GENE CLONING LAB COURSE

2024-2025

Instructions for preparation

- Read the introductory lecture of this lab course and the protocols.
- Represent all the different steps you are going to follow to make sure you understand them.
- Try to answer all questions while you are preparing the course, this will help you to understand what you are going to work on.
- **It is critical to do in advance the sequence analysis and the expected fragments of:**
  - **the digested pG11 plasmid,**
  - **the digested insert (His-GFP DNA molecule)**
  - **the recombinant plasmid obtained after ligation of the digested insert in the digested plasmid.**

For the first day of the lab course session, we ask you to:

*Analyse the sequence of the DNA fragment encoding His-GFP by localizing the nucleotide sequences of interest (initiation codon, His-tag, restriction sites, …).

*Simulate the digest of the DNA fragment encoding His-GFP by the restriction enzymes that you are going to use during the lab course and indicate the expected size.

*Simulate the digest of the pG11 plasmid by the same 2 restriction enzymes and indicate the expected size.

*Simulate the cloning of the digested insert into the digested plasmid and indicate the expected size of the recombinant plasmid. Think about the different possibilities that could happen (no matter the likeliness with which it could happen).

To do so, you are advised to use the software **ApE** (it can be downloaded for free from Internet), or any other software you might know or want to use.

Your cloning preparation (work on the nucleotide sequences) can be summarized as a table or a scheme.

Safety issues

It is the responsibility of each student to manipulate following the appropriate hygiene and safety conditions.

Report

All the questions asked in this document are meant to help you and guide you throughout the protocols. They are some thinking guidelines to help you writing up your lab book. You must be able to answer all of them.

I. OBJECTIVES

I.1. Main objective of the lab course

The main objective of this lab course is to produce a strain of *E. coli* producing a His-tagged GFP protein. To do so, you will clone the His-tagged GFP-encoding gene (called "insert") as a [*Nhe*I-*Eco*RI] fragment into an expression plasmid (called "vector" or "plasmid") and integrate it into a strain of *E. coli*. The different experimental steps to be performed are as follows:

- Digest the insert (previously amplified for you) as well as the plasmid, using *Nhe*I and *Eco*RI.
- Purify the insert and the vector.
- Ligate the insert and the vector: the resulting vector is called "recombinant plasmid".
- This recombinant plasmid will be transferred by transformation in bacterial cells that will be selected on an appropriate culture medium.
- The colonies of interest will be grown in liquid medium to allow the amplification of the recombinant plasmid.
- After extraction and purification of the plasmid, the success of the cloning will be determined.

I.2. Technical objectives

- To manipulate in conditions that are appropriate in terms of sterility, temperature…
- To understand and perform a cloning strategy
- To learn how to search and analyse nucleotide sequences
- To purify DNA plasmid using a commercial kit and to learn the required analysis methods
- To prepare, load and run analytic agarose gels
- To transform an *E. coli* strain with a DNA plasmid and select the resulting transformants of interest
- To set up a strategy allowing the validation of the cloning results, using molecular biology tools.

II. PRINCIPLES AND TECHNIQUES

II.1. GFP

The GFP (Green Fluorescent Protein)-encoding gene was originally isolated from the bioluminescent jellyfish *Aequorea victoria*. It is commonly used as a protein tag to localize specific proteins in cells. *In vivo*, GFP complexes with aequorin which transfers energy to GFP, result in the fluorescence of the protein. *In vitro*, in the absence of aequorin, GFP can be excited by ultraviolet radiation. The unique three-dimensional conformation of GFP causes it to resonate when exposed to UV light and give off energy in the form of visible green light.

The native sequence has been modified genetically to generate different forms of the enzyme that gives more or less fluorescence and that can easily be cloned into various plasmid vectors. This sequence is the one you are provided with.

II.2. Bacterial strains

In microbiology, it is critical to select the bacterial strain that is appropriate for each experiment. Several bacterial strains are commonly used in laboratories. They are different from the equivalent wild strains, as they are genetically manipulated.

In particular, the *Escherichia coli* DH5-α strain is commonly used to perform gene cloning and plasmid amplification. Its genotype is: *fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*. These mutations make the DH5-α strain an excellent tool for molecular biology and genetic studies. Some of them will be important for this lab course:

- a mutation in the *endA* gene renders the strain deficient in endonuclease activity, which improves plasmid yield and therefore the quality of the plasmid purification step.
- the *recA* mutation inhibits the activity of a DNA-dependent ATPase, which limits the recombination of plasmid with the bacterial genome, and therefore increases the stability of the insert.

II.3. Plasmids and screening methods after transformation

Different types of plasmids have been constructed and are used as tools in Biotechnology. The main plasmid types are cloning plasmids, expression plasmids, gene knock-down plasmids and reporter plasmids. In this lab course, you will manipulate an expression plasmid (named pG11). Many expression plasmids contain an inducible promoter (as opposed to constitutive promoters). Two commonly used induction systems are: the use of sugar (*e.g.* arabinose) and IPTG (Isopropyl β-D-1-thiogalactopyranoside).

II.3.a. Regulation of the arabinose operon (in pG11 plasmid)

The 3 structure genes (*araB*, *araA* and *araD*) composing the arabinose operon encode three enzymes that are involved in the breakdown of arabinose (Fig. 1). Transcription of these 3 genes is under the control of a single promoter (P_{BAD}) and requires the presence of 4 components: the DNA template (promoter and operon), the RNA polymerase, a DNA binding protein (AraC) and arabinose. The protein AraC binds to the DNA at the binding site for the RNA polymerase.

- When grown in the **presence of arabinose**, bacteria take it up. Once inside, the arabinose interacts directly with the protein AraC which is bound to the DNA. The interaction leads to a change of conformation of AraC, which in turn promotes the binding of RNA polymerase and the transcription of the 3 genes araB, araA and araD.

- In the **absence of arabinose**, two AraC proteins dimerize and the complex binds to two operating DNA binding site inducing a twist of DNA strands with a loop of 210 bp which disables the binding of the RNA polymerase and thus the transcription of the structure genes of the operon. The system is thus shut off. The originality of the arabinose operon is that the same protein (AraC) could be both a positive or a negative regulator, depending on the presence of the arabinose substrate.

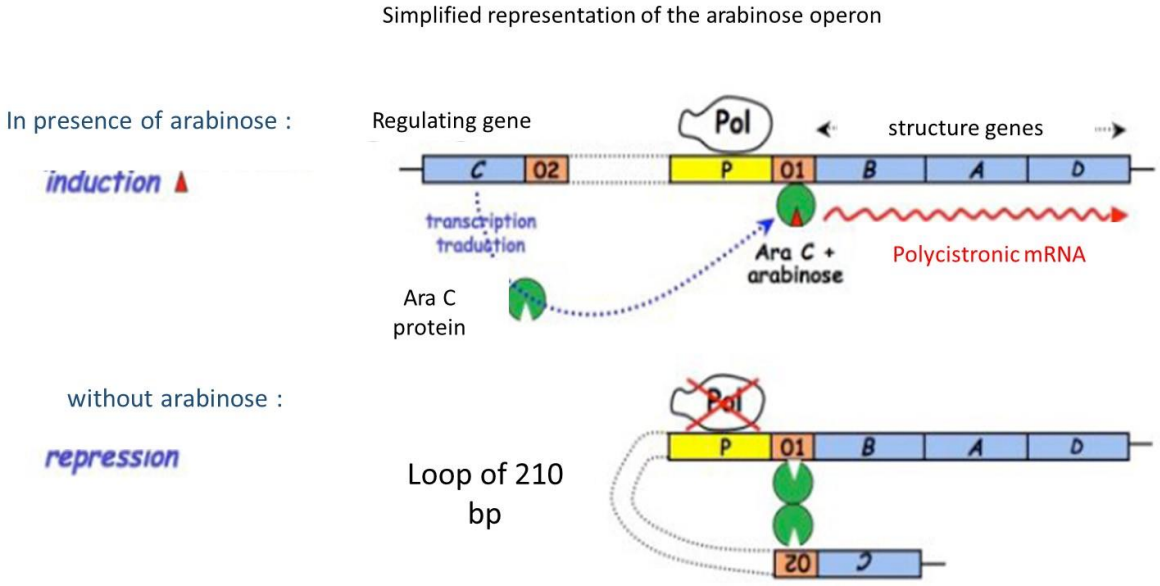

Figure 1. Regulation of the arabinose operon.

II.3.b. Regulation of the lactose operon (only for your information as it is a commonly used technique in many labs)

In bacteria, certain genes are organized in operons. This organisation concerns in particular the co-expression of genes encoding proteins that are involved in the same metabolic pathway, for example, the lactose operon (Fig. 2). This operon contains the 3 genes that are required for the degradation of lactose: *lacZ*, *lacY* and *lacA*, encoding respectively:
- β-galactosidase, an enzyme that cleaves lactose into glucose and galactose.
- Thiogalactoside transacetylase which role is still not fully known. It acetylates β-galactosides that cannot be metabolized, that way they can get out of the cell by diffusion through the cell membrane.
- Lactose permease: this membrane protein allows the entrance of lactose in the cell.

The expression of *lac* genes is controlled by a negative regulation mechanism. Upstream the lactose operon is located a regulator region containing a promoter and an operator, as well as the gene *lacI* encoding a repressor that binds the operator. The expression of *lacI* is constitutive, but the affinity of the repressor for the operator is decreased in the presence of lactose. Consequently, the *lac* operon is repressed in the absence of lactose, and is derepressed in the presence of lactose.

It is possible to induce artificially the *lac* operon using a lactose analog that cannot be metabolised, the isopropyl β-D-1-thiogalactopyranoside (IPTG), in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). The synthesized β-galactosidase catalyzes the hydrolysis of X-gal and releases a blue product.

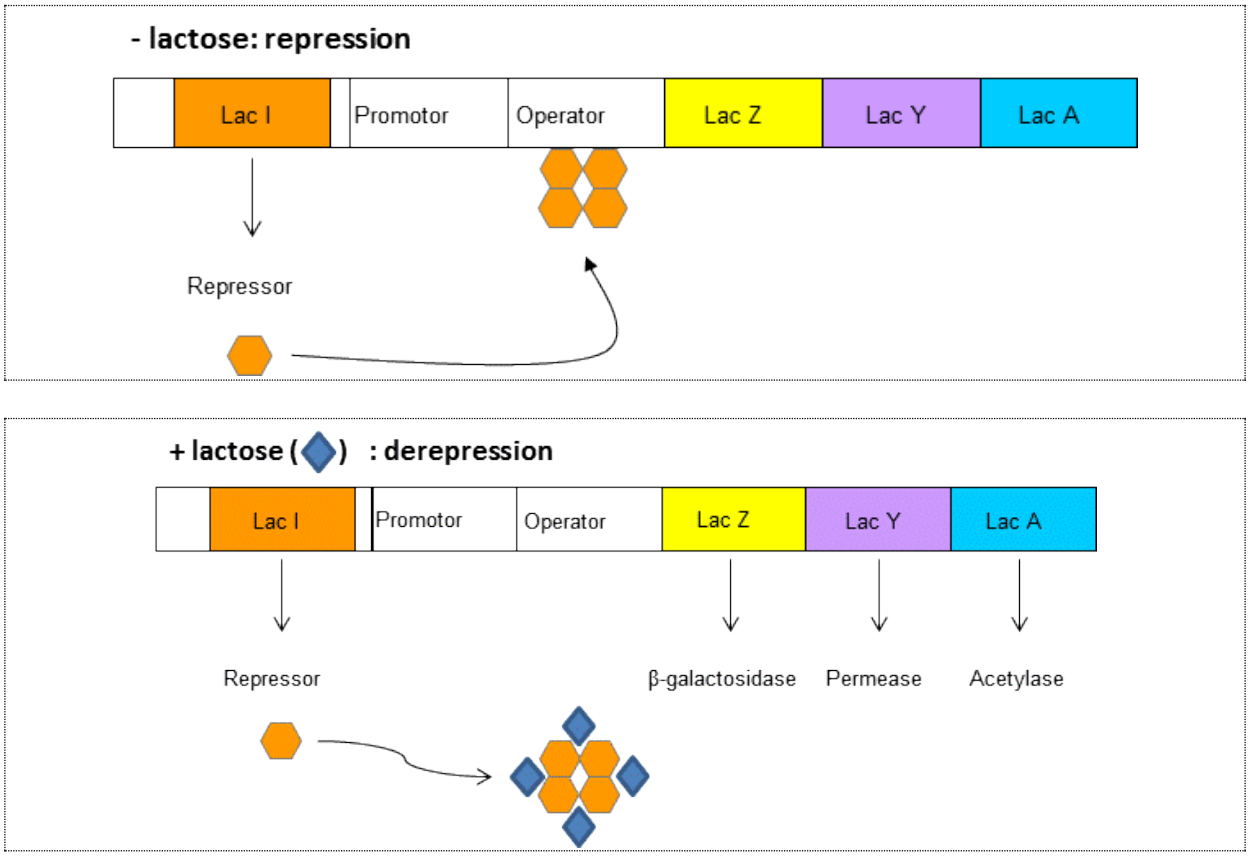

Figure 2. Organization and regulation of the lactose operon in *E. coli*.

Experimental tip: the DH5-α strain, unlike other commonly used strains, does not overexpress the LacI repressor. Therefore, the copy number of plasmids transferred to the bacterial cells exceeds the

number of repressor molecules synthesized by the cells. The induction of the promoter expression does not require the presence of IPTG in this strain.

Alpha-complementation

The *E. coli* β-galactosidase is a homo-tetramer composed of α (N-terminal fragment) and ω (C-terminal fragment) subunits. The *lacZ M15* allele of the bacterial strain encodes only the ω fragment. To obtain a fully functional enzyme, it is then necessary to provide the bacteria with the α fragment via a plasmid: in this case, the β-galactosidase alpha-complementation can occur (Fig. 3).

This biological property is used to perform insertional cloning, as it allows us to discriminate bacteria that contain a recombinant plasmid with those that do not:

- in the absence of insert, the *lacZ* gene is functional; the galactosidase activity gives a blue color to the bacterial colonies.
- in the presence of insert, the *lacZ* gene is not functional; the β-galactosidase is not synthesized, and the bacterial colonies keep their wild white color.

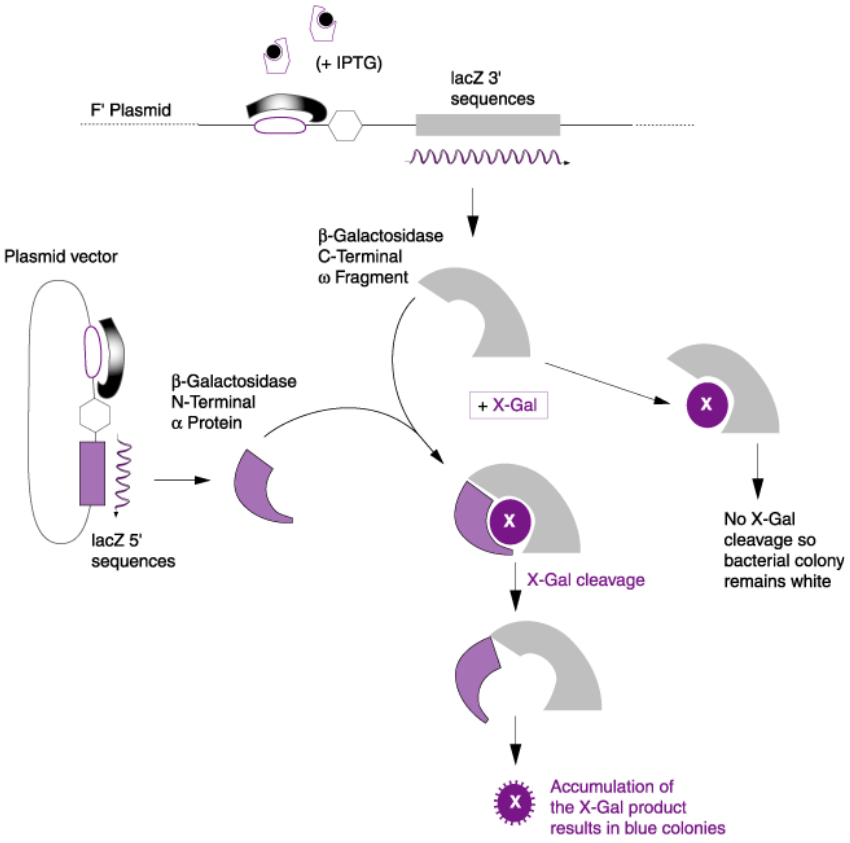

Figure 3. Alpha-complementation of β-galactosidase.

III. PROTOCOLS

! The following order does not follow the chronological order in which the protocols will be realized: this will be established depending on the time constraints.

Parts highlighted in green correspond to experiments that will not be done by yourselves during the lab course, but you must understand their aim and principle.

III.1. Preparation of culture media

All the pairs/trios must organize itself to prepare an appropriate quantity of nutritive LB medium that is required for the whole class (selective and non-selective medium). For that, you must determine in advance the composition of each prepared culture medium.

You must plan **in advance** the number of Petri dishes you must prepare with solid medium and which medium will be poured in each dish, depending on which bacterial suspension you are going to spread after transformation and which ones grows or does not grow on which medium. Keep in mind that you will need to perform some controls (ligation controls and transformation controls). To do so, you are asked to prepare and complete a table that summarizes all the tested conditions, similarly to the following template (add as many lines as required):

Ligation condition	**Transformation condition**	**Composition of the required nutritive medium**	**Number of required Petri dishes**	**Volume of medium (count ~20 mL per Petri dish)**	**Expected colonies (zero / a few / many)**	**Expected phenotype of colonies**
A	**A**					
B	**B**					
C	**C**					
D	**D**					
-	**E**					
-	**F**					

Remember to label all the plates before pouring the culture medium, so that you can identify them.

III.2. Cloning

III.2.a. Enzymatic digestion of the insert and the vector

You will be provided with two solutions containing the insert His-tagged GFP and the pG11 plasmid respectively.

You must understand by which technique the provided insert was obtained.

The pG11 plasmid presents 2 main regions of interest *Amp*^{R} and *ori* (see map in the prelab lecture), describe their role.

► Set up 2 double digestion reactions in a final volume of 20 µl, following the given instructions:

- Reagents must be transferred <u>in the order</u> indicated in the table below. **Why ?**
- The final concentration of each reactive must be strictly respected.

	pG11 plasmid	**Insert**
Ultra-pure H_2O	qsf 20 µl	qsf 20 µl
Restriction buffer (10 X): <u>YOU MUST DETERMINE WHICH ONE IN ADVANCE</u>	1 X	1 X
DNA (pG11 @250 ng/µL; Insert @25 ng/µL)	500 ng	150 ng
***Eco*RI-HF (5,000 U/ml)**	5 U	5 U
***Nhe*I-HF (5,000 U/ml)**	5 U	5 U

⚠ Keep the enzymes at 4 °C and prepare the mixtures at 4°C.
► Mix the contents of each tube and centrifuge at 13,000 rpm for 5 s (shortspin) if necessary.
► Incubate in a water bath at 37 °C for 1 h.
► Shortspin the tubes at 13,000 rpm for 30 s and keep them on ice.

Which types of extremities (generic terms) are generated on the DNA molecules upon digestion using *Eco*RI and *Nhe*I, respectively?

III.2.b. Electrophoresis in an analytic agarose gel

► Volunteers will prepare a sufficient number of 1 % agarose gels using TAE buffer (1 X): this will be done in the presence of teacher.

⚠ Follow precisely the procedure that will be described to you, using only the appropriate working spaces and always using gloves: ethidium bromide is a CMR substance.

► During gel polymerization, prepare your 2 samples (mix of digested DNA and loading buffer):

Sample of digested plasmid or insert	500 ng pG11 or 150 ng insert
Loading buffer (6 X or 10 X)	1 X final
H_2O UP (only if necessary)	qsf 24 µl

► Mix gently using a micropipette to avoid making bubbles.
► Load each sample in a well of the gel (respect the loading plan defined for the whole class).
► Start the migration in the presence of the teacher.
► Observe the results using a transilluminator.

⚠ Make sure you use an anti-UV protection mask and gloves when working with UV rays, to avoid skin and eye burning.

III.2.c. Gel purification of the 2 fragments to be ligated

After migration, on agarose gel:
►Observe and note the relative intensity of the 2 fragments to be purified (*i.e.* the fragments that you will use for the ligation: the cloning vector and the insert), in order to estimate their concentration.
► Cut these 2 fragments on the agarose gel (after the teacher's demonstration).
► Transfer each fragment independently in a **<u>2 ml</u>** microtube.
► Purify these 2 fragments using the Qiaquick kit following the corresponding protocol in appendix 1.

III.2.d. Ligation reactions

► Prepare 3 reaction mixes (A **or** B, as well as C and D) in **200 µl** microtubes:

	Tube Lig A (ratio 1:2)	**Tube Lig B (ratio 1:3)**	**Tube Lig C (ratio idem A or B)**	**Tube Lig D**
H_2O	qsf 20 µl	qsf 20 µl	qsf 20 µl	qsf 20 µl
Ligase buffer (10 X) **! Make sure you vortex it before pipetting it**	1 X	1 X	1 X	1 X
Cloning vector (previously digested and purified)	50 ng	50 ng	50 ng	50 ng
Insert (previously digested and purified)	x ng **(YOU MUST CALCULATE*)**	x ng **(YOU MUST CALCULATE*)**	x ng **(YOU MUST CALCULATE*)**	-
Ligase	1 µl	1 µl	-	1 µl

► Incubate the tubes at 16 °C O/N (or under other appropriate conditions, depending on the time and materials constraints).

Tool recommended for calculation: http://nebiocalculator.neb.com

Tubes A and B: explain the different ratios that are used.
What is the usefulness of the C and D reactions?

III.3. Preparation of competent bacteria

III.4. Bacterial transformation

Before starting this experiment, think of ALL the controls you will need to perform in order to be able to interpret your ligation and transformation results.

The aim of this protocol is to set up 5 transformation conditions. The competent cells will be transformed **separately** with:

- 3 ligation products obtained at the end of III.2.d step (products Lig A **or** B, Lig C and Lig D) → 3 different transformations tubes (tubes A **or** B, C, D)
- **or** native pG11 plasmid (that was previously purified for you and adjusted @1 ng/µL) → tube E
- **or** no DNA → tube F

To do so:

► Homogenize a suspension of competent cells and transfer 100 µl of it in 5 different sterile microtubes (1.5 ml), then add:

Tube "Tranf A" **or** "Transf B"	5 µl of the ligation product from the tube A **or** B
Tube "Tranf C"	5 µl of the ligation product from the tube C
Tube "Tranf D"	5 µl of the ligation product from the tube D
Tube "Tranf E"	5 µl of pG11 plasmid, you must ensure that the quantity of native pG11 must be identical to the quantity of plasmid used for the other transformations
Tube "Tranf F"	5 µl of ultra-pure H_2O

► Mix the contents of the tubes and give a shortspin if necessary.
► Incubate on ice for 30 min.
► Incubate the tubes at 42 °C for 30 s exactly, and transfer them on ice for 5 min.
► Add sterile SOC medium (400 µl) in each tube and incubate immediately at 37 °C for 1h under shaking conditions (130 rpm or maximal speed of the shaker-incubator).
► Spread 150 µl of each suspension on the corresponding Petri dish.
► Incubate at 37 °C O/N.

Explain what are your controls and what are their respective roles.
Calculate the theoretical quantity of recombinant plasmid (*i.e.* the maximum quantity of plasmids obtained after the ligation reaction, if the ligation rate is 100 %).

III.5. Selection of transformed colonies and inoculation

► Select 1 or 2 transformants (*i.e.* 1 or 2 isolated colonies) which you think contain the recombinant plasmid.
► Think of a control cultures you should as well make.
► Inoculate all the selected colonies independently in a suitable nutritive medium (3-4 ml) and incubate at 37 °C for 16-18 h under shaking conditions.

III.6. Purification of the recombinant plasmid and construct validation

For this part, YOU must write up in your **report** your own detailed protocol (that can be different from the protocol of the other pairs of the class). That protocol aims to confirm that your selected bacterial colonies contain the recombinant plasmid that you have tried to construct (*i.e.* if the DNA fragment of interest has been ligated in the right orientation in the plasmid backbone), using an easy molecular biology technique.

To guide you in establishing this strategy, it may include the protocol given as complementary documents.

Advice and data to perform the first protocol of this strategy:
- Follow all the advice given in the protocol by the manufacturer (storage and working temperatures, adding ethanol to the wash buffer, etc…).
- Think about the roe of all the components of the main buffers, knowing that they contain the following reactives:

Buffer 1	**Buffer 2**	**Buffer 3**	**Buffer 4**
50 mM Tris HCl, pH 8 10 mM EDTA	200 mM NaOH 1 % SDS	3 M KOAc, pH 5.5 100 µg/ml RNaseA	75 % EtOH 25 mM NaCl 5 mM Tris-HCl, pH 7.5

Appendix 1: Protocol of DNA gel purification using the Qiaquick kit.

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**
   Minimize the size of the gel slice by removing extra agarose.
2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).**
   For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.
3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**
   IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.
4. **After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).**
   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
   The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.
5. **Add 1 gel volume of isopropanol to the sample and mix.**
   For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.
6. **Place a QIAquick spin column in a provided 2 ml collection tube.**
7. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**
   The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. **Discard flow-through and place QIAquick column back in the same collection tube.**
   Collection tubes are reused to reduce plastic waste.
9. **Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.**
   This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.
10. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**
    Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
11. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x *g* (13,000 rpm).**
    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**

!!!NEVER centrifuge tubes with open lids: remove lids before and keep them aside.

13. **Transfer 30 µl of freshly taken UP H_2O in the column** and let stand for 1 min.
14. **Centrifuge at 13,000 rpm for 30 s to 1 min**.
15. **Transfer the recovered solution back into the column**. Let stand at room temperature for 1 min. Centrifuge at 13,000 rpm for 1 min.
16. **Transfer the recovered plasmid solution into a clean microtube**.