EUSTAR guidelines for preparation and storage of biologic samples in systemic sclerosis

Studies with biological samples in patients with systemic often show contradictory results and general conclusions are often difficult to draw. One of the reasons for the conflicting results is the clinical heterogeneity of the disease, which requires high sample numbers to obtain a homogenous study population. EUSTAR with its large database of clinically well-defined patients offers an excellent screening tool to obtain sufficient numbers of biological samples from patients even with rare clinical events (see rules for submission of basic science projects).

Another problem which accounts for the contradictory results of many studies is the large variety in the methods that are used to prepare and store biological samples. The EUSTAR basic science working group therefore decided to develop guidelines to define “good laboratory practice” in handling of biological samples of systemic sclerosis patients. It has to be emphasized that these guidelines are not strict rules how biological samples must be prepared, rather they are suggestions showing how basic science centers working in the systemic sclerosis field process biological samples in their laboratories. In addition, the measurement of specific factors might require substantial changes to the protocols provided here. Some of the procedures such as the isolation of endothelial cells need a long-standing expertise and close collaboration with groups working in the field is recommendable. Last but not least, biological samples can only be obtained after informed consent was obtained from the patients.

With the guidelines provided here, we hope to contribute to the overall goal of EUSTAR to encourage clinical and clinically-related basic science studies in systemic sclerosis. The equalization of methods to prepare and store biological samples should make the interpretation of studies in patients with systemic sclerosis easier.

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Protocol for explant culture of dermal fibroblasts

Protocol from Royal Free Centre for Rheumatology, London.
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Notes:
This protocol outlines the method that is in routine use for explant culture of dermal fibroblasts from skin biopsies.

Punch skin biopsy technique
Skin biopsies are taken using a 3mm to 5mm diameter sterile biopsy punch. Generally a lesional (involved) and a non-lesional (uninvolved) area of skin is sampled for scleroderma cases. Biopsies distal to the wrist are avoided as healing may be very slow. All biopsies are performed with strict aseptic technique and the skin should be thoroughly cleaned using antiseptic solution. *Each biopsy is placed in DMEM culture medium for transport to laboratory. All containers and medium must be sterile.*

Primary culture protocol
-Prepare laminar flow cabinet/equipment with 70% IMS
-Run laminar cabinet for 5mins before use
-Using a sterile disposable scalpel finely chop the biopsy into 1mm pieces.
-Carefully spread the skin fragments on the surface of a sterile 25ml tissue culture flask.
-Allow the flask to dry at room temperature within the culture hood for 15 minutes
-Add culture medium with antibiotic supplements
-Leave the cultures in standard incubator at 37^{o}C 5% CO2 for 14-21 days
-Trypsinise and split the cells as outlined below.
After primary culture, the subsequent growth can be in antibiotic free medium
-For most experiments fibroblasts should be used between second and fifth passage. Alternatively early passage cells can be frozen and used in future experiments.

Typical fibroblastic morphology by inverted phase-contrast microscopy confirms the fibroblast phenotype.

-Fibroblast growth medium

DMEM (4500 glucose high), glutamine, pyruvate) 500ml
FBS 50mls =>10%
Pyruvate 1mM
L-glutamine 2mM
Penicillin 100U/ml*
Streptomycin 100ug/ml*
Gentamicin 50ug/ml*
Amphotericin B 2.5ug/ml*

* only for initial primary culture of biopsies
-Trypsin/TA – for detachment of cells for freezing or passaging

Passaging fibroblasts

To passage cells
-Check media not cloudy i.e. contaminated
-Discard culture media (pipette)
-Wash with 5-10ml trypsin/serum free DMEM and discard
-Add T/E to detach monolayer: 3-8ml for 75, 5-15ml for 150ml flask, after 5 mins, agitate & check under microscope. Can accelerate in incubator.
-Add 5-8ml 10% FBS to inactivate T/E
-Pipette to centrifuge tube (yellow top)
-Centrifuge @ 1000rcf/10min/temp 22 or 1600/5/22
-Discard supernatant
-Agitate cell pellet
-Add 10% FBS – approx 15ml – vary according to concentration required
-Transfer to flasks (split sample), check conc under scope +/- add further 10% FBS – aim 15-20ml per flask

To freeze fibroblasts for future culture

-Remove media and discard

-Trypsinise – wash 2-3mls TE, then trypsinise with 7-8mls TE 5 mins, add 8mls 10% FBS, centrifuge

-Prepare freezer medium- 14mls 10% FBS, 2mls DMSO, 4mls neat FBS – total 20mls = 20% FBS, 10% DMSO. Filter.

-Add 1ml FM to pellet per cryo-tube. Usually split T75 into 2/3 cryo tubes. 1ml to each tube.

-Put in -80 freezer o/n then transfer to liquid N2.

Protocol for culture of dermal fibroblasts derived by enzymatic digestion

Protocol from the Center of Experimental Rheumatology, University Hospital Zurich, Switzerland

Oliver Distler, Ferenc Pataky

All procedures must be performed under sterile conditions and with sterile solutions

(1) Prepare Dispase II solution: Dissolve 80 mg Dispase II (from Bacillus polymyxa, 0.94 units/mg, e.g. from Gibco BRL, Cat-Nr. 17105-041) in 100 ml PBS (0,8 mg/ml = 0,75 U/ml), perform sterile filtration through a 0.2 μm filter membrane.
(2) Cut the skin biopsy into small pieces (< 1mm) e.g. by using a sterile scalpel. Take care that the tissue does not get dried, e.g. by adding some drops of PBS
(3) Put the minced tissue into a 50 ml Falcon Tube, add 50ml of the Dispase solution and incubate at 37°C for 1 hour with stirring.
(4) Remove supernatant and put into a new Falcon tube. Store at 4°C.
(5) Add the remaining 50 ml of Dispase solution to the remaining tissue. Incubate another 30 min at 37°C with stirring.
(6) Pool supernatant with the supernatant from step 4. Centrifuge the pooled supernatants at 250g for 10 min.
(7) Discard supernatant. Perform a washing step by adding 50ml PBS, followed by agitation of the pellet and another centrifugation at 250g for 10 min
(8) Discard supernatant. The pellet contains cells derived from enzymatic digestion. Dissolve pellet in DMEM and put cells into a culture flask.

We use DMEM with antibiotics as outlined above routinely for all passages. After 2-3 passages the culture usually contains 100% fibroblasts, this can be checked by the typical fibroblast morphology and can be additionally confirmed by FACS analysis to exclude contamination with e.g. macrophages (e.g. CD14 antibodies).

Guidelines for the standardization of the collection and processing of bronchoalveolar lavage (BAL) fluid

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Definition of bronchoalveolar lavage (BAL) according to the American Thoracic Society (1).

Bronchoalveolar lavage (BAL) is a technique that allows the recovery of both cellular and acellular components from the epithelial surface of the lower respiratory tract and differs from **bronchial washings**, which refer to the aspiration of either secretions or small amounts of instilled saline from the large airways.

General remarks

BAL is performed as a part of routine bronchoscopy. It is assumed that centers performing BAL have already had good experience in techniques of bronchoscopy and BAL. Moreover, there are guidelines published by the American Thoracic Society and the European Respiratory Society which precisely describe these techniques (1-3).

Therefore, recent guidelines will only address issues in BAL techniques/handling, which have not been precisely defined (may differ between centers) and which are crucial for obtaining comparable samples allowing standardized studies.

1. Site of lavage

The right middle lobe or lingula.

If possible another site may be lavaged additionally, e.g. lower lobes. **(1)**

For the decision of lavages of additional sites it is recommended to take into consideration the pattern of lung involvement seen on high-resolution computed tomography (HRCT).

2. Volume of fluid instilled

200 ml of sterile, pyrogen free saline solution (isotonic 0.9% NaCl) at room temperature instilled and recovered in 4 aliquots of 50 ml each. **(2)**

3. Processing of BAL samples

a) General remarks

- To avoid artefacts caused be cell activation/damage recovered fluid should be kept and transported on ice using plastic or siliconised glass syringes/tubes and cells should be separated from the fluid as soon as possible.

b) Fluid recovery

- The volumes of all aliquots recovered should be measured and fluid recovery (expressed as a percentage of fluid instilled) should be established.

c) Total and differential cell number

- It is recommended NOT TO pool the first aliquot with the fluid recovered subsequently[3].
- The last three aliquots should be pooled and mixed thoroughly**,** and than filtered through sterile gauze to remove mucous particles.
- The total cell number should be counted in a small aliquot of the pooled BAL fluid using a haemocytometer. Former washing procedure may result in a considerable loss of cells (2).
- Cells should be separated from the fluid by centrifugation at low speed (500 g – 800 g) for 10 to 15 minutes as soon as possible (centrifugation at 4^{o}C is recommended).
- Cytocentrifuge preparations are recommended as a routine procedure to obtain differential cell counts. They may be prepared using pooled BAL fluid or after the cells are resuspended in buffered medium (e.g. Hank's balanced solution) without addition of the serum.
- For differential cell counts cytospins should be stained with May-Grunwald-Giemsa and at least 500 cells should be counted (using random field counting method). It is recommended that differential cell counts are made every time by two independent technicians.

4. BAL supernatant storage

BAL supernatant should be separated from cells as soon as possible and frozen at – 80^{o}C. It is recommended to divide the supernatant before freezing into 1.0-2.0 ml

aliquots that may be used for particular measurements (to avoid repeated thaw and freeze cycles)

5. BAL cell storage

- For storage, cell pellet after centrifugation should be resuspended in RPMI-1640 medium with 20% FCS and 10% DMSO at a concentration of 1-10 x 10^6 cells/ml. If molecular studies are planned DMSO should be avoided. The cells should be rapidly frozen and stored in liquid nitrogen (-80^{o}C).
- Additional cytocentrifuge preparations may be prepared and stored for immunocytochemical or immunofluorescent analysis (after fixation with aerosol-based fixatives).

6. Standardization of the results of measurements of soluble components of BAL fluid

The results of measurements in BAL fluid should be reported per ml of BAL fluid recovered[(4)].

7. BAL data

Every BAL sample should include information regarding:

- patient data
- site of lavage
- fluid recovery (expressed as percentage of fluid instilled)
- if the first aliquot is not pooled, the volume of the fluid processed
- total cell count (expressed as number of cells per millilitre of recovered lavage fluid)
- differential cell count (expressed as percentage of neutrophils, eosinophils, basophils, lymphocytes and macrophages).

8. Final remarks

a. BAL fluids with a recovery of less than 30% should not be included.

b. BAL fluids contaminated with blood should not be included.

c. BAL fluids from patients with respiratory tract infections should not be included (unless intentionally).

(1) *Although controversy exists regarding the site of BAL in diffuse lung diseases, the right middle lobe is recommended as a standard site for BAL due to the following reasons:*

- The right middle lobe is the most convenient one from an anatomical point of view and allows higher recovery of fluid and cells when compared with the lower lobes (about 20% - ref. 2)

- The middle lobe is used as a standard site for BAL in the majority of centers performing BAL (2)

- The report of the European Society of Pneumology Task Group on BAL states that: "...in most patients with diffuse interstitial lung disease lavage at one site should give sufficient clinical information. In general, results obtained at one site should be representative for the whole lung."(2).

- It has been shown that inflammatory indices, such as increased percentage of granulocytes, concentration of albumin and markers of neutrophil activation are present in BAL samples taken from radiologically uninvolved lungs of patients with interstitial lung diseases including SLD (presumably representing early phase of the disease) (3,4).

- In contrast to HRCT-guided BAL, use of a "standard site" does not require HRCT of the lungs, and SSc patients may be included according to standard chest X-ray and pulmonary function tests.

(2) *The volume of fluid used is the point of greatest technical variation in carrying out BAL and varies from 100 ml to 400 ml (1,2). Too small amounts carry the risk that "bronchial washing" components will dominate. Too much fluid instilled will result in greater dilution of acellular BAL components and increases the risk of side effects (2). It has been shown that information about cell types obtained in volumes of 100 ml-250 ml are comparable (2). Therefore, 200 ml in four aliquots of 50 ml each seems to be a reasonable volume which allows to discard the first aliquot (bronchial washing) and provides enough fluid for the study of cellular and acellular components.*

Prewarming of instilled fluids to body temperature (37^{o}C) reduces the risk of bronchial irritation and therefore may improve fluid recovery; however, the majority of centers performing BAL use fluid at room temperature (2).

(3) *The first aliquot represents a disproportionate amount of bronchial airway material rather than alveolar return (bronchial washing) and it differs from the other aliquots in particular in smokers and persons with bronchial inflammation. The first aliquot may be processed separately or discharged. Separation of the first aliquot is particularly recommended when acellular components of BAL fluid are going to be studied (2).*

(4) *There is no reliable denominator or reference substance which can be used to quantitate the dilution of epithelial lining fluid and therefore to make the measurement of soluble components more comparable. Concentration of albumin, which was used in several studies, has been shown to be elevated in interstitial lung diseases, presumable as a result of increased permeability of capillary-alveolar barrier (3, 4). According to the recent "Guidelines for measurement of acellular components and recommendations for standardization of bronchoalveolar lavage (BAL)" published by the European Respiratory Society, "...the investigators should try to standardize the volume of lavage fluid introduced and should report results per ml of bronchoalveolar lavage fluid..."(3).*

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Protocol for serum asservation

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1. Prior to phlebotomy, patient consent form must be signed and archived.
2. Each center needs to have a patient log book, in which the name, date and the individual code for the patient is being registered continuously for each phlebotomy
3. Perform standard phlebotomy with the system generally used for the asservation of serum in the local in/outpatient clinic
3. Asserve at least 10 ml serum for each patient (equivalent to ca. 3-4 ml serum)
4. Centrifuge tube for 400 g for 10 minutes at room temperature.
5. Asserve clear “supernatant”, not the lower cellular phase, with a 500 µl pipet and aliquot the complete serum in 5-8 Eppendorf tubes (with 500 µl serum each).
6. Label each aliquot with the individual patient code number (**not** with the patients’ name) and the date of phlebotomy.
7. Store immediately at -20 ° (better -70°, if available) until further use
(Annotation: most of the molecules in serum are stable at 4°C for a couple of hours, but some critical ones such as TNF need immediate freezing)

Guidelines for biological samples in genetic research in systemic sclerosis: DNA extraction

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After patient's informed consent, DNA can be isolated from a variety of cells including the following three types of samples: Whole blood, lymphoblastoïd cell lines and tissues. DNA extraction can be performed using either commercial kits or standard protocols.

DNA extraction kits are available from many different suppliers. The most commonly used bind DNA while RNA and proteins are removed. Kit based methods provide a rapid DNA extraction and are easily reproduced. The laboratories should choose the method of preparation according to the amount and the quality of the DNA required for subsequent studies. Southern blot analyses requires DNA of high molecular weight, whereas DNA size is not critical for PCR analyses.

As an alternative, a classical inexpensive procedure based on the salting out principle can be used. This procedure gives a good yield and quality of DNA. This procedure is much safer than the phenol-chloroform extraction procedure. The principle of this DNA extraction method is given below (for details see Miller et al, Nucleics Acids Res, 16, 1215, 1988).

1) Whole blood is taken into tubes containing anticoagulants (EDTA, heparin) and stored at -80°C. After having thawed the sample, it is mixed with an hypotonic solution and centrifuged twice in order to lyse red cells. White cells recovered by centrifugation are incubated overnight with a buffer containing a detergent and a proteinase, allowing to separate nuclear DNA from associated proteins. After further centrifugation, the nuclear DNA is precipitated with cold absolute ethanol taking the "DNA jellyfish" form. Then, a tampon volume according to the size of the jellyfish is added before destroying endonucleases by heating. After resuspension, the concentration of the sample is controlled by spectrophotometry. The typical yield is approximately 300-500µg of DNA a from 10 ml whole blood extraction. DNA samples are then stored at -20°C until use.

2) In order to obtain a source for larger amounts of DNA, immortalized lymphocytes can be generated. After purification from peripheral blood, lymphocytes are cultivated with Epstein Barr Virus supernatant during 4 weeks to obtain immortalized lymphocytes. Lymphocytes are conserved at -80°C. Then, the protocol outlined above can be used to isolate DNA (red cells lysis is not necessary). This procedure should only be used, if DNA cannot be generated by other protocols.
3) With tissue samples, there are two differences as compared to lymphocytes: Cellular lysis requires specific tampon and the use of phenol-chloroform is recommended in the case of clots for dilution *.

Limitations and pitfalls:
1) During the whole DNA extraction, all stages are done with gloves and sterile pipettes to avoid DNase contamination.
2) The extraction phase is long and complicated requiring a clear written protocol.
3) *With whole blood, clots could need to use equilibrated phenol and chloroform/isoamylic alcohol mix.
4) If whole blood volume > 10ml, it has to be divided into 2 separated tubes.

Microvascular endothelial cell culture

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Endothelial cells will be isolated from skin biopsies of patients. Briefly, vascularized dermis will be mechanically isolated from skin biopsies, cut in little pieces and digested with 0.3% trypsin-0.1% EDTA in PBS for 60' at 37°C. After the enzymatic treatment, the tissue will be squeezed in culture medium MCDB 131 (e.g. Sigma) to obtain endothelial cells outgrowing from the microvessels. Cells will be cultured on gelatin (1% in PBS, sterilized by autoclave) coated dishes (24 Multiwell) in MCDB 131 supplemented with 30% FCS (e.g. Cambrex), 20 µg/ml endothelial cells growth supplement (ECGS) (e.g. MP Biomedicals), 10 µg/ml hydrocortisone, 15 UI/ml heparin, and antibiotics (100 UI/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml amphotericin). In some cases, it can be necessary to purify the endothelial cells by immuno-magnetic isolation (Mini Macs) with CD105 (endoglin) microbeads (e.g. Miltenyi Biotech). Isolated cells will be identified as microvascular endothelial cells (MVEC) by labelling with anti-factor VIII-related antigen and by re-probing with anti-CD31 antibodies. Cells will be used within the 7th passage in culture.

For freezing: 50% FCS, 10% DMSO, 40% MCDB 131, 1.5 x 106 cells per vial.

GELATIN (e.g. Sigma, G-9391)
For coating dishes: warm gelatin in the incubator for some minutes, meantime wash the multiwell with PBS for twice. Aspirate PBS and add 250 microliters per well of 1% gelatin. After this step, aspirate gelatin and utilize the multiwell.

ECGS (e.g. MP Biomedicals, 152330) dissolve in MCDB 131 medium (2 mg/ml, solution 100x), divide in vials and stock at -80°C.

HYDROCORTISONE (e.g. Sigma, Water soluble H-0396), dissolve in PBS or medium (0,8 mg/ml, solution 100x), divide in vials and stock at -80°C.

HEPARIN (e.g. Calbiochem, 375095), dissolve in PBS or medium (10 mg/ml, solution 100 X), divide in vials and stock at -80°C.

MCDB 131 (e.g. Sigma, M-8537)