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NKP isotec

Isolator User Guide

Basics for isolator
husbandry
planning
procedures
troubleshooting

Contents

Introduction

Without the input and support of several people this handbook would not have been possible. I want to give a special thank you to the TU Munich - Weihenstephan and especially Prof. Dr. Dirk Haller who gave me this opportunity and introduced me to the world of Gnotobiotics.
Another great help was Dr. Jorum Kirundi who taught me so much and was always helping me with lots of advise - I am incredibly grateful for all his insights.

I would also like to thank all my colleagues at the TUM who helped me achieve this!

This guide was originally written in 2015 for my collegues to have a manual of our procedures and to give some background information. Since then I have updated parts as I gained different insights and found alternative approaches through various people and facilites, since leaving my position as technician at the TUM.

I am very happy to be able to make this guide accessible to a wider public and hope it will help others to have a successful start when starting to work with isolators. Under no means is this a complete guide but inspired by my own struggles for information when I started out, I hope it provides a good basic knowledge and ideas to make isolator husbandry work.

It would be fantastic to have a guide which is even more comprehensive than this and if any of my audience is willing to share their knowledge with me to add to this guide I would be very happy to do so.

To leave comments suggestions or let me know if you do things differently, please feel free to contact me on:
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Working with isolators

General considerations

The decision to work with isolators is sometimes not an easy one. Often it is not as easy to get enough information when starting out new, which is improving constantly now, and hopefully this booklet will help as well. Usually the requirements of your research will determine the size and scope of your equipment. Isolators have the great advantage to be a very safe way to avoid contamination or to contain infectious material and minimise the human error, which could cause contaminations. The procedures are not too difficult to learn and allow you long lasting success. Even though it is a substantial investment, there is usually a solution to any budget. Ranging from rigid Isolators which are quite expensive to the flexible film as presented in this manual. There is now also the possibility to use bioexclusion cages in order to give more flexibility when it comes to experiments with a short duration or small cohorts.

To work with isolators requires in general good organization skills and attention to detail. The procedures described in this manual are based on personal experience with flexible film isolators from Harlan/NKP and are not meant to be sufficient in itself, but should be regarded as a guide when starting out to work with isolators for the first time. Different facilities may have different procedures, which are also good practices and work well. In the following, the procedures described, are focussing on flexible film Isolators used under positive pressure. Many of these principals also apply to isolators run at negative pressure, but care should be taken to be aware of the differences. Procedures for rigid Isolators are in principal similar, the main difference being the rigid body of the isolators and therefore the different port options and technical connections. It will also determine the

range of sterilisation methods you can use.

One of many important aspects of working with isolators is the awareness of sterile (protected) and non-sterile (non protected) areas. Gaining a good understanding where your animals or experiments may or may not be compromised is key and will help you to work accurately, efficiently and last but not least successfully. Only with your goals and limitations in mind will you be able to make the correct decisions, check your own work properly and avoid compromises which can lead to contamination and therefore a loss of weeks, months or sometimes even years.

Using an isolator provides you with a good and safe system, enabling you to use simple procedures by using the correct steriliser to achieve your goals. But sterility should never be taken for granted. It is of utmost importance to check the status of your isolator with every import and export. You need to be aware of the fact that every test only gives you the result of the last actions, and that every opening and closing of the isolator presents a new risk. This is the point where good organization comes into play, which not only reduces risks, but also minimises the work load.

Combining as many tasks as possible (e.g. taking samples, importing and exporting animals and material) all at the same time and organising your daily procedures around experimental planning the efforts are kept at a comfortable level. Other risk factors present themselves upon actually working on the isolator.

One major weakness are the gloves of the isolator. Handling material, mice and being exposed to aggressive sterilisation solutions on a regular basis make the gloves prone to tears and holes. This results means that the gloves are to be checked more often than any other part of the isolator: before and after every use.

I recommend additional protection by using separate sterile cotton gloves for each isolator. These should be used sterile and fresh every time work is undertaken in the isolator, or changed at least once a week. This depends on personal preference.

There are a few simple reasons for this:

- putting on the gloves of the isolator is a lot easier
- avoiding cross-contamination from one isolator to another
- providing an additional safety layer between the inside of the isolator and the operator

For other parts presenting a risk, see *Important checks / weak points of the isolator*.

The sterilizing agent used in this manual is the product “P3-oxonia active” (Ecolab). We used a dilution of this product (see *Dilution chart per acetic acid*). Per acetic acid is a very strong disinfectant which has not only very good sterilizing efficiency on direct contact but also as an aerosol, which makes it very effective and easy to use. On the downside it is very corrosive and a risk to personal health. Therefore protective clothing, including a full face mask has to be worn when used.

There are other sterilizing agents that can be used. Special care should be taken to select the right one in order to achieve the disinfection results you require. Every product has a different efficiency and incubation times which need to be adhered to.

One last point I want to say before starting to work with Isolators is possibly the time management. Just s many other types of animal husbandry, it is time consuming and this fact should not be underestimated. Every check needs time, every procedure requires planning and careful checking. Nothing should be done in a hurry, as this will more likely result in losing precious time due to errors made. Taking sufficient time and working accurately is important to make this a long lasting success.

Making mistakes and learning is also a big part of being successful. I hope this guide will help you and gives you insights in making your isolator work a success.

Best of luck!
Melanie

Principal of flexible Film Isolators

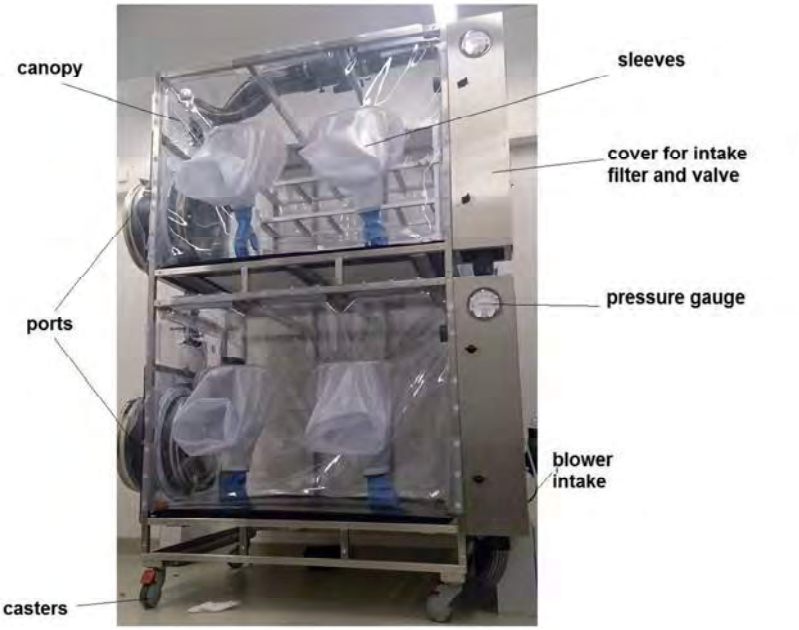
Isolators are used to protect either the contents from potential surrounding contaminants (positive pressure operation) or to keep contaminants confined to protect user or personnel (negative pressure operation).
One great advantage of an isolator is, that even in case of a power failure, the animals itself are not in danger of suffication. Due to the large size of an isolator it would take may hours for the air quality to diminish as far as to be a risk their health. If the isolator is connected to an alarm system usually the situation can be rectified within 12 to 24 hours and the animals will still be fine.

Examples for positive pressure operation:

- germ free
- SPF animals
- SOPF animals
- animals after embryo transfer or surgery
- certain microbiota or experiments which do not harm the user but need protection from contamination from the outside
- animals with an impaired immun system

Examples for negative pressure operation:

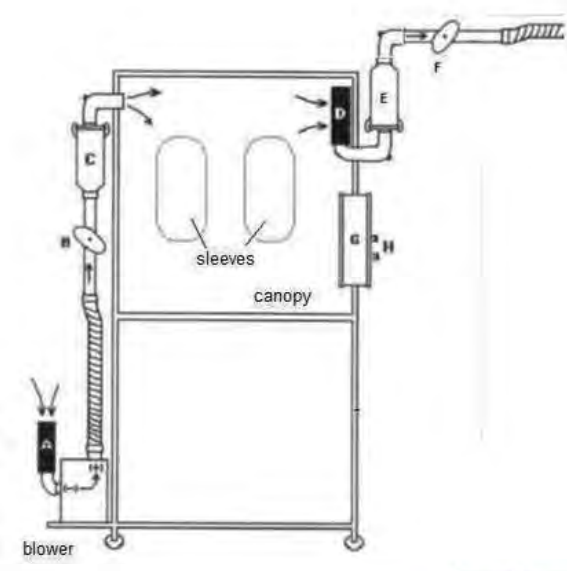
- quarantine
- S2 experiments
- humanisation experiments
- virus or other infectious applications



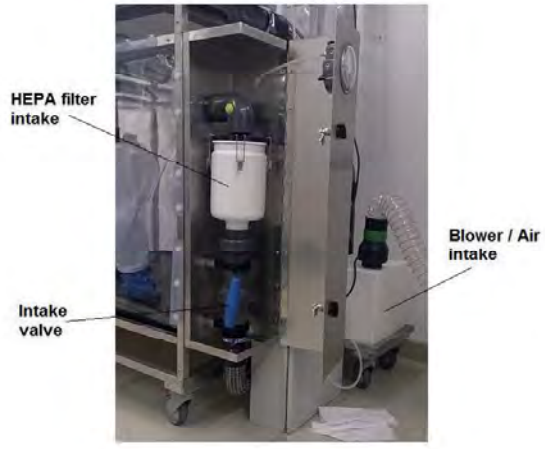
1. Positive Pressure

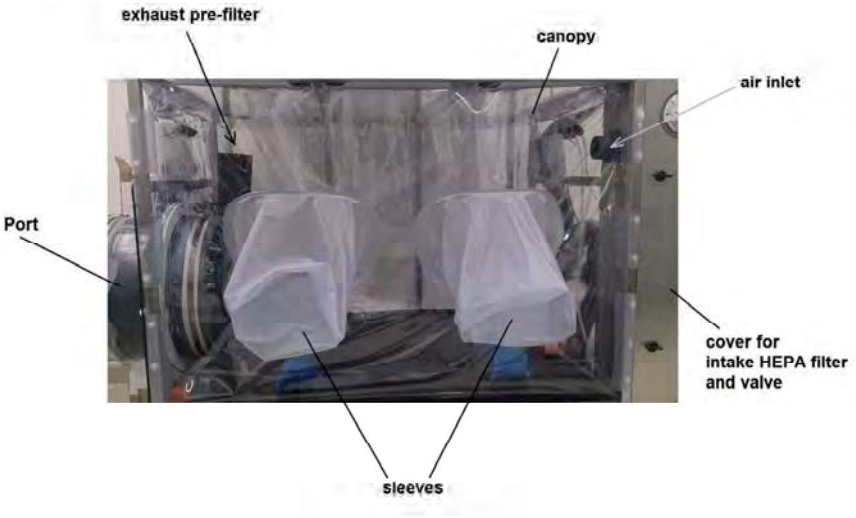
An isolator is used in the positive pressure mode, if the contents (animals) need to be protected from any contamination from the outside. The principal to achieve this is fairly simple. A Fan unit constantly supplies an enclosure made from PVC (the canopy) with air, resulting in positive pressure inside the isolator. In most cases, the air passes upon entry **and** exit of the isolator through HEPA filters, to ensure that only sterile air is able to enter the canopy. In the event of a small breach / hole in the canopy or one of the gloves, sterile air is still passing from the inside to the outside of the isolator, preventing pathogens from entering. If you stick to a good hygiene in the room itself it also reduces the risk of small damage to turn into a contamination. The pressure is regulated by adjusting the valves of the inlet and the exhaust (if applicable). A pressure reading of 80 to

100hPa is sufficient to operate the Isolator without stressing the seams too much. In the case of a power failure and therefore the loss of pressure in the Isolator the contents are still safe, as there are HEPA filters on the air inlet as well as the exhaust. *If you have an isolator where you don't have filters on both air connections, a power failure would be dramatic!* A breach in the canopy however, would present a problem at this point, as the air is not pushed outwards any more.



A – intake pre-filter
B – Air flow valve
C – HEPA filter inlet
D – exhaust pre-filter
E – HEPA filter exhaust
F – Air flow valve exhaust
G – Port





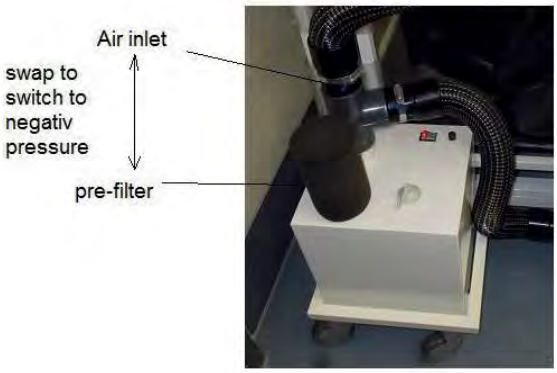
In principal the Isolator provides a good and simple system which makes it easy to control contamination. But the staff and careful working is a key point in spotting errors and working towards a high standard.

2. Negative pressure

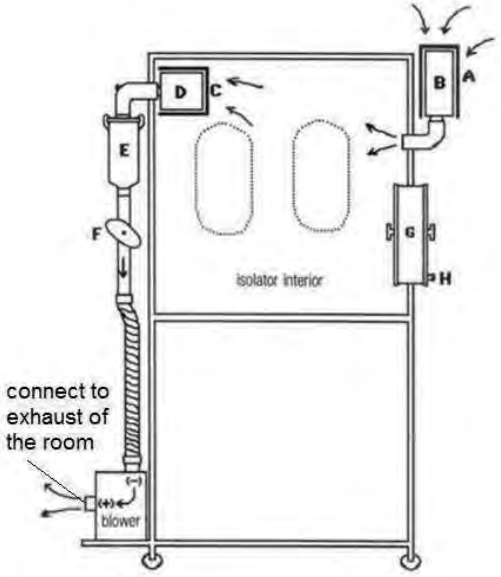
The negative pressure mode is necessary if there are any pathogens inside the isolator and exposure to the personnel needs to be avoided. Speak to your safety officer to determine which experiments are to be run in this mode.

It is more difficult to work in an isolator run at negative pressure. Space is limited, and the risk of damaging the canopy is greater - this is why some people prefer to use rigid isolators in negative pressure. If a breach does happen the air from the outside is sucked into the isolator and contaminates the contents with anything present in the air of the room. To work like this requires more careful planning and good observation of the integrity of the isolator itself.

To switch to a negative pressure mode, the tubing on the blower needs to be swapped so that the air is drawn from the isolator. To be able to adjust the pressure reading, the tubing on the pressure gauge will also need to be changed from high to “low”. The pressure is generally adjusted to a reading between 80 and 100hPa. The new Exhaust on the blower should be connected to the exhaust of the room to avoid the built-up of a smells from the isolator. The previous exhaust tubing serves now as inlet and needs to be fully open to achieve a good air flow. The pressure is regulated by adjusting the new exhaust valve only. Inside the Isolator the pre-filter is also changed to be fitted to the new exhaust.




remove tubing and place pre-filter this is now the air intake
remove pre-filter
if not removed, open valve completely to allow good air-intake
place pre-filter here


A
B
C
D
E
F
G
H
isolator interior
connect to exhaust of the room
blower

Setting up / first sterilisation

First steps:

1. cleaning
2. „leak test“
3. sterilization
4. ventilation
5. import of material
6. testing for sterility

Time schedule:

First day	cleaning, leak-test and first sterilisation
Second day	ventilation
third day	start with import of material

After the isolator is set up (with the last import of material) samples to test for sterility are taken. These samples need to incubate for at least one week before the first animals should be brought in.
Approximate time until first animal import is 2 weeks.

1. cleaning of the isolator

Material:

- tissues / wipes
- mild disinfectant (e.g. Pursept A, 3% Tegodor)
- possibly a mild soap solution

The interior has to be cleared of all material beforehand and cleaned / wiped through with a mild disinfectant in order to remove all traces of dirt and grime. If the dirt is persistent a mild soapy solution can be used. To

avoid residues the surfaces need to be dried off well with a towel. Especially soap or other residues from chemical need to be removed as this may impair sterilisation later on. It is not necessary to work in a sterile manner, but it is a good idea to gradually reduce the amount of germs present. During these procedures all weak areas of the isolator should be thoroughly checked (see also *Important checks / weak points of the isolator*) to make sure everything is in good working order.

Areas which should be checked:

- gloves
- sleeves
- welds / seams
- port-caps

The tubing connections and HEPA-Filters itself for both inlet and exhaust should also be checked in order to determine if they need cleaning or changing. As a general rule the filters should be changed every 1.5 to 2 years, the filters for the intake may want to be kept on a longer, but this is depending on the amount of dust and dirt present in the air of the room.
For the protocol to change the filters see *Change of HEPA-Filters*

After the initial cleaning make sure no soapy residues are left inside the isolator as they adhere to the surfaces and may inhibit your sterilisation solution to decontaminate all areas properly. Clean these off using sterile water and towels.

2. Leak Test of the system

A leak test is performed to establish if the canopy and all of its connections are gas tight. This test should be done prior to the first use and every time when the isolator is used for a new project or experiment. It is possible to perform this test even in use with animals inside when doing annual maintenance or if you would like to check everything is in working order.
Leak test is possible to perform in positive as well as negative pressure, and should be also possible with rigid isolators - please refer to your manufacturer if in doubt.

It is important that both bungs (size 45) are present inside the isolator to perform this test to close off the air connections.

Procedure

1. Visually examine all areas of the isolator canopy for obvious holes or tears, pay particular attention to welds, sleeves and gloves that should be repaired or replaced as necessary.
2. Make sure all connections are tight and closed.
3. Close the inner and outer port caps/doors.
4. Remove the elbow and exhaust pre-filter on the inside.
5. With the fan running and the airflow regulating valve open to allow the isolator to inflate (or deflate) place a bung into the exhaust tube

let pressure build up

connection. above maximal pressure on differential gauge (for both positive or negative pressure).

6. Remove first arm from Isolator and let the sleeve either expand or retract (depending on pos or neg pressure) as much as possible.
7. Place second bung into the inlet filter tube, keeping as much pressure inside the canopy when closing the inlet.

Close air inlet when fully inflated (or deflated in neg-pressure)

fix sleeves for test - nothing should touch or move the sleeves whilst testing

8. Remove you second arm from the isolator.
9. Make sure not there is no more movement on the canopy and nothing is touching any parts thereof, as this would alter the pressure reading.
10. Check the Pascal's pressure reading which has been maintained and note this down. The reading should be as high as possible.
11. Optional - Close the airflow regulating valve and turn off the fan unit.

pressure reading needs to be monitored.

12. Make a note of the pressure and the time. The leak test period is around 10 to 20 minutes.
13. At the end of the allocated time, no more than 10% of the pressure should be lost to deem as a PASS. If more than 10% of the pressure is lost then the isolator has FAILED.

It is usually easy to see if the pressure test is failing, as the pressure will continuously decrease once you removed your arms from the isolator.
If the isolator has failed the pressure leak test, first repeat the check for any obvious loose connections or openings. Give special attention to the tubing, filter connections, doors and seals. Also make sure the bungs are tight inside the tube when closing these. After re-tightening any loose connections try the test again. Should it still turn out unsuccessful, the following procedures will need to be followed:

Introduce 500ml of a soapy solution or sterilisation solution like 0.5% Tegodor into the isolator, increase pressure to maximum, then spray each of the following areas in turn until the leak is found:

a) Inlet and outlet filter attachments.
b) Gloves and sleeves
c) Entry port attachments.
d) All welds.
e) Isolator panels.

While spraying each area, look for bubbles being produced on the outside of the canopy. Once the leak has been found, repair as necessary and repeat the pressure test as described.
Please note that very old canopies (manufacturing date more than 5 years ago) may not hold the pressure very well any more due to natural degradation of the PVC material itself.

3. Sterilisation Procedures

3. 1 with atomizer - liquid steriliser

Assuming the isolator is new or has not been used before, it should be relatively clean. During delivery and construction dust and grit can enter the isolator therefore this must be removed prior to the sterilisation process - see earlier chapter *cleaning of the isolator*.

There are many methods of sterilisation for isolators, described below is a general description using a liquid sterilisation solution in conjunction with a compressor and atomising gun. Please check with your health and safety officer and general regulations which sterilisation method is the best one for your application.

PROTECTIVE CLOTHING / MASK MUST BE WORN

Preparation

Before sterilisation, a leak test should be performed to insure the integrity of the isolator and it's add on components, especially the gloves are intact.

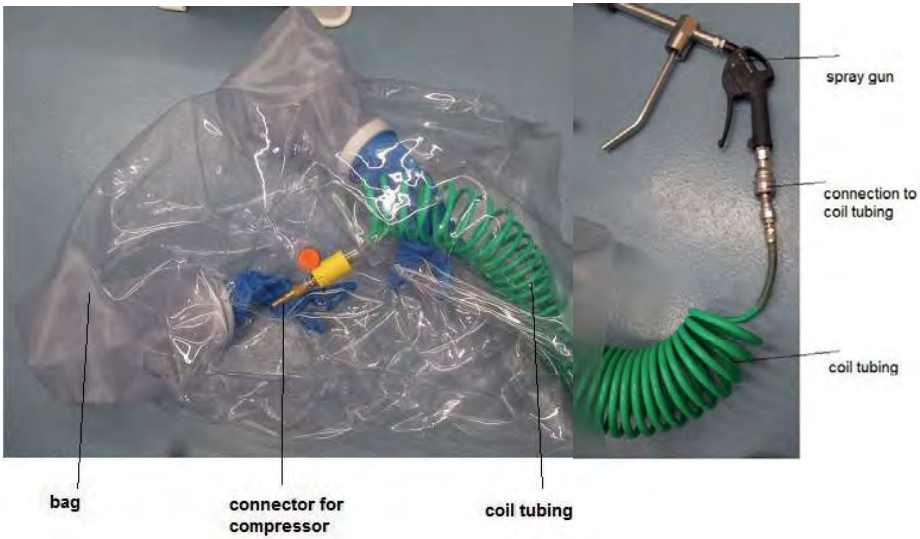
Material:

- 350ml 3% per-acetic acid (see *Dilution chart per acetic acid*)
- Full face mask
- Compressor
- Port-bag for “spraying in” with spray gun and coil-tubing
- Port clamps and rubber bands
- rubber bungs to close air connections
- Inner port cap / 2 rubber bands/ clamp inside the isolator
- Cage rack (if not autoclaved separately)
- Pr-filter exhaust and elbow
- Any Material needed inside the isolator that cannot be autoclaved (Water bottles, scales, etc..)

Pre-sterilisation

In order to stock the isolator as much as possible from the beginning and to reduce the amount of imports at a later stage it is possible to add equipment already at this stage. Any irradiated/autoclaved sundries which are possible to withstand the following sterilisation procedures are possible to introduce now. Please observe general import procedures to work as clean as possible, but to import completely sterile is not necessary at this stage. Gloves and protective clothing is to be worn when handling these goods and sprayed as their entry is made.

To avoid exposure or per-acetic acid fumes into the room itself, the Isolator needs to be closed, being able to use spray gun and per-acetic acid on the inside. To facilitate this, a port bag, coiled tubing and a modified rubber stopper is used to feed the connecting pieces through the spray nozzle of the bag. The tubing is connected to the compressor and spray-gun, which can now be used inside the isolator without exposing per-acetic acid to the room.



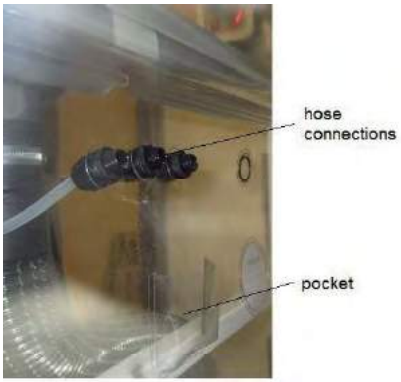
Sterilisation Procedure with atomiser

- Prepare at least 300ml of your sterilisation solution for sterilizing the inside of the isolator.
- Use the compressor and atomiser set ideally with 2 connecting tubing – one of them coiled.(*see picture*)
- Connect the coiled tubing to your atomiser gun and place inside the port/ Isolator together with the bottle of sterilisation solution.
- Feed the end of the tubing through the access nozzles of the port cap /door or port bag as described earlier, and connect to the second tubing which is connected to the compressor.
- Check the inner cap/ door is present inside the isolator.
- Fit the outer port cap/door/port bag to the entry port, and make sure they are securely attached.
- start spraying some of your solution into the air inlet and exhaust and close off securely using the rubber bungs

- spray all surfaces of the isolator carefully, starting with the ceiling and working from areas furthest away until covering the panels which help you see last.

- Special care should be taken to disinfect the hose connections, pockets on the wall, around the port, the sleeves and gloves

port with caps and rubber band inside



- Place the atomiser and tubing back into the port and close the inner cap/door.
- PROTECTIVE CLOTHING / MASK NEED TO BE WORN
- Remove the outer cap/door and remove atomiser and tubing from the outer cap/door.
- Close the Port and spray again through the nozzles and close them tightly.
- Leave for a period of 12 hours.

3.2. Fumigation

If you are able to fumigate the isolator there may be a few options:

- fumigation together with the entire room or chamber
- separate fumigation of the isolator inside only

The most widely used sporicides are chlorine (as in hypochlorite solutions or “bleach”) and formaldehyde, with some use being made of hydrogen peroxide and other oxidizing agents, or glutaraldehyde. At the concentrations necessary to be effective as sporicides, these are potentially hazardous to human health if handled incorrectly.

The difference between the options above are only that the isolator needs to be switched on and running if you are fumigating the entire room with the inner door of the port open, but the outer door closed. Fan unit switched on and Isolator in operation.
If only the inside of the isolator is to be fumigated the hosed to supply the gas need to be attached to the openings in the outer door and the air inlet and exhaust may need to be connected to allow circular gas movement inside the isolator. This will be achieved with the equipment for the fumigation and not with the fan unit supplied with the isolator.

PROTECTIVE CLOTHING / MASK MUST BE WORN

Preparation

Before sterilisation, a leak test should be performed to insure the integrity of the isolator and it's add on components, especially the gloves are intact.
For fumigation the surfaces must be clean an not covered. This means it is not possible to put a lot of material into the inside of the isolator as all surfaces need to be accessible to the gas.

Material:

- Fumigator
- Protective clothing
- tubing connection to set up for fumigation

I will not go into the detail as to the set up and exact protocol and duration as this should be available with the equipment you will use for fumigation and depends on the gas used.
To make sure the fumigation is successful you need to make sure all connections are tight and that the gas supplied into the isolator can reach all surfaces.

4. Ventilation

After 12 hours have expired the isolator can be switched on again and vented to dry off. This will remove the fumes of the sterilisation solution. As this process can be potentially harmful you can follow one of the procedures below to minimise exposure to the room and staff. You do need to check with your supplier if the sterilisation has to be neutralised before letting the gas vent into the general ventilation system.
Please consult your health and safety consultant if in doubt.

1. **Isolators connected to the room exhaust**
   no specific precautions need to be taken if the isolator is already directly connected to the room exhaust as the fumes will be drawn directly into the ventilation system without reaching into the room

2. **Isolators without connection to the room exhaust (only pre-filter present at the exhaust)**
   Fit a fume cover over the outside exhaust filter which is connected to a flexible hose and seal with tape. The hose should be long enough to reach a fume extract system or an open window.
3. **If the sterilisation method is not toxic**
   no specific precautions need to be taken

Procedure

- remove the stoppers first from the exhaust and then from the air inlet inside the isolator (*if you don't stick to this order pressure will built up and make the removal of the stopper from the exhaust difficult*)
- fit exhaust pre-filter and elbow on the exhaust tubing
- switch fan-unit on
- adjust air flow to desired pressure using the valves

After ventilation perform test procedures as you decide for your application.

5. Import of material / equipping the isolator

Before the first mice are imported into the isolator, all necessary equipment should be present. Even though the basic equipment is usually similar, an exact list of materials depends on the use of the isolator. A breeding isolator may require different material to certain experiments. To make sure nothing is missing when needed, the import of materials should be well planned for its specific purpose. You find examples how to stock a drum here in section *Stocking of a drum for import*.

As a general rule it is important not to over-fill a drum in order to let the steam circulate well during the sterilizing process. **It is mandatory to check every autoclaving cycle and every drum with a specific batch-control for this purpose**. This can be done using a bio-indicator or indicators which can be assessed immediately. You can also find a short list of different indicators in the appendix - but these are only suggestions and the list serves only to give you an idea what is out there.

To validate the autoclave cycle it is recommended to use a special validation equipment inside the autoclave drum whilst fully loaded and used in the autoclave. This test equipment is usually very expensive and due to this fact often not used - in this case you need to be aware of this risk and have to rely on experience.

Examples for validation logging equipment.

If a bio-indicator is used, an incubation period is necessary in order to validate the autoclaving process. This does not help if the information is needed at the time of import. Therefore it is necessary to use an indicator which can be evaluated straight away (chemical indicator- see also examples in the appendix). The one used here is MELAcontrol Plus from MELAG.

bioindicator
"sterikon plus"

MELAcontrol PRO

The indicator is placed inside the drum where it can be easily accessed but where steam penetration is difficult (e.g. inside cages, beakers, bags of food). After confirmation that the autoclaving process was successful the material can be imported into the isolator.

There are also two sets to test sterility placed inside one of the drums for the first imports. These are used to swap the inside and the material of the isolator as described Test for sterility on the following pages.

One set contains:

- 4 cotton swaps
- 4x2ml reaction tubes
- 2x5ml reaction tubes

in addition sterile water needs to be present in the isolator

6. Test for sterility

In order to take swaps for testing the sterility, the isolator needs to be dry and free from any remaining steriliser to avoid any accidental sterilizing of the swaps itself. Care needs to be taken that no sterilisation solution gets into contact with the swaps taken. (to avoid false negative results)
All material needed (the stocking of the Isolator), for the import of animals, should also be already inside the isolator, in order to assess the complete set-up of the isolator. Samples are taken by pre-wetting the cotton swaps with sterile water inside the isolator and wiping as many surfaces as possible.

The areas which are of special importance are:

- Inside the tubing for air intake and exhaust
- Black connector pieces for small / pressure tubing
- Pockets on the walls of the isolator
- The cage rack / corners, drilling holes
- gloves
- Bristles on the brush, cages, lids...
- around the inside of the port itself
- any other surfaces / walls of the isolator
- if food is present, soak some with water as a mould trap as well as taking a sample to examine in the lab
- Bedding mixed with water

The swaps are placed into the 2ml reaction tubes (break off the wooden stick to make it fit) food/water/bedding mixture is put into the 5ml reaction tube.***The samples should be processed on the same day as they have been taken***. Therefore it is necessary to take these whilst other material is either im- or exported. **Take special care to export the samples in a clean manner from the isolator - ideally in a sterile bag or container.**
It is possible to import already required growth media to start the "culture" inside the Isolator.

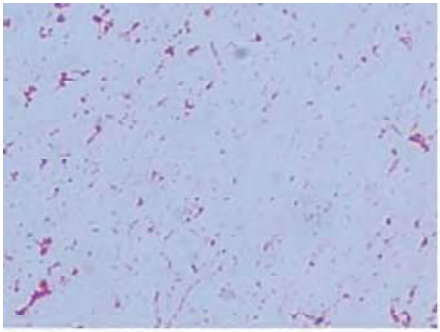
Sample processing:

Material:

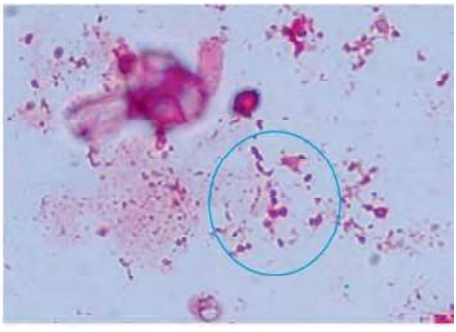
- Sterile working bench / laminar flow
- 3 plates of blood agar
- Sterile forceps
- LB broth (or TG broth)
- Slides for gram staining (label with pencil!)

Handle all samples tubes with special care to avoid false positive results which are caused by sample processing and handling only. Work at a very hight standard of sterility sterilising your gloves regularly.

The reaction tubes containing the swaps are opened in a laminar flow under sterile conditions and removed from the tubes, using the sterile forceps. The swaps are used to inoculate blood agar Petri-dishes. Then placed back into the reaction tube or growth media tube with a volume of at least 500µl broth.



Gram stain - germ free



Gram stain with bacteria

The tubes containing the food/bedding/water mixture are mixed very well and some of the supernatant used to perform a gram staining according to a general protocol.

All tubes and plates are put into a 37°C incubator and left there for at least one week to see if any bacteria is growing. The incubation under aerob conditions is sufficient for tests performed at every import and export, as it

is unlikely to contaminate the isolator with anaerobe cultures. But still the anaerobe cultures and test on the whole mouse should be done on a routine basis in certain intervals!
Mice should be only imported after the test result was negative.
This test is only a short protocol to test the isolator. My advice is to talk to a microbiologist and establish a protocol suitable for the individual requirements.
Testing the mice on a regular basis once they are inside the isolator is very important and should be done with every im- and export. In our experience a gram stain of several faeces samples from different cages proof to be a quick and reliable test, but require a lot of practise to be able to interpret the staining properly.

Husbandry

There are some differences in husbandry inside the isolator compared to other set-ups. I chose mice in this part, as this is the species mainly used and it is where I have gained my experience. For other species this will differ but general experience can be applied with some alterations. (If you have any experience with different species I would be very happy to add these to this manual if you are willing to share)

The main difference is the space available, restrictions in handling, and that all the material needed, has to be inside the isolator well in advance. Constraints of the isolator are the gloves, which makes the handling more difficult, the working positions and the equipment which can be used inside - especially when considering to use any sharps.
The advantage on the other hand is that once everything is inside, the health status is the same throughout. If planned well and imports are kept at a minimum the workload and considerations how to handle the separate

cages are no different to a conventional facility.

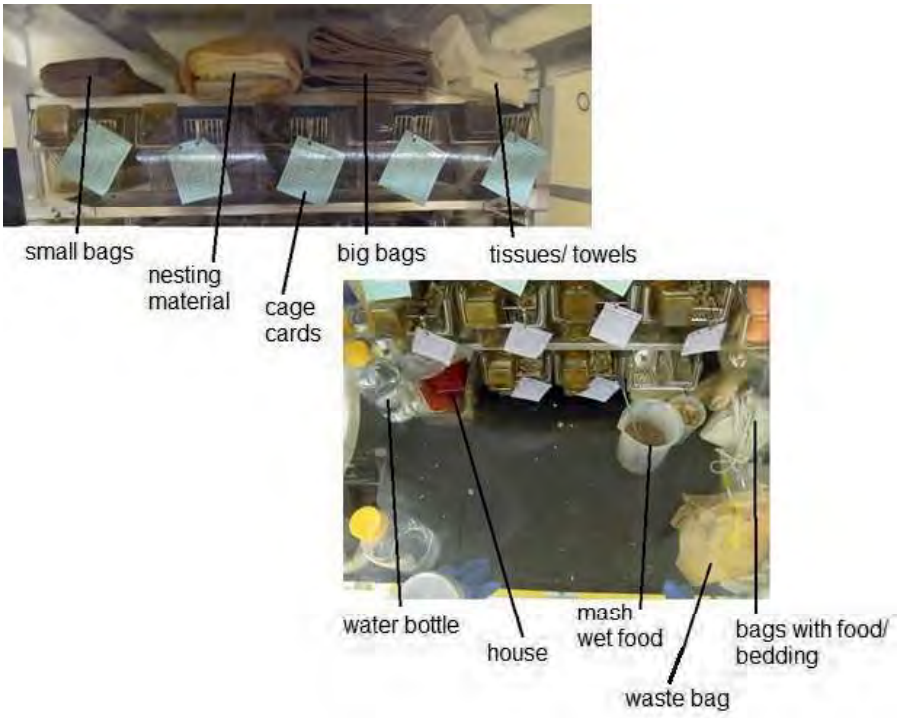
There are a few items that should be always present as they are used very frequently:

- Autoclaved cage cards (made from coloured paper)
- pencil (not varnished is better for autoclaving, two- if one breaks)
- towels (paper towels cause too much dust, it is better to use fabric towels like „Wipall X60“)
- big paper bags, or small autoclave bags for waste (size should be 32x45cm to fit a cage)
- small paper bags (for samples, dead mice etc.)
- tape
- scraper
- mouse handling forceps (to help grab anything, not just mice)
- brush (for cleaning)
- sufficient food, bedding, water
- reaction tubes

The main parts of husbandry:

- Cage change / bedding change
- weaning
- ear-tagging / marking
- weighing mice
- setting up breeding pairs

The procedures described are based on personal experience and should not be regarded as hard rules. They are suggestions and are there to provide help in order to breed mice in an isolator successfully. But as it is with any work with animals, it is advisable to familiarize yourself with the specific strains used and talk to animal caretakers with experience to achieve best results.



Cage / bedding change

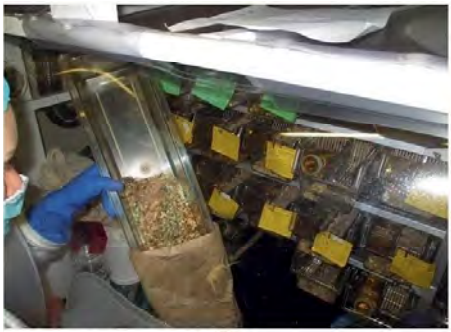
In contrast to most facilities with IVC systems or other cage systems, the cages inside an isolator are not replaced when the bedding needs changing. The amount of imports would be tremendous and it would be very difficult to be able to keep an appropriate hygiene standard, let alone germ free status. Also here the steps of bedding change, weaning, ear-marking can be combined to avoid having to go back to the same isolator later again and to save time.

This means that the cages stay inside the isolator for a long time. They are scraped out, wiped clean, filled with bedding and used again. All the material in the isolator should be sterile as tested with every export and it is

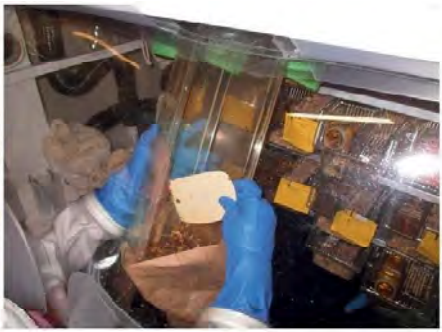
New cage prepared, house, mice, nesting material and a handful of old bedding transferred to new cage. Food and water just filled up

better to use up existing stock inside the isolator compared to new imports. This also reduces the waste, imports needed and workload.

Number and sex of the mice should be checked in a routinely fashion with every cage change.
Using an empty cage (or another container), the mice are "parked" there. The dirty cage is emptied into a paper bag, scraped and wiped and used again for the same mice. Food and water is merely filled up rather than replaced. The cage is then put back into it's position in the rack and the



dirty bedding is emptied into paper/waste bag



stuck on dirt scraped out

next cage is cleaned. This procedure has the positive side-effect of reducing male rival-fights as they are still in their familiar cage and do not need to re-establish their "ranking".

If a cage is particularly dirty it can be wiped out with water and a cloth.
This procedure can differ between strains and decisions should be based on experience to find out what works best. For some lines it may be possible to "rotate" the cages between the animals which can speed up the process.

Females and breeding pairs can be put together after sitting in different cages for weeks. Males from different cages can usually only put together at the point of weaning.

Weaning

The recommended weaning age is 21 days after birth. If the mice are particularly small it is possible to leave them with their mother for an extra week, but only if the female is not heavily pregnant again. After four weeks of age the males and females should be separated in order to avoid unwanted mating. To help the mice adjusting to their new cage, mash/ wet food can be placed inside their cage. This helps with adjusting from suckling to the normal pellet diet and I found it particularly useful for weaker strains. At germ free conditions, I had the wet food constantly prepared in the isolator as it served as a mold trap at the same time. There are different opinions about this and I would not advise this if you are keeping mice which actually have a microbiota.

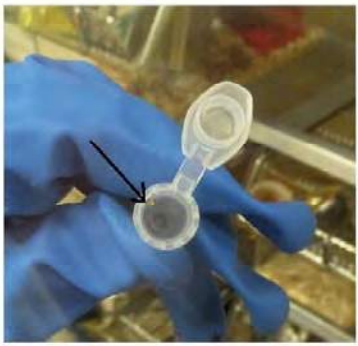
If more than one litter can be weaned at one point, the males and females of different litters can be put together to safe space (keeping in mind that males tend to fight - only do this if you know it works). The mice should be ear-tagged / punched at that point to distinguish them from one another.

There may be differences with different mouse strains and experience should be telling you what you can and cannot do. Once males start to fight is often very difficult to keep them together any more at all - this can become quickly a problem inside the isolator due to the space restrictions. This fact should be considered especially when gender specific experiments are planned.

Ear-tagging / marking

The ear-tagging should happen at the time of weaning in order to help with identification. It also helps to save space as males and females from different litters could be put together in one cage. It is a little bit more difficult than in a normal animal facility as the gloves of the isolator are don't allow the same dexterity as only latex gloves. But with some training and if you take your time it soon becomes routine.

The most important part is the fixation of the mouse. I recommend to use some spare Isolator-gloves and practise this with mice outside the isolator.



Biopsie for genotyping

Also the position of the ear-punch is quite important. Please see the schematic in the appendix (*ear punch schematic*) if you are using the same system. To space the holes slightly further apart makes the punching easier and also later on the identification (which helps also regardless of being punched inside or outside an isolator). If the mouse strain needs to be genotyped, the biopsies need to be taken as early as possible and exported on the same day. It should be planned to coincide with an import / export to avoid opening the isolator just for the biopsies.

Weighing mice / electrical equipment

For many experiments or lines which need monitoring due to health risks, it is required to record the weight gain/loss. To do this in an isolator is not a straight forward task. The greatest challenge is the sterilisation of the scales (or other electrical equipment) in a way to import it into the isolator without compromising its functionality. I would not recommend the import of electrical equipment into a germ free breeding isolator. Packaging and spraying or irradiation (or both) still presents a risk which is not worth taking (in my opinion. Losing the breeding colony will have an detrimental effect on all projects. For experimental purposes the risk is taken and either use irradiated balances or packaged ones can be used. It is also possible to have electric cables installed in an experimental isolator which gives you even more possibilities of the types of equipment that can be used. Special care has to be taken how to sterilise the installation and equipment itself. The easiest will be, if the equipment can be wiped with the sterilisation solution or if it can be irradiated.

What I decided to do is get some small scales and “shrink-wrap” those into small bags to enable us to spray them with per-acetic acid. The accuracy with this is not the best, but with practice it gives fairly reliable readings. A small tray inside the bag helps keep the plastic off the weighing surface when there is no beaker placed on top. With the blunt end of the pencil, the balance is switched on and set to “0” with the beaker on top.

Balance sealed in a plastic bag

The mouse is then picked up and dropped into the beaker. It requires some practise to do this without any mice escaping and to get good readings. But once established the measurements are not far off the ones we get outside of an isolator.

Some facilities use pescola scales on a chemistry stand.

use the blunt end of a pencil to set to "0"

the mouse is dropped in the beaker to get measurement

measurement taken

Setting up breeding pairs

Breeding pair are set up at the age of 7 to 8 weeks. The best breeding results are usually achieved with a “1 to 1” set-up (one male together with one female). If many litters are required at the same time (planning for experiments) it helps to set up as many breeding pairs as possible on the same day. Sometimes it helps to have some breeding pairs set up as a “1 to 2” (one male, two females) in order to get more mice initially. The pregnant females should be separated before they give birth in order to know which female the litter came from.

The planning of the breeding needs to be done very carefully, as space is restricted. The space inside the breeding isolator but also of the isolator receiving the animals and at which time point has to be taken into account. Getting it wrong puts you in a position where you have too many mice and no-where to put them, or you don’t have sufficient mice for planned experiments.

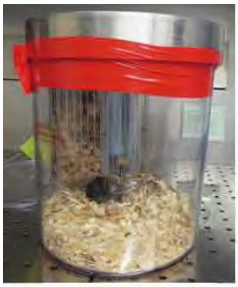
Good communication between scientist and animal caretaker is crucial in order to plan this well.
It helps to do this with someone who has a lot of experience with the strains used in order to get it right.

Import and export procedures

1. Preparation of the drums
2. Export
3. Transportation/ shipping of mice
4. Import

Even though you will normally start with an import, I am describing here first the export to give you an insight how the animals may be packed before they get to you.
There are different ways to packing the animals, depending on the transport "container" used. The most common one for germ-free may be the taconic shipper.
This shipper is connected via the sleeve - transfer sleeves to allow for the size difference between shipper- transfer sleeve and port.
The method in this manual uses a transport cage which is a sealed unit in itself.

Taconic Shipper



Gnotocage

1. Preparation of the drums - export

It needs to be determined, for which purpose the mice are exported from the Isolator:

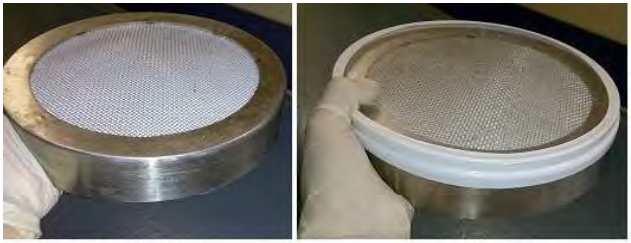
- Ongoing experiment
- import into another isolator
- Transport, sending them to another facility
- Termination of the experiment, used for sampling

The cages used here to export/transfer mice from the isolators are so called “gnotocages”. The amount of cages fitting into a drum depends on the size of the cages itself and the size of the drum. In this case 4 cages fit into one drum (45cm diameter 80cm long). The bases of these cages are made of polycarbonate and will deform if they are put under any physical strain during the autoclaving process. This could cause a loss of shape and may result in the lid not fitting on top of them any more. Therefore it is important to make sure they are put into the drum in an upright position, which will avoid this from happening.
If the mice are used to transfer them into a new isolator or shipping, the amount of mice which can be put into one gnotocage is determined by density regulations and the amount of air present in the cage itself. If mice are exported for termination / sampling, more animals can be put into one cage, as they won’t be kept for more than 24h.
It is important to consider the health status of the animals exported. Are they “clean” (germ free) or are they inoculated with pathogens (S2 experiments).

S2 Experiments

If S2 animals are exported, the lids of the gnotocages have to be taped with an additional plastic lids **prior to autoclaving**.
Exporting S2 organisms require an additional step of decontamination, which means that the cages have to be sprayed with per-acetic acid once

they left the isolator. In order avoid damage to the filter it needs to be protected. As the plastic lid is removed after the sterilizing period, the layer underneath has to be sterile as well. This means, that the lid has to be autoclaved fully prepared and cannot be taped shut inside the contaminated isolator (this would contaminate the area between plastic lid and filter and upon removal expose the user to the pathogens).



filter lid with taped plastic lid

To reduce the frequency of opening the isolator any other material which is needed anyway should be added to the drum for import, e.G. food, bedding, bags, pencils etc. (see Stocking of a drum for import)

Example of material needed:

- gnotocages / transport cages / shipper
- autoclave-batch-control
- Lids/ troughs / empty drink bottles if necessary
- Tape
- For shipping: small autoclave bags to fill later with mash (drink bottles cannot be used for shipping mice as they would leak and endanger the well-being of the mice, for sterility reasons we do not use ready-made gelatine packs)
- Pipette tip or other tool to be used to pierce the mylar foil
- For S2 animals: Filter lids have to be taped with additional plastic lid and a spare autoclave bag needs to be added to close the drum for sterilizing the sleeve at the time of export!!

Once all the material is put into the drum, it is taped closed with mylar film and PVC tape (see also Sealing the drum).
The fully prepared drums are covered by surgical cloths and sterilized.
Autoclaving program: 30min sterilization at 121°C, 20min drying time

Important: Filters are only effective if they are dry!! After the autoclaving cycle, the drum needs to be checked, if the filter has sufficiently dried off, especially around the bottom of the drum!
If the filter is still wet or damp, the drum cannot be used for import!!!

Once the drum is autoclaved it should be used within the next few days. Even though the filter keeps all the contents sterile it is not recommended to leave the drum standing around, being exposed to non sterile air for longer than necessary.

2. The export procedure

Material:

- 200ml steriliser
- Spray gun, compressor
- Face mask
- “blue” pipette tip (to pierce the mylar)
- Sleeve to connect drum to isolator
- Any material to be sprayed in (e.g. water bottles)
- Second person to help with import

The drum is taken to the relevant room and placed on a hoist or appropriate trolley which enables import into the isolator in question. Make sure the drum is placed onto a non-slip surface to avoid it from falling off the trolley.
Before the drum is connected to the sleeve / isolator some important aspects need to be checked:

Drum:

- Is the filter dry?
- Has the filter any tears or punctures
- Are there any creases holes or tears in the mylar?
- Is the tape showing deep creases on the rim of the drum which would be difficult to disinfect properly?
- have the control strips for autoclaving changed colour?

Sleeve:

- Are the gloves properly attached, are there any holes?
- Are the seams / welds still OK?
- Is the material in general in good shape or does it show signs of deterioration?

Isolator:

- Does the pressure gauge show the correct reading?
- Is the outer cap properly attached, showing signs of breakage?
- Is the inner cap attached properly?

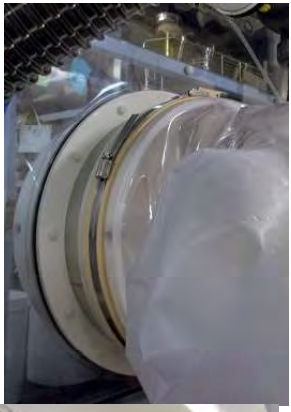
Only if no problems have been identified is the sleeve attached to the drum and secured with the appropriate rubber band and clamp. Making sure the sleeves (especially the gloves should be the correct way round) and the spray nozzles are in the correct position. The trolley with the drum is now placed in a way to enable an easy connection to the isolator.

Then the outer cap of the isolator is removed and the port sprayed in. Special care is to be taken around the rim of the tube to the inner port cap. The cap needs to be pushed in a little and the gap sprayed in carefully to ensure good decontamination.

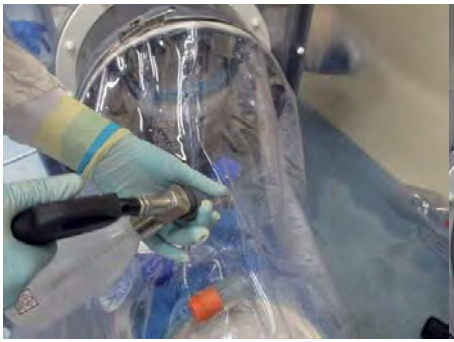
spraying the inside of the port

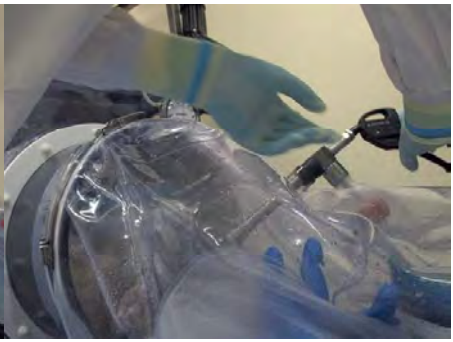
Special care is taken to spray the inner rim of the port cap. Push the cap inwards in order to reach right into the gap.

Any additional material (e.g. water bottles), which is needed inside the isolator can be sprayed in and placed into the port (don't forget the tool to pierce the mylar). Afterwards the sleeve is attached to the port itself and fastened by using a rubber band and clamp.



Once the sleeve is connected to drum and isolator, the surfaces inside the sleeve are sprayed in systematically, using the spray nozzles.





Using short bursts of the spray gun to provide a fine mist of per-acetic acid all surfaces are covered. Special care should be taken spraying the areas where the sleeve is connected to port and drum, as well as the gloves and sleeves. During the spraying in, the sleeve will inflate to some degree. To finish off, the spray gun is removed and the spray nozzles closed swiftly (caps sprayed in) in order to keep the saturated air inside and avoid a loss of pressure. The built up pressure should not decrease much during the incubation time. If it does, it suggests that the sleeve is losing air and has a hole or is not well enough attached to the drum or isolator. The leak needs to be found and corrected, before saturating the air inside the sleeve again. Incubation time will start again.

Incubation time 40min

Potential point of weakness:

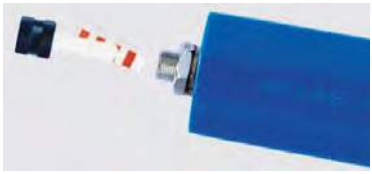
- Connection sleeve-drum
- Connection sleeve-isolator
- Welds on the sleeve
- Caps on the spray nozzles
- gloves

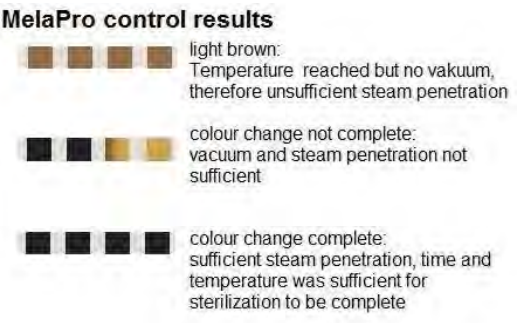
If everything is in working order after the incubation period, the material which was sprayed in and placed in the port and sleeve can be directly imported into the isolator. To do this, use the sterile cotton gloves of the isolator, do the glove check and remove the inner cap of the isolator. If two people are working together, one person can be working in the isolator whilst the other person is working at the sleeve. This makes the import faster and more efficient. All material is put inside the isolator and wiped dry, using one of the towels present in the isolator, to remove most of the per-acetic acid and reduce the exposure for the mice. The wet towel is placed into the port once all the material is inside and the inner port cap is

put back on the port and secured. (see *Mounting a port cap*)

The mylar of the drum is only pierced open with the blue pipette tip, once the inner door is closed!

The first thing to look for in the drum is the autoclaving-batch-control (see also *Import of material / equipping the isolator*).





If the result was satisfactory, the material inside the drum is safe to be imported into the isolator.

Care needs to be taken in order to make sure that the filters of the lids for the gnotocages stay dry!

The same principal as for the filter of the drum itself applies, only a filter which is dry is in working order!!!

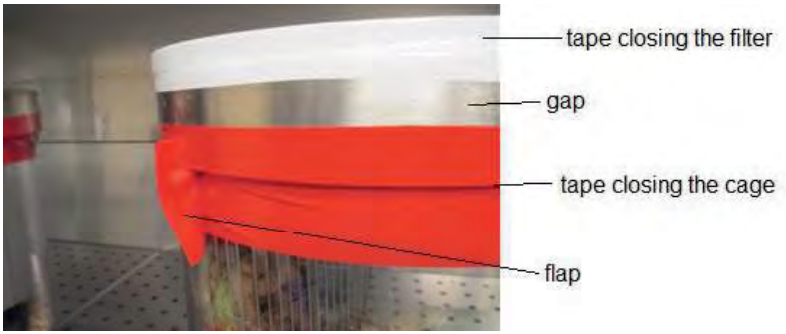
With all the material inside the isolator, the mice which need to be exported can be placed into the gnotocages.

If animals are packed to be shipped /stay inside the gnotocages for more than a few hours, the small autoclave bag needs to be filled with mash/wet food to ensure that they are getting enough liquids during the transfer period. The sides of the bag are folded back to make sure the animals can reach the content. The reason we did not to use ready-made gelatine/ water packs is unfortunately bad experience, the pre-sterilized goods presented

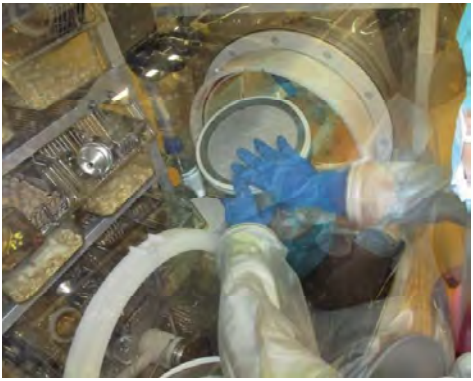
Gnotocage

a risk which can't be controlled and caused on occasion contamination.
The food and water inside the isolator on the other hand, has been tested and used for some time already and presents a much smaller risk for contamination.
Some dry food pellets and bedding material is added to the gnotocage to make the transfer less stressful. If there is a trough, it can be filled with food as well. Water bottles are used only if the cage is waiting for transfer in the same facility, during shipping a water bottle would leak and endanger the animal's welfare.

Once the mice are packed well, the cages can be closed, using autoclaved, PVC tape, leaving a flap on the side to make it easier to open the cage again. It is important to leave a gap at the top big enough to leave



space for the additional plastic lid (needed for the import).
Any material which is not needed any more can be removed from the isolator and placed inside the drum. The gnotocages can be left in the sleeve and the port, just making sure they don't tip over. Now the inner cap is put back on the port.



If the mice are exported from an isolator housing S2 animals (associated with pathogens) the drum has to be closed with a cap or an autoclave bag and the connection sterilized again.
Incubation time 20min

Face mask needs to be used!
Once everything is secured the sleeve can be carefully removed and the gnotocages taken out.

For S2 animals, remove the plastic lid to allow airflow and avoid suffocation of the animals - secure the autoclave bag properly onto the drum and autoclave to kill off any pathogens.

The port is now wiped clean and sprayed in again before the outer cap is put back on. Using the spray nozzles the inside of the port is sterilized again as a precaution to avoid any accidental contamination.

The sleeve is wiped clean and dried to remove residual per-acetic acid. Afterwards it is rinsed off with clear water as per-acetic acid causes the material to deteriorate faster if it is not removed.

For S2 experiments the supply drum needs to be decontaminated with an additional autoclave cycle before the contents can be emptied.

3. Transportation of mice

There are different possibilities to transport the animals in and out of the isolator: The challenge is in general to pack the animals so that a gas tight import/ export can take place, but also the option of a filter in place to allow air circulation without compromising sterility and the health of the animals.

One option are gnotocages. They have a filter lid which allows sterile gas exchange. For shipping they are placed in an additional container to avoid accidental damage and to ensure safe transportation. Shipped together with a clear plastic lid, they can be used directly for import, once they arrive at their destination.
Special care needs to be taken that the correct protocol is followed upon export and import to make sure everything stays safe.

Other shippers - like the taconic shipper - are more difficult to sterilise and assemble by yourself in an empty isolator. It consists of a plastic transfer sleeve which has filter incorporated. This sleeve then needs to be attached to the port for import. (*Pictures see page 40*)

For very quick exports completely gas tight beakers / containers can be used, keeping in mind that the air is restricted for a short period of time!

For large exports and shipping complete transport isolators can be used. This is much more time intensive and the transport is quite expensive. The faclility receiving such an transport isolator has to be compatible with the import connections. Often this is not possible due to hygiene restrictions upon entering the facility.

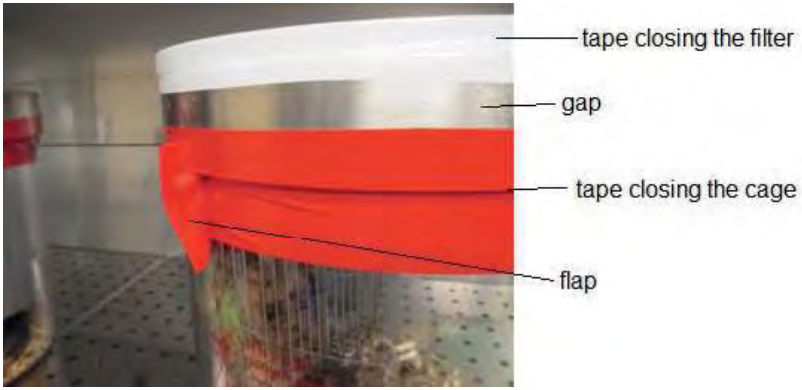
All standard rules and regulations apply when transporting these animals. Only licensed couriers who specialise for animal transport should be used.

4. Import of mice

Material needed:

- 200ml steriliser
- Spray gun / compressor
- Face mask
- Gnotocages, and plastic lids to close filter lid
- Port bag for import, or appropriate sleeve if a drum is imported at the same time
- Port stand, or other support stand to help with the import
- Pipette tip or other tool to pierce mylar if a drum is imported
- Timer, clock to keep track of time
- Second person to help with the import

The import procedure should always be done by two people. One person needs to hold the cages/materials whilst the other person can spray them in, without tipping anything over.
First of all, the sleeve and everything else used, is checked for holes /weakness to see if everything is in working order, just like with any other im- or export. With everything in order, the face mask is put on and the bag is sprayed in, to pre-sterilize before any other material is added.
If a drum is to be imported, it has to be checked as well, the appropriate sleeve attached and sprayed in accordingly. Afterwards the port itself is also sprayed in (see also *The export procedure*).
The gnotocages are closed air tight using the plastic lids and tape. Care needs to be taken in order to avoid the tapes overlapping. The top of the lid, being underneath the plastic lid and tape is not sterile. It is very important that this is never opened inside the isolator!!



Take note of the time when the lid was closed! The mice inside should have enough air to last for an hour, but they need to be monitored at all times. The following spraying-in procedure should be done quickly to avoid a prolonged time for the mice with no fresh air and avoid suffocation.

First cage is placed into the port and sprayed in

The sleeve/drum or bag is now put into the right position to allow an easy connection to the relevant port. A port stand or other suitable equipment is used to support the sleeve/bag to allow the gnotocages to stand upright inside them without falling over.

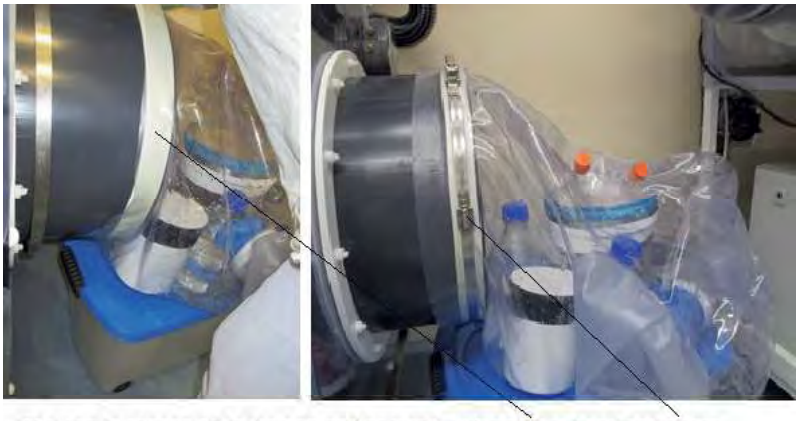
Now starting with the first gnotocage inside the port, the gnotocages are sprayed in, one after another. Special care is to be taken around the lid and the flap!!

Additional gnotocages are placed inside the port bag or sleeve, one at a time, and sprayed in. This is done with one person holding and turning the cage and the second person doing the spraying in.

cages are placed inside the port bag and sprayed - one person holding and turning - second person spraying

support (table or stand)

Once all cages are inside, the sleeve or bag is attached to the port and secured with the rubber band and clamp.



The bag is connected to the port and secured, using rubber band and clamp

To saturate the air inside and cover the all areas inside and especially where the sleeve is connected to the port, some more per-acetic acid is sprayed in, using the spray nozzles.

All areas inside the bag / sleeve are sprayed in via the spray nozzles.

The second person helps moving the cages and other material around to allow all areas to be covered with a fine mist. Once finished the spray gun is removed and the nozzles quickly closed to avoid a loss of pressure.

Incubation time should be at least 30 minutes, without prolonging the duration in which the mice are lacking fresh air. Mice need to be monitored!

After the incubation time is over, the inner cap of the isolator is removed and the gnotocage imported into the isolator. The cage is briefly wiped down to remove excess of per-acetic acid. Afterwards the tape on the bottom of the lid is removed and the lid lifted to allow air getting to the mice.

The lower tape is removed and the lid lifted to allow air flow

Once all cages are inside the inner cap is mounted back onto the port. With the inner cap closed, the mice are put into their new cages. Faeces samples are taken at the same time to test the hygiene standard.

The mice are taken out of the gnotocage and can be placed in previously prepared cages.

If a drum is imported at the same time, it can be opened and the batch control is checked, if the result is satisfactory for import. If everything is fine, the material can be taken into the isolator. Empty gnotocages, samples and any material/waste which is not needed any more, can be removed.

Consider special procedure for S2 experiments!!

At the end of the import the inner cap is put back into place and the sleeve / port bag is removed.
The port is wiped clean and sterilized again for safety reasons. The outer cap mounted and the air inside saturated with per-acetic acid.
The sleeve is wiped to remove excessive amounts of per-acetic acid and rinsed with distilled water to avoid prolonged exposure to the material.

Appendix - Protocols

Daily checks

Every animal facility is required to check on their animals on a daily basis. When animals are kept in an isolator this is no different. Additionally to the mice however, the whole isolator needs a quick check over. If any part is not working properly or damaged it can have a detrimental effect on the welfare of the mice or the hygiene standard inside. Therefore the checks are really important, and part of keeping a certain hygiene standard successfully.
See also *Important checks / weak points of the isolator*

Overall impression – pressure reading

It depends, if the isolators are run at positive or negative pressure. The first check will be the overall appearance of the isolator, if everything looks as expected and check the reading on the pressure gauge.
If the pressure is not as expected it could have one of the following reasons (regarding an isolator run at positive pressure):

- Pressure to low:
    - The blower is switched off (no electricity or switched off by accident, unplugged)
    - The air inlet valve on the isolator was changed / closed too much
    - The isolator tubing which was previously attached to the exhaust of the room is not attached any more (forgotten to put back on after work was done)
    - Room exhaust is not working properly (this can happen after work had to be carried out on the ventilation of the rooms)
    - Filter on the inlet is clogged (pre-filter, or HEPA-filter)

- The isolator has a big hole (a small hole would not lead to loss of pressure)

- Pressure to high:
  - The isolator tubing which was previously attached to the exhaust of the room is not attached any more (forgotten to put back on after work was done)
  - Room exhaust is not working properly (this can happen after work had to be carried out on the ventilation of the rooms)
  - Filter on the exhaust of the isolator is clogged (pre-filter, or HEPA-filter)
  - Exhaust valve of the isolator is closed too much

Checking the welds/seams

Because the isolator is run under pressure, the seams or welds on the isolator are under stress. Over time these may weaken and eventually the seams will tear. For welds around the body this is actually quite rare but the welds around the sleeves are susceptible as they have to withstand the mechanical stress of the movement when working inside the Isolator. This is also true for rigid isolators as they usually have flexible sleeves attached. If the Isolator is run under negative pressure, the seams are not quite as stressed, but as the canopy is constantly sucked in, which makes it more vulnerable to accidental damage. When controlling the seams, check for lighter patches, use soapy spray to find leaks if necessary.

Check the caps on the isolator (if using flexible film caps)

The first check would be to ensure that the rubber bands are aligned well with the clamp, ensuring a good seal. The caps itself need to be checked as well for cuts or damage on the welds. To check if the caps are gas-tight is fairly easy, by opening one of the nozzles of the outer cap and releasing some air, by simply pressing the cap inside the port. Then, the nozzle is closed again. If the outer cap starts to inflate again, the inner cap is not fully gas-tight and needs to be checked.

Glove control, see also more detailed in *Glove-check*

Once the previous checks are done and the isolator is in working order, sterile cotton gloves, specific for the isolator, are put on before putting on the gloves from the isolator itself. These are checked thoroughly for weak spots and holes. Special care should be taken for the areas between the finger, cuffs and any other areas which are usually kinky and exposed to mechanical stress.

Gloves are controlled always before and after working in the isolator!!

If there is a weakness or hole apparent, the glove has to be pulled out of the isolator immediately and the sleeve has to be clamped to protect the inside of the isolator from potential contaminations!! In this case, the mice have to be checked over with just one hand, and the glove has to be changed as soon as possible. Nothing is to be touched with the glove that has a potential breach!!
On rigid isolators it may not be possible to change the gloves whilst the isolator is in use, as they are attached directly to the body.

Only if everything is fine, can the mice be checked to see if they are well and if enough food and water is supplied.

Any abnormalities are recorded in the log-sheet of the individual isolators for future reference!

Important checks / weak points of the isolator

Glove-check:

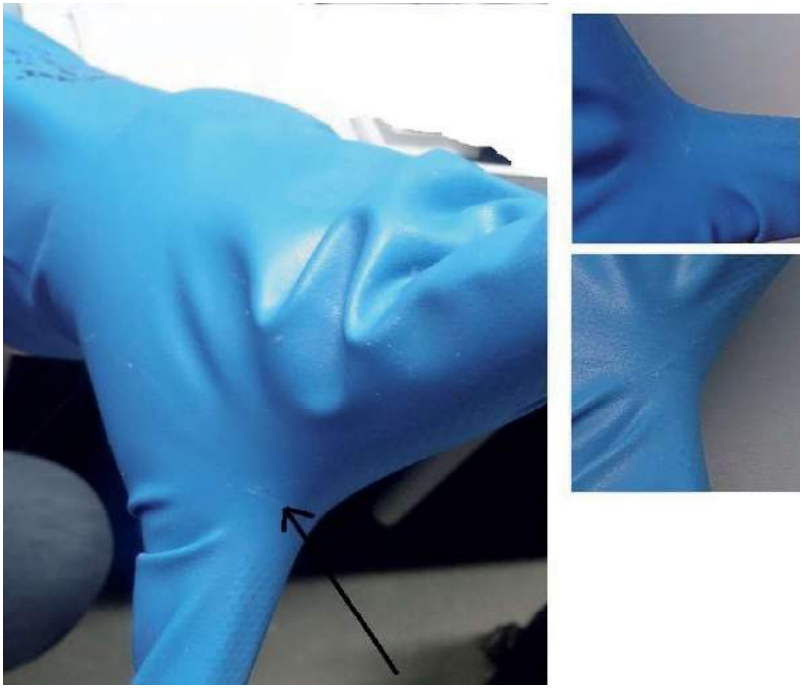
One of the most important controls is done, every time the gloves of the isolator are put on or taken off.

The area between the fingers is to be checked especially carefully. Due to the gloves having a kink at those areas, they usually break first, unless the glove gets caught on a sharp object (cage lids, card holders etc.), which can cause a tear as well.

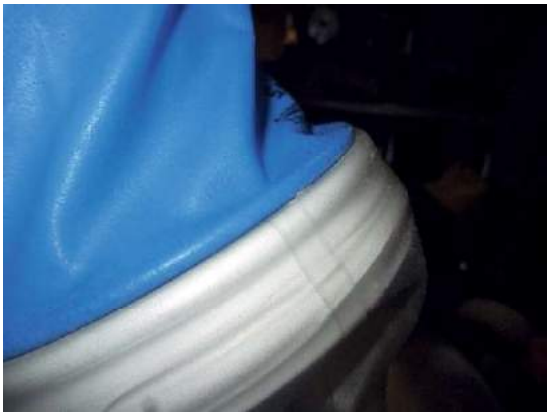
Around the cuffs is another area which is more exposed and therefore prone for holes, tears and cuffs.

In order to check the material properly, it has to be gently pulled and stretched to be able to see anything at all. Holes are usually starting off by small white spots showing trough the coloured material. If this can be seen, the material is not yet fully breached and usually nothing is contaminated yet, but it is important that the glove is changed as soon as possible, as the material can now tear open much easier. To prevent damage to the gloves at an early stage it is important to leave the gloves as crease free as possible after working in the isolator. In time the material of the gloves is becoming fragile and more susceptible to damage. Therefore it is not advisable to have gloves lying around for long periods of time before they are used, as storage is still ageing the material and it becomes brittle.

Check well between the fingers.

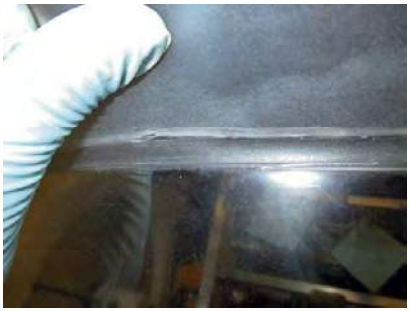


Control the cuffs carefully, where the glove meets the other material and also the sleeve itself needs to be checked!



Checking the welds / seams

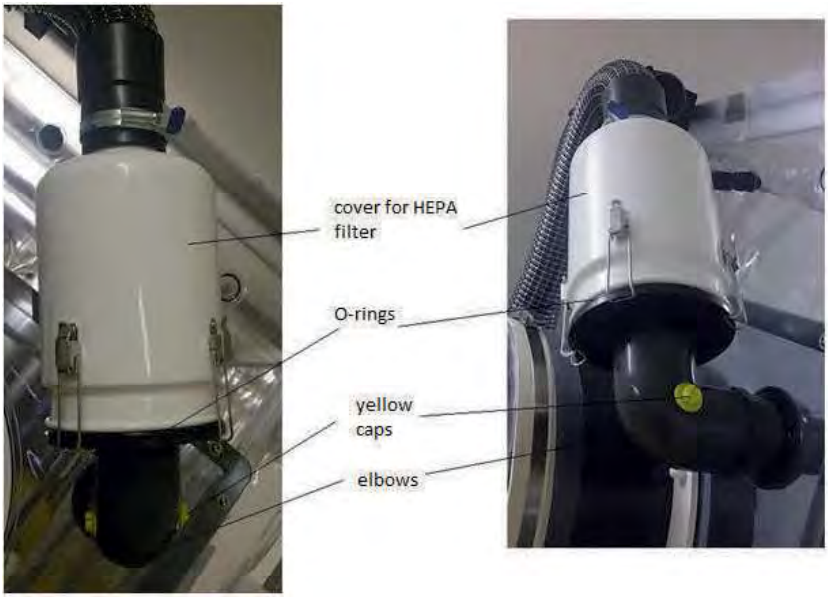
After the gloves, welds and seams are the next weak spot on an isolator. Positive pressure puts constant mechanical stress on those. In time, the seams are stretched and become thinner until they will eventually tear. Therefore they need to be carefully examined, if and how much they are deteriorating. Special force is put onto the seams around the sleeves, as additional stress from moving around in the isolator is put on those.



Checking the air supply connections / HEPA-Filter

There are certain parts on the tubing and filter which are susceptible to damage. One some elbows and tubing are caps which can be over-tightened and the thread may be torn off. This can't be observed by just looking at them but needs to be checked by removing them from the tubing completely. If these caps are broken, they do compromise the isolator, as these parts are still in front of the filter and should remain sterile!

The next part to become brittle in time and break is the rubber seal (O-Ring) on the casing of the HEPA filters. This part however, is already "behind" the filter and doesn't affect the sterility of the Isolator, but changing this seal is best done when the isolator is not in use. Especially for the inlet it is difficult to remove the casing without having to remove the filter. To change this on the exhaust filter however is quite easy.



Glove assembly

Component Parts

Required number of pairs of gloves (preferred brand)
2 per pair two ringed tapered glove cuffs
2 per pair 3" (75mm) 'O' rings
1" (25mm) White Stretch Tape

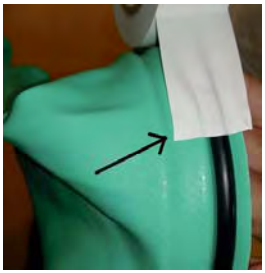
Tools Required

Sharp scissors

Procedure

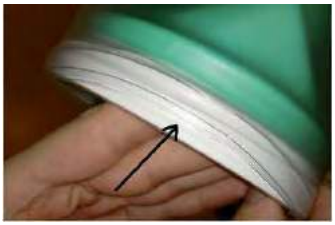
1. Insert into open end of a glove a 2-ringed tapered glove cuff with the narrow end towards the fingers of the glove; push the cuff evenly into the glove until a tight fit is achieved.
2. Fit to the cuff ring furthest away from the fingers a 3" (75mm) 'O' ring.
3. Cut off the excess glove material so that the glove material is flush with the top of the glove cuff (no glove material should protrude past the glove cuff).

4. Apply to the outside of the glove/cuff assembly 1" (25mm) white stretch tape. Ensure the tape is evenly applied without creases or air bubbles. The tape should cover the lower channel of the glove cuff, the 3" (75mm) 'O' ring, the cut part of the glove and the flat section of the glove cuff.

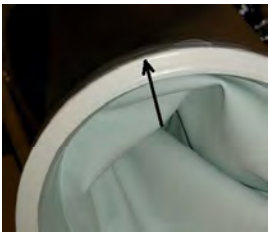


5. Insert a completed glove assembly into the sleeve opening of the isolator. Ensure a left-hand glove is fitted to a left-hand sleeve and so on. Thumb pointing upwards

6. Pull the sleeve so that all twists/creases are removed from the sleeve, with your hand in the glove to be fitted, orientate the glove to the required position.



7. Feed the sleeve material down to the bottom of the glove assembly and secure in place with an ‘O’ ring.

8. Check that all creases are removed.


9. Using the 1” (25mm) white stretch tape to this connection so that the ‘O’ ring of the sleeve and the glove assembly is completely covered and sealed with no creases or air bubbles.


Glove Change (Negative & Positive Pressure) under sterile conditions

The gloves are the weakest part of an isolator and are usually the point where damage can occur first. The check of the gloves should be a routine procedure performed every time the operator is working on the isolator. Once a whole or suspected damage is detected, immediate action should be taken to secure the isolator and replace the glove as soon as possible. Possible causes are normal deterioration of the material, a cut or a severe bite. *Should a mouse bite into the glove during use it is not necessarily the case that the glove has to be changed. The material of the gloves has enough flexibility to cope with this. Very important is on this occasion not to pull away but to stay calm and leave the hand and mouse just where it is. Any sudden movement and pulling away may result in damage!*

First action

Secure the Isolator by clamping the sleeve where the damage has been detected using one of the NKP sleeve clamps.

Component Parts

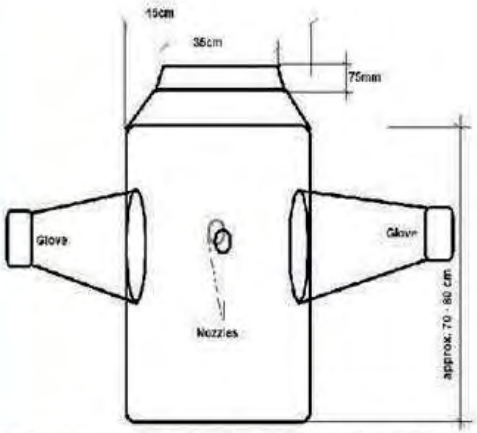
2 Sleeve Clamps (NKP)
Glove change bag (NKP)
Replacement Glove
Replacement Glove Cuff (or existing)
Replacement 3" (75mm) 'O' Ring
1" (25mm) White Stretch Tape (NKP)
Sterilisation solution
Atomiser gun / compressor
Waste Bag and Paper Towels
Personal protective clothing
Scissors

glove-change-kit (scissors, tissues,tape) autoclaved

One way to achieve a glove change in sterile conditions is the use of the "NKP Glove changing Bag":

Procedure

After clamping the sleeve, the Isolator contents are now safe and the damaged glove can be removed, by taking off the tape and simply pulling the glove with its fittings out of the sleeve. The area below the clamp on the sleeve is now "compromised" and not clean! This clamp has to stay in place until the glove change is complete!!!



There are spray nozzles at the front and the back of the glove-change-bag to enable easy spraying of sleeve and clamps

1. Attach sleeve clamp to affected sleeve
2. Remove the tape around the glove cuff to remove the damaged glove
3. Assemble glove with the cuff as described in the section *Glove Assembly* up to step 4 only!
4. Clean the sleeve on the Isolator if any apparent dirt is visible and dry off as much as possible.
5. Using the glove change bag and second clamp place the assembled glove inside the bag and attach to the sleeve. Do not forget the second O-Ring. It helps to start spraying any parts which may be difficult to reach (e.g. inside the sleeve, glove etc) wear protective mask!
6. Clamping it securely ABOVE the current clamp.

7. Spray all Items inside the bag carefully with your sterilisation solution. Take special care to cover the area around the clamp – inside the sleeve covering all areas well
8. Incubate according to your sterilisation procedures (at least 20mins)
9. open the glove change kit and wipe the area where the new glove needs to be attached dry - it takes a bit of practise to dry the areas off and not to soak the wipes straight away.
9. Attach the glove to the sleeve following steps 5 to 8 / *glove assembly* - inside the glove change bag.
10. Making sure the glove has the correct orientation and is securely attached you can carefully remove the second clamp and glove change bag wearing suitable personal protection - or alternatively tape the glove in place still inside the glove change bag. *If you decide to remove the bag and attach the tape outside you should be aware that if you pull the glove out again by accident, you need to sterilise everything again.*

11. Dry the area on the glove assembly carefully without removing the glove from the sleeve until as dry as possible
12. Follow step 9 and secure the glove with the tape.

Dispose of any waste as per your Health and Safety instructions for chemical hazardous waste.

Change of HEPA-Filters

Changing the filters on an active isolator is always a big risk of contamination and should be avoided if possible. If the filter change still has to take place whilst the isolator is in use, it is recommended to take special precautions. In case of a breeding isolator for example, it would be good to have a second isolator with animals as a backup.
The first indication, that a filter needs changing, is a change in pressure in the isolator. If the pressure rises, the exhaust filter may be clogging up due to dirt and dust from the inside of the isolator. If the pressure drops, it could be that the inlet filter is clogged. It needs to be determined if no other reason could be the rise or drop of pressure (see also *Daily checks*)
To change the filter on the air-intake is more critical than changing the filter on the exhaust, as all the air is drawn through the intake filter. Pre-sterilizing all equipment, including the filter is highly recommended!
If at all possible the pressure of the isolator can be re-adjusted for as long as possible using the valves, in order to delay the filter change! If possible it can be delayed for as long as the isolator is taken out of service (experiment finished). Then the filters can be cleaned / changed without risking anything.

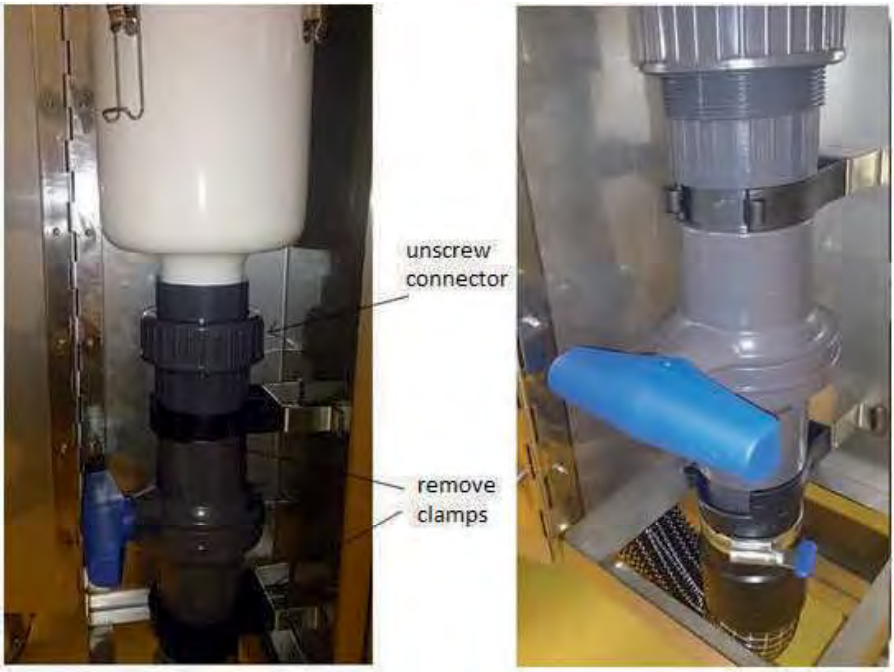
Material:

- 100ml 3% Per-acetic acid
- HEPA Filter (fumigated using H2O2 or irradiated, packed sterile)
- Spray-gun, compressor

Procedure:

- With the blower still on, close the appropriate inlet- or exhaust tubing with the rubber stopper.

- Loosen the connector piece on the pipe attached to the filter and remove the clamps.



- Open the latches on the Filter-cap

- Unscrew piping

- Unscrew HEPA-filter and remove together with cap

- Spray per-acetic acid into the opening, where the Filter was attached to
- Clean the cover and place the new filter inside it (using gloves and disinfectant if necessary)

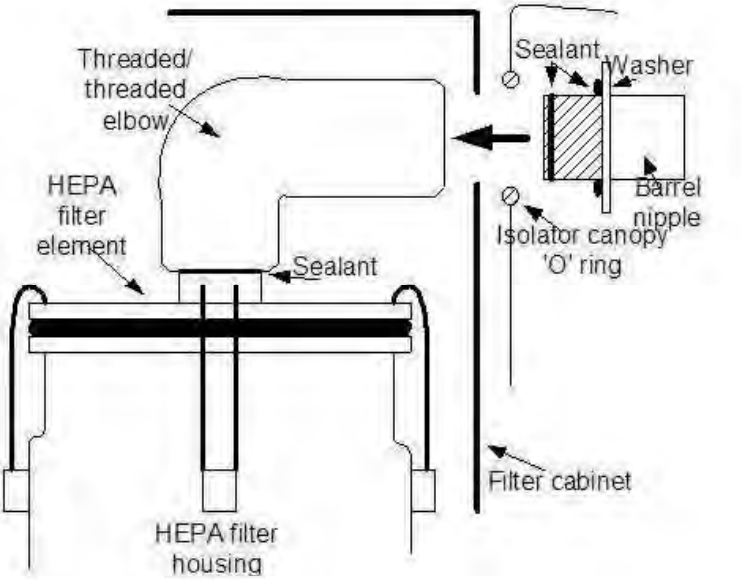
- Place the fastening mechanism on the cover in the “close” position before placing the cover with the filter back on the pipe. (you won’t be able to close those clamps otherwise because of lack of space)

- Remove the yellow cap (if you have a cap on the elbow - if not it has to be sufficient to spray the connection before attaching the filter) from the elbow and spray per-acetic acid into the pipe to sterilize the air inside the filter and pipe.

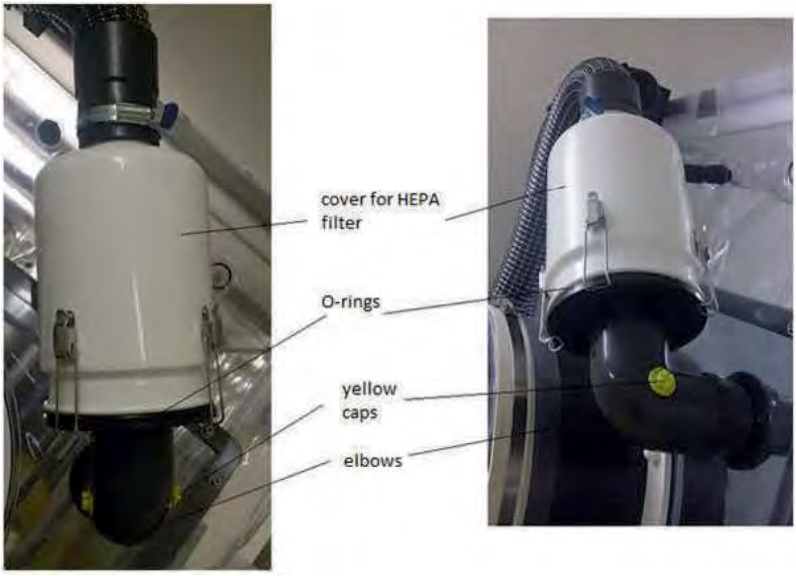
- Close the yellow cap (if applicable), but do not over-tighten it, as the thread may split!
- Incubate for one hour
- Remove the rubber stopper on the inside of the isolator
- Filter change finished, adjust pressure if necessary

Filter positions

Inlet HEPA assembly



Outlet HEPA assembly *with or without casing



Stocking of a drum for import

Any material is additional to the autoclaving batch control!!

First drum:

- 4 - 6 cages (depending on size) with lids
- Drinking bottles and caps accordingly
- Mouse - house
- 1 tray with pellets of food
- 4-6 bags containing bedding
- Paper bags (big and small)
- towels
- reaction tubes (for faeces samples or biopsies)
- 2x Sets for testing sterility:
    - 4 cotton buds
    - 4x2ml reaction tubes
    - 2x5ml reaction tubes
- Nesting material
- brush
- pencils
- mouse handling forceps
- ear-punch and forceps for taking biopsies

Food / Bedding:

- 4 trays with food pellets (only one layer of food on each tray!!)
- 3 empty cotton bags (will be filled with the autoclaved food upon import)
- Up to 8 bags of bedding, arranged to be easily removed,
- Paper bags, cloths, tape or anything else which may be required in the isolator.

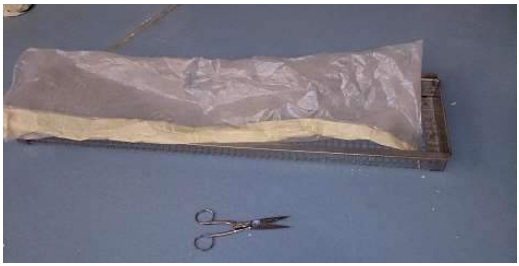
The following suggestions are based on my experience and working with germ free isolators. Using the isolator for other applications may mean that your procedures may not need to be quite as stringent as these. There are always different practices possible:

In order for food to be properly sterilized, only one pellet-layer should be put on each tray to ensure good penetration. Food which is not properly sterilized is one of the main causes for contamination, as it is difficult for the steam to penetrate pellets very well. The time and temperature has to be also adjusted accordingly. Either the time to sterilize is elongated or the temperature is raised to 135°C. Some users prefer both. The program stated here is a minimum requirement:

Autoclaving program: 30min sterilization at 121°C, 20min drying time

To make it easier to fill the pellets into the cotton bags when imported, the pellets can be put into autoclave bags, cut to size for the trays. It is very important to punch plenty of holes into these bags to make sure that the penetration of steam is not reduced!

Autoclaving bags cut to size. Using scissors, plenty of holes are punched through.



Cotton bags with bedding are connected using the drawing string to enable easy removal from the drum:

Transferring mice from one isolator to another using gnotocages

- Up to 4 small Gnoto-cages
- Troughs, bottles and caps if necessary
- Bedding or a layer of food in the gnoto-cages
- Filter-lids / if S2 animals taped with plastic lid!!!
- tape
- Bags with Bedding, paper bags, cloths, etc, depending on needed material and space available.

To be able to fit 4 cages into one drum, only the 2 bottom trays can be put inside it. Otherwise there is not enough space in height to accommodate the cages. It is also possible to put one tray upside-down into the drum and place the cages on top.
If using the through with the cages, make sure it is placed inside the cage and not hung on the rim of the cage. The hook of the trough is sharp and could potentially pierce the mylar film of the drum, or the gloves whilst handling it when importing the cages into the isolator!!

Sealing the drum

there are various ways to close the drum, but in effect I am aware of 2 methods:
1 - Mylar film
2 - autoclavable cap

Procedure 1 - mylar film:

Always handle the mylar film with care to avoid any damage. Examine the foil prior to use!
As preparation, small pieces of tape are prepared in order to secure the mylar film to the drum. The mylar should be cut to be about 1 to 2cm wider and longer than the drum opening, as there needs to be a projecting rim of material in order to be stuck to the drum.
Starting at the bottom, the foil is secured with pieces of tape on 4 sides. As this keeps the mylar into place, scissors can be used to trim the edges off and shape it to fit around the drum. You can also secure the mylar using a rubber band if you find that easier. The tailored foil is secured by adding many small pieces of tape. Making sure the foil is stretched tightly across the open surface of the drum.

small pieces of tape are used to secure the foil tightly to the drum

Now a 5cm wide PVC-tape is used to seal the foil around the drum. Starting behind the small pieces of tape, pulling it tightly around the drum to cover all the small pieces by at least 5mm. (no foil or small tape pieces should protrude the tape layer as these may give rise to air channels and compromise sterility!

After the first round of tape another round is pulled over the front of the drum, leaving a rim to protect the foil from tearing around the edges of the drum. Once the drum is completely closed the tape is pulled slightly back towards the body and cut off. It is important to keep the tape stretched all the time to avoid creases from forming.

tape is used to cover all the small pieces of tape and pull crease free around the drum

a second round of tape covers the edge and leaves an even rim at the front of the drum

Examine the foil and tape fastening after finishing to ensure everything is covered, well stretched and that the mylar film is not damaged.

The mylar foil should be tightly streched across the surface of the drum with the tape showing very few creases.

Folds and recesses are a risk of contamination if they cannot be sterilized properly when spraying in.

Procedure 2 - autoclave-cap:

To cover the drum with a cap is much simpler. It is merely put over the opening and secured with a clamp. Do make sure that the cap is not damaged or cracked.

Filter change of an autoclaving drum

The Filter of a drum is changed every year or if it shows signs of deterioration. To check if the filter has any tears inside the material, place the drum in a well lit area, remove all the trays and everything inside the drum and have a good look at the filter looking from inside the drum. Any tears will let more light through and are therefore easily detectable. It is not always possible to see these tears from the outside, as the outer layers of the filter may be still intact.

Size of the filter (drum size 45m diameter - 80cm long):
4,5 m long (covering the drum in 3 layers of filter material)
55-60 cm wide (The filter hast o be wide enough to cover all the holes of the drum, but narrow enough to have enough space to seal the filter with tape onto the drum. Critical areas of space are at the handles and feet of the drum.)
At the start, the clamp needs to be unscrewed and removed to gain access to the tape and filter. All the old tape and filter is removed and the drum is cleaned.

The new filter is cut to size and secured to the the drum, using the 5cm wide tape. First part pull through the bottom of the drum and around again - this is easier than starting at the top.

The next steps are done by two people, as one person can pull the filter tight on one side and makes sure it is properly aligned, whilst the other person is pulling the filter through and back around the drum.

Once the filter is fully on the drum, the end is again held in place by a long strip of tape.

The sides are now secured with 3 rounds of tape. At the end the clamps are put back on, placing it on top of the tape where it overlaps with the filter material.

Autoclave procedure for water bottles

If no direct watering system for the isolator is possible or wanted, the water is autoclaved separately in bags to keep the outside of the bottles as sterile as possible. The water bottles need to be kept clean and free of grease, dirt and tape residues.
They should not be over-filled to give enough space to boil, without boiling over. The lids are only attached loosely on top and closed properly after the autoclaving procedure whilst the bottles are still hot! If the lids would be screwed on tight the bottle might burst when autoclaved!

Caps are put loosely on top of the bottles max. filling level

It is possible to autoclave the water bottles inside the autoclaving drum. The problem with this method is to achieve a dry filter on the drum after the autoclaving cycle. As the filter is usually very wet after autoclaving water it is not recommended to use these. The only way to prevent this, would be to line the drum with plenty of material soaking up the water to prevent too much water to remain in the filter of the drum. Trial runs need to be performed to find out if the filter dries sufficiently to be able to use these.

Alternatively, bottles can be autoclaved in bags using a normal program for liquids.
It is possible to autoclave the bottles twice to make sure that sterilization is sufficient.

Place the probe into a reference bottle with larger or same volume as the actual bottles autoclaved!

Bottles are placed into cage bottom or tray inside an autoclave bag

Screw the tops tightly on to the bottles **straight after** the autoclaving program has finished, without opening the bag, in which the bottles have been placed. If the bags are closed as shown below, it allows enough space to do so. Any holes and tears should be taped closed after autoclaving, also whilst the bottles are still hot. The outer bag does not guarantee that the outside of the bottles remain sterile but serve as s first protection only. The water should be imported into the isolator as soon as possible after sterilizing - maximum one week later.

Place the bottles in trays or cage-bases and tape it closed at the top of the bag to give enough space for closing the lids later on.
This is easily done by taping it to the legs and pulling the tape accross.

List of materials

This is a list of many essential items needed to be able to run a facility with isolators. It cannot be a fully sufficient list, as every need and facility is different and items should be added or removed accordingly:

“Big” equipment:

- Isolators, size as required
- Racks, cages, lids and bottles etc.
- Sleeves for import (or connecting sleeves isolator to isolator)
- Port-bag
- Port-bag with coiled tubing for sterilizing
- Clamps and rubber bands for fastening caps and sleeves
- Spray-gun (Atomiser)
- Compressor
- Sterilizing drums
- Hoist, height adjustable trolley
- Autoclave-able trolley for drums (ideally at the correct height for import)
- Working platforms or steps to work on the isolator.
- Clamps, different styles!! (see Glove change under sterile conditions S.48)
- Spare HEPA -filters
- Filter material for drums
- Mylar foil / drum cap and fixtures
- Gnotocages for import or export of animals

Smaller equipment / consumables:

- Full face mask for use with per-acetic acid
- PVC tape 150mm and 500mm wide
- Per-acetic acid
- Glass bottles (1l) to autoclave water
- Spare gloves for isolator
- Cotton gloves / autoclave-able
- Spare Rubber bands for Ports
- Cotton bags for bedding and other material
- Big and small Paper bags (Waste bags/ samples)
- Long forceps/ Mouse handling forceps
- Pencils unvarnished
- Ear-punch for taking biopsies
- Nesting material (non dust producing e.g. Sizzlenest)
- towels (e.g. Wipall X60)
- coloured cardboard to be used for cage-cards including clips to hold on to cage-lid
- brush autoclave-able
- scraper, autoclave-able

Sterilisation products

There are many different steriliser available. It is important you chose the correct one for your application. Below you find some examples but there are many more out there.
Important point would be to determine if you want to actually sterilise your surfaces or decontaminate. These two are not the same, as the disinfection will get rid of a lot of bacteria and some viruses and spores but a sterilisation is much more thorough and should be effective against everything.

Liquid fog sterilisation

The one I used in this manual is per acetic acid in the form of

- Oxonia active (Ecolab)

Also commonly use are the following:

- pure per acetic acid (Merck)
- Exspor (ABS global USA)
- Virkon
- Clidox (pharmacal)

Below you find a few links from the internet which give you additional information which may be useful.

https://www.ncbi.nlm.nih.gov/books/NBK310477/
http://www.clordisys.com/whatcd.php
http://www.revoxsterilization.com/sterile-processing/how-peracetic-acid-sterilization-works

Fumigation - gas

- Formaldehyde gas
- Vaporized Hydrogen Vaporized Hydrogen Peroxide
- Chlorine Dioxide Gas

https://www.pda.org/docs/default-source/website-document-library/chapters/presentations/new-england/decon-2-0-emerging-decontamination-technologies.pdf?sfvrsn=6

Sterilisation indicators

There are many products available which help you determine the ones you want to use when autoclaving equipment. Below are again some examples. I did not include basic indicators like autoclave tape in this list as these are not specific enough. Important is to note the difference between bio-indicators and chemical indicators. Bioindicators are the most reliable but can often not be checked immediately after the sterilisation. Chemical inidcators can be checked upon import straight away but are often not as sensitive.

- MelaControl (MELAG)
- BioindicatorSterikon (Merck)
- Steris Bio-indicators

http://certoclav.com/en/support/knowledge/show/chemical-and-biological-autoclave-indicators.htm
https://www.uoguelph.ca/mcb/sites/uoguelph.ca.mcb/files/public/MCB_SOP_Biological_indicators_AODA.pdf
https://en.wikipedia.org/wiki/Autoclave#Quality_assurance

Dilution chart peracetic acid

(Ecolab, oxonia active 17.5%)

	3%	
Total Volume ml	H2O in ml	Peracetic acid oxonia active in ml
20	17	3
40	33	7
60	50	10
80	66	14
100	83	17
120	99	21
140	116	24
160	133	27
180	149	31
200	166	34
220	182	38
240	199	41
260	215	45
280	232	48
300	249	51
320	265	55
340	282	58
360	298	62
380	315	65

General Maintenance

To ensure that the Isolator and it's additional equipment is always in good working condition, it is good to have a habit of keeping everything clean. Generally, a maintenance plan is recommended to check some parts at least once a year, monthly or even daily - your provider may be able to offer a service plan as well.

Frame:

The frame itself does not need a lot of attention - but a general clean to remove dust is helpful.

Canopy:

- daily checks of general conditions - pressure reading to ensure everything is in working order
- keep clean - clean with water or gentle soapy solution or mild disinfectant
- do not use acetone, solvents or strong alcohols as this will damage the PVC material - refer to your manufacturer if in doubt
- check welds on a regular basis
- live expectancy of a flexible film canopy is approx 2-4 years but can last a even longer (if not damaged) - a use for longer than 7 years is not recommended

Filters:

- pre- Filters should be kept clean as this prolongs the life of the Hepa filters
- wash with soapy solution or vacuum clean or both
- HEPA filters should be checked on a regular basis - a tell tale is usually the pressure gage:
  - *if pressure slowly and gradually increases the outlet filter is clogging*
  - *if pressure de-creases the inlet filter is clogging*
- HEPA filter should be changed when too dirty - with normal use

they last minimum 1 year but can be longer depending on air quality

- replace any filter if damaged

Gloves:

- need to be checked upon every use
- need replacing as soon as holes appear or are suspected
- should be changed on a yearly basis (minimum) due to the exposure to harsh chemicals

Fan unit:

- keep pre-filter as clean as possible to stop built up of dust on the inside
- it is possible to vacuum when not attached to get rid of some dust
- change pre-filter on a regular basis

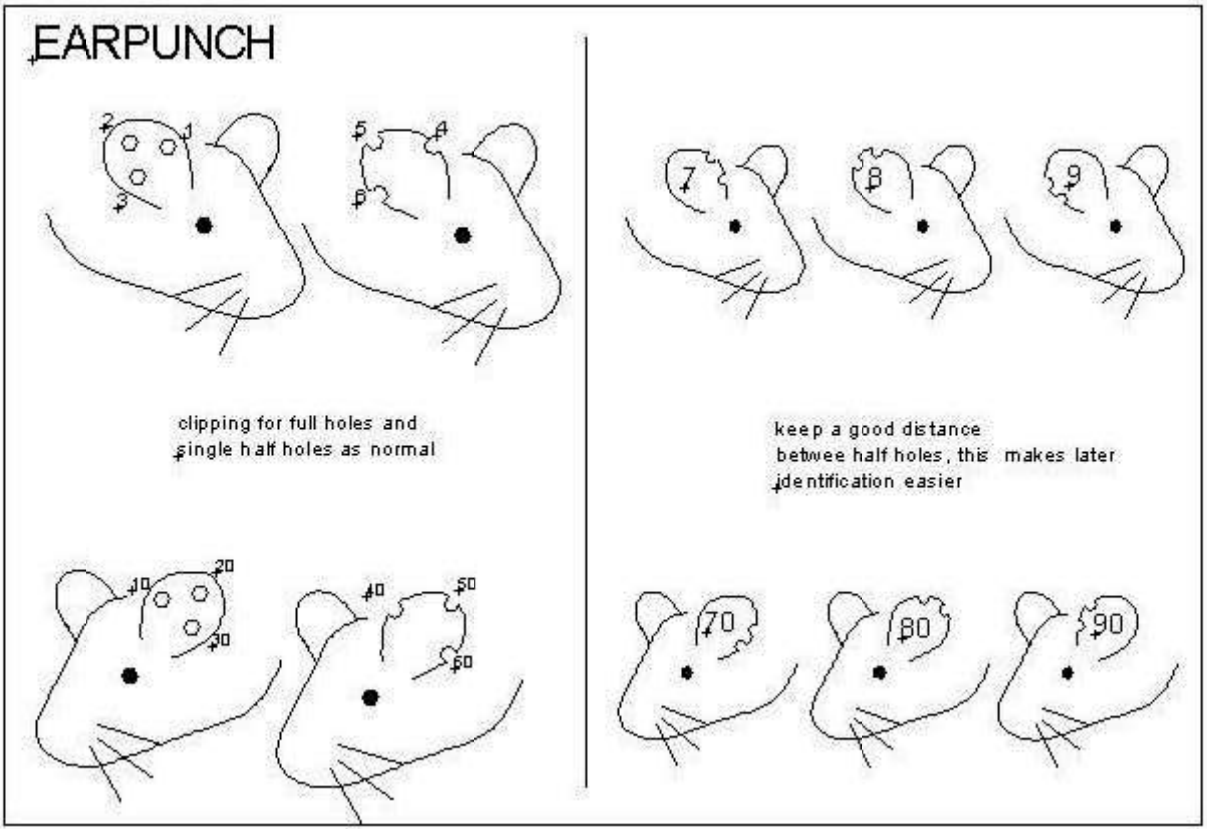
Tubing and connections:

- keep clean on outside
- between uses check if inside the tubing cleaning is necessary
- inlet and outlet reaching into the isolator need to be kept clean (prolongs life of the filter)

Accessories:

This applies to ports - transfer sleeves almost anything you are using with the isolator

- keep clean - always rinse with plenty of water after use with aggressive chemicals
- store dry to prolong life
- keep separate if possible from other equipment for other health status
- control the sterilisation processes
- replace if damaged


EARPUNCH
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keep a good distance
betwee half holes, this makes later
identification easier
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