PATROLS
Advanced Tools for NanoSafety Testing

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Annex 3206

PATROLS Standard Operating Procedures (SOP) Guidance Document for the Deposition of dry powders for Characterisation Studies and *in vitro* Co-Culture lung model Exposure at the Air-Liquid Interface Using the VITROCELL® Dry Powder System

SOP_PATROLS_3206 Vitrocell Dry Powder

This is a SOP recommended for external use by PATROLS

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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1.0	May 2020	Draft Document	David Brown
1.1	July 2020	Draft Document Formatted	David Brown; Vicki Stone
1.2	August 2020	Completion of document	David Brown; Vicki Stone; Barbara Rothen-Rutishauser

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1 Introduction

An alternative to the aerosol mediated exposure is the VITROCELL® Dry Powder Exposure System (https://www.vitrocell.com/inhalation-toxicology/exposure-systems/vitrocell-powder-chamber). It allows deposition of dry powders uniformly and in a dose-controlled manner and uses small quantities of material (typically 20mg) per exposure run. This allows, experimentally, to mimic a common and inevitable form of human ENM exposure, particularly within the occupational exposure scenario. This SOP is intended to provide guidance on the exposure of lung epithelial cells either in monoculture or in combination with other cell types cultured at the air-liquid interface (ALI). The system can also be used for the deposition of particles alone for characterisation studies. The ALI exposure scenario is considered to be more relevant than traditional submerged culture systems. The system is equipped with quartz crystal microbalances (QCM) allowing powder deposition to be measured in real time.To test the system, Calu-3 cells were cultured and set up on 3.0 µm filters and ALI conditions created according to SOP "Guidance Document for cell culture of lung epithelial cell-line" (Annex 4 in Deliverable 3.1). The Vitrocell Dry Powder System was assembled and optimised for exposure, ensuring that temperature settings and microbalances were stable.

1.1 Scope of the protocol

This SOP was created to be used by participants involved in the project PATROLS. This SOP provides instructions on how to culture the cells, deposit the particles using the VITROCELL® Dry Powder System, calculate the deposited dose of particles and how to disassemble and assemble the system for cleaning..

1.2 Limitations of the protocol

The VITROCELL® Dry Powder System must be completely dry and all of the particles used for exposure should be stored in a desiccator. The system is enclosed in a fume hood (not switched on during the particle exposures as this interferes with the operation of the QMCs) maintaining a temperature of 21ºC and an atmosphere of 20% RH (CHECK). The system is not suitable for particles suspended in liquid.

Terms and Definitions

2.1 Nanomaterial

Material with any external dimension in the *nanoscale* or having internal structure or surface structure in the nanoscale.
Note 1 to entry: This generic term is inclusive of *nano-object* and *nanostructured material*.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.4]

2.2 Particle

Minute piece of matter with defined physical boundaries.
Note 1 to entry: A physical boundary can also be described as an interface.
Note 2 to entry: A particle can move as a unit.
Note 3 to entry: This general particle definition applies to *nano-objects*.
[SOURCE: ISO 26824:2013, 1.1]

2.3 Substance

Single chemical element or compound, or a complex structure of compounds.

Principle of the Method

This SOP aims to provide a comprehensive overview of all ALI exposure of Calu-3 and macrophages using the VITROCELL® Dry Powder system.
This protocol will be broken into key stages:

1. Preparing the device, particle exposure and cleaning
2. Read-out measuring

Description of the Method

1.2 Test system used

This SOP should be carried out under controlled laboratory based conditions, with all work following safe handling of ENMs, particles and chemicals. The system is set up in an enclosed fume hood which houses all of the exposure equipment. When handling dry powders to load the exposure chamber, face masks are used. Workers have previously been monitored with personal particle monitors when handling powders and no particle excursions were detected. When an exposure run has been completed, the fume hood is switched on for 15 minutes to ensure that any residual particles are removed. The exposure equipment is also wiped down using alcohol swabs. In addition, cell culturing should be performed under sterile conditions and in a Laminar Tissue Culture Hood.

- VITROCELL® Dry Powder system (VITROCELL®, Germany) equipped with Quartz crystal microbalance (QCM)

- This protocol referes to the use of 12-well format inserts. Only four exposure ports are available on this model.

- For further information: https://www.vitrocell.com/inhalation-toxicology/exposure-systems/vitrocell-powder-chamber

Preparing the device, deposition and cleaning

5.1 System components

The system consists of five main sections

A. Particle release chamber which is loaded with the material for exposure.
B. Sedimentation tubes which are loaded with material for deposition. Tubes of different lengths can be changed to allow different deposition patterns.
C. Exposure tray (base plate) which houses metallic cups to accommodate transwell inserts containing cells cultured at the ALI and microbalances to monitor the deposition over the exposure period.
D. Controller unit which controls different parameters of the deposition process, e.g. flow rate, sedimentation time and exposure time.
E. Computer (not shown) and pre-installed software to collect deposition data from the microbalances.

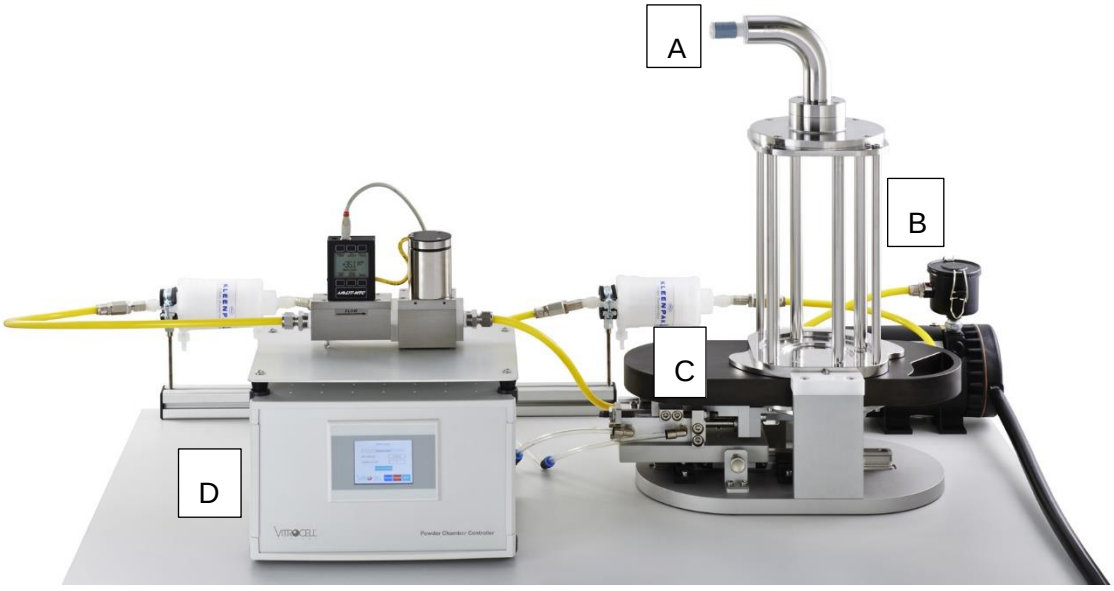


Figure 1. VitroCell Dry Powder exposure system as supplied by the company. Note that in subsequent parts of this document, modifications have been made to this system to optimise the powder deposition.

5.2 Setting up the system

Set up and optimise the Vitrocell Dry Powder system 24 hours prior to the start of exposure (see figures for each step of the assembly process).

Before use ensure that the exposure equipment is clean and dry from previous exposure runs (see appendix for cleaning protocol).

5.3 Microbalance setup (This procedure should be carried out for each particle type as deposition times may vary)

- Switch on the computer and open the Vitrocell software to display the microbalance screen. Four locations for the microbalances are displayed; the values displayed are in red indicating that the microbalance for each position are not yet inserted.
- Using the microbalance locating tool, secure a microbalance by fitting the locating pins of the tool into the location holes on the top surface of the microbalance.

Figure 2 Microbalance assembly (left) and extraction tool (right) showing location holes (arrow) on the microbalance.

- Carefully insert the microbalance into one of the empty locations on the base plate

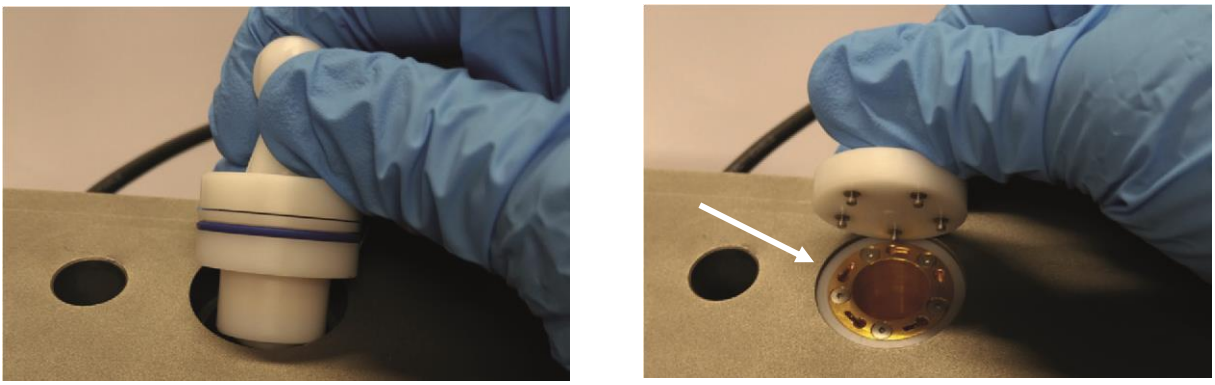


Figure 3 Location of microbalance into the holder in the base plate (indicated by the arrow).

- Each microbalance location in the base plate is labelled with a colour code which corresponds with the colours displayed on the computer screen. For each microbalance, align the side pin on the microbalance with the notch inside the base plate in order to ensure the microbalance is correctly located. Push the microbalance into this location and twist clockwise until resistance is felt.

- At this point, the display for that location on the computer screen should change to black indicating that the microbalance is correctly connected and in place. The microbalance is released from the locating tool by turning the tool anticlockwise (the microbalance is firmly located in the housing and this action will not remove it).
- Repeat this process for all four microbalances.

Figure 4 Baseplate showing all four microbalances in place.

- Assemble the sedimentation tube apparatus on the base plate using the locating pins to correctly secure the complete system.
- Slide the base plate into the pre-exposure position and lock in place.
- Connect the water supply from both circulating water baths to the base plate and to the sedimentation tubes. The water bath temperatures should be set to 40C (base plate) and 48C (sedimentation tubes).
- Set the control unit to the following parameters:
  - Flow rate 30 litres/min
  - Sedimentation time 0 seconds
  - Filling time 1 minute 30 seconds
  - Exposition time 25 minutes

NB These parameters have been selected after many exposures using different particle types and exposure times. Similarly, the filling time and flow rate were optimised for the **particle types** we regularly use for exposures. In the Patrols study, the particles investigated were zinc oxide (ZnO), quartz (DQ12), cerium dioxide (CeO_2) and titanium dioxide (TiO_2.)

- Zero the microbalances by clicking on the 'zero all' button at the top right hand side of the computer screen. Monitor the graphical output until the traces are stable (this may take several hours). Stability is not reached until the temperature in the sedimentation tubes and base plate housing the microbalances has been maintained for several hours or ideally, overnight.

Note: if several exposures are to be made over a prolonged time period, it is more convenient to leave the water continually circulating rather than switching the system off.

- Carry out a 'dry run' exposure to check stability of the microbalances. With the powder chamber empty and using the exposure settings outlined above on the Vitrocell controller, start a 25 minute run (the run does not need to be recorded on the computer). If the traces from each of the microbalances rise significantly over the run, repeat the process. The traces will settle after two or three 'air' exposures. (image of traces to be added)

<u>For the following section, please use appropriate precautions to prevent exposure to laboratory workers of the powder to be studied.</u>

- Select the particle type for exposure and open the powder exposure chamber (unscrew the cone-shaped lid at the top of the apparatus) and placed a rounded shaped amount of material on the central disc. This is approximately 30mgdepending on the particle type.

- Replace the powder chamber lid and set the computer to record the particle deposition over the 25 minute exposure period. Start the exposure from the Vitrocell controller unit.

The flow pump will start to fill the sedimentation tubes (1 minute 30 seconds), after which the pump will switch off, the base plate will automatically slide into the exposure position and the deposition will commence. The deposition can be monitored by observing the traces on the computer screen.

N.B. It is advisable to allow the exposure to continue for 2 minutes and take this as the starting point for the start of deposition. This is to allow the microbalances to recover from the movement of the base plate after it repositions at the start of the exposure. It also allows normalisation to zero so that the exposures monitored from each microbalance start from the same point.

After the 25 minute deposition period, the system will terminate the exposure, the base plate will automatically slide into the pre-exposure position and the powder exposure chamber can be removed for cleaning (if necessary).

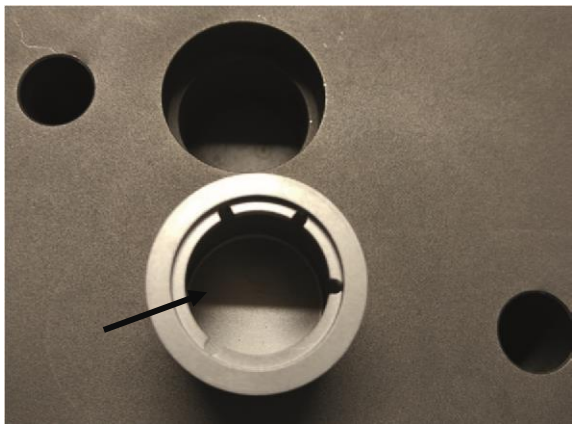
At this point the dose range of the deposited particles from each microbalance can be determined (see below) and the exposure of cells can commence.

5.4 Exposure of cells

After cells have been cultured on inserts and ALI conditions established (Patrols SOP for Calu-3 cell culture), they can then be placed into the system for particle exposure.

- Remove two of the microbalances from the baseplate and clean the remaining two by using a clean cotton bud moistened with 100% ethanol. Carefully wipe the quartz crystal with ethanol and allow to dry.

- Sterilize two of the metal cups using 70% ethanol and allow to dry (in a tissue culture hood). When COMPLETELY dry (10-15 minutes), add 2.7mlcell



culture medium to each cup and place in the incubator (37^{0}C CO_2 humidified) for 25 minutes to allow to equilibrate.

Figure 5 Metal cup holder for inserts and corresponding locating position.

- Remove one of the inserts from the culture plate and place into the metal cup, ensuring that the inserts locate into the notches in the cup (arrow). Repeat with other inserts and maintain in the incubator until required. NB the volume of medium added to the exposure cups (2.7ml) is sufficient to maintain ALI conditions.

Prior to exposure, the traces from the particle only depositions made earlier (above) are re-examined to determine the range of deposition (from 4 microbalances). See below for determination of particle deposition masses.

- Refill the powder chamber with the dry powder for exposure.

- Insert the pre-warmed metal cups containing culture medium and insert/cells into the baseplate and slide the plate into the pre-exposure position. Allow the system to equilibrate for 10 minutes.

Figure 6 Base plate showing four microbalance positions replaced with four metal cups containing cell culture inserts.

- Using the exposure settings used to determine the dose range for this particle type, zero the microbalances by clicking on the ‘zero microbalances’ on the computer screen, start data recording and start the exposure. This set up

consists of two microbalances and two insert exposure chambers (but this can be changed to other combinations).

- As the run progresses, monitor the deposition using the traces provided the computer screen, to check that deposition is continuing within the range previously determined.

At the end of the exposure period, the system will automatically stop and the base plate will move back to the pre-exposure position.

- Remove the metal cups containing the inserts and return the inserts to the 12 well plate and return to the incubator for the desired incubation period (usually 24 hours).

- Incubate the inserts as before maintaining ALI conditions.

- At the end of the incubation period, remove the medium from the basolateral side (BL) of the well (BL side) and split into aliquots in eppendorf tubes and store at -80^{o}C until examination (toxicity and cytokine estimations).
- Fix the cells on the inserts using 4% formaldehyde in PBS (25 minutes4^{o}C), wash in PBS and store in PBS at 4^{o}C. The inserts can be stored indefinitely provided they do not dry out.

NB Other protocols for the inserts/cells may be used.

5.5 Deposition trace determinations

As indicated previously, the traces should be examined 2 minutes after deposition has started. This is to allow calibration (normalisation) so that deposition can be measured from zero. The measurement after 2 minutes is subtracted from each reading over the 25 minute exposure time and the final reading at this point taken as the deposition mass over this time. This is repeated for each microbalance and the range of exposures from the four microbalances is determined.

NB The ranges can differ considerably between each particle types, related to their physicochemical properties.

5.6 Modifications made to the original exposure system

During the period after which the original exposure system was installed, it was realised that the deposition of particles and the monitoring of the deposited particles was not functioning optimally. A series of visits from the Vitrocell team tried to address these problems.

The first modification involved changes to the design of the particle loading chamber. The original design consisted of a horizontal loading tube which was curved at a 90 degree angle which fed into the sedimentation tubes. The change to this configuration consisted of altering the loading chamber to a vertical position, so that the particles were loaded on to a flat surface and then deposited directly into the sedimentation tubes.

This change did not appear to significantly improve the deposition and monitoring of particles. A second modification consisted of constructing a system of circulating water around the sedimentation tubes. The rationale for this was to create a temperature difference between the base plate and the sedimentation tubes (40C and 48C respectively) and to improve the deposition mass. This is a recognised phenomenon related to the physics and density of particles. This intervention improved deposition only slightly, but an additional problem related to the microbalance function was evident. Instead of a steady increase of particle deposition over time (as demonstrated on the output traces), some of the outputs showed a downward trace.

The third modification to the system consisted of improving the inlet valves which allowed particles to fill the sedimentation tubes steadily. This involved construction of a completely different configuration to the originally supplied system (below).

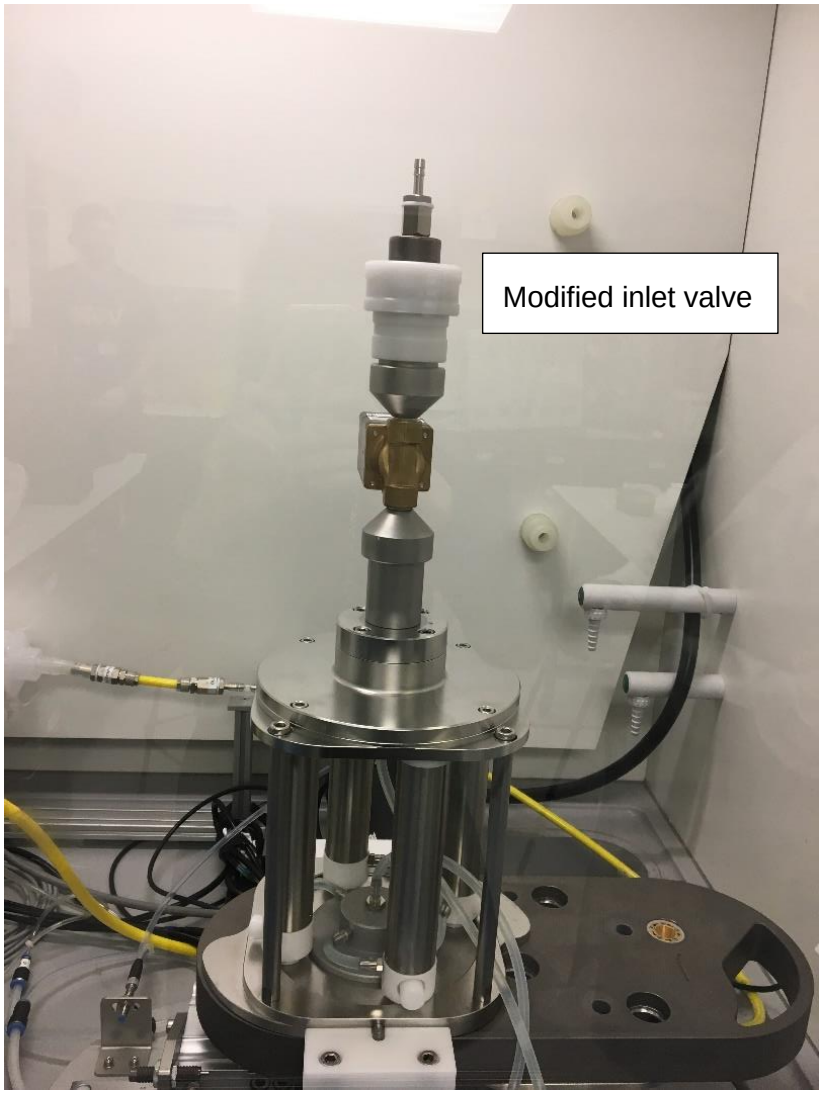


Figure 7 Final modification of the original exposure system. The modifications solved the issues related to deposition, however, to get the system into a usable format took more than 18 months from date of delivery.

Quality control & acceptance criteria

The deposited particles should be checked using TEM after trial exposures, before starting cell culture experiment, and then several times during the experiment.
Visual confirmation of Calu-3 and MDMs shape using light microscope.

Data Analysis and Reporting

Not applicable for this current SOP.

Other Considerations

The dry powder system requires a significant amount of operator training and the specific particle mass deposition can be problematic due to relatively high masses of particles being required. The variation in deposited masses can vary depending on environmental factors sush as temperature and humidity. Optimisation for each particle type requires that several dry runs be carried out in order that a dose range can be identified over a specific time period. This being the case, the behaviour of each particle type related to charge, size and physio-chemical properties means that the dose ranges can be different. There is no prior particle preparation required, the cell inserts are easy to manipulate within the system and cells survive well during the exposure period.

The system does, however, have some drawbacks, notably cleaning the system. Before using the apparatus for different particles, the whole set up must be dismantled and thoroughly cleaned (see cleaning protocol). This takes a considerable amount of time (around 1 hour) as all components and tubing must be completely dry. If any moisture remains in the system or components, this results in poor dispersion and deposition. Secondly, the system must be allowed to equilibrate to allow all of the components to heat up to the optimum temperature (microbalances and sedimentation tubes). As different particle types behave differently, preliminary runs with new material must be carried out to determine the likely dose range over time. This can be time consuming before the actual cell exposure can take place.

9 Publications

Not applicable for this current SOP.

10 References

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***Other useful sources**:*

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APPENDIX

Exposure system cleaning

Materials required:

Absolute ethanol, cotton wool, cotton buds, screwdrivers, forceps, (see images).

Procedure:

Remove the housing containing the sedimentation tubes from the baseplate by disassembling the inlet valve, powder chamber and associated tubular connections. (Images to be added) From the top of the housing, remove the screws holding the impaction plate and connection for the inlet valve. Using a wad of cotton wool moistened with ethanol, insert into the base of one of the sedimentation tubes and push through the tube using a long-stemmed test-tube brush. Repeat three times using a clean wad of cotton wool and three times with a dry wad of cotton wool. Repeat the process for the other three sedimentation tubes.

Using a clean wad of cotton wool moistened with ethanol, clean the impaction plate and associated connecting tubes and 'O' rings (repeat three times) and dry with a clean wad of cotton wool. Reassemble the apparatus ensuring that the 'O' rings have been lightly lubricated using pump oil.

Microbalance Cleaning

Materials required:

Absolute ethanol, quartz crystal microbalances, cotton wool, cotton buds, micro screwdrivers, forceps, microbalance release tool.

Procedure:

Remove the screws around the top surface of the microbalance assembly. Using the release tool, twist and pull apart the plastic housing to reveal the internal platform where the quartz crystals are set. Remove the base platform and all associated 'O' rings. Using a cotton bud moistened with ethanol, carefully clean each component of the microbalance and set aside to dry. Reassemble the balance by first reinserting the brass baseplate into the bottom of the plastic housing.

The baseplate has a side pin which locates into a hole at the bottom of the housing. This procedure requires the use of forceps and by angling the baseplate, the pin easily locates into the hole and rests on the spring anchored at the bottom of the housing. The upper brass connecting piece is reassembled by placing a quartz crystal on the bottom surface and securing with an 'O' ring. This assembly is inserted into the bottom assembly (holding each at an angle) using the assembly tool and twisting so that the

screw holes are in alignment. Secure the whole assembly with the outer screws. Retain the cleaned microbalances in a container to exclude dust and moisture.

A video is currently in preparation outlining the ain processes for handling the equipment components.