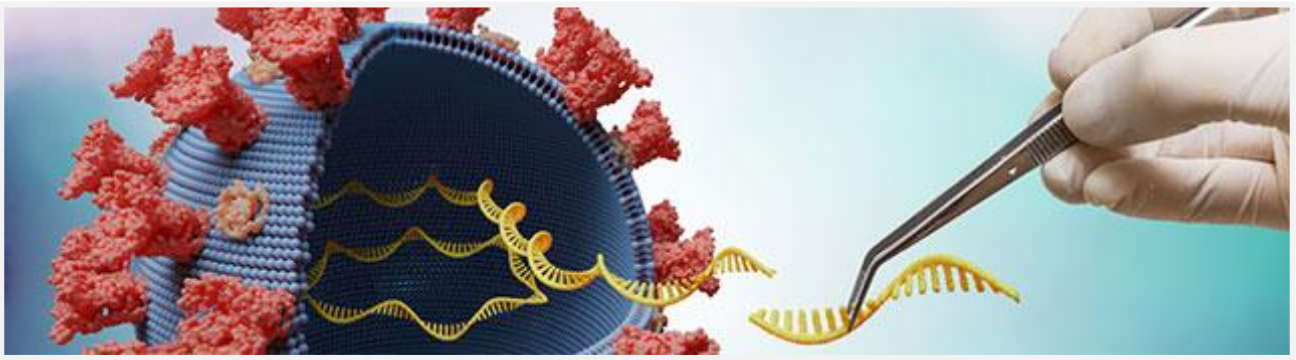
UNIVERSITY OF BOLOGNA

MOLECULAR AND CELL BIOLOGY

MOLECULAR VIROLOGY LAB



https://www.ncbi.nlm.nih.gov/sars-cov-2/

Timeline

Day 1
1.1. Media preparation.
1.2. inoculation of E. Coli, strain SW102, for recombineering.

Day 2
2.1. Preparation of chemical competent E. Coli cells (1).
2.2. Inoculation of E. Coli, strain XL1blue MRF', for M13 titration (1).

Day 3
3.1. Transformation (for recombineering).

Day 4
4.1. M13 titration (1).
4.2. Analysis of the results of the recombineering experiment (3.1).
4.3. Inoculation of positive colonies (4.2) for proteins analysis.

Day 5
5.1. Preparation of the electrophoretic gel (SDS-Page 1).
5.2. M13 titration (2).

Day 6
6.1 Proteins electrophoretic analysis (SDS-Page 2)

Sterile Materials

LB agar plates (w/o antibiotics): 4
LB agar plates (with ampicillin): 4 (50mg/ml)

Liquid LB: 50mls
H_2O: 50mls
1M $CaCl_2$: 2.5mls

Preparation of sterile materials

LB agar plates (with and w/o antibiotics)

5.0 g/l yeast extract
10.0 g/l tryptone
10.0 g/l sodium chloride
15.0 g/l agar

Ampicillin final concentration: 50ug/ml (initial concentration: 50mg/ml)

1. Weight the appropriate amounts of each component (assuming 25mls as the volume required to pour one plate).
2. Transfer the measured powders to an appropriately sized bottle for autoclaving.
3. Transfer MilliQ water to the same bottle and swirl to dissolve possible clumps.
4. Cover the opening of the bottle with a cap or aluminum foil.
5. Label the bottle and tape it with autoclave tape.
6. Place the solution in the autoclave and run it at 121 ℃ for at least 20 min, under 20 psi.
7. During the sterilization process, prepare a plate pouring station.
   a. *Please note that materials handled in this area are supposed to be sterile*.
8. Spray the bench with 70% ethanol solution and wipe it with a paper towel.
9. Remove the required number of plates from the bag and label each individual plate with initials, date, and contents (e. g. antibiotic).
10. Position the working flame in the center of the pouring station.
11. Once the autoclave cycle is complete, slowly open the door and allow any steam to escape from the autoclave.
12. Using insulated gloves, remove sterile materials from the autoclave.
13. Let the molten agar mix cool down to approximately 55℃.
    a. This is usually assessed by holding the bottle without insulating gloves (materials should be warm, but not so hot to hurt: "***touchable hot***").
    b. High temperature results in antibiotics inactivation, while low temperature makes agar harden.
14. Create the sterile environment in the plate pouring station by lighting the working flame.
15. If required, add the antibiotic to the molten gel mix using sterile technique.
16. Swirl the agar bottle to ensure even distribution of the dissolved agar (and of the antibiotic, if present) throughout the solution.
17. Open one plate and directly pour the molten agar mix till the solution covers the entire surface of the bottom half (about 25mls). Close the plate.
    a. Avoid bubbles.

18. Repeat the above step with the remaining plates.
    a. Cap each plate after pouring and stack as you pour.
19. Leave plates on the bench to solidify.
20. Once plates are solidified and dried, they can be inverted and stored at 4°C.

LB medium

5.0 g/l yeast extract
10.0 g/l tryptone
10.0 g/l sodium chloride

1. Weight the appropriate amounts of each component.
2. Transfer the measured powders to an appropriately sized bottle for autoclaving.
3. Transfer MilliQ water to the same bottle and swirl.
4. Cover the opening of the bottle with a cap or aluminum foil.
5. Label the bottle and tape with autoclave tape.
6. Place the solution in the autoclave and run it at 121 °C for at least 20 min, under 20 psi.
7. Once the autoclave cycle is complete, slowly open the door and allow any steam to escape from the autoclave.
8. Using insulated gloves, remove sterile materials from the autoclave.
9. Let the solution cool down on the bench.
10. Store the sterile solution at 4°C.

Preparation of chemical-competent E. Coli (with Calcium Chloride) and induction of the recombinogenic phenotype

Day 1

0. launch an o/n culture by inoculating the bacteria into 1ml LB in a 15ml Falcon tube
   a. from a frozen stock, scrape the top of a -80° frozen vial, **w/o thawing the culture**, and wash the inoculating loop into the medium; from a liquid culture, transfer 5ul into the medium
   b. incubate o/n at 32°C, on a shaker

Day 2

1. inoculate 0.5mls of the o/n culture in 25mls LB medium in a 50mls Falcon tube
2. grow cells on a shaker at 32°C till they get to log phase (OD_{600}=0.2)
   a. it usually takes about 2 hours, depending on the bacteria strain (JM109, X11blue, DH5α,...). **2 hours** is generally fine.
3. Incubate the cultures at 42°C for 15'
   a. prepare 25ml of sterile 0.1M $CaCl_2$ solution and pre-chill it on ice
4. transfer the culture tubes to an ice bucket and incubate for 5'-10'

a. from now on, bacteria should be kept at 4°C

5. centrifuge at 3000 rpm at 4°C for 5'
   a. recovery won't be 100% (still you should clearly see a pellet), but it is critical not to stress the cells. Depending on the centrifuge, you may need to adjust these parameters.
6. discard the supernatant by pouring the supernatant in a container
   a. leave the tubes upside-down for 30-60'' seconds on a paper wiper and then put tubes back on ice
7. add ice cold sterile 0.1M $CaCl_2$ (25mls per tube)
8. gently resuspend bacterial cells by inverting the tubes several times
   a. do not pipette. Try to minimize the time tubes are not on ice. It usually takes about 15', alternating inverting routines with time on ice. Alternatively, bacteria may be gently resuspended on the rocker in the fridge (lay tubes on the rocker, with bacterial pellet on the bottom side)
9. leave the tubes o/n at 4°C

Day 3

   a. prechill 10 eppendorf tubes on ice.

10. without disturbing the white layer at the bottom of the tube, remove the $CaCl_2$ solution from the top, using a 10ml pipette, leaving about 1.4ml in the tube.
Try to minimize the time cells are not on ice

11. gently resuspend the cells with a 2ml pipette
12. Add 0.6ml of sterile glycerol and thoroughly mix the solution
13. Transfer 200ul aliquotes in the pre-chilled sterile eppendorf tubes
14. freeze at -80°C

Transformation efficiency of bacteria obtained following this protocol is comparable to that of commercially available aliquotes. Critical points here are:

- to keep cells sterile and on ice as much as possible
- to avoid (or minimize) mechanical stress while they are in the $CaCl_2$ solution

E. Coli Transformation

1. Label one aliquote of competent bacteria on ice
2. Add 5uls of each DNA and mix well (do not vortex)
3. Leave on ice for 30' (mix occasionally)
4. Incubate the cells/DNA mix at 42°C for 45''.
5. Quickly transfer the transformation tubes to the ice bucket and incubate for about 2'
6. Add 0.8 mls of LB (or SOC)
7. Incubate at 32°C for 1 h, on a shaker
8. Spread the transformed cells on selective plates:
    a. first plate: 10ul
    b. second plate: 100ul
    c. third plate:
        i. Centrifuge the volume left in the tube at top speed on a microfuge for 4''
            1. press the "Pulse" button and hold it till the centrifuge reaches the top speed.
            2. Centrifuge for 4''.
        ii. remove about 800uls of supernatant
        iii. resuspend cells in the volume left in the tube and spread this suspension on the third plate
9. leave plates on the bench till they dry
10. incubate plates upside-down at 32°C, o/n.
11. For clones (colonies) that need to be amplified, prepare individual tubes containing 1ml of LB + appropriate antibiotic.
12. With a sterile toothpick (or micropipette tip), transfer cells from the selected colony into the prepared tube).
13. Incubate o/n on the shaker in the incubator.

List of available samples

Name	Selection	Description
Plasmid	Amp	Acceptor plasmid for recombineering
PCR	-	Insert for recombineering
pEGFP	Amp	Gfp constitutive expression
mC	Amp	mCherry constitutive expression
dTomato	Amp	dTomato constitutive expression
pDsRed2r2	Amp	DsRed2 constitutive expression

M13 phage titration

1. Inoculate 1 ml of LB with a swatch of XL1 Blue-MRF' and incubate at 37°C with shaking to mid-log phase (4-8 hrs; OD_{600} ~ 0.5).

   Cells can be stored at 4°C o/n

2. While cells are growing, prepare sterile Top Agar, 5ml per tube per expected phage dilution. Maintain Top Agar at 45-50°C.

   Top Agar
   5.0 g/l yeast extract
   10.0 g/l tryptone
   10.0 g/l sodium chloride
   7.5 g/l agar

3. Prepare serial 1:10 dilutions of the phage stock using 0.9 ml LB. **The first dilution should be a 1:100 dilution to minimize the volume of the original phage stock used.** Dilutions in the range expected to produce valid plate counts should be 1:10 dilutions (the exact number of tubes will depend on the number of dilutions you have planned in your dilution scheme). **Dilutions must be performed aseptically.**

   For each tested dilution:

4. Transfer 200ul of the XL1 Blue MRF' mid-log phase culture in 15 mls Falcon tubes.
5. Transfer 10ul of the phage dilution in the tube containing the 200ul XL1 Blue MRF' suspension.
6. Mix well and incubate at 37°C for 15'
7. Incubate for additional 15' at 37°C with shaking.
8. Add 5 ml top agar to tubes. Gently mix the tubes, and immediately pour onto prewarmed LB plates. Spread overlay across the plate by tilting and rotating the plate until overlay is evenly distributed. Do not attempt to spread overlay further once it starts to set. This will produce a grainy, opaque overlay, which will make plaques difficult to see.
9. Allow plates to cool until agar has set. Invert the plates (lid side down), and incubate 12-24 hours at 37°C. Count your plates, record the results in your lab book, and calculate the concentration of pfu in the original stock.

SDS Page electrophoresis

Electrophoretic gel preparation

Assemble the glass sandwich following the manufacturer instructions.

Main gel preparation

Choose the appropriate acrylamide concentration (%) for your protein of interest considering its size (kDa) and the linear range of separation. Calculate the amount of 30% Acrylamide/bis and H_2O based on the desired final concentration.

Acrylamide concentration (%)	Linear range of separation (kDa)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

1. In a falcon tube, prepare 5ml of acrylamide solution, according to the following instructions. Please note that 5ml is sufficient to prepare one 0,75mm minigel. For 1.5mm minigels, prepare 10ml of solution.
2. 10% APS and TEMED must be added **immediately before pouring the gel** into the sandwich, as they trigger the irreversible polymerization reaction.
3. Mix well by inverting the tube several times.

Main gel (5ml)

30% Acrylamide/bis	
H_2O	
Tris 1.5M pH 8.8	1.250
SDS 10%	0.050
APS 10%	0.050
TEMED	0.002

4. Transfer enough gel solution into the sandwich, in such a way that about 1cm is left from the bottom of the combs that will be subsequently added on top of the gel. This volume corresponds to about 3.5ml for the 0.75mm minigels.
5. Gently overlay about 500ul of H_2O on top of the gel solution.
6. Let it stand for about 30'-60'. Polymerization is indicated by the appearance of a clear front at the edge of the two solutions (acrylamide-H_2O). Additionally, polymerization can be checked by looking at the status of the solution left in the preparation tube.

7. Pour off the water from the top of the gel by inverting the entire sandwich.

Stacking gel preparation

1. Prepare 2.5ml or 5ml stacking solution for 0.75mm or 1.5mm minigels, respectively.
2. As for the main gel, 10% APS and TEMED must be added immediately before pouring the solution into the sandwich.

Stacking gel (2.5ml)

30% Acrylamide/bis	0.330
H_2O	1.815
Tris 1.0M pH 6.8	0.315
SDS 10%	0.025
APS 10%	0.025
TEMED	0.002

3. Mix well by inverting the tube several times.
4. Fill the glass sandwich with the stacking solution.
5. Insert the comb on top of the sandwich, avoiding trapping any bubble between the solution and the bases of the comb's teeth.
6. Let it polymerize for about 15 minutes.

For storage, gels can be wrapped in transparent microfilm (with a wet napkin on top of the comb, to ensure the proper moisture) and kept at 4°C for few days.

Electrophoretic analysis of proteins expression

Electrophoretic apparatus preparation

1. Assemble the electrophoretic apparatus according to the manufacturer directions.
2. Fill the inner chamber with 1x running buffer.
3. Add 1x running buffer into the outer chamber to cover the bottom of the gel.
4. Gently remove the comb from the top of the gel, paying attention to preserve the wells' walls.

Samples preparation and electrophoresis

5. Transfer 250ul of the o/n culture in one Eppendorf tube.
6. Centrifuge for 4'' at top speed (on most benchtop microfuges, it can be achieved by holding down the "pulse" button for about 15'').
7. Remove the supernatant.
8. Resuspend the pellet in 50ul of 1x loading buffer. Mix well by vortexing.
9. Incubate for 15' at room temperature (RT).

> *The described procedure is adapted from the standard procedure, in which the complete denaturation of samples is obtained by incubation at 95°C for 5'. Complete denaturation is required to avoid migration artifacts coming from proteins 3D structures. As we will visualize the expression of the exogenous protein by taking advantage of its fluorescence, it is critical to maintain its functionality, which would be lost after complete denaturation. The 15' incubation at RT results in mild denaturation conditions that should preserve some functionality.*

10. Consolidate samples by centrifugation at top speed on a benchtop microfuge for few seconds.
11. Load samples (up to 20ul for 0.75mm gels, up to 40ul for 1.5mm gels) in the gel wells, paying attention to avoid the bottom DNA pellet.
12. Close the electrophoretic apparatus and start the run by connecting a power supply set at 160V, constant voltage.

Disassembly of the electrophoretic apparatus and proteins visualization

13. Turn off the power supply and take off the lid from the apparatus.
14. Remove the inner chamber and pour the running buffer into the outer chamber.
15. Disassemble the inner chamber and free the glass sandwich.

16. Gently open the glass sandwich by applying a constant pressure at the edge of the glass plate (it can be done with just your fingers).
17. Carefully, transfer the gel on a piece of transparent microfilm.
18. Place the gel (on the microfilm) on a UV transilluminator.
19. Close the UV transilluminator with the Plexiglas lid for eyes' protection (security shield).
20. Lower the environmental light.
21. Turn on the UV light.