Isolation of Primary Fibroblast Culture from Wildlife: the *Panthera onca* Case to Preserve a South American Endangered Species

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Cell line establishment of somatic cells is a valuable resource to preserve genetic material of rare, difficult-to-find, endangered and giant species like Jaguar (*Panthera onca*), the largest South American felid. This unit focuses on the isolation and culture of fibroblasts from Jaguar skin and muscle biopsies, and ear cartilage dissection immediately after death to preserve one of the several endangered species in this biome. These culture techniques enabled us to contribute 570 samples from 45 autochthonous and endangered species, including Jaguar. The fibroblasts obtained are a part of the Genetic Bank of Buenos Aires Zoo with the 6700 samples, including tissues such as muscle, ovarian, testicular, blood, fibroblast cultures, sperm, hair, and fluids and cells from 450 individuals of 87 different species. © 2016 by John Wiley & Sons, Inc.

Keywords: biobank • cell culture • *Panthera onca*

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INTRODUCTION

Conservation researchers from Brazil (Pontifícia Universidade Católica do Rio Grande do Sul, Brasil) and Argentina (CEiBA-Universidad Nacional de Misiones-CONICET, Argentina) have expressed their concern about the decline of Atlantic rainforest jaguars, *Panthera onca* (Galetti et al., 2013), since it causes a serious imbalance in the most diverse Argentinian biome. Beyond their intrinsic value as a species, big cats are usually selected as focal species since they are considered determinant to regulate the ecosystems where they live (Noss et al., 1996; Miller et al., 1999; Miller and Rabinowitz, 2002; Ray et al., 2005; Cullen et al., 2006). Their disappearance is an early warning of a threat to the ecosystem: they have large territorial requirements which ensure the conservation of many species with lower demands.

The Atlantic rainforest is a highly fragmented biodiversity hotspot, with less than 12% of its original area left (Ribeiro et al., 2009). Although 24% of the remaining areas are large

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enough to support jaguars, jaguar populations can be found in only 7% of the rainforest (Jorge et al., 2013). Researchers estimated that fewer than 250 mature jaguars live in the entire biome, and are distributed among eight isolated populations (Beisiegel et al., 2012). Even worse molecular analyses demonstrate that local effective population size (a critical parameter for the maintenance of genetic diversity) is below 50 animals (Haag et al., 2010).

The cryopreserved biomaterials banks, biobanks, are curated repositories of biological materials to ensure long-term integrity at the molecular level, authenticity, availability and rights management of its samples by adhering to standard operating procedures. Biobanks represent a secure way of preserving the genetic variability of many species, particularly those that are endangered (Leon-Quinto et al., 2009). Therefore, the Biobank of Genetic Resources of Buenos Aires Zoo (BRG) acquires, processes, stores, uses and supplies sperm, oocytes, embryos, tissues and cells such as fibroblasts to conserve wildlife that is viable and available for research. The applications developed so far with fibroblasts from the biobank created with the protocols described here are related to cell cloning (Moro et al., 2014; Moro et al., 2015a,b). These and other applications in artificial insemination, in vitro fertilization, intracytoplasmic sperm injection, embryo transfer, genetic diversity of cellular metabolism (Holt et al., 1996; Wildt et al., 1997; Watson and Holt, 2001; Roldan and Garde, 2004) allow the exchange of genetic material between wild and captive populations, aiding inbreeding and avoiding loss of genetic variability.

Development, isolation and primary culture of fibroblast biopsies through techniques that allow obtaining large amounts of isolated one-of-a-kind cells that have conserved most of their original characteristics is critical for availability of sufficient material to guarantee further genomics, functional, reproductive and genetic analyses. We used the present protocols to generate the materials that constitute the Biobank at Buenos Aires Zoo: currently consisting of over 6700 samples from 450 individuals of 87 different species.

The objective of this unit is (a) to document successful protocols that allow derivation of primary culture of fibroblasts from wild animals and (b) to obtain and ensure data of biodiversity to conserve genetic diversity of an endangered species. The samples to culture fibroblast cells from wild species are taken from biopsies of live and from dead animals. The protocols developed are mainly two variant: one direct, culturing without any sample pretreatment, and other with a collagenase sample treatment to accelerate the time of the culture procedure.

When skin biopsies were taken, the individual was under general anesthesia. The procedure involves shaving part of the inner groin area, cleaning the area with 70% alcohol and letting it dry. The skin biopsy is taken with a sterile hypodermic needle (0, 80 $\times$ 40, 21-G 1 1/2) inserted in a direction parallel to the skin $\sim$2 mm, and pulling up. The skin piece is cut with a sterile scalpel below the needle; four fragments using the same process are obtained and deposited in a cryovial with 1 ml DMEM + 10% FBS. Samples from biopsies should be transported at 5°C in a sterile plastic jar with saline solution supplemented with gentamicin. In order to avoid sample dehydration, solution volume should be enough to cover the whole piece(s) of tissue. To ensure proper transportation, and to avoid freezing of the sample by direct contact with the coolant, the plastic jar should must be wrapped with paper and should be kept in a plastic bag.

NOTE: All cell culture materials and solutions must be sterile. Pliers and scissors should be soaked in 70% ethanol for at least 30 min before use.

Figure 28.7.1 Photograph of skin biopsy.

NOTE: All procedures should be performed inside a sterile cell culture hood. Turn on the cell culture hood 10 to 20 min before starting work and after that time clean it with 70% ethanol.

NOTE: Culture incubations should be carried out in the cell culture incubator with 5 % CO_2 at 37°C.

ESTABLISHMENT OF PRIMARY CELL CULTURE FROM *PANTHERA ONCA* SKIN OR MUSCLE BIOPSIES BY DIRECT CULTURE

BASIC PROTOCOL

This protocol describes the establishment of primary cell culture of fibroblasts from Argentinean Jaguar skin or tissue from muscle biopsies (Figs. 28.7.1 and 28.7.2).

Materials

Tissue biopsy sampling, primary cell culture of fibroblasts from skin or muscle (Support Protocol 1)
Phosphate-buffered saline (PBS; Irvine Scientific, cat. no. 9235), 1×
Dulbecco's modified Eagle medium (DMEM; with red phenol) + supplements (see recipe)
0.25% Trypsin, 1× (see recipe)

20-, 200-, and 1000-µl pipets
Pliers
Pipet tips
60- and 35-mm cell culture dish
Scissors and scalpel
CO_2 incubator
Phase-contrast inverted microscope
50-ml tube

Prepare tissue

1. Put the tissue in a 60-mm dish and add a few drops of sterile PBS. It is not necessary to cover the entire dish area.
2. Using scissors or scalpels, cut the biopsy into fragments measuring 1- to 2-mm on each side (Fig. 28.7.2).

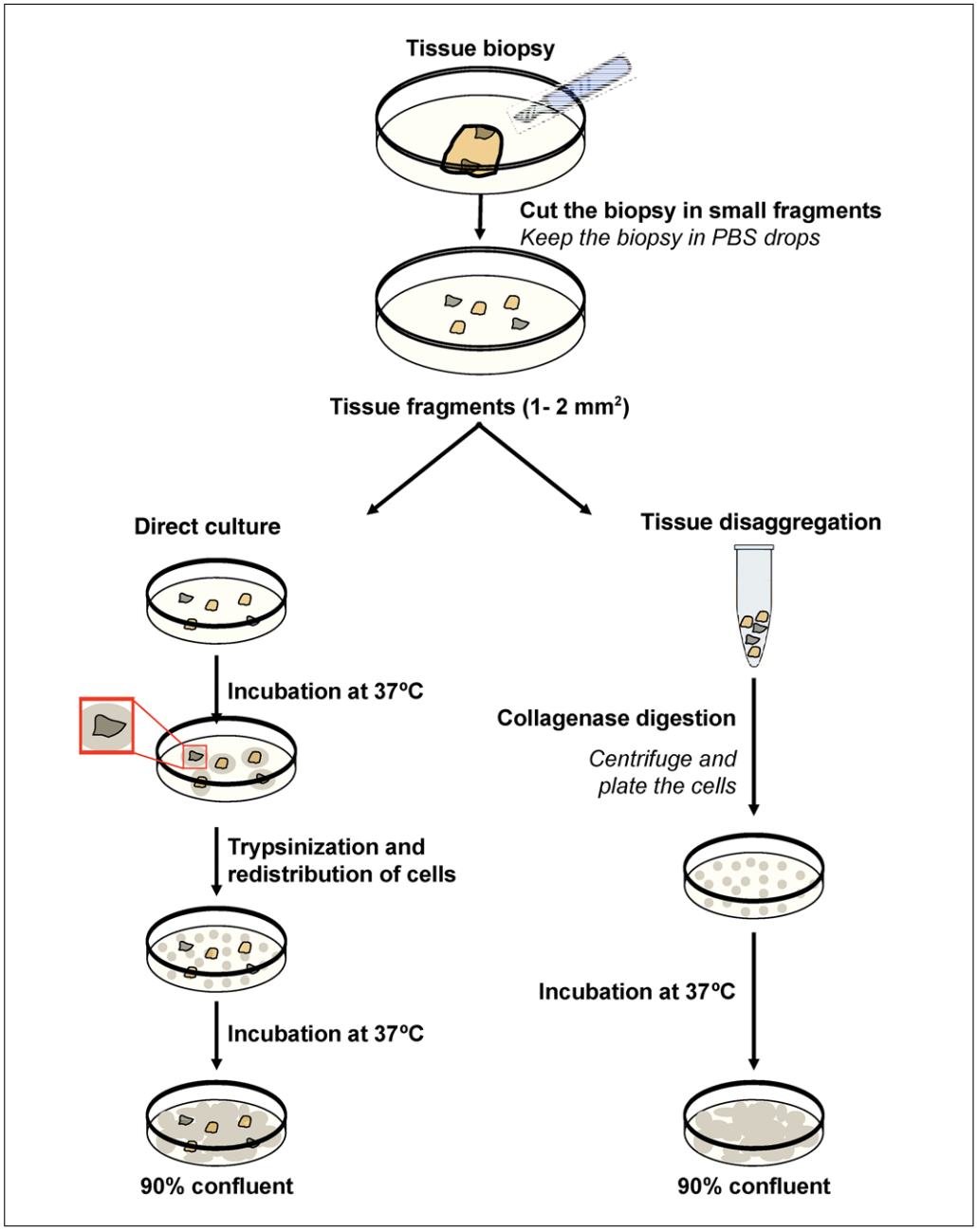

Figure 28.7.2 Scheme of biopsy processing and cell culture establishment. Biopsy should be placed in a 60-mm cell culture dish and cut into small pieces (1 to 2 mm^2) using a scalpel. Tissue fragments can either be cultivated following the Direct Culture protocol or the alternate, Tissue Disaggregation protocol.

Skin biopsies: skin must be shaved before sampling. Remaining short hair is not a problem but it is preferable to remove the hair completely before proceeding (Fig. 28.7.1).

Muscle biopsies: the necrotic tissue [the whole perimeter of the sample that has been in contact with the transport medium (PBS) and looks light brown] must be removed with a scalpel and culture pieces should be obtained from the inner part of the muscle fragment.

Direct culture

3. Separate as many 35-mm dishes as you may need and write the following on the lids:

   Date: Species: ID of the sampling: Type of Tissue:

ID of the sampling = sample specimen identification number of the biobank, identification number used in the laboratory to recognize each sample of each individual.

If one intends to deposit the fibroblasts in a biobank, one needs to record a unique number to each sample of each individual of each species, as in the future this material will be used bearing in mind the genetic diversity contained in samples.

Calculate four to five tissue fragments per 35-mm dish.

4. Put the tissue fragments in each 35-mm dish, making sure they are not touching each other (28.7.2 direct culture).

   Using pliers, take four to five tissue fragments from the 60-mm plate (which were cut and transferred to each of the 35-mm plates identified above), making sure they do not touch each other.

5. Let the PBS drops that keep the tissue hydrated, not evaporate completely the PBS, for 5 to 10 min.

   Allow the tissue fragments to stick to the plate for 5 to 10 min at room temperature, avoiding the complete evaporation of PBS.

6. Add 2 ml of DMEM + supplements, taking care to move the tissue pieces as little as possible.

7. Move the cell culture dishes to the CO_2 incubator and incubate them for 5 to 6 days. Let the fragment cells in the tissue adhere to the bottom of the dish by not touching the dishes during this time.

   Control possible medium contamination. An unclear yellow medium will suggest contamination by fungi or bacteria.

8. Then, after the first 5 or 6 days, change the culture medium for fresh DMEM + supplements every 2 to 3 days.

9. Check cell growth (see 28.7.2 Direct Culture). Observe the culture under phase-contrast inverted microscope at 40× and 100× and define the cell culture density, the percentage of cell growth plate coverage.

   Fibroblasts will start growing and forming a ring of cells next to the pieces of tissue.

10. Grow the cells until the ring around the tissue is visible (~10 days).

11. Add 1 ml of 0.25% trypsin solution to the cells, keep for 10 sec at room temperature, and remove the solution. Place the plate in the CO_2 incubator and incubate the cells 3 to 5 min until all of the cells are unattached. Add 2 ml DMEM + supplements and redistribute the cells on the same plate. Leave the tissue in the same 35-mm dish together with the trypsinized cells.

12. Grow the cells until they cover most of the plate (90% confluent).

13. Wash the cell monolayer twice, each time with 1 ml PBS. Add 1 ml of 0.25% trypsin solution to the cells and incubate 3 to 5 min in the CO_2 incubator. Add 2 ml DMEM + supplements to resuspend the cells. Plate in a new 60-mm dish and incubate.

    All the above is passage 1 (p1). We call all the steps up to this point "passage 1" or p1. See Support Protocol 3 for culture and trypsinization of p1 cells.

14. *Optional:* To obtain larger amount of fibroblasts, leave the tissue fragments in the first 35-mm dish for a second round of cell growth. Add 2 ml DMEM + supplements and incubate as before in step 9 (Fig. 28.7.2).

ALTERNATE PROTOCOL

ESTABLISHMENT OF PRIMARY CELL CULTURE FROM *PANTHERA ONCA* SKIN OR MUSCLE BIOPSIES BY TISSUE DISAGGREGATION

This protocol accelerates cell growth by disaggregating the tissue with collagenase at the beginning of the culture process.

Additional Materials *(see Basic Protocol)*

Collagenase (see recipe)
15-ml tubes
1-ml pipets
Centrifuge

1. Put the tissue in a 60-mm dish and add some drops of sterile PBS. It is not necessary to cover the entire dish area.
2. Using scissors or scalpels, cut the biopsy into fragments 1- to 2-mm each side (Fig. 28.7.2).
3. Collect the fragments in a 15-ml tube containing 1:1 volume of collagenase and DMEM + supplements (28.7.2 Tissue disaggregation).
4. Incubate the tube with tissue fragments in the cell incubator for 16 to 18 hr with 5% CO_2 at 37°C.
5. After incubation, use a 1-ml pipet to take the medium and the pieces of tissue up and down through the pipet. This will help to disaggregate cells.
6. Centrifuge for 10 min at 200 × *g*, room temperature, to spin down the cells and the rest of the tissue and discard the supernatant.
7. Resuspend the pellet (which contains the cells and the rest of the tissue) in 2 ml fresh DMEM + supplements and transfer to a 35-mm dish.
8. Incubate in a CO_2 incubator. Check cell growth daily. Observe the culture under a phase-contrast inverted microscope at 40× and 100 ×. Estimate density of cell culture according to the percentage of the plate that is covered with cell growth (confluence). Change the medium every 2 to 3 days.

   Control possible medium contamination. An unclear yellow medium will suggest contamination by fungi or bacteria.

9. Grow the cells until they cover most of the plate (90% confluent).
10. Wash the cell monolayer twice, each time with 1 ml PBS. Add 1 ml of 0.25% trypsin solution to the cells, incubate for 10 sec at room temperature, and remove the solution. Place the plate in the incubator and incubate the cells 3 to 5 min until the cells are unattached. Add 2 ml DMEM + supplements to resuspend the cells. Plate in a new 60-mm dish and incubate.

    This will be passage 1 (p1). See Support Protocol 3 for culture and trypsinization of p1 cells.

SUPPORT PROTOCOL 1

ESTABLISHMENT OF PRIMARY CELL CULTURE EAR CARTILAGE FROM *PANTHERA ONCA* AFTER DEATH

When jaguars share their territories with humans it is common that they die accidentally or intentionally because they are persecuted for their potential impact on livestock, and their prey have been overhunted even in large protected areas. When an animal dies, it is useful to keep, as much diversity in tissue as possible and the ear is an easy source to obtain and to preserve. Besides muscle samples, ear is a great tissue for fibroblast

isolation with an excellent yield of cell in number and quality. Moreover, refrigerated skin ear tissue remains alive for more than two weeks and the cells derived from such tissues are normal and can be cryopreserved for long-term storage. These are reasons to develop a protocol to obtain cells from the ear of a death animal.

Materials

70% ethanol
Saline solution
40 mg/ml gentamicin (see recipe)
Phosphate-buffered saline (PBS; Irvine Scientific, cat. no. 9235)
Dulbecco's modified Eagle medium (DMEM; with red phenol) + supplements (see recipe)
200 nM L-glutamine, 100×
5 mg/ml Collagenase, 2×
0.25% trypsin, 1×

20-, 200-, 1000-μl pipets
Pipet tips
Animal hair clippers
15- and 50-ml Falcon tubes
Self-sealable zipper storage bags
Pliers
Scissors
Tweezers
35-, 60-, and 100-mm petri dishes
Scalpel
37°C CO_2 incubator

Obtaining ear

1. Shave ear hairs, carefully wash and rinse the ear with sterile saline solution. Rub with sterile cotton soaked in 70% ethanol and wait until it dries.
2. Using scissors, cut the ear off the head and put into a 50-ml Falcon tube with saline solution containing gentamicin (enough to cover piece of ear) at 5°C. Cover the sample with paper to prevent it from withering.
3. Put the labeled tubes (species, collection date, material and material origin) in a self-sealable zipper storage bag.
4. Ship the samples to the culturing site (e.g., the laboratory where the tissue will be processed) at 5°C.

Starting culture

5. Once in the culturing place, sterilize scissors and pliers with 70% ethanol.

   From this point on, work inside a laminar flow cabinet.

6. Sterilize the ear for 1 min in 70% ethanol in a 100-mm petri dish.
7. Using tweezers, transfer the ear into another petri dish with 7 ml PBS for 1 min to remove the 70% ethanol.
8. Dissect the cartilage from the ear fragment. The ear fragment looks like a small sandwich, with a sheet of cartilage wrapped by two layers of skin. Carefully, with the use of tweezers and scissors, separate the skin of the ear cartilage fragment, to obtain a clean, sterile sheet of cartilage (see Fig. 28.7.3).

Figure 28.7.3 Photograph of cartilage ear dissection.

9. Trim the cartilage sheet edges. Keep the central piece and cut it in fragments of 2 × 2-mm^2. Distribute these pieces in at least two 35-mm dishes.

   From here, you can also follow the collagenase digestion (see Alternate Protocol, step 3).

10. Add 2 ml DMEM + supplements to each 35-mm dish containing tissue fragments.

    The pieces should be totally covered and not touching.

11. Label the plates (species, date, protocol type, kind of tissue) to identify the samples in the biobank.

12. Culture in the 37°C CO_2 incubator for 5 or 6 days. Do not move the plates during this time to permit cell attachment. Check the color of culture medium to detect contamination. If contamination is detected, discard the plates.

    Control possible medium contamination. Cloudy yellow medium will suggest contamination by fungi or bacteria.

Cell multiplication and freezing

13. After the first 6 days, change the culture medium every 2 days. Let the cells grow to 90% confluence.

14. Once the cells reach 90% confluency in 35-mm petri dishes, transfer the cells to 60-mm petri dishes (see Basic Protocol 1, step 13).

    This will be passage 1 (p1). See Support Protocol 3 for culture and trypsinization of p1 cells.

BIOPSY SAMPLES AND CELLS FREEZING AND DEFROSTING

SUPPORT PROTOCOL 2

Cryogenic preservation of biopsies and fibroblasts is generally the same as cryopreservation of most continuous cell lines. The addition of dimethyl sulfoxide (DMSO) to a final concentration of 10% permits the long-term preservation of cells at −80°C or in a liquid nitrogen freezer (Sherman, 1965).

Materials

Biopsied skin fragments (see Support Protocol 1)
Freezing solution (see recipe)
Liquid nitrogen
Cells (see Support Protocol 3, step 4)
Ice
Phosphate-buffered saline (PBS; Irvine Scientific, cat. no. 9235), 1×

Cryovials
Ultrafreezer (−80°C)
Mr. Frosty, Nalgene, freezer container (Sigma, cat. no. C1562)
15-ml tubes
Centrifuge
Pliers
20-, 200-, 1000-µl Pipets
Pipet tips
Scalpel

Steps for freezing skin fragments

1. Put two biopsied skin fragments in a cryovial containing 1 ml freezing solution (DMEM + 10 % DMSO). Leave the samples or cells 10 min at room temperature in the cryovial containing freezing solution.

2. Freeze the samples over a period of 10 min by hanging the vial 4 cm over the surface of liquid nitrogen. After that, dip the cryovials in liquid nitrogen tank and store at −196°C for the period needed.

Steps for freezing cells

3. Put the cell pellet (from Support Protocol 3, steps 4i and 4iii) into a cryovial containing 1 ml freezing solution (DMEM + 10% DMSO) and leave 15 min at 5°C.

4. Put the vials containing the cells into Mr. Frosty, put them into an Ultrafreezer at −80°C for 24 hr, and then transfer the cryovials into liquid nitrogen tanks for final storeage at −196°C in liquid nitrogen for an unlimited time.

Steps for defrosting

Prior to starting using the frozen samples as tissue from which to derive primary fibroblasts cultures

5. Quickly defrost the frozen samples at 37°C. Using sterile pliers, transfer the samples into a 15-ml tube with 10 ml DMEM + 10% FBS. Centrifuge for 10 min at 80 × *g*, room temperature.

6. Discard the supernatant and remove the fragments with sterile pliers. Place the tissue pieces on a 35-mm sterile dish and cut with a scalpel into 1-mm fragments. Split into two equal parts. Proceed with each piece according to the Basic Protocol (step 4) or to the Alternate Protocol (step 3).

SUPPORT PROTOCOL 3

CULTURE AND TRYPSINIZATION OF PASSAGE 1 (P1 CELLS)

This protocol describes the routine process used for trypsinization and transfer of cells in culture to the final freezing for storage.

Materials

P1 cells (see Basic Protocol, step 13)
60- and 100-mm dishes
Centrifuge
Phosphate-buffered saline (PBS; Irvine Scientific, cat. no 9235), 1×
Dulbecco's modified Eagle medium (DMEM; with red phenol)
Fetal bovine serum (FBS)
5000 U/ml penicillin/streptomycin, 50×
250 μg/ml Fungizone, 100×
200 nM L-glutamine, 100×
0.25% trypsin, 1×

20-, 200-, and 1000-μl pipets
Pipet tips
100-mm petri dishes
Centrifuge
15-ml Falcon tubes
37°C, 5% CO_2 incubator
1-ml cryovials

1. Grow the p1 cells until they cover most of the 60-mm area (90% confluent).

2. Wash the cell monolayer twice, each time with 2 ml PBS. Add 0.5 ml of 0.25% trypsin-EDTA solution to the cells and incubate 3 to 5 min at 37°C, 5% CO_2. Add 2 ml DMEM + supplements to resuspend the cells and plate again in a new 100-mm dish with a 1000-μl pipet, complete to 7 ml with DMEM + supplements each plate and incubate for the next step.

   This will be passage 2 (p2).

3. Grow the p2 cells until they cover most of the 100-mm dish area (90% confluent). Trypsinize as described above (see step 2) and centrifuge for 10 min.

4. Continue as needed:

   i. To freeze the cells (three to four cryovials) proceed with Support Protocol 2.
   ii. Continue with the cells in culture (one 100-mm dish to three 100-mm dishes).
   iii. Freeze 2/3 in two to three cryovials and plate 1/3 in a new 100-mm dish –passage 3- to continue with the cell culture.

   It is recommended to freeze a proportion of all the passages from now on to preserve all passages. Do not use the cells older than 3 or 4 passages for research applications because after several passages (e.g., 5 or more), the fibroblast growth rate will decrease and cells take longer to reach confluence, or the cells will begin to die by senescence.

Considering that it is storing samples from biological diversity of wild species, it is recommended to freeze the cells from the 3rd or 4th passage to maintain genomic integrity.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

Collagenase, 5 mg/ml

Dissolve 100 mg of Collagenase B (Roche-Boehringer in Argentina, cat. no. 11088807001) in 20 ml phosphate-buffered saline (PBS; Irvine Scientific, cat. no. 9235)
Sterilize by filtration (filter pore size 0.22 μm).
Divide into smalls aliquots (0.5 to 2 ml)
Store up to 2 years at −20°C

DMEM + supplements, 100 ml

To 86 ml DMEM (see recipe) add:
10 ml fetal bovine serum (FBS)
2 ml penicillin/streptomycin (P/E), 50×(see recipe)
1 ml fungizone (100×; see recipe)
1 ml L-Glutamine (100×; see recipe)

Prepare the volume you may need for 1 week of use. You can prepare DMEM + FBS and then add P/E, fungizone and L-glutamine directly to the cell culture plate.

DMEM culture medium

DMEM: Dulbecco's Modified Eagle Medium (Gibco, catalog no. 12100-038) with phenol red. When using DMEM powder, dissolve the powder in 1 liter sterile water and add 3.7 g of $NaHCO_3$. Stir for 30 to 60 min at room temperature and sterilize by filtration (filter pore size 0.22 μm). Store up to 1 year at 4°C.

Freezing solution

10% dimethyl sulfoxide (DMSO) in DMEM (see recipe) + 10% fetal bovine serum (FBS)
Prepare fresh

Fungizone, 100×, 250 μg/ml

Fungizone Antimycotic, Amphotericin B solution 250 μg/ml (Invitrogen, catalog no. 15290)
Divide into 1-ml aliquots
Store up to 1 year at −20°C

Gentamicin, 40 mg/ml

Prepare a stock solution of 40 mg/ml Gentamicin. Divide aliquots and store up to expiration date on the bottle, at room temperature. For working solution, add 0.2 ml of Gentamicin stock solution per 10 ml saline solution. Prepare fresh.

L-Glutamine, 100×, 200 mM

L-Glutamine 100×, 200 mM (Invitrogen, catalog no. 25030). Divide into 1-ml aliquots and store up to 2 years at −20°C.

Penicillin/streptomycin 50×, 5000 U/ml

Penicillin/streptomycin 50 ×, 5000 U/ml (100 mg/ml each; Invitrogen, catalog no. 15070-063). Divide into 1-ml aliquots and store up to 1 year at −20°C.

Trypsin/EDTA solution (0.25%, 0.91 mM EDTA)

1× stock solution (GIBCO, catalog no. 25200-056). Divide into 1-ml aliquots and store up to 18 months at −20°C.

COMMENTARY

Background Information

Although the protocol described here is focused on Argentinean Jaguar biopsies, basically the same protocols of cell isolation and culture could be used for other species. The parameters of cell culture of other species need to be optimize and merit independent study. In the zoo of Buenos Aires and IBYME successful culture of muscle and skin cells from jaguars, among other native as well as non-native fauna species, have been performed. This protocol permitted conservation of Jaguar cell stocks.

Regarding the rescue of post mortem material (cultured from ear cartilage) we have obtained successful results of cell growth through samples collected immediately after the death of a Puma (*Puma concolor*) specimen. The samples were refrigerated for 12 days at 5°C. This time included the time to ship samples to the point of culturing (800 km).

Critical Parameters and Troubleshooting

Use deionized, distilled water when preparing all solutions. Sterilize all solutions immediately after preparation.

1. Contamination of samples is the most frequent problem and can be avoided with strict, careful media preparation and culture handling.

2. Delay in sample transportation leads to a decrease in cell growth rate.

3. The source of the collagenase could be a key factor to obtaining viable cells. Several commercial sources of collagenase have been tested using this protocol. We recommend the one that worked best in our hands, but we suggest testing a set of different ones to find your best results.

Basic Protocol

Cutting the biopsy

We recommend scalpels rather than scissors to cut the tissue because the accuracy of the cut is better and the size of the resulting fragments is smaller. Use a new, sharp scalpel for each new biopsy.

Culturing tissue fragments

It is important to move the tissue fragments as little as possible so that they can attach to the plate fast. Cell culture medium should be changed; taking care not to move the tissue neither before nor after the tissue is attached. While controlling the ring cell formation, culture medium could be changed every 3 days if medium color remains red.

Plates should be moved as little as possible, especially before the tissue becomes attached to the plate floor.

Alternate Protocol

It is important to respect the time of incubation with collagenase as longer periods of activity will end up killing all the cells.

After the collagenase digestion, it is normal to see a lot of tissue and cell debris together with the live cells. This will not interfere with cell attachment and growth. Nevertheless, cell culture media will become yellow faster than if only living cells were plated. If possible, cell culture media should be changed not before two days from the plating date. This is important to allow as many cells as possible to attach to the plate floor. Tissue debris will be eliminated with the first change of medium.

Anticipated Results

The Biobank has a set of 570 samples from 45 autochthonous and endangered species, including Jaguar, which was produced with the culture techniques here described. The fibroblasts obtained are a part of the 6700 samples, including tissues such as muscle, ovarian, testicular, blood, fibroblast cultures, sperm, hair, and fluids and cells from 450 individuals of 87 different species.

Time Considerations

Basic Protocol

The estimated duration of the direct culture protocol is around 1 hr. The growth of the ring of cells around the tissue will take between 10 and 14 days. After the trypsinization of the ring of cells, it will take 3 to 4 days to reach 90% confluency. After each passage, cells will need 3 to 6 days to reach 90% confluency. Therefore, it will take around 20 to 24 days to reach the first 100-mm dish with 90% confluency.

Alternate Protocol

The estimated duration of the collagenase digestion protocol is around 1 hr the first day and 30 min the second day. Cells plated in

the first 35-mm dish will need around 1 week to reach 90% confluency. After each passage, cells will need 3 to 6 days to reach 90% confluency. Therefore, it will take around 18 to 20 days to reach the first 100-mm dish with 90% confluency.

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