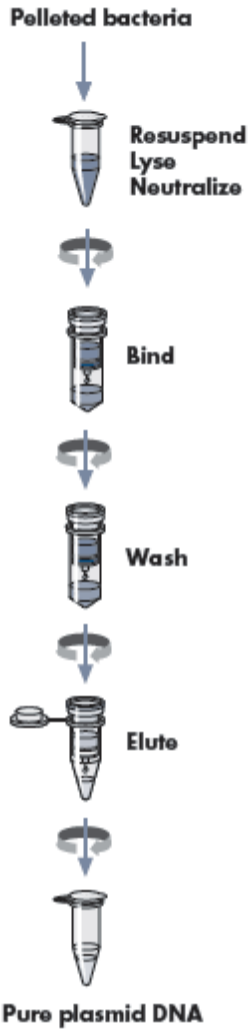
- DA STAMPARE E PORTARE IN LABORATORIO -

Molecular Biology - Lab Experience

Purification and digestion of pGL3-Basic plasmid

Student Workstation

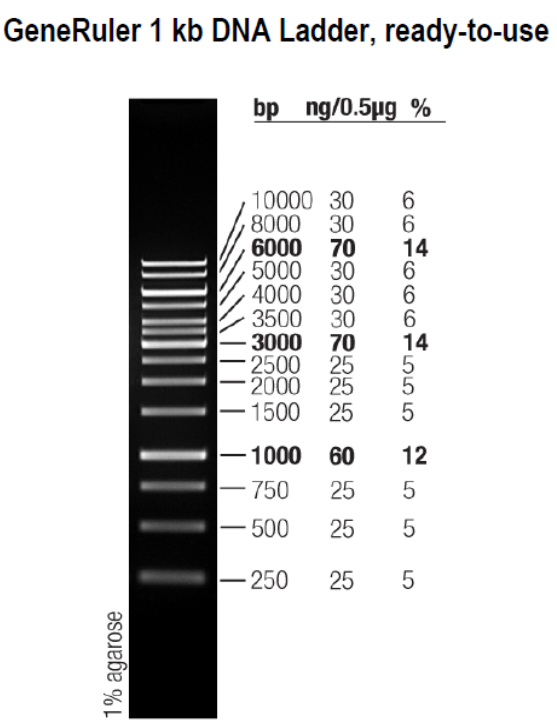
Material	*Quantity*
Empty 2 mL tube (labeled with a dot)	**1**
Empty 1.5 mL tube	**1**
Empty 0.5 mL tube	**1**
Empty 0.2 mL tube	**1**
2 mL tube with colored solution	**1**
1.5 mL tube with ultrapure water ("H_2O")	**1**
1.5 mL tube with bacterial pellet	**1**
QIAprep 2.0 spin column (in 2 ml receiver tube)	**1**
1.5 mL tube with Buffer P1 ("P1")	**1**
1.5 mL tube with Buffer P2 ("P2")	**1**
1.5 mL tube with Buffer N3 ("N3")	**1**
1.5 mL tube with Buffer PB ("PB")	**1**
1.5 mL tube with Buffer PE ("PE")	**1**
1.5 mL tube with Buffer EB ("EB")	**1**
0.5 mL tube with Digestion Buffer ("DIG BUFF") (on ice)	**1**
0.5 mL tube with Loading Buffer ("LOAD BUFF") (on ice)	**1**
UV cuvette for spectrophotometry	**1**
Pipette 2-20 µL (P20)	**1**
Pipette 20-200 µL (P200)	**1**
Pipette 100-1000 µL (P1000)	**1**
Tips 20-200 µL	**1** box
Tips 1000 µL	**1** box
Marking pen	**1**
Bottle for solid waste	**1**
Bottle for liquid waste	**1**
Paper towel	**1**
Tinfoil	**1** piece



Common Materials/Instruments

10 mL pipettes
20-well combs
Agarose
Black waste bags
Buffer TAE 50x
Cuvettes for spectrophotometry
Distilled water

Gel electrophoresis trays and chambers
Glass flasks
Gloves
Graduated cylinders
Green Gel
HindIII in cooling block
Ice
Microcentrifuge
Microwave oven
Molecular weight ruler (on ice)
Parafilm
Pipettes 0.2-2 µl (P2)
Pipettor
Power supply
Scales
Spectrophotometer
Thermal cycler
Tips 2 µL
UV-transilluminator



PROTOCOL

A. PURIFICATION of plasmid pGL3-Basic from bacterial culture by QIAprep Spin Miniprep Kit (Qiagen)

1. Receive Buffer P1 and your 1.5 mL tube containing pelleted bacterial cells. Label the pellet 1.5 mL tube with your position number.

2. Mix Buffer P1 by pipetting without making bubbles. Resuspend the bacterial pellet in **250 µL Buffer P1** by pipetting up and down until no cell clumps remain.

3. Add **250 µL Buffer P2** and mix thoroughly by inverting the tube 6 times. The cell suspension will turn blue. If the suspension contains localized colorless regions, continue mixing the solution until a homogeneously colored suspension is achieved. Do not allow the lysis reaction to proceed for more than 5 min.

4. Add **350 µL Buffer N3** and mix immediately and thoroughly by inverting the tube 6 times. The solution should become cloudy and colorless.

5. Centrifuge for **10 min at 13,000 rpm** at room temperature in a table-top microcentrifuge. A compact white pellet will form.

6. *In the meantime*: unpackage the QIAprep 2.0 spin column and label it with your number; label the clean 1.5 mL tube for the final elution by writing your number + "plasmide" on the cap and on the tube side. Cover the side writing by scotch tape.

7. Without touching the white pellet, apply **800 µL** (in one shot) of the supernatant from step 5 to the QIAprep 2.0 spin column by pipetting, without touching the silica column membrane.

8. Centrifuge for **1 min at 13,000 rpm** at room temperature.

9. Discard the flow-through by pouring it into the bottle for liquid waste and allow the tube dry, in inverted position, on a paper towel (over a tinfoil piece). Place the QIAprep 2.0 column in the same tube again.

10. Add **500 µL Buffer PB** to the column without touching the silica membrane.

11. Centrifuge for **1 min at 13,000 rpm** at room temperature.

12. Discard the flow-through as previously described (see step 9).

13. Add **750 µL Buffer PE** to the column without touching the silica membrane.

14. Centrifuge for **1 min at 13,000 rpm** at room temperature.

15. Discard the flow-through as previously described (see step 9).

16. Discard towel and tinfoil in the black waste bag.

17. Centrifuge at **13,000 rpm** at room temperature for an additional **1 min** to remove residual wash buffer.

18. Discard the tube and place the QIAprep 2.0 column in the previously labeled clean 1.5 mL tube.

19. To elute DNA, add **50 µL Buffer EB** to the center of the QIAprep 2.0 spin column without touching the silica membrane.

20. Incubate for **5 min** at room temperature (on the lab bench). In the meantime: fill your ice bucket with ice.

21. Centrifuge for **1 min at 13,000 rpm** at room temperature.

22. Discard the column and close the tube.

23. Keep your sample on ice.

B. SPECTROPHOTOMETRIC QUANTIFICATION of purified pGL3-Basic

24. *Before measuring the samples (once for each lab session):*

- *Switch the spectrophotometer on.*
- *Choose the correct application.*
- *Transfer 1 mL of ultrapure water (blank) in a clean UV cuvette.*
- *Insert the cuvette into the spectrophotometer and measure the blank sample.*

25. Find your UV cuvette and add:

first, 985 µL of water;
then, 15 µL of your purified pGL3-Basic, after mixing it by finger-vortex.

26. Mix by pipetting with P1000, without making bubbles. Close the cuvette with the red cap.

27. Insert the cuvette into the spectrophotometer and measure your sample.

A_{260} = _____

A_{260}/A_{280} ratio = _____

28. Calculate the CONCENTRATION of your plasmid,

*considering that **1 OD_{260} corresponds to a 50 µg/ml** concentration:*

_____

_____

_____

Resulting **pGL3-Basic concentration**: _____

29. Discuss about the PURITY of your plasmid,

by evaluating the A_{260}/A_{280} ratio:

_____

_____

30. *Question:*

For the digestion you will need **1 µg** of your pGL3-Basic plasmid: how many microliters will be necessary?

_____

_____

_____

C. Preparation of a 0.8% AGAROSE GEL

(1 gel for 18 groups)

31. *For each gel: prepare **1 L** of running buffer **TAE 1x** (40 mM Tris acetate, 1 mM EDTA, pH 8) in the graduated cylinder, starting from buffer TAE 50x.*

_____
_____
_____

32. *Prepare the tray to pour the gel with two 20-well combs.*

33. *Prepare your **0.8%** (w/v) agarose gel.*

- *Dissolve **…….. g agarose** in **150 mL buffer TAE 1x** in a glass flask.*
- *Boil the solution in a microwave oven.*
- *Add **1.5 µL Green Gel** and mix.*
- *Pour the gel into the gel tray and remove possible bubbles.*
- *Allow the gel to solidify at room temperature for at least 20 min.*

D. DIGESTION of purified pGL3-Basic with HindIII

34. Find your 0.2 mL tube and label it by writing your number + "DIG" on the cap and on the tube side.

35. Add the following reagents to the bottom of the 0.2 mL tube, in the indicated order:

Ultrapure water	….. µL	
Digestion Buffer 10x	….. µL	final concentration = 1x
pGL3-Basic (your plasmid, as calculated in step 30)	….. µL	amount = 1 µg
HindIII* 1U/µL	….. µL	amount = 1 U
Total	20 µL	

*add HindIII at last and then immediately place the sample on ice.

36. Mix by pipetting (with P20 set to 10 µL) and cap the tube.

37. Spin down the tubes to force the reactions to the bottom and to dissipate bubbles.

38. Incubate at **37°C for 15 min**.

39. Inactivate the enzyme by incubating at **80°C for 10 min**.

40. Keep samples on ice.

E. GEL ELECTROPHORESIS of not digested and HindIII-digested pGL3-Basic

In the meantime:

41. *Set up your gel electrophoresis apparatus:*

- *Remove the tray and place it onto the electrophoresis chamber in the correct position.*
- *Submerge the gel beneath 2 to 6 mm of buffer TAE 1x.*
- *With both hands carefully remove the combs from the solidified gel.*

Samples to be loaded on the gel:

NON DIG: not digested pGL3-Basic

DIG: HindIII-digested pGL3-Basic

42. Prepare your NON DIG sample (not digested pGL3-Basic):

- Find your 0.5 mL tube and label it by writing your number + "NON DIG" on the cap and on the tube side.

- Add the following reagents to the bottom of the 0.5 mL tube, in the indicated order:

Ultrapure water	….. µL	
Loading Buffer 6x	….. µL	final concentration = 1x
pGL3-Basic (purified from bacteria)	….. µL	amount = 500 ng
Total	12 µL	

The DIG sample (HindIII-digested pGL3-Basic, prepared in step 35) will be directly loaded: 10 µL (500 ng).

For each gel 4 molecular weight rulers (*GeneRuler 1 kb DNA Ladder*) will be directly loaded too: 5 µL for each well.

43. Load **10 µL** of each sample (NON DIG and DIG) onto your gel, after mixing by pipetting.

44. *Someone will load **5 µL** of molecular weight ruler ("M"), according to the gel loading scheme.*

45. *Run the agarose gel with buffer TAE 1x at **100 V** for **30-40 minutes**. Do not let the colored front migrate out of the gel.*
DNA samples will migrate towards the anode during electrophoresis.

46. Check your result at UV-transilluminator.

pGL3-Basic (*4818 bp*)

[…] CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT CCGCGCACAT

TTCCCCGAAA AGTGCCACCT GACGCGCCCT GTAGCGGCGC ATTAAGCGCG

GCGGGTGTGG TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT

AGCGCCCGCT CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG

GCTTTCCCCG TCAAGCTCTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT

AGTGCTTTAC GGCACCTCGA CCCCAAAAAA CTTGATTAGG GTGATGGTTC

ACGTAGTGGG CCATCGCCCT GATAGACGGT TTTTCGCCCT TTGACGTTGG

AGTCCACGTT CTTTAATAGT GGACTCTTGT TCCAAACTGG AACAACACTC

AACCCTATCT CGGTCTATTC TTTTGATTTA TAAGGGATTT TGCCGATTTC

GGCCTATTGG TTAAAAAATG AGCTGATTTA ACAAAAATTT AACGCGAATT

TTAACAAAAT ATTAACGCTT ACAATTTGCC ATTCGCCATT CAGGCTGCGC

AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT TACGCCAGCC

CAAGCTACCA TGATAAGTAA GTAATATTAA GGTACGGGAG GTACTTGGAG

CGGCCGCAAT AAAATATCTT TATTTTCATT ACATCTGTGT GTTGGTTTTT

TGTGTGAATC GATAGTACTA ACATACGCTC TCCATCAAAA CAAAACGAAA

CAAAACAAAC TAGCAAAATA GGCTGTCCCC AGTGCAAGTG CAGGTGCCAG

AACATTTCTC TATCGATA GGTACCGAGC TCTTACGCGT GCTAGCCCGG

GCTCGAGATC TGCGATCTAA GTAAGCTTGG CATTCCGGTA CTGTTGGTAA

AGCCACCATG GAAGACGCCA AAAACATAAA GAAAGGCCCG GCGCCATTCT

ATCCGCTGGA AGATGGAACC GCTGGAGAGC AACTGCATAA GGCTATGAAG

AGATACGCCC TGGTTCCTGG AACAATTGCT TTTACAGATG CACATATCGA

GGTGGACATC ACTTACGCTG AGTACTTCGA AATGTCCGTT CGGTTGGCAG

AAGCTATGAA ACGATATGGG CTGAATACAA ATCACAGAAT CGTCGTATGC […]