Study Title	AIR-NET- Testing anti-inflammatories for the treatment of bronchiectasis
IRAS	1010124
CI	Prof. James D Chalmers
Sponsor	University of Dundee & NHS Tayside
Associated Documents	AIR-NET Sample Logs AIR-NET Assay Log AIR-NET Reagent Log AIR-NET Research Sample Receipt Log AIR-NET Video Protocols AIR-NET NEATstik grading sheet
Version history (and brief summary of changes)	Version 1 14-11-2024 Version 2 17-12-2024 updated research sample packs, SOP E update
Author	Dr Merete Long

Contents

1. Procedures

- Ensure all equipment used is within expiry date
- Obtain blood samples as per local venepuncture Standard Operating Procedure, utilising 21G butterfly needle provided.
- Obtain spontaneous sputum samples as per local Standard Operating Procedure.
- Obtain nasal brushing samples as per instructional video and local Standard Operating Procedure.
- Dispose of all clinical equipment as per local policy.
- Deal with any needlestick injury or body fluid spillage as per local policies.
- Never put blood samples into the refrigerator or on ice.

Laboratories must adhere to Good Clinical Practise (GCP) guidelines, valid GCP/GCLP certification should be obtained by any lab members processing research samples.

All equipment utilised should be regularly maintained and calibrated, including but not limited to fluorescence plate reader, flow cytometer, centrifuges and pipettes utilised, with in-date certification available.

2. Equipment

2.1. Bloods

- **Blood tubes as per visit**
- **Venepuncture equipment (e.g. 21G needle and vacutainer adapter provided to sites.** Tourniquet, cotton wool, alcohol wipes etc. to be utilised locally)
- ***Collection set compatible with vacutainer tubes must be used for PAXgene***
- **Research sample pack note**

For lab processing (summary- please refer to individual SOPs for comprehensive equipment list. Items in bold will be provided by Tayside central site, in addition to reagents listed in individual SOPs):

- **Sample labels**
- **Sample logs**
- **Black permanent marker pens**
- **Sample storage boxes**
- -20°C and -80°C freezers
- Refrigerator (4°C)
- **CoolCell freezing container (if required)**
- **CoolRack freezing block**
- **Cytodelics stabiliser solution**
- **5ml polystyrene tubes for flow cytometry (if required)**
- **1.5 ml fluid X tubes and caps**
- **1.8 ml Cryovials**
- **1.5 ml LoBind Protein Eppendorf tubes**
- **96 well NUNC sterile tissue culture plates (transparent flat bottom)**
- **12 well sterile tissue culture plates (transparent flat bottom)**
- **Sterile disposable reagent troughs**
- p2.5, 10, 20, 200 and 1000 pipettes
- Filter tips for all pipettes (p2.5, 10, 20, 200, 1000)
- Centrifuge (large for 50ml tubes)
- Microcentrifuge (for 1.5ml tubes)

- Vortex
- Lab timer (10 min to 4h)
- Light microscope or other similar cell counting equipment
- 37°C incubator with CO_2 (5%)
- Electronic pipette gun and 10ml and 25 ml sterile stripettes
- **StemCell Easy50 magnet (if required)**
- **StemCell neutrophil isolation kit and accompanying reagents**
- **Reagents for functional assays**
- Fluorometer (fluorescent plate reader) for measurement of 96-well tissue culture plate assays
- Flow cytometer (capacity for APC and FITC fluorophore detection)

2.2. Sputum

- **Sputum sample pots**
- **NEATstik sputum elastase test and colour grade print-out**
- **Murray sputum colour chart**
- Screening only: camera or camera phone (if permitted locally) to photograph NEATstik result

For lab processing:

- **Sample storage boxes**
- **Sample Logs**
- **Pre-labelled 1.5ml vials**
- **Positive displacement pipette and tips (if required)**
- -80°C freezer
- **DNA/RNA Shield reagent**
- p1000 pipette and tips
- ~10 cm sterile petri dish

2.3. Nasal brushing

- **3mm modified bronchoscopy brush**
- **5 ml cryotube containing 1 ml RNAlater buffer**
- **Sample storage box**
- **Sample Logs**
  note, nasal brushing and sample addition into RNAlater buffer should be done during participant visit by clinical team member

3. Sample ID

- Sample ID is made up of:

  NET – site number – participant number – visit number
  e.g. sample ID NET-01-003-02 would be site 01, participant 003, visit 02

  n.b. Use “EX” for visit number for unscheduled visits due to exacerbation.

  e.g. NET-01-003-EX

4. Sample collection

Visit	NHS Labs	Research Blood Samples	Sputum Sample	Other research samples
1 **screening**	Bloods: • FBC • U&Es • LFT Urine: • Pregnancy test if required	Nil	 Min 0.4 g *(for screening test)*	Nil
2 **Day 0 (Baseline)**	Urine: • Pregnancy test if required	 SST II Advanced 5 ml  Sodium Heparin (NH) 4 ml  Lithium Heparin (LH) 4 ml  EDTA (K2E) 24 ml *(2x10ml, 1x4ml)*  PAXgene RNA tube 2.5 ml	 Min 0.4 g	 Nasal brushing (3mm brush and 5ml cryotube with 1ml RNAlater solutiuon)
3 **Day 7**	Urine: • Pregnancy test if required	 SST II Advanced 5 ml  EDTA (K2E) 4 ml	 Min 0.4 g	Nil
4 **Day 14**	Urine: • Pregnancy test if required	 SST II Advanced 5 ml  EDTA (K2E) 4 ml	 Min 0.4 g	Nil

5 **Day 28**	Bloods: • FBC • U&Es • LFT Urine: • Pregnancy test if required							
		SST II Advanced 5 ml	Sodium Heparin (NH) 4 ml	Lithium Heparin (LH) 4 ml	EDTA (K2E) 24 ml *(2x10ml, 1x4ml)*	PAXgene RNA tube 2.5 ml	Min 0.4 g	Nasal brushing (3mm brush and 5ml cryotube with 1ml RNAlater solutiuon)
6 **Day 56**	Bloods: • FBC • U&Es • LFT							Nil
		SST II Advanced 5 ml	Sodium Heparin (NH) 4 ml	Lithium Heparin (LH) 4 ml		PAXgene RNA tube 2.5 ml	Min 0.4 g	
Unscheduled **NHS bloods as clinically indicated** • FBC • U&Es • LFT	Nil						Nil	Nil

5. Sample Processing Summary

5.1. Protocol summary

IMPORTANT NOTE: see section 8 for detailed protocols. Section 5 is for quick-reference purposes only, detailed step-by-step procedures from section 8 should always be followed.

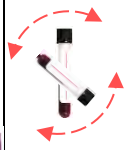
TUBES	INVERT TUBE *(at visit blood draw only)*	ALLOW TO STAND	CENTRIFUGE	TRANSFER	LABEL	FREEZE
 PAXgene RNA tube	 8-10 times	 Stand upright minimum 2 hours from blood draw	n/a		 Add label directly to vacutainer	 Store upright Transfer to -80°C freezer **within 2-24 hours from collection (storage up to 72h permitted for weekends)**
 SSTII Advance	 5 times	 Stand upright 30 mins, **process within a maximum of 2h from draw.**	 1000 xg for 15 mins	Transfer **0.4 ml serum** into each of 3x 1.5ml FluidX tubes, add remaining excess to a 4th tube. Cap tubes.	 Dot the cap of the 4th/excess tube with a permanent marker. Firmly add labels to each of the 4 tubes.	 Store upright Transfer to -80°C freezer
 4 ml EDTA (K2E) ***Visits 3 and 4 only*** ***Do not store 10ml EDTA tubes (use for neutrophil isolation)**	 8-10 times	n/a			 Add label directly to vacutainer	 Store upright Transfer to -80°C freezer **within 1 hour from collection**

TUBE	INVERT TUBE	ALLOW TO STAND	TRANSFER	INVERT	ALLOW TO STAND	LABEL	FREEZE 1	FREEZE 2
 Sodium Heparin (NH)	 5-10 times ***(at visit blood draw only)***	 Stand upright 15 mins (if already 15 min post-draw, continue immediately)	 **Within 1 hour** from draw, transfer **0.5 ml blood** to each of 4x cryovials containing stabiliser	 Invert filled cryovials 15 times. *1 full inversion = 180° degree turn and back to original position again*	 Stand upright 10 mins at room temp	Label the 4 cryovials	Store upright in round CoolCell freezer container, with filler vials in empty spaces. Add to -80°C freezer. 	Transfer to storage box in -80°C freezer **4-72 hours** after storage in CoolCell 

TUBE	INVERT TUBE	TRANSFER	INVERT TUBE	INCUBATE	CENTRIFUGE	TRANSFER	LABEL	FREEZE
 Lithium Heparin (LH)	 5-10 times ***(at visit blood draw only)***	**Within 1 hour** from blood draw, add 750µl blood to each of 4 Eppendorf tubes containing pre-warmed zymosan (x2) or HBSS (x2)	Cap tubes very tightly  5 times	 Incubate samples upright in a tube rack at <u>37°C</u> for 30 min <u>exactly</u>	Invert tubes 2 times then immediately centrifuge  600 xg for 10 mins	<u>Immediately</u> after centrifuge completion, aliquot 200µl plasma into one 1.5ml fluidX tube per original Eppendorf tube (4 total). <u>Do not transfer any buffy coat/RBCs</u>.	Label 1.5ml FluidX tubes: 2x Zymosan 2x HBSS	 Store upright in FluidX box Transfer to -80°C freezer

*note for clinical team: LH tube filling upon blood draw can be slow, allow time for the vacutainer to fill sufficiently

SAMPLE	LABEL	COLLECT *Note, this step should be done by the clinical team member performing the participant visit*	LABEL AND FREEZE
 Nasal Brushing (3mm)	Pre-label a 5 ml cryotube containing 1 ml RNALater buffer (as supplied).	Immediately after obtaining nasal brushing sample, vigorously agitate brush in RNAlater buffer for 30 seconds to release cells from sample brush. Lower the plastic sheath of the brush to remove any remaining cells Dispose of the brush in a sharps bin. ***See video protocol for brushing procedure***	In the lab, add sample directly into storage box in -80°C freezer. Complete log sheet. 

TUBE	INVERT TUBE	TRANSFER, AND BEGIN ISOLATION	MAGNET 1	TRANSFER	MAGNET 2	TRANSFER, MAGNET 3
Visit 2 and 5 only  2x10 ml EDTA (K2E) plus 1x4ml EDTA (K2E)	 8-10 times ***(at visit blood draw only)***	**Within 1 hour** from blood draw, gently pour blood (45° angle) into blue-lidded 50ml Falcon tube. Add 50µl Ab cocktail and 50µl pre-vortexted bead solution, per ml whole blood. Gently invert 4X to mix. Incubate 5 min, room temp.	Top up to final volume 50ml with DPBS/EDTA. Gently invert 3X to mix. Incubate on StemCell Easy50 magnet 10 min, without lid. 	Transfer the neutrophil-rich plasma layer to a new 50ml falcon. Add beads (same vol as first addition). Invert 3X to mix. Incubate in a rack for 5 mins. 	Remove tube lid. Add tube to magnet. Incubate in magnet for 5 mins. 	Again transfer the purified neutrophil-rich plasma to a new 50ml falcon. Remove lid, add to magnet. Incubate a final time for 10 min. 
TRANSFER, COUNT, CENTRIFUGE		**WASH CELLS**	**RESUSPEND NEUTROPHILS**	*PART OF CELL SUSPENSION: TRANSFER, CENTRIFUGE*	*PART OF CELL SUSPENSION: STORE*	**FUNCTIONAL ASSAYS**
Transfer neutrophil-rich plasma to a new 50 ml tube. Note the final volume. Add 10 µl cell suspension to haemocytometer. Centrifuge cells. Perform count.  300 xg for 6 mins low brake RT		Discard supernatant. Gently resuspend cell pellet in 10ml DPBS (no EDTA). Centrifuge cells to wash and pellet.  300 xg for 6 mins low brake RT	Discard supernatant. Resuspend pellet at $5x10^6$ cells/ml in DPBS according to cell count calculation. *Priority for use:* *-1x Pellet storage* *-1x RNALater storage* *-Functional work* *-2x further pellet storage*	Add 1ml cell suspension to each of 3x pre-labelled 1.5ml LoBind Eppendorf tubes (if count allows). Pellet cells.  300 xg for 5 mins RT	Discard supernatant from 1.5ml Eppendorfs. Store 3x tubes in pre-cooled block at -80°C. 3rd tube: Resuspend cells in 100µl DPBS, add 500µl RNALater. Pipette 10X. Store in -80°C freezer. Log all samples.	Use live neutrophil suspension at $5x10^6$ cells/ml for functional assays: - NETs - Phagocytosis - ROS - In-plate cell stimulation

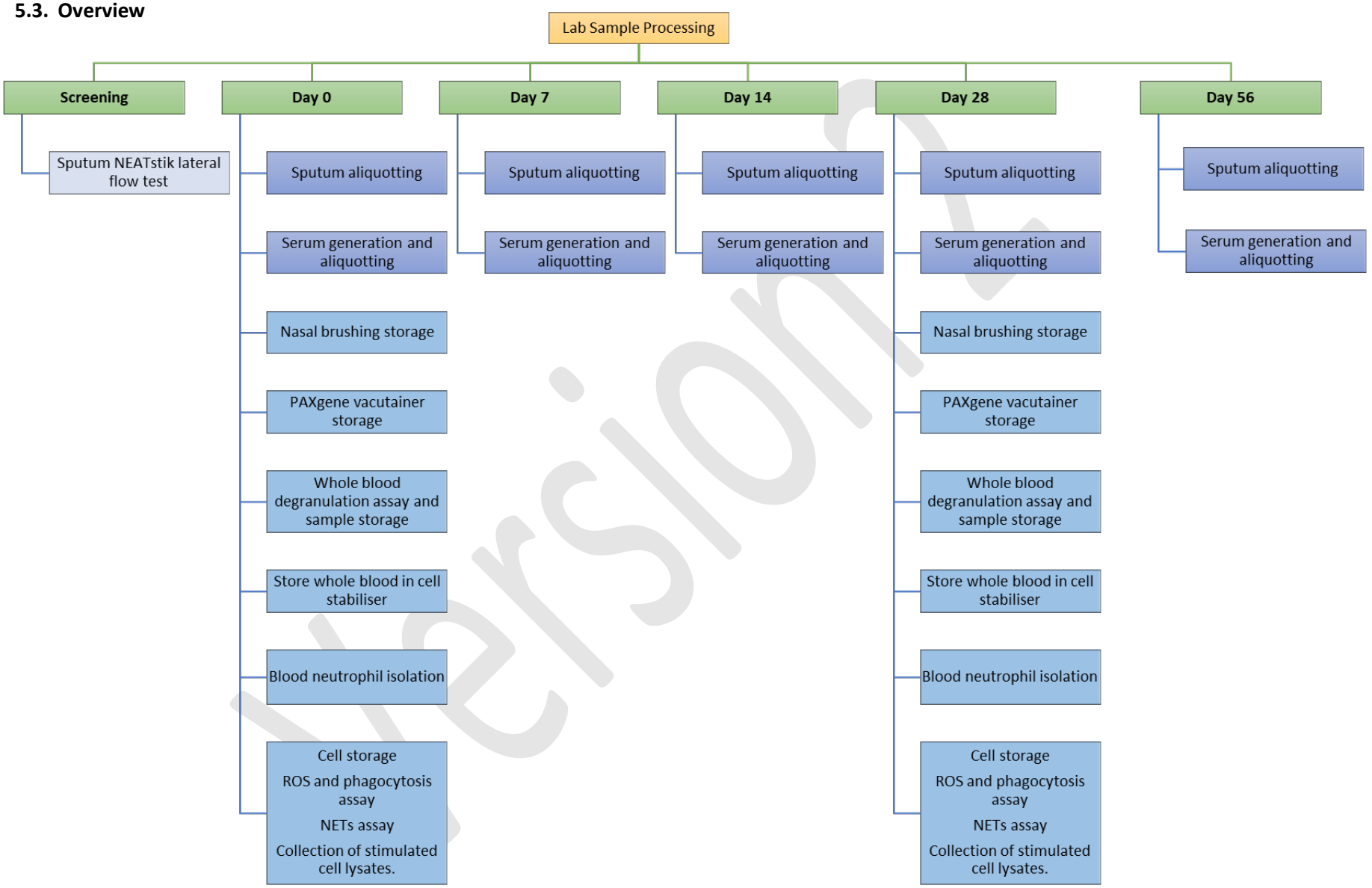
5.2. Preparation summary

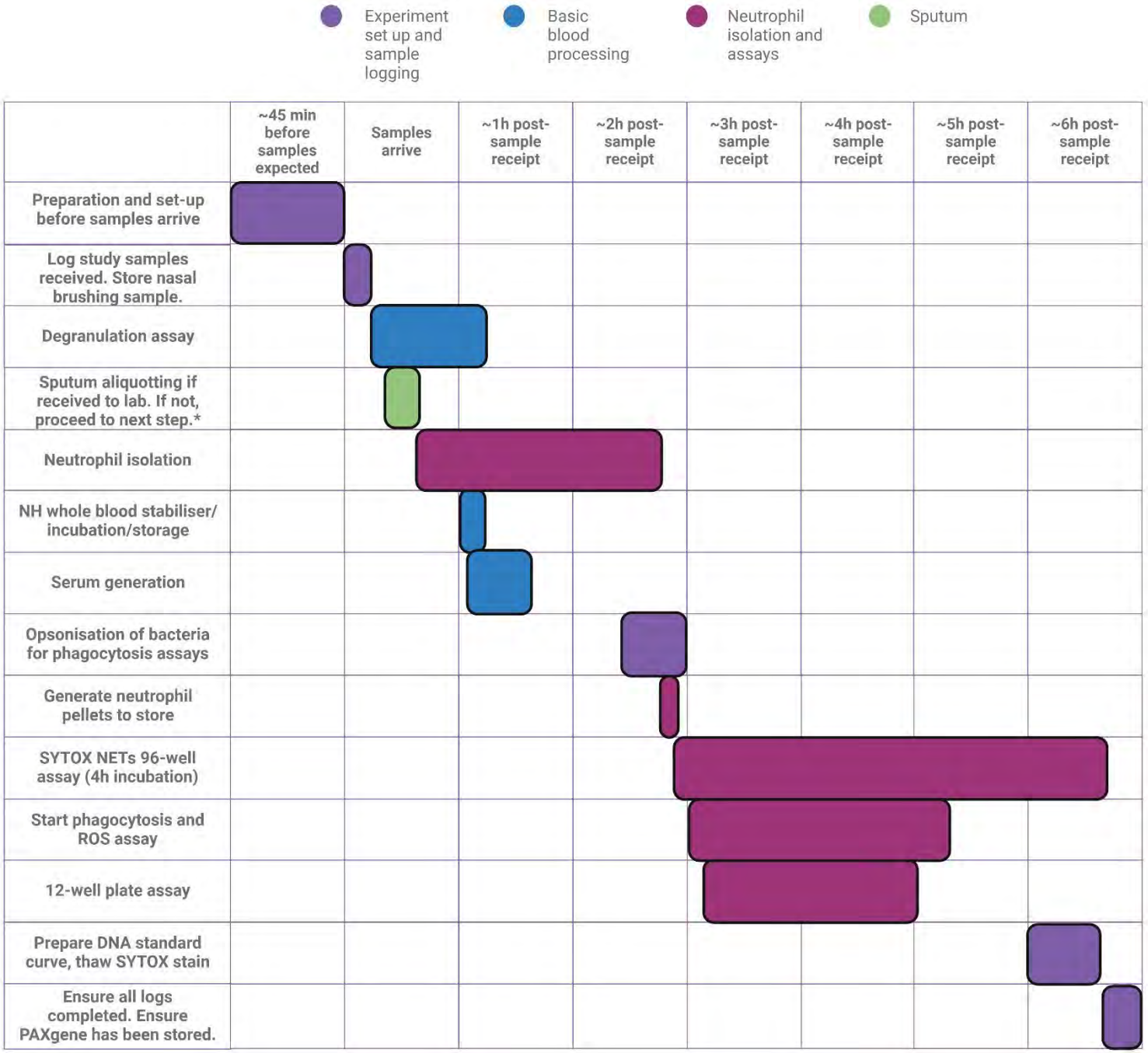
Before sample receipt, run through preparation checklist:

- Ensure centrifuges are at room temperature (i.e. no pre-cooling)
- Nasal brushing:
    - Ensure brush and 1x 5 ml cryotube containing RNAlater buffer are available, pre-label tube.
- 4ml EDTA (visit 1/screening only):
    - Complete 1x label ready for vacutainer addition with participant details and date
- SSTII Advance:
    - Label 4x 1.5ml FluidX tubes with participant details and date and keep sterile in hood. Ensure septum caps are available.
- Sodium heparin:
    - Remove 4x 1.5ml cryovials containing 0.5ml aliquots of stabilisation buffer from the fridge (or generate these aliquots from stock bottle if required). Allow aliquots to warm to room temperature at least 15 min (maximum 2h) before use.
    - Label the 4 cryovials with stabilised blood labels, participant details and date.
    - Check the round CoolCell freezer container is at room temperature (after any previous samples are removed and stored). If CoolCell is still in the freezer due to previous sample processing, appropriately store any previous frozen samples at -80°C, then take out freezing container and keep at room temperature at least 1 hour before use.
- Lithium heparin:
    - Remove 2x zymosan and 2x HBSS aliquots (1.5ml Eppendorf tubes containing 75ul reagent) from -20°C freezer. Allow to warm to room temperature minimum 10 mins (max. 30 mins) before use.
    - Label 4x 1.5ml FluidX tubes (2 per condition) with the appropriate experimental condition labels (2xzymosan, 2xHBSS) and add participant ID and date.
- 2x10ml EDTA tubes:
    - Ensure sufficient volume of necessary reagents is available, generate more if needed:
        - DBPS
        - DPBS/1mM EDTA
        - RPMI/10mM HEPES
        - RPMI/10mMHEPES/10%FBS
    - Warm RPMI/HEPES/FBS media to room temperature minimum 30 mins and maximum 1h before use.
    - Fill cool box (polystyrene box is acceptable) with ice.
    - Check cold block is in the -80°C freezer and is empty of any previous samples (store these as appropriate if present).
    - Make up complete RIPA/SDS/PMSF/PI/PhosSTOP and store in fridge or on ice until use.
    - Generate 96-well plate with media and stimulants for NETs assay and store in fridge until 30 mins before use.
    - Add media to 12-well plate appropriate wells and label (don’t add stimulants yet). Store in the fridge until 30 min before use.

- Label 1.5ml LoBind Protein Eppendorf tubes:
  - 3x Neutrophil pellets (2 “spare” if enough cells)
  - 1xNeutrophils in RNAlater
  - 2x Neutrophil RIPA lysate - PBS
  - 2x Neutrophil RIPA lysate – TNF
  - 2x Neutrophil RIPA lysate - LPS
- Label 1.5ml FluidX tubes:
  - 2x Neutrophil supernatant – PBS
  - 2x Neutrophil supernatant – TNF
  - 2x Neutrophil supernatant – LPS
  - 2x Neutrophil supernatant – PBS (no cells)

5.3. Overview





Recommended timings and order of processing/assays for day 0 and day 29 sample sets

*Sputum- if sputum sample is not received at the same time as blood samples and is brought to the lab later in the day, keep sputum sample on ice and process as soon as time is available (e.g. during functional assay incubation periods)

<u>Never put blood tubes in the fridge/on ice for any amount of time</u>

6. Labelling Research Samples

- Sample pack note should be included in the visit pack with sample tubes and should be completed at the visit by clinical team member. Vacutainers need only to be labelled with the sample ID, date and time using the manufacturers label by clinical team.

AIR-NET research samples

Participant ID: NET- __ __ - __ __ __ - __ __

Date: __ __ - __ __ - __ __

Blood and nasal sampling time: __ __ : __ __

Sputum production time: __ __ : __ __

Staff initials: __ __

- All research samples (i.e. all samples not for NHS analyses) including vacutainers for storage should be labelled in the lab with the labels provided by the lab team member, with the exception of the Nasal Brushing (see SOP E).

AIR-NET Whole Blood (4ml EDTA)
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Serum
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Sodium heparin, stabilised 0.5 ml
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Degran. Plasma Zymosan-treated
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET PAXgene
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Neut. pellet
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Neut. RNAlater
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Degran. Plasma HBSS-treated
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Neut. RIPA lysate PBS
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Neut. RIPA lysate LPS
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Neut. RIPA lysate TNF
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Nasal brushing RNAlater
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Neut supernatant PBS
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Neut supernatant LPS
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Neut supernatant TNF
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Neut supernatant PBS (no cells)
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Excess Sputum
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Sputum 100 uL
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Sputum 200 uL
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

AIR-NET Sputum DNA/RNA shield
NET - _ _ - _ _ _ - _ _
Date: _ _ - _ _ - _ _

- The appropriate sample label should be used for whole blood, serum, sodium heparin stabilised blood, PAXgene, lithium heparin degranulation assay plasma, neutrophil pellets, neutrophils in RNAlater buffer, nasal brushing or sputum samples.
- Ensure labels are attached **prior** to freezing. Ensure that the label is **firmly rubbed** on to the tube to prevent it coming off.
- Complete each label with full sample ID and date as below.
- Date to be filled in day-month-year i.e. 30-01-24
- All samples should be logged on the appropriate AIR-NET Sample Log in the lab.

7. Storing Research Samples

- The samples should be stored in the storage boxes provided.
- Study samples should be stored in separate boxes according to sample type.
- The storage boxes should be labelled with study title, site number, sample type, and box number.
- Sample receipt log, storage log, and assay log should be completed.
- No identifiable patient details (e.g. name, CHI number, date of birth) should be associated with processed research samples when shipping back to Tayside.

8. Detailed sample processing protocols

8.1. SOP A Sputum NEATstik (screening/v1 only)

Equipment/ reagents required:

Nitrile gloves	Microbiological Safety Cabinet MSCII
Lab coat	Sterile petri dishes
Clinical waste bags & autoclave tape (or as per local procedures)	Timer
1x NEATstik pack (ProAxsis) (stored at room temp)	Vortex
Camera/camera phone	P1000 and p200 pipette and filter-tips
2ml screw-capped tube	Printed disposable grading sheet (in lab folder)
Ice and small ice box	P100 positive displacement pipette and tips

Notes

No patient detail, (name, CHI number) should be associated with processed samples in the lab.

NEATstik pack should contain:

1x lateral flow test

1x 20ml sample dilution buffer

1x sputum dilution pot *(not required for this protocol)*

1x dual-bulb pipette *(not required for this protocol)*

1x graduated pipette *(not required for this protocol)*

1x instruction leaflet

Procedure:

1. Upon receipt of sputum sample to the lab, keep on ice until use.
2. Add sputum sample to a sterile petri dish to allow selection of sputum and elimination of any contaminating saliva present in the sample. Sputum can be distinguished from saliva typically by it's colouration and higher viscosity.
3. Using a p100 MicroMan positive displacement pipette set to 100 µL, select out the sputum, combining into a separate area of the petri dish if needed (e.g. if sample has high saliva content and small separated bits of sputum). Transfer exactly 100 µl of sputum to the sterile 2ml screw-capped tube.

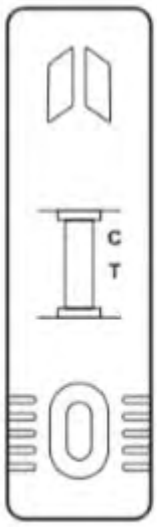
4. Add NEATstik sample dilution buffer from the kit, to produce a 10x dilution (i.e. to 100 µl sputum, add 900 µl of the buffer)
5. Cap the sample tightly and vortex at high speed (approx. 20,000 rpm) for 15 seconds. Pipette the sample up and down with a p1000 pipette to break up the sputum sample 10 times, or until no large lumps are observed. Allow sputum to settle for 1 min by placing in a rack (room temperature).

Perform the test:

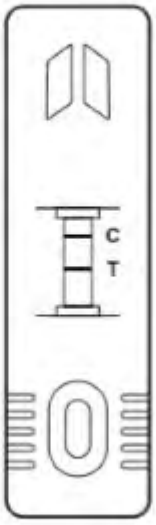
6. Remove the NEATstik lateral flow test from the foil packaging and place on a level surface with viewing window facing upwards.
7. Use a p200 pipette to add 125µl of sputum/buffer mixture to the sample port, ensuring no lumps of sputum are transferred (if lumps are very hard to avoid, carefully remove lumps from the tube by pipetting lumps out into a new petri dish to remove them, then discard to waste after loading test).
9. Once sample is added, incubate at room temp in a hood for 10 mins (set a timer).
10. After 10 min, read and record the result (see result guide below for how to read and QC assay). Add the sample ID and the date to the test with permanent marker, circle the final score decided upon on the printed grading sheet, then take a photograph of the lateral flow test result placed on top of a disposable printed grading sheet. Dispose of the test and all associated equipment into the appropriate waste stream.

Reading the result and Quality Control

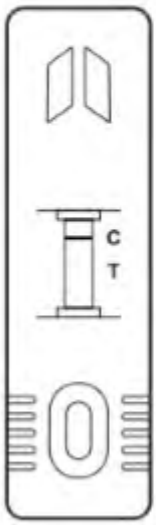
If the test has been performed successfully, the CONTROL (C) line will be visible as a red line (colour intensity may vary). If the CONTROL (C) line is not visible, the results are invalid and the test should be repeated with a new sample and a fresh test kit.



If the TEST (T) line is visible, this confirms the presence of active NE greater than the pre-set threshold, in the sputum sample.

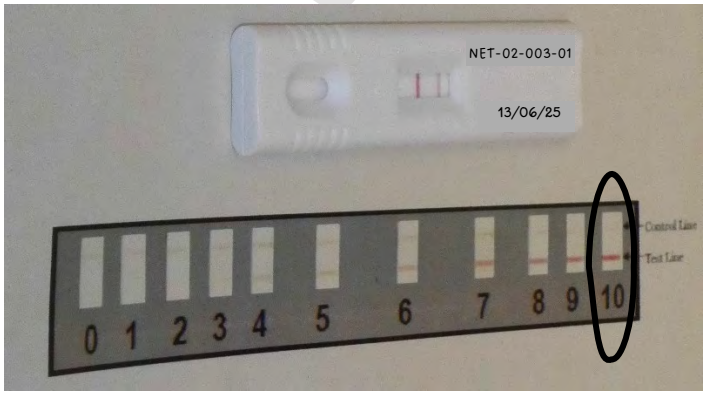


If the TEST (T) line is not visible, any active NE present in the sputum sample does not exceed the pre-set threshold.



test result scoring guide (utilise print out provided, if needed print further copied from Appendix 3, always print in colour and ensure no discolouration is transferred from the printer)

Example photograph and labelling (positive test is shown)



8.2. SOP B Sputum aliquotting (all visits)

Equipment/ reagents required:

Nitrile gloves	Microbiological Safety Cabinet MSCII
Lab coat	Sterile petri dishes (ideally 10 cm)
Clinical waste bags & autoclave tape (or as per local procedures)	P100 positive displacement pipette and corresponding piston tips
1.5ml sterile screw-capped tubes	Ice and small ice box/container
DNA/RNA shield (stored at room temp)	Sputum sample labels (use appropriate 100 or 200 µl, DNA/RNA shield, or Excess)

Procedure:

1. Upon receipt of sputum sample to the lab, remove sputum pot from the delivery packaging, wipe the outer surface of the vacutainer with either 10% terminex, 10% ChemGene, or appropriate decontamination reagent as per local procedures and dispose of the tissue. Ensure pot has dried off before opening.
2. In the event of contamination of packaging (i.e. sample has leaked into packaging), attempt to rescue as much uncompromised sample as possible, decontaminate the delivery packaging as per local SOPs, and make a note of this in the log.
3. Keep sputum sample on ice until use.
4. Add sputum sample to a sterile petri dish to allow selection of sputum and elimination of any contaminating saliva present in the sample (note: saliva contamination will impact assay results and study endpoint measurements, take care to ensure saliva is not transferred for storage).
4. Using a p100 MicroMan positive displacement pipette set to 100 µL, select out the sputum, combining into a separate area of the petri dish if needed (e.g. if sample has high saliva content and sputum sections are small).
5. Transfer 100 µl of selected sputum to sterile 2ml screw-capped tube, with the following order of priority:
    1. 2x 100 µl aliquots (i.e. two tubes, final sputum volume of 100 µl per tube)
    2. 1x 200 µl aliquot (use the p100 to pipette two lots of 100 µl sample into these tubes for a total of 200 µl sputum per tube)
    3. 1x 100 µl aliquot plus 900 µl DNA/RNA shield buffer (method: add 900 µl DNA/RNA shield buffer to aliquoted sputum sample, cap tightly and vortex for 15 seconds at high-speed (approx. 20,000-30,000 rpm) to mix. Pipette up and down 10X with a p1000 pipette to disperse lumps.
    4. 1x further 200 µl aliquot (use the p100 to pipette two lots of 100 µl sample into these tubes for a total of 200 µl sputum per tube)

5. Add any remaining excess sputum into as many 1.5 ml tubes as required to store all sputum, adding a maximum final volume of 1 ml per tube to avoid over-filling.

If sample volume is too low for any of the study visits and aliquots for 1. or 2. above cannot be achieved, store any sputum available, by reducing the pipette volume setting and record the accurate final volume in the log sheet notes section.

6. Store all samples in AIR-NET sputum box in the –80°C freezer immediately after aliquotting is completed. Record sample details in the sputum log sheet. Add any notes (including approximate excess sputum volumes or any volumes below 100 or 200 µL if sample volume was low).

8.3. SOP C 4ml EDTA vacutainer tube storage (visit 1/screening only)

Equipment/ reagents required:

Nitrile gloves	Microbiological Safety Cabinet MSCII (or as appropriate for locally approved procedures)
Dedicated lab coat	EDTA 4ml vacutainer tubes containing blood
Clinical waste bags & autoclave tape (or as per local procedures)	Sample label (1 per participant)
Cardboard tube storage box	

Procedure (processing should be completed within maximum 1hr of collection):

1. Remove the 4mL EDTA vacutainer from the delivery packaging, wipe the outer surface of the vacutainer with either 10% terminex, 10% ChemGene, or appropriate decontamination reagent as per local procedures.
2. In the event of contamination of packaging (i.e. sample has leaked into packaging), attempt to rescue as much uncompromised sample as possible, decontaminate the delivery packaging as per local SOPs, and make a note of this in the log.
3. Add the sample label to the blood tube and fill out sample log. Then place immediately in cardboard storage box provided (upright) in -80°C freezer.
4. Record details in the AIR-NET log sheet, including notes such as low blood volume in tube if noticed.

8.4. SOP D Serum generation and aliquotting (all visits)

Equipment/ reagents required:

Nitrile gloves	P1000 Pipette and Filter tips
Dedicated lab coat	1x SSTII Advance 5ml vacutainer tube containing clotted blood
Centrifuge (room temp)	Serum sample labels (4 per sample/visit)
Microbiological Safety Cabinet MSCII	FluidX 1.4mL tubes (4 per sample) and yellow septum caps (4 per sample)
Clinical waste bags & autoclave tape (or as per local procedures)	FluidX 96-well box for serum

Procedure (processing should be completed within maximum 2hrs of collection):

1. Remove the 5mL SSTII yellow-topped vacutainer from the delivery packaging, wipe the outer surface of the vacutainer with either 10% terminex, 10% ChemGene, or appropriate decontamination reagent as per local procedures.
2. In the event of contamination of packaging (i.e. sample has leaked into packaging), attempt to rescue as much uncompromised sample as possible, decontaminate the delivery packaging as per local SOPs, and make a note of this in the log.
3. Allow the tube to stand upright for 1 h at room temperature (i.e. 1h from time of blood draw). If 1h has already passed from time of venepuncture when the sample is received to the lab, proceed directly to further processing.
4. Turn on centrifuge. Follow safe use of the rotors and lids for this unit paying particular attention to correct balancing of samples in the appropriate rotor for each sample process. Ensure centrifuge is at room temperature.
5. Place the vacutainer tube in the rotor bucket with the appropriate blood tube adaptor and vacutainer counterbalance. Centrifuge the tubes at 1000 xg for 15 mins.
6. Label 4x FluidX tubes (white freezer-safe labels) with sample ID, visit number, and date.
7. Remove the vacutainer from the centrifuge carefully and transfer back into the MHCII hood (or as per local procedures).
8. Pipette 400 µl aliquots of serum using a P1000 and sterile filter tips into four 1.4mL FluidX tubes. Aliquot any excess serum into the final (i.e. 4th) tube. *If excess is more than 800 µl, also add in 400 µl increments to the 3rd tube, 2nd tube, 1st tube, in that order, if required, to avoid over-filling.*

In the case than <400 µl is available, aliquot this anyway and make a note of the approximate volume in the log sheet.

9. Cap the samples with yellow septum caps. Add one dot with permanent marker to the cap of the “excess” tubes to indicate extra volume. Also add a dot to the cap if a tube has <400 µl in the case of low volume.
10. Record details in the sample log sheet, including any important notes (for example if haemolysis is noticed or if timing was not possible to keep to).
11. Place all aliquoted sera in clearly labelled FluidX 96 well boxes and store at -80°C.

8.5. SOP E Nasal epithelial cell brushing and storage (visits 2 and 5)

Equipment/ reagents required:

Nitrile gloves	1.5ml LoBind Eppendorf tube containing 1ml of 1X RNAlater buffer (stored at room temp)
Dedicated lab coat	Nasal brush (modified 3mm bronchoscopy brush)
Cardboard sample storage box for brushings	Brushing sample label (1 per sample)
	Sample bag and absorbent material

Notes:

For nasal brushing procedure to obtain this sample, please refer to instructional video.

Procedure:

1. Label the tube before performing the brushing procedure, including participant ID and date.

2. At the study visit, immediately after obtaining brushing, **the clinical team member** should vigorously agitate the brush in RNAlater solution in a 1.5 ml LoBind Eppendorf tube for 30 seconds to ensure all cells have been detached from the brush into the solution. The plastic sheath of the brush can be lowered over the brush to remove remaining cells.

3. Cap the tube very tightly and securely.

4. Add sample to the sample bag together with absorbent material.

5. *Upon receipt in the lab*, wipe the outer surface of the vacutainer with either 10% terminex, 10% ChemGene, or appropriate decontamination reagent as per local procedures).

6. Transfer the 1.5ml tube to the permanent storage box in a -80°C freezer.

7. Record details in the sample log sheet, add any important notes, including any presence of red blood cells.

8.6. SOP F PAXgene tube storage (visits 2, 5, 6)

Equipment/ reagents required:

Nitrile gloves	Microbiological Safety Cabinet MSCII
Dedicated lab coat	2.5mL PAXgene vacutainer tubes containing blood
Clinical waste bags & autoclave tape (or as per local procedures)	1x Sample label
Cardboard boxes for tube storage	-80°C freezer

Procedure (process ideally within 4h from blood draw):

1. Remove the PAXgene RNA vacutainer from the delivery packaging, wipe the outer surface of the vacutainer with either 10% terminex, 10% ChemGene, or appropriate decontamination reagent as per local procedures.
2. In the event of contamination of packaging (i.e. sample has leaked into packaging), attempt to rescue as much uncompromised sample as possible, decontaminate the delivery packaging as per local SOPs, and make a note of this in the log.
3. Add a sample label with site, participant ID, visit number, and date. Store the PAXgene tube upright for at least 2 hours and no more than 6h (from time of venepuncture) at room temperature before transferring to -80°C freezer storage.
4. Record details in the log sheet, including any notes (e.g. low blood sample volume, if this occurs).

8.7. SOP G Lithium heparin whole blood degranulation assay (visits 2, 5, and 6)

Equipment/ reagents required:

Nitrile gloves	10 mg/ml Zymosan in HBSS
Designated Lab coat /safety glasses	FluidX 1.4mL (4 per participant)
Centrifuge	FluidX blue septum caps (4 per participant)
Microbiological Safety Cabinet MSCII	Tube labels (4 per participant)
Clinical waste bags & autoclave tape (or as per local procedures)	Incubator (37°C, 5% CO_2)
P1000 and P200 pipettes and filter tips	4ml LH vacutainer with whole blood
HBSS (2x 75 µl aliquots in 1.5ml tubes) (stored at -20°C)	Zymosan (2x 75 µl aliquots in 1.5 ml tubes) (stored at -20°C)

Notes

Process within maximum 2h of venepuncture, ideally within 1h.

Assay timings below **must** be exact, do not leave samples in the incubator or centrifuge once completed as neutrophils will continue to degranulate and significantly impact endpoint measurements.

Procedure

1. Remove the 4ml lithium heparin (LH) vacutainer from the delivery packaging, wipe the outer surface of the vacutainer with either 10% terminex, 10% ChemGene, or appropriate decontamination reagent as per local procedures. Ensure the correct LH tube has been selected (distinct from NH sample)
2. In the event of contamination of packaging (i.e. sample has leaked into packaging), attempt to rescue as much uncompromised sample as possible, decontaminate the delivery packaging as per local SOPs, and make a note of this in the log.
3. Remove 2x stored Zymosan aliquots and 2x HBSS aliquots containing 75 µl (per 1.5ml tube) of appropriate reagent from –20°C freezer. Ensure Z or H is clearly labelled on tube lid and allow to thaw and bring to room temp for 10 min before use (maximum 30 min). Label the Eppendorf tubes with the participant ID.
4. Label 2 x FluidX 1.4ml tubes for plasma from Zymosan-treated blood and 2 x FluidX 1.4ml tubes for the control plasma aliquots.
5. Add 750 µl of blood into the 4 labelled Eppendorf tubes. Use a new p1000 tip for each tube.
6. Invert the tubes 5 times to mix, ensuring the white zymosan pellet is no longer visible, and incubate the tubes for 30 minutes at 37°C in an incubator (5% CO_2). Use the lab timer for precision.
7. At exactly 30 mins, invert the tubes twice to resuspend the blood cells, then immediately centrifuge at 600xg for 10 mins at room temperature.

8. As soon as the centrifuge cycle has finished (note: timing **must** be exact), immediately aliquot the separated plasma supernatants (200 µl per tube) from the Eppendorf tubes into a 1.4mL FluidX tubes for each original sample tube (4 FluidX tubes in total).
   DO NOT DISTURB THE WHITE BUFFY COAT LAYER *between the plasma and the red blood cells- this will result in cell contamination and affect plasma protein assays.*

9. Store plasma aliquots at -80°C immediately. Update freezer sample log.

10. Remaining red cell pellets in Eppendorf tubes can be discarded to waste bag or liquid waste, according to local procedures.

8.8. SOP H Sodium heparin whole blood stabilisation and storage (visits 2, 5, and 6)

Equipment/ reagents required:

Nitrile gloves	Microbiological Safety Cabinet MSCII
Dedicated lab coat	Green topped sodium heparin (NH) 4ml vacutainer tubes containing whole blood
Clinical waste bags & autoclave tape (or as per local procedures)	Sample labels (4 per participant)
Cardboard tube storage box	Stabilisation buffer (Cytodelics; from whole blood processing kit/Gen 2); 500 µl/tube (Stored at 4°C)
4 x 1.8 ml cryovials	8x "filler vials" for unoccupied CoolCell spaces (i.e. 8x 1.8 ml cryovials, filled with 1 ml PBS per vial)

Notes:

On /before the first use, generate 8x "filler vials" which should be placed into any unoccupied spaces in the CoolCell freezing container when samples are being frozen. For this, add 1ml of sterile PBS into 8x 1.8 ml cryovials. Re-use filler tubes as many times as needed, but check volume remains at 1ml over multiple uses and top-up tubes or remake as required. Label these clearly with black permanent marker as "FILLER" and add a dot to the cap to distinguish easily from stored blood samples.

Cryovials with 0.5ml stabilisation buffer to be stored in fridge, prepare aliquots in advance as required. **Before use, buffer must come up to room temperature**.

Round CoolCell freezer containers to be stored at room temperature after any samples have been appropriately removed and stored. If CoolCell is still in the freezer due to previous sample processing, appropriately store any previous frozen samples at -80°C, then take out freezing container and keep at room temperature at least 1 hour before use.

Procedure (within 1h from blood draw):

1. Remove stabilisation buffer aliquots from fridge (500 µl per tube) and bring to room temp (~10-15 min before use, maximum 30 mins before).
2. Remove the 4ml sodium heparin (NH) vacutainer from the delivery packaging wipe the outer surface of the vacutainer with either 10% terminex, 10% ChemGene, or appropriate decontamination reagent as per local procedures. Ensure the correct tube (NH, *not* LH) has been selected from the sample pack.
2. In the event of contamination of packaging (i.e. sample has leaked into packaging), attempt to rescue as much uncompromised sample as possible, decontaminate the delivery packaging as per local SOPs, and make a note of this in the log.
3. Label 4 x 1.8 ml cryovials containing stabilisation buffer with the ID, visit, and date using white label provided.

4. Just prior to aliquotting, gently invert the NH vacutainer twice to mix to ensure plasma and blood cells are not separated.

4. Add 500 µl NH whole blood to 500 µl room-temperature stabilisation buffer in each of the four cryovials.

5. Cap the cryovials and gently invert 15 times to mix - **DO NOT VORTEX**

6. Incubate in the hood at room temperature (ideally ~20°C) upright for 10 mins (use lab timer).

8. Place in CoolCell cryobox (upright) in -80°C freezer. Add details to log sheet.

9. After a minimum of 4h (maximum is indefinite), transfer the frozen cryovials to a permanent storage box. Ensure this is done rapidly so that samples do not thaw.

10. Record sample location in log sheet.

8.9. SOP I Neutrophil isolation with EasySep kit and neutrophil storage (visits 2 and 5)

Equipment/ reagents required:

Nitrile gloves	StemCell EasySep Neutrophil Isolation Kit (cat no 196666) (stored at 4°C)
Dedicated lab coat	Blue lid 50ml Falcon Tube (4 per participant)
Centrifuges (for 50ml tubes and 1.5ml Eppendorf tubes) at room temp	DPBS + 1mM EDTA (no Ca^{2+} or Mg^{2+}) *Provided pre-made to site, stored at room temp*
Microbiological Safety Cabinet MSCII	1X DPBS (no Ca^{2+} or Mg^{2+}) *Provided pre-made to site, stored at room temp*
Clinical waste bags & autoclave tape (or as per local procedures)	Electronic pipette (for 10-25 ml stripettes)
Haemocytometer (glass or plastic)	Microscope (or equivalent cell counting equipment)
2x Purple-topped 10ml EDTA blood vacutainers, 1x 4ml EDTA vacutainer	Easy 50 magnet
Disposable sterile 25ml and 10 ml stripettes	4x Eppendorf 1.5ml LoBind tubes, 4x white labels (3x neutrophil pellets, 1x RNAlater)
RNALater (provided to site as 5ml tubes) (stored at room temp)	P10, 200, 1000 pipettes and tips
Pre-cooled Corning CoolRack (-80°C)	

Notes:

Please familiarise yourself with the instruction materials provided by StemCell - Product Information Sheet and Safety Data Sheet.

Minimum expected yield from 1ml EDTA blood is typically 1 million isolated neutrophils.

Neutrophils are very sensitive and are easily activated. Do not vortex any samples containing neutrophils, treat the cells very gently, and avoid bubbling through any of the cell solutions at any step.

Procedure (process within maximum 2h from blood draw, ideally no more than 1h):

1. Remove the 2x 10ml and 1x 4ml purple-topped EDTA vacutainers from the delivery packaging, wipe the outer surface of the vacutainer with either 10% terminex, 10% ChemGene, or appropriate decontamination reagent as per local procedures.
2. In the event of contamination of packaging (i.e. sample has leaked into packaging), attempt to rescue as much uncompromised sample as possible, decontaminate the delivery packaging as per local SOPs, and make a note of this in the log.

3. Retrieve StemCell kit from fridge, add 4x 50ml blue-lidded Falcon tubes to the hood. Invert EDTA vacutainers 2 times gently if separation has occurred.

4. Gently pour the collected blood from all of the vacutainers at a 45° angle into one combined 50 ml blue-lidded Falcon tube, carefully so as not to activate the neutrophils and avoid incorporating any bubbles. Leave vacutainers inverted for ~10 sec to ensure as much blood sample as possible has been added to the falcon tube. Record the approximate total volume of blood in the lab book and study log sheet, utilising the volume markers on the 50ml tube as a guide.

5. Add 50µl of Isolation Cocktail per mL of whole blood (colourless tube; e.g. add 1ml if 20ml blood was noted in the 50ml falcon tube for this preparation, add 900 µl if there is 18 ml blood, etc.). Record volume of cocktail added in the log sheet. Adjust according to total blood volume available.

Isolation kit typically has more bead volume than cocktail volume, so use this carefully and take any liquid that may have collected in the reagent tube cap if possible.

6. Vortex the RapidSpheres from the EasySep kit for 30 seconds (brown tube in kit) to resuspend them and add 50 µl/ml blood (use the same volume as in step 5) to the blood preparation.

7. Very gently invert end over end 4 times to mix, avoiding generation of bubbles (remove with p1000 if large bubbles form). Incubate for 5 minutes upright at room temperature in the hood (use lab timer). *Do not add to magnet yet.*

8. After 5 min, very gently pour or pipette pre-prepared 1X DPBS/1mM EDTA to top the blood sample up to a final volume of 50 ml. Securely re-cap the falcon tube. Carefully and slowly invert the tube 3 times to mix evenly.

9. Unscrew the falcon tube lid and place the tube of cells into the EasySep Magnet (silver Easy50 magnet). Incubate at room temperature for 10 minutes in the magnet.

A yellow-ish neutrophil-rich layer and darker red layer should form. Occasionally separation is not as clear on the first magnet step and the top layer remains pink, but should clarify with subsequent separation steps.

10. With a new 25ml stripette, slowly pipette up the top layer of cells keeping the stripettes tip at the very top of the liquid layer and moving down with it, avoiding any bubbles, and taking a maximum of 25ml each time. Transfer the top neutrophil-rich layer into a new 50ml blue lidded Falcon tube, taking care not to disturb the red cell layer. Dispose of the Stripette and the old blood tube as appropriate into the solid or liquid waste.

11. Add the Rapid Spheres at the same volume as in step 6 (vortex beads for 30 seconds before use to resuspend) to the newly transferred cells. Gently invert the suspension 3 times to mix and then incubate (<u>not in magnet</u>) for a further 5 minutes at room temperature. Return the Stem cell reagent kit to the fridge.

12. After 5 min, place the tube of cell suspension onto the EasySep Magnet. Remove the lid (or place on lightly) and incubate the cells on the magnet at room temperature for 5 minutes.

13. Repeat step 10 and transfer the neutrophil-rich plasma solution to a new 50ml falcon tube (<u>do not</u> add any further beads/RapidSpheres). Immediately add the transferred cell suspension to the magnet.

14. Incubate the tube on the magnet for 10 minutes at room temperature.

15. Collect purified Neutrophils into a new tube using a 25 ml stripettes. Remove 10 µl of cell suspension and load this directly into a haemocytometer slide for cell counting. *No trypan blue addition is needed.*

16. Note the total volume of cell suspension in the 50 ml tube for later cell number calculations, then centrifuge the cells at 300 *xg* for 6 minutes with Acceleration 7, Brake 7 (setting depends on centrifuge at the site; medium acceleration and medium brake are required). Ensure the centrifuge is at room temperature.

17. Whilst the cells are undergoing centrifugation, conduct cell counts. Use the total cell volume from step 16 to calculate the final cell number. Then, calculate the volume of DPBS needed to resuspend the cells at a concentration of $5x10^6$ cells/ml (calculations below; note all cell numbers and calculations in log sheets).

18. After the centrifugation cycle completes, using a 25mL Stripette, gently remove the plasma supernatant and discard into liquid waste. Leave behind a small volume of buffer (max ~1ml) if needed to avoid accidentally removing and discarding cells from the pellet.

19. Gently resuspend the white cell pellet in 10 ml of DPBS to wash using a 10 ml stripette, pipetting up and down 3 times.

20. Pellet cells once again by centrifuging at 300 *xg* for 6 min.

21. Remove and discard the supernatant by gently pouring this into liquid waste, leaving the tube inverted for ~2 seconds, and then resuspend the cell pellet in the calculated volume of DPBS (without EDTA) to achieve the desired cell concentration ($5x10^6$/ml); for this, use a p1000 pipette to gently and evenly resuspend the pellet in 1 ml of DPBS initially, then use a stripette to top up the suspension to the correct final volume. Cap the tube securely and invert gently end-over-end 3 times to mix cells evenly. Cells can now be used for further live assays and storage according to the priority list below.

Order of priority for isolated neutrophil usage:

1. *1x pellet storage ($5x10^6$ cells)*
2. *1x RNALater storage ($5x10^6$ cells)*
3. *Functional work*
    i. *SYTOX NETs assay ($2x10^6$ cells; assay uses $8x10^5$ cells, but some excess is required for accurate plate loading)*
    ii. *Phagocytosis assay ($2x10^6$ cells)*
    iii. *ROS assay ($2x10^6$ cells)*
    iv. *Cell stimulation and storage ($9x10^6$)*
4. *2x further pellet storage ($5x10^6$ cells)*

22. **For cell pellet storage**, aliquot 1ml of neutrophil suspension into a pre-labelled (participant ID, visit, date) 1.5 ml Eppendorf LoBind protein tube, in triplicate, and centrifuge 300 *xg* for 5 mins to pellet.

To allow pellet generation before moving on to functional work, use the below as a guide to determine the number of 1.5ml LoBind tubes to fill and centrifuge:

Neutrophil count	Number of 1.5 ml LoBind neutrophil tubes to generate before proceeding to functional work
29.9 million or less	2 (1 pellet, 1 RNAlater lysate)
30 to 34.9 million	3 (2 pellets, 1 RNAlater lysate)
35 million or more	4 (3 pellets, 1 RNAlater lysate)

23. Remove and discard supernatant from the Eppendorf tubes. Except for the tube reserved for RNAlater, immediately snap freeze up to 3 tubes/cell pellets (if cell number permitted) by putting these into a pre-cooled Corning CoolRack block in the –80°C freezer. Transfer to permanent storage after minimum 2h in the block (maximum time is indefinite, but recommend doing this within 24-48h to avoid losing sample tubes due to other freezer usage, as block is not lidded).

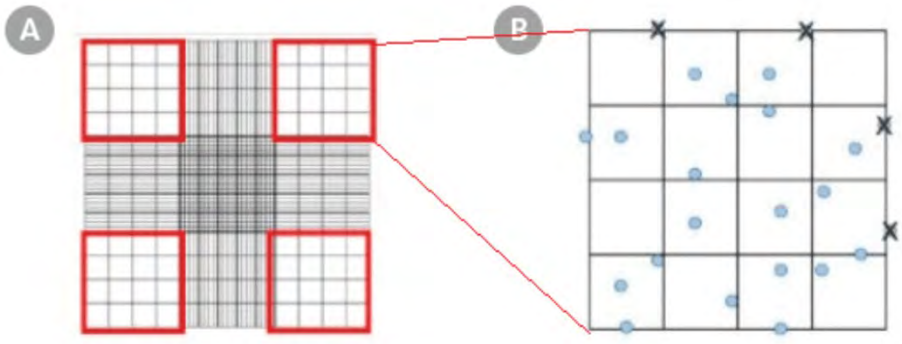
For permanent storage and shipping, pellets are very sensitive to rapid thawing, ensure samples are always handled on dry ice and storage boxes are never out on the bench at room temp for any time period.

24. To the other RNALater Eppendorf tube (sample #2 in priority list), gently resuspend the cells in 100 µl DPBS by pipetting 3 times. Add 500 µl RNAlater buffer, then pipette up and down with a p1000 pipette vigorously 10 times to homogenize and lyse cells. Store immediately at –80°C in appropriate box.

25. Proceed to live neutrophil functional assays with remaining cells.

Calculation for cell counting

- 1. No. of cells in a 4x4 grid on the haemocytometer multiplied by 10^4 = **cells/ml**
- 2. **Cells/ml** multiplied by the **volume of the original cell suspension** = **total cell number**
- 3. **Total cell number** divided by 5 million = **volume of DPBS (without EDTA) needed** to resuspend total neutrophil pellet to achieve $5x10^6$ cells/ml



Count any one of the grids outlined in red in A. Counting more than one 4x4 grid is not necessary. However, if this is done, ensure the average count per 4x4 grid is used for the first calculation above. Do not include the cells crossing two outer-most edges of the grid, as per B above (indicated by X).

8.10. SOP J *In vitro* neutrophil extracellular trap (NETs) assay with SYTOX green (visits 2 and 5)

Equipment/ reagents required:

Nitrile gloves/Long cuff gloves	P1000, P200 and p10 multichannel pipettes, p10 single channel pipette tips
Lab coat	Incubator (37°C) with 5% CO_2 with humidity *(get in touch with Tayside site if humidity is not maintained for alternative solutions)*
Fluorescence plate reader for 96-well plates	Clinical waste bags & autoclave tape (or as per local procedures)
Microbiological Safety Cabinet MSCII	2x Disposable reagent trough/well
PA10 LPS (Sigma L9143-10MG) (provided as 0.5 mg/ml stock in dH_2O) (stored at -20°C)	1x 96-well TC-treated NUNC plate with lid
PMA (Sigma P1585-1MG) (provided as 10 µM stock) (stored at -20°C)	RPMI with 10mM HEPES (no phenol red, NO SERUM) (stored at 4°C)
Salmon sperm DNA standard (provided as 5 µL 10mg/ml stock aliquots, stored -80°C) and plate control sample (provided as 1.5ml pre-made aliquots)	Sytox green DNA dye (provided to sites as 5µM stock ready to use and made up in dH_2O) (stored at -20°C)
Nuclease-free water (provided as 13ml in 15ml falcon tubes) (stored at -20°C)	Sterile PBS (stored at room temp)
Vortex	8x sterile nuclease-free 15 ml tubes

Notes:

Fluorescence plate reader must be regularly serviced and maintained with in-date certificate.

RPMI media preparation: Add 0.25 ml HEPES to 24.75ml Phenol-red-free RPMI. Keep any excess in the fridge and use within 1 month. Warm to room temp for use ~30 min before required.

Procedure:

1. Label the plate lid with the plate conditions and plate plan shown below using a permanent marker (all conditions have quadruplicate wells). Vortex all stock reagents and working solutions before pipetting.

i. Unstimulated control cells (2 µl PBS per well) (row A, wells 1-4)
ii. LPS – 5 µg/ml (2µl per well of 500 µg/ml LPS stock) (row A, wells 5-8)

iii. PMA – 100 nM (2µl per well of 10 µM PMA stock) (row A, wells 5-8)
iv. Cells only (no SYTOX) (2 µl PBS per well) (row B, wells 1-4)
vi. Media only (no neutrophils or sytox green) (row B, wells 5-8)
vii. Sytox Green only (no neutrophils) (row B, wells 9-12)

Plate layout:

	Column #: 1, 2, 3, 4	5, 6, 7, 8	9, 10, 11, 12
Row A	**Unstimulated cells**	**LPS**	**PMA**
Row B	*Cells WITHOUT SYTOX*	Media only (no cells or SYTOX)	**SYTOX only (no cells)**

2. Add reagents listed above (i-iv) in quadruplicate to a 96-well plate. *Add PBS, LPS and PMA to the plate before adding media, to visualise addition clearly and prevent errors. Check 2 µl spots are present in wells before proceeding.*

3. Add appropriate volume of sterile RPMI media containing 10mM HEPES (no phenol red, no serum) to each well as below (final well volume should be 200µl):
    - Unstimulated cells, cells without SYTOX, and LPS/PMA conditions: add 188 µl media
    - SYTOX only: 200 µl media

4. All steps above can be prepared in the morning on day of use, refrigerate prepared plate after reagent addition. **~30 min before use** (as an estimate a good time to do this is on the first centrifugation step after the last StemCell magnet step in the cell isolation), remove the prepared plate from fridge and store at room temp inside the hood to allow to come to room temp. If two participants are expected at the same time, only one "Sytox only/no cells" control and only one sperm DNA standard curve is required per plate.

5. Isolate neutrophils using EasySep Kit. SOP I Neutrophil isolation with EasySep kit and neutrophil storage.

CONTINUE HERE AFTER NEUTROPHIL ISOLATION:

6. Using a white plastic disposable reservoir, add isolated neutrophils at 5×10^4 cells per well (i.e. 10 µl per well of the $5x10^6$ cells/ml suspension in DPBS) to plates using a 10 µl multichannel pipette, for all conditions except
    v. "Media only" (no neutrophils or sytox green) (row B, wells 5-8)
    vi. "Sytox green only" (no neutrophils) (row B, wells 9-12)

Pipette up and down once when adding the 10 µl cell suspension, without introducing bubbles. Ensure all solution is removed from every tip by visual inspection. Use fresh tips for each well/addition.

7. Incubate plate for 3 hours and 50 min at 37°C, 5% CO_2, with humidity, then proceed to step 9.

8. **At 3h and 35 mins**, thaw an aliquot of the DNA stock solution to room temperature for 5 mins. Also remove the SYTOX green stain from the freezer to thaw to room temperature at this time. Then proceed to generate a DNA standard curve as follows:
    i. Set out 8x 15ml DNase-free sterile falcon tubes, carefully ensuring no DNA contamination during set up. Number the tubes 1 to 8.
    ii. To the 5 µl DNA stock aliquot in 1.5ml tube, add 620 µl of Nuclease-free water (provided as 13 ml aliquots) using a p1000 pipette. Vortex for 5 seconds to mix.
    iii. To tube 1, add 2.7 ml nuclease-free water. To tubes 2-7, add 1 ml of water. To tube 8, add 2 ml water.
    iv. To tube 1, add 300 µl of DNA solution prepared in step ii.
    v. Vortex the tube for 5 seconds to mix.
    vi. Take 2 ml of the newly generated solution from tube 1 and add to tube 2, pipetting up and down ~4 times.
    vii. Vortex tube 2 for 5 seconds, take 2 ml from tube 2 and add this to tube 3.
    viii. Repeat this serial dilution process iteratively to generate subsequent dilutions until **tube 7**. At the end of the process, tubes 1-6 should have 1 ml DNA standard and tube 7 should have 3 ml.
    ix. DO NOT ADD ANY DNA TO TUBE 8. Tube 8 will be the blank.
9. At 3h and 50 mins, remove the 96-well plate from the incubator, add the DNA standard curve to the plate according to the layout below, in quadruplicate, loading 200 µl of each standard or blank per well.

	Column #: 1, 2, 3, 4	5, 6, 7, 8	9, 10, 11, 12
Row A	**Unstimulated cells**	**LPS**	**PMA**
Row B	*Cells WITHOUT SYTOX*	Media only (no cells or SYTOX)	**Media and SYTOX only (no cells)**
Row C	***DNA 800 ng/ml (tube 1)***	***DNA 533 ng/ml (tube 2)***	***DNA 355 ng/ml (tube 3)***
Row D	***DNA 236 ng/ml (tube 4)***	***DNA 158 ng/ml (tube 5)***	***DNA 105 ng/ml (tube 6)***
Row E	***DNA 70 ng/ml (tube 7)***	***DNA blank (0) (tube 8)***	*DNA blank (0) (tube 8), NO SYTOX*
Row F	***Plate control sample***		

If two participants are being processed in parallel at the same time for either visits 2 or 5, repeat the conditions shown in rows A and B in rows G and H for the second donor's cells.

10. At 4h exactly, add 25 µl fully-thawed Sytox green to all wells shown in bold text above using a disposable plastic reservoir and P100 multichannel pipette. Use fresh tips for each well to avoid contamination.

11. Read plate at excitation/emission 490/537 nm on the plate reader, removing the plate lid before reading. Keep plate inside the reader, and read again exactly 10 mins later (use a saved programme created in advance for the assay/study if possible).

12. Record the plate reader use and save file with time point, date, and sample ID (NET-site-participantID-visit). Include plate plan in the file if deviating from SOP plate layout. Store/send file into appropriate "AIR-NET NETs assay" folder, ensure cloud-based back up of the file is made either by copying to a secure online platform (e.g. SharePoint or Google Drive) if not done automatically on the plate reader computer.

    Before disposing of the assay plate and before closing the original data read-out, immediately open the stored results file and check wells A1-4 fluorescence readings match those on the original read-out to any prevent saving errors. Initial the corresponding box in the log sheet to confirm this has been done.

13. Discard the plate into the biohazard waste bag after use or appropriate local waste stream.

8.11. SOP K *In vitro* neutrophil phagocytosis and reactive oxygen species assay (visits 2 and 5)

Equipment/ reagents required:

Nitrile gloves/Long cuff gloves	2x **FITC-labelled** heat-killed *P. aeruginosa* (PAO1) aliquots (see Tayside-only SOP N - FITC labelling of *P. aeruginosa*) with 10% normal human serum 2x **non-labelled** heat-killed *P. aeruginosa* (PAO1) aliquots with 10% normal human serum 4x HBSS vehicle-control containing 10% serum *All stored at -20°C and provided as combined bacteria or vehicle and serum pre-prepared 30 µl aliquots*
Dedicated Lab coat/Face Guard	5 ml round-bottomed flow tubes with lids
GTR Centrifuge	4% PFA/PBS (stored at 4°C)
Microbiological Safety Cabinet MSCII – see Appendix	Tube labels
Clinical waste bags & autoclave tape (or as per local procedures)	Incubator (37°, 5% CO_2)
CellROX deep red reagent (stored at -20°C)	Sterile DPBS (stored long-term at room temp)
Sterile HBSS (stored at room temp)	2% BSA/DPBS (stored at -20°C)

Notes:

Heat-killing of bacteria has been previously validated and is checked for every batch of PAO1 prepared for AIR-NET. See appendix 1 for confirmation of current batch successful heat-inactivation and killing. No bacterial growth is observed after the process has been completed.

Procedure:

1. During the neutrophil isolation process, when the isolated cells are being centrifuged for the first time (i.e. cells centrifuged in their own plasma), start to opsonise the pre-prepared, FITC-labelled heat-killed *P. aeruginosa* and the non-labelled *P. aeruginosa* with human serum. For this, remove:
    1. 2x FITC-PA tubes
    2. 2x unlabelled PA tubes
    3. 4x vehicle control tubes

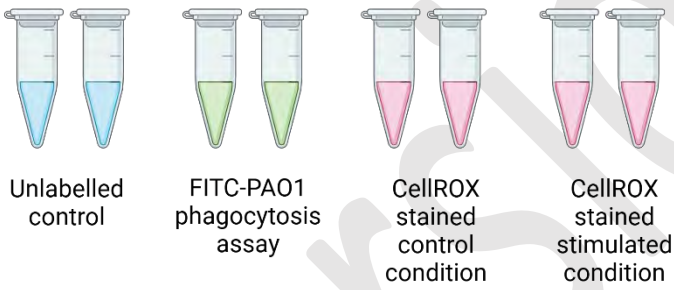
from the -20°C freezer and immediately add the tubes into a rack in a 37°C incubator for 30 minutes (all tubes provided already contain serum). **Keep samples protected from light for the whole procedure to avoid beaching the fluorescence.**

<u>Also add an aliquot of 2% BSA/DPBS to the fridge to slowly thaw for later use. Do not vortex BSA.</u>

2. Once opsonisation is completed, <u>(in a hood with the light turned off if possible)</u> label the 1.5ml Eppendorf tubes containing bacteria or vehicle control with the participant ID.
3. A few minutes before the assay, remove the CellROX stock from the freezer to that for ~5 mins.
4. Generate a CellROX concentrated solution by adding 2 µl of full-thawed CellROX stock to 198 µl HBSS in a 0.5 or 1.5ml Eppendorf tube. Vortex for 5 seconds to mix.
5. Add 100 µl room-temperature HBSS (from the larger stock HBSS bottle) to each of the 8 Eppendorf tubes, then add 100 µl of the final isolated neutrophil suspension to each of the 8 tubes (cells should have been prepared in DPBS at a concentration of $5x10^6$ neutrophils per ml just after isolation was complete, therefore $0.5x10^6$ neutrophils in total are being added to each Eppendorf tube in this assay).
6. To the 2x <u>unlabelled *P. aeruginosa*</u> tubes (shown in pink below) and to 2x HBSS vehicle control tubes, add 20 µl of the concentrated CellROX stain (generated in step 4) to each tube.

   4 tubes in total should now contain CellROX stain. **Add a star (*) to the lids of these tubes with permanent marker to distinguish them from the rest.**

Tubes should now be as follows:



7. Invert all of the Eppendorf tubes gently 2 times to mix. <u>Never vortex the cells</u>. Incubate the samples at 37°C, 5% CO_2 for 30 min.
8. After 30 min incubation, add 300 µl of cold (4°C/refrigerated) 2% BSA/DPBS to each tube. Invert twice gently to mix.
9. Use a microcentrifuge to wash and the pellet cells (400xg for 5 mins, room temperature).
10. The cell pellet should be visible as a small white dot towards the bottom of the tube. Visually locate the pellet (do this for all wash steps), then carefully remove and discard the supernatant into liquid waste with a p1000 pipette, avoiding the edge with the pellet to prevent disturbing the cells with the tip or taking off the supernatant too quickly.
11. Resuspend the cell pellet in 500 µl cold DPBS (4°C/refrigerated) using a p1000 pipette. *If the pellet is accidentally removed at any step, pipette out the solution containing the cells back into the tube, add the DPBS to wash as above, and proceed to step 10. Make a note of this in the log sheet.*
12. Pellet cells once more (400xg for 5 mins, room temp).
13. Resuspend cells in 150 µl of 4% paraformaldehyde (PFA). Incubate protected from light at 4°C for 1 hour to fix and stabilise the cells.

14. After 1 hour, pellet the cells at 400xg for 5 mins.
15. Remove the PFA supernatant as described in step 10 and discard into special/hazardous waste as appropriate per local procedures.
16. Resuspend cells in 0.5ml cold 2% BSA/DPBS to wash, pellet by centrifugation at 400xg for 5 mins.
17. Remove and discard the supernatant. Resuspend cells in 400 µl 2% BSA/PBS and transfer to 5ml round-bottomed polystyrene flow cytometry tubes (with lids).
18. Ensure 5ml tubes are clearly labelled with permanent marker, adding a large star (*) again to those with ROS stain. Cover samples with foil to protect from light, and store at 4°C until analysis (maximum 28h after preparation).

For flow cytometry analysis, see SOP M

8.12. SOP L *In vitro* neutrophil stimulation in 12-well plates (visits 2 and 5)

Equipment/ reagents required:

Nitrile gloves	Microbiological Safety Cabinet MSCII
Dedicated Lab coat	Blood-isolated neutrophils ($9x10^6$)
Clinical waste bags & autoclave tape (or as per local procedures)	Sample labels
50ml Falcon tubes	RIPA buffer (10X stock; Abcam #ab156034)
SDS	Sterile water
6x 1.5 ml LoBind Eppendorfs , lysate labels, and lysate storage box	Eppendorf/lysate box
8x FluidX tubes, supernatant sample labels, and FluidX SPN box	TNF-Alpha 10 µg/ml 100X stock (R&D; 210-TA-020/CF; stock in PBS)
RPMI media (Fisher sci; 10363083) with 10mM HEPES and 10% HI low-endotoxin FBS (Thermo; A4766801)	LPS 0.5 mg/ml stock 100X (Merck; L4391-1MG)
Protease Inhibitor Cocktail (Merck; P8340-1ML)	PhosSTOP phosphatase inhibitor (100X stock)
PBS	PMSF 100mM/100X (Thermo; 36978)
12-well sterile tissue culture plate	Ice (and ice box)

Preparation notes:

RPMI media preparation: Add 5ml thawed (from -20°C freezer) heat-inactivated FBS to 0.5ml HEPES and 44.5ml Phenol-red-free RPMI. Keep any excess in the fridge and use within 1 month. Warm to room temp for use ~30 min before required.

On the day of use for RIPA: Aliquot 1.47 ml of 1XRIPA/SDS from the fridge into a 1.5ml Eppendorf tube, add 15 µl of PMSF (1:100; 20ul aliquots stored at -80°C), 15 µl protease inhibitor cocktail (1:100; 20ul aliquots of original stock stored at -20°C), and 15 µl PhosSTOP (1:100; 20ul aliquots of original stock stored at -20°C). Do not re-use solution once inhibitors have been added.

Procedure:

1. Add 690 µl RPMI/10mMHEPES/10%FBS media to **8** wells of a 12-well plate.
Add 300 µl of isolated neutrophils in DPBS (1.5 million cells since cells are at $5x10^6$/ml after isolation) to **6** of these wells (shown in bold on the plate layout).

2. Incubate the plate for 60 min at 37°C, 5% CO_2 to allow cells to settle and adhere to wells.
3. In duplicate, to each of 2 wells with neutrophils per condition add:
    10 µl of sterile PBS (vehicle)

10 µl of 100X TNF

10 µl of 100X LPS

Also add 10 µl PBS (vehicle) to the two wells containing media only (no cells), as per the scheme below.

Plate layout:

	Column 1	Column 2	Column 3	Column 4
Row A	**Cells + media + PBS**	**Cells + media + PBS**	Media + PBS (no cells)	Media + PBS (no cells)
Row B	**Cells + media + TNF**	**Cells + media + TNF**		
Row C	**Cells + media + LPS**	**Cells + media + LPS**		

Bold text indicates wells to which neutrophils should be added. Non-bold text indicates no cells.

4. Incubate the plate for a further 30 min, 37°C, 5% CO_2 to allow cell stimulation to occur.
5. Remove plate from incubator, collect 800 µl of supernatant per well into pre-labelled 1.5ml FluidX tubes tube for each well.
6. Wash cells in the plate very gently by adding and then gently removing 1 ml of cold (ice-chilled) DPBS to each well. Pipette at the sides of the wells to prevent scratching adherent cells.
7. When DPBS has been added to all 6 cell-containing wells in bold text in the plate plan above (do not process the cell-free control well any further as no lysate can be obtained), then start to gently remove and discard the supernatant from each well sequentially, discarding all the liquid from the well.
8. After the first well supernatant has been removed, add 200 µl complete, cold complete RIPA buffer to that well, before proceeding to remove all DPBS supernatant from the next well and adding RIPA buffer. Repeat the process until all wells have been washed and have RIPA added. Ensure cells are not left dry for more than ~10 sec.
9. Bend the end of a p1000 tip by pushing it down into an empty well of the plate, and use the flattened end to scrape the wells to detach the cells (scrape 5x diagonally in one direction, then repeat in the opposite direction 5x, then scrape 5x in a circle around the well including the edges).
10. Use the tip with a p1000 pipette to collect the cell lysate into 1x pre-labelled Eppendorf tube per well.
11. Repeat with a new tip for each well until all lysates are collected.
12. Incubate the cell lysate in Eppendorf tubes on ice for 30 min to allow lysis to occur.
13. Meanwhile, store and log the supernatant samples in FluidX boxes.
14. After 30 min, store the Eppendorf tubes in the -80°C freezer and complete the log.

8.13. SOP M Flow cytometry analysis of ROS and phagocytosis assay samples (visits 2 and 5)

Equipment/ reagents required:

Nitrile gloves	8 x prepared samples in 5ml flow cytometry tubes
Dedicated lab coat	Trypan blue
P1000 pipette and tips	4x extra 5ml flow cytometry tubes
Biolegend Rainbow Calibration Particles, 8 peaks (3.0-3.4 µm; # 18631311). 5ml (stored at 4°C)	2% BSA/DPBS
Invitrogen™ AccuCheck™ ERF Reference Particles, 3 peaks (#17166388) (stored at 4°C)	AccuCount Blank Particles **(for initial set up only)** (stored at 4°C)

Notes:

See appendix 2 for flow cytometry plot set up.

Flow cytometer must be regularly serviced and maintained, in addition to appropriate in-date calibration performed.

Rainbow calibration beads provided for AIR-NET are utilised in each experiment to generate an 8-peak standard-curve based on fluorescence values to normalise the final sample across all participating trial sites and machines. AccuCheck beads are used as an assay control to determine variability.

The beads provided by Tayside site for this assay should not be used locally to calibrate the flow cytometer.

Run all samples on the "low" speed setting to avoid misidentification of single cells.

Follow local SOPs for flow cytometer use, including best practises such utilising a 30 mi warm-up time for the machine to allow laser and delivery system set up before use.

Procedure:

1. **Ensure flow cytometer has received daily calibration (e.g. CS&T beads run and passed). If not, run this before beginning any further analysis.**
2. Prepare the 5x new 5ml tubes by initially adding 1 ml 2% BSA/DPBS per tube. Label these as 1, 2, 3, 4, and 13.
3. Generate a new experiment in the flow cytometry software utilising the template generated in Appendix 2.
4. Name the new experiment as the sample ID (NET-site-participantID-visit) and the date of the sample run (dd.mm.yyyy).
5. Ensure there are empty sample "tubes" within the experiment for each of the following, and add the sample ID to the name:

1. NET-site-participantID-visit 8-peak beads A
2. NET-site-participantID-visit 8-peak beads B
3. NET-site-participantID-visit 3-peak beads A
4. NET-site-participantID-visit 3-peak beads B
5. NET-site-participantID-visit unlabelled control A
6. NET-site-participantID-visit unlabelled control B
7. NET-site-participantID-visit FITC-PAO1 A
8. NET-site-participantID-visit FITC-PAO1 B
9. NET-site-participantID-visit CellROX unstimulated A
10. NET-site-participantID-visit CellROX unstimulated B
11. NET-site-participantID-visit CellROX STIMULATED A
12. NET-site-participantID-visit CellROX STIMULATED B
13. NET-site-participantID-visit 2% BSA/DPBS only

6. Once the flow cytometer and experiment have been set up and samples are ready to be run, vortex the **Biolegend 8-peak beads** for 10 seconds, then add <u>three drops</u> to one of these beads to tube 1.
7. Vortex the new solution for 5 seconds. <u>Protect all samples including beads from direct light throughout to avoid photobleaching.</u>
8. Immediately acquire and then record the sample in the flow cytometer. Set the stopping gate to 25000 beads.
9. Once the sample has almost completed running, vortex the **8-peak bead** stock tube again for 10 seconds, and repeat steps 8 to 8 to analyse this duplicate tube.
10. Repeat this process for the **AccuCheck 3-peak beads**, in duplicate, this time adding just <u>one drop</u> of bead solution to 1ml BSA/DPBS.
11. Make up the bead solution freshly just before running each sample to ensure that bead aggregates are avoided.

Examples of the bead plots are shown in appendix 2. Ensure 8 or 3 bead populations can be observed as applicable in the APC vs. FITC dot-plot.

12. After the 4x bead tubes have been run, begin to run the neutrophil samples, in the order shown above starting from the unlabelled control sample.

13. <u>Do not vortex any of the cell samples</u>, but resuspend by gently flicking/tapping the tube approx. 5 times just before running.

14. For samples 5-8 only, just before running, add 100 µl trypan blue to the 5ml flow cytometry tube to quench membrane fluorescence. *Note: trypan must be added <u>immediately</u> before the running for each tube individually. As cells are fixed they may be more permeable and trypan blue may enter the cell over time, affecting the signal of internalised fluorescent bacteria.*

Make a note in the lab book of any deviations, for example, if there are not enough cells to complete the run, if any sample is lost, any notes about the flow cytometer (for example slow/unexpectedly fast running of the sample, any errors).

15. Acquire and then record the sample as above, this time setting 15000 single cells as the stopping gate.

16. Run tubes 9-12 as normal, without trypan blue.

Examples of the cell plots for each assay are shown in appendix 2.

17. Once all samples have been run, finally run the cell-free buffer-only tube (tube 13) to determine background events. For this, keep the stopping gate set for the cells, but run the sample for 60 seconds, then manually stop the run. *As there are no cells or beads in the sample, the run will not stop automatically.*

18. Export the fcs files for all tubes in the experiment and the batch analysis file (i.e. pdf file) for all tubes onto the computer or a secure storage device. Suggested file naming is "AIR-NET" for all files and a sub-folder names as the sample ID and date.

19. Also upload all files into a secure cloud-based storage system to ensure the files are saved in two locations and backed-up.