1

Basic Cell Culture

Jeffrey W. Pollard

1. Introduction

This article describes the basic techniques required for successful cell culture. It also acts to introduce some of the other chapters in this volume. It is not intended to describe the establishment of a tissue culture laboratory, nor to provide a historical or theoretical survey of cell culture. There are several books that adequately cover these areas, including the now somewhat dated, but still valuable volume by Paul *(1)*, that of Freshney *(2)*, and the multi-authored volumes edited by Jakoby and Pastan *(3)*, Davis *(4)*, and Celis *(5)*. Instead, this chapter focuses on the techniques for establishing primary rodent cell cultures from embryos and adult skin, maintaining and subculturing these fibroblasts and their transformed derivatives, and the isolation of genetically pure strains. The cells described are all derived from Chinese hamsters since to date, these cells have proved to be the most useful for somatic cell genetics *(6,7)*. The techniques, however, are generally applicable to most fibroblast cell types.

I only discuss growing fibroblastic cells in semidefined media. A very detailed consideration of serum-free culture and the maintenance of epithelial cells can be found in Chapter 4. Methods for culturing many other non-fibroblastic cell types are described in Chapters 2 through 24.

2. Materials

1. Alpha minimum essential medium (α-MEM) containing penicillin and streptomycin: for economy, we buy prepared medium as powder in 20-L aliquots. A 44-g quantity of sodium bicarbonate is added, the powder is made up to 20 L in deionized distilled water, the pH adjusted to 7 4, and the media sterile-filtered through a 0.22-μ*M* filter using a pressure vessel coupled to a filtration apparatus and driven by a pressurized 95.5% air CO_2 gas mix This gas mix maintains pH

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on preparation and storage The 500-mL bottles are stored at 4°C in the dark until use (*see* Notes 1 and 2). Prepared media can also be purchased from many suppliers.

2 Growth medium: α-MEM plus 15 or 7 5% (v/v) fetal calf serum This is made up as required and stored at 4°C.

3. Fetal calf serum (FCS) should be pretested to ensure it supports optimal growth (*see* Section 3.6.). It can also be heated at 56°C for 30 min to destroy complement if it is to be used for cultures where the presence of complement can cause complications. Sera should be aliquoted and stored at –20°C

4 $Ca^{2+}Mg^{2+}$-free phosphate-buffered saline (Dulbecco's PBS). 8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 2.16 g/L Na_2HPO_4 $7H_2O$), pH 7 2.

5 PBS citrate· PBS + sodium citrate at 5.88 g/L.

6. Trypsin· One vial of lyophilized Difco (Detroit, MI) Bacto-trypsin in 400 mL of PBS citrate (0 125% trypsin) or 10 times this concentration for the isolation of embryonic fibroblasts (*see* Note 3)

7 Counting fluid: PBS + 0 2% (v/v) FCS.

8. Formalin fixative: 10% (v/v) commercial formaldehyde (comes as a 40% [v/v] solution).

9. Methylene blue stain: 0.1% (w/v) methylene blue in distilled water filtered through a Whatman No. 1 filter.

10 Trypan blue: 0.5% (w/v) in PBS

11 Colcemid: 10 μg/mL, store at 4°C

12. Karyotype fix· Methanol acetic acid (3:1) made up on the day of use and kept on ice in a tightly stoppered bottle

13 Giemsa stain: Use commercial Giemsa concentrate diluted 3.47 parts in commercial Gurr's buffer (one tablet to 1 L distilled water). Alternatively, 10 m*M* potassium phosphate, pH 6.8, can be used as the buffer. The diluted stain is only stable for 2–3 mo.

3. Methods

3.1. Establishment of Primary Chinese Hamster Fibroblast Cultures

3.1.1. Embryo Culture

1. Kill a 12-d old pregnant Chinese hamster with ether.
2 Wash the animal in tap water and then with 70% ethanol
3. Make a surgical incision on the dorsal side to expose the uterus using sterile instruments (these can be dipped in ethanol and flamed to maintain sterility during the operation)
4 Remove the uterus *in toto*, and transfer it to a sterile Petri dish. Dissect the embryos, and place them in a new sterile Petri dish (*see* Note 4).
5. Mince the embryos very finely, and while still in the Petri dish, wash the pieces with 5 mL of 0.125% Bacto-trypsin at 37°C.
6. Tilt the Petri dish so that embryo pieces go to the side Remove the pieces into a 50-mL centrifuge tube using a wide-bore pipet.

7. Add 40 mL of fresh 1.25% Bacto-trypsin, and incubate at 37°C for 5 min
8 Regain the embryo pieces by centrifugation at 100*g* for 3 5 min, and discard the supernatant.
9 Resuspend the pieces in 40 mL of fresh 0 125% Bacto-trypsin, and incubate at 37°C for 25 min (this can be performed in a roller apparatus).
10. Neutralize the trypsin with 4 mL of FCS
11. Deposit the supernatant through a 100-μm sterile mesh into another centrifuge tube.
12 Centrifuge the supernatant for 5 min at 300*g* at room temperature
13 Resuspend the pellet in 10 mL α-MEM plus 15% FCS, and count the cells in a hemocytometer (*see* Section 3.3.) at about 1/100 dilution.
14 Lay down 1.5×10^7cells in 40 mL of a-MEM plus 15% FCS into a 75 cm^2 flask, and place it in a 37°C tissue-culture incubator
15 The next day, replace the medium with an equal volume of α-MEM plus 15% FCS.
16 Forty-eight to 72 h later, the monolayer should be confluent, and at this point, the cells are ready for subculture. This is performed by incubating the monolayer with 4.5 mL of 0.125% Bacto-trypsin at 37°C until the cells detach. Cell detachment can be visualized either by observing the cell monolayer in oblique light or directly under the microscope. When the cells have detached (~80%), add 0.5 mL FCS (10%), pipet up and down five times, and transfer contents to a 15 mL centrifuge tube.
17. Centrifuge the cells at 300*g* for 3.5 min at room temperature.
18. Remove the supernatant, resuspend the cell pellet in 5 mL α-MEM plus 15% FCS, and determine the cell concentration.
19 Resuspend the cells at 4×10^6/vial in α-MEM plus 15% FCS plus 10% (v/v) sterile dimethyl sulfoxide (DMSO), and freeze at –135 or –176°C The cells will remain viable for several years
20. The cells may also be subcultured at one-third to one-tenth dilutions They have doubling times of approx 36 h At this point, start to calculate the number of mean population doublings by keeping careful records of subculture number and split ratio (*see* Chapter 3 for details).

3.1.2. Skin Fibroblasts

See Chapter 2 of this volume for human explants.

1. Kill and wash an animal as described for the isolation of embryonic fibroblasts (*see* Section 3 1.1., steps 1–3). In fact, it is often convenient to prepare skin fibroblasts from the same animal as the one from which the embryos were obtained.
2. Cut small pieces (1–2 mm^2) of dermis from the exposed skin flaps using sterile instruments, avoiding any fur.
3. Place several (5–10) small pieces (*see* Note 4) into a 25-cm^2 flask, and allow them to adhere for 30 min in a very thin film of medium (0.5 mL) at 37°C.
4. Once adhered, add 5 mL of growth medium to the opposite surface (i.e., top surface) of the flask to avoid washing off the skin pieces. Place the flask in the incubator in the upside-down position for 24 h (the surface tension holds a thin film of medium to the upper surface and stick the explants to the flask surface).

5 Once the explants are firmly stuck, gently invert the flask and return to the incubator
6 The next day, it is often advisable to change the medium to remove any debris and unattached explants.
7. After several days, first "epithelial" type cells and then fibroblast will grow out of the explants (*see* Chapter 2 for details) Let this process continue until most of the surface is covered with fibroblasts or until obvious necrosis is observed in the explant. It may be necessary to change the medium every week until substantial outgrowth is observed.
8. Remove the explanted material (*see* Note 5) with a Pasteur pipet attached to a vacuum line leaving the adherent fibroblasts
9 At this stage, depending on the density, the fibroblasts can either be trypsinized (>50% confluent) or allowed to continue to grow to form a monolayer before they are trypsinized, subcultured, and frozen as described in Section 3 1 1

3.2. Maintenance and Subculture of Transformed Cell Lines

Many transformed cell lines will grow both as monolayers and in suspension culture. The CHO-S cell line is one such line having been selected for suspension growth by Thompson from the original K1 CHO cell line isolated by Puck (*see* Note 6, ref. *7*). Because CHO cells are transformed, they do not require as much serum as normal diploid fibroblasts, and we routinely culture them in 7.5% (v/v) FCS. Despite the relative ease with which transformed cells can be cultured, however, unlike normal diploid fibroblasts, they do not enter a stationary phase of long-term viability *(8,9)*. In this phase, they rapidly lose viability, and therefore must be subcultured during the exponential phase of growth and cannot be maintained as arrested cultures in reduced serum

1 CHO cells are stored frozen at ~4 × 10^6 cells/mL at –135 or at –176°C (liquid nitrogen) in growth medium containing 7 5% FCS and 10% (v/v) DMSO A single vial is removed from the frozen stock, rapidly defrosted in a 37°C water bath, and the cells regained by centrifugation at 300*g* at room temperature for 3.5 min
2 The supernatant is discarded and the pellet resuspended in 1 mL of prewarmed medium and placed into a 25-cm^2 flask or a 60-mm diameter dish containing 4 mL of growth medium
3 Approximately 2 d later, the cells should be almost confluent and ready for subculture (*see* Note 7) They are trypsinized as described for the primary diploid fibroblasts. After cell detachment, FCS is added to 10% and the cells resuspended as single cells by pipeting up and down about five times with a 5 mL pipet An aliquot of this cell suspension (up to a total of 10% of the recipient volume of the medium) can be added directly to a new tissue culture vessel containing growth medium and returned to the incubator until the next subculture Alternatively, the cells may be regained by centrifugation, resuspended, and the concentration/mL determined (*see* Section 3.3). Known concentrations of cells may then be subcultured by appropriate dilution. In a 25-cm^2 flask with 5 mL of growth medium,

CHO cells should yield about 2 5 × 10^5/cm^2 but yields are variable depending on serum batch and media used

4 At this stage, cells maybe transferred to a magnetically stirred spinner flask (commercially available) containing pregassed (95% air/5% CO_2) growth medium; usually a 250-mL spinner flask is seeded to give a density of ~8 × 10^4 cells/mL. These flasks are then placed in a warm room or in a temperature-regulated water bath (Heto), and stirred at 100 rpm (full details of spinner culture and scale up are described in Chapter 5, *see also* Note 8) CHO cells grown in suspension should give ~10^6 cells/mL at saturation density, at which point the medium will be very yellow (acid).

3.3. Determination of Cell Number

This can be performed either using an electronic particle counter (e.g., Coulter Electronics Inc.) or a hemocytometer. The former is the more accurate and can be used to count low concentrations of cells (~10^3 cells/mL); the latter requires higher density and is more prone to sampling error, but allows a visual estimation of the "health" of the cells and, combined with Trypan blue exclusion, can be used to estimate cell viability.

1 Resuspend cells to give a uniform cell suspension by pipeting up and down against the side of the plastic centrifuge tube
2 If the cells have been trypsinized, as described in Section 3 1 1 , 0 2 mL of the cell suspension to 7.8 mL of counting fluid in a 15-mL Falcon snap-cap tube will give a statistically reliable cell count (1000–14,000 particles/0 5 mL counted). Count three aliquots with the Coulter counter set to count 0 5 mL, sum the three counts, divide by 3, and multiply by 40 (for dilution) and 2 to calculate the cells/mL (*see* Note 9)
3 The cells can then be appropriately diluted for the experimental setup or subculture
4 Alternatively, the cells can be counted on a hemocytometer. The cells need to be resuspended at 3–5 × 10^5 cells/mL A drop of a cell suspension is added to either side of the hemocytometer, taking care not to overfill it and making sure that the coverslip is firmly in place
5. Each large square on the hemocytometer (improved Neubauer type) gives an area of 1 mm^2 and a depth of 0.1 mm (i e , the volume is 10^{-4} mL). Count the cells in the square (usually using the one bounded on each side with triple lines) on either side of the counter, average the counts, and divide by 2 and multiply by 10^4 to give the number of cells/mL If there are too many cells (>1000), just count the 5 diagonal squares and multiply by 5 to give the number to be multiplied by 10^4 If there are too few cells, count more than one complete square on each side of the chamber, and divide the total cell number accordingly.
6 This procedure can also be used to determine cell viability, since prior to placing the cells in the hemocytometer, they can be diluted 1 1 with 0 5% Trypan blue

The number of cells that can then exclude the stain (i e , have intact cell membrane) can be determined by counting the cells as described in steps 3–5

3.4. Isolation of Genetically Pure Cell Lines

The isolation of somatic cell mutants is outside the scope of this chapter, and the reader is referred to Thompson *(10)* for the considerations necessary to isolate such mutants successfully. All cell lines will genetically alter over time, however, and periodically the parental type will need to be purified from variants or revertants. The easiest way to do this is to isolate a single clone. This causes some potential problems, however, since a clone may itself be a variant, and thus several clones will need to be isolated and tested to ensure the phenotype selected is the required one. To overcome this problem of clonal variability, it is usually better to contract the cell population to about 100 cells and then expand this to the mass culture. This contraction should statistically remove any variants from the population. It is worth remembering, however, that any variant that has a growth advantage over the parental type will soon overgrow the whole culture. Once a mass culture is obtained, it should be frozen in a large number of vials (20–50) to provide a base for future experiments. This enables the investigator to grow a culture for approx 3 mo before discarding it, and then to return to the frozen stock for the next set of experiments. This protocol reduces the genetic drift in the culture and avoids the necessity of frequent genetic purification using the following methods.

1 Trypsinize a culture, recover the cells, and determine the cell number as described in Section 3 1 1
2 Dilute to 2.5 cells/mL with 20 mL of growth medium.
3 Plate out 0 2 mL/well into a 96-well tissue-culture plate
4. Incubate plates at 37°C in an humidified incubator for 10–12 d Do not move or disturb the plates, mitotic cells will float off and form satellite colonies.
5 Examine every well with a microscope, and ring those that have a single clone. These may be pure clones but a second cloning ensures that you end up with populations derived from a single cell
6. Trypsinize two to three of these individual clones with 0.2 mL of trypsin and, once detached, transfer the well's contents into 4 mL of growth medium in a snapcap tube.
7. Pipet this up and down to ensure a single cell suspension, and then plate it again at one-tenth serial dilutions (i e., 0.4–3 6 mL) and 0 2 mL/well into a 96-well tissue-culture dish (*see* Note 10).
8. Return these new plates to the incubators Add medium from a different batch to the trypsinized wells of the old plates, and also return this to the incubator This provides a backup in case the new plates are contaminated Again, do not move the plates.

9. After 10–12 d, select individual clones in the new plates, and expand them up to mass culture (remember to always keep a backup culture).
10. Freeze a large stock (20–50 vials) as described in Section 3.1 1 , since at this stage, you will have a genetically pure line (except for the mutations that may have occurred during the clone's expansion). Split the frozen stock between a liquid N_2 store (long-term) and a –70 or –35°C store (short-term experimental stock)
11 Alternatively, the mass culture that needs to be genetically cleansed can be plated into 60-mm dishes containing 5 mL growth at 100 cells/dish
12 Leave these to grow for approx 10 d. Trypsinize the ~100 clones from each plate and expand them together to a mass culture in the normal way.
13 Freeze 20–50 vials of these cultures as described in step 10.

3.5. Karyotyping

It is often desirable to karyotype your cells. Full details for banding and identifying karyotypes are given in Chapter 27. This chapter, therefore, deals with a simple method, derived from Deaven and Petersen *(11)* for producing karyotypes of Chinese hamster cells.

1 A culture growing in the exponential phase of growth (i e., having a high mitotic index) in a 10-mL suspension culture (2×10^5 cells/mL) or as a monolayer (10^6 cells/60-mm plate) is treated with colcemid at 0 06 μg/mL for 2 h to accumulate cells in mitosis
2. For the monolayer culture, tap the plate and remove the medium containing detached mitotic cells Trypsinize the remaining monolayer, pool with the medium, and proceed
3 Regain cells by centrifugation at 300*g* for 3.5 min at room temperature.
4. Resuspend cells in 1 mL of growth medium, add 3 mL of distilled water, and invert to mix (do not pipet because the cells are fragile)
5. Leave for 7 min to allow the cells to swell (this time can be altered if satisfactory spreads are not obtained)
6. Add 4 mL of freshly prepared ice-cold fixative (methanol:acetic acid, 3:1) directly to the hypertonic solution to avoid clumping
7. Regain the cells by centrifugation at 300*g* for 3.5 min
8. Disperse the pellet gently by agitation (do not pipet) in 10 mL of fixative
9 Repeat this procedure three times. At this point, the fixed cells can be stored for a week at 4°C or slides can be made immediately.
10. Using a Pasteur pipet, drop two to three drops of the resuspended cells onto a chilled slide from about 20 cm. Blow gently onto the surface, and place the slide onto a hot plate at 60–65°C (just too hot to keep the palm of one's hand on the plate).
11. Leave the slide to dry for 5 min and then place in a staining chamber (a Coplin jar) ensuring that the surfaces do not touch (*see* Note 11).
12 Stain the karyotypes with Giemsa for 3 min.

13 Wash the slides by dipping the slides through three additional Coplin jars each containing 50 mL of water
14 Dry the slides and count the chromosome number under the microscope, or process for banding (*see* Chapter 27)

3.6. Serum and Media Testing

Before a new batch of serum or media is purchased, it is advisable to obtain a sample from the manufacturer and test its growth-supporting characteristics. This is particularly important for serum. I usually select two of the most used cell types in the lab—currently these are a human diploid fibroblast strain and CHO cells—to test their growth and plating efficiencies (*see* Note 12).

1 Make up individual aliquots of growth media, all containing the same media batch, but with the different test sera and including the serum batch currently being used (or vice versa if you are testing media batches)
2. Plate the cells into 15 dishes for each test media at 5×10^5 cells/60 mm tissue-culture dish and containing 5 mL of the media
3 Every day for 5 d thereafter, trypsinize the cells from triplicate plates and determine the cell number/plate
4 Plot a growth curve (log cell number vs time), and calculate the doubling time and saturation density
5 At the same time as setting up the growth curves, seed in triplicate 60-mm dishes containing 5 mL of the appropriate media with 100 and 200 cells (6 plates/test)
6 After 10–12 d fix the culture for 15 min by flooding with formalin
7 Tip the media and formalin down the drain, and stain the clones with methylene blue
8 Leave the stain for 15 min, and then wash it away with water
9 Leave the plates stacked up against each other to dry in a 37°C room
10 Count the colonies
11. The three parameters of doubling time, saturation density, and plating efficiency should allow the section of a serum (or media) that gives optimal growth (*see* Note 13)

4. Notes

1 The shelf-life of a powdered medium is several years Once reconstituted, however, this is reduced to 2–3 mo, mainly because glutamine is unstable If older medium is used, the glutamine should be replenished (292 µg/mL) The pH of a medium, on storage, should not be allowed to rise, and to achieve this, good plastic caps with close-fitting rubber inserts should be used I also find it useful to seal the caps with a strip of Parafilm®, since this prevents condensation around the cap rim and, thus, minimizes the risk of fungal contamination. Medium containing HEPES can also be used to avoid bicarbonate buffering. I have never been entirely happy, however, with the cell's long-term growth characteristics in HEPES-containing medium.

2. α-MEM is a rich, multipurpose medium developed by Stanners et al *(12)* to grow hamster cells I have not had the experience of any mammalian cell type that will not grow in this medium, including hybridomas It is slightly more expensive than most media, however, and many cells will tolerate less rich and, therefore, cheaper media
3 Purified trypsin can also be used and is sometimes necessary, e.g , for macrophage cell lines *(13)*, but it is much more expensive and usually not necessary The citrate chelates Mg^{2+} and Ca^{2+} and replaces EDTA (Versene) in the buffer
4 It is advisable to keep fibroblast cultures from individual animals distinct, since it may be required to distinguish between individuals genetically
5 If the explant is not necrotic, it is possible to remove it with sterile forceps and transfer it to a new culture flask for further outgrowth of cells.
6 The detailed derivation of the various CHO stains is given in Gottesman *(7)*. It should be noted that CHO is a proline auxotroph and should always be maintained in proline-containing medium.
7. CHO cells can maintain viability, providing the medium pH does not become alkali, at 4°C for extended periods of time (7–10 d). Cultures in capped bottles can therefore be moved to the cold room to avoid subculture under desperate circumstances
8 Primary cell cultures may also be grown on microcarriers in suspension culture Full details of this technology are given in Chapter 5
9 The Coulter counter should have a 140-μ*M* aperture and the thresholds set as described in the machine's Instruction Manual. Serum in the PBS prevents cells from aggregating and giving unreliable counts The counter sometimes gets partially blocked, only experience of the time taken for each count and for the cell's particular display on the spectroscope will indicate problems with counting. Gentle brushing of the orifice with a camel-hair brush will unblock the counter. The Coulter counter can also give a visual display of cell volumes This, when combined with a pulse height analyzer, can be used quantitatively to measure cell volume or to determine cell viability by estimating the amount of cell debris in a sample.
10 To maintain genetically pure cell lines, it is absolutely essential not to cross-contaminate cultures To ensure this, fresh pipets must *always* be used at every step. Do not re-enter a media bottle with a pipet that has been near a culture Similarly, *never* pour from a media bottle into a culture. Splash-backs can occur. If you have more than one culture at a time in a tissue culture hood, only one of these should be opened at any one time. Meticulous attention to these small details will prevent the cross-contamination scandals (e.g., HeLa cells in all cultures!) that one so often reads about
11 It is usual to prepare one slide and check it with phase contrast microscopy so that adjustments can be made on subsequent slides. If there are many nuclei without cytoplasm and a few metaphases, reduce the swelling time. If there are many scattered chromosomes, blow less vigorously. If the metaphase spreads are overlapping, either swell for a longer time (up to 40 min) or blow more vigorously

All these parameters need to be adjusted according to the local environment conditions and cell type (*see* Chapter 27 for greater detail and ref. *14*).

12. If many cell lines are being used, it is often impractical to test the serum out on all the cell types. Usually the most difficult to grow are chosen for the test, but caution needs to be exercised since I once had a batch of serum that supported the cloning and growth of primary diploid fibroblasts but failed to allow cloning of CHO cells!
13. This procedure need only be performed about once every year Enough serum can then be ordered for the next year, since the serum is stable at –20°C for at least 2 yr We used to check our serum using [^{3}H]-thymidine incorporation 1 d after seeding the cells, but given the hazard of using radioactive thymidine, we abandoned this procedure. It is less labor-intensive, however, than measuring growth curves and gives perfectly adequate results. Details of measuring radioactive isotope incorporation into acid insoluble material may be found in Chapter 9, Volume 1 of the *Methods in Molecular Biology* series

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2

Establishment, Maintenance, and Cloning of Human Dermal Fibroblasts

Gareth E. Jones and Clare J. Wise

1. Introduction

The widespread use of human diploid fibroblasts in many tissue-culture-based systems has its origins in the pioneering work on cellular senescence by Hayflick *(1)*. He established a reliable protocol for the maintenance of fibroblast strains that was also favorable for stimulating cell proliferation. The reproducibility of the cell cultivation system he developed allowed the realization that normal diploid cells exhibit a limited proliferative potential in vitro. Depending on the age of the donor and the biopsy site, human dermal fibroblasts can reach over 50 population doublings before senescence sets in *(2)*. This high value is of great advantage to laboratories wishing to study some aspect of cell function in a normal diploid cell population. With the development of reliable cryopreservation protocols, it is possible to build up a substantial bank of early to mid passage fibroblasts from a single primary culture derived from a biopsy. This bank of stable diploid cells thus has many attributes usually only found in transformed cell lines where homogeneity of cell population over a prolonged period of time is often crucial to the experimenter. Unlike a transformed line however, human fibroblasts maintained, as described here, generally retain the normal diploid karyotype.

A second consequence of the proliferative potential of human fibroblasts is that they can be successfully cloned. In most other species, and especially in rodents, a culture crisis will arise after some 5–7 passages in which diploid cells will cease undergoing mitosis. Subsequent random events will often throw up a spontaneously transformed clone that will take over the culture dish *(3)*. Although this transformant may well be useful, it is no longer a normal diploid cell and it is thus unsuitable for many purposes. Given such behavior, cell clon-

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ing with the aim of using stable diploids is out of the question, but human fibroblasts have been found to be quite agreeable to the rigours of cloning. This allows the experimenter to derive clonal populations of cells, as for example in the cases of females who are heterozygous for an X-chromosome linked phenotype of interest *(4)*.

This chapter describes the basic procedures that enable successful establishment of human diploid fibroblasts and gives two methods by which early passage cells may be cloned. The success of our cloning method is largely the result of the use of adsorbed fibronectin as a seeding substratum; fibroblastic cells bind avidly to fibronectin via cell surface integrins resulting in high cell seeding efficiency.

There are a few problems unique to collecting human biopsy material. First, the tissue sample is usually taken for sound clinical reasons, such as aiding diagnosis. The needs of the pathologist must be met first, and it is essential that your wishes should have been communicated to the clinician in charge well in advance of the biopsy. Second, there is the problem of repeat sampling; multiple biopsy of one human is rare since it often cannot be clinically justified. Similarly, one cannot always be confident that the site of biopsy is consistent between patients. For these and other reasons, it is virtually obligatory to obtain the active participation of clinical colleagues who are well versed in the requirements of a prolonged research program plus the backing of the hospital ethical committee for your project. Lack of attention to these early considerations will probably render your research efforts useless.

2. Materials

1 Basal salt solution, with Hank's balanced salt solution (HBSS, ICN Flow, Thame, Oxfordshire, UK) (*see* Appendix)
2. Growth medium containing: 10% fetal calf serum (heat inactivated) (Globepharm), 1% penicillin/streptomycin (ICN Flow), 1% L-glutamine (ICN Flow) (*see* Note 1).
3 Calcium- and magnesium-free phosphate buffered saline (PBS-A) (Oxoid, Basingstoke, Hampshire, UK)
4 0.04% EDTA (Sigma, St Louis, MO) in PBS-A.
5. 0 25% Trypsin/0 02% EDTA solution in HBSS (Gibco BRL, Paisley, Scotland, UK).
6 Stock solution of human plasma fibronectin (Sigma, St Louis, MO) prepared following the instructions of the supplier, diluted to 10 µg/mL in PBS-ABC (with calcium and magnesium salts).

3. Methods

3.1. Collection of Biopsy

1 Provide a labeled container of medium to your clinical collaborator. A 20-mL sterile universal tube, with leak proof cap is ideal, about half full of complete culture medium.