Protocol: Influenza virus propagation in embryonated chicken eggs

General risks & prevention measures:

❗Attention:

- Eggs inoculated with influenza viruses increase the risk of exposure to influenza viruses mostly via the airways due to breakage of eggs during transport or transit from incubator to the bench and vice versa.
- The use of sharp items such as scalpels, needles or scissors that get in contact with virus containing solutions increase the risk of accidents and inoculation of virus via wounds.

General Prevention **Measures:**

- During all steps involving egg inoculation and virus harvest, only the person performing the experiment and a trained assistant are allowed to be in the virus lab. They have to wear in addition to the virus lab coat and nitrile gloves, the additional surgical gown, two pairs of gloves, sleeves, googles, lab shoes, single-use shoe cover, and an FFP2 mask.
- Perform all procedures involving manipulation of the egg under sterile conditions. Pre-clean all equipment with Meliseptol before use. In general, influenza virus inoculation and harvest should be conducted in a BSL-2 laboratory. The Biological Safety Cabinet (BSC) should be booked prior to entering the virus lab.
- A sign indicating egg inoculation and/or virus harvest is placed on the outside virus lab door to prevent accidentally entering of persons not involved in the experiment.
- All used consumable (tubes, pipette tips, masks, gloves, syringes, etc.) and infected eggs must be disposed in the S2 biohazard waste bag, and then autoclaved.
- Collect all sharp items in a sharps disposal containers that is autoclaved when it is full.
- For all contaminated instruments, such as forceps and spatula, leave them to soak in Korsolex basic 3% for 1 hour, then autoclave and clean.
- After each use of the benches, disinfect the working area with Meliseptol.

Measure in Case of **Accidents:**

- In case of an accident with infected eggs, protect yourself by wearing personal safety equipment mentioned above, make sure, no one else enters the lab or steps in the contamination, get rid of big amounts of liquid by using paper towels (collect contaminated towels in the biowaste bins), wet with freshly prepared Incidine active (final concentration 3 %) and let act for 1h, use paper towels to clean up and finally clean again with Incidin active 3%.
- In case of an accident with a contaminated needle or scalpel:
    1. Get support by a first aid helper (030 838 56354)
    2. Remove your gloves, wash your hand, let wound bleeding out.

3. In case of any symptoms such as dizziness, difficulty in breathing, or pain make an emergency call (112) consult a doctor and get there accompanied by a colleague:
    - Dr. Boldt, Hainbach, Schloßstr. 111, 12163 Berlin, 030 791 8087
    - Unfallbehandlungsstelle der Berufsgenossenschaften, Hildegardstr. 28, 10715 Berlin, Mo-Fr 8-18 Uhr, 030 857 7140
    - Charité, Hindenburgdamm 30, 12203 Berlin, 030 844 50
4. Watch local symptoms (swelling, rash, pain), and body symptoms (temperature, breathing, dizziness) until you see a doctor.
5. Inform Katharina Achazi (replacement Daniel Lauster) and submit an accident protocol to Carlo Fasting.

1. Preparation of eggs for inoculation

a. Upon arrival, mark the name and date on eggs with a pencil, and place them in a humidified egg incubator (37 °C and 55-60% humidity) with an automatic egg turner to rotate eggs regularly. If an automatic egg turner is not available, manually rotate eggs at least 2-3 times a day.

❗**Attention:** When transferring eggs from original packages to the incubator in step 1a, there is a risk that eggs may fall to the ground.

Measures: Be careful to put eggs inside the incubator. If eggs are broken, clean with paper towel and 70 % ethanol properly.

2. Egg Candling

a. Use an egg candler to determine if it is fertile or infertile by candling after about 7 or 8 days of incubation. (NOTE: In fertilized eggs, thin blood vessels leading to a bean-shaped embryo should be clearly visible. Unfertilized eggs will appear as a small blood spot with a visible egg yolk).

b. Discard unfertilized eggs, and return the viable eggs to the incubator. (NOTE: Do not leave eggs outside of the incubator for more than 30 min. If the status of an egg cannot be confirmed, mark it with question mark, and observe it later).

❗**Attention:** When candling and returning eggs to the incubator in step 2a and 2b, there is a risk that eggs may fall to the ground.

Measures: Put eggs in a specific egg holder or empty egg box carefully, and hold on to the box during transport. If eggs are broken, clean with paper towel and 70% ethanol.

3. Preparation of Virus Inoculum

a. Dilute influenza virus stock to about 10^3 -10^4 PFU/ml in sterile PBS. (NOTE: Dilute the virus stock immediately before use and keep the diluted virus on ice all the time)

4. Influenza Virus Inoculation via the Allantoic Route.

When conducting the experiment make sure that the lab door is closed so that there is no air flow happening at the moment.

a. On the day of virus inoculation (day 10 and 11), candle the eggs again and eliminate any dead embryos. (NOTE: Distinguish live embryos by looking for movement inside the egg).

b. Mark the air sac by drawing a line with a pencil.

c. Put eggs in a separate holder with the air sac up.

d. Disinfect the eggshell above the air space with 70% ethanol.

e. Transfer eggs into a biosafety hood (BSL-2) in a special egg holder for one hand handling.

❗**Attention:** In the above steps (Step 4a-e), there is a risk that eggs may fall to the ground.

Measures: Put eggs in a specific egg holder or empty box carefully, and hold on to the box during transport. If eggs are broken, clean with paper towel and 70 % ethanol.

f. Use only one hand to punch a small hole in the shell over the air sac of each egg using a sterile 18G needle or egg shell egg. (NOTE: Be careful not to insert the needle too deeply to avoid stabbing the embryo or yolk.)

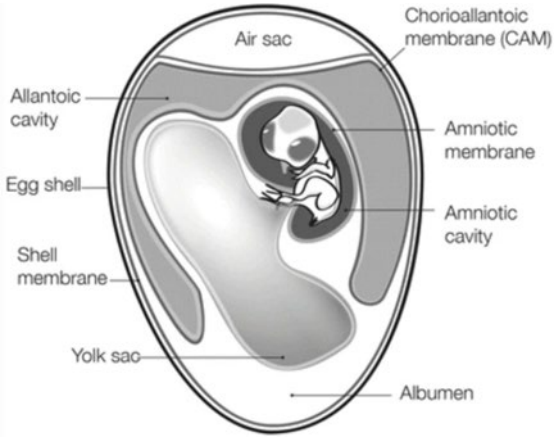

Figure 1. The anatomy of the embryonated egg.

g. Draw up the diluted influenza virus into a sterile 1 ml syringe and attach a 22G needle.

h. Carefully advance the syringe with needle at a 45° angle into the allantoic cavity.

i. Use only one hand and inject 100-300 µl of the diluted influenza virus in the punched hole; then discard the syringe and needle.

❗**Attention:** Since we cannot avoid using sharp items, there is a risk for a needle stick in Step 4f-i.

Measures: Be careful, when handling needles, and wear special needle-safe hand protection during inocculation. Immediately after use, place needles or other sharps into a sharp waste disposal container that is autoclaved when it is full. Do not recap. If accident happens, get help by a first aid helper, let the wound bleed, get to the doctor accompanied by a colleague or call the emergency and inform Katharina Achazi (replacement Daniel Lauster) and complete an accident protocol (submit to Carlo Fasting).

Figure 2. Egg infection by one hand.

j. Seal the hole with glue or melted paraffin wax. (NOTE: Be careful that glue does not leak inside the egg).

k. Place the eggs back into the egg incubator with the air space pointed upward.

Attention: In the above steps (Step 4j-k), there is a risk that eggs inoculated with influenza virus may fall to the ground.

Measures: Put eggs in a specific egg holder or empty egg box carefully, and hold on to the box during transport. If eggs are broken, directly dispose of the broken egg with tissue into the autoclave bin. Disinfect contaminated area with Incidine active (final concentration 3%) and let it incubated for 1 hour. Then clean the surface with paper towels and put them into the autoclave bin.

5. Incubation Time

a. Incubate the infected eggs without turning at 37 °C and 55-60% humidity.

b. Choose optimal incubation time depending on the influenza virus type. (NOTE: Incubate influenza A for 48 h; incubate influenza B and C for 72 hr.).

6. Influenza Virus Harvest

During viral harvest process put a label on the door so that other lab members are informed.

a. After incubation period, chill the eggs at 4 °C for a minimum of 4 hr (or O/N) to kill the embryo and constrict the blood vessels to reduce the risk of contaminating the infected allantoic fluid with blood. Alternatively, chill eggs for 30 min at -20 °C; However, this increases the risk of blood contamination.

b. After the eggs have been sufficiently chilled, transfer the eggs to a biosafety hood. Place the egg into a firm holder with the air sac up. Clean the egg surface with 70% ethanol.

Attention: In the above steps (Step 6a-b), there is a risk that eggs may fall to the ground.

Measures: Put eggs in a specific egg holder or empty box carefully, and hold on to the box during transport. If eggs are broken, clean with paper towel. Disinfect contaminated area with Incidin active (final concentration 3%) and let it incubated for 1 hour. Then clean the surface with paper towels and put them into the autoclave bin.

c. Put egg holders or cartonage in a container to reduce spill over in the bench. Wear needle and cut prove gloves and open the eggshell above the air sac with sterile scissors. Remove the shell around the air sac while being careful not to destroy the chorioallantoic membrane.

d. Open the chorioallantoic membrane with sterile blunt forceps. Gently move aside the embryo and the yolk sac with a small spatula or spoon. Take care not to rupture the yolk.

e. Using a 1 ml Eppendorf pipette, carefully collect the allantoic fluid. If needed, tilt the egg a bit to the side to collect as much fluid as possible.

f. Combine the allantoic fluid from all the eggs into a 50 ml plastic falcon tube. Keep tubes on ice at all times during virus harvest. One egg yields 5-10 ml of a slightly yellowish fluid.

❗**Attention:** There is a risk that the allantoid fluid containing viruses may spill and cause contamination.

Measures: Collect allantoic fluid carefully. If accident happens, disinfect contaminated area with Incidine active (final concentration 3%) and let it incubated for 1 hour. Then clean the surface with paper towels and put them into the autoclave bin.

g. Spin the virus-containing allantoic fluid at 1,000 x g for 10 min at 4 °C to pellet debris. Transfer the clear fluid into a new 50 ml tube kept on ice. Dispose eggs into a BSL-2 waste container and autoclave. Collect the waste with eggs and embryo residues separately as the inactivated waste needs to be collected and discarded by a special company latest the next day.

❗**Attention:** There is a risk that allantoid fluid containing viruses may spill and cause contamination during centrifugation.

Measures: Close the falcon tubes tightly and seal it with the parafilm. Use aerosol tight centrifuge lids with special aerosol tight lids. In case of broken centrifuge tubes, take the whole rotor out of the centrifuge and inside the biosafety hood and open the centrifuge rotor lids inside the biosafety hood. Disinfect contaminated area and rotor with Meliseptol and let it incubated for 30 minutes. Then, clean the surface with paper towels and put them into the autoclave bin.

7. Storage

a. Immediately aliquot the clear allantoic fluid in a biosafety hood.

b. Store at -80°C for further use.

c. Document storage in the appropriate folder and also in the wiki.