Ex-Vivo Lung Perfusion (EVLP)

Standard Operating Procedure (Version 3.0: 5th July 2013)

Location:

EVLP is performed in a suitably designated clinical area that fulfils the necessary infection control precautions. In most cases this will be in an operating theatre but it could be in an air-locked ICU cubicle with operating theatre standard positive pressure ventilation.

Equipment:

- **Vivoline evaluation unit**: An integrated roller pump (Jostra), heater cooler unit, gas system, monitor and control unit with associated software package.
- **Vivoline disposable lung set**: Includes an oxygenator (Capiox), LDF leukocyte filter, pressure sensors and temperature probes.
- **93% Nitrogen and 7% CO2 gas mixture cylinder on a trolley**: A full cylinder contains 10000 litres and each EVLP run requires at least 2000 litres of gas mixture (3 hours).
- **Oxygen supply**: From the wall outlet.
- **ICU standard ventilator:** Model that measures minute and tidal volumes, lung compliance / resistance and airway pressure.
- Arterial blood gas machine.
- **Standard thoracic surgical instrument tray**.
- **Sterilised bronchoscope**: With suction tubing and lavage traps, 5, 10 and 20ml syringes.
- **Sample collection disposables:** Includes Duet-stapler devices for lung biopsies, specimen pots for tissue, test tubes for perfusate and ice bucket or refrigerator for sample storage (please refer to sample SOP).

Medications:

- **Saline 0.9%:** 240mls for 2xBAL and additional 100mls for flushing pressure transducers.
- **Steen Solution:** At least 4 x 500ml bottles.
- **Packed Red Blood Cells:** Request 3 packs of red blood cells from the blood bank, either universal donor (O negative) if >1 potential recipient identified or cross matched to the single known recipient.
- **THAM/TRIS solution:** Use a preparation containing Trometamol 3.0mmol/ml in a quantity as directed by base deficit to a Base Excess +/- 3 and pH of 7.35 – 7.45 (see Appendix 1).
- **Heparin:** 10,000IU unfractionated.
- **Methylprednisolone:** 500mg.
- **Antibiotics:** Meropenem 500mg is the default antibiotic if there is no documented recipient allergy to ß- lactams. If donor airway cultures known, other antibiotics can be used after discussion with microbiology.
- **Amphotericin B:** 10 mg for injection (Amphocin, Fungizone or equivalent).
- **Actrapid Insulin and 10% Glucose Solution:** Possible requirement for 10ml of 10% Glucose solution and 20 IU Actrapid Insulin but not routinely used.

Priming the Circuit:

- Connect the disposable kit to the evaluation unit according to user manual instructions.
- Flush the pressure transducers.
- Connect the water to the heater-cooler unit, open the water bag.
- Start the ***priming phase*** on the screen. Check the oxygenator for leakage.

- Calibrate the pressure transducers.
- Fill the system with 2.0L Steen solution (4 x 500ml bottles).
- Calibrate the pump occlusion by following the screen instructions.
- Recirculate perfusate at 15°C set flow to 2.0 L/minute and maximum perfusion pressure at 10 mmHg.
- Connect the gases.
- Add 10,000IU of unfractionated Heparin to circulating Steen solution.
- Add 1-2 units of Packed Red Blood Cells.
- Prepare 500mg dose of Methylprednisolone.
- Make up Antibiotics and Amphotericin B according to manufacturers' instructions.
- Add Methylprednisolone, Antibiotics and Amphotericin B to the circulating perfusate.
- Do a perfusate blood gas analysis and correct the base deficit with THAM to a Base Excess +/- 3 and pH of
  7.35 – 7.45. Add 3 mmol THAM per minus unit in base deficit please see Appendix 1.

  The blood gas needs to be temperature corrected for 15^{O}C.
- Note haematocrit of perfusate on blood gas. Target is 10-15% so add more red cells if needed.
- Check perfusate glucose and potassium concentration via blood gas analysis; if glucose <5 or >20 mmol or potassium >7mmol correct with Insulin as per Appendix 2.
- Repeat the perfusate gas prior to connecting the lung for any further corrections.
- The perfusate, pharmaceuticals and gases should be circulated for ≥15 minutes before connecting the lung.
- If satisfactory, start the ***reconditioning phase***.

Reconditioning Phase:

- Surgical dissection is performed to allow placement of the donor lung onto the Vivoline EVLP circuit in the covered organ bath to maintain humidity.
- Before connecting the lung to the circuit, take ***Lung Biopsy #1*** from the RML or Lingula and collect ***Perfusate Sample P0*** (See separate Sample collection SOP).
- Cannulate the main pulmonary artery with the quick-fix pre-fashioned cannula and open the shunt to the inflow cannula.
- The left atrium is left open and visualised to ensure a smooth flow of perfusate.
- The LA temperature probe and sampling line is secured in place equal distance from the 4 pulmonary veins.
- Where possible the trachea remains clamped with lungs partially inflated with 50% FiO2 while the quick-fix ventilation tube is secured in place. This prevents collapse of the lungs and development of atelectasis prior to the ventilation.
- Set the pulmonary artery (PA) pressure to a maximum of 15 mmHg.
- Perform de-airing of the circuit with the shunt open at a flow of 0.5L / minute for approximately 2 minutes until fully de-aired. Leave the shunt open.
- Set temperature to 32^{O}C. The system will warm up the lung automatically with a maximum delta temperature of less than 8°C.
- If initial perfusion is uneventful, increase the PA pressure limit to 20 mmHg and flow to maximum.
- The recommended max flow is 70 ml/kg IBW /minute (see Appendix 3). With a cold lung the pressure will limit the flow, when the resistance in the lung goes down the flow will increase over time.
- When temperature has reached 25^{O}C close the shunt.
- At 32^{O}C remove the clamp on the trachea and before commencing ventilation, perform a

bronchoscopy and collect ***BAL sample #1*** from either RLL or LLL using 120 ml 0.9% NaCl with the bronchoscope in a wedged position (See separate Sample collection SOP).

- Commence mechanical volume-controlled ventilation at 32^{O}C with a protective ventilation strategy:
    - Set inspired oxygen (FiO_2) to 0.5 (50%)
    - Set respiratory rate (RR) initially to 5 or 8 breaths/min according to donor IBW (see Appendix 4)
    - Set Minute volume (mV) initially to 1 L/min
    - Set PEEP at 5cmH_2O
    - Set I:E ratio 1:2 and inspiratory pause at 10%
    - Keep peak airway pressure <20 cmH_2O
- Increase temperature from 32^{O}C to 37^{O}C.
- Increase the mV in 1 L/min increments gradually as lung warms to 37^{O}C; **mV should not exceed 1.5X the flow**. Continue to keep peak airway pressure (Paw) < 20 cmH_2O. Increase the RR, as mV increases, up to a maximum of 15 breaths/min to keep tidal volume <7ml/kg IBW (see Appendix 4).
- At 32°C the perfusate flow is usually lower than the set value, please note the flow as the lungs warm to 37°C as the **minute volume should not exceed 1.5X the flow**.
- If uneventful and once 37°C reached, mV can be increased **gradually** up to a maximum of 100ml/kg IBW/ min but the tidal volume should not exceed 7ml/kg IBW (see Appendix 4).
- If persistent atelectasis present, perform a recruitment manoeuvre by transiently increasing PEEP from 5cmH_2O in increments of 1cmH_2O for a few breaths to a max of 12cmH_2O while always keeping Paw <25cmH_2O. Note flow will fall significantly during increase in PEEP. After recruitment return PEEP to 5cmH_2O.
- Flow, PVR, Lung Compliance and PA pressure to be documented once lungs reach 37°C and flow stabilised.
- Once lungs are at 37°C with stable flow and ventilation settings and satisfactory appearance, shift to the ***evaluation phase***.

Evaluation Phase:

- Once re-warming is complete and target perfusion established, the function of the donor lungs undergoing EVLP can be assessed as specified in the study protocol.
- Disconnect the oxygen from the perfusion system prior to evaluation.
- Once the perfusate is deoxygenated and confirmed on blood gas analysis, perform recruitment manoeuvres as above and set the ventilator for evaluation as below:
    - Increase FiO_2 via the ventilator from 50% to 100%.
    - PEEP can be increased to 8cmH_2O for a short period.
    - Maximum mV should not exceed 100mls/kg/min (donor IBW)
    - Keep peak airway pressure Paw <25cmH_2O
- RR can be adjusted between 12 and 15breaths/min to maintain V_T up to a maximum of 7ml/kg (donor IBW)
- Perform blood gas analysis 15 minutes after FiO_2 is increased to 100% to assess venous and arterial pO_2 values. Blood gas analyses should be performed from each pulmonary vein as well as a mixed LA sample.
- Flow, PVR, Lung Compliance and PA pressure should be carefully documented on the data sheets.
- Perform a lung deflation test by disconnecting the tracheal tube at the end of inspiration. **Remember to first reduce perfusate flow to maximum of 1.5L/min to avoid alveolar**

oedema. Recoil of the lungs is evaluated subjectively; global collapse of the lungs is defined as normal.

- If transplant suitability criteria have been achieved, move immediately to ***cooling phase*** for organ preservation.
- If transplant suitability criteria have not been achieved, return to the reconditioning phase.
- Perform hourly clinical assessments as documented in the protocol until a decision on suitability of the lungs for transplantation or 240 minutes of EVLP perfusion has been reached (from the time of reaching 37°C).
- During perfusion if pH <7.35 administer additional THAM to the Steen Solution™.
- During perfusion do not automatically replace Steen Solution™.

Cooling Phase:

- Reduce mV by a 30% reduction in tidal volume and lowering respiratory rate to 8 breaths/min
- Set temperature to 32°C
- Reconnect the oxygen to the perfusate.
- Before discontinuing ventilation, perform a bronchoscopy and collect ***BAL sample #2*** from the same lobe, but from a different segment than BAL sample #1, using 120 ml 0.9% NaCl with the bronchoscope in a wedged position (See separate Sample collection SOP).
- Stop ventilation at 32°C, clamp trachea with lungs partially inflated with 50% FiO2.
- Set temperature to 12°C and continue to cool lungs until perfusate temperature 12°C.
- Collect ***Perfusate Sample PX***.
- Disconnect PA cannula and plug PA with special bung.
- Once perfusion has stopped and the lung is disconnected, take ***Lung Biopsy #2*** from the same lobe as Lung Biopsy #1 (See separate Sample collection SOP).
- Commence topical cooling.
- Place mat under lungs and wrap towels over lungs so that they touch the mat all around lungs.
- Connect the Y shaped hose from the cooling assembly to the lung perfusion quick connection and cover the highest point of each lung. Connect the remaining hose from the cooling assembly to the shunt quick connection and place over the trachea and PA.
- In the preservation phase set pump to 2.5 L/ min. Check fluid level and add more Steen Solution if necessary.
- Maintain the lungs in topical Steen solution at 6 - 8 °C on the circuit (preservation phase) until ready for transplant.

Adaption for Single lung Reconditioning

- For cannulation, if feasible staple the contralateral PA (right PA if it is a left lung transplant or vice versa) at least 2 cm above its first branch to facilitate as much length as possible. If the pulmonary artery is too short and/or the surgeon is unable to fit the quick-fix pre-fashioned cannula, use a part of the donor's aorta to augment the cuff.
- For connection of the bronchus, please note there are 3 sizes of connection available in the disposable kit to aid achieving an effective connection. If it is a right lung EVLP, staple the left main bronchus at the level of the carina and cannulate the trachea. If it is a left lung EVLP, the left main bronchus from the level of the carina should be long enough to facilitate attachment. Ensure a seal that prevents either ineffective ventilation of fluid entering the airway.
- Set to 50% target flow i.e. 35 ml/kg body weight/minute.
- When starting ventilation, start at a minute volume of 0.5 L/min and increase to a

maximum of 1.5 times the perfusate flow.

Appendices:

Appendix A

Use of THAM/TRIS to buffer Steen Solution: THAM is available in various dilutions (ranging from 3.6% (Abbott, Köhler) to 36.5% (Braun) or 40% (Fresenius-Kabi or Addex-THAM) so caution is advised in measuring the correct amount. **PLEASE CHECK THE CONCENTRATION OF YOUR THAM PREPARATION!**

A 3.6% solution of THAM contains a concentration of Trometamol (active ingredient) of 0.33mmol/ml A 40% solution of THAM contains a concentration of Trometamol (active ingredient) of 3.0mmol/ml When buffering Steen solution in the circuit use 3mmol of THAM per minus unit in base deficit.

This will be 1ml per minus unit base deficit for the 40% THAM preparations and 10ml per minus unit base deficit if the 3.6% THAM preparation is used.

A THAM preparation of between 3.0 – 3.3 mmol/ml is strongly recommended and use of lower concentrations strongly discouraged due to the dilutional effects on Steen Solution of adding large volumes of a lower concentration THAM.

Appendix B

During perfusion administer 10ml of 10% Glucose if the perfusate glucose concentration falls below 5mmol/L. If the glucose concentration is above 20mmol/L then add 10 IU Actrapid Insulin to perfusate. Recheck glucose 5 min after any intervention. If potassium is >7mmol then administer 10 IU Actrapid Insulin and recheck potassium and glucose 5 min after intervention.

Appendix C

The ideal body weight calculation formulae used to determine the tidal volumes in protective lung ventilation are:

IBW (kg) for men = [(height (cm) -154) x 0.9] + 50

IBW (kg) for women = [(height (cm) -154) x 0.9] + 45.5

If you copy/paste below link you'll get an online IBW calculator

http://www.ukmicentral.nhs.uk/resource/calcs/ibw.asp?group=m

1. Check Ideal body weight (IBW) as per formula and note maximum minute volume

Donor Ideal Body Weight / kg	40	45	50	55	60	65	70	75	80	85	90	95	100
Maximum Minute Volume L/min	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5	10
Maximum Perfusate Flow L/min	2.8	3.15	3.5	3.85	4.2	4.55	4.9	5.2	5.6	5.95	6.3	6.65	7

Note minute volume must not exceed 1.5X the perfusate flow

2. Dial tidal volume and respiratory rate to achieve the target minute volume and target V_T/kg for the appropriate donor IBW

If Donor IBW 60kg or greater increase minute volume by 1L/min for each degree from 32 to 37 degrees keeping Peak airway Pressure less than 20cmsH_2O

If Donor IBW 59kg or less increase minute volume by 1L/min for each 2 degrees from 32 to 37 degrees keeping Peak Airway Pressure less than 20cmsH_2O

Target Minute Volume	Breaths/min	Tidal volume (ml) for given donor ideal body weight													Target V_T ml/kg
		40kg	45kg	50kg	55kg	60kg	65kg	70kg	75kg	80kg	85kg	90kg	95kg	100kg	
1L/min	5						200	200	200	200	200	200	200	200	2-3 ml/kg
32°C	8	120	120	120	120	120									
2L/min	8								300	300	300	300	300	300	3-4 ml/kg
33°C	10			200	200	200	200	200							
34°C	12	170	170	170	170										
3L/min	10					300	300	300	300	300	300	300	300	300	3-5 ml/kg
34°C	12			250	250	250	250	250	250	250					
36°C	15	200	200	200	200	200	200								
4L/min	10								400	400	400	400	400	400	3-5 ml/kg
35°C	12						330	330	330	330	330	330	330	330	
37°C	15	260	260	260	260	260	260	260	260	260	260				
		MAX	MAX												
5L/min	10										500	500	500	500	4-6 ml/kg
36°C	12							420	420	420	420	420	420	420	
37°C	15			330	330	330	330	330	330	330					
				MAX	MAX										
6L/min	10										600	600	600	600	5-7 ml/kg
37°C	12								500	500	500	500	500	500	
	15					400	400	400	400	400					
						MAX	MAX								
7L/min	12										580	580	580	580	6-7 ml/kg
37°C	15							470	470	470	470				
								MAX	MAX						
8L/min	12												670	670	6-7ml/kg
37°C	15									530	530	530	530		
										MAX	MAX				

A Study of Donor Ex-vivo Lung Perfusion
In United Kingdom Lung Transplantation

RECIPIENT INCLUSION / EXCLUSION CRITERIA

Inclusion Criteria

- Male or female patients
- Adult patients (aged over 18 years)
- Patients already on or added to the active waiting list for first lung transplant while the DEVELOP-UK study is in its recruitment phase
- Patients providing informed consent for participation in the DEVELOP-UK study at the time of study commencement or time of listing for transplant*
- Patients in EVLP treatment group re-confirming informed consent for the DEVELOP-UK study on the day of lung transplant*

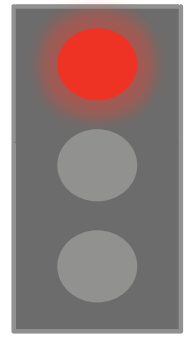
**** If Informed Consent Form was signed on the day of transplant re-confirming consent is not required. Patients in standard control group are not required to re-confirm informed consent on the day of transplant if they have signed the Expression of Interest Form or the Informed Consent Form prior to the transplant.***

Exclusion Criteria

- Patients aged less than 18 years
- Patients listed for lung re-transplantation
- Patients listed for heart-lung transplantation
- Patients listed for live donor lobar transplant
- Patients not in possession of patient information sheets for the DEVELOP-UK study prior to the day of lung transplant
- Patients in EVLP treatment group not re-confirming consent for the DEVELOP-UK study on the day of lung transplant*
- Patients in the ITU requiring invasive ventilation, ECMO or Novalung support
- Patients enrolled in Trials within the preceding 12 months (please discuss with principal and chief investigators before exclusion on this basis).* ***If Informed Consent Form was signed on the day of transplant re-confirming consent is not required.***

ABSOLUTE CONTRA-INDICATIONS TO DONOR ORGAN USE FOR TRANSPLANT (BASED ON NHS BT GUIDELINES)

Donation after Brain Death (DBD)



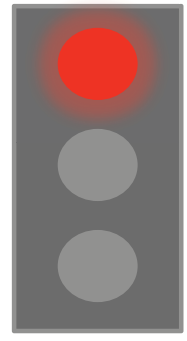
- Age >85 years
- Cancer with evidence of spread outside affected organ (including lymph nodes) within 3 years of donation (however, localised prostate, thyroid, *in situ* cervical cancer and non-melanotic skin cancer are acceptable)
- Active melanoma
- Choriocarcinoma
- Active haematological malignancy (myeloma, lymphoma, leukaemia)
- Definite, probable or possible case of human TSE, including CJD and vCJD, individuals whose blood relatives have had familial CJD, other neurodegenerative diseases associated with infectious agents
- TB: active or within 6 months of treatment*
- Malaria: if not fully treated*
- Meningoencephalitis for which no infection has been identified*
- HIV disease (but not HIV infection)

**** in exceptional cases***

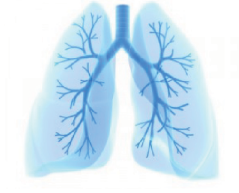
Donation after Cardiac Death (DCD) (NHSBT Guidelines)

- As above but age >80 years

ABSOLUTE CONTRA-INDICATIONS TO DONOR LUNG USE FOR STANDARD TRANSPLANT OR FOR EVLP



- Donor age >65 years
- Donor HIV positive or other contra-indicated infection risk eg Hepatitis
  B or C unless being used for a HIV, Hepatitis B or C positive recipient
- Chest trauma with extensive bilateral lung contusions
- Convincing evidence of bilateral pneumonic consolidation on inspection
- Pre-existing structural lung changes (e.g. emphysematous or multiple large bullae)
- Previous complex intra-pleural thoracic surgery or dense adhesions prohibiting safe lung procurement
- Confirmation of malignancy within 5 years (excluding central nervous system malignancies)

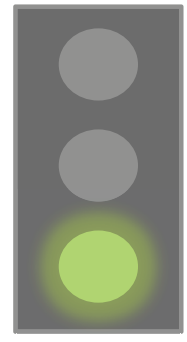


DONOR LUNG PROCUREMENT FOR ALL LUNGS

The standard lung procurement procedure will be followed for donor lungs to be used for EVLP.

- Flush the organs ante-gradely with supplemented (3.6% THAM 3.3 mls, 0.6 ml CaCl +/- 2.5 mls Prostacyclin / litre) Perfadex®:
  - the first 1 litre at room temperature,
  - the rest at 4^{o}C. Give a minimum volume of 60ml/Kg.
- After the antegrade dose, give 200 ml down each pulmonary vein as a final retrograde flush.
- An adequate portion of main pulmonary artery (PA), left atrial cuff and particularly at least 4cm of trachea will be taken by the retrieval surgeon.
- A piece of aorta will be required to extend a deficient main PA (divided in close proximity to the bifurcation) to allow for successful cannulation and bilateral perfusion.

CRITERIA FOR STANDARD TRANSPLANT (DBD AND DCD DONOR LUNGS)



Using Donation after Brain Death (DBD) donor lungs

- Satisfactory Chest X-ray reviewed by retrieval surgeon
- Systemic arterial PO_2 > 35-40 kPa on 100% FiO_2 and 8cm H_2O PEEP
- Selective Pulmonary Vein (PV) Gases >30kPa on 100% FiO_2 and 8cm H_2O PEEP
- Peak airway pressure < 30 cmH_2O
- Bronchoscopy – no severe inflammation of the airway, or recurrent secretions in the distal airway after adequate bronchial toilet
- Easily recruited atelectasis
- Satisfactory deflation test on disconnecting endotracheal tube
- Satisfactory palpation of the lung to exclude undetermined masses, nodules or gross oedema
- Satisfactory inspection of the lung after administration of the preservation flush and procurement

Using Donation after Circulatory Death (DCD) donor lungs

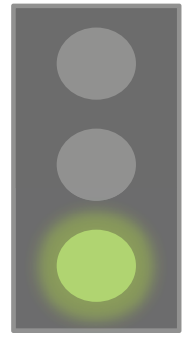
- Satisfies criteria as for standard DBD donor lungs (if information available)
- DCD Donors from Maastrict Category 2, 3 or 4
- Systemic arterial PO_2 > 40 kPa on 100% FiO_2 and 8 cmH_2O PEEP, or equivalent FiO2:PaO2 within 12 hours
- Warm ischaemic time (WIT) < 30 minutes
  (WIT starts when donor systolic BP < 50 mmHg and / or O_2 sats < 70%)
- Withdrawal of life support (WLS) time < 120 minutes

CRITERIA FOR EVLP ASSESSMENT AND RECONDITIONING (DBD AND DCD LUNGS)

Using DBD or DCD lungs
Any one or more of the following:

- Warm Ischaemic Time (WIT) > 30 minutes for DCD donors but < 60 minutes
- Chest X-ray findings prohibitive to standard transplantation
- Systemic arterial PO_2 < 35-40 kPa and / or selective PV gas < 30 kPa on 100% FiO_2 and 8 cmH_2O PEEP
- History of aspiration with bronchoscopic inflammation/soiling of the airway, or recurrent but not prohibitive secretions in the distal airway after adequate bronchial toilet
- Difficult to recruit atelectasis
- Sustained peak airway pressure > 30 cmH_2O
- Unsatisfactory deflation test on disconnecting ET tube
- Unsatisfactory palpation of the lungs identifying undetermined masses, nodules or gross oedema
- Deterioration or cardiac arrest in the donor prior to retrieval such that uncertainty over assessment remains
- Unsatisfactory inspection of the lung after administration of the preservation flush and procurement
- Logistical reasons that will extend donor lung ischaemic time >10-12 hrs and prevent donor organ use, such as:
  - Viral studies awaited
  - HLA compatibility studies
  - Pathology assessment of indeterminate mass in any donor
  - Awaiting recipient admission

CRITERIA FOR TRANSPLANT AFTER SUCCESSFUL EVLP ASSESSMENT AND RECONDITIONING



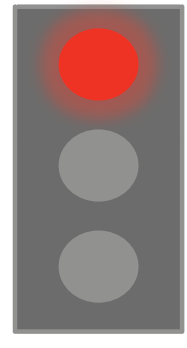
- Any DBD or DCD donor lungs meeting previously stated criteria for standard transplant
- Pulmonary artery pressure < or equal to 20 mmHg, whilst achieving stable perfusate flow of up to 70 ml/kg IBW /minute at 37^{o}C.
- Peak airway pressure < 25 cms H_2O while achieving adequate ventilation (tidal volumes up to a max 7 mls/kg IBW)
- Oxygen capacity shown by deltaPO_2 of > 40 kPa (perfusate LA PO_2 – perfusate PA PO_2) / FiO_2
- Selective PV gas > 30 kPa on 100% FiO_2 and 5 cm H_2O PEEP
- Stable or improving lung compliance and stable or falling lung resistance
- No pulmonary oedema build-up in the ET tube
- Satisfactory assessment on inspection and palpation
- Confirmed re-consent of potential matched recipient to receive an EVLP reconditioned lung*

**** If Informed Consent Form was signed on the day of transplant re-confirming consent is not required***

CRITERIA FOR FAILED EVLP ASSESSMENT AND RECONDITIONING

Transplant will not proceed if:

- Any DBD or DCD donor lungs not meeting stated criteria for standard transplant
- Not satisfying criteria for transplant after successful EVLP assessment and reconditioning



REPORTING OF SERIOUS ADVERSE EVENTS
PROTOCOL SPECIFICATIONS

Serious adverse events requiring urgent reporting include:

- Death within 90 days of lung transplantation
- Severe Primary Graft Dysfunction requiring ECMO/Novalung support
- Bronchial anastomotic dehiscence
- Any unexpected SAE felt to be probably or definitely causally related to EVLP

Serious adverse events excluded from urgent reporting:

- Death on the waiting list prior to transplant
- Death greater than 90 days after lung transplantation
- Primary Graft Dysfunction grade 1 to 3 not requiring ECMO/Novalung support
- Severe sepsis associated with consolidation, necrosis or cavitation of lung tissue within 30 days of transplant
- Renal failure necessitating renal replacement therapy
- Gastrointestinal complications
- Central nervous system complications
- Infections requiring an addition or change in anti-microbial therapy
- Bronchial stricture whether or not requiring bronchial stenting
- Acute rejection requiring augmented immunosuppression
- Development of post-transplant lymphoproliferative disease
- Development of obliterative bronchiolitis
- Deterioration of pre-existing medical conditions both pre and post transplant

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***Disclaimer:** The purpose of this flyer is to act as an aid memoir and in no way replaces the trial protocol. Please refer to the main protocol for further information. The protocol may be revised periodically. If so participating centres will be informed.*

Sample collection, processing and storage

Standard Operating Procedure

Give each sample a **unique identifying code** comprising the following: **Centre / EVLP number / Type of Sample and number / Date (DDMMYY)**

Centre Codes

NCL = Newcastle
HAR = Harefield
PAP = Papworth
BMH = Birmingham
MAN = Manchester

Sample Codes

BAL = Bronchoalveolar Lavage
PERF = Perfusate
BIO = Lung Biopsy

For example **NCL/17/PERF3/030912** represents the code for perfusate sample 3 collected during EVLP run 17 performed in Newcastle on 3rd September 2012.

Collection and storage of biological samples from donor lungs is limited to those donor lungs exposed to EVLP assessment and reconditioning and no samples are collected from standard donor lungs.

Equipment:

Equipment and Reagents	***Procedure***
Sterile normal saline (0.9% NaCL solution)	**BAL, BIO**
Crushed Ice	**BAL, BIO**
Sterile universal containers	**BAL, PER**
Sterile gauze	**BAL, BIO**
Centrifuge (refrigerated if possible)	**BAL, PER**
PBS (phosphate buffered saline)	**BAL**
Improved Neubauer Haemocytometer 0.1 mm depth	**BAL**
Cytospin with Cytospin funnels and glass slides	**BAL**
1ml Storage tubes (NUNC)	**BAL, PER**
-20ºC freezer	**BAL, PER**
-80ºC freezer	
Covidien Duet (absorbable buttressed) endo-GIA stapler	**BIO**
Sample pots	**BIO**
Liquid nitrogen in Dewar storage flask	**BIO**
Formalin	**BIO**
Glutaraldehyde (see Appendix 1)	**BIO**
Dulbeccos Phosphate buffered saline. (Sigma D5773)	**BAL**
Acetone (VWR 20066.321)	**BAL**
Virkon (Du Pont)	**BAL**
Trigene (Medichem)	**BAL**
RNA later	**BAL**

BAL- BAL collection and processing

PER- Perfusate collection and processing

BIO- Donor Lung Biopsy collection and processing

Bronchoalveolar Lavage (BAL)

BAL Collection:

- Under flexible bronchoscopic guidance perform a standardised BAL using **120 mls** of **sterile normal saline (0.9% NaCl solution)** from: Either the left or right lower lobe of the donor lung on two occasions (BAL1 and BAL2).
- Perform **BAL2** in a different segment from the same lobe as **BAL1**
- The timing of each BAL is detailed below:
  - **BAL1**: At the beginning of the EVLP process after perfusion has commenced and the lung temperature has reached at least 30^{o}C but before ventilation of the lung is initiated
  - **BAL 2**: At the end of EVLP process once the final assessment is complete but before ventilation is discontinued
- For **BAL1** and **BAL2** record data on sample collection data sheet for:
  - the duration of perfusion before the sample is taken
  - the lobe and segment the BAL is performed in
  - the volume of saline administered
  - the volume retrieved
  - the volume after filtration
- Store **BAL1** on ice until **BAL 2** is performed and placed on ice. The 2 BAL samples can then be processed together at the end of the EVLP run.
- The samples after processing can be placed straight into a -80^{o}C freezer if accessible. If not immediately accessible the samples can be placed at -20^{o}C until transfer to the -80 within the following 48 hours.

BAL Processing:

- Place a minimum of 2mls to a maximum of 5mls of the total BAL sample into a sterile container and send to your hospital laboratory for gram stain and formal microbiological assessment.
- Keep the remaining BAL on ice until ready to process, this should be started as soon as is feasible and ideally within 6-8 hours of it being taken.
- Filter the BAL fluid through a single layer of gauze to remove excess mucus. The gauze can be moistened with sterile saline to aid adherence to the funnel. Measure and record the volume on the work sheet.
- Centrifuge to separate the cellular component from the acellular supernatant at 180g for 6 minutes at 4^{o}C

- Decant the supernatant into a clean tube, taking care not to disturb the cell pellet.
- Centrifuge the supernatant at 700g for 6 minutes at 4^{o}C.
- Divide the supernatant into 1ml aliquots (maximum 12 from each BAL sample) and freeze at -20^{o}C before transfer to -80^{o}C the next working day for long term storage.
- Add Dulbeccos Phosphate Buffered Saline* (D-PBS) to the **cell pellet** to produce an opaque suspension. Mix gently.

  *(*The choice of the initial volume of D-PBS added to the pellet comes with experience and depends on the volume and visual characteristic of the starting BAL sample and the size of the cell pellet. For example a 10ml BAL sample which appears dense and opaque suggests a high cellularity sample, so in this scenario start with 10mls of Dulbeccos PBS added. If the BAL is 10ml but clear and translucent suggesting a lower cellularity sample, add just 5mls of D-PBS initially. In essence the initial volume added is a best guess and the final volume can then be adjusted to get the correct final cell concentration.)*

- To find the total cell concentration, use an Improved Neubauer counting chamber (depth 0.1mm). Count the cells in 4 large squares. (4 mm^2).
- Divide this figure by 4 then adjust the volume to give a final cell concentration of 0.5 million cells per ml.

$$\frac{\text{Total number of cells x } 10^6}{0.5} = \text{x ml DPBS}$$

- Use the diluted cell suspension to prepare cytospins x 6, 100ul per cytospin at 300 rpm (9g) for 3 minutes. Check the quality of the cytospins microscopically. If they are too dense repeat using a more dilute cell suspension. If the cytospins are too sparse increase the volume to 150ul.
- Fix 1 cytospin in acetone at room temperature for 10 minutes then air dry.
- The remaining 5 cytospins should be air-dried and then wrapped in foil and placed in the freezer. These can be stored at -20^{o}C before transfer to -80^{o}C the next working day.
- Stain with Geimsa (or Diff Quick) and perform a differential count. (This can be done the next day if necessary). Perform differential cell count to determine the percentage of:
  - Neutrophils; macrophages; lymphocytes; eosinophils and epithelial cells
- Once the quality of the cytospins has been assessed the cell suspension is centrifuged at 180g for 6 minutes at 4^{o}C. Decant the supernatant from the cell pellet and resuspend in 2mls of RNAlater. This concentrated cell suspension can then be divided to give approx 3 x 10^6 cells per tube. Snap Freeze and store at -20^{o}C before transfer to -80^{o}C.
- Place used cytofunnels in 1% Virkon for sterilisation. Discard all pipettes, tubes etc to clinical waste. Sterilise work surfaces with 1:50 Trigene.

Perfusate

Perfusate Collection:

Collect samples of perfusate solution longitudinally during the EVLP process from the perfusate sampling port on the back of the Vivoline machine.

Place collected perfusate samples in an ice filled insulated box to keep at 4°C.

- Collect 5mls from the perfusate sampling port at the following times:
    - **Perfusate 0**: Taken from the primed EVLP circuit before the donor lung perfusion is started
    - **Perfusate 1**: Taken 15 minutes after perfusion is started
    - **Perfusate 2**: Taken 30 minutes after perfusion is started
    - **Perfusate 3 to a maximum of Perfusate 8**: Taken every 30 minutes during perfusion
    - **Perfusate X**: Taken at the end of the perfusion immediately before the perfusion is stopped

Perfusate Processing:

- The perfusate samples can be stored on ice for the duration of the EVLP run and then placed in a fridge at 4°C until processed.
- The samples should be processed all together at the end of the EVLP run and ideally within 8 hours of collection.
- Centrifuge the perfusate samples at 180g for 6 minutes at 4°C to remove cellular debris
- If a refrigerated centrifuge is not available please keep the samples on ice immediately before and after centrifugation.
- Carefully remove the supernatant and aliquot equally into 5x1ml tubes for each time point sample before freezing at -20°C.
- Transfer them to -80°C as soon as feasible. The next working day ideally but within the following 48 hours for longer term storage.

Donor Lung Biopsy

Biopsy Collection

Take small biopsies of lung tissue using a Covidien Duet (absorbable buttressed) endo-GIA stapler from either the right middle lobe or lingular at two time points:

  - **Biopsy 1**: Taken prior to the commencement of the EVLP process at the recipient hospital
  - **Biopsy 2**: Taken at the end of the EVLP process once perfusion has stopped
- Place biopsies on sterile gauze.
- Dampen them with 0.9% Saline in a sample pot.
- Store the pot on ice until processing.
- It is understood that there will be different storage times on ice for the two biopsies. Once cooled to 4 degrees it is unlikely to affect the cellular processes in the tissue.
- At the end of the EVLP run both biopsies can be processed at the same time. It is important is to make sure the first biopsy is kept moist in the pot but not wet.

Biopsy Processing

From each of these biopsies:

- Fix a small amount of tissue in glutaraldehyde for electron microscopy studies.
  - See detailed protocol below
- Snap freeze small amount of tissue in liquid nitrogen for subsequent mechanistic studies.
  - See detailed protocol below
- Place the remaining tissue in Formalin fixative pots.
- Sterilise the work area with 1:50 Trigene. Dispose of gloves, apron etc to clinical waste.
- Transfer Formalin fixed blocks to Pathology for paraffin (FFPE) embedding as soon as possible.
- Subsequently cut FFPE sections for routine histological evaluation (Haematoxylin and Eosin staining).

Snap freezing Procedure

- 2-3 small pieces of tissue should be prepared for freezing. Each piece should be ideally 5-10mm in diameter.
- Store biopsies / blocks at 4^{o}C until ready to be quenched.
- In a Dewar flask collect 1litre of liquid nitrogen.
- Place approx 60ml isopentane* in a plastic beaker and gently suspend in the flask of nitrogen, leave until the isopentane is almost solid, approx 10 – 15 mins.
- Remove the beaker from the Dewar flask.
- The isopentane should now be a half liquid / half frozen slurry. Place the pieces of tissue on squares of tin foil approx 1.5 x 1.5 cms using medium forceps plunge the tin foil and

tissue into the isopentane moving it through the liquid phase for approx. 30 seconds until the tissue is solid. Repeat this for each piece of tissue.

- Remove the frozen block from the isopentane and drain off any liquid then securely wrap in a 5cm square of tin foil to exclude all air. Place the wrapped tissue in a labeled unicassette, seal and plunge into the liquid nitrogen.
- Using tongs transfer the unicassette to the –20^{o}C freezer for later transfer to –80^{o}C storage on the next working day.
- Place all instruments in 1:50 Trigene for sterilization.
- Transfer the Dewar flask containing the Liquid nitrogen to the fume hood. Attach the hazard label and allow the nitrogen to evaporate.

(Snap freezing in isopentane slurry is considered the best methods to avoid freezing artefact. As the frozen tissue will be used mainly for cell biology purposes. The isopentane step is not compulsory part of the protocol but is recommended.)*

Preparation of lung biopsy for Electronic Microscopy (EM) examination

- 3-4 small pieces of approximately 4x4x10 mm are CAREFULLY (try not to squash) taken with a sharp scalpel or scissors from the removed lung tissue. Take these away from the stapled edges where there might be crush artefact.
- Place IMMEDIATELY in glutaraldehyde fixative as delays will lead to artefact which can be interpreted as tissue injury.
- Gently without squeezing the tissue push to the bottom of the fixative and agitate to try to get the air out of the lung. Residual air will make the tissue float and not fix resulting in artefact.
- Agitate the closed bottle to try to remove further air.
- Once the sample is adequately placed in glutaraldehyde it can be stored in the fridge until posted away for processing in Birmingham on the next convenient working day.
- See Appendix 1 for method to prepare glutaraldehyde stock solution. This is best done by an experienced pathology lab.

Appendix 1: Method for the preparation of a standard 2.5% glutaraldehyde fixative in 0.1M sodium phosphate buffer - for electron microscopy.

Contains a **Hazardous** chemical, read COSHH data sheet before preparing fixative for the first time. This is best done in an experienced pathology laboratory.

Materials and Equipment:

pH meter
Balance

500ml measuring cylinder
100ml measuring cylinder
Gloves
Clean 500ml bottles with well-fitting stoppers

Chemical Suppliers: *Glutaraldehyde EM grade (25%) Catalogue no. R1011*

Agar Scientific Ltd
Unit 7, M11 Business Link, Parsonage Lane,
Stansted, Essex, CM24 8GF
Tel: [redacted]
Email: [redacted]

di-Sodium hydrogen orthophosphate
Sodium di-hydrogen orthophosphate
VWR International Ltd.
Hunter Boulevard
Magna Park
Lutterworth
LE17 4XN
Customer service centre: [redacted]

Procedure:

Preparation of 0.2M sodium phosphate buffer

To make up stock solutions

Acidic solution (A): 31.2g/l sodium di-hydrogen orthophosphate is an 0.2M solution

Weigh out 15.6g of A; put powder in 500ml measuring cylinder, half fill with distilled water, shake until dissolved and top up to 500ml. Transfer to a clean 500ml bottle and stopper tightly. Label with 3 months expiry date.

Basic solution (B): 28.4g/l di-Sodium hydrogen orthophosphate is an 0.2M solution

Weigh out 14.2g of B. Prepare and label 'Solution B' as above.

To make up 0.2M phosphate buffer

To make 100ml of 0.2M sodium phosphate buffer measure out 23mls of solution A and 77mls of solution B in to a beaker. Mix thoroughly. This should give a pH of 7.3.

To check the pH

Check pH. Bring solution to pH 7.3 using small quantities of A or B, measured out with a pipette. Stir thoroughly. Keep in fridge until required.

NOTE: When making up buffer from older stock solutions.
Ensure any crystals formed at the bottom of the stock bottles are dissolved thoroughly before making up the buffer. Stand bottles in warm / hot water to speed the process.

Preparation of fixative

Work is conducted in a fume cupboard and wearing gloves.
Add 10mls of 25% glutaraldehyde to 50mls 0.2M sodium phosphate buffer and dilute with 40ml distilled water and mix well.
Label with contents, date made up and expiry date (one month) and "Harmful" warning label
Keep "*PGP*" well stoppered in fridge.
Decant into specimen vials. Ensure specimens are immersed in sufficient fixative – the volume of fixative should be at least 10 to 20 times the volume of the specimens. Ensure all lids are tightly closed.

Cleaning up

Rinse glassware in fume cupboard before transferring to lab sink for further washing. Wash using detergent and rinse well, giving final rinse in distilled water.
Label all vials with the appropriate 'HAZARD' label plus one which states the concentration of glutaraldehyde and 'EM FIXATIVE'.
Discard all unused fixative after 1 month and replace with fresh.
Always discard down the sink in the fume cupboard with extraction on and plenty of running water.

For any further advice on EM preparation and processing contact:

Dr Liz Curtis
Clinical; Scientist
Electron Microscopy Unit
Queen Elizabeth Hospital Birmingham
Birmingham
[redacted]
Tel: [redacted]

Addresses for Dispatch of EM Samples

EM samples in glutaraldehyde to be sent to:

Dr Desley Neil
DEVELOP-UK study
Electron Microscopy Unit
Department of Cellular Pathology
Level -1 Queen Elizabeth Hospital Birmingham
Edgbaston
Birmingham
B15 2WB

EVLP NUMBER	
DATE (dd/mm/yyyy)	☐☐/☐☐/☐☐☐☐
TIME (hh:mm)	☐☐:☐☐
TRANSPLANTED	☐ **YES** ☐ **NO**
Donor Consent for Research	☐ **YES** ☐ **NO** ☐ **UNKNOWN**

		BAL 1 Pre-EVLP	BAL 2 Post-EVLP
The duration of perfusion before BAL 2 is taken			
The lobe and segment the BAL is performed in			
The volume of saline administered			
The volume of BAL retrieved			
Beginning of sample processing: Date (dd/mm/yyyy) Time (hh:min)			
Volume of BAL after filtration (ml)	**A**		
Volume D-PBS added to cell pellet (ml)	**B**		
Total Cell Count	**C**		
Total Cell Count / 4 x 10^4	**D**		
Cell Count x Volume D-PBS	**D x B = E**		
Cell Count / Volume of BAL	**E / A**		
Number of 1 ml supernatants (max 12)			
Number of Cell pellets for RNA			
Number of Cytospins produced			

Cell Differential				
	Pre Number of Cells	**%**	**Post Number of cells**	**%**
Macrophages				
Neutrophils				
Lymphocytes				
Eosinophils				
Total				
Ciliated Epithelia				
Metaplastic Epithelia				
Total				

Perfusate Samples										
	P0	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**Px**
Number of Perfusate samples (stored at -80)										

Donor Lung Biopsy			
List numbers of lung tissue specimens stored			
	Formalin	**EM**	**Snap Frozen**
Biopsy 1 prior to EVLP			
Biopsy 2 post EVLP			
List specimens transferred and centre to which moved			
EM transfer date (dd/mm/yyyy)	/ /		
Frozen transfer date (dd/mm/yyyy)	/ /		
Paraffin transfer date (dd/mm/yyyy)	/ /		
Responsibility			

Method for the preparation of a standard 2.5% glutaraldehyde fixative in 0.1M sodium phosphate buffer - for electron microscopy.

Contains a **Hazardous** chemical, read COSHH data sheet before preparing fixative for the first time.

1. Materials and Equipment

1.1. Suppliers:

Glutaraldehyde EM grade (25%) Catalogue no. R1011

Agar Scientific Ltd
Unit 7, M11 Business Link, Parsonage Lane,
Stansted, Essex, CM24 8GF
Tel: [redacted]
Email: [redacted]

---

di-Sodium hydrogen orthophosphate
Sodium di-hydrogen orthophosphate

VWR International Ltd.

Hunter Boulevard

Magna Park

Lutterworth

LE17 4XN

Customer service centre: [redacted]

---

balance

500ml measuring cylinder

100ml measuring cylinder

gloves

clean 500ml bottles with well fitting stoppers

Procedure

2.1 Preparation of 0.2m sodium phosphate buffer

1.1.1. To make up stock solutions

Acidic solution (A): 31.2g/l sodium di-hydrogen orthophosphate is an 0.2M solution

Weigh out 15.6g of A; put powder in 500ml measuring cylinder, half fill with distilled water, shake until dissolved and top up to 500ml. Transfer to a clean 500ml bottle and stopper tightly. Label with 3 months expiry date

Basic solution (B): 28.4g/l di-Sodium hydrogen orthophosphate is an 0.2M solution

Weigh out 14.2g of B. Prepare and label 'Solution B' as above.

1.1.2. To make up 0.2m buffer

To make 100ml of 0.2m sodium phosphate buffer measure out 23mls of solution A and 77mls of solution B in to a beaker. Mix thoroughly. This should give a pH of 7.3.

1.1.3. To check the pH

Check pH. Bring solution to pH 7.3 using small quantities of A or B, measured out with a pipette. Stir thoroughly.

Keep in fridge until required.

1.1.4. NOTE: When making up buffer from older stock solutions.

Ensure any crystals formed at the bottom of the stock bottles are dissolved thoroughly before making up the buffer. Stand bottles in warm / hot water to speed the process.

1.2. Preparation of fixative

Work is conducted in fume cupboard

Wear gloves

Add 10mls of 25% glutaraldehyde to 50mls 0.2M sodium phosphate buffer and dilute with

40ml distilled water.

Mix well.

Label with contents, date made up and expiry date (one month) and "Harmful"

Keep "*PGP*" well stoppered in fridge.

Decant into specimen vials. Ensure specimens are immersed in sufficient fixative – the volume of fixative should be at least 10 to 20 times the volume of the specimens. Ensure all lids are tightly closed.

2. Cleaning up

Rinse glassware in fume cupboard before transferring to lab sink for further washing. Wash using detergent and rinse well, giving final rinse in distilled water.

Label all vials with the appropriate ‘HAZARD’ label plus one which states the concentration of glutaraldehyde and ‘EM FIXATIVE’.

Discard all unused fixative after 1 month and replace with fresh.

Always discard down the sink in the fume cupboard with extraction on and plenty of running water.

Dr Liz Curtis
Clinical; Scientist

EM Unit
Level -1
Cellular Pathology Department
Queen Elizabeth Hospital Birmingham
Mindelsohn Way
Edgbaston
Birmingham B15 2WB

[redacted]

Any other SOPs?

There are:
- Donor offer pathway document
- Sample collection data sheet
- ex vivo Vivoline machine protocol
- Guide to Primary Graft Dysfunction