling of tissue explants. Left: Illustrative image showing the relative diameters of a standard blood collection needle, a 1-mm punch and a 4-mm punch. Corresponding skin wounds are visible at the bottom. Right: 15-ml conical Falcon tubes containing 1-mm TE prepared for culture.

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fluctuations remained within known biological ranges and did not induce notable deviations in the isolation and culture protocol execution. The robustness of the method, considering the clinical and genetic heterogeneity of the 51 donors, lends further credence to its validity.

To further validate the functionality and purity of the derived HDFs cultures, phenotypic characterization was performed through the analysis of growth kinetics, expression of fibroblast-specific markers by immunofluorescence, and profiling of cell populations across passages by flow-cytometry (Fig 3). These results are further detailed in the following sections.

3.1 Growth curves

Given the reduction in TE size to 1 mm, it was essential to verify whether the resulting HDFs cultures provided sufficient cells for downstream molecular biology applications and to assess whether they retained robust proliferative capacity and viability. To address these questions, we evaluated the cumulative population doubling level (PDL) and the growth kinetics of the derived HDFs cultures.

We estimated the cumulative PDL based on standard flask capacities and culture progression. Each HDFs line typically underwent four expansion cycles following the sequence [TE → 1xT25 → 1xT75 → 3xT75 → cryopreservation]. Considering an estimated confluence density of $2.5 \cdot 10^5$ cells per explant and approximately $5 \cdot 10^6$ cells per T75 flask, the theoretical cumulative yield across these passages would be at least $6 \cdot 10^7$ cells. Using the standard formula for population

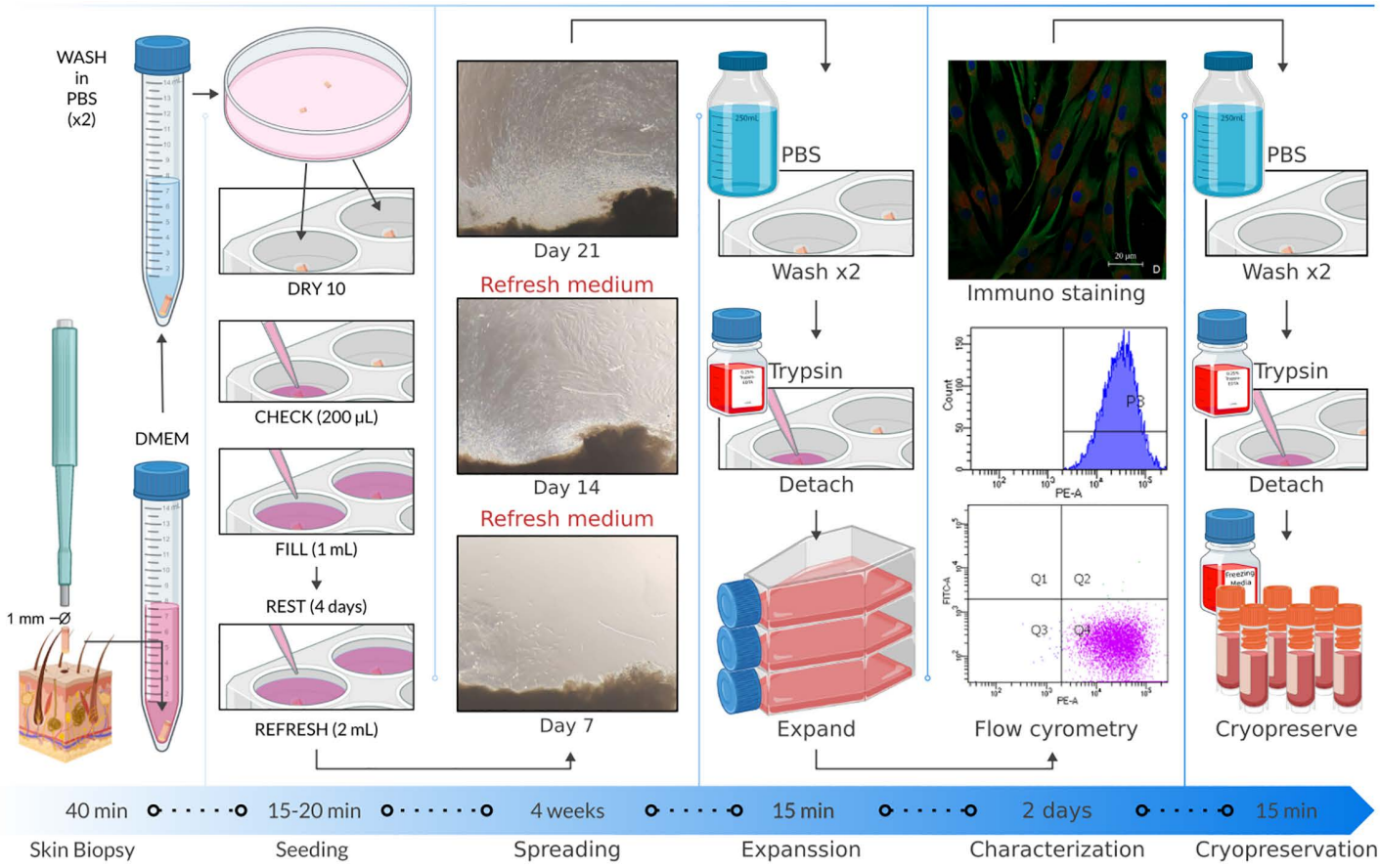

Fig 3. Summary of the protocol, including the four main steps: 1) biopsy extraction, 2) seeding and spreading, 3) expansion, and 4) cryopreservation; followed by the characterization of primary fibroblast.

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doublings ($N_H/N_I = 2^X$, where N_I is the inoculum number, N_H the harvested cell number, and x the population doubling level), this corresponds to a minimum cumulative PDL of approximately eight. These estimates indicate that even from a 1-mm skin biopsy, sufficient fibroblasts may be expanded for multiple experimental assays and/or cryogenic storage. Cultured fibroblasts experience progressive gene expression changes with increasing passage number. Therefore, some considerations should be taken into account before adopting a protocol requiring 4 passages, depending on the intended application. Specifically, studies aimed at characterizing cellular identity and population heterogeneity by scRNA-seq, or at epigenomic profiling of the original tissue, should be performed on ex vivo tissue or at passage 1 at the most, as culture-induced transcriptional convergence and epigenetic drift have been shown to occur even at early passages [26,27]. However, for the majority of molecular biology applications, passage 4 remains within an acceptable range, as the main documented confounding factors — widespread replicative senescence and cumulative epigenetic drift — have not yet manifested to a substantial degree at this stage, according to the available evidence [26,28].To experimentally confirm fibroblast growth and viability, third-passage fibroblasts were seeded at $2{,}5{\cdot}10^4$ cells per cm^2 in 24-well culture plates and maintained in DMEM supplemented with 20% FBS. Two wells per individual were harvested and counted daily over 8 days using Trypan Blue staining and a Bio-Rad TC20™ Automated Cell Counter. The cultures exhibited a lag phase followed by robust proliferation, with an average doubling time of 1.5 days during the exponential growth phase (days 2–6), consistent with the natural growth observed in HDFs cultures [29] (Fig 4). Cell viability remained high throughout the experiment (average of viable cells = 92.1% ± 2.5), indicating that HDFs maintained functionality without evidence of early senescence, supporting their applicability for downstream molecular analyses within the standard passage range.

3.2 Immunofluorescence

The identity of HDFs was assessed by immunofluorescence, using vimentin and human fibroblast surface protein as positive markers and EpCAM/TROP as a negative marker (Fig 5). HDFs were seeded in 12-mm poly-D-lysine coated coverslips, fixed with paraformaldehyde 4% for 15 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. Primary antibodies were incubated overnight at 4ºC. After thoroughly washing, secondary antibodies were applied for 2 hours and

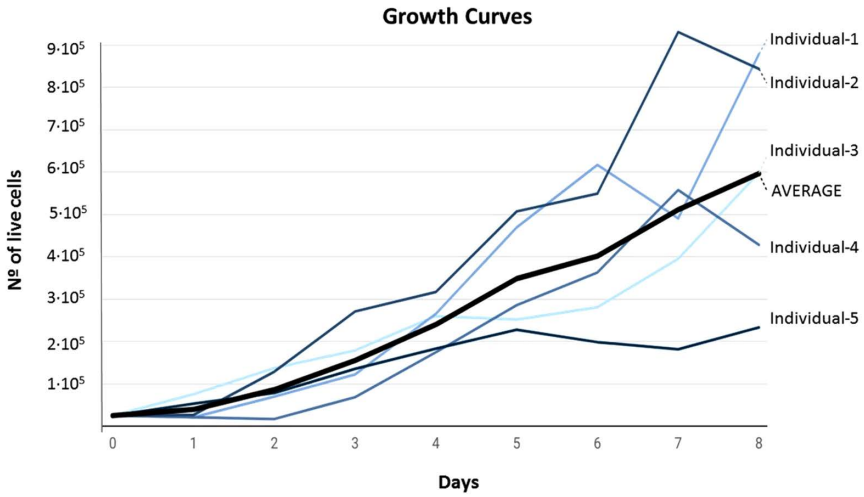

Fig 4. Growth curve of human fibroblasts obtained from five different individuals. Third-passage human fibroblast cultures were seeded in 24-well plates and maintained in complete DMEM. Two wells per individual were counted daily. The mean cell growth is shown as a black thicker line. During the first day after seeding, cell numbers tended to remain constant, indicating a short lag phase. From the second day, cells entered an exponential growth phase lasting until day 6, reaching a PDL of 3.2, corresponding to an average doubling time of approximately 1.5 days.

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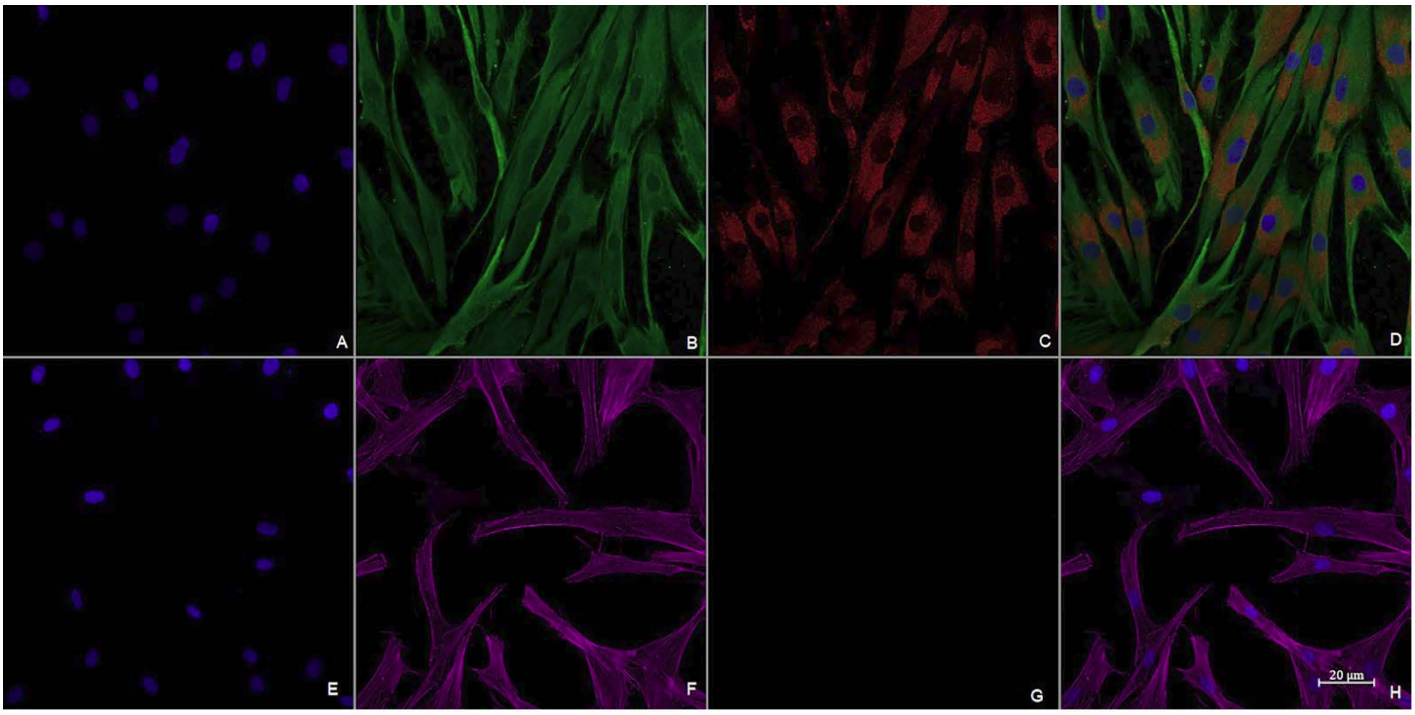

Fig 5. Characterization of HDFs derived from skin biopsy by immunofluorescence. Nuclei were counterstained by DAPI **(A, E)**. Fibroblast-specific markers: anti-vimentin (green) **(B)** and anti-human fibroblast surface protein (red) **(C)**. Merged images A-C **(D)**. F-actin detected with Phalloidin-iFluorTM 647 conjugate (purple) **(F)**. Fibroblast negative marker EpCAM/TROP. **(G)** Merged images E-G **(H)**. Images were acquired with 20x magnification.

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nuclei were counterstained with DAPI. Coverslips were mounted using Vectashield, and images were acquired using a ZEISS LSM 900 confocal microscope. Phalloidin staining revealed the characteristic spindle-shaped morphology of HDFs. Images confirmed that cells were positive for fibroblast-specific markers, with a high signal from vimentin and human fibroblast surface protein. Moreover, cells were negative for EpCAM/TROP, confirming the absence of epithelial contamination and validating the fibroblast identity and purity of the culture.

3.3 Flow cytometry

To ensure the purity of the HDFs primary culture, we conducted flow cytometry analysis, acknowledging that the initial skin biopsy may contain other cell types, including keratinocytes, melanocytes, and immune cells such as lymphocytes or macrophages. We tracked the evolution of cellular subpopulations from the tissue explant stage through successive passages, achieving >99% fibroblast purity by passage 3. At the biopsy stage, cells were passaged upon reaching 100% confluence as described in the online protocol [23], with 1×10^6 cells reserved for flow cytometry analysis using a BD FACSCelesta™ SORP cytometer. Monoclonal antibodies S100A4-PE and integrin α6-FITC were employed as positive and negative fibroblast markers, respectively, following 15 minutes fixation with PFA 4% and 30 minutes permeabilization with 0,2% Triton X-100. After washing, the cellular mass was predominantly made up of fibroblasts, with ~88% of cells positive for S100A4. Over successive passages, HDFs purity progressively increased, reaching >99% by passage 3 (Fig 6). These findings confirmed that the culture conditions effectively favored HDFs enrichment while minimizing contamination from other cell types.

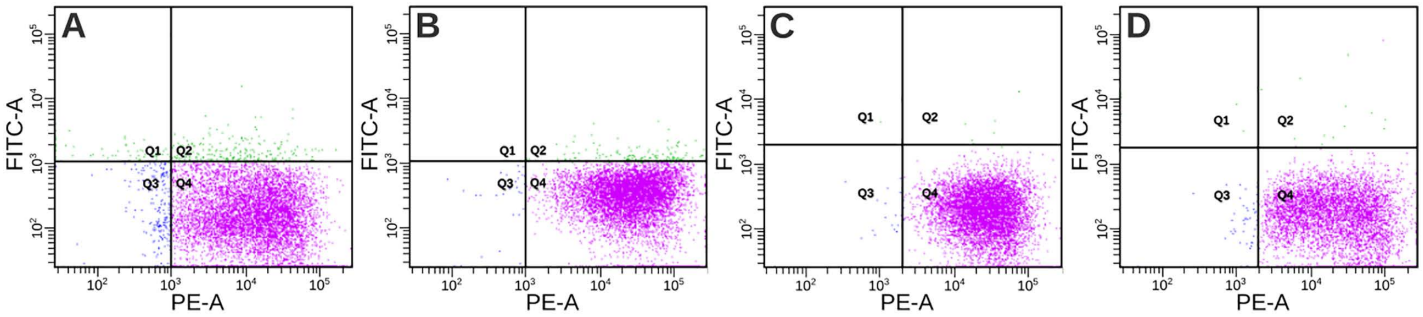

Fig 6. Phenotypic characterization of cell populations derived from skin biopsies across passages 0-3. Experiments were performed on five different donors. **(A-D)** Representative flow cytometry dot plots showing cell populations positive for CD49f (FITC-A) and S100A4 (PE-A). The mean percentages (± SD) of S100A4 + cells were 87.76 ± 14.07% in the cell mass derived from the biopsy **(A)**, 91.52 ± 4.96% in passage 1 **(B)**, 99.5 ± 0.23% in passage 2 and 97.84 ± 1.25% **(C)** in passage 3 **(D)**. Each dot plot corresponds to the most representative sample for the respective passage; therefore, fluorescence thresholds may differ among panels, as they were independently determined for each donor based on its corresponding negative control.

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Supporting information

S1 File. A detailed step-by-step protocol.
(PDF)

S2 File. A detailed list of reagents and materials.
(ODT)

S3 File.
(PDF)

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Writing – review & editing: Raúl Montañez, Alfonso María Lechuga-Sáncho.

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