Telethon Network of Genetic Biobanks (TNGB)

STANDARD OPERATING PROCEDURES (SOPs)

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DNA EXTRACTION FROM BLOOD OR LYMPHOCYTES WITH PHENOL/CHLOROFORM - AUTOGEN®AUTOMATED

The cell and nuclear membranes are destroyed by the combined action of SDS and proteinase K. The cell debris from lysis, mainly protein in nature, is captured by organic solvents such as phenol and chloroform.

The DNA, which is insoluble in the organic phase, is present in the non-miscible aqueous phase.

The DNA is precipitated, washed, dried and redissolved in solution in an aqueous buffer.

Equipment and Materials

- 50 ml SARSTEDT tubes
- Nunc tubes 4.5 ml
- An aspiration system including : a pump, a vacuum trap, a bottle.
- 2-3 DONASET tubules and 1-2 needles (lumbar puncture type)
- Centrifuge
- Agitator incubator at 37°C
- AutoGen® extractor, NA-2000 (Geneworx)
- Hole tubes units, AGPT 5000 (Geneworx)
- Nunc tubes 4.5 ml
- Disposable gloves

1. Reagents, media & solutions

To be kept at room temperature :

- TRIS Base PM 121.1g (1kg)
- Magnesium Chloride PM 203.3 g (1kg)
- NaCl PM 58.44 g (1kg)
- EDTA PM 372.4 g (1kg)
- HCl 37% (1L)
- NaOH (PASTILLES) (500 g)
- SDS 20% (500 ml)
- Absolute ethanol
- Phenol / KOAc - AUTOGEN
- Ethanol / Butanol - AUTOGEN
- Propanol
- TE 10¹
- Ethanol 70%

To be stored at 4°C :

- Proteinase K (500 mg)

2. Preparation of stock solutions

These stock solutions should be stored at 4°C and are used for the preparation of working solutions.

TRIS 2M pH 7.6
- Weigh 242.2g of TRIS Base
- 800ml distilled water

Adjust to pH 7.6 with HCl (~ 95 ml)
- Distilled water to 1000ml

$MgCl_2$ 1M
- Weigh 203.3g of $MgCl_2$
- Distilled water to 1000 ml

NaCl 3M
- Weigh 175.32g of NaCl
- Distilled water to 1000 ml

EDTA 0.4 M PH 8
- Weigh 148.96 g of EDTA
- Weigh 18 g of NaOH
- Water 800 ml
- After dissolving, adjust to pH 8 by adding NaOH, pastille by pastille
- Distilled water to 1000 ml

3. Preparation of working solutions

These working solutions are prepared in sterile graduate cylinders using sterile distilled water (cover with parafilm to mix) and stored at +4°C.

SLR

TRIS 2M pH 7.6	10 ml
$MgCl_2$ 1M	10 ml
NaCl 3M	6.6 ml
Distilled water to	2000 ml

SLB

TRIS 2M pH 7.6	10 ml
EDTA 0.4 M pH 8	50 ml
NaCl 3M	34 ml
Add 500 ml water, shake (parafilm)	
SDS 20%	20 ml
Distilled water to	2000 ml

SLB Solution / Proteinase K

Gloves must be worn

Dissolve 500 mg of proteinase K powder (stored at +4°C) in 10 ml SLB. Pour this solution in a 2 L graduated cylinder containing 500 ml of SLB, rinse the flask with 10 ml of this mixture, and adjust the volume of SLB to 1250 ml.

Freeze this solution at -20°C in small aliquots.

TE 10[1]

TRIS 2M - pH7.6	5 ml
EDTA 0.4 M - pH8	2.5 ml
Distilled water	to 1000 ml

Saline

NaCl 3M	50 ml
Distilled water	to 1000 ml

Ethanol Solution 70 %

TE 10[1]	600 ml
Absolute ethanol	to 2000 ml

Procedure

1. Lysis of red blood cells

1.1 Discarding plasma

- Pool the tubes of blood in a 50 ml GREINER tube
- Centrifuge for 10 min at 2000 rpm
- Aspirate the plasma without touching the leukocyte layer (buffy coat)
- Mix the red blood cells and leukocytes

1.2 Lysis of red blood cells

- Lyse in a 50 ml final volume with a solution of SLR
- Centrifuge for 10 min at 2000 rpm
- Aspirate about 45 ml of lysed red blood cells. Re-suspend the pellet using a sterile pipette, and make up to 50 ml with SLR
- Centrifuge for 5 min at 2000 rpm
- Remove the supernatent
- If red blood cells still remain, resuspend the cellular pellet in 5 ml of SLR
- Proceed to a third centrifugation and remove the supernatant
- Freeze the pellet at -80°C

2. Lysis of leukocytes

- Defrost the pellet in a water bath at 50°C for one minute
- Lyse the leukocytes in one volume of SLB + proteinase K (see 3.3); the volume to use depends on the size of the pellet:
    - very small pellet: 5 ml
    - small pellet: 10 ml
    - nice pellet: 15 ml
- Homogenize the tube with gentle rotation
- Incubate overnight at 42°C with agitation

3. Extraction of proteins by AutoGen®

- Check the level of reagents in the apparatus

- Place the lysate in the extractor tubes
- Turn on the apparatus; the cycle lasts for about one hour, during which the lysate is mixed with the phenol/chloroform/potassium acetate reagent and the DNA is then precipitated with the ethanol/butanol mixture. The DNA is then collected in new extractor tubes
- Transfer the precipitated DNA "medusa" to a 4.5 ml Nunc tube
- Wash the "medusa" with 1 ml of isopropanol, then 3 times with 1 ml of 70% ethanol
- Let the medusa dry until it becomes translucid
- Add TE 10^1 (0.1 to 1 ml depending on the size of the medusa)
- Release the medusa by shaking the tube
- Incubate overnight at 37°C with rotation
- Leave the tubes at 4°C for about one week
- Then you can store the tube at -20°C for a long period of time

4. Check quantity/quality of DNA

(see QUALITY CONTROL OF DNA protocol)

DNA EXTRACTION FROM LYMPHOCYTES

Cells should be washed and treated under the same conditions as for whole blood (see MANUAL DNA EXTRACTION FROM BLOOD OR LYMPHOCYTES WITH PHENOL/CHLOROFORM PROTOCOL).

Equipment and Materials

- Saline solution:

NaCl 3M	50 ml
Distilled water	to 1000 ml

- SLR:

TRIS 2M pH 7.6	10 ml
$MgCl_2$ 1M	10 ml
NaCl 3M	6.6 ml
Distilled water to	2000 ml

- 50 ml SARSTEDT tubes
- Centrifuge

Procedure

The cell suspension must be treated on the day it arrives, imperatively.

1. Homogenize the culture flask with gentle manual agitation
2. Transfer the culture to a 50 ml tube
3. Centrifuge at 2000 rpm for 10 min
4. Decant the supernatant
5. Wash the pellet with 10 ml saline solution
6. Centrifuge for 5 min at 2000 rpm
7. Decant the supernatant
8. Wash the pellet with 10 ml SLR
9. Centrifuge for 5 min at 2000 rpm
10. Decant the supernatant
11. Cells may be frozen at -80°C at this stage, or be lysed for DNA extraction

DNA will be extracted from the cells using the same procedures as for cells from whole blood (see corresponding protocols: Salt Method, Phenol/Chloroform, Autogen®)

MANUAL DNA EXTRACTION FROM BLOOD THROUGH SALTING OUT PROCEDURE

One of the obstacles encountered when extracting DNA from a large number of samples is the cumbersome method of DNA extraction with the organic solvents phenol and chloroform.

This method is rapid, safe and does not require expensive and environmentally hazardous reagents and equipment.

Equipment and Materials

- Polypropylene tubes 15ml
- Lysis buffer (10mM Tris-HCL,400mM NaCl, 2mM Na_2EDTA, pH 8.2)
- SDS 10%
- Proteinase K solution (1 mg proteinase K in 1% SDS and 2 mM Na_2 EDTA)
- Centrifuge
- Absolute ethanol
- TE buffer (10mM Tris-HCL, 0.2mM Na_2 EDTA, pH 7.5)
- Disposable gloves
- Gilson pipette

Procedure

1. Resuspend the buffy coats of nucleated cells obtained from blood with anticoagulants (ACD or EDTA) with 3ml of nuclear lysis buffer
2. Digest the cell lysates, with 0.2 ml of 10% SDS and 0.5 ml of proteinase K solution, overnight at 37 °C
3. Add 1ml of saturated NaCl (6M) to each tube and shake vigorously for 15 seconds
4. Centrifuge for 15 minutes at 2500 rpm
5. Transfer the supernatant containing the DNA to another 15ml polypropylene tube, the precipitated protein pellet is left behind at the bottom of the tube
6. Add 2 volumes of absolute ethanol and invert the tubes several times until the DNA precipitates
7. Remove the precipitated DNA with a plastic spatula or pipette and transfer to a 1.5ml microcentrifuge tube containing 100-200 microliters TE buffer
8. Dissolve the DNA for 2 hours at 37°C
9. Store the tube at +4 or -20°C.
10. Check quantity/quality of DNA (see QUALITY CONTROL OF DNA protocol)

Reference

Miller S.A, Dykes D.D, Polesky H.F : A simple salting out procedure for extracting DNA from human nucleated cells._ Nucleic Acids Research 1988; V16 Number 3:1215

MANUAL DNA EXTRACTION FROM BLOOD OR LYMPHOCYTES WITH PHENOL/CHLOROFORM

The cell and nuclear membranes are destroyed by the combined action of SDS and proteinase K. The cell debris from lysis, mainly protein in nature, is captured by organic solvents such as phenol and chloroform.

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- Nunc tubes 4.5 ml
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- NaCl PM 58.44 g (1kg)
- EDTA PM 372.4 g (1kg)
- HCl 37% (1L)
- NaOH (PASTILLE) (500 g)
- SDS 20% (500 ml)
- Absolute ethanol
- Phenol
- Chloroform (1l)
- Propanol (1l)

To be stored at 4°C :

- Proteinase K (500 mg)

2. Preparation of stock solutions

TRIS 2M pH 7.6

- Weigh 242.2g of Tris Base
- 800ml distilled water
- Adjust to pH 7.6 with HCl (☐ 95 ml)
- Distilled water to 1000 ml

$MgCl_2$ 1M

- Weigh 203.3g of $MgCl_2$

- Distilled water to 1000 ml

NaCl 3M
- Weigh 175.32g of NaCl
- Distilled water to 1000 ml

EDTA 0.4 M PH 8
- Weigh 148.96 g of EDTA
- Weigh 18 g of NaOH
- Water 800 ml
- After dissolving, adjust to pH 8 by adding Na OH, pastille by pastille
- Distilled water to 1000 ml

These stock solutions should be stored at +4°C and are used for the preparation of working solutions.

Working solutions are prepared in sterile graduate cylinders using sterile distilled water (cover with parafilm to mix) and stored at +4°C.

3. Preparation of working solutions

3.1 SLR

TRIS 2M pH 7.6	10 ml
$MgCl_2$ 1M	10 ml
NaCl 3M	6.6 ml
Distilled water to	2000 ml

3.2 SLB

TRIS 2M pH 7.6	10 ml
EDTA 0.4 M pH 8	50 ml
NaCl 3M	34 ml
Add 500 ml water, shake (parafilm)	
SDS 20%	20 ml
Distilled water to	2000 ml

3.3 SLB Solution / Proteinase K

Gloves must be worn

Dissolve 500 mg of proteinase K powder (stored at +4°C) in 10 ml SLB. Pour this solution in a 2 L graduated cylinder containing 500 ml of SLB, rinse the flask with 10 ml of this mixture, and adjust the volume of SLB to 1250 ml.

Freeze this solution at -20°C in small aliquots.

3.4 TE 10[1]

TRIS 2M pH7.6	5ml
EDTA 0.4 M pH8	2.5 ml
Distilled water	to 1000 ml

3.5 Saline

NaCl 3M	50 ml
Distilled water	to1000 ml

3.6 Ethanol Solution 70 %

TE 10[1]	600 ml

Absolute ethanol	to 2000 ml

Procedure

1. Lysis of red blood cells

1.1 Discarding plasma

- Pool the tubes of blood in a 50 ml GREINER tube
- Centrifuge for 10 min at 2000 rpm
- Aspirate the plasma without touching the leukocyte layer (buffy coat)
- Mix the red blood cells and leukocytes

1.2 Lysis of red blood cells

- Lyse in a 50 ml final volume with a solution of SLR
- Centrifuge for 10 min at 2000 rpm
- Aspirate about 45 ml of lysed red blood cells -Resuspend the pellet using a sterile pipette, and make up to 50 ml with SLR
- Centrifuge 5 min at 2000 rpm
- Remove the supernatent
- If red blood cells still remain, resuspend the cellular pellet in 5 ml of SLR
- Proceed to a third centrifugation and remove the supernatant.
- Freeze the pellet at -80°C

2. Lysis of leukocytes

- Defrost the pellet in a water bath at 50°C for one minute
- Lyse the leukocytes in one volume of SLB + proteinase K (see 3.3); the volume to use depends on the size of the pellet:
    - very small pellet: 5 ml
    - small pellet: 10 ml
    - nice pellet: 15 ml
- Homogenize the tube with gentle rotation
- Incubate overnight at 42°C with agitation

3. Extraction of proteins

- Step 1:
- Add one volume of phenol-chloroform equal to the volume of SLB-Proteinase K-
- Stir for 10 min
- Aspirate the lower phase after stabilization or after centrifugation for one minute at 2000 rpm
- Repeat Step 1, with 5 minute-agitation
- Depending on Step 1, proceed to the extraction with chloroform, with 5 minute-agitation at each step.

4. DNA precipitation & wash

- Precipitate the DNA with 1 to 1.5 volumes of absolute isopropanol (propanol 2) in the presence of NaCl, 60 mM final concentration
- Homogenize with gentle rotation until the DNA precipitate (medusa) appears
- Discard the liquid by transferring the medusa to a 5 ml Nunc tube
- Wash the medusa twice with 3 ml of isopropanol.
- Wash the medusa three times with 3 ml of 70% ethanol in TE 10^1
- Remove the liquid between each wash
- Dry the medusa at room temperature

5. Resuspension of DNA

- When the medusa is translucid and therefore dry, resuspend it in TE 10^1(depending on the size of the medusa, suspend the DNA in 0.5 to 2 ml of TE 10^1).
- Incubate overnight at 37°C with rotation
- Place it at 4°C for one week
- Then you can store the tube at -20°C for a long period of time

6. Check the quantity/quality of DNA

(see QUALITY CONTROL OF DNA protocol)

AUTOMATED DNA EXTRACTION FROM BLOOD WITH CHEMAGICSTAR® PLATFORM

Fully-automated genomic DNA extraction from large (3-5 ml) blood samples is performed using the ChemagicSTAR® robotic system [Hamilton (Carnforth, United Kingdom) and Chemagen (Baesweiler, Germany)] and the Chemagic DNA Blood 5k kit (Chemagen). This platform is a Microlab STAR (Hamilton) liquid handler coupled with a Chemagen Magnetic Separator Module, allowing the simultaneous extraction from 12 samples in about 45 minutes.

The extraction steps are performed in 50 ml Falcon tubes, which are organized in 4x3 buckets in order to fit into the Chemagic Magnetic Separator Module. The module consists of an electromagnet and a metal 12-rod head. The magnetisable rods are dipped into the Falcon tubes, which contain the samples and a magnetic bead suspension. When the electromagnet is switched on, the rods become magnetic and the beads are separated.

Equipment and Materials

- ChemagicSTAR® platform
- Falcon tubes 50 ml
- Deep-well plates
- Disposable gloves

1. Reagents, media and solutions

All reagents are provided with the Chemagic DNA Blood 5k kit (Chemagen).

To be kept at room temperature:

- Magnetic Beads
- Lysis Buffer
- Binding Buffer
- Wash Buffer 3
- Wash Buffer 4
- Wash Buffer 5
- Elution Buffer 6 (10 mM Tris-HCl pH 8.0)

To be stored at 4°C:

- Protease

Procedure

- Briefly vortex the blood sample and place 5 ml into a 50 ml tube.
- Add 20 µl Protease and then 7.5 ml of Lysis Buffer to the tube, mix by pipetting, and incubate 5 min at RT.
- Add 600 µl of well-resuspended Magnetic Beads and then 19 ml Binding Buffer to the sample, mix thoroughly by pipetting and incubate 5 min.
- Place the tube in a Chemagic Stand and leave 4 min to separate all of the beads. Discard the supernatant and then remove the tube from the Chemagic Stand.
- Add 10 ml Wash Buffer 3 to the tube, thoroughly resuspend the pellet by vigorous

vortexing for 1 min and incubate for 2 min.

- Place the tube in a Chemagic Stand and leave 3 min to separate all beads.
- Discard the supernatant and remove the tube from the Chemagic Stand. Repeat the washing procedure using 10 ml of Wash Buffer 4 and then using 10 ml of Wash Buffer 5. After removing the last traces of Wash Buffer 5, leave the tube in the magnetic separator.
- While leaving the tube against the magnet, and the beads attracted to the side of the tube, gently add 30 ml of Wash Buffer 6, being careful not to disrupt the pellet. Incubate for 1 min and immediately discard the supernatant and then remove the tube from the Chemagic Stand.
- Add 0.5-1 ml Elution Buffer and then completely resuspend the Magnetic Beads/DNA complex pellet by vortexing for 20 s.
- Incubate the suspension for 10 min at room temperature with gentle agitation to facilitate DNA elution.
- Place the tube in a Chemagic Stand 50k and leave 3 min to attract all of the beads and then carefully transfer the eluate containing the purified DNA in a clean tube or deep-well plate.
- Check quality/quantity of DNA.

All manipulations are performed using disposable equipment and sterile solutions to avoid sample contamination.

The quality of the extracted DNA is always verified by measuring adsorbance at 260, 280, and 230 nm (with the calculation of 260/280 and 260/230 ratios) using the NanoDrop ND-1000 spectrophotometer, which is also used for DNA quantitation. Bad-quality DNAs (in terms of <1.5 260/280 ratio values) are subjected to a manual phenol/chloroform standard purification.

All DNAs are routinely standardized for concentration (40 ng/uL) and arrayed into 96-well plates. A back-up copy of the standardized plate is conserved.

The isolated DNA can be used directly in a variety of downstream applications, such as PCR, MLPA, Sanger and next-generation sequencing.

MANUAL EXTRACTION OF TOTAL RNA FROM WHOLE BLOOD

The isolation of total RNA from 2.5 ml human whole blood samples is performed using the PAXgene Blood miRNA Kit (Qiagen, Hilden, Germany), which allows the purification of all different RNA species longer than 18 nucleotides. Hence, the purified RNA includes both mRNAs and small RNAs such as microRNAs (miRNAs).

Blood samples are collected in PAXgene Blood RNA Tubes (Qiagen), which contain a proprietary reagent that lyses blood cells and immediately stabilizes intracellular RNA, thus preventing degradation by RNases and minimizing *ex vivo* changes in gene expression. RNA stabilization is critical for reliable downstream gene expression analysis. Without stabilization, degradation of RNA and upregulation or downregulation of transcripts start soon after blood is drawn. Blood samples collected in PAXgene Blood RNA Tubes can be safely stored or transported at 15-25°C for up to 72 hours, at 2-8°C for up to 5 days, or at -20°C or -70°C for at least 50 months without showing any significant RNA degradation or changes in transcript levels.

All manipulations are performed using disposable equipment and sterile solutions to avoid sample contamination with RNases. Purification is carried out manually using silica-based RNA purification technology in a spin-column format, following the manufacturer's protocol.

Equipment and Materials

- PAXgene Blood RNA Tubes
- PAXgene RNA Spin Columns
- PAXgene Shredder Spin Columns
- Processing Tubes 2 ml
- Microcentrifuge Tubes 1.5 ml
- Refrigerated benchtop microcentrifuge
- Thermomixer (Eppendorf)
- Disposable gloves

1. Reagents, media and solutions

- Buffer BM1 (resuspension buffer)
- Buffer BM2 (binding buffer)
- Buffer BM3 (wash buffer concentrate)
- Buffer BM4 (wash buffer concentrate)
- Buffer BR5 (elution buffer)
- RNase-Free Water
- Proteinase K
- RNase-Free DNase I
- Buffer RDD (DNA digestion buffer)
- Isopropanol (99%)
- Ethanol (95-99%)

2. Preparation of working solution:

Buffers BM2 and BM3 may form a precipitate upon storage. If necessary, warm to 37°C to dissolve before use.

Buffers BM3 and BM4 are supplied as a concentrate. Before using for the first time, add the appropriate volume of ethanol (96-100%, RNase-free), as indicated on the bottle, to obtain a working solution.

Add 10 µl DNase I stock solution to 70 µl Buffer RDD in a 1.5 ml microcentrifuge tube. Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.

Procedure

- Equilibrate the PAXgene Blood RNA Tube to room temperature (RT), and then store it at RT for 2 hours before starting the procedure.
- Centrifuge for 10 min at 3,000-5,000 x g using a swing-out rotor.
- Remove the supernatant, add 4 ml RNase-free water to the pellet and close the tube with a clean cap.
- Vortex until the pellet is visibly dissolved, and centrifuge for 10 min at 3,000-5,000 x g using a swing-out rotor.
- Remove the entire supernatant, add 350 µl Buffer BM1 to the pellet and mix by vortexing.

2. Protein removal

- Transfer the sample into a 1.5 ml microcentrifuge tube.
- Add 300 µl Buffer BM2 and 40 µl proteinase K. Mix by vortexing for 5 s, and incubate for 10 min at 55°C in a thermomixer at 400-1,400 rpm.
- Pipet the sample into a PAXgene Shredder spin column placed in a 2 ml processing tube, and centrifuge for 3 min at full speed.
- Carefully transfer the entire supernatant of the flow-through from the PAXgene Shredder spin column to a new 1.5 ml microcentrifuge tube without disturbing the pellet.
    - Add 700 µl of isopropanol, and mix by vortexing.

3. DNA isolation & wash

- Pipet 700 µl sample into the PAXgene RNA spin column, and centrifuge for 1 min at 8,000-20,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through. If the volume of the sample is greater than 700 µl, repeat this step until the entire sample is processed.
- Add 350 µl Buffer BM3 to the PAXgene RNA spin column, and centrifuge for 15 s at 8,000-20,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old one.
- Add 80 µl of DNase I directly onto the PAXgene RNA spin column membrane, and incubate at RT for 15 min.
- Add 350 µl Buffer BM3 to the column, and centrifuge for 15 s at 8,000-20,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old one.
- Add 500 µl Buffer BM4 to the column, and centrifuge for 15 s at 8,000-20,000 x g. Discard the flow-through and place the spin column in a new 2 ml processing tube.

- Add another 500 µl Buffer BM4 to the PAXgene RNA spin column, and centrifuge for 2 min at 8,000-20,000 x g.
- Discard the flow-through, and place the PAXgene RNA spin column in a new 2 ml processing tube. Centrifuge at 8,000-20,000 x g for 1 min. It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions.

4. DNA elution

- Discard the flow-through. Place the PAXgene RNA spin column in a new 1.5 ml microcentrifuge tube, and pipet 40 µl Buffer BR5 directly onto the spin column membrane, and centrifuge for 1 min at 8,000-20,000 x g.
- Repeat the elution step as described, using 40 µl Buffer BR5 and the same microcentrifuge tube.
- Incubate the eluate for 5 min at 65°C to denature the RNA for downstream applications, then chill immediately on ice.
- Store RNA samples at –20°C or –70°C.

The integrity of the extracted RNA is always verified by agarose gel electrophoresis and the RNA quantitated using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

The purified total RNA is ready to use and is ideally suited for any downstream application, including:

- RT-PCR and real-time RT-PCR
- Differential display
- cDNA synthesis
- Northern, dot, and slot blot analyses
- Primer extension
- Poly A+ RNA selection
- RNase/S1 nuclease protection
- Microarrays
- RNA sequencing

ISOPENTANE FREEZING PROTOCOL FOR MUSCLE BIOPSY

Equipment and Materials

- Polystyrene container for liquid nitrogen
- Floating platform (polystyrene) with punched hole for plastic cold-resistant container (see diagram below)
- Adhesive gum (or tissue teck)
- Cork supports
- Isopentane
- Cryotubes (2 ml) for conditioning tissues

Procedure

1. Prepare the polystyrene container. Fill to three-quarters mark
2. Take the floating platform with the punched hole, and put the "isopentane" container on it. Then pour the isopentane into the container up to the mark
3. Carefully place the floating platform with the isopentane on top of the nitrogen (it will float), allow the isopentane to cool until white crystals appear at the bottom of the container
4. Place a drop of adhesive gum or "Tissue-Teck" on the cork support, place the tissue on the gum, and drop the whole thing into the cooled isopentane. Leave for 2 minutes
5. Label the cryotubes
6. Cool the cryotubes in liquid nitrogen (30 seconds) BEFORE placing the tissue-cork-gum constructions in them
7. Store the cryotubes at -80°C or in a liquid nitrogen container

Reference

Dubowitz V., Sewry C.A. Muscle Biopsy: A Practical Approach, 3rd Edition, Saunders Elsevier, 2007

NITROGEN FREEZING PROTOCOL FOR MUSCLE BIOPSY

Equipment and Materials

- Cryotubes (2 ml) with screw-top and silicon joints
- Liquid nitrogen
- Dry ice
- "Transport form-human tissue specimens"[1] containing the information necessary for optimal utilization of tissues (this information will be rendered anonymous ; the donor cannot be identified)

Procedure

- Place the specimen in a dry tube
- Close the tube and label it (please note that certain types of ink are erased in liquid nitrogen)
- Plunge the tube in liquid nitrogen
- Fill out the specimen fact sheet if necessary
- Store the tube in a freezer at -80°C or in dry ice until sending it, or collection by our services

Reference

Dubowitz V., Sewry C.A. Muscle Biopsy: A Practical Approach, 3rd Edition, Saunders Elsevier, 2007

1 The specimen fact sheet is provided by the bank

FREEZING AND THAWING OF VITAL MUSCLE BIOPSY FOR CULTURE

Equipment and Materials

- Laminar flow hood
- CO_2 incubator
- Inverted microscope
- Petri dishes 60 mm
- Sterile surgical Instruments for microdissection
- Medium for collecting human muscle biopsy
- Conditioning medium
- Ham's F14 proliferating medium
- Freezing medium
- Human plasma
- Cryotubes
- Freezer (minus 20-25°C)
- Freezer (minus 70-90°C)
- Dewar 1-2 litres
- Liquid N_2
- Tank for liquid N_2

Medium for collecting human muscle biopsy

DMEM	500 ml
Penicillin-streptomycin solution 100x	6.25 ml
Gentamicin solution (50 mg/ml)	0.5 ml
Fungizone (250 microg/ml)	5 ml
Filter and store at +4°C	up to 2 months.

Conditioning medium

Medium 199	49 ml
Foetal bovine serum	30 ml
Penicillin-streptomycin solution 100x	1 ml
Fungizone (250 microg/ml)	0.8 ml
Filter and store at +4°C	up to 2 months

Ham's F14 Proliferating medium

Nutrient mixture Ham's F14	390 ml
Foetal calf serum	100 ml
Penicillin-streptomycin solution 100x	5 ml
Insulin	5 ml
L-glutamine	5 ml
Human basic fibroblast growth factor:	25 ng/ml
Epidermal growth factor	10 ng/ml
Filter and store at +4°C	up to 1 month.

Insulin solution
Dissolve 100 mg of insulin in 12 ml of HCl (1N). Add 88 ml of ultrapure water, filter through 0.22 □m filters and aliquot (5 ml) into sterile tubes.

Freezing medium
to be prepared just before use:

DMEM	70 %
Foetal bovine serum	20 %
DMSO	10 %

Procedure

Freezing
Immediately after collection, muscle specimens are placed in medium for collecting human muscle biopsy. They are then cleaned of adipose tissue and blood vessels (see PRIMARY MYOBLAST CULTURE FROM FRESH HUMAN MUSCLE BIOPSY).

The tissue is then cut into small fragments (about 3 mm^3) and transferred to a 60 mm Petri dish containing 5 ml of conditioning medium.

The fragments are maintained in this medium overnight at 37°C in the CO_2 incubator.

The next day the specimens are transferred to cryotubes (3-4 tissue fragments per tube) containing 1 ml of freezing medium B, and left at -20°C per 1.5-2 hours, then transferred at -80°C and left overnight. Finally, they are placed in liquid nitrogen for storage.

Thawing
Muscle specimens stored in freezing medium under liquid nitrogen are thawed quickly at 37°C and rinsed in conditioning medium to remove residual DMSO.

The specimens are then incubated in a freshly-prepared mixture of 80% conditioning medium and 20% human plasma (from donors) and filtered directly on the dish using a syringe with a 0.22 micrometer filter.

About 5-7 days after plasma clot formation, myoblast proliferation will be observed.

At this point the specimens are washed in PBS, trimmed into pieces about 1 mm3 and placed on gelatine-coated dishes.

Gelatine-coated dishes are prepared from 0.1 % bovine gelatine solution which is filtered, poured into 60 mm Petri dishes (3 ml per dish) and left to stand for an hour, then the excess liquid is discarded.

Evenly distribute 10-15 pieces of tissue over the surface of the gelatine.

Add a few drops of Ham's F14 proliferating medium and incubate for an hour.

Add 3 ml of Ham's F14 proliferating medium.

Change the medium twice a week.

After 6-8 days, or when cell growth is sufficient, trypsinize and subculture.

The tissue fragments can be re-used by placing them in new Petri dishes with a few drops of Ham's F14 proliferating medium (see PRIMARY MYOBLAST CULTURE FROM FRESH HUMAN MUSCLE BIOPSY).

Reference

Askanas V. and Engel W.K.: A new program for investigating adult human skeletal muscle grown aneurally in tissue culture. Neurology 25 (1): 58-67, 1975.

DETECTION OF CONTAMINANTS IN HUMAN CELL CULTURE

A- DETECTION OF MICROBIAL AND FUNGAL CONTAMINATION

Cells for research or cell banks must be kept healthy and free of contamination. Continuous use of antibiotics and antifungal agents may sometimes mask the presence of these organisms and can lead to the development of resistant strains that are difficult to eliminate. It is important therefore to check periodically for the presence of contamination.

The first steps in detecting bacterial or fungal contamination are macroscopic and microscopic checking. Visual inspection of the culture flasks or Petri dishes may reveal a turbid medium or yellowish colour (acid pH) immediately leading to the suspicion of contamination. Fungal colonies, when present, are usually clearly evident on visual inspection. Both microbial and fungal contaminations are readily confirmed microscopically.

Once contamination has been ascertained the medium and all other reagents used for this cell line must be discarded. The contaminated culture is destroyed with sodium hypochlorite.

B- DETECTION OF VIRAL CONTAMINATION

As long as serum and natural trypsin are used to supplement media as well as for subculture, respectively, there will always be a risk of infection from these agents. Viral contamination can also arise from the source tissue or from growth media contaminated from other infected cultures. Elimination of viral contamination is difficult and there are no simple universal diagnostic tests to identify viral contamination. Viruses are identified by using a panel of immunological or molecular probes. Such testing is usually restricted to human pathogens such as HIV, HBV and EBV, using PCR techniques. Screening for these viruses should be performed every 3-4 months.

Equipment and Materials

- Deoxynucleotides
- Taq polymerase
- 10x Taq reaction buffer
- Specific primers for the various viral particles
- PCR apparatus
- Disposable gloves
- Gilson pipette

Procedure

HIV

DNA is isolated from cell lines using routine methods. Specific oligonucleotide pairs from the gag region of HIV-1 (SK 38 and SK 39) and from the pol region of HLTV-I/-II (SK 110 and SK 111) are used in a hot start PCR reaction. After 30 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, the amplified products are identified by

agarose gel electrophoresis and visualized by ethidium bromide (Kellogg and Kwok, 1990).

EBV
DNA is isolated from cell lines using routine methods and amplified by PCR using primers from the EB2 gene under the following conditions: 95°C for 30 sec, 65°C for 30 sec., 72°C for 1 min., for 35 cycles. The amplified products are identified by agarose gel electrophoresis and visualized by ethidium bromide (Loughran et al., 1993).

HBV and HCV
The active form of the human pathogenic virus HBV is tested on DNA extracted from the cell culture by routine methods. HCV RNA is prepared from cells by organic extraction followed by DNase digestion. PCR or RT-PCR for HBV and HCV respectively, is carried out as for EBV using primers coding for conserved sequences of the X gene of HBV and for the 5'-UTR of HCV, respectively. (Pontisso et al., 1993).

References

Kellogg D, Kwok S: Detection of human immunodeficiency virus. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR Protocols. A Guide to Methods and Applications. Academic Press, San Diego, 1990, pp. 337-347.

Loughran TP Jr, Zambello R, Ashley R, Guderian J, Pellenz M, Semenzato G, Starkebaum G*:* Failure to detect EBV DNA in peripheral blood mononuclear cells of most patients with large granular lymphocyte leukaemia. Blood 1993; 81: 2723-2727)

Pontisso P, Ruvoletto MG, Fattovich G, Chemello L, Gallorini A, Ruol A, Alberti A: Clinical and virological profiles in patients with multiple hepatitis virus infection. Gastroenterology 1993; 105: 1529-1533

C- DETECTION OF MYCOPLASMA CONTAMINATION

Mycoplasma are sub-microscopic organisms that pass through the 0.22 micrometer filters commonly used for the sterile filtration of media and reagents. Unlike ordinary bacterial contamination, mycoplasma infection does not result in culture medium turbidity and specific tests are required. Several PCR-based methods are now available for the routine detection of mycoplasma (e.g. Mycoplasma Plus PCR Primer Set or Takara PCR Mycoplasma detection set). Recently, a rapid and easy luminometric detection assay (MycoAlert) has become available. Routine tests for mycoplasma contamination should be performed every two months.

METHOD 1 by PCR Timer Set

Equipment and Materials

- Mycoplasma Plus PCR Primer Set or Takara PCR Mycoplasma detection set
- Deoxynucleotides
- Taq polymerase
- 10x Taq reaction buffer
- PCR apparatus
- Disposable gloves

- Micropipettes

Procedure

1. Transfer 100 µl of medium from the test cell culture to a microfuge tube, hold at 95° C (water bath) for 5 minutes, then spin briefly.
2. Add 10 microliter of Strataclean Resin (a phenol-free technique for DNA purification: the solid phase silica-based resin contains hydroxyl groups that react with proteins in much the same manner as the hydroxyl group of phenol).
3. Transfer an aliquot of the treated medium to a fresh tube, leaving all resin behind. This will be used as template in the PCR which is performed according to the manufacturer's instructions.
4. Electrophorese the PCR product on high-grade 2% agarose gel.

References

Hay RJ, Macy ML, Chen TR, Mycoplasma infection of cultured cells. Nature 1989; 339: 487-488.

Wirth M, Berthold E, Grashoff M, Pfutzner H, Schubert U, Hauser H, Detection of mycoplasma contaminations by the polymerase chain reaction. Cytotechnology. 1994;16(2):67-77.

Uphoff CC, Drexler HG, Detection of Mycoplasma contaminations. Methods Mol Biol. 2004;290:13-24.

METHOD 2 by MycoAlert assay kit

MycoAlert Assay☐ Kit is a selective biochemical test that exploits the activity of certain mycoplasma enzymes. The viable mycoplasma are lysed and the enzymes react with the MycoAlert substrate catalysing the conversion of ADP to ATP. By measuring the level of ATP in a sample before and after addition of the MycoAlert substrate, a ratio can be obtained indicating the presence or absence of mycoplasma. If mycoplasma enzymes are not present, the second reading shows no increase over the first, while reaction of mycoplasmal enzymes with their specific substrates in the MycoAlert reagent, results in elevated ATP levels. This increase can be detected by a bioluminescent reaction based on luciferase activity. The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer.

Equipment and Materials

- MycoAlert mycoplasma detection assay
- Luminometer
- Multi-well plate for luminometer
- Bench centrifuge
- Disposable gloves
- Micropipettes

Procedure

1. Bring all the reagents of the kit at room temperature.
2. Reconstitute the MycoAlert Reagent and MycoAlert Substrate in the MycoAlert

Assay Buffer and leave for 15 minutes at room temperature.

3. Transfer 2 ml of cell culture into a centrifuge tube and centrifuge at 1500 rpm for 5 minutes.
4. Transfer 100 microliter of supernatant into a luminescence compatible plate;.
5. Program the luminometer to take a 1 second integrated reading as the default setting.
6. Add 100 microliter of the MycoAlert Reagent to each sample and wait 5 minutes.
7. Place the plate in the luminometer and start the reading (A).
8. Add 100 □l of MycoAlert Substrate to each sample and wait 10 minutes.
9. Place the plate in the luminometer and start the reading (B).
10. Calculate the ratio = reading (B)/reading (A)

The ratio of reading B to reading A is used to determine whether a cell culture is contaminated by mycoplasma. A ratio of less than 1 is produced by the ongoing consumption of ATP over the time course of the assay and is considered as negative. A ratio above 1 indicates contamination. A ratio around 1 is borderline and the sample should be retested.

FREEZING, CRYOPRESERVATION, STORAGE AND REACTIVATION OF CELL LINES

A- FREEZING, CRYOPRESERVATION AND STORAGE OF CELL LINES

Cultures for cryopreservation should be healthy, free of contamination and in log phase growth for several days before freezing.

Equipment and Materials

- PBS without Ca^{+2} and Mg^{+2}
- Trypsin-EDTA 1X
- Petri dishes 100 mm
- DMSO
- Falcon tubes 15 ml
- Refrigerated centrifuge
- Haemocytometer
- Cryotubes
- Freezing medium
- Freezer (minus 20-25°C)
- Freezer (minus 70-90°C)
- Dewar 1-2 litres
- Liquid N_2
- Tank for liquid N_2

Freezing medium
to be prepared just before use:

A:

Foetal bovine serum	90%
DMSO	10%

or

B:

Dulbecco's modified Eagle medium	70 %
Foetal bovine serum	20 %
DMSO	10 %

Procedure

Suspension cultures

1. Count the number of viable cells which should be in log phase (as described in "Routine Cell counting and assessment of viability"). Centrifuge for 7-10 minutes at 200 g (1000 rpm) to pellet cells. Use a pipette to remove the supernatant without disturbing the pellet

2. Re-suspend the cells in freezing medium to a concentration of 5×10^6 to 1×10^7 cells/ml
3. Transfer to sterile cryotubes and close tightly
4. Write date, cell line code and passage number on cryotube
5. Place tubes briefly on ice: start the freezing procedure within 5 minutes
6. The cells should be frozen slowly (at -1°C/min). This is most easily done using a programmable cooler. If such a cooler is not available, transfer the tubes from ice to an insulated pre-cooled box (or wrapped in cotton wool) and place for 2 hours in a freezer at minus 20-25°C; then place in a minus 70-90°C freezer for one or two days. Finally transfer the cryotubes to the liquid nitrogen tank for long-term storage

Adherent cultures

1. Detach cells from 100 mm dish with trypsin-EDTA solution. Subsequently pipette up and down gently and transfer to a 15 ml centrifuge tube
2. Add complete 5 ml of growth medium to inhibit trypsin
3. Take sample to determine viability (as described in "Routine Cell counting and assessment of viability")
4. If sufficiently viable, centrifuge at 1000 rpm for 7-10 minutes; discard the supernatant avoiding disturbing the pellet
5. Suspend the pellet in freezing medium to a concentration of 5×10^6 - 1×10^7 cells/ml.
6. Aliquot into sterile cryogenic tubes
7. Write date, cell line code and passage number on cryotubes
8. Place tubes on ice and begin the freezing procedure within 5 minutes (as described above)

B- REACTIVATION OF FROZEN CELL LINES

Cryopreserved cells are fragile and require gentle handling.

Equipment and materials

- Proliferating medium pre-warmed at 37°C
- Water bath 37-56°C
- Centrifuge
- Laminar flow hood
- CO_2 incubator
- 100 mm Petri dishes
- Pipettes
- Dewar, 1-2 litres
- Proliferating medium
- Haemocytometer

DMEM Proliferating medium

DMEM	390 ml

Foetal bovine serum	100 ml
Penicillin-streptomycin solution 100x	5 ml
Insulin (10 microg/ml final)	5 ml
L-glutamine	5 ml
Human basic fibroblast growth factor	25 ng/ml
Epidermal growth factor	10 ng/ml

Filter and store at +4°C, up to 1 month.

Insulin solution
Dissolve 100 mg of insulin in 12 ml of HCl (1N). Add 88 ml of ultrapure water, filter through 0.22 micrometer filters and aliquot (5 ml) into sterile tubes.

Procedure

Cells are kept in liquid nitrogen until they are ready to be thawed, and the thermostated water bath is at 37°C.

Centrifugation method
1. The cryotubes are placed in the water bath at 37°C and the cells allowed to thaw (requires a few minutes).
2. Pipette gently up and down the suspension to homogenize. Transfer to a 15 ml plastic tube with 5 ml of proliferating medium.
3. Centrifuge at 4°C, 200 g (1000 rpm) for 7 minutes.
4. The cells are placed on ice and the supernatant discarded.
5. The pellet is suspended in 1 ml proliferating medium.
6. 25 □l of cell suspension is transferred to an Eppendorf tube for cell counting (not always necessary).
7. The remaining cells are transferred to one or two 100 mm Petri dishes (depending on the viable cell count: there should be at least 3×10^5 cells/ml) containing 8 ml of proliferating medium.
8. The Petri dishes are placed in an incubator at 37°C and cells allowed to adhere to the surface.

Direct plating method
1. The cells are removed from storage and thawed quickly in a 37°C water bath.
2. The cells are then placed directly in complete growth medium (10 ml per 1 ml of frozen cells).
3. The cells are cultured for 12-24 hours. The medium is removed (to remove cryo-preservative) and fresh complete growth medium added, as described in "Primary myoblast culture from fresh human biopsy".

GOOD PRACTICES FOR CELL CULTURE TECHNIQUES

A- ASEPTIC AND GOOD CELL CULTURE TECHNIQUES

Good cell culture practice is achieved when all procedures are carried out to a standard that precludes contamination by bacteria, fungi and mycoplasma and cross-contamination with other cell lines.

Equipment and Materials

Instruments and tools used for manipulating and maintaining cell cultures are:

- Laminar flow hood (Microflow biological safety cabinet)
- CO_2 incubator
- Inverted microscope with phase contrast
- Refrigerator
- Freezer -20 -25°C
- Freezer -70-90°C
- Centrifuge
- Water bath 37-56°C
- Dewar (1-2 litres capacity) for handling of frozen cells
- Liquid nitrogen tank for storage
- Haemocytometer
- Micropipettes 20-100-1000 microliter, with tips (sterile, nucleic acid and nuclease free)
- Pasteur pipettes
- Disposable sterile filters: Millex-GV 0.22 micrometer (Millipore)
- Wheaton flasks
- Disposable plastic ware:
- Disposable Millipore Sterilcup 0.22 micrometer, 250 ml and 500 ml
- Pipettes 5-10 ml
- Petri dishes 35-60-100 mm
- Culture flasks 25-75 cm^2
- Centrifuge tubes 15-50 ml Falcon
- Eppendorf tubes 1.5 ml safe-lock, autoclaved
- Cryotubes 1.5 and 5 ml
- Syringes 5-10 ml, sterile
- Micro-dissecting surgery instruments:
    - Tweezers with very fine tips
    - Scissors

- Scalpels
- 70% ethanol in distilled water, or other suitable disinfectants
- Disposable gloves

Procedure

Keep all work surfaces clean and tidy.

1. Before commencing work, disinfect the hood cabinet with 70% ethanol. Disinfect gloves by washing them with 70% ethanol and allow to air dry before starting work
2. Disinfect the surfaces of all materials and equipment before putting them into the cabinet
3. Bottles with growth media and reagents are opened only inside the hood. Before closing the bottles, their necks and caps are flamed on a Bunsen
4. To avoid cross contamination, cell lines should be manipulated one at a time under the hood (and all pipettes etc. changed for each new cell line)
5. Cultures and media should be examined daily for evidence of gross bacterial or fungal contamination
6. After completing work, equipment and materials should be disinfected before they are removed from the hood. Work surfaces inside the hood must be sprayed with 70% ethanol; the gloves must be discarded
7. Incubators, cabinet, centrifuges and microscopes must be cleaned regularly

B- SAFETY ASPECTS OF CELL CULTURE

Experience from the earliest days of microbiological research has shown that most agents manipulated in the laboratory are pathogenic. Exposure to infectious agents in the laboratory is recognized as the most common occupational hazard for microbiology laboratory workers. Consequently, guidelines have evolved to protect microbiology workers based on an understanding of the risks associated with the manipulation of agents transmissible by different routes. These guidelines are based on the premise that safe working depends on a combination of structural provisions, management policies, working practices and procedures, and occasionally medical intervention. The hierarchy of biosafety levels defined for microbiological and biomedical facilities provides increasing levels of personnel and environmental protection in relation to the risk[2].

Use of various types of specialized equipment that serve as primary barriers between the micro-organism and the laboratory operative is crucial for safe working. Such equipment ranges from simple gloves and other personnel protective appliances through simple containment devices (sealed centrifuge heads), to complex containment devices (biosafety cabinets and containment facilities).

Biosafety Level 1

BSL-1 is appropriate for working with micro-organisms that are not known to cause disease in healthy humans. This is the type of laboratory found in municipal water-testing laboratories, in schools, and in colleges teaching introductory microbiology

2 Biosafety in Microbiological and Biomedical Laboratories (BMBL), U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Institutes of Health, Fourth Edition, May 1999, http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

classes, where the agents used are not considered hazardous.

The door should be closed to keep visitors out of the laboratory during manipulation of the agents is in progress. Hazard warning signs must be posted on the door indicating any hazards that may be present, including radioactive materials, lasers, high noise levels, and toxic chemicals. A hand-washing sink should be available, preferably near the door. Waste materials are segregated according to hazard type, and there is an appropriate chemical decontamination tray for receiving contaminated implements. Work is done on the open bench, and plastic-backed absorbent pads can be placed on the work surface to collect splatter or droplets. Bench tops should be impervious to acid and all furniture should be sturdy. If there are openable windows, they should be fitted with screens.

The laboratory should be constructed in such a manner that it can be easily cleaned and decontaminated. At BSL-1 there is no specific recommendation that the laboratory should be isolated from other parts of the building.

Standard microbiological practices in BSL-1 facilities include the use of mechanical pipetting devices; a prohibition on eating, drinking and smoking, and the necessity that all personnel wash their hands after finishing their work or, in any event, before leaving the laboratory (even temporarily). Lab coats should always be worn to protect clothes. It is recommended practice to wear gloves while manipulating agents. Additional protective equipment may include working behind a splatter shield or wearing eye or face protection.

Biosafety Level 2

This is typically the biosafety level of cell culture facilities. The agents manipulated in a BSL-2 facility are often those to which personnel have been exposed in the community (typically as children) and for which an immune response has been developed. Unlike the guidelines for BSL-1, immunizations are recommended before working with certain agents in a BSL-2 facility. Notably, immunization against hepatitis B is recommended for those who work with blood and blood products or are at high risk of exposure to them. Agents manipulated in a BSL-2 facility are transmissible by ingestion, exposure of mucous membranes, or intradermal exposure.

As biosafety level increases, microbiological practices and procedures required at a lower level are carried forward to the next level. Thus, the standard microbiological practices used in BSL-1 are required at BSL-2, with emphasis on glove wearing, use of mechanical pipetting devices, and attention to handling needles and other sharp instruments. Eating, drinking and smoking are prohibited in BSL-2 laboratories as in BSL-1.

Access to the laboratory is restricted, and determined by the laboratory supervisor, who establishes the biosafety level, the need for specified personal protective equipment, and the need for training. The door to the laboratory is kept closed to minimize access by casual visitors.

Some work may be done on the open bench by personnel wearing appropriate protective clothing or gear. Any manipulations that may produce aerosols of infectious material must be performed in containment. The most usual containment device is a biological safety cabinet (BSC), and the most commonly employed cabinet is a Class II, type A BSC.

In a BSC, room air drawn in at the face opening is immediately ducted down through the front grille and under the work surface, so it does not contaminate the work surface. This air is then blown up through the rear air plenum to the top of the cabinet where it divides. Thirty percent is exhausted out of the cabinet through a high

efficiency particulate air (HEPA) filter back into the room or externally. The remaining 70% of the air is directed down through another HEPA filter and passes over the work surface in a laminar flow, to be removed through the front and back grilles. A typical HEPA filter removes 99.97% of all particles that are 0.3 micrometers or larger in size, which means that all microbial agents are trapped in the filter. The air returned to the laboratory and delivered to the work surface is therefore virtually sterile.

Other special practices include decontaminating work surfaces after completing work with infectious materials, keeping non-research animals out of the laboratory, and reporting all spills and accidents. Before materials are introduced into the BSC, they should be wiped with 70% alcohol to remove any external contaminants. Good management of the work within the BSC is essential for preventing cross-contamination. Rapid air movements outside the BSC (e.g. by co-workers walking past, air vents directed across the face of the BSC, etc.) will disrupt the rather fragile down-flowing air curtain, and this may result in air-borne contaminants in the cabinet being drawn into the lap of the worker, or conversely may result in non-sterile air from the room eddying over the work surface.

The following agents must always be cultured/manipulated in BSL-2 or higher containment facilities:

- All cell lines (primary or established) of animal origin; in particular:
- All cell lines (primary and established) of human and other primate origin
- All cell lines derived from lymphoid or tumour tissue
- All cell lines exposed to or transformed by any oncogenic virus
- All cell lines exposed to or transformed by amphotropic packaging systems
- All cell lines new to the laboratory (until shown to be free of all adventitious agents)
- All mycoplasma-containing cell lines
- All human clinical material (samples of human tissues and fluids obtained surgically or by autopsy)

Biosafety Level 3

BSL-3 is suitable for work with infectious agents which may cause serious or potentially lethal diseases as a result of exposure by inhalation. BSL-3 laboratories should be located away from high-traffic density areas.

The secondary barriers required by BSL-3 facilities generally mean they should be in a separate containment building. BSL-3 facilities have a double-door entry and because the agents manipulated are transmissible by aerosol, BSL-3 facilities are designed to control air movements. Air always moves from areas of lesser contamination to those of higher contamination, e.g from a corridor into a laboratory. Furthermore, exhaust air is not re-circulated into other rooms. Exhaust air does not have to be HEPA filtered, unless local conditions are such that the air from potentially contaminated areas unavoidably has to re-enter the building air supply system.

All manipulations that may create aerosols or splatter are performed inside a BSC. The wall, ceiling and floor penetrations of the cabinets are sealed to keep both aerosols and gaseous decontaminants in. The floor of the laboratory is monolithic, and there are continuous cove mouldings that extend at least 10 cm up the wall. Ceilings should be waterproof for easy cleaning. Centrifuge tubes are placed into containment cups in the BSC, transferred to the centrifuge, spun, then returned to the BSC for unloading. In some laboratories centrifuges are placed in a vented area to minimize aerosol exposures due to centrifuge failure. Vacuum lines are protected with HEPA

filters so that maintenance personnel are not exposed to infectious aerosols.

Microbiological practices in BSL-3 facilities are the same as those in BSL-1 and BSL-2. Class II type A biological safety cabinets are suitable for BSL-3 laboratories. Sometimes Class II type B3 cabinets are installed, requiring a "thimble" connection to building exhaust systems. Depending on the nature of the work being done, additional protective devices, e.g. respirators, may need to be worn. When lung protection is required, personnel require medical assessment and training in the fitting, testing and care of their respirators.

Supervisors of BSL-3 laboratories should be competent scientists experienced in working with the agents used. They establish criteria for laboratory entry, restrict access, develop appropriate practices and procedures, and train the staff. They are also responsible for developing the laboratory safety manual. Laboratory personnel must rigorously follow established guidelines, participate in specified medical surveillance programs, and report all incidents that constitute potential exposures.

Handling frozen biological material

Storage and retrieval of frozen biological material from liquid nitrogen requires appropriate personal protection equipment. The three major risks associated with liquid nitrogen (-196° C) are frostbite, asphyxiation and exposure. Gloves thick enough to insulate but flexible enough to allow ampoule manipulation should be worn. When liquid nitrogen boils off during routine use, regular ventilation is sufficient to remove the excess, but when nitrogen is being dispensed, or a lot of material is being inserted into the freezer, extra ventilation is necessary.

When ampoules are submerged in nitrogen, the pressure difference between the outside and inside of the ampoule is high; if it is not perfectly sealed, liquid nitrogen will enter, so that when thawed the ampoule will explode violently. Ensure that ampoules are perfectly sealed, but in any event wear eye protection and a face shield. Thawing from storage under liquid nitrogen must always be allowed to occur in a container with a lid, such as a plastic bucket, and eye protection and face shield must be worn.

ROUTINE CELL COUNTING AND ASSESSMENT OF VIABILITY

Assessing medium suitability, determining population doubling times and monitoring cell growth in culture, all require a mean of quantifying cell population. Cell quantitation also allows standardization for manipulations such as transfection or cell fusion. Cell numbers are usually determined with a haemocytometer, an instrument for estimating the number of cells in a given volume under a microscope. The Improved Neubauer haemocytometer is a thick glass slide with two counting chambers, each 0.1 mm deep. Each chamber is divided into nine large squares delineated by triple white lines. The centre square is further divided into 25 squares. These 25 squares are further subdivided into 16 squares. The entire reticulated part has an area of 9 mm^2.

Using a vital stain, such as trypan blue, cell viability can also be determined. Only non-viable cells absorb the dye which appear blue and asymmetrical under the microscope, while healthy viable cells are refractory to the dye and rounded. However even viable cells absorb trypan blue over time. Hence dilutions and dye addition should be performed immediately prior to counting.

Equipment and materials

- Neubauer haemocytometer
- Trypan blue solution 0.5%
- Phosphate buffered saline x1
- Eppendorf tube 1.5 ml
- Pasteur pipette
- Inverted microscope
- 70% ethanol in water

Procedure

1. Prepare a uniform cell suspension of the cell culture to be counted (if cells are adherent detach by trypsin treatment)
2. Transfer 25 microliter of the cell suspension to an Eppendorf tube and add 62.5 microliter of 0.5% trypan blue and 37.5 microliter PBS x1 giving a total volume of 125 microliter. The dilution of the cell suspension is therefore 1:5
3. Mix thoroughly and allow to stand for 5 minutes
4. Place a cover-slip over the two chambers of the haemocytometer. Using a Pasteur pipette transfer 10-15 ml of the cell suspension to one of the haemocytometer chambers. The solution will pass under the cover glass by capillary action and fill the chamber
5. Place the haemocytometer on the stage of an inverted microscope at x 100 magnification and focus

Count the cells in the central square and in the four squares at the corners. Count separately viable (opaque) and non-viable (blue-stained) cells

Important: Count the cells touching the mid line of the triple line, on the top and left of each square. Do not count cells touching the mid line of the triple line, on the

bottom or right side of the square.

Calculate the number of cells per ml and total number of cells:
cells/ml = number of cell counted/number of squares counted x 104 x dilution factor (5)
total cells = cells/ml x vol. of original suspension

The percentage of viable cells is calculated as:
% viability = (number of viable cells counted/ total number of cells counted) x 100

STERILIZATION AND FILTRATION OF MEDIA AND REAGENTS - SERUM THAWING, INACTIVATION AND TESTING

A-STERILIZATION AND FILTRATION OF MEDIA AND REAGENTS

The media (DMEM, medium 199, SGM, Nutrient mixture Ham's F14, Basal Medium Eagle) used in the cell culture laboratory are purchased as gamma ray-sterilized solutions and certified as endotoxin-free. They are purchased without L-glutamine for increased stability. L-glutamine, purchased as a sterile endotoxin-free solution, is added immediately prior to use.

The foetal bovine serum used is certified as virus (BVD-IBR-PI3) and mycoplasma-tested and, since 1988, also bovine spongiform encephalopathy (BSE)-tested. The main method for eliminating mycoplasma contamination is filtration (0.1 micrometer pore size), while gamma-irradiation is used to destroy viruses.

Human fibroblast growth factor and the epidermal growth factor are purchased as sterile filtered solutions with certified endotoxin concentration less than 0.1 ng/microg. Antibiotics (penicillin-streptomycin 10000 IU) are purchased as filtered and endotoxin-free solutions 100x. Insulin is purchased as a gamma-sterilized lyophilised powder. Before addition to the medium, 100 mg of powdered insulin is dissolved in 12 ml HCl 1N and 88 ml of distilled water; this solution is then filtered (Millipore Sterilcup, 0.22 micrometer). Aliquots of insulin solution are stored at -20° C.

Complete growth medium is prepared under the hood using disposable sterile plastic ware, and filtered using 500 ml Millipore Sterilcup (0.22 □m pore size). The cap and neck of the bottle containing the sterilized medium are protected with Parafilm and stored at 4°C.

NB: No media or reagents are sterilized by autoclaving. Most complete media are stored at 4°C for 1-2 months.

B- SERUM THAWING, INACTIVATION AND TESTING

Animal sera are the most important source of nutrients for cultured cells. They contain lipids, salts, vitamins, minerals, amino acids and other necessary components for cell growth. In order to preserve these properties for long periods of time, careful handling is crucial.

Equipment and Materials

- Laminar flow hood
- Water bath 37-56°C
- 50 ml sterile centrifuge tubes
- Foetal bovine serum

Procedures

1. Storage

Animal sera should be stored frozen at -20°C and protected from light. It is also

important to avoid multiple thaw/freeze cycles because this will hasten degradation and result in formation of insoluble precipitates. To avoid this, small aliquots are prepared and immediately refrozen for future use.

2. Thawing
The serum bottle is removed from the freezer and left to stand at room temperature for 10 minutes. The bottle in then placed in a 37°C water bath, with the bottle cap clear of the water (not submerged). The bottle is shaken occasionally until the serum is completely thawed.

3. Heat inactivation of sera
This is the most commonly used procedure for destroying complement and ensuring that antibody binding will not lyse cells. The serum inactivation process consists of heating to 56°C and holding at that temperature for 30-35 minutes, with occasional gentle shaking (every 10 minutes) (Important: before heating to 56°C, the serum must be completely thawed at 37°C).

C- SERUM TESTING - CELL PLATING EFFICIENCY ASSAY

Before ordering a new serum lot, a sample of the lot should be tested on the cell lines under study. This must be done well before the old (the one in use) has run out. The testing procedure is the cell plating efficiency assay. The assay should employ both the new serum lot and the current serum lot, and the results compared.

Equipment and Materials

- Laminar flow hood
- CO_2 incubator
- Growth medium pre-warmed at 37°C
- Haemocytometer
- Inverted microscope
- Petri dishes 35 mm
- Aliquot of foetal bovine serum to be tested
- Aliquot of foetal bovine serum in use

Procedure

1. The cell line of interest (growing on plates or in suspension) is counted by the haemocytometer cell counting method (see Protocol "ROUTINE CELL COUNTING AND ASSESSMENT OF VIABILITY")
2. 200, 400 and 800 cells per dish are seeded, in triplicate, with 5 ml of proliferating medium onto 35 ml Petri dishes. The inverted microscope is used to check, every 2-3 days, for the presence of dark aggregates (serum sediments) in the medium
3. At 12-14 days, the numbers of colonies are determined (when cells are seeded at low density they tend to form colonies) and the cells are examined for morphology alterations, such as formation of pseudopodia or vacuoles
4. The number and morphology of colonies in the new serum are compared with those formed in the current serum lot. If there are no major differences, the new serum lot can be used

COLLECTION OF INNERVATED MYOTUBES USING THE SANDWICH TECHNIQUE

For rapid immunocytochemical analysis, innervated myotubes are prepared using the sandwich technique. Using this technique, cross-sections of innervated myotubes are suitable for immunocytochemistry, as they do not require membrane permeabilization, which might compromise the integrity of the plasmalemma.

Equipment and materials

- Petri dishes 35 mm with innervated myotubes
- Fresh rat or mouse skeletal muscle samples, about 2-3 mm thick and 5 mm in diameter
- Sterile surgical instruments for microdissection
- Dewar 1-2 litres
- Isopentane
- Liquid N_2
- Tank for liquid N_2

Procedure

1. Select areas of innervated and contracting myotubes under an inverted microscope
2. Remove any debris of rat spinal cord, taking care not to disturb the myotubes
3. Collect the innervated myotubes by delicate and careful excision, with microdissection scissors
4. Place myotubes between two layers of previously prepared fresh mouse or rat muscle
5. Freeze this sandwich in isopentane (pre-cooled in liquid nitrogen) as for muscle biopsy and place it into pre-identified cryotubes

Reference

Askanas V, Shafiq SA, Adamchak D: Sandwich embedding of multiple layers of muscle tissue culture for serial frozen sectioning. Stain Technol 1971; 46: 323-324.

MUSCLE CELL INNERVATION WITH FOETAL RAT SPINAL CORD (METHOD 1 of 2)

Equipment and Materials

- Petri dishes 35 mm
- Sterile surgical instruments for microdissection
- Disposable syringe 1 ml
- Bovine gelatine
- Ham's F-14 proliferating medium
- Innervating medium
- Medium for collecting biopsies
- Foetal bovine serum
- Sprague Dowley or Wistar gravid female rats (12-13 day after mating (Contact Charles River or other supplier in advance and specify exact day of mating)
- Dissecting microscope
- Laminar flow hood

Innervating medium

Nutrient mixture Ham's F14	440 ml
Foetal bovine serum	50 ml
Insulin	5 ml
Penicillin-streptomycin solution 100x	5 ml
Human basic fibroblast growth factor	25 ng/ml
Epidermal growth factor	10 ng/ml

Filter and store at +4°C, 15 days maximum.

Ham's F14 or DMEM Proliferating medium

Nutrient mixture Ham's F14 or DMEM	390 ml
Foetal calf serum	100 ml
Penicillin-streptomycin solution 100x	5 ml
Insulin	5 ml
L-glutamine	5 ml
Human basic fibroblast growth factor	25 ng/ml
Epidermal growth factor	10 ng/ml

Filter and store at +4°C, up to 1 month.

Insulin solution

Dissolve 100 mg of insulin in 12 ml of HCl (1N). Add 88 ml of ultrapure water, filter through 0.22 micrometer filters and aliquot (5 ml) into sterile tubes.

Medium for collecting human muscle biopsy

DMEM	500 ml
Penicillin-streptomycin solution 100x	6.25 ml
Gentamicin solution (50 mg/ml)	0.5 ml
Fungizone (250 microg/ml	5 ml

Filter and store at +4°C, up to 2 months.

Procedure

1. Seed myoblasts 3-4 days before innervation into 35 mm Petri dishes previously treated with gelatine, at a concentration of 12-25 X 104 depending on growth rate. Gelatine-coated dishes are prepared from 0.1 % bovine gelatine solution which is filtered, poured into 60 mm petri dishes (3 ml per dish) and left to stand for an hour, then the excess liquid is discarded
2. Add Ham's F-14 or DMEM proliferating medium and change medium every 2-3 days as usual
3. For optimal innervation the myoblasts should be ready to fuse but only a few myotubes should have formed
4. The day before innervation replace proliferating medium with innervating medium, which stimulates differentiation rather then proliferation
5. Just prior to innervation, set up the tools necessary for sacrifice (guillotine, ethanol 70%, plastic bag for cadavers, and sterile surgical instruments)
6. Carefully clean all surfaces, including the microscope, with 70% ethanol
7. Briefly expose the rat to ether vapour and guillotine. One animal may suffice but a second must be kept ready in the event that the first is non gravid or contains too few foetuses
8. Disinfect the belly with ethanol and open the abdominal cavity with sterile scissors. Expose the uteri (chains of 10-12 uteri in each gravid female) and collect them
9. Place the uteri in 100 mm Petri dishes containing medium for collecting muscle biopsies (rich in antibiotics) and transfer to the flow hood
10. Clean the guillotine with detergent and 70% ethanol
11. Under the hood, extract the foetuses from the uteri and place them into a new 100 mm Petri dish containing medium for collecting muscle biopsies
12. Under the dissecting microscope use the cutting edge of insulin needles and tweezers with very fine tips, to dissect out the entire spinal cord of each foetus including the dorsal ganglia
13. Cut the spinal cords plus ganglia into fragments containing 1 or 2 pairs of ganglia and transfer them to the flow hood
14. Remove the medium from the 35 mm Petri dishes containing the myoblasts/myotubes and add 4-5 spinal cord fragments distributing them equidistant from each other
15. Add a few drops of innervating medium and incubate for 1 hour at 37°C in CO_2 incubator to allow the fragments to adhere.
16. Add sufficient innervating medium to completely cover spinal cord fragments (about 1ml) and place the dish in the incubator
17. Change medium every 2-3 days
18. After 7-10 days, myotubes will start to contract asynchronously. After 20-30 days, FBS concentration can be reduced to 5%; after 2-3 months it can be further reduced to 2%

References

Kobayashi T, Askanas V, Engel WK: Innervation of human muscle cultured in

monolayer by rat spinal cord: importance of dorsal root ganglia for achieving successful functional innervation. J Neurosci 1987; 7: 3131-3141.

MUSCLE CELL INNERVATION WITH FOETAL RAT SPINAL CORD (METHOD 2 of 2 - IN MICROWELL)

Equipment and materials

- Hanks' balanced salt solution (HBSS) without Ca^{++} and Mg^{++} (1X)
- DMEM
- L15 Leibovitz medium
- Foetal bovine serum (FBS)
- Bovine Serum Albumin (BSA)
- DMSO
- Gelatine type B from bovine skin
- Ultrapure distilled H2O
- Minimum essential medium (MEM) Earle's without L-Glutamine
- M199 Earle's salts
- L-glutamine 100X 200 mM
- Antibiotic-antimycotic 100X
- Insulin from bovine pancreas
- Basic Fibroblast growth factor (b-FGF)
- Epidermal growth factor (EGF)
- Laminar flow hood
- Inverted microscope
- Disposable plastic ware

Medium for muscle cell culture
This medium is for satellite cell isolation and muscle cell growth

MEM	65 ml
Medium 199	25 ml
FBS (10 % final)	10 ml
L-glutamine 100X (2X final)	2 ml
Antibiotic-antimycotic solution 100X (1X final)	1 ml
Insulin solution 5 mg/ml (10 microg/ml final)	200 microliter
EGF solution 0.2 microg/microliter (10 ng/ml final).	5 microliter
B-FGF solution at 0.1 microg/microliter (5 ng/ml final)	5 microliter

Store at 4°C for 15 days.

Gelatin solution 1%
Weight 1 g +/- 0,2 g of gelatine in a glass flask

Dilute gelatine in cell culture water

Warm at 37°C until complete dissolution

Sterilise solution for 30 minutes in autoclave

Homogenise and aliquot in 50 ml tubes.

Store at 4°C

Gelatin solution 0,1%
For amplification of muscle cells, prepare a gelatine solution at 0,1%

Warm up at 37°C the solution of gelatine 1% until clarification of the solution

Dilute in cell culture water.

Conditioned medium for embryo dissection
This medium is poor in Ca^{++} and Mg^{++}. It is used to immerge embryos as soon as they are collected:

HBSS 1%

BSA 1%

Antibiotic-antimycotic solution 1%

Filter on 0.22 µm and store at 4°C for 1 month.

Dissection medium
This medium is used to dissect embryos and to prepare spinal cords. It is buffered at pH 7.4.

For 500 ml of L15 medium

BSA 1%

Antibiotic-antimycotic solution 1%

Filter on 0.22 µm and store at 4°C for 1 month

Muscle cell innervation medium
Stage 1 : basic medium preparation

For 600 ml

MEM 420 ml

M199 150 ml

FBS 5% (30 ml)

Antibiotic-antimycotic solution 1X final

Store at 4°C for 1 month

Stage 2 : preparation of cell muscle innervation medium

For 100 ml of basic medium

L-glutamine 2X final (1ml)

Insulin 10 microg/ml final

Store at 4°C for 15 days

Procedure

1. Embryo preparation
Collect Wistar 15-day-old rat embryos, from the amniotic bag and place in a Petri dish containing HBSS dissection medium. Just before the dissection, transfer embryos to L15 dissection medium for spinal cord isolation.

2. Spinal cord preparation

Dissect spinal cords from the embryos, place in the innervation medium and cut into small slices containing the 2 corresponding dorsal root ganglia.

3. Human muscle cell culture and innervation

Thaw muscle cells rapidly at 37°C and seed into 24 well microplates in muscle cell innervation medium. Immediately after satellite cell have fused to form myotubes with no contractile activity, place on the cell monolayer one slice per well of embryo spinal cord with the 2 dorsal root ganglia. Innervated cultures are maintained in neural medium containing 5 % foetal bovine serum and the medium is changed every 2 days.

After one day of co-culture, neurites are seen growing out of the explant; they make contact with the myotubes and, after 5 days, induce the first contractions. After 3 weeks in co-culture, the innervated muscle fibres become cross-striated, have well-differentiated neuromuscular junctions and display other biochemical and pharmacological marker of maturation.

The validation of the model is focused on functional innervation such as contractile activity and on the presence of different neuron and muscle cell markers.

4. Counting contraction frequency

Contractions are visible from day 4 to day 36 of co-culture and can be counted using an analysis software. Contraction frequency can be filmed with a video camera linked to the microscope and counted for 30 seconds.

5. Immuno-characterization of neurons and muscle cells

Fix cells with 4 % paraformaldehyde.

Stain for choline acetyl transferase or Beta-tubulin, both markers of neurons or for myosin heavy chain, marker of skeletal muscle, by incubation for 24 hours at 4 °C in a phosphate-buffered saline solution (PBS) containing 0.3 % Triton X100 (PBT medium) and 5 % foetal calf serum. After extensive washings with PBT, incubate cultures in the CY3 (red dye)-conjugated secondary antibody for 24 hours in PBT. Wash, and keep stained cell cultures at 4 °C until analysis. Observations are made using a fluorescence-equipped inverted microscope.

MYOBLAST IMMORTALISATION

Primary skeletal muscle myoblasts have limited proliferation capacity in culture and cease to proliferate after several passages. Although several immortal rodent myoblast cell lines are in common use, immortal human myoblast lines have not been isolated. The molecular basis for this species difference is unclear but it may reflect the presence of more stringent cell cycle regulation in human cells. Rodent myoblasts can be readily immortalized with genes from DNA tumour viruses, such as that for SV40 large T antigen, which targets p53 and retinoblastoma protein (pRB), key components of the cell cycle regulatory machinery at the G1/S checkpoint. Human myoblasts stably transfected with a plasmid driving inducible expression of the SV40 large T antigen are reported to have extended lifetimes, but are not immortalized. Infection of human myoblasts with polyoma large T antigen or a retroviral vector expressing a temperature-sensitive SV40 large T antigen also fail to immortalize at the permissive temperature. Origin-defective SV40 virus has been used to immortalize human myoblasts, but the cells retain little capacity to differentiate.

Expression of the E6E7 early region from human papillomavirus type 16 can greatly extend the life span of both foetal and satellite cell-derived myoblasts and release them from dependence on the growth factors normally necessary for their proliferation.

Equipment and materials

- Polybrene (hexadimethrine bromide), Sigma H9268; stock solution 1mg/ml, filtered sterile, stored at 4°C
- G418 (Geneticin), Gibco, Cat. No. 11811-064, stock solution 100 microg/ml, filtered sterile, stored at -20°C.
- To prepare the G418 stock solution, dissolve 1 g G418 in e.g. 7.17 ml PBS if biological activity is e.g. 717 microg/mg. This figure corresponds to the biological activity or potency (written on the tube) that varies with each G418 lot. The stock solution is stored in 500 ml aliquots at -20°C.
- Virus stock (supernatant of transfected cells containing human papillomavirus type 16 retroviral particles, available upon request from Hanns Lochmüller, MTCC[3])
- DMEM
- Foetal bovine serum
- SMG proliferating medium
- Laminar flow hood
- CO_2 incubator

SGM Proliferating medium

Commercial medium (Promocell) for muscle cell culture	500 ml
Supplement Mix (sold with SGM, to be stored as aliquots at - 20°C)	25 ml
Foetal bovine serum	50 ml
Gentamicin (50 mg/ml) (40 □g/ml final)	0.4 ml
Glutamax 100 X (to be stored as aliquots at -20°C).	7.5 ml

[3] mtcc@fbs.med.uni-muenchen.de

Store at +4°C, 10-15 days maximum.

Procedure

For virus transfection start with a 100 mm plate containing myoblasts 30-50% confluent and an equivalent control plate for mock control without virus.

Day 1:

1. Remove medium and add 1 ml of the virus stock plus 3 ml of serum-free medium containing 4 µg/ml polybrene
2. Incubate at 37°C for 2 hours
3. Add 5 ml more medium with polybrene and continue incubating at 37°C for 5 more hours or till the end of the day
4. Remove medium from cells, rinse once with medium, then feed cells with 10 ml of fresh medium without polybrene

Day 2:

1. Optional: At this point the cells can be divided onto several plates. It is useful to make a serie of dilutions if you want to end up with colonies that are well isolated

Day 3:

Start G418 selection:

1. Remove medium and add medium containing G418 (e.g. 400µg/ml)
2. Selection should be continued for 7 to 10 days

Days 5-12:

1. Change medium every other day; replace with medium containing G418 (e.g. 400 µg/ml)
2. Collect individual G418-resistant myoblast clones and expand them
3. Cells in the mock control should not survive G418 treatment for longer than 7-10 days

Reference

Lochmuller H, Johns T, Shoubridge EA. Expression of the E6 and E7 genes of human papillomavirus (HPV16) extends the life span of human myoblasts. Exp Cell res. 1999, Apr 10;248(1):186-93.

MYOBLAST PURITY ASSESSMENT BY FACS

Cell purity assessment

Many cells lines of different origins and biological characteristics appear identical and cannot usually be distinguished by morphology or culture characteristics. In addition, contamination with virus or mycoplasma may significantly change the characteristics of the cells, although contamination will not be apparent. Cell lines also change with time in culture. Mislabelling or cross-contamination may also occur. Once introduced, an error will be disseminated.

The integrity of a cell line has to be maintained actively by a well-organized cell bank with structured quality assessment and testing. In particular the identity and purity of a cell line must be considered doubtful in the absence of a well-recorded culture history and recent test data. Cell line characterization relies on immunochemical protein expression analysis, biochemical isoenzyme analysis and DNA profiling.

A primary muscle cell culture normally consists mainly of myoblasts, but also of some fibroblasts. The percentage of the latter varies depending on the provenience of the cell line (a culture from a DMD patient will contain more fibroblasts than usual because muscle is replaced by fibrous connective tissue). If fibroblasts are numerous their proportion will tend to increase with time. Therefore it is recommended that muscle cell lines be used and that their purity be checked in the first 3-4 passages of subculture. If a pure cell line is required, the best way to obtain it is by cell sorting, either by FACS or by microbead isolation (see also MYOBLAST PURITY ASSESSMENT BY MICROBEADS).

For myoblast cultures, cell purity is usually assessed by analysing the expression of markers such as desmin or N-CAM (CD56); when muscle cells are made to differentiate into myotubes, biochemical determination of creatine-kinase activity or expression of foetal myosin heavy chain, sarcomeric actin or other skeletal muscle specific proteins, may be used for purity assessment.

Sorting of human myoblasts using FACS

Equipment and materials

- Laminar flow hood
- CO_2 incubator
- Proliferating medium
- Washing medium
- Mg and Ca free PBS
- Bovine serum albumin (BSA)
- Trypsin-EDTA solution 1X
- PBS/BSA (PBS + 0.5%BSA = 0.25 g BSA in 50ml sterile PBS filtered through a 0.22 micrometer filter)
- Supernatant of hybridoma-cell line producing anti-NCAM (also known as CD56 or 5.1H11)
- Biotinylated anti-mouse IgG secondary antibody
- Texas red-avidin or fluorescein-streptavidin

- Eppendorf tubes
- Microfuge
- Fluorescence-activated cell sorter (FACS)

Procedure

1. Harvest cells with trypsin-EDTA, and wash twice in PBS containing 0.5% BSA with centrifugation for 2 min at 1500 rpm
2. Count cells (see ROUTINE CELL COUNTING AND ASSESSMENT OF VIABILITY)
3. Transfer cells to Eppendorf tubes and incubate at room temperature for 20 min in anti-NCAM supernatant, then for 20 min in biotinylated secondary anti-mouse IgG (7mg/ml), and finally for 20 min in Texas red-avidin or fluorescein-streptavidin (10 microg/ml)
4. Between each incubation wash cell three times in PBS/BSA with 15 second centrifugation at 15000 rpm in a microfuge
5. To assess viability (facultative) during the 5 last min of the final incubation add propidium iodide (1 microg/ml final concentration)
6. Do a sterile sort of the cells by FACS following standard procedures taking care to correctly calibrate the fluorescence and light scatter channels
7. Collect cells into proliferating medium, either in 96-well tissue culture plates (one cell per well for clonal propagation) or in 6 ml tubes for replating into Petri dishes (for polyclonal mass culture)

Reference

Webster C, Pavlath GK, Parks Dr, Walsh FS and Blau HM: Isolation of human myoblasts with the Fluorescence-Activated Cell Sorter. Exp. Cell Res. 1988; 174: 252-265.

MYOBLAST PURITY ASSESSMENT BY MICROBEADS

Cell purity assessment

Many cells lines of different origins and biological characteristics appear identical and cannot usually be distinguished by morphology or culture characteristics. In addition, virus or mycoplasma contamination may significantly change the characteristics of the cells, although contamination will not be apparent. Cell lines also change with time in culture. Mislabelling or cross-contamination may also occur. Once introduced, an error will be disseminated.

The integrity of a cell line has to be maintained actively by a well-organized cell bank with structured quality assessment and testing. In particular the identity and purity of a cell line must be considered doubtful in the absence of a well-recorded culture history and recent test data. Cell line characterization relies on immunochemical protein expression analysis, biochemical isoenzyme analysis and DNA profiling.

A primary muscle cell culture normally consists mainly of myoblasts, but also some fibroblasts. The percentage of the latter varies depending on the provenience of the cell line (a culture from a DMD patient will contain more fibroblasts than usual because muscle is replaced by fibrous connective tissue). If fibroblasts are numerous their proportion will tend to increase with time. Therefore it is recommended that muscle cell lines in the first 3-4 passages of subculture be used and that their purity be checked. If a pure cell line is required, the best way to obtain it is by cell sorting, either by FACS or by microbead isolation (see also MYOBLAST PURITY ASSESSMENT BY FACS).

For myoblast cultures, cell purity is usually assessed by analysing the expression of markers such as desmin or N-CAM (CD56); when muscle cells are made to differentiate into myotubes, biochemical determination of creatine-kinase activity or expression of foetal myosin heavy chain, sarcomeric actin or other skeletal muscle specific proteins, may be used for purity assessment.

Magnetic sorting of human myoblasts (MACS)

Equipment and materials

- Laminar flow hood
- CO_2 incubator
- Proliferating medium
- Washing medium
- Bovine serum albumin (BSA)
- PBS/BSA (PBS + 0.5%BSA = 0.25 g BSA in 50ml sterile PBS filtered through a 0.22 micrometer filter)
- Trypsin-EDTA solution 1X
- Supernatant of hybridoma-cell line producing anti-NCAM (also known as CD56 or 5.1H11, available from Hanns Lochmüller, MTCC, München46).
- Petri dishes 100 mm
- Rat anti-mouse IgG-conjugated microbeads

- MACS (Magnetic Cell Sorting) apparatus.

Washing Medium

DMEM	500 ml
Foetal Bovine Serum	50 ml
Gentamicin (50 microg/ml)	0.4 ml

Filter and store at +4°C, up to 1 month.

SGM Proliferating medium

Commercial medium (Promocell) for muscle cell culture	500 ml
Supplement Mix (sold with SGM, to be stored as aliquots at - 20°C)	25 ml
Foetal bovine serum	50 ml
Gentamicin (50 mg/ml) (40 microg/ml final)	0.4 ml
Glutamax 100 X (to be stored as aliquots at - 20°C)	7.5 ml

Store at +4°C, 10-15 days maximum.

Procedure

1. Trypsinize cells (ca. 70% confluent) in a 100mm dish, rinse with 8 ml washing medium and centrifuge for 5 min. at about 500g
2. Discard the supernatant and re-suspend the pellet in 1 ml of PBS/BSA
3. Transfer suspension (1 ml) to 2 ml Eppendorf tube. Spin at 4°C, 500g for 5 min. Discard the supernatant and re-suspend the pellet in 1ml PBS/BSA
4. Add 100 µl supernatant of anti-NCAM producing hybridoma-cell line, mix and incubate in the dark on ice with gentle shaking for 45 min. After 45min, spin the tube for 5 min at 4°C and 500g
5. Wash pellet 3x with 1 ml PBS/BSA and with re-centrifugation
6. Re-suspend the pellet in 160 microliter PBS/BSA
7. Add 40 microliter rat anti-mouse IgG1 with microbeads, mix and incubate in the dark on ice for 15 min, preferably on a shaker
8. Spin tube and re-suspend pellet in 500 microliter PBS/BSA
9. Attach the miniMACS magnet to the miniMACS stand. Attach MACS-column to the magnet and equilibrate the column with 500 microliter PBS/BSA, discarding effluent
10. Transfer the cell suspension to the column, allow it to run through and collect in a 100 mm dish (MACS (-) dish (= negative fraction with fibroblasts)). Wash the column 2 - 3 times with 500 microliter PBS/BSA and collect the effluent in the MACS (-) dish
11. Disconnect the column from the magnet, add 1 ml PBS/BSA and flush out the positive fraction into the 100 mm dish MACS (+) dish, exerting gentle pressure with the plunger supplied
12. Add 10 ml SMG medium to the 100 mm dish MACS (+) dish, and allow cells to grow in a CO_2 incubator

PRIMARY MYOBLAST CULTURE FROM FRESH HUMAN MUSCLE BIOPSY

Primary cultures are derived directly from excised human tissue and cultured either as explants or after dissociation into a single cell suspension by enzyme digestion.

The protocol describes the different steps for obtaining a primary cell line from biopsies of human muscle: culture of the muscle biopsy, maintenance of cell culture, routine subculture of adherent cell lines, differentiation of muscle cells.

Equipment and materials

- Laminar flow hood
- CO_2 incubator
- Inverted microscope
- Sterile surgical instruments for microdissection
- Medium for human muscle biopsies
- Proliferating medium pre-warmed to 37°C
- PBS 10x without Ca^{+2} or Mg^{+2}
- Petri dishes 100 mm
- Pasteur pipettes
- Sterile forceps
- Sterile scalpel and blade
- Culture flask, 25 cm^2
- 15 ml sterile plastic tube
- Wheaton flask
- Solution A
- ATE solution

Medium for collecting human muscle biopsy

DMEM	500 ml
Penicillin-streptomycin solution 100x	6.25 ml
Gentamicin solution (50 mg/ml)	0.5 ml
Fungizone (250 microg/ml)	5 ml

Filter and store at +4°C, up to 2 months.

DMEM Proliferating medium

DMEM	390 ml
Foetal bovine serum	100 ml
Penicillin-streptomycin solution 100x	5 ml
Insulin (10 microg/ml final)	5 ml
L-glutamine	5 ml
Human basic fibroblast growth factor	25 ng/ml
Epidermal growth factor	10 ng/ml

Filter and store at +4°C, up to 1 month.

Procedure for culturing the muscle biopsy

1. After having obtained the fresh muscle biopsy (one or two small pieces about 1 mm3), place immediately in a 15 ml sterile plastic tube containing 10 ml of medium for biopsies
2. In the laboratory, prepare under the hood two 100 Petri dishes with 10 ml of proliferating medium, one dish with 10 ml of sterile PBS (with no calcium and no magnesium salts) and one empty dish
3. Place the sterile surgical instruments ready for use in the hood
4. Empty the specimens plus medium into one of the empty Petri dishes
5. Place a specimen on the lid of the Petri dish
6. Using the sterile tweezers, scissors and scalpel remove as much as possible of the adipose tissue, connective tissue and blood vessels from the biopsy material
7. Wash in PBS once or twice
8. Wash in proliferating medium and again place the specimen on Petri dish lid and seek to remove any remaining adipose, connective tissue and blood vessels
9. Wash the specimen, now free of extraneous tissue, in proliferating medium
10. Place the specimen on the Petri dish lid and use the scalpel to cut finely into very small fragments
11. Collect the minced tissue on the scissor tips and place in the 25 cm^2 culture flask
12. Using a sterile Pasteur pipette with flame-rounded tip (round the tip in Bunsen flame and cool in PBS) distribute the small tissue fragments over the bottom surface of the flask
13. Pass the flask rapidly and carefully through the Bunsen flame in order to evaporate the medium so that the minced tissue pieces adhere to the plastic surface, but so as not to heat-damage the minced tissue. Take care not to cook the tissue!
14. Carefully add culture proliferating medium, firmly close the lid of the flask and place in CO_2 incubator
15. The next day slightly unscrew the lid of the flask so that the tissue can "breathe." Leave in incubator for a week
16. After a week replace the culture medium with fresh medium. From this point on replace the culture medium three times a week (usually Mondays, Wednesdays and Fridays)
17. The muscle cells will start to grow from the minced fragments in 8-15 days. When there are sufficient cells, they are detached enzymatically and plated in Petri dishes for proliferation (see below "Maintenance of cell cultures in dishes and flasks" and "Routine Subculture of adherent cell lines"). The minced fragments in the flask will continue to produce cells for a while and remain useful for a month or two

MAINTENANCE OF CELL CULTURES IN DISHES AND FLASKS

In culture, cells grow either as a single cell layer attached to specially treated plastic surfaces or in suspension. In order to keep adherent cells healthy and actively growing, it is usually necessary to subculture them at regular intervals.

Equipment and Materials

- Laminar flow hood
- CO_2 incubator
- Proliferating medium pre-warmed to 37°C
- Petri dishes 100 mm
- Inverted microscope

DMEM Proliferating medium

DMEM	390 ml
Foetal bovine serum	100 ml
Penicillin-streptomycin solution 100x	5 ml
Insulin (10 microg/ml final)	5 ml
L-glutamine	5 ml
Human basic fibroblast growth factor	25 ng/ml
Epidermal growth factor	10 ng/ml

Filter and store at +4°C, up to 1 month.

Procedure

1. The general morphology and growth of a cell population, and the presence of any microbial contaminants, should be checked regularly under an inverted microscope in phase contrast
2. Dishes or flasks with cells at about 70% confluence are treated with trypsin; the cells are then harvested and either frozen or divided for further proliferation (see below "Routine Subculture of adherent cell lines"). For dishes with non-confluent cells the medium is discarded and replaced with fresh medium:
   7 ml for 100 mm Petri dishes
   5 ml for 60 mm Petri dishes
   5 ml for 25 cm^2 flasks
3. Medium has to be changed three times a week, usually Mondays, Wednesdays and Fridays

NB: When introducing medium to flasks, a new sterile pipette must be used for each flask; when changing medium in Petri dishes, one pipette may be used for 2 or 3 dishes if the cell line is the same, but the tip must be flamed at each passage. Lids of flasks containing cells must to be slightly unscrewed after being placed in the CO_2 incubator.

ROUTINE SUBCULTURE OF ADHERENT CELL LINES

Subculturing requires prior rupture of intercellular and cell-to-substrate connections using proteolytic enzymes such as trypsin. After the cells have been dissociated into a suspension of mainly single cells, they are diluted and transferred to new culture dishes containing fresh medium or to cryotubes containing freezing medium.

How often a cell line is subcultured depends on its growth properties which are determined by observation of cell growth under the microscope and by count

Equipment and Materials

- Laminar flow hood
- CO_2 incubator
- Proliferating medium
- PBS
- Trypsin-EDTA solution 1X
- Petri dishes 100 mm
- Inverted microscope

DMEM Proliferating medium

DMEM	390 ml
Foetal bovine serum	100 ml
Penicillin-streptomycin solution 100x	5 ml
Insulin (10 microg/ml final)	5 ml
L-glutamine	5 ml
Human basic fibroblast growth factor	25 ng/ml
Epidermal growth factor	10 ng/ml

Filter and store at +4°C, up to 1 month.

Procedure

Thaw trypsin at 37°C and allow PBS and proliferating medium to reach room temperature.

Flask cultures containing tissue fragments

1. Using a sterile pipette remove medium from flask and replace with 5 ml PBS in order to eliminate serum residue that could inactivate the trypsin
2. After a few minutes remove PBS (pipette) and add 1 ml trypsin
3. Place flask in incubator at 37°C for 3-5 minutes
4. Observe the cells under the microscope: if they are seen to be rounded they are detached, if most are not rounded leave the suspension in the incubator for a further minute or two (until rounded)
5. Add 5 ml or more of proliferating medium (add volume equal to or more than that of volume of trypsin) to inhibit enzyme activity
6. Use the tip of the pipette (or gently pipette up and down) to detach cells from the

bottom of the flask, but be careful not to touch or detach the tissue fragments

7. Transfer the cells (pipette) to a 100 mm Petri dish containing 7 ml of proliferating medium and place in incubator for 20 min. This is the pre-plating step which helps to reduce fibroblast contamination since fibroblasts adhere to the dish surface more readily than myoblasts
8. After 20 min transfer the medium with unattached cells to another Petri dish and place it in incubator. Write the date, number of passages, and cell line code on the Petri dish lid
9. The initial flask (treated with trypsin) is refilled with proliferating medium and placed in incubator with cap slightly unscrewed

Petri dish cultures:

1. Remove medium from Petri dish and add PBS (7 ml to 100 mm Petri dish, 4 ml to 60 mm Petri dish)
2. After a few minutes, remove PBS and add trypsin (1 ml to 100 mm dishes, 0.5 ml to 60 mm dishes)
3. Place in incubator at 37°C for 3-5 minutes
4. Check under the microscope that cells have detached and proceed as trypsinization of flask cultures (above)
5. Add proliferating medium (volume equal to or greater than volume of trypsin added) in order to inhibit trypsin activity
6. Mechanically detach cells from dish surface with the help of a pipette tip, then gently pipette the cell suspension up and down so as to obtain a suspension of individual cells
7. Dispense appropriate aliquots of the cell suspension to new 100 mm Petri dishes and add 6 ml of growth medium
8. Label each new dish with cell line code, date and passage number. Place the dishes to the CO_2 incubator at 37°C

NB: When trypsinizing cells from Petri dishes, pre-plating is optional: it is recommended if the cells are mainly fibroblastoid rather than fusiform. The following day, check under the microscope that the cells have reattached and are growing.

DIFFERENTIATION OF MUSCLE CELLS

Myoblasts differentiate into myotubes by fusing to form multinucleated cells. In vitro, myoblast fusion is obtained by withdrawing growth factors and by replacing foetal bovine serum with horse serum.

Equipment and Materials

- Laminar flow hood
- CO_2 incubator
- Proliferating medium
- Inverted microscope
- Petri dishes 35, 60 or 100 mm
- Differentiating medium

DMEM Proliferating medium

DMEM	390 ml
Foetal bovine serum	100 ml
Penicillin-streptomycin solution 100x	5 ml
Insulin (10 microg/ml final)	5 ml
L-glutamine	5 ml
Human basic fibroblast growth factor	25 ng/ml
Epidermal growth factor	10 ng/ml

Filter and store at +4°C, up to 1 month.

Differentiating medium

DMEM (or Ham's F14)	93 ml
Horse serum	5 ml
Insulin	1 ml
Penicillin-streptomycin solution 100x	1 ml

Filter and store at +4°C, up to 1 month.

Procedure

1. Seed myoblasts into 35 Petri dishes at a concentration of 12-25 X 10^4 (36-75 X 10^4 in 60 mm dishes, 4-8 X 10^6 in 100 mm dishes), depending on growth rate
2. Add DMEM proliferating medium and change medium every 2-3 days as usual.
3. When myoblasts reach about 70% confluence, replace proliferating medium with differentiating medium
4. After a few days, check under the microscope: if cells are mostly myoblasts (fibroblast contamination should be negligible), several myotubes will form. Note that a myotube is considered as such if the cell contains at least 3 nuclei
5. Myotubes will increase in number and size to cover the entire surface of the dish. They will last for a short period of time (from several days to a few weeks) and then will die, unless innervated (See Muscle Innervation with Foetal Rat Spinal Cord)

PRIMARY FIBROBLAST CULTURE FROM HUMAN SKIN BIOPSY

This protocol describes the steps for obtaining a primary fibroblast cell line from human skin biopsies. Fibroblasts are derived directly from excised skin as explants; enzyme digestion by collagenase may help obtain cells in a shorter time. This protocol describes the different steps for obtaining a primary cell line from a skin biopsy.

Equipment and materials

- Laminar flow hood
- CO_2 incubator
- Inverted microscope
- Sterile surgical Instruments for microdissection
- BME fibroblast medium
- PBS 10x without Ca^{+2} or Mg^{+2}
- Collagenase type II (4mg/ml)
- Petri dishes 100 mm
- Pasteur pipettes
- Culture flask, 25 cm^2
- 15 ml sterile plastic tube

BME Fibroblast medium

BME	80 ml
Foetal bovine serum	20 ml
Penicillin-streptomycin solution 100x	1 ml

Filter and store at +4°C, up to 1 month.

The skin biopsy sample should be shaped as a diamond and about 5-10 mm in diameter. Collect the tissue sample in sterile BME fibroblast medium.

Procedure 1

1. Rapidly wash the skin biopsy in PBS in a Petri dish, cut into small fragments and transfer these to a flask.
2. Using a sterile Pasteur pipette with flame-rounded tip, distribute the small tissue fragments over the bottom surface of the culture flask.
3. Pass the flask rapidly and carefully through the Bunsen flame in order to evaporate the medium so that the minced tissue pieces adhere to the plastic surface, but so as not to heat-damage the minced tissue.

   Take care not to cook the tissue!
4. Carefully add BME medium for fibroblast growth, firmly close the lid of the flask and place in CO_2 incubator.
5. The next day, slightly unscrew the lid of the flask so that the tissue can "breathe."
6. Replace the culture medium after two days and, from this point on, replace it three times a week.

7. The fibroblasts will start to grow from the minced fragments in 2-3 days. When there are sufficient cells, they are detached enzymatically and plated in Petri dishes, or 75 cm^2 culture flasks, for proliferation (see next steps: "Maintenance of cell cultures in dishes and flasks" and "Routine subculture of adherent cell lines").The minced fragments in the flask will continue to produce cells for a while.

Procedure 2

1. Add collagenase to BME medium containing 20% FBS; so that the ratio medium : collagenase is 6:1, and filter.
2. Place the skin biopsy in a Petri dish, mince it into a coarse slurry using sterile scalpel and transfer to a 15 ml sterile plastic tube containing the BME-collagenase solution.
3. Place the tube in incubator at 37°C for 24 hours.
4. The next day centrifuge at 1 600 g for 10 min.
5. Using a sterile pipette, remove supernatant and wash pellet twice with PBS.
6. Prepare 1.5 ml of BME fibroblast medium in 2 flasks.
7. Suspend the pellet in 1 ml of BME fibroblast medium, transfer 500 microliter of suspension to each of the two flasks and distribute it over their bottom surface.
8. Place flasks overnight in CO_2 incubator at 37°C tightly closed.
9. The next day, slightly unscrew the lid of the flasks.
10. After a few days, fibroblasts should start to grow; if cells have developed, replace the culture medium with fresh medium after one week. From this point on, replace the culture medium three times a week.

T LYMPHOCYTES ACTIVATED WITH HUMAN INTERLEUKIN-2 (hIL-2)

Equipment and materials

- RPMI
- DMSO
- PBS
- Foetal bovine serum
- Human Interleukin 2 (hIL-2)
- phytohemaglutinin
- Accuspin System 1077 (centrifuge tubes for cell separation)
- Cell culture tube
- Culture flasks 25 cm2
- Sterile disposable 0.45 micrometer filter
- 2 ml cryogenic vials
- Bench centrifuge

Growth Medium

RPMI	90 ml
Fetal Bovine Serum (FBS)	10 ml
hIL-2	10%

Procedure

Purification of lymphocytes from peripheral blood:

1. Centrifuge Accuspin 1077 tubes for 30 sec. at 2500 rpm at room temperature
2. Place 3 ml fresh heparinized blood on the top of filter of Accuspin 1077 tube
3. Centrifuge for 15 minutes at 2500 rpm, at room temperature
4. After centrifugation remove plasma carefully
5. Transfer cells suspended in physiological solution to a 15 ml tube and centrifuge for 15 minutes at 1200 rpm at room temperature
6. Wash with physiological solution, and centrifuge a second time for 15 minutes at 1200 rpm at room temperature
7. Wash the pellet with physiological solution and centrifuge at 800 rpm for 10 minutes at room temperature
8. Resuspend cells in 3 ml of medium RPMI1640 containing 10% FBS and 10 microg/ml phytohemaglutinin
9. After 4-5 days add 2 ml of fresh medium RPMI1640 containing 10% FBS and 0,5 ml of hIL-2
10. Check the culture twice a week for cell growth and when cell clumps are clearly visible transfer the cell suspension in 25 cm2 flask
11. Incubate the culture, horizontally at 37°C in a 5% CO2 in air atmosphere. Maintain the cell density of the culture as suggested under the subculture

procedure

Subculturing Procedure

1. Cultures can be maintained by addition of fresh growth medium supplemented with 10% hIL-2. Alternatively, cultures can be established by centrifugation with subsequent resuspension at 3x 105 viable cells/ml
2. Maintain cultures at a cell concentration between 3 x 105 and 1 x 106 cells/ml. Do not allow density to exceed 3 x 106 cells/ml. Add medium, depending on cell density, every 2 to 3 days
3. Pool sufficient flasks for freezing a seed stock. Dissociate the cell clumps by trituration and count the viable cells. Calculate the total number of viable cells. Centrifuge the culture for 10 minutes at 1200 rpm at room temperature. Resuspend the cell pellet in the appropriate volume of cold (4-10°C) freeze medium [RPMI 1640 with 20% FBS and 10% DMSO] to yield approximately five million viable cells/ml. Dispense the cell suspension in 1-ml aliquots into plastic or glass cryovials. Freeze at -1°C/min to -80°C (either in microprocessor controlled freezer or passively in an isopropanol bath placed in a -80°C freezer overnight). Store in liquid nitrogen (vapor or liquid phase as appropriate)

EBV IMMORTALISATION OF LYMPHOCYTES

Lymphocytes are converted to lymphoblastoid cells by immortalisation. This is a convenient way of obtaining unlimited quantities of patient DNA or RNA. For this purpose, it is necessary to purify lymphocytes and obtain a source of Epstein Barr Virus (EBV) to infect the cells. Virus should be produced in a facility with adequate equipment for manipulating viruses (see GOOD PRACTICES FOR CELL CULTURE TECHNIQUES). Once the cells are transfected, no special precautions are required to handle them.

Method 1: B lymphocytes immortalization by EBV and cyclosporine

Equipment and materials

- B95-8 monkey cell line
- DMEM
- Ham's F12
- DMSO
- Cyclosporin
- PBS
- Foetal bovine serum
- Accuspin System 1077 (centrifuge tubes for cell separation)
- Culture flasks 25-75 cm^2
- Sterile disposable 0.45 micrometer filter
- 2 ml cryogenic vials
- Bench centrifuge

Growth Medium

DMEM or Ham's F12	90 ml
Fetal Bovine Serum (FBS)	10 ml
Cyclosporin	2 microg/ml

Procedure

Production of Epstein Barr Virus supernatant for lymphocyte transformation:

4. 2 ml supernatant is required for one transformation

5. Grow B95-8 monkey cells (EBV producers) to a density of 2.5 x 10^5 cell/ml as suspension cultures in 25 cm^2 flasks in 20 ml of DMEM/Ham's F12 supplemented with 10% FBS. Subculture the cells to obtain sufficient medium containing EBV for the transformation

6. When the culture has reached the desired density, transfer to a 75 cm^2 flask and add 40 ml medium. Leave in CO_2 incubator for 5 days

7. Spin suspension at 2000 rpm for 10 minutes

8. Filter through 0.45 micrometer filter

9. Freeze in ice and thaw in a 37 °C water bath (to denature proteins) three times,

very quickly

10. Aliquot in 2 ml cryogenic vials and freeze in liquid nitrogen

Purification of lymphocytes from peripheral blood:

1. Centrifuge Accuspin 1077 tubes for 30 sec. at 2500 rpm at room temperature
2. Place 3 ml fresh heparinized blood on the top of filter of Accuspin 1077 tube
3. Centrifuge for 15 minutes at 2500 rpm, at room temperature
4. After centrifugation remove plasma carefully
5. Transfer cells suspended in physiological solution to a 15 ml tube and centrifuge for 15 minutes at 1200 rpm at 4°C
6. Wash with physiological solution, and centrifuge a second time for 15 minutes at 1200 rpm at 4°C
7. Wash the pellet with physiological solution and centrifuge at 800 rpm for 10 minutes at 4°C
8. Resuspend cells in 8 ml of medium DMEM/Ham'sF12 containing 10% FBS and 2 microg/ml cyclosporin
9. Start the cell culture in two 25 cm^2 flasks as a suspension culture in 4 ml of medium and add 1 ml EBV supernatant
10. Check the culture twice a week for cell growth, visible as formation of cell clumps
11. After 4 weeks, there should be sufficient clumps and subculture should be performed to eventually obtain 4 X 25 cm^2 flasks (see SUBCULTURE OF SUSPENSION CELL LINES):
    Use one flask for karyotyping
    Use the other flasks for experiments, further growth or freezing
    Freeze cells in 2 ml of DMEM/Ham's F12 with 15% FBS and 5% DMSO at 10^6/ml.

Method 2: B lymphocytes immortalization by EBV and phytohemaglutinin

Equipment and materials

- B95-8 monkey cell line
- RPMI 1640
- DMSO
- phytohemaglutinin
- Foetal bovine serum
- Accuspin System 1077/Ficoll (centrifuge tubes for cell separation)
- Culture flasks 25-75 cm2
- 24 well cell culture plates
- Sterile disposable 0.45 micrometer filter
- 2 ml cryogenic vials
- Bench centrifuge

Growth Medium

RPMI 1640	80 ml
Fetal Bovine Serum (FBS)	20 ml
Phytohemaglutinin	10 ml

Procedure

Production of Epstein Barr Virus supernatant for lymphocyte transformation:
See Method 1

Purification of lymphocytes from peripheral blood:

1. If using Accuspin 1077 tubes, centrifuge for 30 sec. at 2500 rpm at room temperature. Alternatively, using Ficoll, carefully layer up to 5 ml of the sample onto of 2,5 ml of Ficoll, taking care not to cause mixing. Centrifuge tubes for 20 minutes at 2500 rpm at room temperature.
2. Place 3 ml fresh heparinized blood on the top of filter of Accuspin 1077 tube
3. Centrifuge for 15 minutes at 2500 rpm, at room temperature
4. After centrifugation remove plasma carefully
5. Transfer cells suspended in physiological solution to a 15 ml tube and centrifuge for 15 minutes at 1200 rpm at room temperature
6. Wash with physiological solution, and centrifuge a second time for 15 minutes at 1200 rpm at room temperature
7. Discard the supernatant. If the cells are to be frozen as ***peripheral blood lymphocytes (PBLs)*** *resuspend in 1 ml of RPMI 16140 +20% FCS +10%DMSO in a 1 ml cryotube*. If they are to be transformed follow step 8
8. Resuspend the cell pellet in 1ml of the following media:RPMI1640 + 20%FBS + antibiotics at 100 units/ml + 2mM L-glutamine+ phytohemaglutinin 10 microg/ml + 20% by volume EBV supernatant
9. Pipette the suspension into one of the eight central cells of a 24-well plate or alternatively into a tissue culture tube
10. Incubate at 37C, 5% CO2
11. After 4 days, check the cultures microscopically, looking for clumps of cells. Change the media by removing half the supernatant and replacing it with fresh media (RPMI+ 2% glutamine + 1% antibiotics + 20%FBS) taking care not to disturb the cells
12. Repeat steps every 2 or 3 days for 2 to 3 weeks. At some point during this time, foci of B cells will become visible and the initial proliferation of T cell will die down. The suspension is now ready to be transferred to a 25 cm2 flask with 4 ml of media RPMi+10%FBS + 2% glutamine + 1% antibiotics
13. Twice weekly, examine flask for a change to an acidic pH. Adjust the volume of medium in the flask by removing spent medium and adding more or less fresh medium to maintain a slightly acidic pH. Be sure to let the cells settle to the bottom of the flask before adjusting the volume of the medium
14. By 21 to 35 days in culture, the volume should have increased to approximately 20 mls. Cells should be growing in loose aggregates that can be broken apart by gentle trituration. At this time the lymphocytes can be subcultured at a seeding density of not less than 2x105 viable cells per ml
15. When cell density reaches 8x105 to 1x106 cells per ml, the culture should be split at not less than 2x105 cells per ml or cell stocks should be cryopreserved

16. Pool sufficient flasks for freezing a seed stock. Dissociate the cell clumps by trituration and count the viable cells. Calculate the total number of viable cells Centrifuge the culture for 10 minutes at 1200 rpm at room temperature. Resuspend the cell pellet in the appropriate volume of cold (4-10°C) freeze medium [RPMI 1640 with 20% FBS and 10% DMSO] to yield approximately five million viable cells/ml. Dispense the cell suspension in 1-ml aliquots into plastic or glass cryovials. Freeze at -1°C/min to -80°C (either in microprocessor controlled freezer or passively in an isopropanol bath placed in a -80°C freezer overnight). Store in liquid nitrogen (vapor or liquid phase as appropriate)

References

Bolton, BJ. Spurr, NK. B-Lymphocytes. In Freshney, RI and Freshney, MG (ed): Culture of Immortalized Cells; Wiley-Liss, New York, 1996, pp 283-297

Tumilowicz JJ. Gallick GE. East JL. Pathak S. Trentin JJ. Arlinghaus RB. Presence of retrovirus in the B95-8 Epstein-Barr virus-producing cell line from different sources. In Vitro 20: 486-492, 1984

Miller G. Shope T. Lisco H. Stitt D. Lipman M. Epstein-Barr virus: transformation, cytopathic changes, and viral antigens in squirrel monkey and marmoset leukocytes. Proc. Natl. Acad. Sci. USA 69: 383-387, 1972

SUBCULTURE OF SUSPENSION CELL LINES

Cells should be cultured in the exponential growth phase when they appear bright and rounded under the microscope, and refractory to vital dye. If phenol red is added to the medium and turns yellowish, the pH is on the acid side of neutral, indicating that the population has passed the exponential phase and is not in the best condition for subculture or storage.

Equipment and Materials

- Laminar flow hood
- CO_2 incubator
- Growth medium
- Flasks
- Inverted microscope

Procedure

If the pH of the medium is acid:

1. Transfer the cell suspension to a centrifuge tube and centrifuge for 5 minutes at 2000 rpm
2. Remove the supernatant but do not discard it; suspend the pellet in 5 ml of fresh medium and add 10-20% of the supernatant as conditioning agent
3. After a few hours or the day after, count the cells and, depending on the number, either let the cells grow in the same medium or subdivide them in further flasks with fresh proliferating medium according to the type of cell

If the pH is neutral:

1. Gently pipette the cell suspension several times to break up any clumps of cells and take an aliquot for cell counting and cell viability determination (see ROUTINE CELL COUNTING AND ASSESSMENT OF VIABILITY)
2. Calculate the cells/ml and re-seed the desired number of cells into new flasks without centrifugation, just by diluting the cells

PROTOCOL FOR THE CONDITIONING AND TRANSPORT OF TISSUES AT 4°C

Preparation of the packaging

Equipment and Materials

The following packaging material[4] is necessary:

- Primary packaging: A leak-proof tube (stop valve or screw cap) containing the transport medium (limited to 50 ml) and the tissue sample
- Absorbent paper (kitchen paper)
- Bubble wrap
- Secondary packaging: Hard plastic container with a screw-top lid
- A frozen pack (freezpack®) to maintain the sample at 4°C during transport
- Outer packaging: An approved cardboard package-box for the transport of biological material

Keep the freezpack in the freezer before using it for the transport of the sample(s)

Procedure

A- Preparation of the packaging : this procedure is in conformity with the regulation(s) in force

1. Wrap the leak-proof tube containing the sample in absorbent paper (kitchen paper), then in bubble wrap, and place it in a plastic container
2. Tightly close the screw-top lid of the plastic container
3. Then put the plastic container in the package-box together with the frozen pack (or freezpack®)
4. Please enclose the following documents :
5. the Transport Document
6. the Safety Instructions Document
7. Label the package-box clearly so that it is easy to read and to identify the sender's and recipient's addresses, on two separate labels, and give all the information necessary for the delivery.
8. The label must indicate: "DIAGNOSIS SAMPLE(S) -UN3373"

B- Transport of Samples

1. Samples shall be picked up at the sender's institution
2. The carrier must be accredited for the transport of dangerous goods
3. For transport to foreign countries, both the sender and the recipient must have an import-export authorization for biological material[5]

4 The packaging material is approved by the Regulation on Transport of Biological Material (ADR) in force as of January 1st 2005.

5 Authorisations are delivered by the national or local authority

SAFETY INSTRUCTIONS IN THE EVENT OF AN INCIDENT OR AN ACCIDENT

The "Transport Document" should be affixed to the outer packaging and provide information on the products transported. The "Safety Instructions Document" must be attached to it.

IF THE OUTER PACKAGING BREAKS

1. Please check that the secondary packaging has not been damaged and that there is no leak.
2. Recondition the package, taking the temperature into account, as specified in the Transport Document
3. Call the sender using the phone number indicated on the Transport Document and the package label

IF BOTH THE OUTER PACKAGING AND THE SECONDARY PACKAGING BREAK

1. Isolate the damaged package, do not touch it and do not walk on any spilled fluid
2. Keep any non-authorized personnel clear of the risk zone
3. Treat the damaged package wearing latex gloves

For a package at 4°C

- Absorb any spilled fluid from the primary packaging with absorbent paper, if you have some, or with the soil near it
- Recover the soiled absorbent paper and put it either in a container that you can close, or in the package-box
- Confine the package in a plastic bag
- Do not dispose of it in an ordinary rubbish-bin, but in a bin specially designed for the incineration of Class 6.2 products. Please contact the sender to organize the incineration
- Spray bleach, or ethyl alcohol 70% or else any known antiseptic product, on the soiled zone

For a package on dry ice

- Do not touch any spilled dry ice. Dry ice may be soiled. It will evaporate by itself.
- Then follow the same procedure as for a package at 4°C

For a package in liquid nitrogen

- Do not touch the liquid nitrogen as you may get seriously burnt. It will quickly evaporate by itself
- Then follow the same procedure as for a package at 4°C

Report the incident to the sender using the phone number indicated on the Transport Document and the package label.