Experiment 22	Isolation of plasmid-DNA from bacteria and PCR
Advisor	Konrad Egli: kegli@botinst.unizh.ch
Reading	Chapters in BBOM 10th: 10.8 BBOM : Madigan M.T., J.M. Martinko and J. Parker: "Brock - Biology of Microorganisms", 10th Edition (2003), Prentice Hall.
Objectives	• Isolation of plasmid-DNA from different bacteria clones • Handling of bacteria clones • PCR-experiment
Background	The typical plasmid is a circular double-standed DNA molecule less than 1/20 the size of the chromosome. Most plasmids are circular, but linear plasmids are also known. Naturally occuring plasmids vary in size from 1 to more than 1000 kilobase pairs. Most of the plasmid-DNA isolated from cells is in the supercoiled configuration, which is the most compacted form within the cell. Isolation of plasmid-DNA can generally be accomplished by making use of the physical properties of supercoiled DNA molecules. Although chromosomes are also supercoiled inside the cell, isolation of chromosomal DNA almost always leads to breakage of the strands and consequent loss of supercoiling. Separation can then proceed by a variety of techniques, including ultracentrifugation and electrophoresis on agarose gels. Among the most widespread and well-studied groups of plasmids are the resistance plasmids which confer resistance to antibiotics and various other inhibitors of growth. Today, there are are number of well established techniques commercially available as kits which make DNA extraction and purification easy. Although kits offer fast and reliable methods for extracting plasmid DNA with high yields, kits are expensive. Since a PCR is performed afterwards, which amplifies certain DNA fragments, we use miniaturized extraction methods; the extracted amount is large enough for further processing. **PCR**, a method to amplify DNA: For details see lectures in molecular biology. Exponential increase of amount of DNA (every cycle doubles the amount of product). The primers set the specificity of the reaction.
Literature	The chapters of BBOM 10th mentioned above
www. Links	http://www.accessexcellence.org/AB/GG/plasmid.html http://histmicro.yale.edu/mainfram.htm http://gweb1.ucsf.edu:8080/shared_databases/plasmids/
Practical work	You will isolate DNA fragments of 16S ribosomal RNA genes from different bacteria which had previously been cloned into a plasmid of *E.coli*

Material and Methods

Composition of PBS (phosphate buffered saline, per liter):
8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4
pH 7.4

Easy extraction of plasmids containing a cloned gene (16SrDNA) from *E.coli*
1. Grow *E.coli* (approx. 5 ml LB) in glass tubes at 37 ¡C over night.
2. Centrifuge 1 ml in Eppendorf-tube (2 min at approx. 10 K)
3. Resuspend cells in 0.5 ml PBS-buffer (phosphate buffered saline)
4. Heat cells for 4 min at 94 ¡C
5. Centrifuge tube to remove cell debris, transfer supernatant to a new tube
6. Add 3 ml of plasmid-containing solution to the 20 ml of prepared PCR-reaction mixture (composition see below: points 1 to 3), fill up to 25 ml with diluted Taq-Polymerase
7. Start PCR reaction (see protocol below)

PCR (polymerase chain reaction)

1. Preparation of a "master-mix":

	per sample
dH_2O (steril)	18.175 ul
Buffer (10 x)	2.5 ul
dNTP (2.5 mM)	2 ul
forward primer (5 uM)*	1 ul
reverse primer (5 uM)**	1 ul
BSA (bovine serum albumin, 20 mg / ml)	0.125 ul
Taq DNA Polymerase (5 U/ul)	0.2 ul
Total:	25 ul

Primers used:

* M13 Forward: 5'-GTA AAA CGA CGG CCA G-3' Tm: 50 ¡C

** M13 Reverse: 5'-CAG GAA ACA GCT ATG AC-3' Tm: 50 ¡C

2. Mix the master-mix well on a vortex
3. Put 1-3 µl sample (from DNA extraction) and 25 µl Master-Mix into a 200µl tube. Centrifuge only for a short time. Stop again, as soon as the centrifuge has reached a moderate speed; this avoids sedimentation of the polymerase.

Typical PCR program on the Techne Progene / Genius thermocyclers used in the practical course (**Program 84-86)**.
1 cycle of: 94 ¡C 1min 30 sec
30 cycles of: 94 ¡C 15 sec
50 ¡C 30 sec
72 ¡C 1min 14 sec + 1 sec per cycle
1 cycle: 72 ¡C 8 min
Hold 10¡C

	4. Put the 200 l tubes containing the samples into the thermocycler and press the start button. Watch the first 20 seconds whether it starts at the right position of the program. 5. Prepare a 1% agarose gel (in TAE-buffer). We will need this to check whether we obtained PCR products. 6. Load 4 l of your PCR products onto the gel (in loading buffer). **How PCR can be improved:** - dilute the template DNA - set the annealing temperature higher or lower - add BSA (1 l of 2.5 mg/ml per reaction) or DMSO (1% v/v per reaction) - purify the template DNA - set the $MgCl_2$ concentration higher or lower (standard 1.5 mM final concentration) - try different polymerases - check all used solutions for contaminations - use different primer concentrations
Experinces gained	• Handling antibiotic resistant bacteria • Learning how to isolate plasmid DNA
Laboratory Rules and Precautions	Use good laboratory practice! Do not contaminate yourself, others or the laboratory environment. All waste must be sterilized before disposal. It is necessary to work cautiously and aseptically. Use gloves for all steps of the procedure. Wash your hands before you leave the room and desinfect bench surfaces with 70% ethanol.
Timing	90 minutes
Reporting	Report the different functions of the different buffers used and the success of your DNA extraction
Questions to be answered	How is it possible to separate chromosomal DNA from plasmid-DNA?