MODULE 2: PLASMID-MINIPREPARATION AND RESTRICTION DIGEST/PCR

Plasmids are small circular double-stranded DNA-molecules. They exist in most of bacteria, some yeast but not in higher eukaryotes. Within these cells, plasmids replicate autonomously.

In the 50's, scientists studied the phenomenon that antibiotics resistance was rapidly transferred from one bacterium to another. They found, that the necessary genetic information for the resistance and the transfer was often located on plasmids. Later, at the end of the 60's, restriction enzymes could be isolated from bacterial cells. These enzymes cut double-stranded DNA within specific recognition sequences. Normally, they are a defence mechanism against infection by viruses. Both, plasmids and restriction enzymes were important discoveries for the emerging gene technology. On the basis of these tools it was for the first time possible to combine DNA-fragments from different species outside a cell. With the help of restriction enzymes a DNA-fragment can be cut from a genome and inserted into a plasmid. For the insertion, another enzyme called DNA-ligase is required. It seals the gap between the DNA-fragments by making a phosphodiester bond. The recombined plasmid can be used as a vector to bring a specific gene into a bacterial cell. Depending on the construction, the inserted gene may be expressed.

Bacterial cells grow at an exponential rate by cell division. After each division both daughter cells have copies of the plasmid with the inserted DNA in their cytoplasm. When the bacteria grow in a liquid culture medium, we can easily amplify the recombined plasmid. The bacteria cultures consist only of a few milliliters and we can only isolate a few micrograms of plasmid-DNA from the cells. This is sufficient for our analysis. We call it a plasmid-minipreparation in contrast to large preparations (maxi-prep) that yield milligram quantities of DNA.

The plasmid:

We use a plasmid called pET-14b into which a gene encoding the enzyme β-galactosidase has been inserted. β-galactosidase catalyzes the hydrolysis of lactose (milk sugar) into its monosaccharids glucose and galactose. Therefore the enzyme can be used to produce milk products that are free from lactose.

By inserting the ß-galactosidase gene into pET-14b by ligation, you will also obtain some "wrong" forms, for example the plasmid pET-14b without the gene for β-gal or with the insert in the "wrong" orientation.

When plasmids have been transformed into bacteria cells, you have to make sure that they incorporated the right DNA fragment in the right orientation.

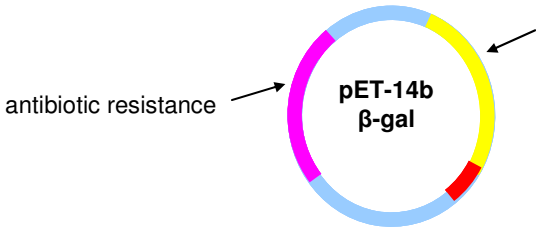


Figure 1: our plasmid pET-14b β-gal
purple: antibiotic resistance
yellow: included Gen (β -gal)

And that's your job today:

Do a plasmid-minipreparation to isolate the plasmids from the bacterial cells. Then you perform a restriction digest and a PCR reaction. After agarose-gelelectrophoresis we can determine whether the bacteria have received the right plasmid pET-14B with the β-gal gene.

Important: Always label your Eppis carefully, otherwise you are lost!

The pipettes:

1000µL pipette (blue): use blue tips and only pipette 100-1000 µl

200µL pipette (yellow): use yellow tips and only pipette 20-200 µl

20µL pipette (yellow): use yellow tips and only pipette 2-20 µl

Legend:

Instructions | Additional information | Questions

Step 0: preparation of two different bacteria cultures / Harvesting the bacterial cells

☑ ▶ Pick one white and one blue clone with a toothpick from the agar plate. Incubate each toothpick in an 1,5 mL eppi with 1 mL LB-amp-medium for a few seconds.

☑ ▶ Use sharp forceps and pierce a little hole in the cap of the eppi to make sure, that the *E. coli* cells get enough oxygen. Incubate the two cultures over night at 37°C on a shaker.

(We have already done these steps.)

☐ ▶ Harvest the bacterial cells by centrifugation at 4000 rpm (1700 rcf) for 5 min. Pour off the supernatant.

1. Isolation of plasmid-DNA

Step 1: resuspend the bacterial cells

☐ ▶ Add 100 µL solution S1 to the pellet of bacteria in your eppi. Snip your finger against the eppi until the pellet has disappeared and the bacteria are resuspended in the liquid.

Solution S1: *Solution S1 is a buffer that keeps the pH-value constant. The bacteria cells that were concentrated in the pellet are now dispersed in the diluted solution. The bacteria cells are still intact. That's the reason why we call this a suspension.*

? *What does the suspension of bacteria look like?* _____

_____

Step 2: lysis of the cells

☐ ▶ Add 200 µL solution S2. Close the cap of your eppi and mix carefully. Wait for 5 min.

Solution S2: *Solution S2 contains:*

SDS *(sodium-dodecyl-sulfate): SDS is a detergent that is used e.g. in cleaning agents or tooth paste. It breaks the cell wall of bacteria and proteins, chromosomal DNA, plasmid DNA and RNA are free now. The solution becomes viscous. SDS also denatures proteins and they loose their characteristic three-dimensional structures.*

Sodium hydroxide *(NaOH) causes the denaturation of DNA-molecules. Chromosomal DNA as well as plasmid-DNA are separated into single strands since the hydrogen bonds between the bases are broken. The whole solution becomes very alkaline (high pH).*

? *How does the suspension in your eppi appear now?* _____

_____

Step 3: precipitation of proteins

☐ ▶ Now add 150 µL of cold solution S3, carefully turn the eppi over a few times and put it on ice for 10 minutes.

Solution S3: *Solution S3 contains* **potassium acetate**.at pH 4,7. *This causes the neutralisation. Both the plasmid-DNA and the chromosomal DNA renature and form double strands. Since the chromosomal DNA is much longer, it cannot rehybridize correctly and forms mispaired networks instead. Proteins also denature and form large aggregates together with the DNA network. When you add S3 you see these networks as a slimy mass. The small plasmids, however, rapidly form correct double strands and remain soluable (i.e. they stay in solution).*

What happens to the solution? _____

_____

*Do you know this phenomenon from everyday life?*_____

_____

The eppi is now centrifuged for 10 minutes at 10000xg.

By centrifugation precipitated proteins and chromosomal DNA are pelleted. The plasmid-DNA remains in the supernatant.

Step 4: precipitation of plasmid-DNA

Transfer about 350 µl of the supernatant into a new labelled eppi. Add 1 mL of 100% EtOH, shake the eppi and then put it back on ice for 5 minutes.

The plasmid-DNA is insoluable in 70% EtOH and precipitates. The precipitation of DNA works better when you put your eppi on ice.

Take care that you don't transfer anything of the pellet to the new tube!

Now, shake your eppi again and centrifuge for 10 minutes at 10.000xg.

During the centrifugation the insoluble plasmid-DNA is collected at the bottom of the Eppi. Often the pellet is very small and can hardly be seen. If you get a large white pellet, you have probably transferred protein and chromosomal DNA into your tube. Make a note what your pellet looks like.

Carefully pour off the supernatant.

Don't worry! The plasmid-DNA remains in the eppi. Usually, a tiny little droplet of liquid remains in the eppi.

Step 5: washing the plasmid-DNA

Add 1 mL 70% EtOH and centrifuge for 5 minutes at 10.000xg. After carefully pouring off the supernatant, you might (!) see a small pellet at the bottom of the tube. The pellet is then dried at 60°C in a heating block (leave the eppi open).

In the 70% EtOH the plasmids are also insoluable. In the 30% of water residual potassium acetate is dissolved.

What happens in the washing step? _____

_____

Step 6: solution of plasmid-DNA in UV-water

Dissolve the pellet in 20µL UV-water.

UV-water has been irradiated with ultraviolet light. Any DNA that may have been in the water has been destroyed.

To get a better solution of the plasmid-DNA in water, the eppi is heated to 60°C on the heating block (this time, close the eppi).

*The eppi now contains the **isolated plasmid-DNA**, disolved in UV-water!*

2a. Amplification of selected plasmid-DNA fragments

During the polymerase-chain-reaction (PCR) a specific plasmid DNA-section is copied and amplified.

Step 7: preparing the master-mix

We prepare the *master mix* together. The *master mix* contains all components necessary for the PCR-reaction. The master mix (23 µL) is combined in special tubes („PCR-tubes“) that fit into the thermocycler. In the end, add 2 µL of the isolated plasmid-DNA.

Attention: *Don't snip the PCR-tube with your fingers. Droplets on the tube wall cannot be centrifuged down because the tubes are too small.*

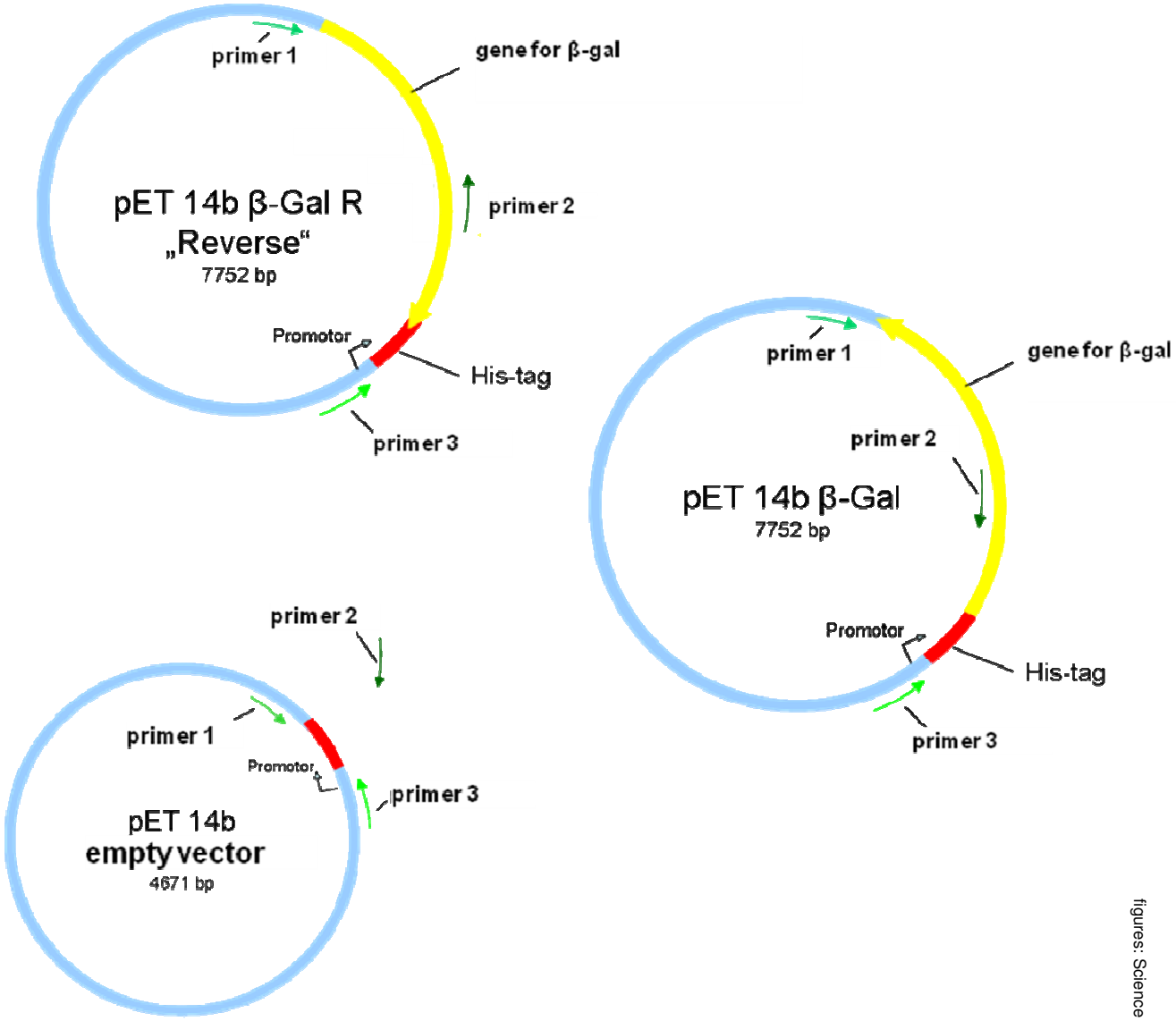
Master-Mix		
UV-water	15 µL	
PCR-buffer with $MgCl_2$	2,5 µL	*The incubation buffer causes optimal conditions for the Taq-polymerase.*
dNTP-mix	2,5 µL	*The dNTP-mix contains all nucleotides necessary for the DNA-synthesis*
Primer-mix (3 primers)	2 µL	*The primer-mix contains three primers (short single-stranded DNA). The Taq-polymerase needs the primer as a starting point for the synthesis. Dependent on the form of the plasmid you will get a PCR-product with a particular length.*
Taq-polymerase	1 µL	*The Taq-polymerase is thermally stable. The enzyme extends the primers by adding nucleotides. A new strand is synthesized. So, the Taq-polymerase copies the target sequence.*
plasmid-DNA in solution	2 µL	
total volume	25 µL	

Now place your tube into the PCR machine and start the program.

PCR-program:

	step	**temperature**	**time**	**comment**
	Denaturation	95 °C	3 min	*We want to make sure that the target-DNA is separated into single strands.*
30 cycles	Denaturation	95 °C	30 sec	*The double-stranded DNA is separated into single strands.*
	Annealing	70 °C	20 sec	*At this temperature the primers bind to the single stranded DNA.*
	Extension	72 °C	40 sec	*The Taq-polymerase extends the primers by adding nucleotides. A double-stranded copy of the target DNA is formed.*
	Extension	72 °C	2 min	*The final extension time helps to finish the elongation of some PCR-products.*

Further information about our plasmid: pET14b-β-gal



figures: Science Bridge 2008, Sara Müller

If you have isolated an empty vector you will get a fragment of 309 bp. If you have plasmids where the β-gal gene has been inserted in the wrong direction you will get a fragment of 400 bp. If you have isolated plasmids where the gene for β-gal has been inserted in the right direction you will get a PCR-product of 600 bp.

Why is it so important to know in which direction the gene for β-gal has been inserted in the plasmid?

Which primers (1,2,3) generate the 309bp, the 400bp and the 600bp fragment?

2b. Restriction digest of plasmid-DNA

Step 7: preparing the restriction digest

Give the following solutions into a new labelled eppi and mix them carefully. If there are droplets on the inner wall of the eppi after mixing, collect the solutions by a short spin in the centrifuge. Put the eppis in the heating block at 37°C for 1h.

component	amount	Comment
plasmid-DNA	5 µL	
buffer for incubation	2 µL	*The incubation buffer gives optimal salt and pH conditions for the restriction enzyme(s).*
RNAse A	1 µL	*RNAse A is an enzyme that degrades RNA*
Restriction enzyme	1 µL	*You get the restriction enzyme from the tutor.*
UV-water	11 µL	
total volume	20 µL	

*If you want to make a restriction digest with two different enzymes you will have to adjust the components. Use 1 µL of each enzyme but add only 10 µL of UV-water.

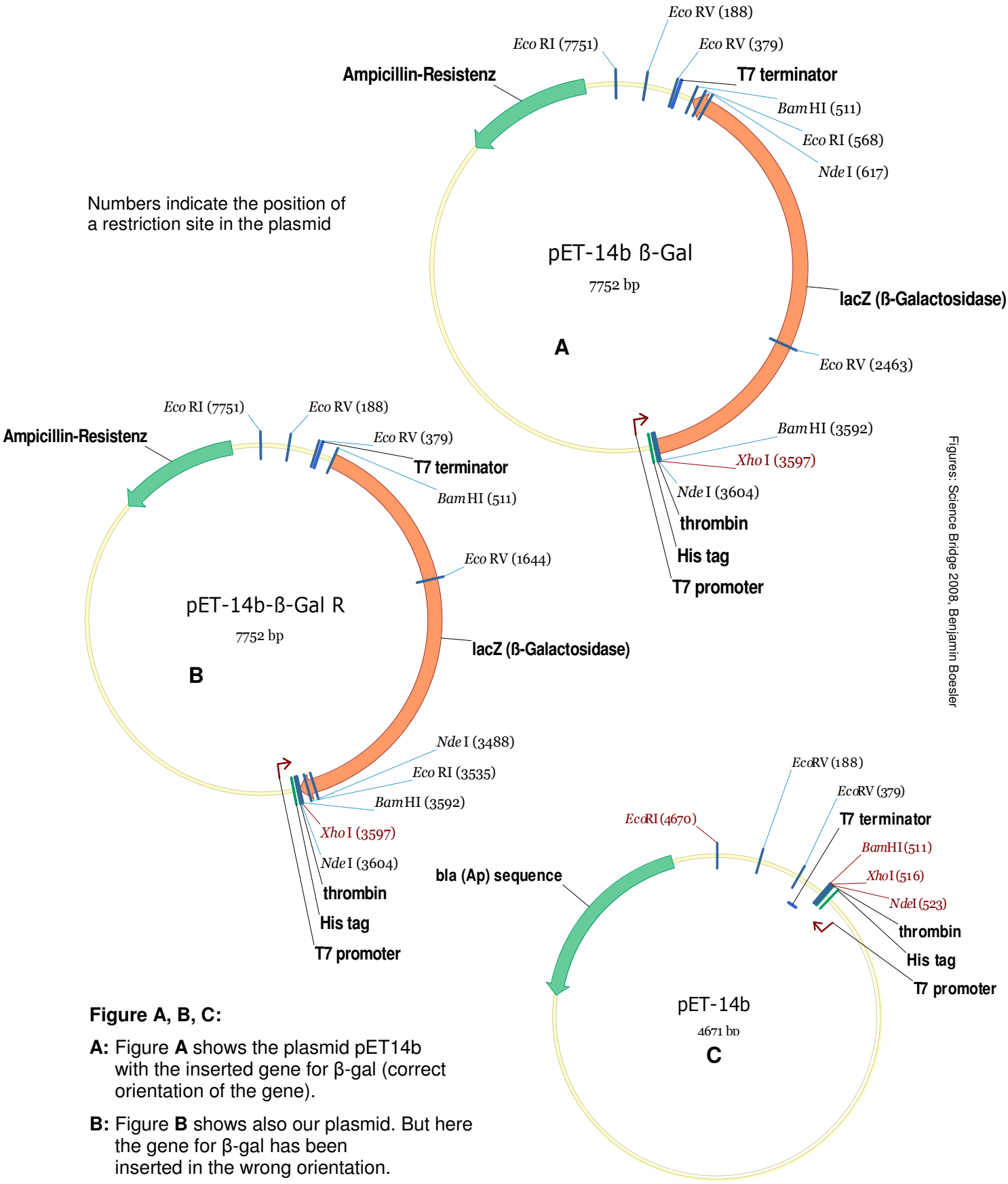
Restriction sites in our plasmid (you find a more detailed plasmid map below).

restriction enzyme	**buffer**	**cutting site**	**expected fragments**	**obtained fragments**
Eco RI	*Eco* RI buffer	1 in Plasmid 1 in Insert	569 bp/ 7183 bp or 3536 bp/ 4216 bp	
Bam HI	*Bam* HI buffer	2		
Nde I	Red oder Orange	2		
Xho I	Red	1 in insert		
Eco RV	Red	2 in plasmid 1 in insert		

Complete the table above using the information on the plasmid maps on the next page.

Further information about our plasmid: pET14b-β-gal

Numbers indicate the position of a restriction site in the plasmid



Figures: Science Bridge 2008, Benjamin Boesler

Figure A, B, C:

A: Figure **A** shows the plasmid pET14b with the inserted gene for β-gal (correct orientation of the gene).

B: Figure **B** shows also our plasmid. But here the gene for β-gal has been inserted in the wrong orientation.

C: Figure **C** shows the empty vector: pet-14b

Agarose gel-electrophoresis

a) Separation and visualization of plasmid-DNA fragments after PCR.
b) Separation and visualization of plasmid-DNA fragments after the restriction digest.

Step 8: casting the agarose gels

a) 2% agarose for the PCR-fragments
b) 1,2% for the restriction digest

☐ ▶ 2 g agarose (for 100 mL) and 1,2 g agarose (for 100 mL) are filled into a beaker. 1x TBE buffer is added to make an agarose suspension of 2% and of 1,2%. The suspensions are boiled at 250°C on a heating plate (or in a microwave oven if available). To prevent the solutions from boiling over, they are agitated with a magnetic stirrer at approx. 500rpm. In hot water, the agarose is dissolved.

Agarose is a powder from the Agar-Agar plant, which produces a gel when cooled down. The puffer is required to keep the pH value on a constant level.

☐ ▶ The solutions have to cool down a bit before you pour them into the **electrophoresis chambers,** where they will finally solidify. After **hardening** (ca. 30 min later), remove the ridges (spacers) and the combs carefully. Add 1 times concentrated TBE buffer into each chamber, until the gel is completely covered.

The comb forms pockets in the gel, into which you can fill your samples.

Step 9: loading the gel

☐ ▶ Fill 10 µL of your PCR product into a new labelled eppi, add 5 µL of the blue loading buffer for small fragments and mix.

☐ ▶ Fill 10 µL of your digested plasmid-DNA into a new labelled eppi, add 5 µL of the blue loading buffer" and mix. We will also mix 5 µL of undigested plasmid-DNA and 5 µL loading buffer as a control in a separate tube.

*The **loading buffer** contains:* **glycerol**. This is heavier than the 1x TBE buffer, and your sample will sink to the bottom of the pocket. Two **blue dyes** (Bromophenolblue and Xylencyanol) are in the loading buffer so that you can easily control where you pipet the sample. IMPORTANT: These dyes do not stain the DNA! **Ethidium-Bromid** or **SYBR-Green** are dyes that intercalate in the DNA double helix. They can be used to visualize the DNA, because they fluoresce under UV light but are invisible in daylight.

☐ ▶ Pipet your 15 µl sample into a pockets of the gel.

*The first pocket is filled with a **marker**.*
***IMPORTANT** Take a note in which pocket your DNA was loaded! Otherwise you will not be able to identify your sample later!*

Step 10: start of gel electrophoresis

☐ ▶ *Switch on* the power supply (ca. 100 V – max. 120 V) to start the electrophoresis. The gels should be run for 30-45 minutes. We then take the gels out of the puffer and look at them on the **UV-box** (or the Dark Reader) to see the DNA bands.

*The DNA fragments are separated by length. **During the whole process they are invisible!!!** You can take a first look at the gel now, but you will also get a picture of it**!***

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