**Thesis project:**

# Mucosal rheology in the airways of patients with severe lung disease

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**DOE2:**

This appendix, which is referred to in Chapter n. 4, aims to present the materials and methods developed for the second DOE, showing tools, apparatus, components and basic steps for organizing the reconstruction of synthetic lung mucosa.

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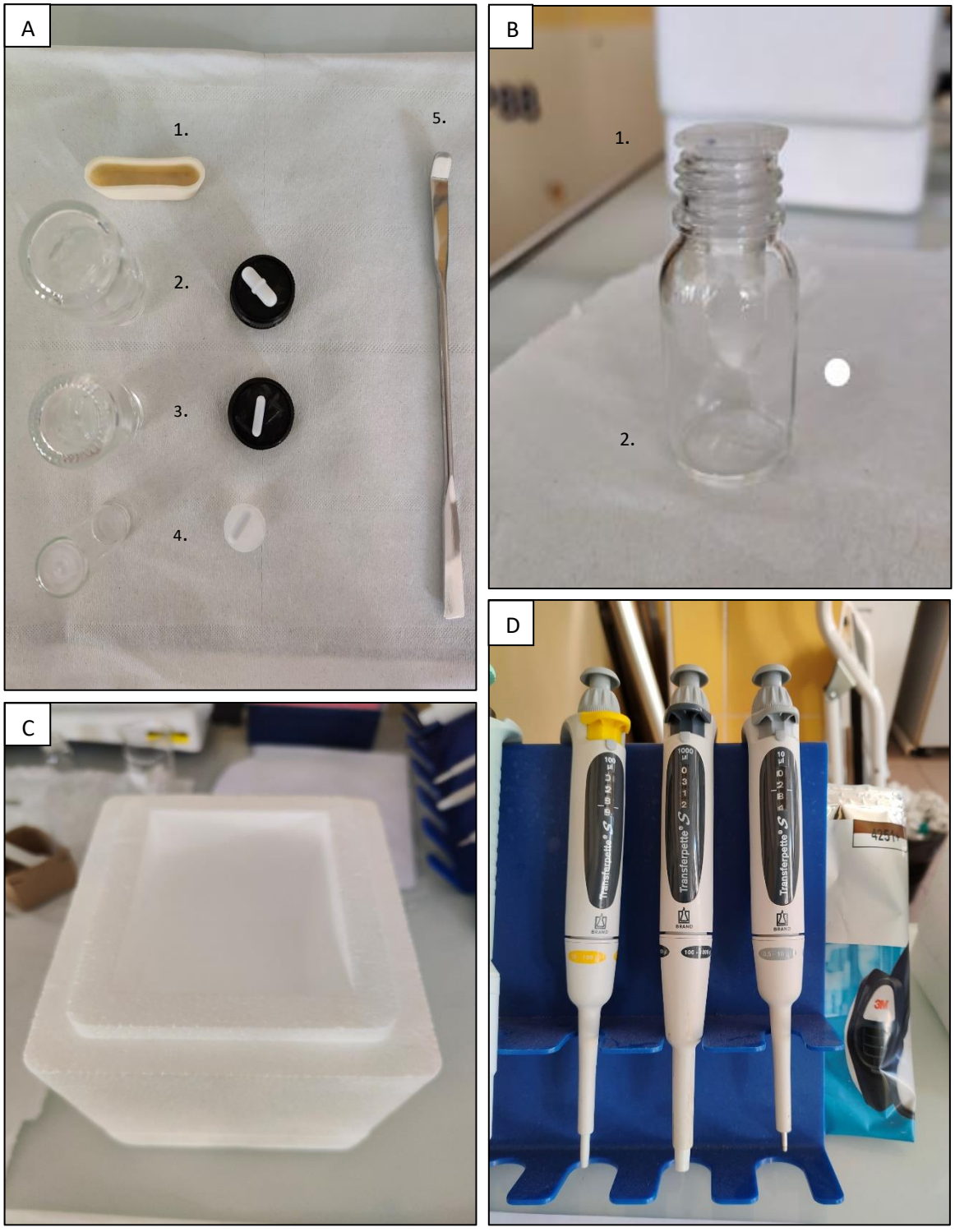
## MATERIALS AND METHODS USED FOR THE RECONSTRUCTION

Here-below the following figures display all the tools and apparatus used during DOE 2**.**



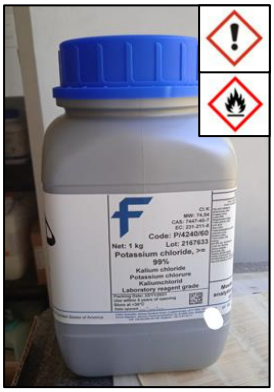

*(Fig.A) 1. Ceramic pot to weight the component 2. Bottle (T) complete of stirrer and cup 3. Bottle (-) complete of stirrer and cup 4. Glass vial complete of stirrer and cup. - (Fig.B) 1. Plastic vial 2. Support - (Fig.C) Box of Styrofoam as T isolated environment - (Fig.D) Micropipette 1000, 100, 10 µl.*


*(Fig.E) Precision Balance - (Fig.F) Magnetic Stirrer at controlled rpm and T – (Fig.G) Oscillator Mixer - (Fig.H) Precision Rheometer "Anton Pear MCR 302".*

# CHEMICAL COMPONENTS of RECONSTRUCTED MUCUS


*Sodium chloride chemicals.*
*TSCHEM*
*(code: 27810295)*


*Mucin from bovine submaxillary glands.*
*Sigma Aldrich*
*(code: M3895)*




*Potassium Chloride, Extra Pure, SLR, Eur. Ph.*
*Thermo Fisher scintific*
*(code: P/4240/60)*


*Deoxyribonucleic acid from fish sperm.*
*Sigma Aldrich*
*(code: 74782)*


*Caseine hydrolysate from bovine milk.*
*Sigma Aldrich*
*(code: C9386)*


*Alginic acid.*
*Thermo Fisher Sienticic*
*(code: 177775000)*


*Mucin from porcine stomach.*
*Sigma Aldrich*
*(code: M2378)*

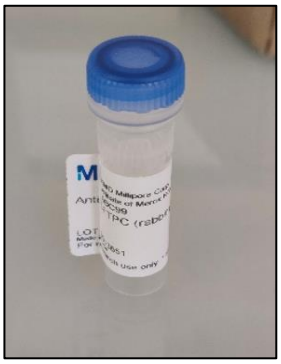

*Poly(acrylic acid).*
*Sigma Aldrich*
*(code: 306231)*


*Tris Base (White Crystals or Crystalline Powder/Molecular Biology).*
*Fisher BioReagents*
*(code: BP152-1)*


*Deionised water.*
*(taken from the laboratory deioniser)*
*Demineralised water.*
*(taken from the tank filled by the laboratory distiller)*

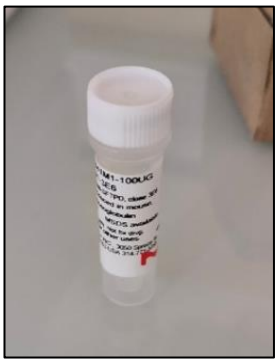
## COMPONENTS of ARTIFICIAL SURFACTANT


*16:0 PC (DPPC).*
*Sigma Aldrich*
*(code: 850355C)*

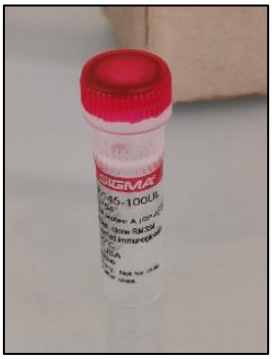


*Anti-SFTPC Antibody.*
*Sigma Aldrich*
*(code: ABC99)*

*05:0 PC.*
*Sigma Aldrich*
*(code: 850304C)*



*Monoclonal Anti-SFTPD antibody produced in mouse.*
*Sigma Aldrich*
*(code: WH0006441M1)*




*Anti-Surfactant protein A (SP-A) antibody, Rabbit monoclonal.*
*Sigma Aldrich*
*(code: SAB5600145)*

# EXPERIENCES PROCEDURE

## RECONTRUCTED MUCUS (Part 1) – FIRST DAY

***Operative condition 25 °C , 1 Atm***

1. Weight a bottle with inside the stirrer and a cap ( g).

2. With the help of a micropipette of 1000 µl fill the bottle at pt.1 with ( ml) of DEIONIZED $H_2O$ (Better fill with a quantity of nearly 500 µl less than how we had to add, perform a check with the balance and then add the Δ resulting from this measure and the value desired).

3. Briefly add following the order reported under constant stirring at 800 rpm :
   - NaCl (Sigma) (POWDER little spatula) ( mg)
   - KCl (Sigma) (POWDER little spatula) ( mg)
   - Casein Hydrol. (POWDER little spatula) ( mg)
   - MUC5AC (POWDER little spatula) ( mg)
   - MUC5B (POWDER little spatula) ( mg)
   - DNA (POWDER little spatula) ( mg)
   - Alginic Acid (POWDER little spatula) ( mg)

   **VIP.** Use ceramics cup to weight the element with little spatula
   **VIP. VIP.** Before to add Casein hydrolysed is better washing and DRYING carefully the ceramics cup because those powders became particularly sticky even with really low lv. of humidity trapped in the pored ceramic surface. In addition Casein hydrolysed is really subjected to electrostatic adhesion and for this reason it is important to verify that the border of the ceramic cap will be free of it before going over with MUC5AC.
   **VIP. VIP. VIP** For the MUC5AC morphology, similar to a cotton filament (really light and volatile), it is better to weight it performing compact little balls, so pressing it a little bit, managing to better enter it into the bottle avoiding the stickiness of the mucin on the bottle wall and preventing lost in the transfer of it.

4. After 1 h of continuous stirring, perform the **first pH regulation** adding Trizma Base (POWDER little spatula) ,measuring the pH values, add the base (taking note and add on the excel file the quantity of base added and the pH value before and after the addition), stir for 5 min. at 800 rpm, measure again the pH and so on till the neutrality of the sample.

5. Weight an other bottle, similar to the one of pt.1, complete of cap and stirrer ( g).

6. Transfer the half of the sample present in the bottle at pt.4 in the bottle at pt. 5 and perform again the weight of them.

   **VIP.** It is essential to take note of the actual weight of the two bottles in other to understand the quantity of all the other components to add.
   **VIP. VIP.** From this moment on the reconstruction of the two bottle will go on in parallel as operations and time of reconstruction but independently. For this reason, from this moment on, we will call the bottle firstly weighted at pt.1 (T) = Treated with High LV. of Alginate & the bottle firstly weighted at pt.5 (-) = Treated with Low LV. of Alginat

**BOTTLE (-)**

7. Perform the Δ between the weight registered at pt.6 and the one registered at pt.5 and multiply this quantity for 0.009. This will result to be the value of PAA to add in the following pt.

   **VIP.** This passage is done to have approximately the 0.9% [w/v] of PAA in the reconstructed.
   **VIP. VIP.** By experimental evidence in this moment the reconstructed has quite the same ratio w/v of the pure $H_2O$ and for this reason the density of it is considered ≅ 1 kg/l.
   **VIP. VIP. VIP.** Entering the value obtained in the support excel file is also possible to determine the value of Alginate to add in the following pt.

8. Add under constant stirring at 800 rpm the quantity of PAA (POWDER little spatula) calculated at pt.7.

9. After 30 min. of stirring add without stop stirring (800 rpm) the quantity of Alginate (POWDER little spatula) calculated at pt.7.

10. After 1h of stirring, perform the **second pH regulation** with the same modality and shrewdness followed at pt.4 reaching the optimum value of 6.9 with the gradual addition of Trizma Base (POWDER little spatula).

11. Set the bottle (-) in the fridge.

**BOTTLE (T)**

7. Perform the Δ between the weight registered at pt.6 and the one registered at pt.1 and multiply this quantity for 0.009. This will result to be the value of PAA to add in the following pt.

   **VIP.** This passage is done to have approximately the 0.9% [w/v] of PAA in the reconstructed.
   **VIP. VIP.** By experimental evidence in this moment the reconstructed has quite the same ratio w/v of the pure $H_2O$ and for this reason the density of it is considered ≅ 1 kg/l.
   **VIP. VIP. VIP.** Entering the value obtained in the support excel file is also possible to determine the value of Alginate to add in the following pt.

8. Add under constant stirring at 800 rpm the quantity of PAA (POWDER little spatula) calculated at pt.7.

9. After 30 min. of stirring add without stop to stir (800 rpm) the quantity of Alginate (POWDER little spatula) calculated at pt.7.

10. After 1h of stirring, perform the **second pH regulation** with the same modality and shrewdness followed at pt.4 reaching the optimum value of 6.9 with the gradual addition of Trizma Base (POWDER little spatula).

11. Set the bottle (T) in the fridge.

## RECONTRUCTED Artificial Surfactant, A.S. – SECOND DAY

***Operative condition 25 °C , 1 Atm***

1. Create an environment at T ≅ 0°C that is as coinbent as possible. Use, for example, a Styrofoam box filled with fridge ice lollies.

   **VIP.** SP-D has to be conserved into the freezer but at T ≅ 0°C it freezes for this reason before to start the pt. 1 is necessary to set it into the fridge.

2. Insert the vial of plastic that will contain the A.S. mixture and the media into the freezer.

3. Once a T ≅ 0°C has been reached by vial and support, place them in the environment created in step 1 and seal it.

4. Add the following components to the vial:
   - DPPC (LIQUID micropipette 10 µl) ( µl)
   - PC (LIQUID micropipette 10 µl) ( µl)
   - SP-A (LIQUID micropipette 10 µl) ( µl)
   - SP-C (LIQUID micropipette 10 µl) ( µl)
   - SP-D (LIQUID micropipette 10 µl) ( µl)

   **VIP.** During this operation is essential to keep the vial and the support as more as possible at T ≅ 0°C, for this reason is necessary to maintain them into the conditioned environment as long as possible.
   **VIP. VIP.** By the time that the components up listed are really sensible at Δ >20°C it is essential to keep them out to the fridge as short as possible, with this objective is a saving of time regulate and dispone the micropipette and all the facilities needed for the operation before to exit the component from the freezer.
   **VIP.** SP-D has to be conserved into the freezer but at T ≅ 0°C it freezes for this reason before to start the pt. 1 is necessary to set it into the fridge.

5. Shake the vial with the components inside for 1 min 30 s on a vibrating plate.

6. Set the A.S. so reconstructed into the fridge and wait 30 min before to use it.

## RECONTRUCTED MUCUS (Part 2) – SECOND DAY

***Operative condition 25 °C , 1 Atm***

For repeatability reasons and in order to have the less degradability of the added components and the most homogeneity dispersion of the ones that are required in this second day, it is essential to pay attention to the following rules:

- Once set into the fridge the sample has to stay there for at least 15 min. before being taken again to be manipulated .
- Once exit from the fridge the sample has to stay 10 min. at room temperature than stirred for 5 min at 800 rpm before being manipulated.
- A.S. during all the operation has to stay just for brief time in an environment at T > 0°C.

It is to consider that the sample present in the Bottle (-) will be called (-) Mother Solution, (-) M.S. and the sample present in the Bottle (T) will be called (T) Mother Solution, (T) M.S.

### BOTTLE (-)

1. Weight a vial of glass with inside the stirrer and a cap ( g).

2. Take a quantity of (-) M.S. (LIQUID micropipette 1000 µl) performed as result of the first day of reconstruction and set it in the glass vial of pt.1.

3. Weight again the glass vial at pt.1 , complete of stirrer and cap with 2 or 300 µl of (-) M.S. inside ( g).

4. Performing the Δ between the weight measured at pt.1 and pt.3, enter the value in the excel file and calculate the value of A.S. to add in the following pt.

5. Add under continuous stirring (250 rpm) the value of A.S. gained in the previous pt. (LIQUID micropipette 10 µl) ( µl).

   **VIP.** The stirring velocity in this case is 250 rpm instead 800 rpm not to brake the structure of A.S. , present mainly at the surface of the mucus and not to embed lot of air in the sample.

6. Stir for 5 min. at 250 rpm before performing the analysis.

### BOTTLE (-)

1. Weight a vial of glass with inside the stirrer and a cap ( g).

2. Take a quantity of (T) M.S. (LIQUID micropipette 1000 µl) performed as result of the first day of reconstruction and set it in the glass vial of pt.1.

3. Weight again the glass vial at pt.1 , complete of stirrer and cap with 2 or 300 µl of (T) M.S. inside ( g).

4. Performing the Δ between the weight measured at pt.1 and pt.3, enter the value in the excel file and calculate the value of A.S. to add in the following pt.

5. Add under continuous stirring (250 rpm) the value of A.S. gained in the previous pt. (LIQUID micropipette 10 µl) ( µl).

   **VIP.** The stirring velocity in this case is 250 rpm instead 800 rpm not to brake the structure of A.S. , present mainly at the surface of the mucus and not to embed lot of air in the sample.

6. Stir for 5 min. at 250 rpm before performing the analysis.

**BOTTLE (-)**

7. Weight again the bottle at containing the remaining (-) M.S. and take note of it in the excel file ( g).

8. Perform the Δ between the weight measured at previous pt. and the one registered at pt.5 of the first day of reconstruction, enter the value in the excel file and calculate the value of all the components to add in the following pt.

9. Briefly add, following the order reported and under constant stirring at 800 rpm :
   - NaCl (Sigma) (POWDER little spatula) ( mg)
   - KCl (Sigma) (POWDER little spatula) ( mg)
   - Casein Hydrol. (POWDER little spatula) ( mg)
   - MUC5AC (POWDER little spatula) ( mg)
   - MUC5B (POWDER little spatula) ( mg)
   - PAA (POWDER little spatula) ( mg)
   - Alginic Acid (POWDER little spatula) ( mg)
   - DNA (POWDER little spatula) ( mg)

10. After 1h of stirring, perform the **third pH regulation** with the same modality and shrewdness followed at pt.4 of the first day of reconstruction, reaching the optimum value of 6.9 with the gradual addition of Trizma Base (POWDER little spatula).

11. Weight a vial of glass with inside the stirrer and a cap ( g).

12. Take a quantity of (-) M.S. (LIQUID micropipette 1000 µl) performed as result of the pt.10 and set it in the glass vial of pt.11.

13. Weight again the vial of glass at pt.1 , complete of stirrer and cap with 2 or 300 µl of (-) M.S. inside ( g).

14. Performing the Δ between the weight measured at pt.13 and pt.11, enter the value in the excel file and calculate the value of A.S. to add in the following pt.

15. Add under continuous stirring (250 rpm) the value of A.S. gained in the previous pt. (LIQUID micropipette 10 µl) ( µl).

16. Stir for 5 min. at 250 rpm before performing the analysis.

**BOTTLE (T)**

7. Weight again the bottle at containing the remaining (T) M.S. and take note of it in the excel file ( g).

8. Perform the Δ between the weight measured at previous pt. and the one registered at pt.5 of the first day of reconstruction, enter the value in the excel file and calculate the value of all the components to add in the following pt.

9. Briefly add, following the order reported and under constant stirring at 800 rpm :
   - NaCl (Sigma) (POWDER little spatula) ( mg)
   - KCl (Sigma) (POWDER little spatula) ( mg)
   - Casein Hydrol. (POWDER little spatula) ( mg)
   - MUC5AC (POWDER little spatula) ( mg)
   - MUC5B (POWDER little spatula) ( mg)
   - PAA (POWDER little spatula) ( mg)
   - Alginic Acid (POWDER little spatula) ( mg)
   - DNA (POWDER little spatula) ( mg)

10. After 1h of stirring, perform the **third pH regulation** with the same modality and shrewdness followed at pt.4 of the first day of reconstruction, reaching the optimum value of 6.9 with the gradual addition of Trizma Base (POWDER little spatula).

11. Weight a vial of glass with inside the stirrer and a cap ( g).

12. Take a quantity of (T) M.S. (LIQUID micropipette 1000 µl) performed as result of the pt.10 and set it in the glass vial of pt.11.

13. Weight again the vial of glass at pt.1 , complete of stirrer and cap with 2 or 300 µl of (-) M.S. inside ( g).

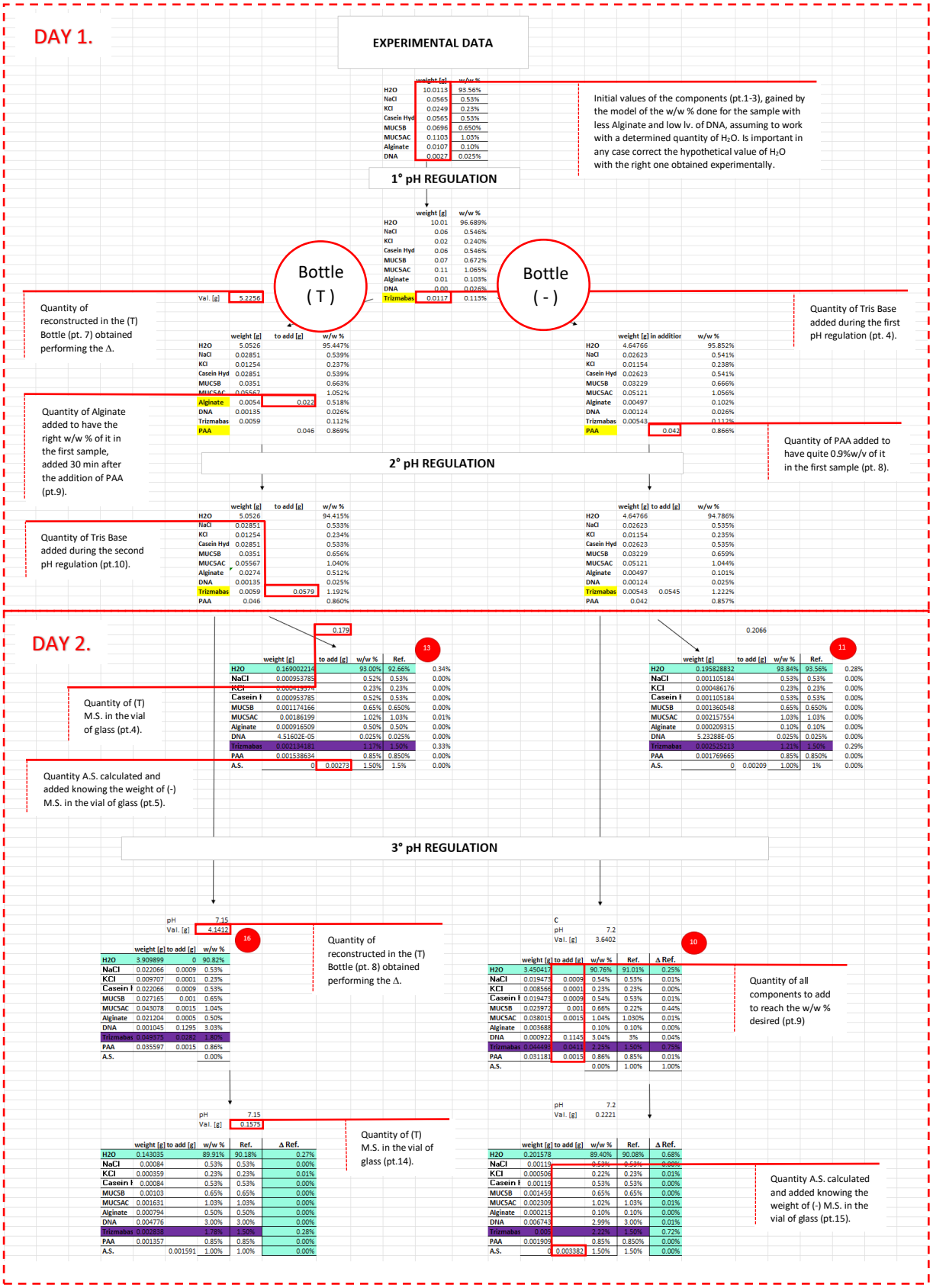
14. Performing the Δ between the weight measured at pt.13 and pt.11, enter the value in the excel file and calculate the value of A.S. to add in the following pt.

15. Add under continuous stirring (250 rpm) the value of A.S. gained in the previous pt. (LIQUID micropipette 10 µl) ( µl).

16. Stir for 5 min. at 250 rpm before performing the analysis.

# SUPPORT EXCEL FILE

In order to be as clear as possible and to give a general view of how the reconstruction works, as follow is reported an image of the excel file to complete while the reconstruction is going on.



# CONSIDERATIONS

To gain a good repeatability of the measurements and in order to maintain in an optimum working state the Rheometer, itis necessary to pay attention to the following points:

- The analysis has to be performed immediately after the end of the 5 min. at pt6.
- The analysis has to be performed at 32 °C and 1 Atm.
- The $H_2O$ to use to maintain constant the humidity around the measuring plate has to be DISTILLATE.
- The humidity in the chamber of the Rheometer has to be constant.
- The two half of the chamber must not touch the superior plate of the Rheometer.
- Once regulated in temperature, before performing the SAOS test, wait 5 min. so that the components into the sample will resume a stable configuration.
- Between an analysis and one other it is necessary to wait 15 min. so that the components into the sample will resume a stable configuration.
- Immediately after the analysis both superior and inferior plates, the two half of the cap and the glass wall of the chamber have to be cleaned and sterilised with DEIONIZED $H_2O$, Methanol, DEIONIZED $H_2O$ and dried carefully with an absorbent paper.

Even if it would be possible to finish to perform and analyse half of the samples generated by a trial of reconstruction in the first day of manipulation, it has been chosen to perform the analysis of all the samples on the same day in order to reduce the interval of time with respect to the analysis of the sample. It is important because of the suspected fast degradability of the Mother Solutions. Thanks to the excel file and the experience gained by DOE 1, this time it has been possible to reduce the composition delta between projected and reconstructed pulmonary mucus to 0.03%, improving the DOE repeatability.