Experiment 23	**PCR-RFLP (PCR-Restriction Fragment-Length Polymorphism) of 16S-rDNA from pure cultures or clones**
Advisor	Konrad Egli kegli@botinst.unizh.ch
Reading	Chapters in BBOM 10th: 10.12 BBOM: Madigan M.T., J.M. Martinko and J. Parker: Brock - Biology of Microorganisms , 10 th Edition (2003), Prentice Hall.
Objectives	• Digesting plasmid-DNA. • Calculating fragment sizes of digested plasmid-DNA • Identifying different bacteria on the basis of their plasmid-DNA • Separation of different DNA-fragments by gel electrophoresis
Background	The function of the restriction enzymes is to recognize certain sequences of DNA and cut the DNA at these specific sites. Restriction enzymes have proven enormously valuable in many of the techniques of *in vitro* DNA manipulation. Indeed, the discovery of these enzymes was the beginning of what in now called genetic engineering. Because the base sequences recognized by restriction enzymes are mostly four to six nucleotides long, there will generally be only a limited number of such sequence motifs in DNA piece. After cleaving the DNA, the fragments can be separated from each other by gel electrophoresis. The number of fragments, which is the number of bands obtained on the separating gel, depends on the number of cutting sites present on a particular DNA piece. The size of the fragments gives a first indication about the spacing of cutting sites. The gel pattern is this a rough characterization of the DNA which allows to distinguish DNA from the same and from different organisms. Electrophoresis is the procedure by which charged molecules migrate in an electric field, the rate of migration being determined by the charge on the molecule and by its size. Small or compact DNA molecules migrate more rapidly than larger DNA molecules. After a defined period of migration time the DNA fragments on the gel can be made visible by staining with a compound that binds to DNA. Because a given restriction enzyme always cuts at the same site, the banding pattern of a given DNA is reproducible and, using a standard to compare with, the size of the fragments can be determined.
Literature	The chapters of BBOM 10th mentioned above
www Links	• http://www.people.virginia.edu/~rjh9u/restdna1.html • http://www.erin.utoronto.ca/~w3bio/bio215/lecture03_2002/
Practical work	• Digestion of PCR products with *Rsa* I (cutting motif: 5'-GT/AC-3') &*Hpa*II (cutting motif: 5'-C/CGG-3') or • Digestion of PCR products with *Hinf* I (cutting motif: 5'-G/ANTC-3') & *Hae*III (cutting motif: 5'-GG/CC-3')

Material and Methods

1. Preparation of RFLP:

Assay	per sample
dH_2O (steril)	1 ul
Appropriate buffer (M) for endonuclease (10x)	1 ul
*Hinf*I (10 U/ml) and *Hae*III (6 U/ml)	0.1 and 0.16 ul
DNA (PCR-products): add later!	8 ul
Total:	10.26 ul

2. Mix dH_2O with buffer and enzyme (depending on number of samples)

3. Take 1.5 ml sterile tubes or PCR-tubes, mark them and take 2 ul of the master mix solution.

4. Take 8 ul of the DNA (PCR products) of each sample, mix well by pipetting up and down.

5. Incubate all tubes in 37¡C incubator (PCR-machine, waterbath or hybridisation oven) for at least 2 hours.

6. Mix 6 ul loading dye with sample and load 4 ul on the gel (acrylamide or 2 % agarose) and run the acrylamide gel at room temperature during 2 h at 130 V.

7. Stain the gel in 1 ug/ml Ethidium Bromide for about 3 minutes and take the picture for documentation.

Experiences gained

- learning how to calculate fragment sizes of digested DNA
- loading an electrophoresis gel
- detect different bacteria groups with Restriction Enzyme Analysis

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.

Ethidium bromide is extremely toxic. Wear gloves and handle the solution and the gels only in the hood which is assigned for this procedure.

Wash your hands before you leave the room and disinfect bench surfaces with 70% ethanol.

Timing

90 minutes

Reporting

Note the different fragment sizes of digested DNA and the number of different DNA types.

Questions to be answered

How many DNA fragments do you expect for a DNA-sample?
How long are these fragments ?
Discuss virtual digestion patterns.